Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE E TECNOLOGIE AGRARIE, AMBIENTALI E ALIMENTARI

Ciclo XXXIII

Settore Concorsuale: 07/B1
Settore Scientifico Disciplinare: AGR/02

EMERGING PLANT-BASED PROTEINS: COMPARISON FROM EXTRACTION TO FUNCTIONAL PROPERTIES AND FOOD APPLICATION

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General Introduction

In this thesis, results of two projects concerning plant proteins will be discussed in separate sections. More in detail, in one project the focus was to obtain a soy-extract rich of a bioactive peptide, whereas the main research was about plant proteins concentration for food industry.

The first section concerns the extraction of Lunasin, a peptide discovered in soy and described in literature as an antitumoral agent. Preliminary results were obtained within a collaboration with CSIC (University of Madrid) and the peptide content in 20 soybean and soy flour samples appeared promising. The aim of the experiments was to test two protein enrichment processes in order to obtain Lunasin-rich extracts from soybean and soy flour and to test them for antioxidant and anti-inflammatory activities. The research was developed in collaboration with professor Giovanni Dinelli of the University of Bologna and the results confirmed previously researches of *Dinelli et al*. Indeed, significant amount of the Lunasin free form was not found in the tested samples, leading to focus more on the main project, following described.

In the main section of this thesis the core is represented by the plant proteins other than soy that were concentrated with the purpose of being used as ingredient for food productions. This work wants to compare two wet protein extraction processes in terms of protein content, mass and protein yields as well as functional properties of the derived extracts. The plant sources used for the comparison were oleaginous seeds (sunflower and canola) and legumes (chickpea and lentils). It is to underline that an additional source was included in the study, the camelina meal, an emerging oleaginous seed interesting for its high omega 3 content. Moreover, dry protein enrichment processes were preliminary tested during a 3-months collaboration at Wageningen University & Research (WUR). Several tests were conducted with sunflower flour and camelina meal differently milled using air classifier and electrostatic separator devices. The protein flours obtained were then texturized using the shear-cell, a technology developed by WUR to produce plant-based meat analogues.

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Abstract

The topic of this thesis concerns the plant proteins, intended both to obtain bioactive molecules for nutraceutical field and to obtain protein concentrates/isolates for food application. In particular, the work focuses on Lunasin (a bioactive peptide discovered in soybean) and on protein concentration and functional characterization of legumes and oil-industry by-products.

Lunasin is a peptide of 5.5 kDa well described in literature due to its antitumoral and antioxidant activities properties. Lunasin was firstly discovered in soybean as part of a 2S albumin but several studies found the peptide also in other plant sources, such as wheat and barley. In this study, the presence and the quantity of Lunasin were investigated in soybean samples and other plant sources, using two different protein extraction processes. The aim of the project was to produce protein extracts rich in Lunasin, intended to nutraceutical/cosmetic markets. Western blotting with customized antibody did not reveal the presence of the free form of the peptide, confirming those studies that deny the presence of Lunasin in cereals. However, in some soy samples a band around 10 kDa emerged, suggesting the possibility of a dimeric form of the peptide.

The increasing demand for alternatives to animal derivatives and meat food products, which is linked to ethical and environmental reasons, highlights the necessity of using different protein sources. Plant proteins provide a valid option, thanks to the relative low costs, high availability and wide supply sources, such as cereals, pulses, oleaginous seeds. Plant proteins could be used in food production as protein flours (30-50 % proteins), protein concentrates (50-85 %) and protein isolates (> 85 %). The key point is the process used to produce the protein ingredients: besides to determine the protein content and the yields, the proteins have different functional properties based on the technology adopted. The functional properties eventually determine the food application of the proteins. The current industrial process used to produce plant concentrates and isolates is the alkaline extraction followed by isoelectric precipitation. However, despite the high purity of the obtained proteins, it presents some drawbacks. First

of all, the huge quantity of water and energy required, that makes the process low sustainable. Furthermore, the proteins are often denatured due to the strong pH shifting, causing loss of functionalities. Innovative protein extraction processes are emerging, with the aim of reducing the environmental impact and the costs, as well as improving the functional properties. Among the innovative processes, the dry protein separation has gained attention thanks to its advantages (no water requirement and no denaturation of the proteins). By the way, compared to the alkaline process, dry separation allows to obtain protein concentrates and can't reach protein isolates.

In this study, the traditional wet protein extraction and another simplified wet process were used to obtain protein-rich extracts out of different plants. The sources considered in the project were de-oiled sunflower and canola, chickpea, lentils, and the camelina meal, an emerging oleaginous seed interesting for its high content of omega 3. The extracts obtained from the two processes were then analysed for their capacities to hold water and fat, to form gel and a stable foam. Results highlighted strong differences concerning the protein content, yield and functionalities. The extracts obtained with the alkaline process confirmed the literature data about the four plant sources (sunflower, canola, chickpea and lentils) and allow to obtain a camelina concentrate with a protein content of 63 % and a protein recovery of 41 %. The second easiest process was not effective to obtain a protein enrichment in oleaginous sources, whereas an enrichment of 10 and 15 % was obtained in chickpea and lentils, respectively. The functional properties were also completely different: the easiest process produced protein ingredients completely water-soluble at pH 7, with a discrete foaming capacity compared to the extracts obtained with alkaline process. These characteristics could make these extracts suitable for the plant milk-analogue products.

The supernatants co-products obtained during alkaline process were also dried and analysed; surprisingly they possessed a good amount of proteins and showed functionalities similar to the extracts obtained with the second process.

Preliminary data about dry protein enrichment were also collected using the air classification and electrostatic separation devices at Wageningen University & Research. Camelina meal and sunflower flour were tested at diverse milling

conditions, and generated protein fractions with different yields and protein contents. The sunflower sieved at 0.5 mm and milled using impact miller at 7.000 rpm provided a promising result using electrostatic separation (protein enrichment of 10,9 %).

First section: Lunasin project

Chapter 1 Introduction

1.1 Bioactive molecules

Nowadays there is more and more attention to human food, focusing on all those constituents that could modify the risk of developing or aggravating human disease conditions. Indeed, functional food and nutraceuticals are used to maintain the healthy state and as adjuvant in some therapies. Into the wide world of functional food, interest in food-protein derived bioactive peptides (BAPs) is growing very fast. BAPs are defined as protein fragments derived from animal or plant food proteins, that exert a positive impact on a specific human body function or condition, improving health status (Sharma et al., 2011).

In vivo, encrypted peptides can be liberated by the digestive or microbial enzymes, whereas in vitro BAPs can also be released during food processing or ripening by microbial enzymes (Korhonen & Pihlanto, 2006; Sharma et al., 2011). Once the peptides are released from the native protein, their sequence, length and aminoacidic composition determine their functional properties. Bioactive peptides may possess many activities as antimicrobial, antithrombotic, antihypertensive, immunomodulatory, antioxidative and antitumoral, and have demonstrated potential for application as health-promoting agents against numerous human health and disease conditions, including cardiovascular disease, inflammation, and cancer (Sharma et al., 2011; Udenigwe & Aluko, 2012).

The application of these peptides as pharmacological therapy depends on their absorption and bioavailability in target tissues (Udenigwe & Aluko, 2012). Currently, BAPs are studied and used for cancer prevention and cure, since they possess the ability to reduce tumor progression through multiple mechanisms including apoptotic, anti-proliferative, anti-angiogenic and immunomodulatory activities (Hernández-Ledesma & Hsieh, 2017). Plant proteins and peptides with cytotoxic activity against cancer cells have become promising alternatives for the

development of new anti-cancer drugs. Soybean (*Glycine max*) for example contains many bioactive molecules, the most studied for their cancer-preventive and antioxidant effect are the protease inhibitors such as the Bowman Birk Inhibitor (BBI) and the isoflavones (De Lumen, 2005; Hyung Jin Jeong, Jeong, Kim, & De Lumen, 2007). Many studies report the positive effects of the administration of soy concentrate, attracting the researchers to deepen the knowledge about soy bioactive molecules. In the late '80, a small cancer-preventive peptide, called Lunasin was discovered in soybean by a research team at Berkley University (USA).

1.2 Lunasin peptide

Lunasin is a 43-amminoacid peptide (5.5 kDa) derived from a 2S-albumin of soy, discovered at the end of 90th. The gene Gm2S-1 codes for a signal peptide, Lunasin (a small subunit), a linker peptide and a large subunit methionine-rich protein (Hernández-Ledesma & de Lumen, 2008; Hyung J. Jeong et al., 2003) (Figure 1).

The Lunasin sequence is:

SKWQHQQDSCRKQKQGVNLTPC**EKHIMEKIQ**G**RGD**DDDDDDDD.

The peptide contains a poly D carboxyl end with 8 aspartic acid residues responsible of the binding to chromatin, a cell adhesion motif RGD (arginine, glycine, aspartic acid), and a helix fragment (EKHIMEKIQ) structurally homologues to a conserved region of chromatin-binding proteins (De Lumen, 2005; Hernández-Ledesma & de Lumen, 2008; Lule et al., 2015).

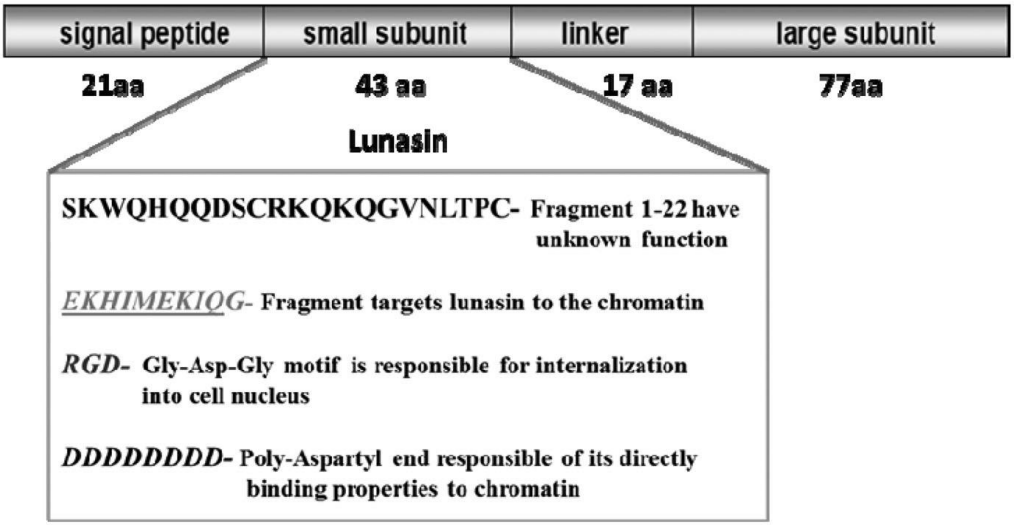


Figure 1 Gm2S-1 gene encoded (Lule et al., 2015)

It has been demonstrated that Lunasin gene transfected in mammalian cells, leads to mitotic arrest and cell death (Hernández-Ledesma & de Lumen, 2008; Hyung J. Jeong et al., 2003). The poly D carboxyl-end of Lunasin binds the kinetochore of the centromere (hypoacetylated histones) and prevents attachment of the microtubule to the centromere, resulting in mitotic arrest (De Lumen, 2005). Lunasin peptide, has chemo preventive properties both in vitro and in vivo, in mouse model for skin cancer, where it prevents transformation of mammalian cells caused by chemical carcinogens and viral oncogenes (De Lumen, 2005). An epigenetic mechanism was proposed to explain Lunasin action, whereby the peptide selectively kills cells that are being transformed by disrupting the dynamics of histone acetylation-deacetylation (Galvez et al., 2001; Hyung Jin Jeong, Jeong, Kim, & De Lumen, 2007). According to this hypothesis, the peptide binds specifically to deacetylated core histones H3 and H4, inhibiting their acetylation (Hernández-Ledesma & de Lumen, 2008).

Besides its potential anticancer activity , Lunasin has cholesterol lowering properties, antioxidant and anti-inflammatory activity (Lule et al., 2015). The anti-inflammatory property of the peptide has been demonstrated in RAW264.7 cells stimulated by lipopolysaccharide (LPS) and also on 3T3-L1 adipocytes, resulting in the inhibition of proinflammatory cytokine production (Hernández-Ledesma et al., 2009a; Hsieh et al., 2017).

Lunasin seems to be very stable , retaining its properties even after high temperature exposure (De Lumen, 2005). Nevertheless, in vitro digestibility studies demonstrated that the peptide is almost totally hydrolysed during digestion, although the presence of protease inhibitors as Bowman Birk and Kunitz trypsin (BBI, KTI) protect Lunasin from enzymatic hydrolysis (Cruz-Huerta et al., 2015; Price et al., 2016).

Free form of Lunasin in literature is generally identified and quantified in soy samples through western blot, mass spectrometry, HPLC and ELISA assay.
Peptide concentration in soy products and derivatives is very wide, depending on soybean cultivar, growth conditions and post-harvest processes. In soybean seeds, the range is between 1 and 13 mg of Lunasin per gram of material tested (De Mejia et al., 2004; Hyung J. Jeong et al., 2003). In defatted flour (generally called protein flour, about 45/50 % of proteins) the content of Lunasin varies from 5 to 14 mg/g, whereas in soy protein concentrate (60/70 % of proteins) and soy isolates (80/90 % of proteins) the content is higher, 9-28 mg/g and 6-38 mg/g respectively (De Mejia et al., 2004; Hyung J. Jeong et al., 2003). Considering soy protein concentrate and isolates, the range is higher also depending on the production process and the solvent used.

1.3 Lunasin-enrichment processes

Different methods are used in literature to isolate and purify Lunasin for experimental purposes, including solvent-extraction methods, chromatography processes and ultrafiltration. Most of these procedures are suitable for laboratory tests, but for large-scale production are too expensive and time-consuming.

Two methods are described in literature to obtain a large-scale production of Lunasin peptide from soy material. The first method applies a sequence of processes that allow to reach a biologically active Lunasin extract, with purity >99% and a yield of 442 mg/kg of defatted soy flour (Seber et al., 2012). In this method, a PBS solution is used to solubilize Lunasin from defatted soy flour, and anion exchange chromatography, ultrafiltration and reversed-phase

chromatography are applied to generate the extract. The described process surely enables to obtain a high pure Lunasin concentrate, but it is expensive. The second method is easier and cost-effective, it allows to obtain 3,2 g of LPIC (Lunasin Protease Inhibitors concentrate) from 100 g of defatted soy flour (Krishnan & Wang, 2015). The process, that includes a protein enrichment with ethanol 30% solution and a calcium chloride precipitation, generates an extract rich in Lunasin, BBI and KTI. The LPIC could be used in nutraceutical since BBI and KTI, besides to protect Lunasin from digestion, are themselves biologically active compounds. Moreover, LPIC could be used in a further step of chromatography purification if high pure Lunasin is required.

The second enrichment process described was tested in the present study.

1.4 Lunasin products

In the United States market, Lunasin supplements has existed for many years. The range of healthy claims cited to sponsor those products is really wide. The company Reliev for example, commercializes a product called LunaRichX (https://reliv.com/p/lunarich-x), described as the “first epigenetic superfood”. They acquired a patent to produce ultra-pure Lunasin and the supplement is adviced for cholesterol management, inflammation reduction, antioxidant benefits and immune support. The company Simplesa Nutrition, produces the supplement LunaCell (https://www.simplesanutrition.com/products/lunacell-Lunasin-capsules), featured by a quantity of the bioactive peptide, more than twice if compared with other brands. Even in this case, the indications for the use of the product is general and it is described to be helpful to support neurodegenerative diseases and motor neuron diseases therapies. Others companies are LunaCode (http://luna-code.com/), that produces the homonymous product, and CareFast (http://carefast.com/products/pqq), that sell a vitaminic supplement with addition of Pyrroloquinoline quinone (PQQ) and Lunasin to support cellular function.

Concerning European market, to date no companies are producing this kind of supplement. In Italy, the company Giuliani produces a cosmetic line for sensitive skin, with the addition of fermented soy rich in Lunasin (patent n° US 2013/0231291 A1).

Chapter 2 aim of the study

Soybean seeds and soy protein flours are the main raw material and products that represent the core business for CD company. Aim of this study was firstly to detect the real presence and the quantity of the peptide Lunasin in soybean seeds and derivatives, and subsequently in other seeds. A preliminary study derived from a collaboration with the Professor Blanca Hernandez Ledesma of CSIC, Univesidad Autonoma de Madrid, and the results of the 20 samples analysed are reported in table 1.

Table 1. List of the samples analysed in this study and by CSIC (Madrid University) and related quantity of Lunasin/ g of material

	TYPE	DESCRIPTION	Lunasin mg/g flour		TYPE	DESCRIPTION	Lunasin mg/g flour
1	Soybean seed	cv Dekabig	3.81 ± 0.21	11	Soybean seed	National edamame	3.39 ± 0.32
2	Soybean seed	cv Iroco	3.22 ± 0.29	12	Soy protein flour	Chemical de-oiling from national seeds	6.02 ± 0.03
3	Soybean seed	cv M10	3.91± 0.13	13	Soy hulls	National soy hulls	0
4	Soybean seed	cv M10	2.8 ± 0.39	14	Soybean seed	cv Luna	2.98 ± 0.31
5	Soybean seed	cv M22	3.72 ± 0.07	15	Soybean seed	national organic seed (unknown cv)	3.63 ± 0.33
6	Soybean seed	cv M22	2.67 ± 0.61	16	Soybean seed	Seed from Niger (unknown cv)	1.66 ± 0.04
7	Soybean seed	cv Asca Subi	1.54 ± 0.22	17	Soybean seed	cv Planta Magnum	3.99 ± 0.38
8	Soy protein flour	Chemical de-oiling from Canadian OGM seeds	0	18	Soybean seed	cv Nikko	4.1 ± 0.6
9	Soy protein flour	Chemical de-oiling from national seeds	3.82 ± 0.24	19	Soybean seed	cv M35	4.32 ± 0.26
10	Soy protein flour	Chemical de-oiling from paraguayan OGM seeds	5.73 ± 0.75	20	Soybean seed	High protein seed (unknown cv)	4.3 ± 0.21

Protein extraction and western blot were performed in 15 soybean seeds (different cultivars/ origin), 4 protein flours and soy hulls. The results were promising, showing a Lunasin content in seeds ranging from 1.54 ± 0.22 to 4.3 ± 0.26 mg/g of tested material. Soy flours were 2 derived from GMO seeds and 2 from non-GMO seeds. The Lunasin content in the flours was 0 for one of the two GMO flours, and ranged from 3.82 ± 0.03 to 6.02 ± 0.75 mg/g for the other flours. No Lunasin content was found in soy hulls.

In this study, the protein profiles of various cereals, pulses and oleaginous seeds were investigated to display the 5.5 kDa band correspondent to Lunasin peptide. Subsequently, soybean was the major focus of the study, in particular those samples that showed a good amount of Lunasin in the preliminary results: cultivars M35, Planta Magnum, high protein seed (unknown cv) and non-GMO protein flour. Two different processes to enrich in protein content were used for all samples, and the protein profiles displayed with SDS-PAGE. One method used phosphate saline buffer (PBS) as is the solvent most used in literature to enrich in Lunasin. The second method used ethanol 30% solution as solvent (described in 1.3 section), as Lunasin is water-soluble and protease inhibitors are alcohol-soluble. The mentioned process in fact, allows to enrich in Lunasin peptide and BBI and KTI, that have also antitumoral activity and protect Lunasin by digestion. Both these methods could be used at industrial scale, since the by-product generated (wet soybean/ soy flour) still contains proteins and once dried could be used as feed.

Western blot using customed antibody was then performed to confirm the presence/absence of Lunasin, and DPPH assay to test the radical scavenging properties of the peptide and the soy extracts.

*Protein extracts from the preliminary study (Madrid University) were also included in these experiments to compare the protein enrichment methods and results.

The long-term aim of the study was the scale-up of the process to obtain a soy-extract rich in Lunasin to be used in nutraceutic and cosmetics.

Chapter 3 Materials and methods

3.1 Samples

Samples used in this study to test Lunasin presence and content are soy seeds (varieties M35, M10, Planta Magnum), high protein seeds (cv unknown, 40% proteins), soy protein flour (defatted, about 48% proteins), soy germ. Furthermore, seeds of wheat, barley, pea, yellow lupin, sunflower flour and camelina cake were tested.

Soybean and the other protein sources have been collected directly from farmers and seed companies. Soy and sunflower flours were provided by CD company and derived from industrial chemical oil extraction using hexane as solvent.

In order to enhance the protein enrichment during the process and to standardize the particle size of the starting material, all the sample have been grinded at the same size (0,5 mm) using a rotor mill (ZM 200, Retsch).

3.2 Protein extraction- ethanol method

Protein extracts enriched in peptides and proteins of small molecular size were obtained using Krishnan et al. 2015 method, with minor modifications (Krishnan & Wang, 2015). The described method was used to isolate abundant quantities of Lunasin and protease inhibitors as BBI (Bowman Birk Inhibitor) and KTI (Kunitz Trypsin inhibitor), the method is simple, quick and uses food-grade solvents. The protease inhibitors protect Lunasin during monogastric digestion, making the protocol potential scalable and useful to create an ingredient for cosmetic and nutraceutical purposes.

1 ml of EtOH 30% v/v was added to 100 mg of grinded sample, mixed with a vortex and then with a glass rod to break the tissue. Tubes were heated at 37°C and shaken at 1.000 rpm for 60 minutes using Thermomixer C (Eppendorf). Tubes were then centrifuged (Centrifuge 5804 R, Eppendorf) at 15.800 g and 4°C

for 10 minutes. 20 ul of 100 mM calcium chloride was added to each 200 ul of supernatant collected after centrifugation. The tubes were mixed with vortex and let at room temperature for 5 minutes. After centrifugation at the same condition, the supernatant was discarded, and the protein pellet resuspended in deionized water. The protein extracts were kept at -20°C or freeze dried.

3.3 Protein extraction- PBS method

A very simple method using food grade solvent (PBS, Phosphate Saline Buffer, pH 7.8) was tested to obtain plant extracts enriched in soluble proteins (Hameed et al., 2009; Ranjan & Matcha, 2012).

3 ml of PBS 50 mM were added to 600 mg of grinded sample, mixed with a vortex and then with a glass rod to break the tissue. Tubes were heated at 37°C and shacked at 1.000 rpm for 60 minutes using thermomixer. The supernatant collected after centrifugation at 16.000 g and 4°C for 10 minutes was refrigerated at -20°C and used for the subsequent tests.

3.4 Protein quantification-bradford assay

Bradford assay was used to determine the protein concentration of the extracts. The dye contained into the Bradford solution is the Coomassie blue, able to bind the proteins causing a colour shifting from red to blue, measurable by spectrophotometer (Bradford, 1976).

Standard curve was prepared using BSA (Bovine Serum Albumine, Bio-Rad) as standard reference at the concentrations of 0, 125, 250, 500, 750, 1000 µg/ml. PBS 50 mM or deionized water were used to dilute BSA. The assay was assessed in microplate, adding 245 µl of Bradford solution (Bio-Rad) and 5 µl of standard or sample to each well. Absorbance was measured at 595 nm using the microplate reader Sinergy H1 (Biotek), the data exported as csv file and elaborated to calculate the concentrations of the samples.

3.5 SDS-Page

Electrophoresis gel SDS-PAGE was used to view the protein profiles of the extracts. Three kinds of precast polyacrylamide gel were used:

- 4-20 % tris-glycine gel (mini-PROTEAN TGX, Bio-Rad), with a separation range of 10–200 kD

- 16,5 % tris-tricine gel (mini-PROTEAN, Bio-Rad), with a separation range of 1.5–30 kD, optimal for peptides and small proteins

-4-15 % tris-glycine gel stain free (mini-PROTEAN, Bio-Rad), with a separation range of 20–250 kD.

3.5.1 SAMPLES PREPARATION

The samples were prepared at the concentration of 1 µg/µl, using the same diluent of extraction process (PBS or deionized water). Sample buffer was added to each sample at the 1:1 ratio and the total volume loaded into the gel was 25 µl. For 4-20 % tris-glycine gel the sample buffer was Laemmli sample buffer (Bio-Rad) + 0,5 % v/v of β-mercaptoethanol (Sigma Aldrich). The sample buffer used for 16,5 % tris-tricine gel was Tricine sample buffer (Bio-Rad) + 0,2% v/v of β-mercaptoethanol. The tubes were then heated at 95°C for 5 minutes in the thermomixer, to denature the proteins.

3.5.2 RUNNING CONDITION

The summary of reagents and running conditions used during the experiments is showed in table 2. The electrophoresis chamber used was Mini-Protean Tetra System (Bio-Rad).

Table 2. Running conditions of SDS-PAGE

GEL USED	RUNNING BUFFER	VOLTAGE	TIME	STANDARD
4-20 % tris-glycine	TGS Tris-glycine 1 X (Bio-Rad)	300 V costant	20 minutes	6 µl Precision Plus Protein, Dual X-Tra, Bio-Rad (2-250 kD range)
16,5 % tris-tricine	Tris-tricine SDS 1 X (Bio-Rad)	100 V costant	100 minutes	6 µl Precision Plus Protein, Dual X-Tra, Bio-Rad (2-250 kD range)
4-15% tris-glycine STAIN FREE	TGS Tris-glycine 1 X (Bio-Rad)	300 V costant	20 minutes	6 µl Precision Plus Protein unstained, Bio-Rad (10-250 kD range)

3.5.3 COOMASSIE BLUE STAINING

The gel after electrophoresis running was stained to display the band protein patterns. The gel was firstly fixed with a solution containing 50% methanol, 10% acetic acid and 40% deionized water for 30 minutes, constantly agitated. After fixing step, the gel was incubated constantly agitated for 60 minutes with staining solution. The staining solution had the same composition of the fixing solution + 0.25 % w/v of Coomassie brilliant blue R-250 (Bio-Rad). After staining, the gel was washed 3 times changing the de-staining solution, composed by 5% Methanol, 7.5 % Acetic acid, 87.5% deionized water. The duration of the de-staining step was an average of 12 hours. The pictures of the gel after Coomassie-staining were taken using Chemidoc Imaging System (Bio-Rad).

The pictures are edited using the software Image J.

3.6 Western blotting

Western blotting analysis was conducted to detect the presence and the quantity of the Lunasin in the protein-enriched extracts. Lunasin peptide standard and the Lunasin antibody were synthesized by GenScript Biotech. The sequence of the peptide was taken from the literature (Chemistry & Krishnan, 2017; Hernández-Ledesma & de Lumen, 2008).

Lunasin peptide standard was resuspended in ultra-pure water (Generon) and the final concentration was 4,85 mg/ml. Lunasin polyclonal antibody (rabbit) was conjugated with Horseradish Peroxidase (HRP), the concentration was 0,958 mg/ml. The aliquots of both peptide and antibody were stored at -20°C. Firstly, antibody and peptide were tested to determine the optimal concentrations to work with and SDS-page gel were run with the peptide at the concentration of 0.06, 0.125, 0.25, 0.5, 0.75, 1, 1.5 µg/µl. Western blots were performed at the same condition but at different concentrations of Ab (0.25, 0.5, 0.75, 1 µg/ml). Western blots of protein-enriched extracts were performed as following: the gel after electrophoresis running was washed with distilled water and proteins transferred on a 0,2 µm Nitrocellulose membrane using the ready-to-use kit Tans-Blot Transfer-Pack (Bio-rad). The gel was blotted using LOW MW protocol (5 minutes) using Trans-Blot Turbo Transfer System (Bio-Rad). After blotting, the membrane

was washed with deionized water and then incubated 1 hour in 20 ml of TBST + 1% casein. TBS solution was prepared with TRIS 20 mM, NaCl 150 mM and distilled water, then the pH was adjusted to 7.5 using HCl 6M. TBST was prepared adding 1% Tween 20 (Bio-Rad) to TBS solution. After blocking incubation, the membrane was washed three times (15 minutes each) with TBST solution. The membrane was then incubated 1 hour with 20 ml of TBST + 1% casein + 0,25 µg/ml Lunasin antibody. After antibody incubation, the membrane was washed three times as before and 2ml of luminol + peroxide solution (Clarity Western ECL Substrate, Bio-Rad) were distributed over the membrane just before the chemiluminescence reading, using Chemidoc Imaging System (Bio-Rad).

3.7 Antioxidant activity

Antioxidant activity of protein-enriched extracts was investigated through DPPH assay. DPPH (1, 1-diphenyl-2-picrylhydrazyl) is one of the free radical mostly used for testing preliminary radical scavenging activity of plant extracts(Bhuiyan & Hoque, 2010; Cumby et al., 2008). The purple colour of the DPPH in solution shifts to light yellow in presence of antioxidant agents. The change of the colour is measured spectrophotometrically.

Standard curve was prepared twice using ascorbic acid as standard reference (Sigma-Aldrich) and ethanol 60 % v/v as solvent. The standard concentrations tested were 1, 5, 10, 12, 15, 17, 20, 30, 40, 50 µg/ml. DPPH solution was prepared at the concentration of 0.004 % (Sigma-Aldrich), dissolved in ethanol 60%. Samples were tested twice at the concentration of 50 µg/ml. 100 µl of standard/ sample were added to 300 µl of DPPH solution, mixed with vortex and centrifuged at 2.000 g and 4°C for 5 minutes. Blanks were made adding 100 µl of EtOH 60% to 300 µl of DPPH solution. After 30 minutes at room temperature, 300 µl of each tube were transferred to the microplate and the absorbance was red at 517 nm. The antioxidant activity % of the standard/samples was calculated with the formula:

$$\%\ antioxidant\ activity = \frac{(blank\ abs - sample\ abs)}{blank\ abs} * 100$$

Chapter 4 Results

4.1 SDS-Page

SDS page gels were used to check the extracts obtained from different methods and to compare different protein sources to the Lunasin standard. In figure 2 the ethanol and PBS protein extraction methods are compared. As the protein profiles suggest, ethanol method (lanes from 1 to 4) tend to enrich in smaller proteins (<20 kDa) compared to PBS protocol (lanes 5, 6, 7). In some samples (especially soy protein flour and M35 soy cultivar, lanes 3 and 4) an about 5 kDa light band is visible.

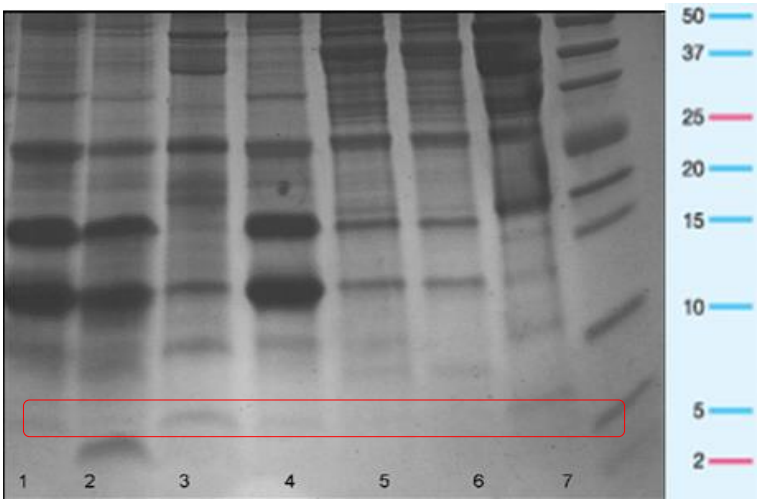


Figure 2. SDS tris-tricine 16,5% gel. Lanes 1-4 ethanol extraction method, respectively: protein soy seed, soy germ, protein soy flour, M35 soy cv. Lanes 5-7 PBS extraction method, respectively: M35 soy cv, M10 soy cv, protein soy flour.

In figure 3, ethanol extracts of wheat, camelina, sunflower, barley, pea and lupin are compared to Lunasin standard (lane 4). The protein profiles resulted in this gel suggest that no protein sources (including soy, lane 3) has a band at the same size of the Lunasin standard.

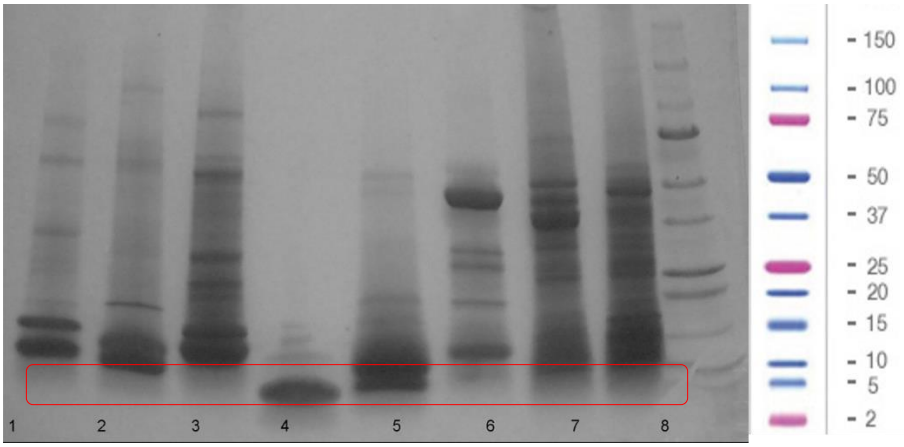


Figure 3. SDS tris-glycine 4-20% gel. Ethanol extraction method: lane 1 wheat, 2 barley, 3 M35 soy cv, 4 Lunasin soy peptide 1µg/µl, 5 sunflower flour, 6 camelina cake, 7 yellow lupin, 8 protein pea.

In figure 4 soy ethanol extracts are compared with the same samples extracted by Univeristy of Madrid (Chapter Aim of the study). The protein extracts from the collaboration are similar with PBS extracts of this study (lanes 1 and 2), and are different from the ethanol extracts, that show bigger bands at about 10 and 15 kDa (lanes 3, 6, 8). However, also in this gel no extracts show a band at the same size of the Lunasin standard (lanes 4 and 5 at different concentration), with the exception of the protein soy seed (lane 6). To notice, Lunasin standard shows another band at about 10, suggesting the possible presence of Lunasin dimers (11 kDa).

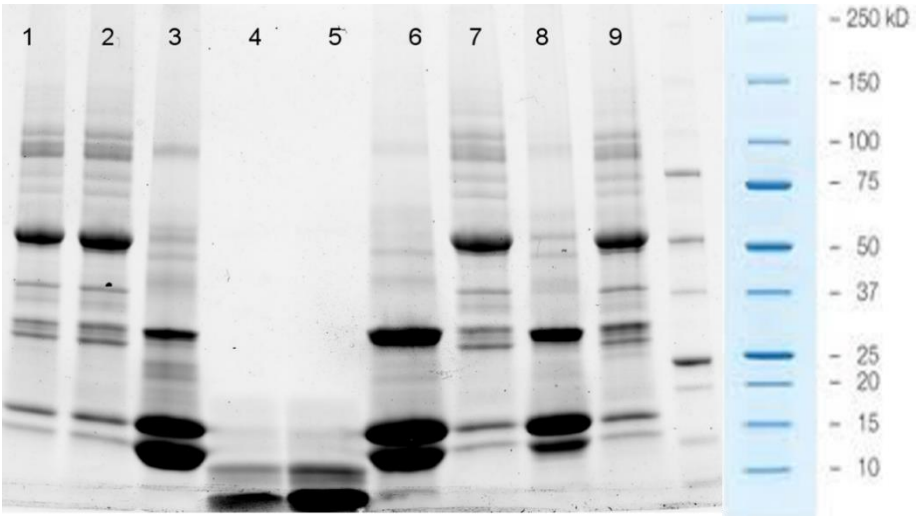


Figure 4. SDS stain-free tris-glycine 4-15% gel. Lane 1 M35 soy cv PBS method, lane 2 M35 soy cv, lane 3 M35 soy cv ethanol method, lanes 4-5 Lunasin soy peptide 0,5 and 1 µg/µl, lane 6 protein soy seed ethanol method, lane 7 protein soy seed*, lane 8 Planta Magnum soy cv, lane 9 Planta Magnum soy cv*.*
**extracts from University of Madrid*

4.2 Western blotting

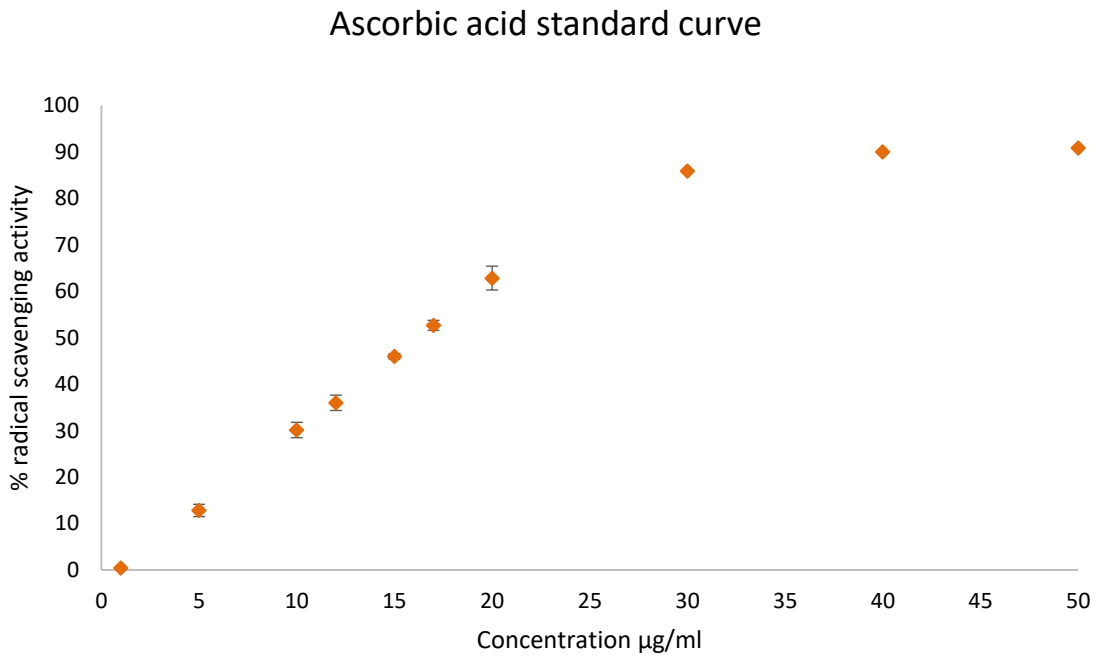
Western blotting was performed at different experimental conditions, to verify the presence of the peptide Lunasin in the protein extracts, using a polyclonal customized antibody and the Lunasin peptide standard as reference. In each western blotting run, the Lunasin peptide used as standard exhibited two bands at 5 and 10 kDa (figure 5), confirming what showed in SDS page (figure 4). All the protein extracts tested, showed no bands at the 5 kDa region, suggesting the absence of the peptide. Some samples tested, as showed in figure 4 for M35 and soy protein flour ethanol extracted, display a light band at about 10 kDa.

Figure 5. Western blotting. Lanes 1, 2, 3 Lunasin soy peptide at the concentrations of 0,25, 0,125, 0,06 µg/µl. Lane 4 protein soy flour ehtanol method extract, lane 5 M35 soy cv extracted from Madrid university, lane 6 M35 soy cv ethanol method extract.

4.3 Antioxidant activity

DPPH assay has been used as preliminary test to detect antioxidant activity of the protein extracts at the concentration of 50 µg/ml (based on Bradford assay calculation, as the interest was to correlate the antioxidant activity to proteins/Lunasin peptide concentration). Ascorbic acid was used as standard reference at increasing concentrations from 1 to 50 µg/ml. Ascorbic acid radical scavenging activity increased linearly at increasing concentrations, and reached its maximum activity at concentrations of 40/50 µg/ml (graph 1). Soy samples from different proteins concentration methods were compared. In particular, M35 cultivar, soy germ and high protein soy seeds from PBS and ethanol extraction were tested at 50 µg/ml. M35 extract of Madrid University was also included into these trials. Moreover, Lunasin standard peptide was used at the same concentrations to investigate its antiradical properties. At the concentration tested, all the samples had no significant radical scavenging activity (Table 3).

Soy germ ethanol extract and the Lunasin peptide showed an activity of 1% and 3.86% respectively.



Graph 1. DPPH assay. Ascorbic acid standard curve

Table 3. Antioxidant activity % of protein extracts

SAMPLE [50 µg/ml]	ABS VALUES	STANDARD DEVIATION	ABS AVERAGE	% ANTIOXIDANT ACTIVITY
M35 cv PBS method	0.873	0.015	0.863	0.00
	0.852			
M35 cv EtOH method	0.861	0.008	0.867	0.00
	0.872			
M35 cv Madrid extracts	0.859	0.005	0.863	0.00
	0.866			
Soy germ EtOH method	0.839	0.009	0.846	1.05
	0.852			
Soy germ PBS method	0.857	0.008	0.863	0.00
	0.868			
Soy protein seed EtOH method	0.874	0.002	0.873	0.00
	0.871			
Soy protein seed PBS method	0.861	0.010	0.868	0.00
	0.875			
Lunasin peptide	0.827	0.008	0.822	3.86
	0.816			

Chapter 5 Discussion and conclusions

The goal of this study was to detect and quantify the peptide Lunasin (5.5 kDa) in soy samples and derivatives, and to verify the efficacy of the lab-scale process described by Krishnan et al (Krishnan & Wang, 2015), useful to concentrate the target peptide and other bioactive molecules to be used in nutraceutical field. In literature, many properties are attributed to Lunasin (antitumoral, anti-inflammatory, antioxidant) and several studies showed great amount of this peptide both in soy seeds and in its derivatives as soy flour, milk, concentrates and isolates. Ledesma et al for example, analysed through western blot 12 commercial soy milk and found a Lunasin content that ranged from 10.7 to 18.9 mg/100 ml of milk (Hernández-Ledesma et al., 2009b). Gonzalez de Meja et al, in their study, used ELISA assay to quantify Lunasin in different soybean cultivars and soy commercial products. They found a Lunasin range of 3.3-9.5 mg/g in soy modern cultivars, and a range of 12-44 mg/g in commercial soy flours, concentrates, isolates and hydrolysed (De Mejia et al., 2004). In another work, the Lunasin content was 5.4, 5.9 and 9.1 mg/g in soy flour, soy isolate and soy concentrate respectively (Hyung J. Jeong et al., 2003).

Moreover, aim of this study was to confirm the presence or absence of the peptide in other pulses, cereals and oleaginous seeds, since in literature exist different opinions concerning the issue. Indeed, Lunasin was identified through western blot and LC-ES-MS assays in barley and wheat seeds as well as in amaranth and Solanaceae (Hernández-Ledesma & de Lumen, 2008; Hyung J. Jeong et al., 2002; Hyung Jin Jeong, Jeong, Kim, Park, et al., 2007; Silva-Sánchez et al., 2008). Nevertheless, in other studies, the results are different: Mitchel et al searched for the sequence of Lunasin and its precursor in the most common databases and there is no matching in species different from soy (Mitchell et al., 2013). Dinelli et al demonstrated the absence of the peptide in different cultivars of wheat, using LC-ES-MS and genetic approaches (Dinelli et al., 2014). In another work, researchers used two specific antibodies that recognize the N and

C-terminal of Lunasin: the blots confirmed the total absence of Lunasin in all the seeds different from soy (Alaswad & Krishnan, 2016).

In this study, two different protein enrichment methods were used to compare the protein profile of the extracts: one uses phosphate saline buffer as solvent, and the other ethanol 30% and a subsequent $CaCl_2$ precipitation. Soy extracts of the same samples used in this study have been provided by Madrid University during a collaboration, they were also tested to quantify the petide. In SDS-page (figure 1), the two methods to enrich proteins used in this study are compared and could be notice that the extracts have very similar profile. Nevertheless, as described by Krishnan et al (Krishnan & Wang, 2015), ethanol method produces enriched in small proteins and peptides-extracts, compared to PBS method. The mentioned work demonstrated that this process is useful also to enrich in Bowman Birk and Kunitz-trypsin inhibitors (14 and 21 kDa respectively): Lunasin is an albumin e thus water soluble, whereas the protease inhibitors BBI and KTI are alcohol-soluble. These molecules, besides to protect Lunasin during digestion, have also chemo preventive effect (De Lumen, 2005). In this study BBI and KTI were not quantified in the extracts, it could be interesting to perform a western blot to confirm a BBI and KTI increasing using ethanol process. In figure 1 a light band around 5 kDa is displayed by M35 cv and protein flour ethanol-extracted (lanes 3 and 4). In figure 3, it is possible to compare also the profiles of Madrid-university extracts: as predictable, they are equal to the PBS-extracts profiles obtained in this study, as the lab-processes are very similar. In figure 2, the protein profiles of ethanol-extracts of soy and other seeds (wheat, barley, pea, lupin and sunflower) are compared to Lunasin standard, emerging that none of the protein sources investigated show a band at the same height of the peptide. Comparing the profiles to Lunasin standard, the ethanol extract of high protein soy seed seems to be the only sample that shows a very light band at the same height of synthetic Lunasin (figure 3, lane 6). Western blotting using Lunasin customized antibody were than performed to confirm the results of SDS-page: all the experiments show that 5.5 kDa band was not displayed in all the soy extracts tested (figure 4). Some of the soy extracts tested showed a band around 10 kDa, as the Lunasin standard: this result could suggest the possibility Lunasin naturally exist in dimeric form. Considering soy bean and derivatives, the results found in

this study are in contrast with the results of Madrid-university and the literature. Indeed, the group of Blanca Ledesma found a huge amount of Lunasin in M35 cultivar, protein flour and high protein seed, respectively 4.32 mg, 6.02 and 4.3 mg/ g of material tested. In figure 6, a western blot performed by Madrid University: as found in this study, some samples show a light 10 kDa band, but the most important difference is the 5 kDa band, clearly displayed in all samples (lanes 8-12).

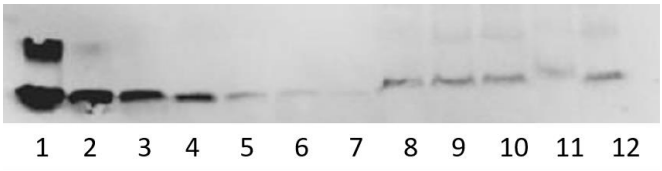


Figure 6. Western blot performed by Madrid University. Lanes 1-7: scalar concentrations of Lunasin standard. Lanes 8-11 extracts of soybean different cv. Lane 12 soy protein flour.

One of the Lunasin properties is the radical scavenging activity. In a work of B. Ledesma, antioxidant activity of Lunasin synthetic peptide was tested using ABTS and other assays, highlighting a strong activity of the peptide (Hernández-Ledesma et al., 2009a). In this study, a preliminary DPPH assay was used to test the antioxidant activity of Lunasin standard and ethanol extracts at the concentration of 50 µg/ml, concentration based on the protein measurements of Bradford assay. The results were completely negative except for a very weak activity of Lunasin standard (3,9 %).

Concerning seeds different from soy, SDS-page results of this study agree with those works that did not find Lunasin in other protein sources such as cereals, pulses and oleaginous seeds. Instead, results obtained with soy samples, suggesting that Lunasin does not exist in its free form, or in very small quantity. The discrepancy of the results could be due to technical differences as protein enrichment processes, detection methods and antibody nature. Moreover, as described in Gonzalez de Mejia works (De Mejia et al., 2004; W. Wang et al., 2008), Lunasin presence and quantity is strongly influenced by cultivars, environmental-growth conditions and post-harvest processes. The peptide-quantity variability is confirmed by Madrid-University results, in which the various

seed cultivars and flours seem to contain different amount of Lunasin. Furthermore, considering the results obtained from western blot, the second band at around 10 kDa could indicate that the Lunasin free form exist only after processes that cause proteolysis. In Rizzello et al (Rizzello et al., 2015) work for example, they found that fermentation of soy flour with lactic-acid bacteria increased Lunasin content of 2 times. In this study, enzymatic hydrolysis of three soy samples were performed, but none of them showed the 5.5 kDa and neither 10 kDa band (data not reported). The enzyme used was Pancreatin and the incubation time was 3 hours, probably too long. It would be interesting repeat the experiment at shorter incubation times and with other kind of enzymes, to obtain a less-intensive hydrolysis.

Considering radical scavenging properties, to confirm an antioxidant activity of Lunasin it would be necessary repeat the DPPH assay at higher concentrations (as for example 100 and 200 µg/ml). Moreover, it would be interesting to test other assays to compare the results (ABTS, cell culture tests).

Summarizing, results obtained in this study don't confirm the presence of Lunasin peptide free form both in soy and in other seeds. Further experiments are necessary to confirm the total absence and the possible presence of a dimeric form in soy and derivatives. Further interesting point is to test the protein extracts for antitumoral and anti-inflammatory bioactivity: it would be possible indeed that the bioactivity is a result of a combination of different proteins. Moreover, it would be interesting to test other soy cultivars from different countries and soy products obtained from concentration/isolation processes.

Second section: plant proteins project

Chapter 1 Introduction

1.1 Food production sustainability

The concept of human food need nowadays to be completely changed. There are two big issues: from one side, the huge difference between developing and developed countries, in which the opposite problems of starving and overweight-related diseases emerged (Mirabella et al., 2014). From the other side, the increase of world population is making unsustainable for environment the consumption of animal meat and derivatives (Day, 2016, Henchion et al., 2017). The answer to both problems is a revolution of food industry, that comprises first of all the utilisations of new protein sources alternatives to meat, and the reduction of the food waste. Indeed, the food waste is generated in all stages of food life cycle: from agriculture phase, up to industrial manufacturing and processing, retail and household (Mirabella et al., 2014). An estimation calculated a total food waste in Europe of about 179 Kg of food per capita in the 2006 (Mirabella et al., 2014), whereas globally FAO calculated that one third of total produced food is wasted (Morone et al., 2019).

Food industrial waste, besides the ethical aspects, is an economical issue, since often the companies must pay to dispose of these materials. The industrial food wastes could still be re-destined to human food or also to biorefinery to produce energy or bio-fuel (Chéreau et al., 2016). Indeed, global sourcing of pure ingredients with a long shelf life are requested, meaning transformations and processes that require a huge amount of water and energy to dry and sterilize these products. This production model generates many undervalued fractions or by-products, often still rich of nutrients and bioactive molecules (Pelgrom, Boom, et al., 2015). It is thus necessary to revise the entire proteins production system, shifting from the concept of “pure ingredients” to the more sustainable “protein enriched fractions”, using energy-efficient processes (Van Der Goot et al., 2016). This aim is also driven by consumers, that are willing to invest more resources for products that contribute to protect environment and human healthy.

In the last years, massive consumption of animal proteins became unsustainable for environment: 66% of agricultural production is intended to livestock and only 15% of vegetable proteins are converted into animal proteins (Day, 2016). Moreover, animal proteins production requires 100 times of water more than the same quantity of vegetable proteins production (Day, 2016, Thavamani et al., 2020). In the next years, we will need 70% more food, intensifying the land degradation, with serious consequences for the environment in terms of biodiversity loss, energy, water, climate change, human health and animal welfare (Aiking, 2011). It is calculated that following the current consumption of meat, in the 2050 the meat demand will be the double (Henchion et al., 2017). In this scenario, it is necessary to find alternative and more sustainable protein sources. Nowadays many researchers and producers are studying and investing in protein ingredients alternatives to traditional meat and animal derivatives, such as the plant proteins, insect proteins, mycoproteins and bacteria proteins. Furthermore, cultured meat is also being developed by different groups. The mentioned protein sources are currently at different stage of development. Insects proteins for example, have similar nutritional value of meat and their production require much less water and energy, by the way the critical point is the consumers acceptance and the lack of regulatory. Mycoprotein and single-cell protein are now produced at pilot scale from start-up, but the costs are still prohibitive and the production in some cases requires high quantity of water. Plant proteins are well known and are produced since years, the issue concerns the protein concentration processes, that are expensive and low sustainable. Cultured meat shows many advantages (same taste, texture and nutritional profile of meat, low environmental impact) but is still at laboratory scale, and the consumer acceptance is not very high (Thavamani et al., 2020).

1.2 Plant proteins

Proteins are complex polymers of more than 20 amino acids linked through peptide bonds. The structural and functional differences in the proteins are the result of the sequence in which the amino acids are linked, their size and type and the size of the peptide chain. Proteins are biomolecules very important for human and animal diet: besides to their nutritional value, they possess a key role in structural formation of food, thanks to their functional properties (Pam Ismail et al., 2020). Plant proteins are widely present in nature and their production is cheaper and more sustainable compared to the meat. Nevertheless, the plant proteins utilisation in human food as meat replacer is still limited, due to the costly process used to extract the proteins, and the low nutritional quality compared to real meat (Sá et al., 2020; Thavamani et al., 2020). Protein quality is described as the ability of the protein to provide essential amino acids in the required amount and pattern (Danihelová & Šturdík, 2012; Hertzler et al., 2020). The issue could be bypassed combining two or more different plant sources into the same meal, as for example cereal and legume (Hertzler et al., 2020). Another drawback, depending on the protein source considered, is the poor digestibility due to the presence of insoluble fibres and antinutritional factors (Danihelová & Šturdík, 2012): in some cases, could be overcomed during toasting, de-hulling, de-oiling, protein extraction and cooking processes. Furthermore, a common issue of plant proteins is the presence of off-flavours, depending on the source considered and the post-harvest processes used. Beisdes to the costs and the sustainability, plant proteins are the focus of many researchers as many sources such as pea, soy etc. present health-promoting proteins and peptides (Hertzler et al., 2020). These bioactivities include the reduction of cardiovascular diseases, anti- inflammation and anti-cancer (Udenigwe & Aluko, 2012). It is likely that the bioactive components of a plant diet are often attributed to whole food sources than isolated protein.

The Osbourne classification divides plant proteins into 4 classes based on their solubility: albumins, which are soluble in water, globulins soluble in salty solution, prolamins soluble in alcoholic solution, and glutelins, that are soluble in alkali or acidic solution (Osborne, 1924; Shewry et al., 1995). The albumins are globular

proteins constituted by two polypeptide chains forming a disulphide interchain linked protein, Albumin are the major source of sulphur in leguminous seeds. Globulin proteins contain relatively low levels of the sulphur-ammino acids (cysteine and methionine). The most globulins represented in legumes are the 11S and 7S, based on their sedimentation cofficent (Chéreau et al., 2016). Prolamins are really wide concerning their molecular weight within and between plant species, and the name reflect the high content in proline and glutamine. The main prolamin proteins are the gliadins and glutenins in wheat, hordeins in barley, kafirins in sorghum and zeins in maize. Glutelins have high molecular weights (from 45.000 to 150.000 Da), and are the major protein fraction of rice. This classification is still used, though recently complex proteins that determine an important overlapping between classes have been discovered (Day, 2016). The most important vegetable proteins sources are cereals and pulses, but in the last decades the industrial food by products gained attention as sustainable protein source for humans (Wadhave et al., 2014).

1.3 Cereals proteins

Cereals comprises plant seeds used worldwide for human and animal nutrition, such as corn, rice and the most known wheat. With the exception of rice, the most storage proteins of cereals are the prolamins.

Wheat (*Triticum durum*) contains 8-15 % of proteins, depending on grain variety and environmental-farming conditions. The main storage proteins (85 %) are the gluten proteins: half of them are monomeric gliadins, that can associate with each other and with glutenins proteins through non-covalent hydrogen bonds and hydrophobic interactions (Guerrieri & Cavaletto, 2018, Day, 2016). Gluten is well-known in food industries, especially bakery, thanks to its capacity to form a visco-elastic network essential to produce leavened products (Guerrieri & Cavaletto, 2018). Wheat is consumed mainly as flour and flakes, but gluten concentrate and isolate produced through chemical and enzymatic processes are commercially available, and are used in bakery and meat analogues (Day, 2016). By the way,

in the last years, gluten free products are growing due to the emerging gluten-sensitivity.

Rice (*Oryza sativa*) is the second largest cereal crop in the world and has a low protein content: between 7 and 9 % (Guerrieri & Cavaletto, 2018). Rice is mainly consumed as grain, but protein isolates exists (Day, 2016). Rice protein isolates has the advantage of the lack of typical off-flavours, but protein extraction is low sustainable due to the very low protein content.

Corn (*Zea mays*) protein content ranged between 9 and 12% and half of its world production is used ad feed. Corn is also used in food industry for many purposes, as oil production, chips, flakes and baby food. Corn and rice are the most cereals used in gluten free-products.

1.4 Pulses proteins

Pulses comprise various species of seeds rich in dietary proteins (18-32 %) at relative low cost (Karaca et al., 2011b). The protein content varies with genotypes, germination, environmental conditions, and application of fertilizers during growth and development (Agarwal, 2017). Pulse proteins comprise mainly globulins and albumins, while prolamin and glutelins represent less than 5 % of the proteins (Shevkani et al., 2019). Globulins represent indeed the 70-80 % of the proteins, and legumins (11S) and vicilins (7S) are the main storage proteins (J. Boye et al., 2010). The albumin: globulin and legumin: vicilin ratios are really different between pulses. Albumins from pulses are generally high in cysteine and methionine whereas legumin proteins generally have higher amounts of the limiting sulphur-containing amino acids (methionine and cysteine) compared to the vicilin fraction (J. Boye et al., 2010).

Soybean (*Glycine max*) is the most important oil-pulse crop worldwide, became a commodity due to the large use of the derived oil and the high protein content (about 35 % in the seed) (Day, 2016). The defatted soy flour is indeed largely used both for feed and food as soy concentrates, isolates, textured and flakes-grits. Soy drink, commonly named soy milk, is used as milk-replacer. Soy proteins

are currently the most used vegetable proteins for plant-based food production, and an emerging market request is to find protein sources alternatives to soy.

Pea (*Pisum sativum*) is another pulse seed largely consumed, due to its easy cultivation all over the world, and its hull that could be easily removed (Day, 2016). The protein content ranges from 23 to about 30 % and the protein classes-ratio is very different between varieties (J. Boye et al., 2010). After soy, pea concentrates and isolates are the second plant proteins commercially available for vegan food.

Another emerging pulse is the chickpea (*Cicer arietinum*), that has a protein content similar to the pea. As for pea, different varieties of chickpea have different ratio of albumins, globulins, prolamins and glutelins (J. Boye et al., 2010). Chickpea is mainly farmed in Mediterranean countries and is consumed as grains or flour after cooking (Sánchez-Vioque et al., 1999). The chickpea proteins are better appreciated compared to the proteins from other pulses due to their high biological value, high bioavailability, well balanced amino acids content and low content in antinutritional factors (Ionescu et al., 2009). The oil content of chickpea (5-7 %) seems to be responsible, together with protein composition, of high functional properties of chickpea protein ingredients (Ionescu et al., 2009; Zhang et al., 2007).

Lentils (*Lens culinaris*) is a legume seed high in fibres and low in fat. The protein content is similar to the other mentioned pulses, ranging from 20 to about 30 % (Jarpa-Parra et al., 2014). Storage proteins of lentils consist of about 80% of total seed proteins and are mainly composed of globulins (Joshi et al., 2011). The cotyledons can be yellow, red, or green and are consumed as cooked grains, split and dehulled (Corradini & Julian McClements, 2019). Lentils are currently limited consumed as food grains, and as for chickpea, the is lack of protein-concentrates/isolates in the market (Joshi et al., 2011).

Further pulse still underexploited is faba bean (*Vicia faba*). Faba contains on average 29 % of proteins and 39 % of starch (Jiang et al., 2016; Vioque et al., 2012). As for the other legumes, faba proteins are formed of two main fractions, albumins and globulins. The first are proportionally rich in sulphur amino acids

and lysine, whereas globulins consist of convicilin, vicilin, and legumin (Vioque et al., 2012). The functionality of faba bean protein for food uses, especially as protein isolate, has been studied at the laboratory scale, and has shown good solubility, emulsifying, foaming and gelling properties (Cepeda et al., 1998; Jiang et al., 2016).

1.5 Oleaginous seeds proteins

The protein fraction obtained after oil extraction of plant seeds is currently undervalued, used in small portion as feed. The valorisation of these flours or meals, depending on the de-oiling technique used, is now the challenge in a circular economy perspective (Pilorgé & Muel, 2016; Rodrigues et al., 2012). Oil extraction processes indeed could cause protein denaturation, due to the high temperatures or the chemical solvents used. After soybean, the most important oil seed crops are canola and sunflower.

Canola (*Brassica napus*) is an oleaginous seed containing between 40 and 45 % of oil and about 30- 40 % of protein after oil extraction (Janitha P.D. Wanasundara, 2016; Khattab & Arntfield, 2009; Manamperi et al., 2007; Tan et al., 2011). The proteins napin and cruciferin constitute respectively the 20 and 60 % of the canola proteins seed. Napin is a 2S albumin of low molecular weight (12.5-14.5 kDa) with good foaming properties, and cruciferin is a 12S globulin with high molecular weight (300-310 kDa) and several subunits and high gelling capacity (Manamperi et al., 2007). Canola proteins possess a well-balanced amino acid composition, but the meal is mainly used for animal nutrition due to the presence of antinutritional factors. Indeed, canola meal contains glucosinolates, erucic acid, phytates and phenolics (Khattab & Arntfield, 2009; Manamperi et al., 2007). However, many studies focused on the processes that could reduce these antinutritional factors, such as heating, solvent and enzymatic treatments (Tan et al., 2011).

Sunflower seeds (*Helianthus annuus*), as canola seeds, contains about 40% of oil and 20 % of proteins (Kachrimanidou et al., 2015; Salgado et al., 2011; Shchekoldina & Aider, 2014; Vidosavljević et al., 2019). The protein fraction is

highly digestible and source of essential amino acids, even if the oil extraction could cause protein denaturation (Kachrimanidou et al., 2015). Sunflower meal is currently used in low quantities in animal nutrition, due to the high insoluble fibres content, about 18-23 %, and the presence of antinutritional factors (Vidosavljević et al., 2019). Among the antinutritional factors of sunflower meal, the majority is represented by chlorogenic acid with small amounts of caffeic acid. These compounds contribute also to reduced protein solubility and unwanted organoleptic characteristics (Salgado et al., 2011). Many processes have been proposed to reduce the antinutritional factors, even if they possess important antioxidant properties that could have a positive impact in human health (Salgado et al., 2011; Shchekoldina & Aider, 2014).

Camelina (*Camelina sativa*) is an emerging oil-seed crop (Cheng et al., 2015; Gesch & Archer, 2013; Zanetti et al., 2017). Despite being somewhat new, camelina does have a long history of cultivation in northern Europe and a considerable amount of selection and development for agricultural use has been done (Gesch & Archer, 2013). In addition to its relatively high seed oil content, camelina requires lower agricultural production inputs than most commodity crops (Gesch & Archer, 2013; Zanetti et al., 2017). Camelina has an interesting oil composition with a high content of α-linolenic acid (20 to>35%), eicosanoic acid (11‑19%) and tocopherols, and has low content of the undesirable fatty acid erucic acid (< 4%) (Zanetti et al., 2017). These characteristics make the camelina oil well-suited for a variety of food, feed or non-food applications. Camelina meal contains a good amount of proteins (35-40 %) and is currently used in feed trials (Cheng et al., 2015).

1.6 Protein enrichment processes

Plant protein ingredients such as protein flours (30-50 % proteins), protein concentrates (50-85 % proteins) and protein isolates (85-98 % proteins) are used to produce plant-based food in combination with other ingredients. Generally, high protein content is more requested by food producers, even if higher protein content ingredients have some drawbacks. First of all, the price is higher, since

higher protein purity means lower yield. In addition, pure ingredients require costly processes, decreasing the sustainability. Furthermore, protein concentrates despite to protein isolates, contain other molecules than could improve the food structuring or have healthy bioactivities.

Currently most of plant proteins at industrial level are obtained by wet fractionation, that has the advantage to produce protein isolates or concentrates (thus high purity), but consumes a large amount of water and energy to dry the obtained product (J. Boye et al., 2010; Chéreau et al., 2016; Hadnadjev et al., 2017; Pojić et al., 2018). In addition, two wet co-products are generated during the process, generating wastes to manage. Furthermore, the proteins are often denatured by acids and bases used during process to shift the pH, causing loss of native functionality. In figure 7, the main steps included in the traditional wet extraction, called alkaline extraction and isoelectric precipitation. An alkaline solution (pH about 8-8.5) is added to the plant material in order to solubilize the proteins. A first gently centrifugation is used to remove the insoluble material, then the supernatants is acidified until the isoelectric point of the proteins (pH 4-4.5) to induce the precipitation. The protein pellet is recovered after high-speed centrifugation, and supernatant is discarded (J. Boye et al., 2010; Chéreau et al., 2016; Hadnadjev et al., 2017).

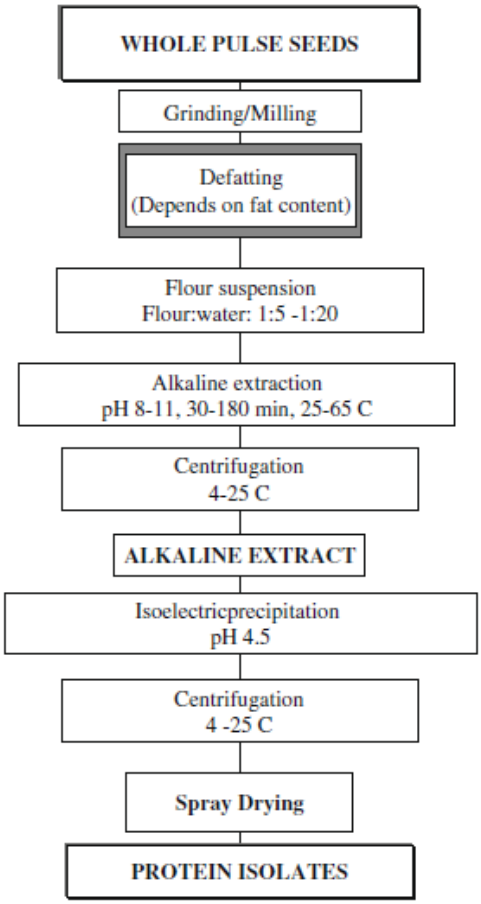


Figure 7. Steps of traditional wet protein extraction, that produces protein isolates and concentrates (J. Boye et al., 2010)

Other wet processes are described in literature, such as micellization, ultrasounds/microwave assisted extraction, subcritical water extraction, enzymatic extraction, but they are still at early stage (Hadnadjev et al., 2017; Pojić et al., 2018; Sari et al., 2013). Indeed, the issues of the mentioned technologies concern the low yields, the large amount of water, the costs of the materials or the damage of the proteins during the extraction.

A sustainable alternative to produce plant proteins is dry fractionation (Chéreau et al., 2016; Mayer Laigle & Barakat, 2017; Pojić et al., 2018)Ionescu et al., 2009; Van Der Goot et al., 2016). The evident advantages of these processes are: no utilisation of water, stabilization and dry post-process are not required and the easily scalability (Schutyser & van der Goot, 2011). The drawback of dry separation is a lower yield and purity compared to wet fractionation. However,

this is compensated by the maintenance of proteins functionality. Moreover, the fraction obtained contains other components (such as oil, fibre, bioactive molecules, etc.) that could improve the rheological characteristics of the final products, and positively impact in human health (Van Der Goot et al., 2016). Dry fractionation consists of dry milling and elusive or electrostatic separation. Elusive separation is based on particle sizes and density differences (air classification), while electrostatic separation is based on differences in triboelectric charging of components. Concerning air classification, improvement in purity and yield could be obtained with a degree of milling in which the non-protein components, like starch and fibre, should remain larger than the protein bodies (Pelgrom, Boom, et al., 2015). Other treatments could increase purity and yield of air classification: for example, before milling, variation of moisture or hulls/oil removal are effective depending on plant seed considered (Pelgrom, Boom, et al., 2015; Pelgrom, Wang, et al., 2015; Xing et al., 2018) . Another treatment that increases yield is using electrostatic separation after air classification (Pelgrom, Wang, et al., 2015). The advantage of electrostatic separation is that the particle generated during milling could be similar, they are separated based on their charge (proteins are usually positive charged and fibre and carbohydrates are negative charged) (Mayer Laigle & Barakat, 2017; Pelgrom, Wang, et al., 2015; J. Wang et al., 2016; Xing et al., 2018).

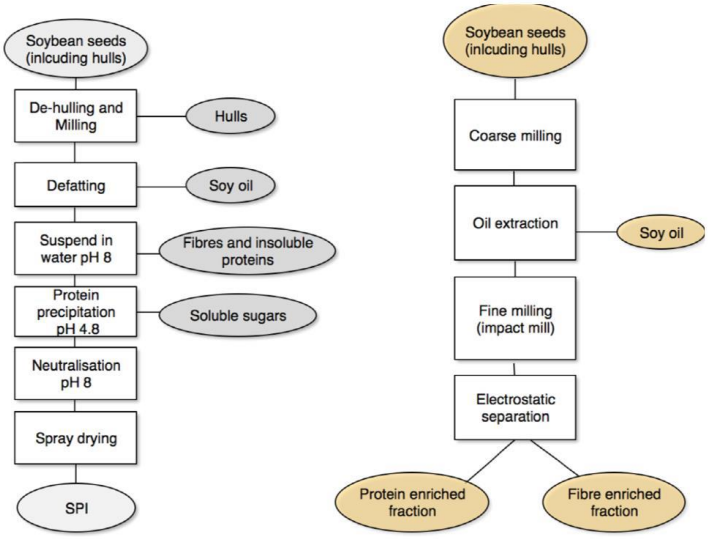


Figure 8. Comparison of traditional wet protein extraction (left) and dry electrostatic separation (right), (Protein for Life Symposium, Wageningen University and Research)

1.7 Functional properties and food application

A way to reduce animal proteins consumption is the availability of plant products that can replicate meat in terms of mouthfeel, texture, taste, colour and smell (Dekkers et al., 2018; Pam Ismail et al., 2020). Currently, many companies are producing this type of meat-analogues, and the main process used to obtain them is the extrusion of protein fractions, or the 3D printing. Extrusion is a thermomechanical process that uses a combination of pressure, heat, and mechanical shear. There are two types of extrusion processes, the low-moisture extrusion in which 20–40% of water is added, and high-moisture extrusion in which the water added could reach the 80 %. Low-moisture textured proteins must typically be rehydrated prior to use, often in combination with other ingredients, whereas high-moisture extruded products may not require any further processing prior to use (Pam Ismail et al., 2020). 3D printing used to produce plant-based meat is now being developed by two start-ups. Structured products very similar to fibrous meat could be achieved with application of intensive-shear and heat in Shear-cell or Couette-cell devices (Krintiras et al., 2015,Krintiras et al., 2016). The application of simple-shear and heating produce an anisotropic-fibrous product: for this purpose, a “protein enriched fraction” obtained by dry separation, compared to a pure protein ingredient, is even better than the use of protein isolates (Grabowska et al., 2014; Krintiras et al., 2015, 2016). Shear-cell technology was developed at laboratory and pilot scale by the food engineering group at Wageningen University and Research (figure 9).

Figure 9. Fibrous textured soy-gluten product obtained using couette-cell device (Krintiras et al., 2016)

Besides to meat analogues, plant proteins are used to produce other kind of food, as for example the plant-based milk and the vegan egg. Plant-food that are similar in taste and structure to the related animal-derivatives, allows to produce other kind of vegan meal, as bakery products, baby foods, cheeses and pastry products.

The product obtained using the mentioned structuring processes depends on the protein ingredients used. Besides to the source and its protein content, the important characteristics that influence the texturization are the functional properties of proteins. Functional properties are defined as the physical and chemical properties which affect the behaviour of proteins in food systems during processing, storage, preparation and consumption, and thus define their ultimate application in various food systems (J. Boye et al., 2010; Shevkani et al., 2019). Those functional properties are influenced by the molecular size of proteins/peptides, structure (primary amino acid sequence, secondary and tertiary arrangement), charge distribution and the presence of other molecules as carbohydrates and oil (Bessada et al., 2019). Moreover, the conditions of processes (temperature, pH, etc) could change the protein functionalities (J. Boye et al., 2010). It is important to investigate the functional properties of protein ingredients since the protein extraction processes modify these characteristics.

Protein solubility is a desired parameter for food application and also a pre-requisite for other related functional properties such as foaming, emulsification, and gelation. The solubility depends mainly on the polarity of molecules and their arrangement, being directly affected by temperature, pH, type of solvent, and ionic strength (Bessada et al., 2019). Usually, solubility is lower at pH values close to the isoelectric point because the net charge of the protein is zero, which reduces the intermolecular electrostatic repulsion and ionic hydration, leading to precipitation.

Water holding capacity (WHC) and oil holding capacity (OHC) corresponds to the maximum water/oil volume that can be absorbed by 1 g of protein material (Bessada et al., 2019; Shevkani et al., 2019). WHC is particularly a critical property of proteins in viscous foods, such as, soup, dough, custard and baked foods, which are supposed to imbibe water without dissolution of the proteins

(Shevkani et al., 2019). OHC is an important parameter because it is associated with the sensory quality of food, as mouthfeel and flavour retention and thus the palatability (Bessada et al., 2019). WHC has been associated to polar AA in protein-water interaction sites, while OHC is mainly determined by nonpolar AA at the protein-oil interaction sites. The WAC/FAC ratio is determinant to the development of foods with high acceptability (Bessada et al., 2019).

The emulsifying activity indicates the oil quantity that a protein can emulsify per unit of water, while emulsion stability evaluates the maintenance of the emulsion over a period of time(Shevkani et al., 2019). The emulsifying properties of proteins represent their most important functional characteristic as multi-purpose ingredients for different food applications. Emulsifiers are compounds with the ability to form interface films (emulsions) between immiscible liquids, and proteins could be generally good emulsifiers due to their amphiphilic nature(Bessada et al., 2019).

Foaming capacity is an indicator of the increase in volume after whipping and it depends on the ability of proteins to diffuse to the interface, reorient, and form a viscous film without excessive aggregation. Foam stability reflects the ability of proteins to maintain the foam in a certain time (J. Boye et al., 2010). Foaming properties determine the applications of proteins in food products where aeration and overrun is essentially required, as for example chiffon cakes, fudges, confectionery products, whipped toppings, souffle´s and mousses, ice-cream mixes. Globulins have low ability to unfold/reorient at the interface, which limits encapsulation of air bubbles and thus have low foaming capacity. Albumins, in contrast, have high solubility and high ability to foam due to enhanced protein unfolding (Shevkani et al., 2019).

Gelation is an important functional property of proteins in viscous foods such as puddings, soups, gels, curds, meat analogues. The protein gels can be formed by protein denaturation caused by heating, pressure and ions (Shevkani et al., 2019). Generally, higher protein concentrations and high globulins fraction increase the gelation capacity, as heating process of globulins may lead to their dissociation and re-association in different shapes, forming gels (Bessada et al., 2019).

Chapter 2 Aim of the study

Food industries that use protein ingredients in their products are looking for protein sources alternatives to soy. In general, the proteins must have high functional properties, high availability and low price. Consequently, the interest of protein ingredients producers is the availability of new and costly protein sources. The key point for producers is the protein enrichment process: the technique used in fact, highly influence the yields in terms of mass, protein concentration, and functional properties of the ingredient. Besides to the yields and the final characteristics of the protein products, the enrichment processes are really different based on the equipment and the amount of energy/water required, in few words they vary on the investment necessary to produce these ingredients. To date, the most used industrial process to produce protein isolate is the alkaline extraction followed by a precipitation at isoelectric point (following abbreviate in alkaline extraction). This method has the advantage to produce protein isolates (very high in protein purity), but it presents some drawbacks. First of all, the technique is really expensive as requires huge quantities of water to solubilize proteins, and energy to centrifuge at high speed and to dry the obtained product. Moreover, most proteins lost their properties due to the strong pH shifting. Another important point is that the process could not be used for organic production, since the use of some of chemicals is not allowed. In this scenario, many researchers and companies are testing alternative protein sources and alternative processes both wet and dry to improve the protein enrichment and the functionalities of the final products.

With all this in mind, the general aim of the project was to compare the wet alkaline process extraction with other processes wet and dry of various protein seeds. The comparison, based on both literature/company data and on experimental data, is referred to the yields of the processes and the functional characteristics of the obtained proteins.

More in detail, the goals of this study were:

1) Comparison of two wet processes (alkaline and PBS methods, described in materials and methods) used to produce protein extracts on lab scale. The protein sources used are sunflower, canola, camelina, chickpea and lentils. In the case of oil seeds, de-oiled materials were used. For the sunflower and canola, flours and cakes were both tested, to investigate the influence of oil content on the protein extraction process. The meals in fact derived from mechanical oil extraction and have an oil content between 7 and 10%, whereas flours derived from chemical de-oiling process and the oil content is very low (< 2 %). The mass and protein yields were therefore calculated for every protein source and for the two processes performed. Camelina meal is a relatively new protein source, and data about its protein extraction are still not present in literature.
2) Test of the functional properties as water/oil absorption capacity, gel formation and foaming activity of the different protein extracts. The first comparison was between the starting material and the relative protein extract, in order to understand if the extraction process could improve or worsen the protein functionalities. The second comparison was between the extracts derived from the two processes, to define the functional characteristics derived from the two techniques. Functionalities of protein concentrates/isolates derived from pilot or industrial processes were also tested to make the third comparison with those produced in this thesis. The samples were sunflower isolate, chickpea concentrate, soy isolate, faba concentrate and isolate.
3) Alkaline extraction was performed to compare 5 different cultivars of chickpea farmed in USA, samples also donated during a collaboration with a biotech company. The goal was to identify the cultivar(s) with the highest yields and functionalities.

Further goal of the project was to preliminary test the dry separation processes and the shear-cell technology (Chapter 5), using the equipment of Wageningen University Research (NL). Both electrostatic separation and air classification were performed to compare the two technique and understand their strengths

and limitations. The protein extracts obtained from the dry enrichment were tested for fibres formation meat-like using the shear-cell technology.

In this thesis, camelina meal is used to produce protein-rich extracts to compare with the other protein concentrates and isolates, however it could not be still commercialized since it is necessary to produce all the documents about its safety, as it is considered “novel food”.

Chapter 3 Materials and methods

3.1 Samples

Different plant protein sources interesting for industrial application were compared in terms of yields of protein enrichment and functional properties of their extracts. The co-product (supernatant) of the alkaline extraction was also included to compare functionalities. In the table 4, the list of the samples used and their chemical composition: 3 oil seeds (sunflower, canola and camelina) and 2 pulses (chickpea and lentils). Concerning oil seeds, both flours and cakes were used for canola and sunflower, whereas for camelina only cake was available for the study. The flours were obtained from industrial chemical de-oiling with hexane in the plant of CD company (VI), the cakes derived from industrial mechanical oil extraction in the plant of CD company using organic seeds. The organic chickpea was kindly donated by Montanaro S.r.l. company (PZ) and the organic lentils micronized flour by CerealVeneta S.r.l. (PD). 5 different cultivars of chickpea farmed in the USA were also used in the study, donate from a company during a collaboration. The names of the 5 cultivars are covered by secret agreement and cannot be disclosed in this thesis. In order to enhance the protein enrichment during the process and to standardize the particle size of the starting material, all the samples (except for lentils that was just milled) have been grinded at the same size (0.5 mm) using a rotor mill (ZM 200, Retsch).

Table 4. Samples used in this thesis and their chemical composition

CODE	DESCRIPTION	HUMIDITY %	OIL CONTENT %	PROTEIN CONTENT %	TOTAL FIBERS %
30	sunflower flour (chemical de-oiling)	9.41	< 2	24.84	29.3
36	organic sunflower cake (mechianical de-oiling)	4.3	12.34	25.53	28.71
32	canola flour (chemical de-oiling)	14.49	< 2	32.98	10
37	organic canola cake (mechianical de-oiling)	8.82	7.29	30.99	11.26
28	camelica cake (mechanical de-oiling)	9.3	10.27	33.41	10.06
29	dehulled red lentils flour	10.04	< 2	22.76	7.4
38	organic chichpea seeds	9.46	4.56	22.12	3.6
44	chickpea seeds from USA	9.97	2.94	20.97	7.24
45	chickpea seeds from USA	10.08	4.05	21.94	2.99
46	chickpea seeds from USA	9.74	2.9	23.72	4.83
47	chickpea seeds from USA	9.84	3.75	21.99	3.29
48	chickpea seeds from USA	9.79	2.83	21.68	3.7

Samples of protein concentrates and isolates derived from pilot or industrial plants were also included in this thesis to compare their functionalities. In table 5 are listed the mentioned samples and their composition in terms of humidity, protein and lipid contents. Sunflower isolate was donated from a company during a collaboration, and derived from a process similar to the alkaline extraction described in this thesis (3.4). Chickpea concentrate were also obtained within a collaboration with an Israeli start-up, and derived from a wet process not better explicated. Soy and faba isolates were purchased from Fenchem Biochemie GmbH and are obtained from wet separation, also in this case the nd of process

was not explicated. Faba concentrate was kindly donated by Vestkorn Milling AS, and the process in this case is dry, but the type of dry method is unknown.

Table 5. List of commercial samples and their chemical characteristics used to compare functionalities with the extracts obtained in this thesis

CODE	DESCRIPTION	HUMIDITY %	OIL CONTENT %	PROTEIN CONTENT %
33	sunflower isolate 80%	5.47	< 2	78.83
54	chickpea concentrate 70%	3.5	10.3	69.9
66	soy isolate 90%	4.9	< 2	91.5
68	faba isolate 90%	6.78	< 2	90.2
57	faba concentrate 65%	5.4	< 2	65

*All the samples are coded to enhance the reading of the thesis.

3.2 Moisture content

Water content of the flours was measured weighing about 10 g of the material and letting in the heater at 116°C for about 8 hours. The samples were then placed in a lab dryer and let cooling down. The samples were weighted again and when the weigh was constant, total moisture was calculated as following:

$$Moisture\ \% = \frac{(g\ of\ starting\ material - g\ after\ drying)}{g\ of\ starting\ material} * 100$$

3.3 Solubility

The protein solubility in aqueous solutions is dependent on the balance of hydrophilic and hydrophobic residues in the structure, surface charge, pH of the system as well as type and concentration of salts. Protein solubility is an important property of food proteins because of its association with emulsifying, foaming and gelation properties. Besides, the knowledge of protein solubility of a starting material (seeds, flours) is important to optimize the protein extraction

using precipitation at isoelectric point process. Indeed, knowing the solubility in relation to the pH of the solution, allows to adjust the pH at solubilization and precipitation phases.

In this study, protein solubility in relation to 5 different pH was investigated in starting material (before protein extraction) and in two protein extracts. Solubility of 5 chickpea cultivars and camelina cake was performed first of all to know the correct pH to use in the subsequent isoelectric point extraction (protocol below 3.4). Concerning the 5 chickpea cultivars, the further aim was to compare them each other.

Solubility protocol was taken from various papers with minor modifications (Jarpa-Parra et al., 2014; Khattab & Arntfield, 2009; Samanta & Laskar, 2010; Suliman et al., 2006). The samples to test were prepared in 50 ml tubes at the concentration of 8% w/v adding deionized water. Each sample was prepared 5 times as the pH to test. The tubes were mixed with vortex, and the pH was adjusted to 2, 4, 7, 8 and 10 using NaOH and HCl. Once reached the correct pH, the tubes were stirred at room temperature for 30 minutes using a shaker (Rotamax 120, Heidolph). After the incubation, pH was measured and fixed if necessary, and the tubes were centrifuged at 4.000 g for 30 minutes (Centrifuge 5804 R, Eppendorf). Kjeldahl analysy (protocol below 3.6) to calculate the total nitrogen was then conducted into 10 ml of the supernatant obtained (Figure 10) and in 0.8 g of the starting material. The solubility % was calculated for every samples at each working pH as following:

$$Solubility\ \% = \frac{total\ N\ in\ the\ supernatant}{total\ N\ in\ the\ tested\ material} * 100$$

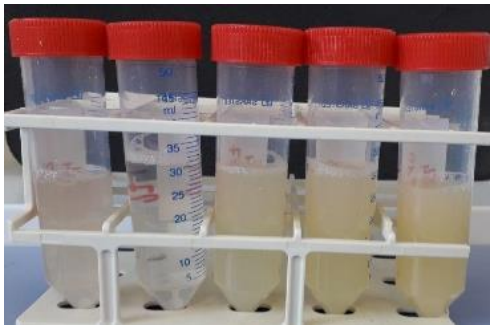


Figure 10. Protein solubility of grinded chickpea. At pH 4, in which the protein solubility is lower, the supernatant is transparent (second falcon from left)

3.4 Protein enrichment-PBS method

A very simple and cost-effective method using food grade solvent (PBS, Phosphate Saline Buffer, pH 7.8) was tested to obtain plant extracts enriched in proteins(Hameed et al., 2009; Ranjan & Matcha, 2012).

35 ml* of PBS 50 mM were added to 7 g of grinded sample, mixed with a vortex and then with a glass rod to break the tissue. The tubes were heated at 37°C and shaken at 1.000 rpm for 60 minutes using thermomixer (the tubes were mixed with vortex every 10 minutes). The supernatant collected after centrifugation at 16.000 g and 4°C for 10 minutes was refrigerated at -20°C (Figure 11) and stored until the subsequent tests.

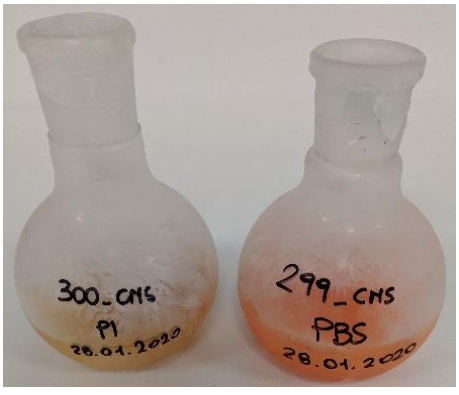


Figure 11. Frozen PBS extracts just before to be freeze-dried

*For same samples (for example camelina cake) it has been necessary to modify the sample/solvent ratio, based on water holding capacity of the material.

3.5 Protein enrichment-alkaline extraction

At industrial scale, the most used technique to concentrate/isolate proteins is the alkaline extraction/isoelectric precipitation. The procedure comprises a protein solubilization in alkaline conditions followed by a centrifugation to remove the insoluble material. The proteins in the supernatants are precipitated shifting the pH in acidic conditions, near to the protein isoelectric point. At the protein isoelectric point, there are no repulsion between the charged molecules and the protein–protein interaction favoured aggregation(J. Boye et al., 2010; Pojić et al., 2018). The protein pellet recovered after a second centrifugation is then freeze-dried. There is a wide range of protocols based on starting protein sources, mainly due to different pH solubilization and differences in the isoelectric point. Generally, highly alkaline conditions led to a better solubilization, but also a high denaturation of the proteins. Also timing, speed centrifugation and working temperature affect the extraction yield and purity of the extracts (Hadnadjev et al., 2017; Pojić et al., 2018).

The protocol used in this study is taken from literature (J. I. Boye et al., 2010; Karaca et al., 2011a; Salgado et al., 2011; Tan et al., 2011; Vioque et al., 2012; Zhang et al., 2009). A mix of 1:5 flour/deionized water* was shaken and the pH adjusted to 8.5 using NaOH 1M. The tubes were incubated at 40°C for 30 minutes, shaking at 1.000 rpm with thermomixer (Thermomixer C, Eppendorf) to solubilize the proteins. After incubation, the tubes were centrifuged at 4.500 g for 15 minutes at the temperature of 4°C. The pH of the supernatants collected was shifted at 4.5 using HCl 1M and the tubes were left at room temperature for 20 minutes. After the incubation, tubes were centrifuged at 20.000 g for 15 minutes, at the same temperature of the first centrifuge to promote proteins precipitation (Figure 12). The supernatants were removed and placed in others tubes, and then both supernatants and pellets were refrigerated at -20°C.

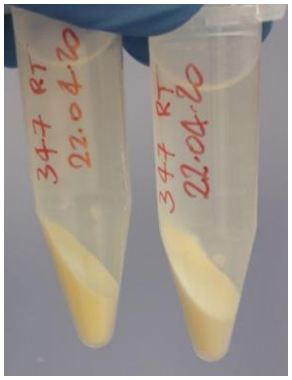


Figure 12. Protein pellet and supernatant formed after the high-speed centrifugation

*As in the PBS method, for some samples the flour/water ratio has been adjusted based on their water holding capacity.

3.6 Freeze drying

All the protein extracts and their co-products, after refrigeration of at least 24 hours at -20°C, were freeze dried for about 5 days (Lyovapor 200, Buchi). The lyophilisation started at pressure of 1 mbar for about 30 minutes, then the pressure was reduced regularly until 0,2 mbar pressure was reached.

Figure 13. Protein extracts during freeze-drying

3.7 Total nitrogen quantification and yields calculation

The total nitrogen content of the starting materials, extracts and co-products was calculated with Kjeldahl method, universally recognized for raw proteins quantification in organic samples. The Kjeldahl method is divided into three main steps: the digestion, distillation, and titration. During digestion phase, the sample is heated in the presence of sulphuric acid, that breaks down the organic

substance via oxidation, and nitrogen is liberated in the form of ammonium sulphate. During distillation, sodium hydroxide is added to convert the ammonium salt to ammonia, that is then titrated with acids to calculate the nitrogen content.

20 ml of 98% H_2SO_4 (sulfuric acid) were added to 0,8 g of sample to test (10 ml in case of liquid samples) and heated at 400°C using a digestor (K-439, Buchi). Potassium sulphate and copper sulphate tab was added as catalyst to each tube, the reaction generally lasted 3 hours. After cooling down the tubes under the fume hood, 100ml of distilled water were added to each sample, and ammonia was separated adding 50 ml of 32% NaOH during steam-distillation (distiller Vapodest, Gerhardt). 50 ml of 2% boric acid was used as receiving solution, forming ammonium-borate complex. Ammonia was then quantified through direct titration using H_2SO_4 10N and the ml of acid needed to shift the colour from green to red were used to calculate the % of raw proteins as following:

$$\%\ total\ N = \frac{(ml\ of\ \text{sulfuric acid used during titration} * 0.14) * 6.25}{g\ or\ ml\ of\ the\ sample}$$

Where:

0.14 is the amount of nitrogen in mg bound by 1 ml of 0,01 N sulfuric acid

6.25 is the conversion factor for food proteins

To verify the efficacy of the extraction processes and their applicability at industrial-scale, mass and protein yields were calculated for each extract. Mass yield represents the quantity of extract resulting after the protein enrichment compared to the quantity of the starting material used in the extraction. The protein yield represents the quantity of proteins in the extract compared to the grams of the proteins contained in the starting material used in the extraction.

The yields were calculated as following:

$$Mass\ yield\ \% = \frac{(g\ of\ extract * 100)}{g\ of\ starting\ material}$$

Where:

g of extracts are the grams obtained after lyophilisation

g of starting material are the grams of the flour used for protein extraction process (adjusted on dry matter)

$$Protein\ yield\ \% = \frac{(g\ of\ proteins\ in\ the\ extract * 100)}{g\ of\ proteins\ in\ starting\ material}$$

Where:

g of proteins in the extract are the grams of total N obtained after lyophilisation

g of proteins of starting material are the grams of the of total N contained in the flour used for the extraction process (adjusted on dry matter).

3.8 Water and oil holding capacity

Water holding capacity may be defined as the ability of tested material to physically absorb water against gravity. WHC of pulse protein products is mainly due to the hydrophilic parts like polar and charged side chains of proteins as well as of carbohydrates which have an affinity for water molecules. FHC/OHC as the WHC, may be defined as the amount of fat/oil that can be absorbed by tested material. In general, proteins interact with lipids through the binding of non-polar side chains of amino acids with aliphatic chains of oils/fats. Water and fat/oil absorption of proteins are related with texture, mouthfeel and flavour retention of food. Water holding capacity (WHC) is a critical property of proteins in viscous foods which are supposed to imbibe water without dissolution of the proteins, whereas high fat/oil holding capacity (FHC/OHC) of protein is required in meat replacers in which fat/oil contribute to the texture of finished foods. In general, pulse protein isolates show higher water and fat absorption capacities as compared to the corresponding flours (Shevkani et al., 2019).

The protocol used in this study is taken from literature (J. I. Boye et al., 2010; Samanta & Laskar, 2010; Suliman et al., 2006). WHC of starting materials and extracts was tested adding about 1 g of material in a pre-weighed 15 ml tube. 10 ml of pH 7 water were added in each tube and shaken using vortex for about 2 minutes to ensure that all the material was wet. After 30 minutes at room temperature to rehydrate the material, the tubes were centrifuged at 3.000 g for 20 minutes. The supernatant was discarded and tubes containing the sediment were weighed, and WHC was calculated as following:

$$WHC\ ml/g = \frac{w_2 - w_1}{w_0}$$

Where:

- w_0 is the material weight (g)
- w_1 is the material weight + test tube weight (g)
- w_2 is the sediment weight + test tube weight (g)

Since the capacity of water absorption is strongly influenced by pH, the test could be repeated adding water at target pH.

To determine OHC, 1 g of tested material was placed in a pre-weighed 15 ml tube and 10 ml of refined sunflower oil was added. After shaking for 2 minutes to ensure that the material was in contact with oil, the tubes were left at room temperature for 30 minutes to allow the material to absorb the oil. After centrifugation at 3.000 g for 20 minutes, the oil supernatant was collected in a 10 ml cylinder and OHC was calculated with the formula:

$$OHC\ ml/g = \frac{v_1 - v_2}{w_0}$$

Where:

- w_0 is the material weight (g)
- v_1 is the volume of added oil (ml)
- v_2 is the volume of supernatant collected (ml)

3.9 Gelling capacity

Gelation is an important functional property in viscous foods such as puddings, soups, gels, curds, heated-minced meats, etc. Food proteins, particularly globular proteins, are able to form gels when they are denatured by heat in aqueous solutions (Day, 2016). The protein gels can be formed by application heat, pressure and ions, though heat-induced gelation is most commonly involved. However, excessive heating of proteins at high temperatures (>100°C) may cause scission of peptide bonds which may prevent gelation (Shevkani et al., 2019). Gel formation temperature and the resulting gel properties are determined by the protein molecular structure, protein-protein and protein-solvent interactions. The gelation behaviours of the two major legume proteins, i.e. 11S glycinin (legumin) and 7S conglutin (vicilin), are quite different and result in different gel properties such as gel fracture behaviour, firmness and/or elasticity. pH and salt can influence the protein's gelation behaviour greatly as they affect the net charge of the proteins, interactions between protein molecules, stability of protein structure and dissociation of subunit polypeptides. The LGC test allows to determine the critical concentration of testing material below which no firm gel can be formed. LGC is therefore used as preliminary screening about gelling properties of a material, and can be performed at different pH conditions, to determine the different behaviour of the tested material.

The protocol used in this study is taken from literature (J. I. Boye et al., 2010; Raikos et al., 2014). Gelation activity was firstly tested at the concentrations of 15 and 20 % w/v for every material and related extracts, using pH 7 water. The tubes prepared at the correct concentrations were shaken and left at room temperature for 10 minutes. The tubes were then placed for 60 minutes in the thermomixer pre-heated at 100°C. After 1 hour, the samples were immediately cooling down in an ice-box, and placed in the fridge at 4°C overnight. To determine the gel formation, the tubes were inverted and those gels that did not flip down were considered completely gelled. To facilitate the results collection, to every concentration tested a score from 1 to 5 was assigned, were 5 indicate a completely gel formation (Figure 14) and 1 when no gel was formed. In case of

a gel formation at 15%, the experiment was repeated at lower concentrations (12.5, 10, 7.5, 5 %) to determine the LGC.

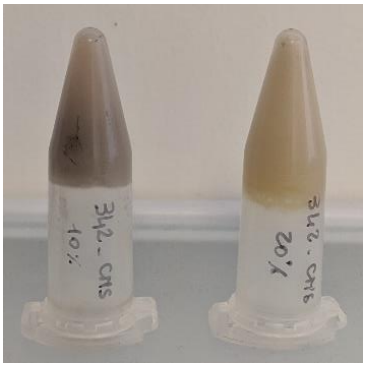


Figure 14. LGC test, completely gel formation (score 5)

3.10 Foaming capacity

Foaming properties determine the applications of proteins in food products where aeration and overrun is essentially required, such as chiffon cakes, fudges, confectionery products, whipped toppings, souffle´s and mousses, ice-cream mixes, etc. The ability of food proteins to stabilise foams is related to the propensity of proteins to be adsorbed onto air/water interfaces and their ability to reduce surface tension and form strong interfacial membranes via protein e protein interactions at air/water interfaces. Native plant proteins, because of their compact structure, have limited foaming properties. Plant protein fractions that are rich in albumins have shown good foaming properties that are equivalent to that of egg white (Day, 2016). Globulins show lower foaming properties, attributed to their reduced ability to unfold/reorient at the interface, which limits encapsulation of air bubbles (Shevkani et al., 2019). The soluble pulse proteins can migrate to the interface quickly to accommodate change in conformation for formation of stable foam. Foaming properties of pulse proteins are measured as foaming capacity (FC) and foam stability (FS). FC is an indicator of the increase in volume after whipping. FS reflects the ability of proteins to maintain the foam after a certain period.

The protocol to test the foaming capacity and stability of materials and extracts is taken from literature (Raikos et al., 2014; Samanta & Laskar, 2010; Suliman et

al., 2006; Tounkara et al., 2013). As for the other functionalities, the test could be performed at different pH conditions, in this case FC and FS is tested at neutrality.

All the flours and their protein concentrates/isolates were tested at concentrations of 1 and 2 % w/v, adding the correct amount of pH 7 water. After 10 minutes of rehydration in constantly agitation, the solution was placed in a graduate cylinder and whipped using a milk frother (Fenvella, Amazon) at 13.000 rpm for 70 seconds. The volume of the formed foam was recorded and the foam capacity was calculated as following:

$$FC\ \% = \frac{foam\ volume}{volume\ before\ whipping} * 100$$

The foam stability was determined after 1 and 2 hours, the volume of the foam was recorded and used the formula:

$$FS\ \% = \frac{foam\ volume\ after\ 1\ or\ 2\ hours}{initial\ foam\ volume} * 100$$

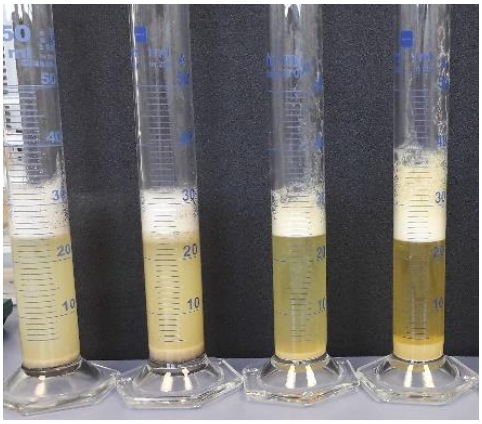


Figure 15. Foaming capacity of chickpea and canola

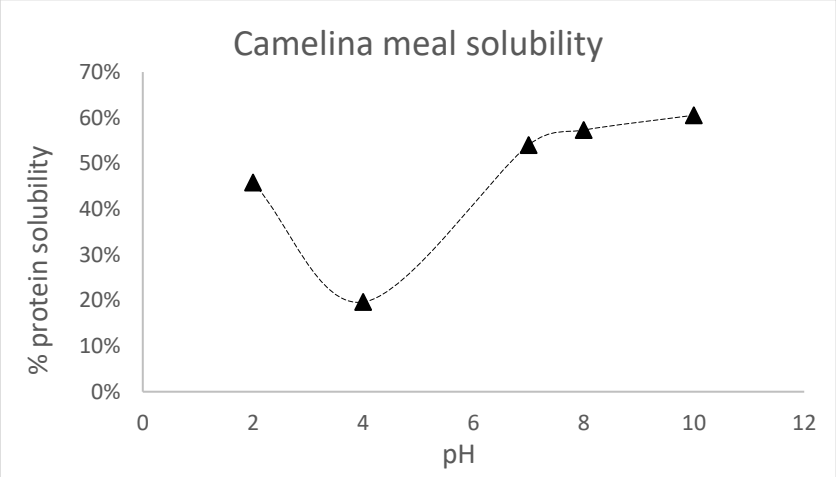
3.11 Statistic analysis

All statistical analyses were conducted using Statistica 7.1 software (2005, StatSoft, Tulsa, OK, USA). Differences between mean values were compared by least significant difference (LSD) in a one-way, two-way or three-way analysis of variance (ANOVA), according to the different experimental scheme used in the different trials.

Chapter 4 Results

4.1 Camelina solubility

The pH conditions used during alkaline extraction tests were taken from literature, and generally work for different protein sources. Camelina is a new protein seed and not yet used for human nutrition, therefore the protein extraction is not described in literature. For this reason, protein solubility at different pH was verify for this meal. In graph 2 the protein solubility trend is described. As for the most plant seeds, the highest solubility is reached at extreme pH, in particular at pH 2 the solubility is almost of 50 % and at 10 pH is about 60 %, whereas the lowest solubility rate was at 4 pH (20 %). The pH conditions used during alkaline extraction of camelina meal were the same used for the other plant seeds, as too extreme pH lead to protein denaturation.



Graph 2. Protein solubility of camelina meal at different pH values

4.2 Protein enrichment

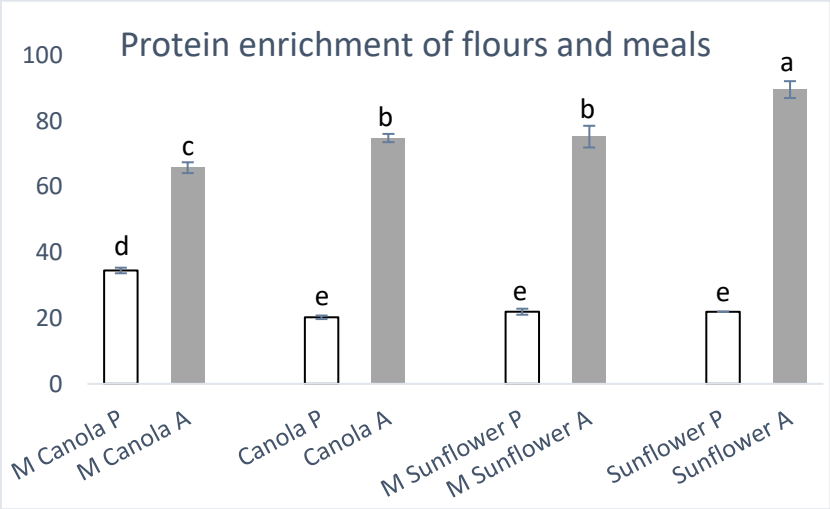
Two wet processes were used in this study to produce protein-rich extracts of different plant seeds. More in detail, the traditional alkaline process was compared to a simpler process that uses phosphate saline buffer as solvent. The plant sources were sunflower flour and meal, canola flour and meal, grinded chickpea, dehulled lentils flour and camelina meal.

In the table 6 the protein content and protein-mass yields of each extract obtained with the two processes are reported.

Table 6. Protein concentration, mass and protein yields of the two protein enrichment processes (A: alkaline process, P: PBS process)

CODE	TRADITIONAL WET (A)			PBS PROTOCOL (P)		
	% proteins	% mass yield	% protein yield	% proteins	% mass yield	% protein yield
30 sunflower flour	89.54 ± 2.57 (a)	5.89 ± 0.52 (gh)	21.25 ± 2.48 (de)	21.93 ± 0.01 (g)	24.15 ± 0.71 (de)	20.93 ± 0.08 (de)
36 sunflower meal	75.23 ± 3.30 (b)	4.88 ± 0.49 (gh)	14.40 ± 2.08 (fg)	21.95 ± 0.92 (g)	25.96 ± 1.35 (d)	22.29 ± 0.22 (de)
32 canola flour	74.79 ± 1.26 (b)	3.85 ± 0.42 (h)	8.86 ± 1.32 (g)	20.26 ± 0.54 (g)	34.50 ± 0.28 (c)	20.98 ± 0.45 (de)
37 canola meal	65.78 ± 1.64 (cd)	8.13 ± 0.18 (g)	17.24 ± 0.94 (ef)	34.50 ± 0.85 (ef)	41.40 ± 0.14 (b)	45.90 ± 1.41 (c)
38 chickpea	69.81 ± 0.45 (bc)	20.52 ± 0.39 (ef)	64.77 ± 0.81 (b)	31.59 ± 0.02 (ef)	46.88 ± 1.60 (a)	66.93 ± 2.24 (ab)
29 lentils	86.91 ± 0.12 (a)	17.04 ± 0.52 (f)	65.08 ± 2.09 (b)	37.55 ± 0.37 (e)	43.57 ± 1.82 (ab)	71.86 ± 2.29 (a)
28 camelina meal	63.19 ± 3.15 (d)	21.78 ± 0.01 (de)	41.44 ± 1.68 (c)	28.88 ± 0.78 (f)	30.67 ± 2.24 (c)	26.86 ± 2.69 (d)

The alkaline extraction of sunflower flour and meal led to different extracts (graph 3): the protein content derived from sunflower flour is definitely higher (89.5 %) compared to those derived from meal (75.2 %). Mass yield result similar (between 5 and 6 %) and protein yields of the meal lower (14.4 % compared to 21.5 % of the flour). Concerning PBS process, the extracts obtained from sunflower flour and meal are really similar, resulted in a protein content of 22 %, a mass yield comprises between 24 and 26 % and a protein yield from about 21 and 22%. Alkaline extracts derived from canola flour and meal have a different protein content (around 75 % for the flour and 66% for the meal), as found in the sunflower extracts. However, looking at the yields, the trend is different: in the extract derived from the meal are higher than those derived from the flour (8 % of mass yield compared to 5 %, and 17 % protein yield compared to 9%). On the contrary, PBS extract derived from canola meal had a higher protein content (34.5 %) then those derived from canola flour (20.2 %). The mass and protein yields of canola meal extract are also definitely higher than flour extract: 41.4 % compared to 34.5 % of mass yield and 46 % compared 21 % of protein yield.

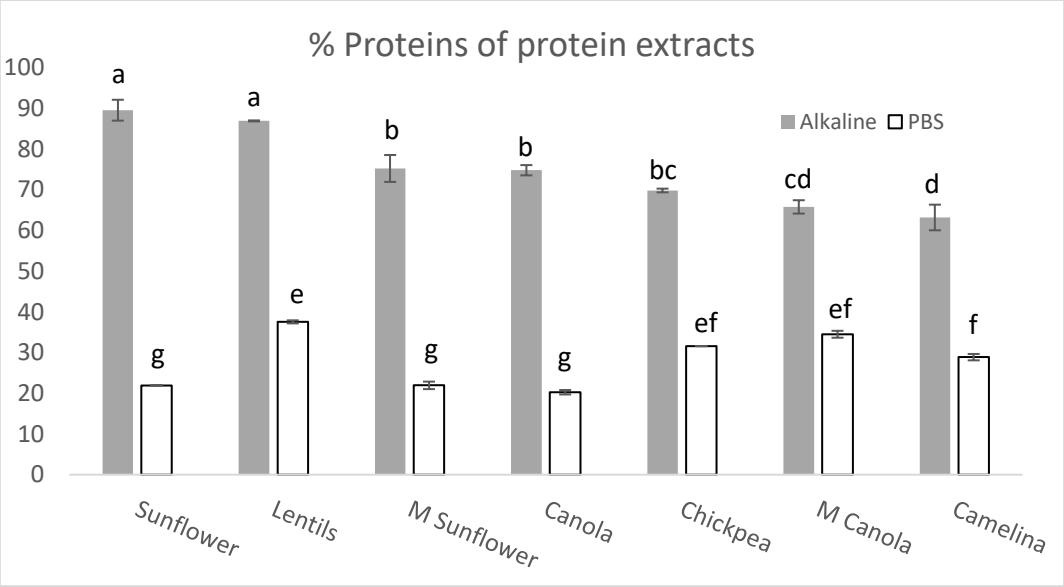


Graph 3. Protein % of sunflower and canola extracts using alkaline process (A) and PBS process (P). Meals derived from mechanical oil removal (M canola and M sunflower) are compared to the flours obtained from chemical oil removal.

In graph 4, the protein purity of the two extracts of the different species are reported. The extracts obtained from camelina meal confirmed the trend found in sunflower and canola: the alkaline extract has a protein content of 63 % and PBS extract 29 %. Mass and protein yields of alkaline extract are 22 and 41.4 % respectively, whereas for PBS extract are 31 and 27 %.

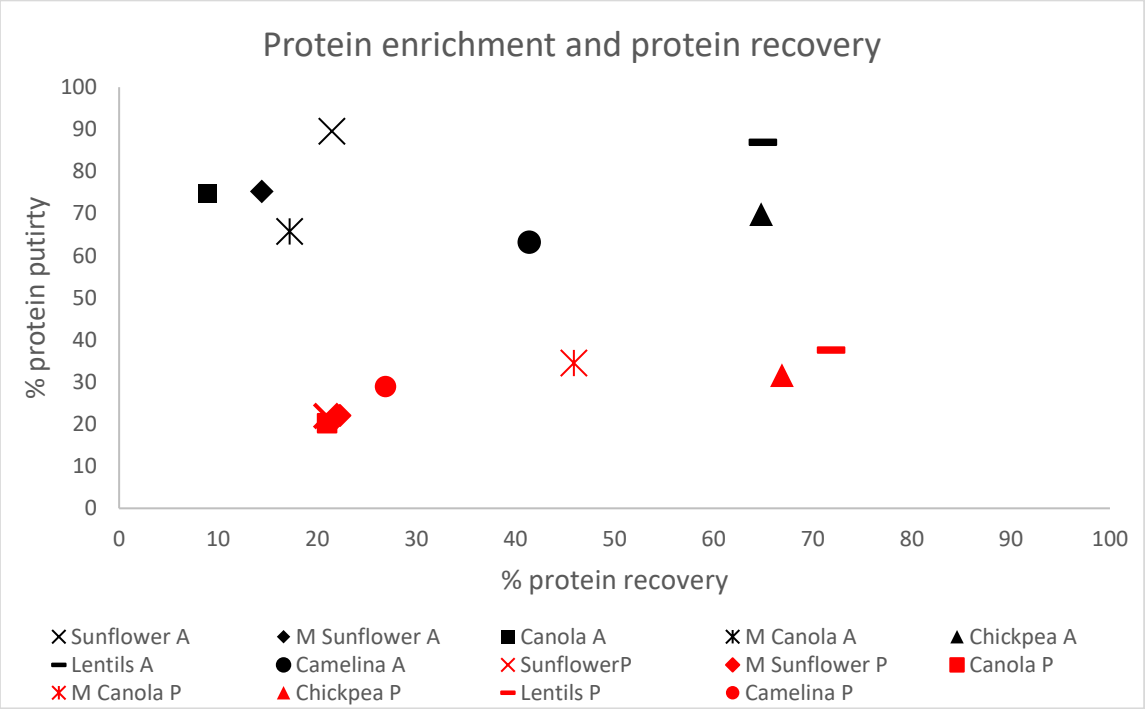
Alkaline enrichment of chickpea has a protein content of about 70 % with a mass yield of 20.5 % and a protein yield of about 65 %. The PBS extraction, as for the other protein sources, results in a protein content lower (31.6 %), but the mass and protein yields are higher (47 and 67 %).

Lentils extracts confirm that alkaline process generate protein ingredient with a higher purity: alkaline extract in fact has a protein content of about 87 %, versus the 37,6 % of PBS extract. As for chickpea, mass and protein yields of PBS extract were higher than those derived from alkaline (43.6 and 71.9 % compared to 17 and 65 %).



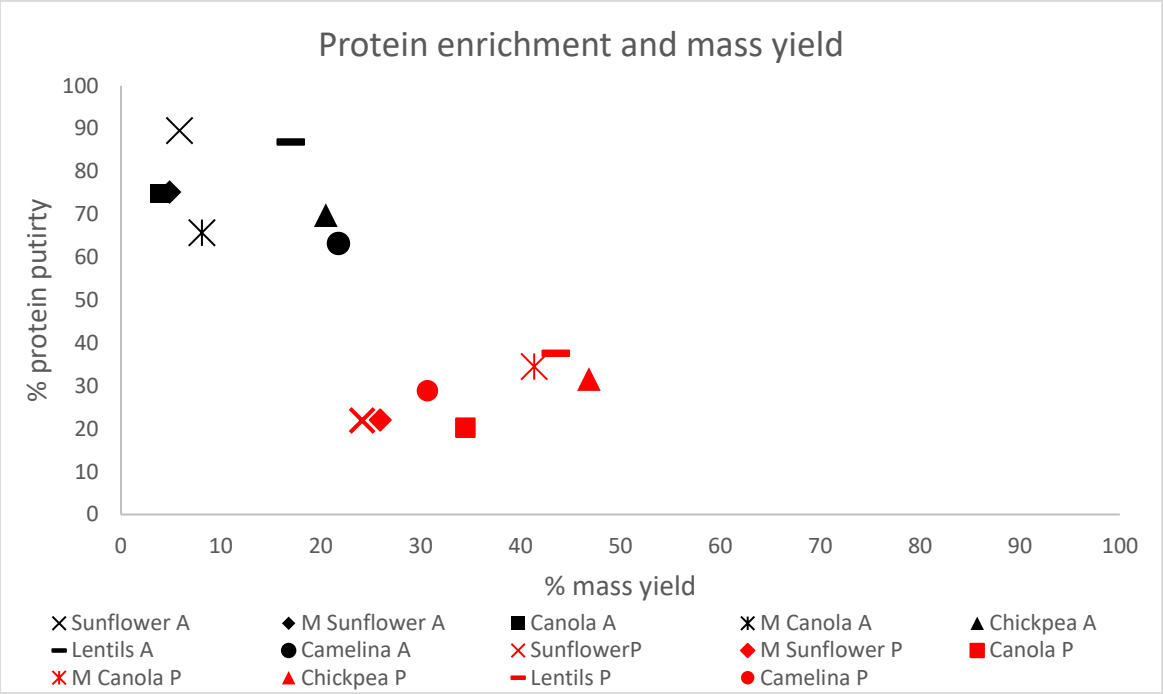
Graph 4. Protein purity of the extracts derived from alkaline extraction (grey) and PBS extraction (white)

In graph 5, alkaline extracts (A, in black) and PBS extracts (P, in red) are compared based on their protein content and protein yield. As it is shown in the graph, there is a marked difference in the protein content reached through the two processes, as the alkaline extraction results in a higher protein content in each plant source considered, producing protein isolates/concentrates. The protein recovery of alkaline extracts is lower in some samples (as canola flour and meal, sunflower meal) compared to PBS process.



Graph 5. Protein enrichment and protein yield of the two methods. In black the alkaline extraction samples (A), in red extracts derived from PBS method (P). Extracts from alkaline method had higher protein content, though the yield is very low, except for lentils and chickpea. M sunflower and M canola are the extracts obtained from mechanical de-oiled meals.

In Graph 6, the protein purity is plotted with the mass yield of the extracts derived from the two processes. As shown in graph 5, the protein purity is always higher in the alkaline extracts (black), but the mass yield is higher in PBS extracts (red). The mass yield of sunflower and canola derived from alkaline process is very low (<10 %).



Graph 6. Protein enrichment and mass yield of the two methods. In black the alkaline extraction samples (A), in red extracts derived from PBS method (P). M sunflower and M canola are the extracts obtained from mechanical de-oiled meals.

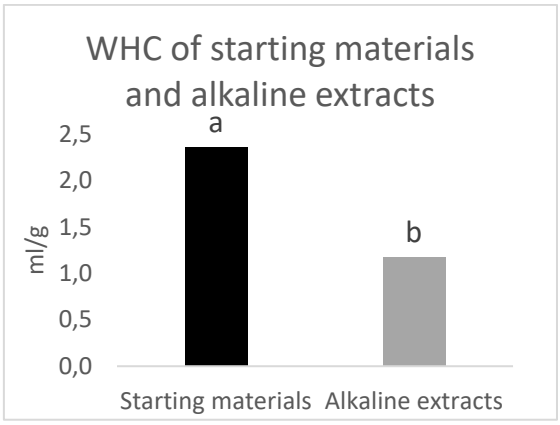
4.3 Functional properties of protein extracts

Functional properties as water/oil holding capacity and gel and foam formation were tested to compare the characteristics of the protein concentrates produced through the enrichment processes. The starting flours/cakes used for protein extraction were also tested for functional properties, in order to determine if the processes increase or decrease these activities.

4.3.1 WATER HOLDING CAPACITY

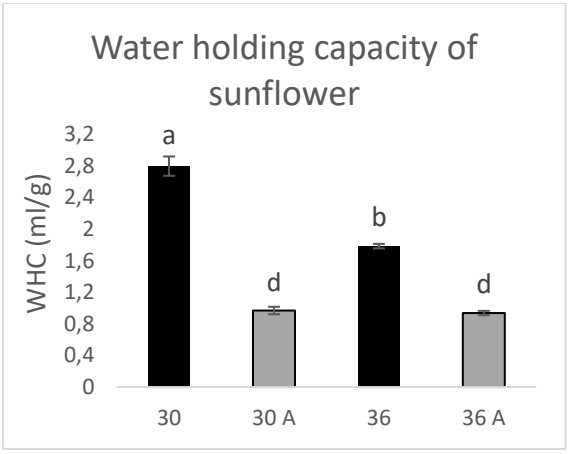
Water holding capacity of flours, meals and relative alkaline extracts are reported. WHC of PBS extracts were 0 ml/g for every sample considered, as the products of this process are completely water soluble.

Graph 7 reports the WHC of starting materials and alkaline extracts: the process in general decrease the retain water property, with the exception of lentils extract.



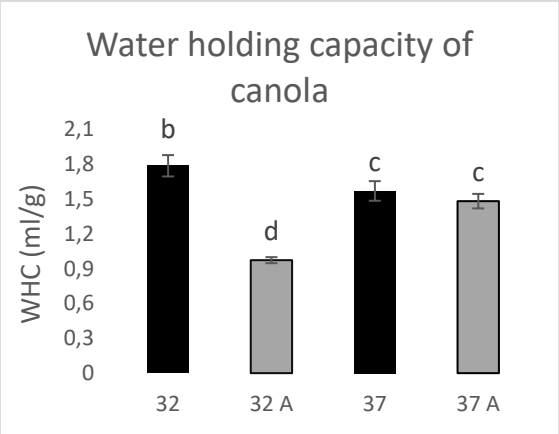
Graph 7. WHC of starting materials and alkaline extracts: the protein enrichment process leads to a decrease of this property

In graph 8, the water holding capacity of sunflower flour, sunflower meal and alkaline extracts. In both samples, the alkaline extract shows a WHC lower than the corresponding starting material. Sunflower flour has a WHC of 2.8 ml/g, that decreases to 0.96 ml/g in the alkaline extract. WHC of sunflower meal is lower (about 1.8 ml/g) and decreases to 0.93 ml/g in its extract.



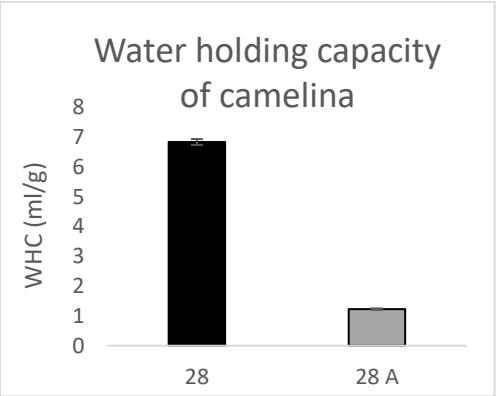
Graph 8. Water holding capacity of sunflower flour (30) and meal (36) and their corresponding extracts from alkaline enrichment method

Water holding capacity of canola meal and flour is showed in the graph 9. As for sunflower, also the alkaline concentrates derived from canola flour present a lower WHC compared to the starting material (0.97 and 1.8 ml/g). WHC values of canola meal and its extract are similar (1.57 and 1.48 ml/g).



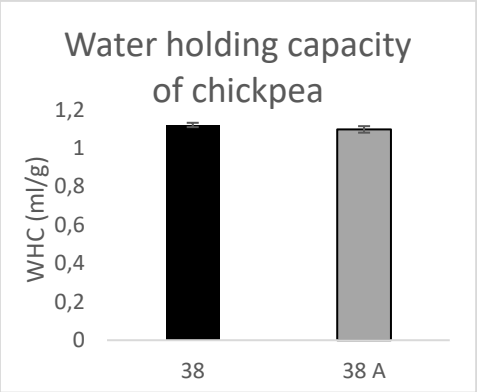
Graph 9. Water holding capacity of canola flour (32) and meal (37) and their corresponding extracts

Water holding capacity of camelina meal is significantly higher comparing with the other protein sources (6.8 ml/g, graph 10). The alkaline process strongly decreases this property, in fact the extract shows a WHC of 1.2 ml/g.



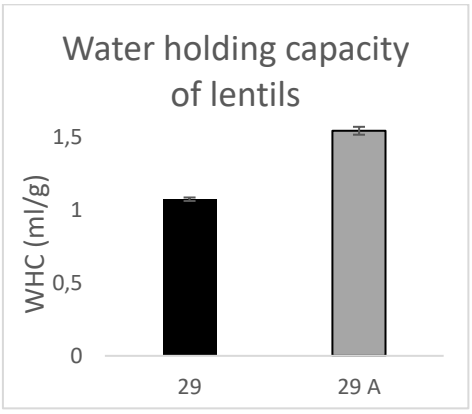
Graph 10. Water holding capacity of camelina meal and its alkaline extract

Water holding capacity of grinded chickpea and the corresponding alkaline concentrate (graph 11) are basically equal (1.12 and 1.09 ml/g): in this case, the alkaline process does not affect the WHC property**.**



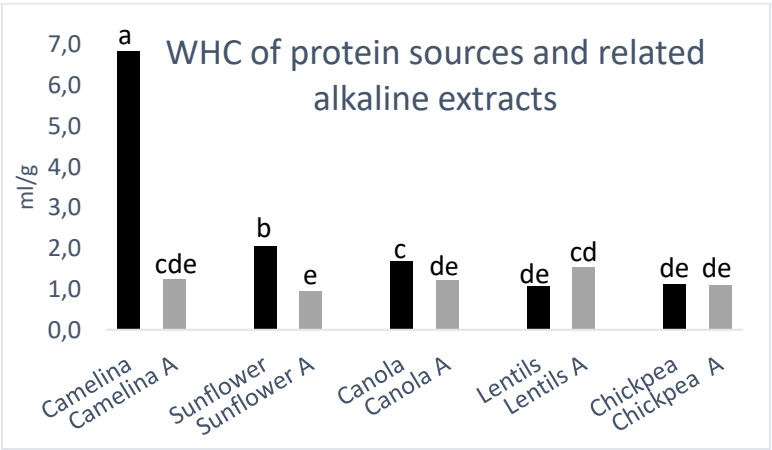
Graph 11. Water holding capacity of grinded chickpea and its alkaline extract

Unlike the protein sources just discussed, water holding capacity of lentils concentrate is higher than the lentils flour (starting material). In graph 12, WHC of lentils flour indeed is about 1 ml/g and the value of isolate is 1.54 ml/g.



Graph 12. Water holding capacity of dehulled lentils flour and its alkaline extract

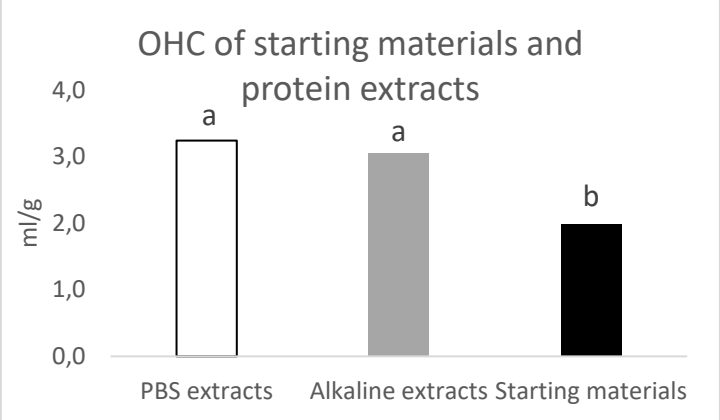
Graph 13 summarize the WHC of the different protein sources tested and their related alkaline protein-rich products. Statistical differences are also reported.



Graph 13. WHC of the protein sources used in this study and the related alkaline extracts

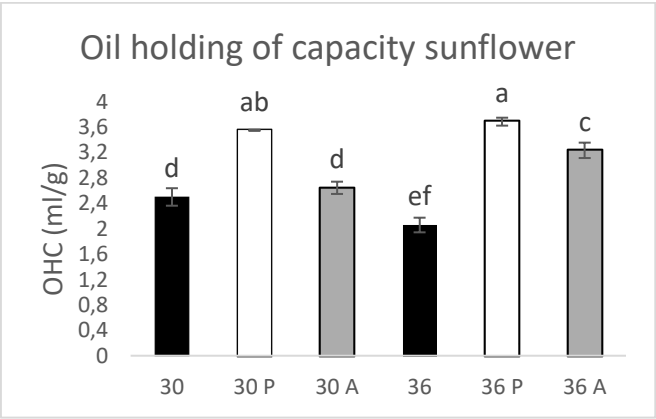
4.3.2 OIL HOLDING CAPACITY

Oil holding capacity was tested for every sample and its alkaline and PBS extracts. Almost every protein extract derived from the two processes showed an OHC value higher than the corresponding starting flour/meal (graph 14).



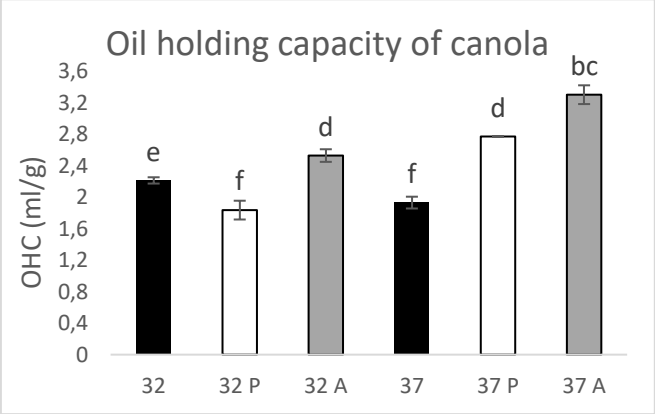
Graph 14. OHC of starting materials and protein extracts: both processes lead to an increase of this property

In graph 15, oil holding capacity of sunflower flour and meal. In both cases, OHC of the alkaline and PBS products is higher than the starting material. OHC value of sunflower flour is 2.5 ml/g and the extracts are 3.6 (P) and 2.6 (A) ml/g. OHC of sunflower meal is similar to the flour (2.1 ml/g) and OHC of the extracts is 3.7 (P) and 3.2 (A) ml/g.



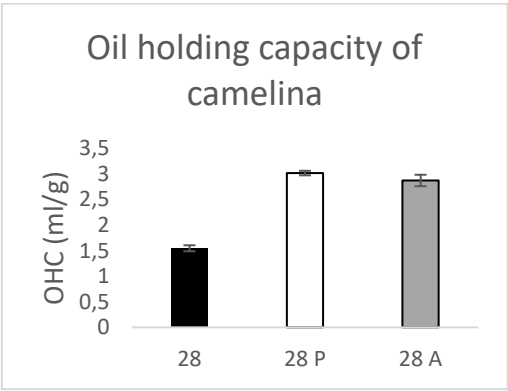
Graph 15. Oil holding capacity of sunflower flour (30) and meal (36) and their corresponding extracts (A from alkaline method, P from PBS method)

Oil holding capacity of canola samples is showed in Graph 16. OHC value for canola flour is 2.1 ml/g, the values of the extracts are 1.8 ml/g for PBS extract and 2.5 ml/g for alkaline extract. OHC of canola meal is 1.9 ml/g and the value increased after protein concentration processes (2.8 ml/g for PBS extract and 3.3 ml/g for the traditional wet extraction).



Graph 16. Oil holding capacity of canola flour (32) and meal (37) and their corresponding extracts (A from alkaline method, P from PBS method)

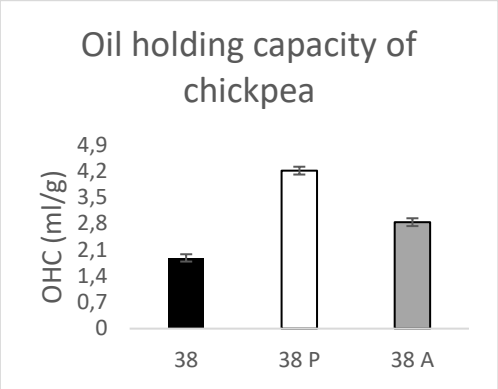
Even in the case of camelina meal, the protein enrichment processes caused an increasing of oil holding capacity property (graph 17). OHC value of camelina meal is 1.5 ml/g, the value increase at 3 ml/g in PBS extract and 2.89 ml/g for alkaline extract.



Graph 17. Oil holding capacity of camelina meal and its extracts (A from alkaline method, P from PBS method)

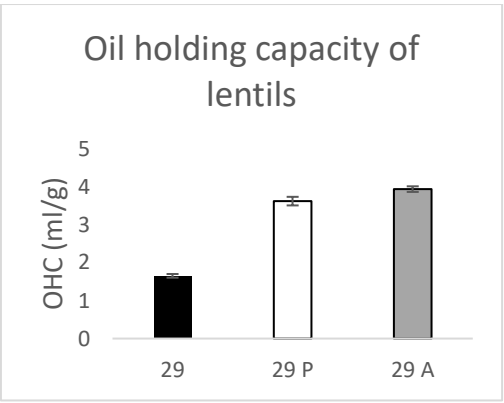
Both extracts derived from chickpea have oil holding capacity values higher than the starting material (graph 18). Grinded chickpea has indeed an OHC of 1.9 ml/g

and for the protein extracts increases to 2.8 ml/g in case of alkaline process and to 4.2 ml/g in PBS product.



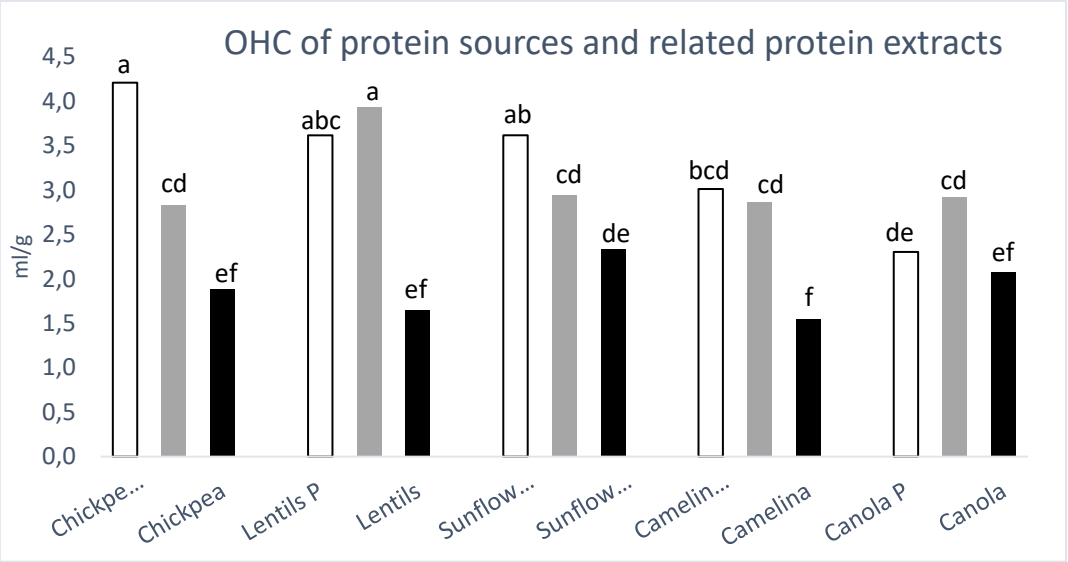
Graph 18. Oil holding capacity of grinded chickpea and its extracts (A from alkaline method, P from PBS method)

Lentils flour OHC values are showed in graph 19. The lentils flour before protein extraction presents an oil holding capacity of 1.6 ml/g, this value increases at 3.6 and 3.9 ml/g after protein enrichment processes.



Graph 19. Oil holding capacity of grinded chickpea and its extracts (A from alkaline method, P from PBS method)

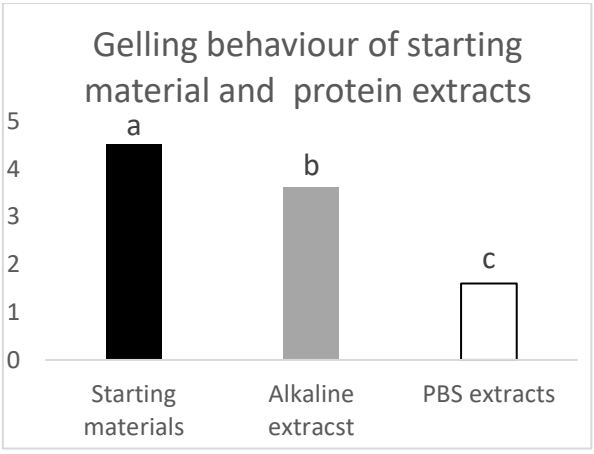
Graph 20 summarize the OHC of the different protein sources tested and their related protein extracts derived from the two processes. Statistical differences are also reported.



Graph 20. OHC of the protein sources used in this study and the related protein extracts (A alkaline, P PBS)

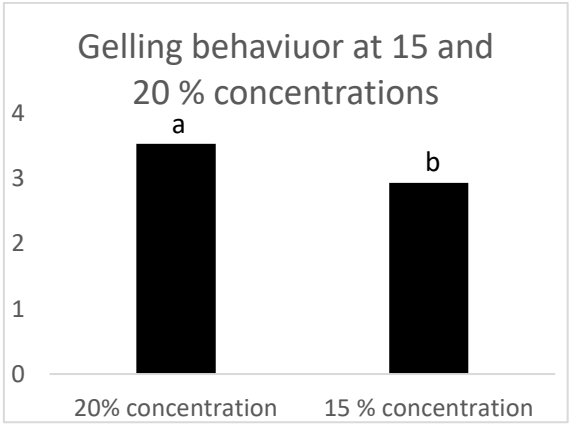
4.3.3 GELLING CAPACITY

As for the WHC and OHC just described, also gelling property was investigated before and after protein extraction processes. In graph 21, the gelling score of starting material are compared with alkaline and PBS extracts: the protein enrichment processes generally determine a decrease of this property. Most of PBS extract showed total absence of gelation (score 1) at the tested conditions (15 and 20 %).



Graph 21. Gelling score of starting materials and alkaline and PBS extracts

The gelling score of the materials tested at 15% is significantly lower than the same materials tested at 20 % (graph 22), meaning that generally increasing the concentration of the material tested, the gelling property increase.



Graph 22. Statistical difference among 15 % and 20 % of material concentration tested

In table 7 are reported the gelation score of 15 and 20 % (w/v) of sunflower flour and meal and their protein extracts. In case of sunflower flour, the protein enrichment processes led to a decrease of gelation capacity. Indeed, the lower gelling concentration of sunflower flour is 20 % (score 5), whereas the protein extracts at 20 % concentration have lower score (2 and 1). Sunflower meal at 20 % concentration does not completely gel (score 4) and also in this case the protein extracts show lower gelation score (3 and 1). The LGC is therefore >20 % concentration.

Table 7 Gelling capacity of 15% and 20% concentrations of sunflower flour and meal and their corresponding A and P extracts. The score of gelling and the lower gelling concentration are reported for each experiment.

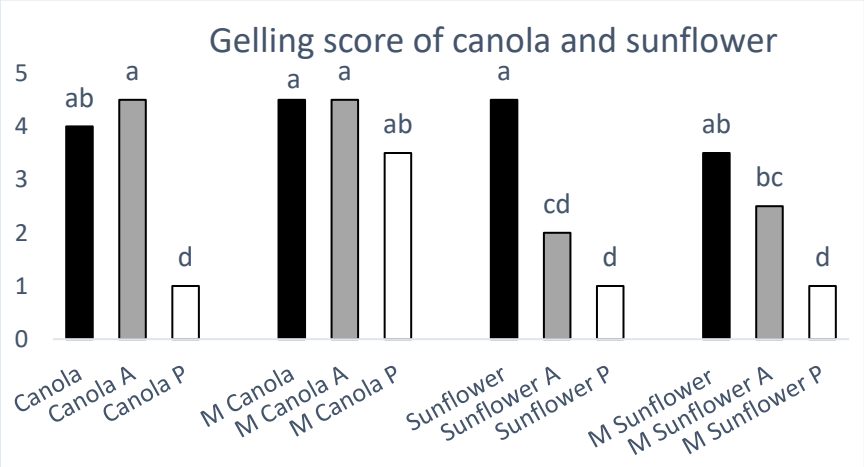
CODE	CONCENTRATION (w/v)	SCORE	Lower Gelling Concentration
30	15%	4	20%
30	20%	5	
30 A	15%	2	> 20%
30 A	20%	2	
30 P	15%	1	> 20%
30 P	20%	1	
36	15%	3	> 20%
36	20%	4	
36 A	15%	2	> 20%
36 A	20%	3	
36 P	15%	1	> 20%
36 P	20%	1	

Canola gelling property is described in table 8. Canola flour at 20 % was not completely gelled (score 4), the property increases in alkaline protein extract (score 5) and decrease in PBS extract (score 1). Canola meal and its alkaline extract have the same LGC (20 %), whereas for PBS extract is >20 %, showing a score of 4 (almost completely gelled).

Table 8. Gelling capacity of 15% and 20% concentrations of canola flour and meal and their corresponding A and P extracts. The score of gelling and the lower gelling concentration are reported for each experiment

CODE	CONCENTRATION (w/v)	SCORE	Lower Gelling Concentration
32	15%	4	> 20%
32	20%	4	
32 A	15%	4	20%
32 A	20%	5	
32 P	15%	1	> 20%
32 P	20%	1	
37	15%	4	20%
37	20%	5	
37 A	15%	4	20%
37 A	20%	5	
37 P	15%	3	> 20%
37 P	20%	4	

In graph 23 are reported the differences in gelling property among the sunflower and canola flours derived from chemical oil removal and cakes derived from mechanical oil removal.



Graph 23. Statistical differences of gelling score of sunflower and canola flours and meals

Camelina meal and its PBS extract show an LGC value of 20 % (table 9). The alkaline extract LGC value is > 20 % highlighting a score of 4 (almost completely gelled).

Table 9. Gelling capacity of 15% and 20% concentrations of camelina meal and its corresponding A and P extracts. The score of gelling and the lower gelling concentration are reported for each experiment

CODE	CONCENTRATION (w/v)	SCORE	Lower Gelling Concentration
28	15%	4	20%
28	20%	5	
28 A	15%	3	> 20%
28 A	20%	4	
28 P	15%	3	20%
28 P	20%	5	

Dehulled lentils flour exhibits a very high gelation property (table 10). Indeed, at the concentrations of 15 and 20 % was completely gelled (score 5), and lower concentrations were tested (10 and 7,5 %), resulting in an LGC of 10%. Both protein extracts showed a decrease of this property, in fact the LGC is >20 % (score 4 for alkaline extract and 1 for PBS extract).

Table 10. Gelling capacity of 15% and 20% concentrations of lentils flour and its corresponding A and P extracts. The score of gelling and the lower gelling concentration are reported for each experiment.

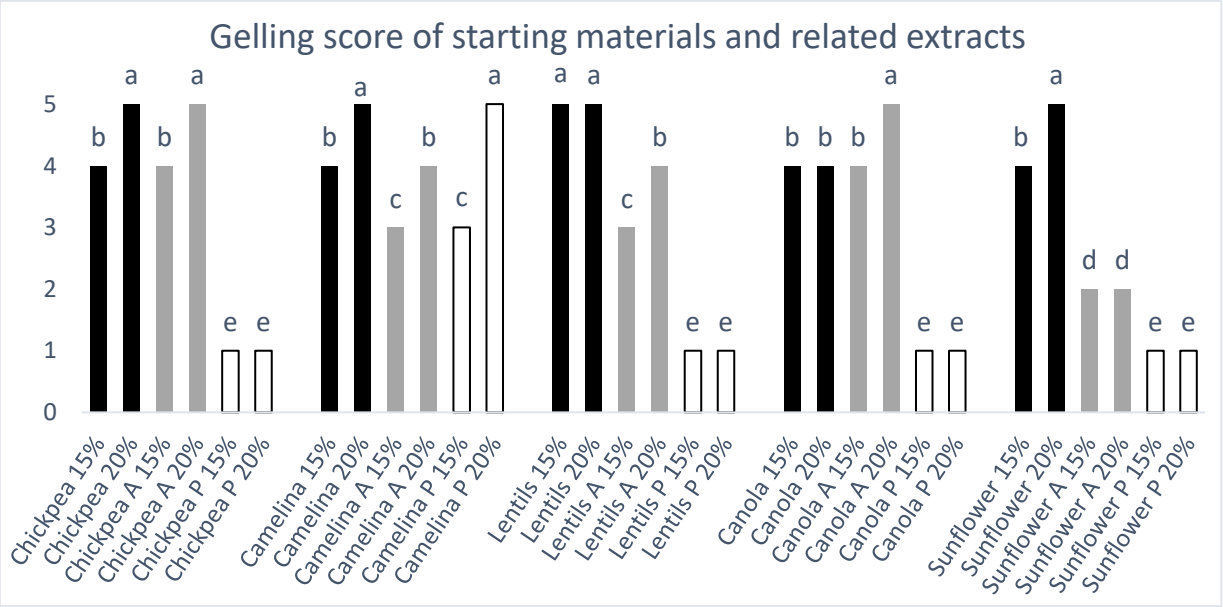
CODE	CONCENTRATION (w/v)	SCORE	Lower Gelling Concentration
29	7,5%	4	10%
29	10%	5	
29	15%	5	
29	20%	5	
29 A	15%	3	> 20%
29 A	20%	4	
29 P	15%	1	> 20%
29 P	20%	1	

Gelation values for chickpea and its protein rich extracts are reported in table 11. Grinded chickpea before extraction and alkaline extract showed the same gelation scores, with al LGC of 20 %. PBS extract lost completely the gelling property, and the score at the highest concentration tested is 1.

Table 11. Gelling capacity of 15% and 20% concentrations of chickpea flour and its corresponding A and P extracts. The score of gelling and the lower gelling concentration are reported for each experiment.

CODE	CONCENTRATION (w/v)	SCORE	Lower Gelling Concentration
38	15%	4	20%
38	20%	5	
38 A	15%	4	20%
38 A	20%	5	
38 P	15%	1	> 20%
38 P	20%	1	

In graph 24 are summarized the gelling scores of the starting materials and protein extracts (alkaline and PBS), tested at 15 % and 20 % of concentrations. Statistical differences among the protein sources are also reported.

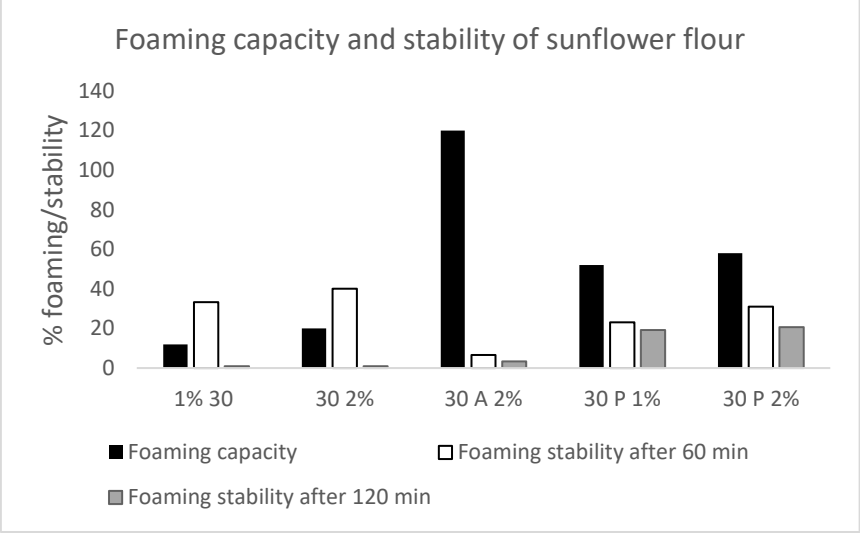


Graph 24. Gelling score of starting materials and protein extracts at the two concentrations

4.3.4 FOAMING CAPACITY

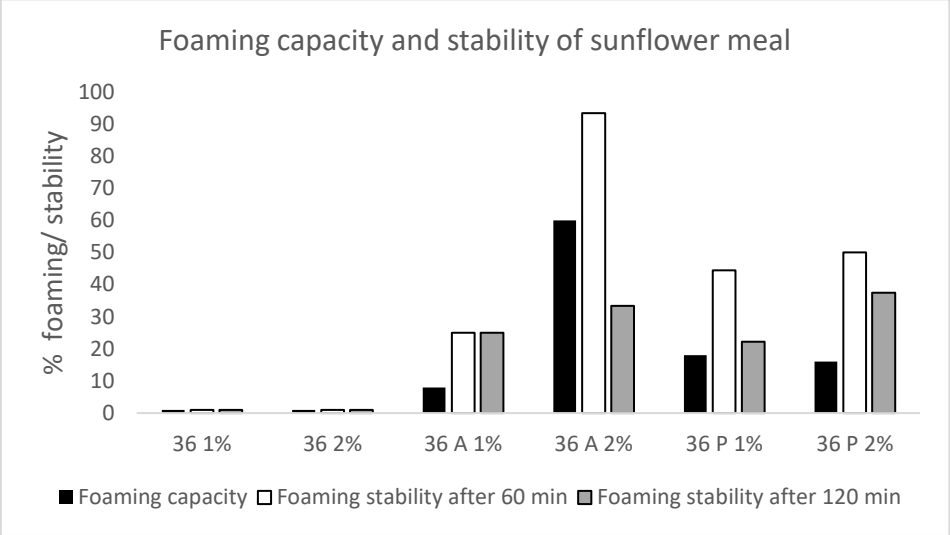
Another functional property investigated was the capacity to produce foam and the stability of the produced foam after 1 and 2 hours. All the samples before and after protein enrichment processes were tested at the concentrations of 1 and 2 % (w/v), for the exception of some samples that was not possible and were tested only at 2 %. Statistical differences could not be investigated since duplicates of each values were not available.

Graph 25 shows the results about the foaming capacity of sunflower flour. The foaming capacity of the protein extracts at 2 % increases (120 % and 58 %) compared to the flour before processes (20 %). The stability of the formed foam is different based on the sample considered and on the elapsed time (1 or 2 hours). Alkaline extract for example, shows a high foaming capacity (120 %), but the stability is very low (7 % after 1 hour and 4 % after 2 hours). PBS extract have a lower foaming capacity (58 %) and the stability after 1 hour is 31% and after 2 hours 21%. The flour shows an acceptable stability after 1 hour (40%), but the foam was not stable after 2 hours.



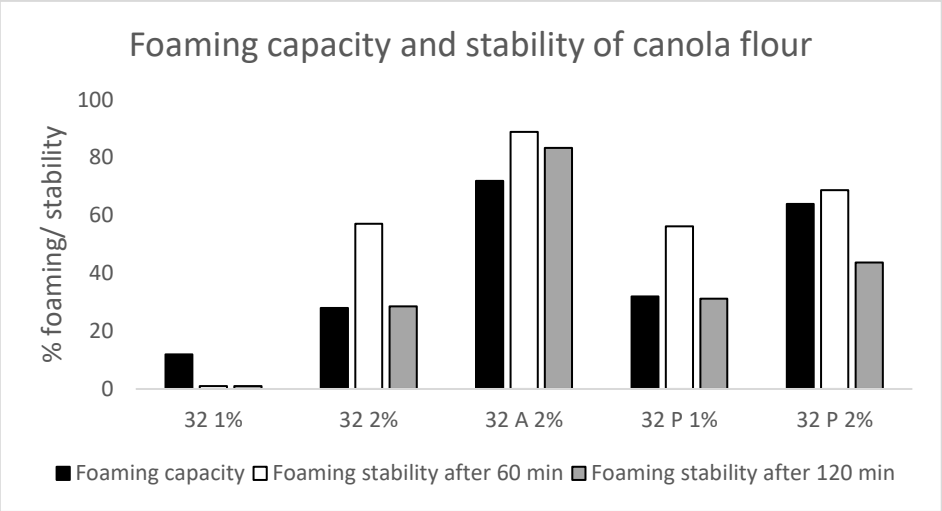
Graph 25. Foaming capacity and stability of sunflower flour and related A and P extracts. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Sunflower meal (graph 26) did not form foam neither at 1 or 2 % of concentration. The alkaline extract exhibited the highest foaming capacity at 2 % (60 %) and a good stability (94 % after 1 hour and 34 % after 2 hours). PBS extract shows a similar foaming capacity and stability both at 1 or 2 % (17 % of foaming and 50 % of stability after 1 hour and 37% after 2 hours).



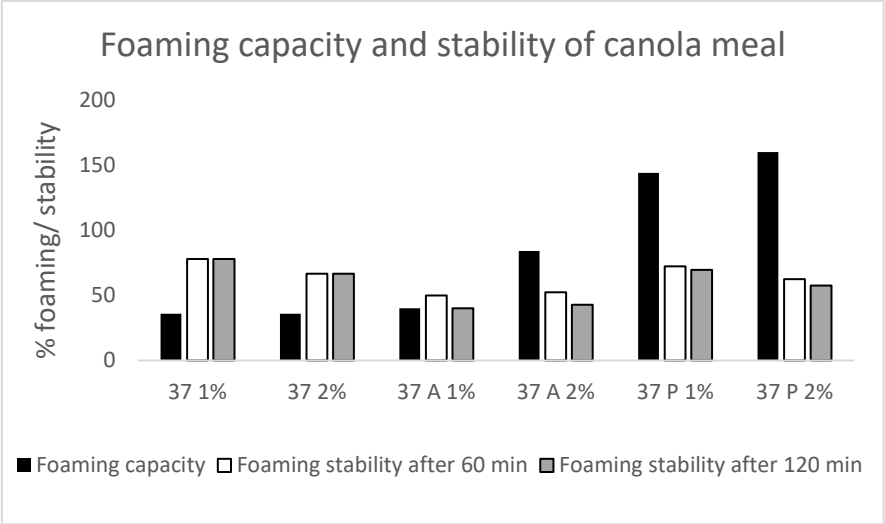
Graph 26. Foaming capacity and stability of sunflower meal and related A and P extracts. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Graph 27 represents the foaming capacity and stability of canola flour and its protein extracts. The foam produced by the starting material is very low (12 % at the concentration of 1 % and 28 % at 2 %) and the stability of the foam produced at 2 % is acceptable (57 % after 1 hour and 28 % after 2 hours). The FC of the protein extracts at 2 % is higher, in particular for the alkaline extract: 72 % of foaming with a stability of 89 % after 1 hour and 84 % after 2 hours. PBS extract also shows a good foaming capacity at 2 % (64 %) and a stability of 69 % after 1 hour and 44 % after 2 hours.



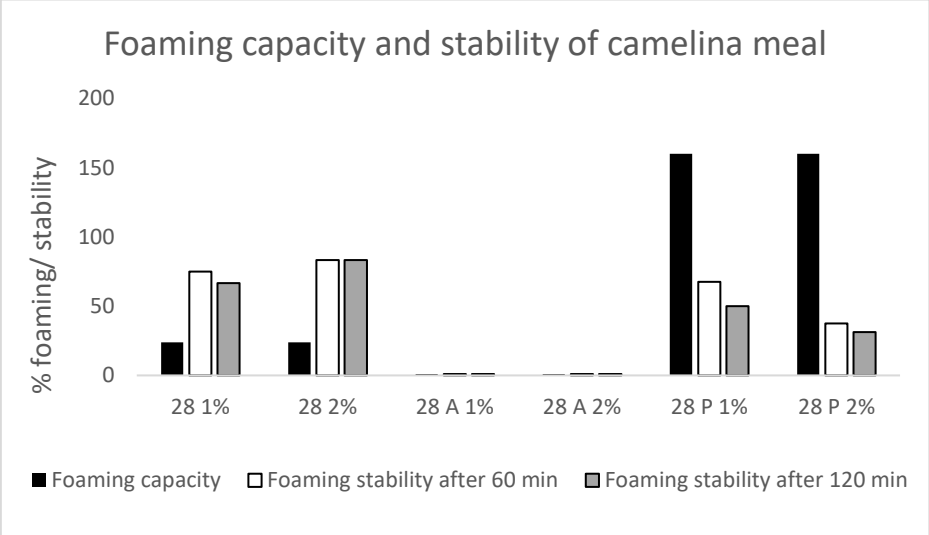
Graph 27. Foaming capacity and stability of canola flour and A and P extracts. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Foaming property of canola meal is reported in graph 28. Foaming capacity value of canola meal is the same (36 %) testing the material at 1 and 2 %. The stability is equal after 1 or 2 hours and is slightly lower at testing condition of 2 % (67% versus 77%). Alkaline extract at 1 % has similar results compared to the starting material, however at the concentration of 2 % the foaming capacity is double (84%). The stability of the foam formed by alkaline extract is slightly lower than the meal (52 % after 1 hour and 43 % after 2 hours). PBS extract shows a foaming capacity very high, 140 % at the testing concentration of 1 % and 160 % at 2 % of concentration. The stability is similar to the meal values (about 65 % after 1 and 2 hours).



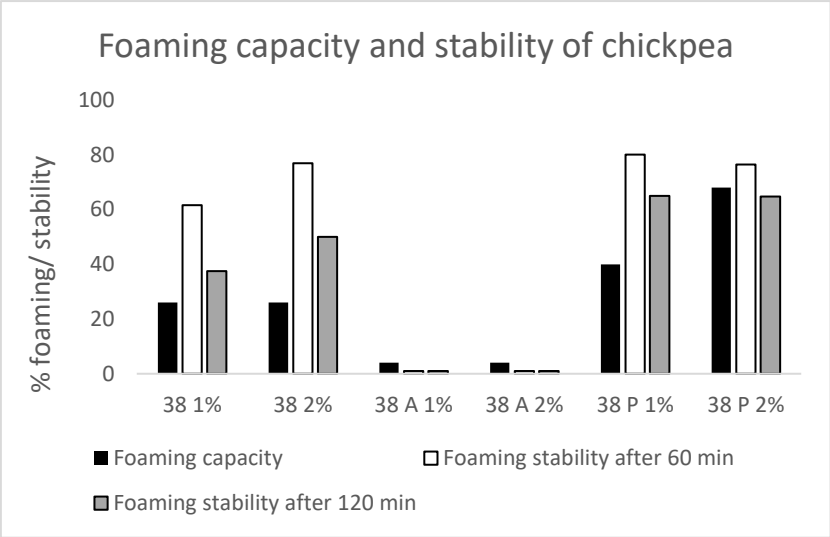
Graph 28. Foaming capacity and stability of canola meal and related A and P extracts. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Foaming capacity of camelina meal (graph 29) is equal if tested at 1 or 2 % (24 %). The stability is also similar and after 1 hour is 75 and 84 % and after 2 hours is 67 % and 84 %, resulting slightly more stable the foam formed at the concentration of 2 %. Alkaline extract, did not form foam neither at 1 or 2 %. The FC of PBS extract is very high, 160 % at both concentrations. The stability instead is higher at 1% (67,5 % versus 37.5 % after 1 hour, 50 % versus 31 % after 2 hours.



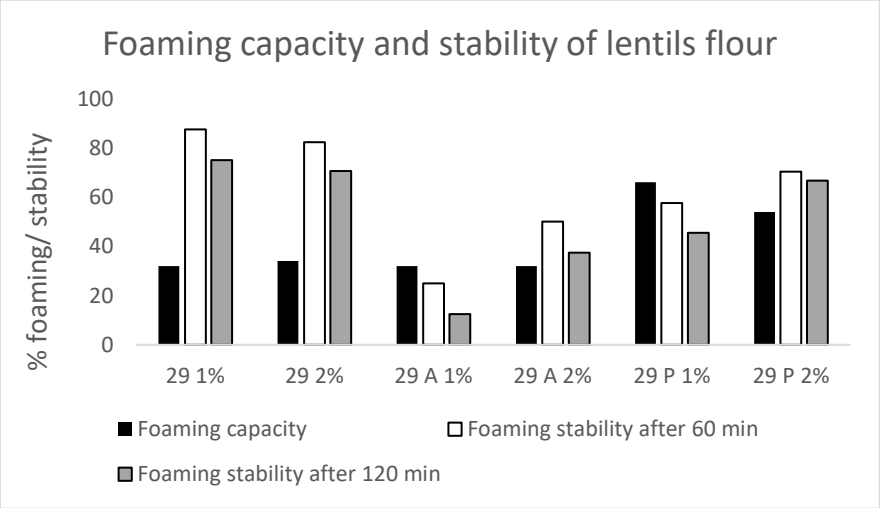
Graph 29. Foaming capacity and stability of camelina meal and related A and P extracts. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

As for camelina meal, the extract derived from alkaline process of chickpea shows very low foaming capacity (4 %) and zero stability at both concentrations tested (graph 30). Foaming capacity of grinded chickpea is equal (24 %) at 1 and 2 % with a stability higher at 2 % (77 % versus 61 % after 1 hour and 50 % versus 37,5 % after 2 hours). FC of PBS extract is higher than the starting material: 40 % at the concentration of 1 % and 68 % at 2 %. The stability is very similar between the 2 concentrations (about 80 % after 1 hour and 65 % after 2 hours).



Graph 30. Foaming capacity and stability of grinded chickpea and related A and P extracts. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Lentils flour foaming capacity and stability is represented in the graph 31. Lentils flour and its corresponding alkaline extract show the same foaming capacity (32 %) at both concentrations (1 and 2 %), but the stability is different. The stability of the flour in fact is higher, about 85 % after 1 hour and 73 % after 2 hours, whereas for alkaline extract at 2 % is 50 % after 1 hour and 37,5 % after 2 hours. The stability of alkaline extract at 1 % is lower, 25 % and 12,5 % after 1 and 2 hours. Foaming capacity of the protein extract form PBS process is higher (66 % at the lower concentration and 54 % at the 2 % of extract), and stability is good at 2 %, 70 % after 1 hour and 67 % after 2 hours.



Graph 31. Foaming capacity and stability of lentils flour and related A and P extracts. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

4.4 Functional properties of supernatants

The traditional wet protein enrichment produces two co-products that currently are not valued. One of these co-products is the supernatant discarded after the second centrifugation, and it could be interesting since it contains a discrete amount of proteins and could possess bioactivities/functionalities. The main drawback is the huge amount of water, meaning that need to be dried before an eventual use in food or other fields.

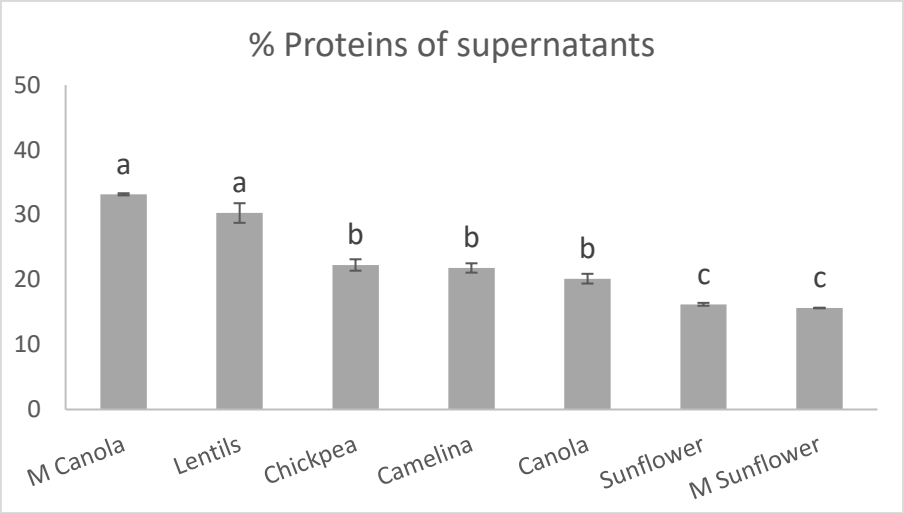
In this study, the supernatants were collected and freeze-dried as the protein pellet, the protein content and any functional properties were determined in order to evaluate a possible destination of use.

Data about water holding capacity are not reported, as the supernatants (as PBS extracts) are completely water soluble, therefore the WHC values were 0 ml/g. In table 12 are reported the protein contents % and mass- protein yields of the supernatants collected after alkaline enrichment.

Table 12. Protein concentration, mass and protein yields of the supernatants, co-product of the alkaline extraction process

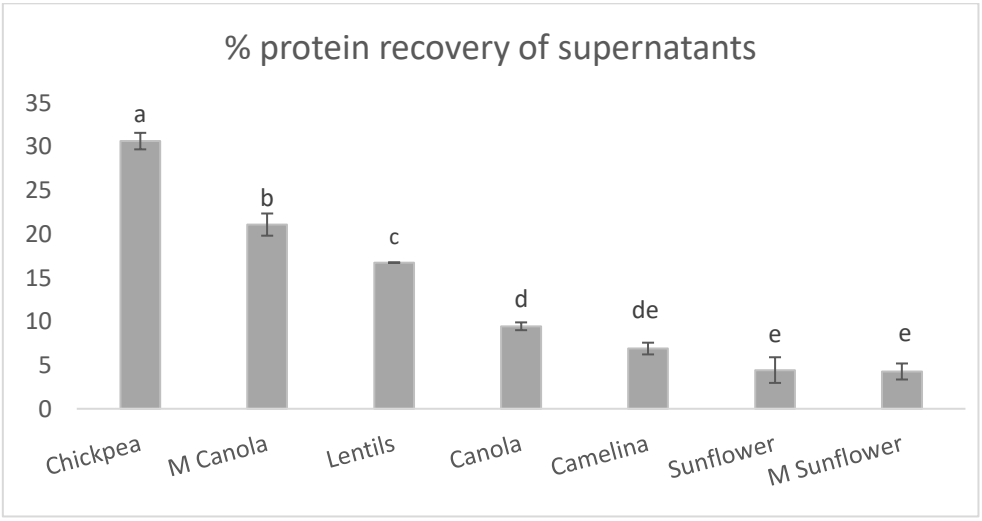
CODE	% proteins	% mass yield	% protein yield
30 S	16.22 ± 0.22, (c)	6.81 ± 2.34, (e)	4.43 ± 1.47, (e)
36 S	15.65 ± 0.05, (c)	6.97 ± 1.48, (e)	4.27 ± 0.92, (e)
32 S	20.16 ± 0.75, (b)	16.13 ± 1.12, (bc)	9.45 ± 0.45, (d)
37 S	33.17 ± 0.16, (a)	19.66 ± 1.15, (b)	21.09 ±1.27, (b)
28 S	21.81 ± 0.72, (b)	10.48 ± 0.77, (de)	6.89 ± 0.67, (de)
29 S	30.28 ± 1.51, (a)	12.56 ± 0.64, (cd)	16.74 ± 0.02, (c)
38 S	22.27 ± 0.88, (b)	30.03 ± 0.33, (a)	30.65 ± 0.94, (a)

The protein content is wide (graph 32) and range from 15.65 % (sunflower meal) to 33.17 % (camelina meal). The mass yield ranges from 6.8 % for the supernatant collected from sunflower flour extraction, to 30 % for the supernatant of chickpea.



Graph 32. Protein purity of supernatants, co-product of alkaline extraction

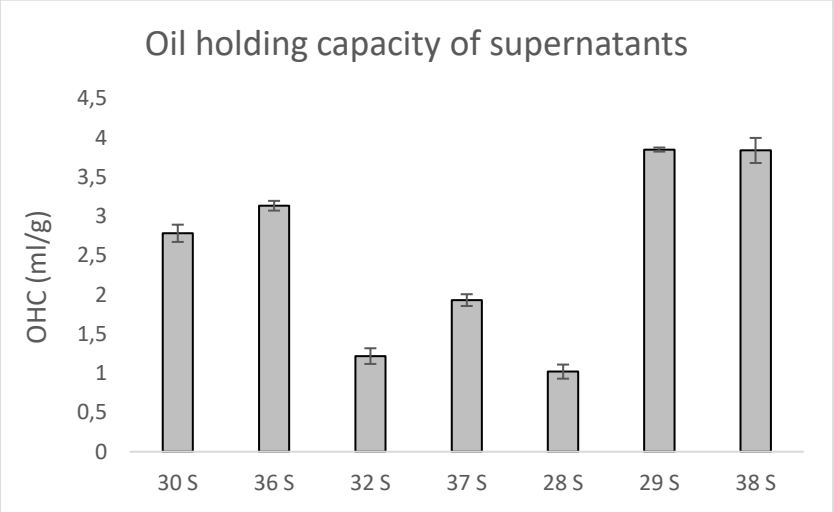
Protein yield, as mass yield, is also very different among the protein source considered: the minimum value is 4.27 % of sunflower meal and the highest 30.38 % of chickpea (graph 33).



Graph 33. Protein recovery of supernatants, co-product of alkaline extraction

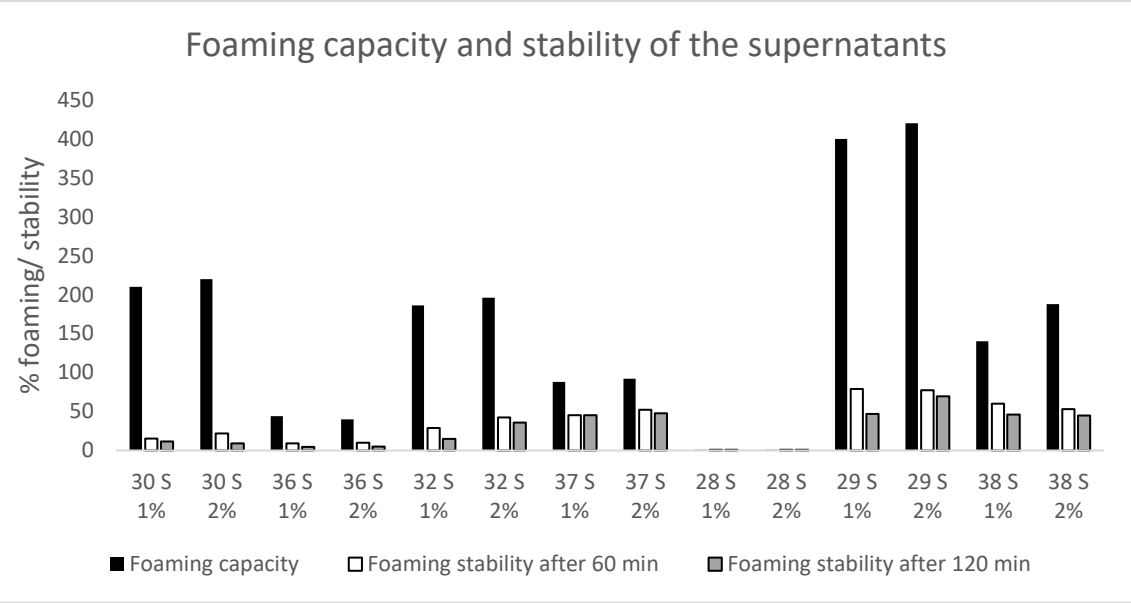
Oil holding capacity supernatants-values are really wide (graph 34). Supernatants derived from the extraction of canola flour and camelina meal show the lower values: 1.22 and 1.02 ml/g respectively. Canola meal supernatant has a higher OHC compared to canola flour (1.9 ml/g). Supernatants derived from sunflower flour and meal extractions are similar: 2.8 and 3.1 ml/g respectively.

The highest OHC values derived from the supernatants of lentils (3.85 ml/g) and chickpea (3.84 ml/g).



Graph 34. Oil holding capacity of the supernatants, co-product of the alkaline extraction method

Foaming capacity and stability were investigated at the concentrations of 1 and 2 % of supernatants (Graph 35). In general supernatants showed a very high foaming capacity, with the exception of the supernatants derived from camelina meal and sunflower meal alkaline enrichment. Indeed, camelina supernatant showed no foaming capacity, whereas the foaming capacity value for sunflower meal supernatant is about 40 %, whit a low stability (about 10 % after 1 hour and 5 % after 2 hours). Sunflower flour supernatant instead presents a high foaming capacity (210 % at 1 % and 220 % at 2 % of concentration), but the stability was low (about 17 % after 1 hour and 10 % after 2 hours). Canola meal supernatant show a similar FC at both concentrations (about 90 %) and similar stability (50 % after 1 hour and 46 % after 2 hours). Foaming capacity of canola flour supernatant is higher (196 %) whit a moderate stability at the concentration of 2 % (42 % after 1 hour and 35 % after 2 hours). Chickpea supernatant had a good FC (140 % at 1 % and 188 % at 2 % concentration). The stability at 1 and 2 % is similar, about 53 % after 1 hour and 45 % after 2 hours. The foaming capacity of lentils supernatant is the highest, at 1 % showed an FC of 400 % and at 2 % the value was 420 %. The stability after 1 hour was similar among the two concentrations (about 78%), whereas after 2 hours the stability at 1% concentration was 47 % and at 2% was 70%.

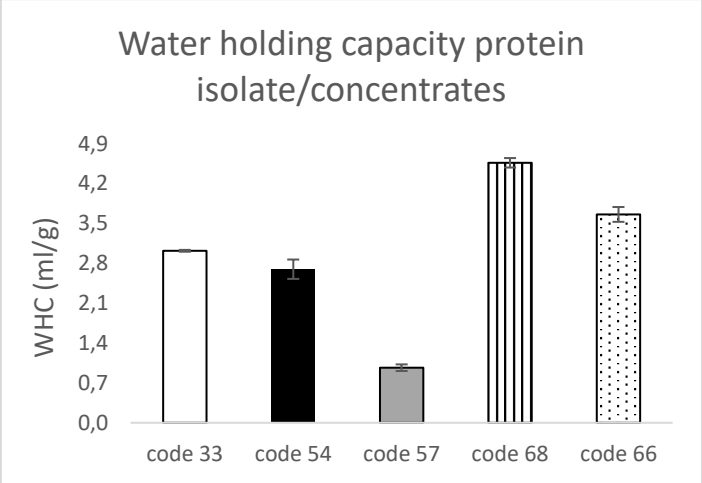


Graph 35. Foaming capacity and stability of the supernatants, co-product of the alkaline extraction method. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

4.5 Functional properties of commercial protein concentrates/isolates

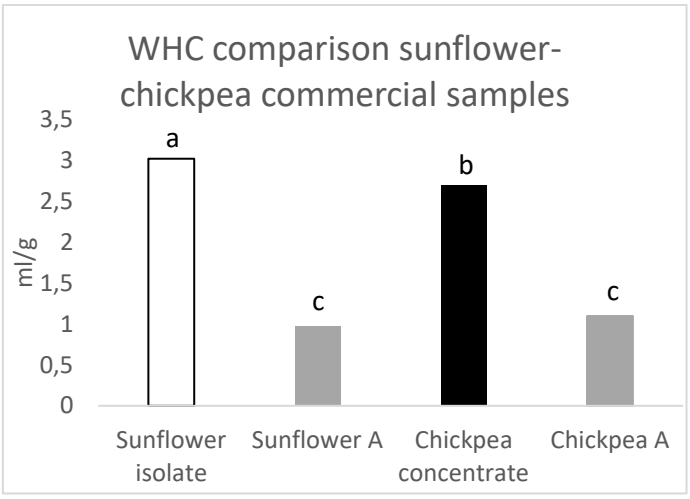
The properties of protein isolates/concentrates commercially available were also verify to compare them with those of protein extracts produced within this project. In particular, WHC, OHC, LGC and FC FS were investigated in samples of sunflower isolate (code 33), chickpea concentrate (code 54), faba concentrate (code 58), faba isolate (code 68) and soy isolate (code 66).

Water holding capacity of protein products is reported in the graph 36. The samples showed a good water absorption, for the exception of faba concentrate that has a WHC of 0.96 ml/g, in contrast with faba isolate that has a high WHC (4.56 ml/g). Soy isolate has a water holding capacity of 3.7 ml/g, sunflower isolate of 3.02 ml/g and chickpea concentrate of 2.7 ml/g.



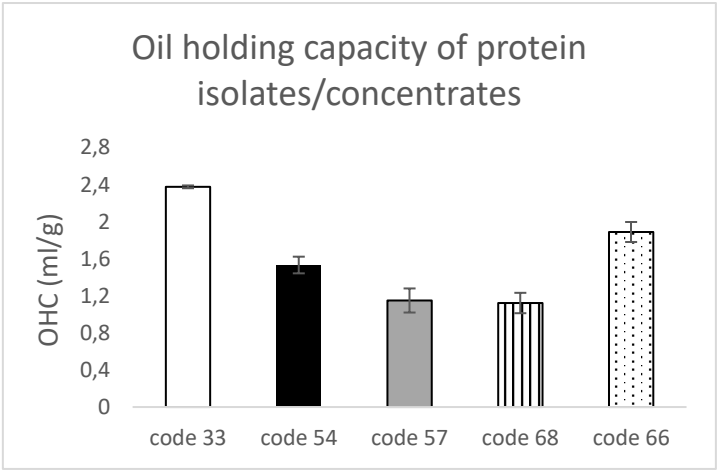
Graph 36. Water holding capacity of samples of protein isolates and concentrates (code 33: sunflower isolate, code 54: chickpea concentrate, code 57: faba concentrate, code 68: faba isolate, code 66: soy isolate)

In graph 37, a comparison of WHC values of commercial products (sunflower isolate and chickpea concentrate, code 33 and 54 respectively) and the alkaline extracts of the same species produced in this thesis. In both species, the WHC value of commercial products is significantly higher than the value of alkaline extracts.



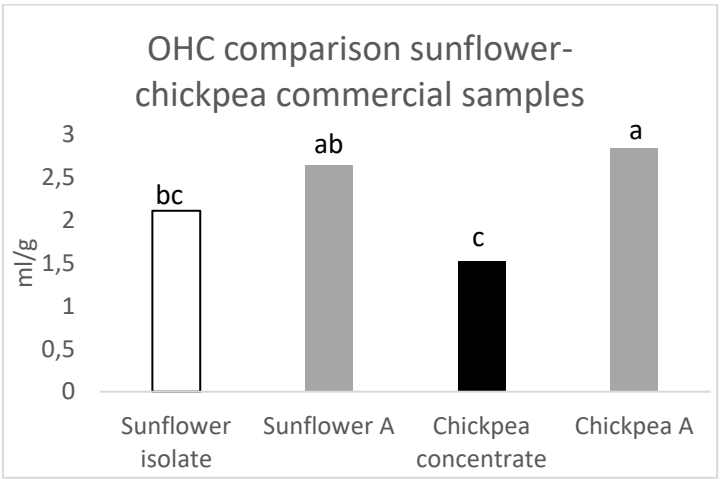
Graph 37. WHC of commercial protein products (sunflower isolate and chickpea concentrate) and the same species processed in this thesis (sunflower and chickpea alkaline extracts)

Concerning oil holding capacity test, faba concentrate and faba isolate show the same value, about 1.14 ml/g (graph 38). Chickpea concentrate and soy isolate have a slightly higher OHC (1.5 ml/g and 1.9 ml/g respectively), whereas sunflower isolate has a good OHC value of 2.4 ml/g.



Graph 38. Oil holding capacity of samples of protein isolates and concentrates (code 33: sunflower isolate, code 54: chickpea concentrate, code 57: faba concentrate, code 68: faba isolate, code 66: soy isolate)

In graph 39, a comparison of OHC values of commercial products (sunflower isolate and chickpea concentrate, code 33 and 54 respectively) and the alkaline extracts of the same species produced in this thesis. The OHC value of sunflower alkaline extract is slightly higher than the value of the commercial sunflower, whereas the OHC value of chickpea alkaline extract is significantly higher than the value of the commercial chickpea proteins.



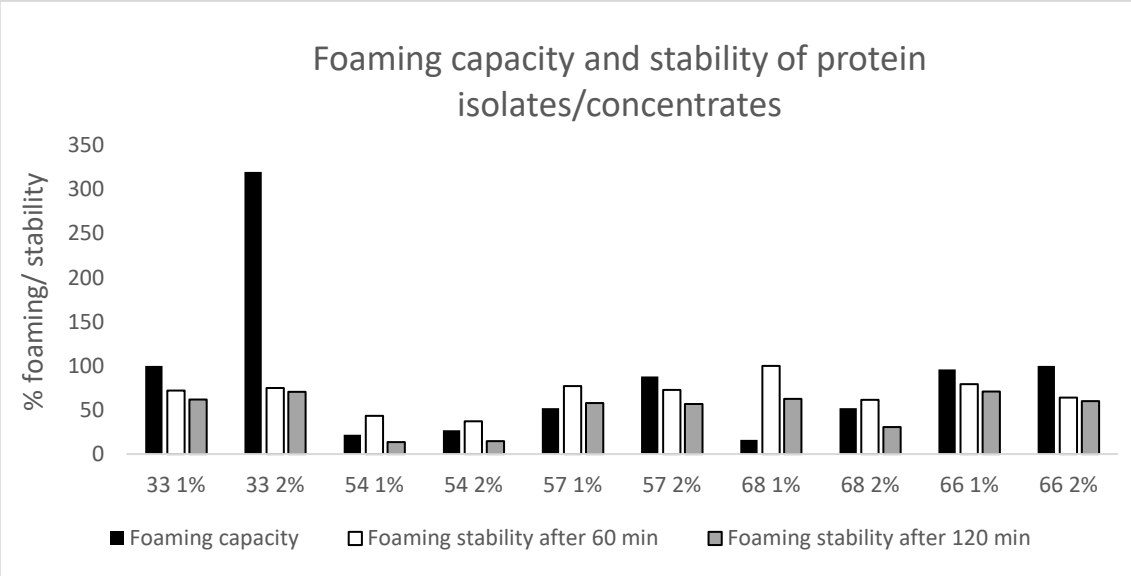
Graph 39. OHC of commercial protein products (sunflower isolate and chickpea concentrate) and the same species processed in this thesis (sunflower and chickpea alkaline extracts)

Gelation capacity of the protein products at 15 and 20 % of concentrations is reported in the table 13. Faba isolate showed an incomplete gelation (score 4) at 20 %, determining an LGC of >20 %. Faba and chickpea concentrates instead reached the score 5 at 20 %, since they were completely gelled. For soy and sunflower isolates, a lower concentration (12.5 %) was tested, since at 15 % they were completely gelled. However, at 12.5 % the gelation was not complete (score 4), thus the LGC for sunflower and soy isolates is 15 %.

Table 13. Gelling capacity of 15% and 20% concentrations of protein isolates and concentrates (code 33: sunflower isolate, code 54: chickpea concentrate, code 57: faba concentrate, code 68: faba isolate, code 66: soy isolate). The score of gelling and the lower

CODE	CONCENTRATION (w/v)	SCORE	Lower Gelling Concentration
33	12.5%	4	15%
33	15%	5	
33	20%	5	
54	15%	4	20%
54	20%	5	
57	15%	4	20%
57	20%	5	
68	15%	2	> 20%
68	20%	4	
66	12.5%	4	15%
66	15%	5	
66	20%	5	

Foaming capacity and stability at 1 and 2 % of concentration of each protein product is showed in the graph 40. Sunflower isolate stands out since at the concentration of 2 % its foaming capacity is more than double of the value found at 1 % (320 % and 100 % respectively). The stability is instead similar between the two concentrations (about 74 % after 1 hour and 67 % after 2 hours). Chickpea concentrate has approximately the same values of foaming capacity and stability at 1 and 2 % of concentration. The FC is 25 % and stability after 1 hour is 40 %, after two hours decreases at 13.5 %. Faba concentrate and isolate show different foaming capacity. Indeed, FC of faba concentrate is higher, 52 % testing the product at 1 % and 88 % testing it at 2 %. The stability is the same at both concentrations, and is 74 % after 1 hour and 57 % after 2 hours. Foaming capacity of faba isolate at 1 % is 16 % and at 2 % is 52%. The stability decreases at 2% of concentration: after 1 hour is 62 % against the 100 % found testing the 1 %, after 2 hours is 31 % against 63 % found at 1 %. The foaming capacity values of soy isolates are similar testing 1 and 2 % of concentrations, whereas the stability is slightly lower at 2 %. The FC is about 98 %, and stability after 1 hour is 79 % testing the 1 % and 64 % testing 2 %. Stability after 2 hours is 71 % at the concentration of 1 % and 60 % at the 2 %.



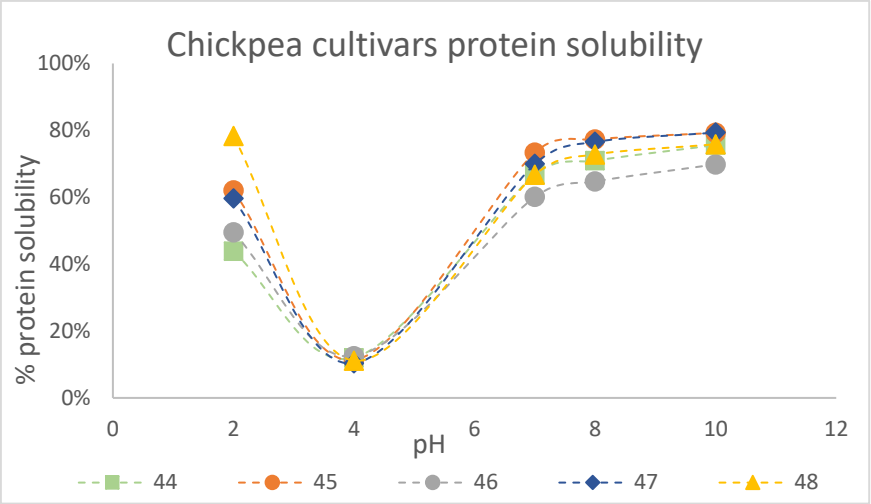
Graph 40. Foaming capacity and stability of protein isolates and concentrates (code 33: sunflower isolate, code 54: chickpea concentrate, code 57: faba concentrate, code 68: faba isolate, code 66: soy isolate). The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

4.6 Chickpea cultivars comparison

Alkaline protein-enrichment process and functional properties investigation of 5 different cultivars of chickpea were performed in order to preliminary compare them about protein-mass yields and functionalities of alkaline extracts.

4.6.1 CHICKPEA SOLUBILITY

Solubility of grinded chickpea cultivars was tested to determine the correct pH to use during alkaline process. As described in graph 41, the trend of solubility depending on the pH shifting is the same between the cultivars: at pH 4 the solubility is very low and increases with the increase (or decrease) of the pH. However, the % of solubility is wide between the cultivars: at pH 2 for example, solubility of 44 cv is 43 % whereas 48 cv has a solubility of 78 %. The solubility % at pH 8 is 65 for the 46 cv and 77 for the 45 cv.



Graph 41. Protein solubility at different pH of 5 chickpea varieties

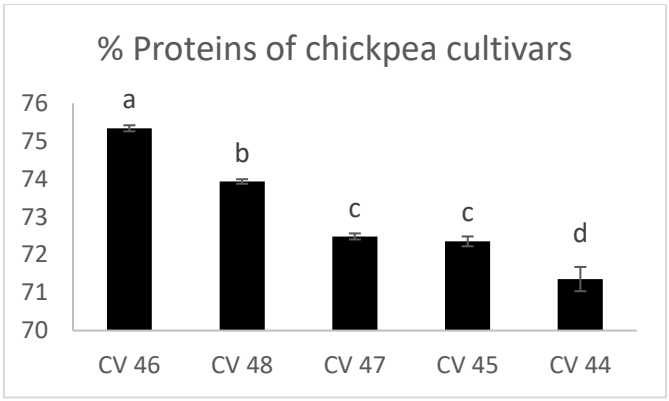
4.6.2 CHICKPEA ALKALINE EXTRACTION

The grinded chickpea cultivars were used to produce protein concentrates using alkaline process. The protein concentration and the mass- protein yields are reported for each cultivar in the table 14.

Table 14. Protein concentration, mass and protein yields after alkaline extraction of 5 different chickpea cultivars

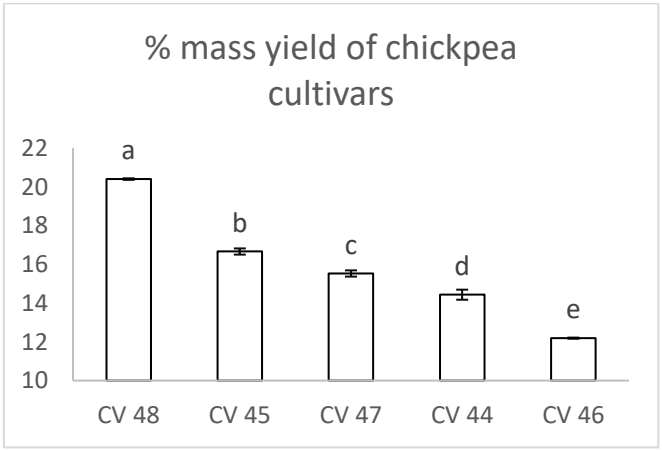
CODE	% proteins	% mass yield	% protein yield
44	71.36 ± 0.32 (d)	14.44 ± 0.26 (d)	49.22 ± 0.59 (d)
45	72.36 ± 0.13 (c)	16.67 ± 0.16 (b)	55.02 ± 0.08 (b)
46	75.34 ± 0.08 (a)	12.19 ± 0.03 (e)	38.71 ± 0.05 (e)
47	72.49 ± 0.08 (c)	15.53 ± 0.16 (c)	51.19 ± 0.06 (c)
48	73.94 ± 0.06 (b)	20.41 ± 0.04 (a)	69.56 ± 0.06 (a)

Alkaline process produced 5 chickpea concentrates (graph 42) that presented similar protein content. The % of proteins indeed vary form 71.36 % of 44 cv to 75.35 % of 46 cv. The differences obtained in the protein content resulted statistically significant.



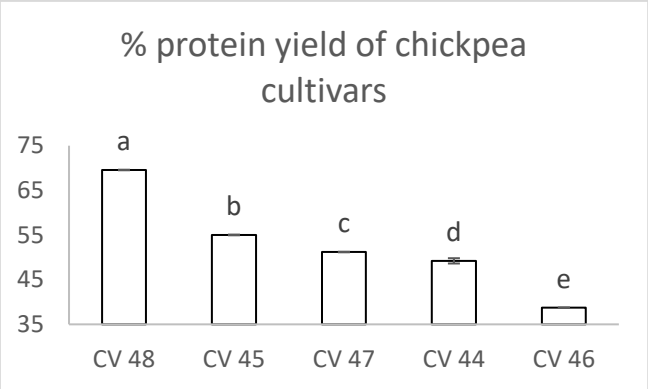
Graph 42. Protein content of the 5 chickpea cultivar extracts

In graph 43, mass yield after protein-enrichment process is reported for each chickpea cultivars. The yields are statistically different, the lowest mass yield is 12,19 % of 46 cv, whereas the higher mass yield is 20.4 % of 48 cv.



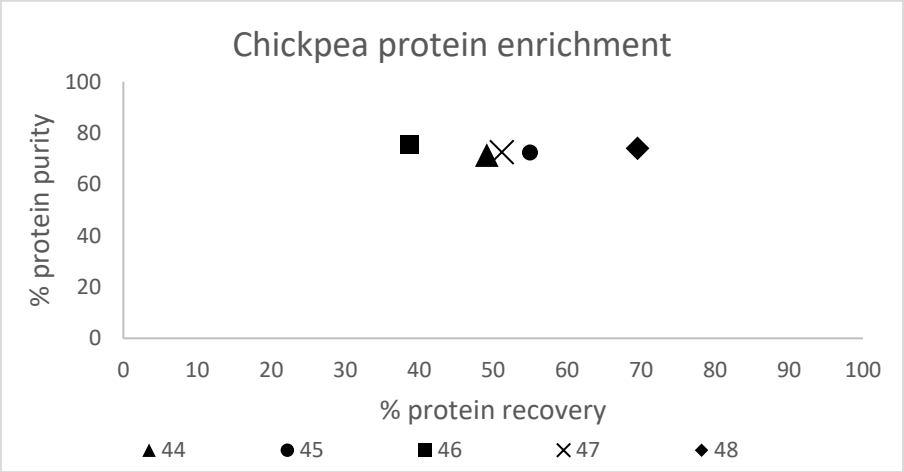
Graph 43. Mass yield of chickpea cultivars after alkaline protein enrichment process

As for the mass yields, the protein yields are significantly different among the cultivars (graph 44): the lowest value is that of 46 cv (38.71 %) and the highest that of 48 cv (69.56 %).



Graph 44. Protein yield of chickpea cultivars after alkaline protein enrichment process

In graph 45, the protein content is plotted with the protein yield: it stands out that the chickpea concentrates reached about the same protein content but have different yields: in fact, cv 46 has the highest protein content, but the lowest yields.



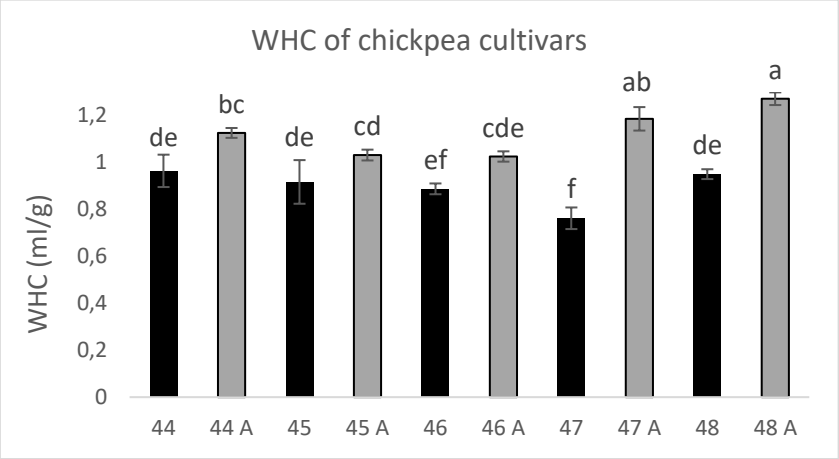
Graph 45. Protein enrichment of 5 chickpea cultivars. in x axis the protein recovery % and in y axis the protein purity %

4.6.3 CHICKPEA WATER AND OIL HOLDING CAPACITY

Water and oil holding capacity were investigated for all the grinded chickpea (starting material) and the related protein concentrates obtained after protein extraction (graphs 46 and 47).

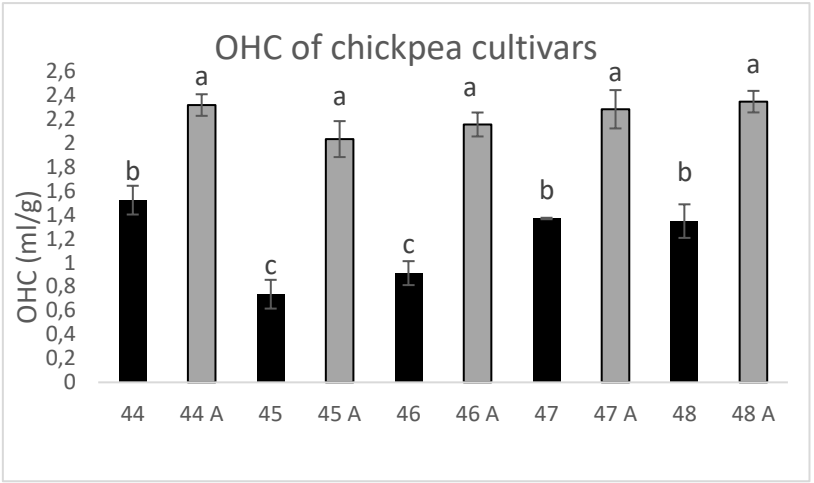
For all cultivars, the alkaline extract shows a slightly higher WHC than the related chickpea flour. The 5 cultivars WHC ranges from 0.76 ml/g (cv 47) to 0.96 ml/g

(cv 44), whereas the extracts WHC range from 1.02 ml/g (cv 46) to 1.27 ml/g (cv 48). The protein concentrate derived from 48 cultivar shows the highest WHC.



Graph 46. Water holding capacity of 5 chickpea cultivars and their alkaline extracts (A)

Concerning oil holding capacity (graph 47), the range of values is wider and as for water holding capacity, the extracts show a higher OHC than the related chickpea flour, meaning that the enrichment process improve these functionalities. The range of chickpea OHC is from 0.7 ml/g (cv 45) to 1.5 ml/g (cv 44). Oil holding capacity of alkaline extracts are similar and do not result significantly different, the lowest is 2.03 ml/g (cv 45) and the highest is 2.34 ml/g (cv 48).



Graph 47. Oil holding capacity of 5 chickpea cultivars and their alkaline extracts (A)

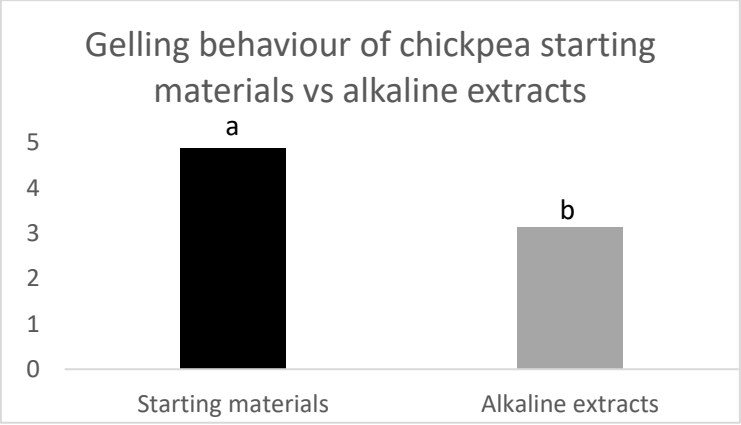
4.6.4 CHICKPEA GELLING CAPACITY

Gelation capacity of grinded chickpea cultivars and related alkaline protein extracts was investigated, and data about scores and LGC are reported in table 15.

Table 15. Gelling capacity of 15% and 20% concentrations of 5 chickpea cultivars. The score of gelling and the lower gelling concentration are reported for each experiment

CODE	CONCENTRATION (w/v)	SCORE	Lower Gelling Concentration
44	7.5%	4	10%
44	10%	5	
44	15%	5	
44	20%	5	
44 A	7.5%	4	10%
44 A	10%	5	
44 A	15%	5	
44 A	20%	5	
45	7.5%	4	10%
45	10%	5	
45	15%	5	
45	20%	5	
45 A	15%	3	> 20%
45 A	20%	4	
46	7.5%	4	10%
46	10%	5	
46	15%	5	
46	20%	5	
46 A	15%	3	> 20%
46 A	20%	4	
47	10%	4	15%
47	15%	5	
47	20%	5	
47 A	15%	3	> 20%
47 A	20%	4	
48	10%	4	15%
48	15%	5	
48	20%	5	
48 A	15%	3	> 20%
48 A	20%	4	

In general, the alkaline process leads to a decrease of gelation property (graph 48), with the exception of 44 cultivar. In fact, extracts 45, 46, 47 and 48 have an LGC >20 %, showing an incomplete gelation at 20 % concentration (score 4). The LGC of these 4 cultivars before protein extraction range from 10 % to 15 %. Cultivar 44 shows the lowest LGC (10 %) and the property is maintained after alkaline extraction.



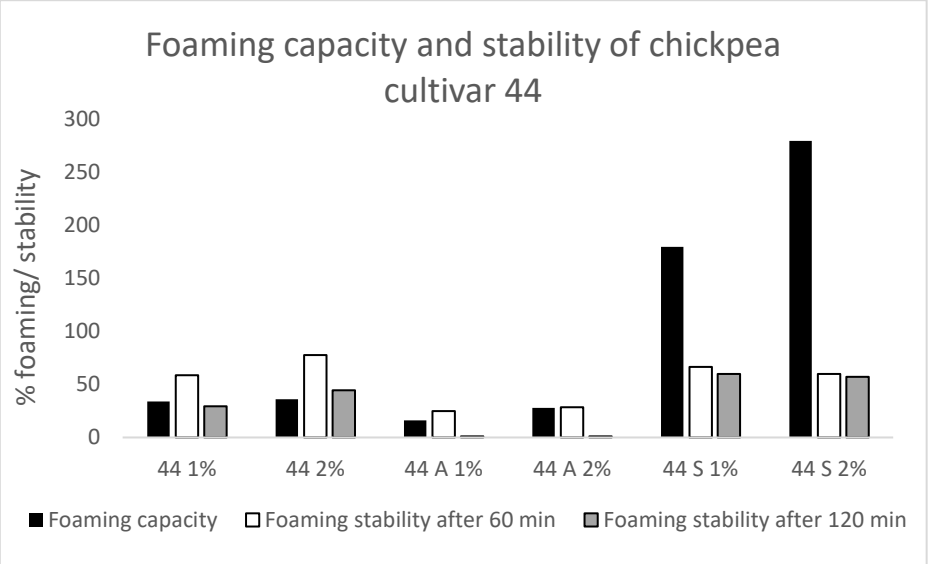
Graph 48. Gelling score of grinded chickpea and protein concentrates

4.6.5 CHICKPEA FOAMING CAPACITY

Foaming capacity and stability were investigated at the concentrations of 1 and 2 %. Chickpea flours and the related alkaline protein extracts and supernatants were tested to detect an increasing or decreasing of this property after the protein enrichment process. For every cultivar tested, alkaline extracts showed an FC lower that the related chickpea flour, whereas all the supernatants had very high foaming property.

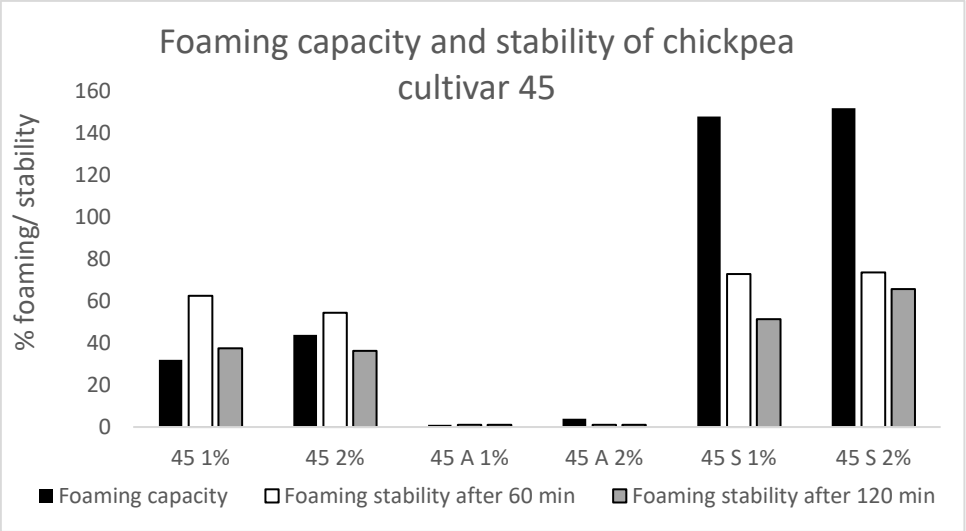
Graph 49 reports the foaming capacity and stability of the cultivar 44. The starting flour at 1 and 2 % has similar foaming capacity (about 35 %) but the stability is slightly higher testing the 2 %: after 1 hour is 77.8 % (testing 1 % is 59 %) and after 2 hours is 44.5 % (testing 1 % is 29.4 %). The foaming capacity is reduced in the alkaline extracts: at 1 % is 16 % and at 2 % is 28 %. The stability at both concentrations is very low: about 26 % after 1 hour, and no stability after 2 hours. The FC values of supernatants is very high: 180 % testing 1 % of supernatant,

280 % testing it at 2 %. The stability is similar between the 2 concentrations, 63 % after 1 hour and about 58 % after 2 hours.



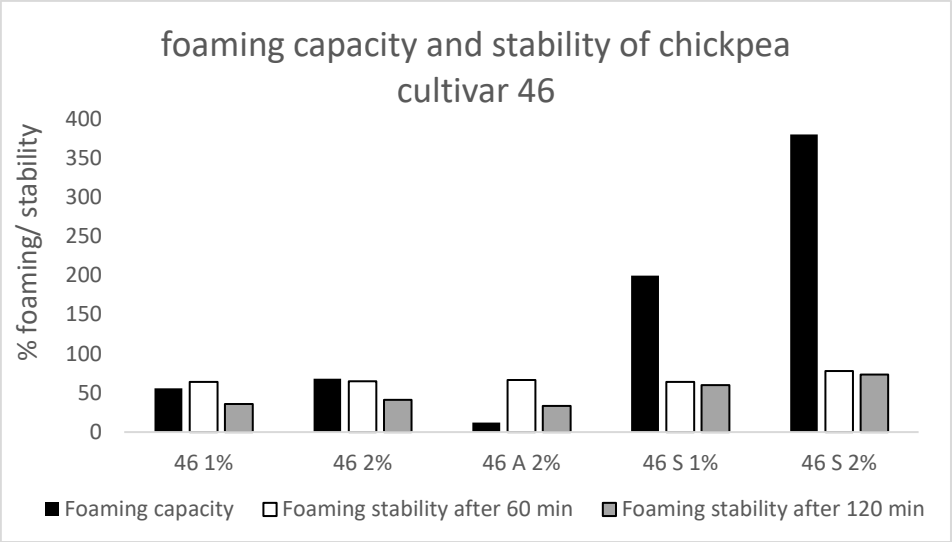
Graph 49. Foaming capacity and stability of 44 chickpea cultivar. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Data about foaming property of chickpea cultivar 45 are reported in the graph 50. The starting flour shows an FC of 32 % at the concentration of 1 %, and 44 % at the 2 %. The stability of the foam is similar between the 2 concentrations, after 1 hour is 62.5 % at 1 % and 54.5 % at the concentration of 2 %. After 2 hours the stability is the same, about 37 %. Basically, no foaming capacity was found testing the protein extract 45. The supernatant FC and stability values are high and similar between the two concentrations tested. FC value is about 150 %, the stability after 1 hour is 73 % and after 2 hours is 51 % at 1 % and 65 % at the 2 % concentration.



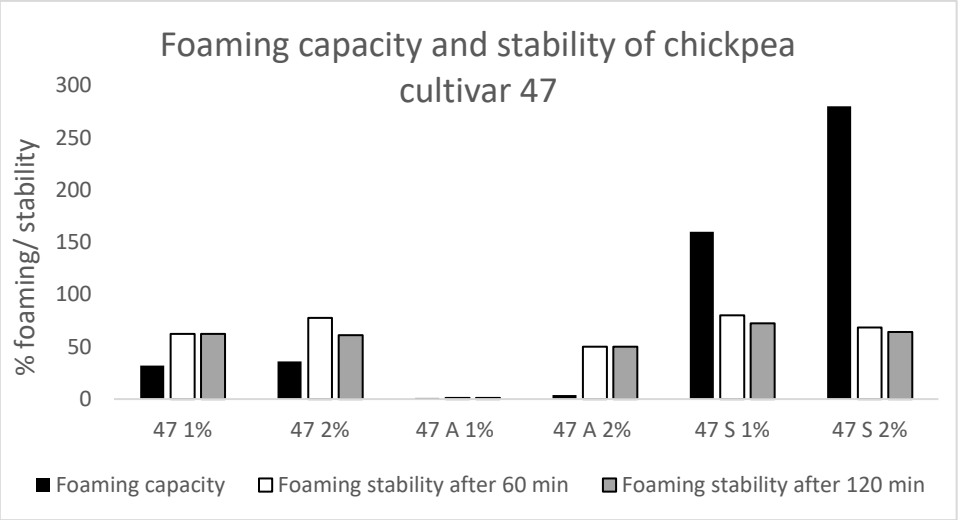
Graph 50. Foaming capacity and stability of 45 chickpea cultivar. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

In the graph 51 are reported the foaming data about the cv 46. Testing the flour at 1 and 2 %, the FC values and stability are similar. The FC in fact is 56 % at 1 % of concentration, 68 % at 2 %. The stability after 1 hour is 64 % and after 2 hours about 38 %. The alkaline extract was tested only at the concentration of 2 %: the foaming capacity was 12 % and the stability after 1 hour was 66.7 % and after 2 hours 33.4 %. The foaming capacity of supernatant was the highest between the chickpea cv supernatants. The foaming capacity at 1 % was 200 %, at 2 % was almost the double, 380 %. The stability testing 1 % was 64 % after 1 hour and 60 % after 2 hours. Testing the 2 %, stability after 1 hour was 77.9 % and 73.7 % after 2 hours.



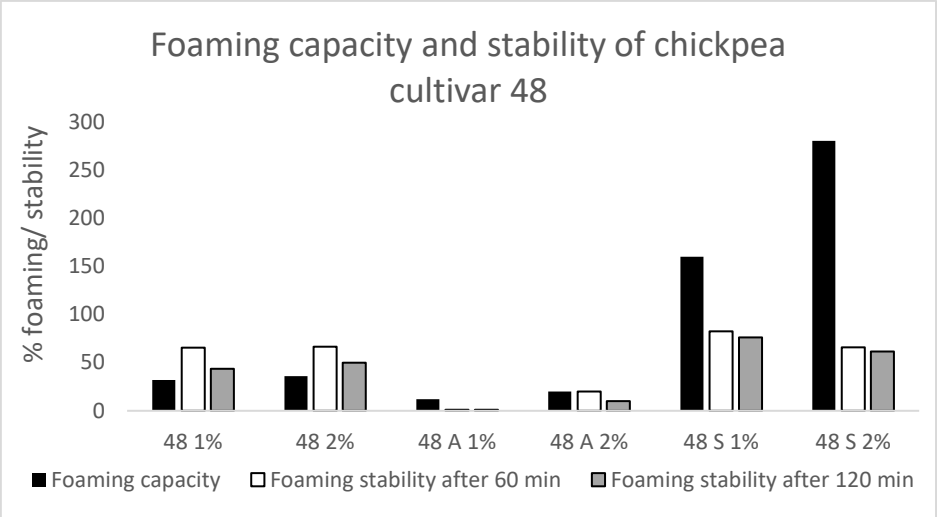
Graph 51. Foaming capacity and stability of 46 chickpea cultivar. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Graph 52 reports data about foaming property of cv 47. The foaming capacity of 47 flour was 32 % testing it at 1 % and 36 % testing at 2 %. The stability after 1 hour was 62.5 % and 77.8 % at 1 and 2 % concentration respectively. The stability after 2 hours was similar between the 2 concentrations and was 62 %. The protein extract showed no foam formation at 1 %, whereas at 2 % the foaming capacity was very low (4 %). The small amount of foam produced had a good stability (50 %) after 1 and 2 hours. As for cv 46, the foaming capacity of the supernatant is almost the double at the concentration of 2 % (280 %) compared to the 1 % (160 %). The stability after 1 hour was 80 % (1 % concentration) and 68.6 % (2% concentration). The stability after 2 hours was similar between 1 and 2 %, 72.5 % and 64 % respectively.



Graph 52. Foaming capacity and stability of 47 chickpea cultivar. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Foaming capacity and stability values of chickpea cv 48 flour are similar to those of cv 47, and are reported in graph 53. The foaming capacity in fact, are 32 % at 1 % of concentration tested and 36 % at 2 %. The stability of the foam is similar testing the 2 concentrations: 66 % after 1 hour and about 47 % after 2 hours. The protein extracts of 48 cv showed a slightly higher foaming capacity compared to the other chickpea extracts: 12 % testing 1 % of extract, 20 % testing it at 2 %. However, at 1 % of extract there was no stability of the foam, whereas at 2 % the stability was 20 % after 1 hour and 10 % after 2 hours. The supernatant values of foaming property are basically the same of cv 47. At 1 % of supernatant in fact, the foaming capacity is 160 %, with a stability after 1 hour of 82.5 % and after 2 hours 76,3 %. The FC at 2 % of concentration was 280 %, with a stability of 65.7 % after 1 hour and 61.4 % after 2 hours.



Graph 53. Foaming capacity and stability of 48 chickpea cultivar. The black bars indicate the ability to create foam, the white bars indicate the stability of the formed foam after 1 hour and the grey bars the foam stability after 2 hours

Chapter 5 Dry protein enrichment and texturizing

5.1 Aim of the experiments

Currently most of plant proteins are obtained by wet fractionation, that has the advantage to produce pure protein isolates, but consumes a large amount of water and energy to dry, producing many wastes. Furthermore, the proteins are often denatured by acids and bases used during process, causing loss of native functionality.

A sustainable alternative to produce plant proteins is dry fractionation. The evident advantages of this process are: use of very low amount of water, stabilization and dry post-process are not required and it is easily scalable (Schutyser & van der Goot, 2011). The drawback of dry separation is a lower yield and purity compared to wet fractionation. However, this is compensated by the maintenance of proteins functionality. Moreover, the fraction obtained contains other components (such as oil, fibre, bioactive molecules, etc.) that could improve the rheological characteristics of the final products, and positively impact in human health (Van Der Goot et al., 2016).

Protein concentrates and isolates are used to produce meat-like products plant based, that can replicate meat in terms of mouthfeel, texture, taste, colour and smell. The market of this kind of food is growing very fast, many companies are producing this type of meat-analogues, and the main process used to obtain them is the extrusion of protein fractions. Other technologies are emerging to produce structure-meat like products, for example the shear-cell technology. Shear cell technology is a technique developed at Wageningen University & Research in which heating and shearing are used to produce anisotropic fibres in the protein products.

In this project, both dry fractionation techniques (air classification and electrostatic separation) were preliminary tested with the aim to enrich in proteins the sunflower flour and camelina cake. The parameter mostly varied during experimental session was the particle size of the starting material, obtained

through sieving and two different type of milling. Protein and mass yields were calculated in order to compare with wet extraction. Further goal was to test the obtained protein fractions using the shear-cell laboratory scale device, in order to verify if the proteins, alone or mixed with gluten or other flours, generate a structured fibrous product.

5.2 Materials and methods

The following described experiments were performed at Wageningen University Research (NL) and provided preliminary results about dry protein enrichment and texturizing of sunflower and camelina. The results need to be confirmed with further tests at the same conditions.

5.2.1 SAMPLES

Two kinds of sunflower flour and camelina seeds were used to test the dry protein enrichment and the structuring process shear-cell technology. The main sunflower flour used (code 30) was produced by CD company and derived from chemical oil extraction, and its protein content was 26.8 %. The second sunflower flour is also derived from chemical oil extraction, then sieved at 0.5 mm, extruded and milled at 0.15 mm (code 130). The protein content of this particular flour was 38 %. Camelina seeds (code 27) were kindly donated by Bologna University (DISTAL department).

Additional experiment of dry protein separation and structuring process were conducted using commercially available flours of red lentils (25 % proteins), faba bean (26% proteins) and lupin (39% proteins).

5.2.2 MECHANICAL OIL EXTRACTION

The oil from the camelina seeds was removed to improve the subsequent protein enrichment. The cake was obtained using a lab-scale screw press (KK20F Universal, Kern Kraft, Figure 16). The screw was pre-heated at 80°C for 20 minutes to prevent the screw block adding the seeds. The pressing was then started using the drill jet number 11 and the feed rate between 15 and 20 rpm. During the oil extraction the temperature of the screw was constantly monitored and was about 90°C. The mentioned conditions were established after some tests.

Figure 16. Screw press used for mechanical oil extraction

The residual oil of the camelina cake was measured with automatic soxhlet equipment and was about 8%. The cake was then roughly milled using a simple miller (figure 17).

Figure 17. camelina cake pellet after oil removal and after coarse milling.

5.2.3 SIEVING AND MILLING

Sunflower flour was sieved using three different sizes (0.4, 0.5 0.8 mm) to partially remove fibres and compare the protein enrichment of no-sieved samples. For the sieving process an air-jet sieve was used (E200 LS, Hosokawa Alpine).

Since the particle size of the starting material is the crucial feature implicated in the dry separation processes, two kind of milling were performed. In details:

- sunflower flour and sunflower flour sieved at 0.8 mm were milled with a rotomiller (figure 18, Pulverisette 14, Fritsch) at 0.5 mm with a speed of 15.000 rpm;

Figure 18. Rotomiller used to grind sunflower flour at 0.5 mm

- Impact miller (figure 19, 50 ZPS, Hosokawa Alpine) was used to obtain very thin and humogen material. This mill contains an internal rotating classifier wheel that allows the passage of fine particles, while coarse particles are returned and further milled. Sunflower flour sieved at 0.8 mm was milled at ZPS speed of 10.000 rpm, sunflower sieved at 0.5 mm at the speeds of 4.000 and 7.000 rpm. Camelina cake was milled at ZPS speed of 7.000 rpm.

Figure 19. Impact miller used to grind the material at very small particle size

5.2.4 PROTEIN ENRICHMENT-AIR CLASSIFICATION

Air classification is a dry method to obtain protein-enriched material starting from flours. This technique is based on the size and density of the particles of the starting flour, that is the reason why milling is the crucial pre-treatment. Indeed, improvement in purity and yield could be obtained with a degree of milling in which the non-protein components, like starch and fibres, should remain larger than the protein bodies (Pelgrom, Boom, et al., 2015). Other treatments could increase purity and yield of air classification: for example, before milling, variation of moisture or hulls/oil removal are effective depending on plant seed considered (Pelgrom, Wang, et al., 2015). The parameters of classifier that could be varied based on protein source and the degree of milling are the air flow and the wheel speed.

The flour is placed into the feeder and is taken up in the classifier chamber by air flow, the small and light particles will be taken higher than heavy and large particles. At the top, a classifier wheel with slits rotates: the small particles go through the slits and arrive in the left bottle (fine fraction), larger particles leave the classifier at the bottom and arrive into the right bottle (coarse fraction) (figure 20). The size of the particles that can pass the slits decreases with the speed of the classifier.

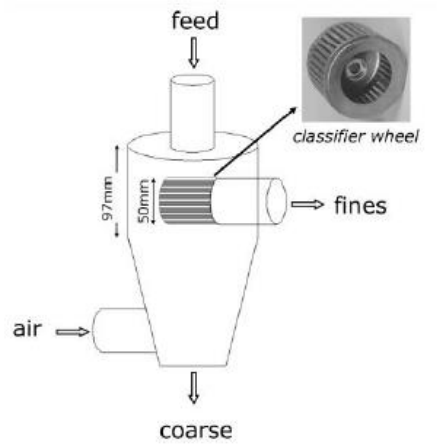


Figure 20. Rappresentation of functioning of air classifier ATP50.

In this study, an amount between 150 and 300 g of flour was placed into the feeder of the air classifier (figure 21, ATP 50, Hosokawa Alpine). The air flow was kept constant for all the experiments at 60 m^3/h and the wheel speed varied between 2.500 and 5.000 rpm. In table 16 are listed the experiments performed in duplicate.

Figure 21. Air classifier ATP 50. In detail in the right picture, the fine fraction (left) and coarse fraction (right)

Table 16. Summary of the air classification experiments performed

STARTING MATERIAL	SIEVING	MILLING	PROTEIN CONTENT %	WHEEL SPEED (rpm)
Sunflower flour code 30	0.8 mm	/	30	2.500
Sunflower flour code 30	0.4 mm	/	37	2.500
Sunflower flour code 30	/	0.5 mm rotomiller	28.8	5.000
Sunflower flour code 30	0.8 mm	0.5 mm rotomiller	33	5.000
Sunflower flour code 30	0.5 mm	impact mill ZPS 4.000rpm	33	5.000
Sunflower flour code 30	0.5 mm	impact mill ZPS 7.000rpm	41	5.000
Sunflower flour code 130	/	/	38	2.500
Camelina cake	/	/	41	2.500
Camelina cake	/	impact mill ZPS 7.000rpm	41	3.000
Camelina cake	/	impact mill ZPS 7.000rpm	41	5.000

5.2.5 PROTEIN ENRICHMENT-ELECTROSTATIC SEPARATION

Electrostatic separation is a dry protein enrichment method based on differences in triboelectric charge of the components. Due to the presence of the electrical field between the electrodes, positively charged particles move towards the grounded electrode and the negatively charged particles towards the positive electrode. Protein bodies are positively charged and move to the negative electrode.

The advantage of electrostatic separation compared to air classification is that the particles generated during milling could be similar as they are separated based on their charge. Anyway, milling is a crucial step also for this technique, since too coarse or thin materials could clog the charging tube. Another important parameter is the charging tube, where flour particles are tribo-electrically charged due to particle-particle and particle-wall collisions.

In this study, a custom-built bench scale electrostatic separator was used (Figure 22). A variable amount of the flour to test (from 30 to 150 g) was placed into the feeder and passed into the linear steel charging tube. The charged particles arrived into the separation chamber, and based on their charge were attracted to the opposite electrode. Each electrode has a rotating belt where the particles adhered, and a brush enhance to collect the material in the two boxes (figure 22, right). The material that did not adhere to the belts, were collected in two bag filters below the separation chamber. The feed rate and the voltage used were kept constant in each experiment and were 0,5Kg/h and 20.000 V respectively.

Figure 22. Electrostatic separator 1 Material feeder, 2 charging tube, 3 separation chamber containing electrodes. In detail to the right, the separation chamber containing the electrodes, the rotator belts and the collection boxes.

In the table 17, the list of the samples and their pre-treatments used to test the electrostatic separation technology.

Table 17. List of the samples used for electrostatic separation tests.

STARTING MATERIAL	SIEVING	MILLING	PROTEIN CONTENT %
Sunflower flour code 130	/	/	38
Sunflower flour code 30	/	0.5 mm rotomiller	26.8
Sunflower flour code 30	0.8 mm	impact mill ZPS 10.000rpm	32.7
Sunflower flour code 30	0.5 mm	impact mill ZPS 4.000rpm	33
Sunflower flour code 30	0.5 mm	impact mill ZPS 7.000rpm	41
Camelina cake	/	impact mill ZPS 7.000rpm	41

5.2.6 TOTAL NITROGEN CONTENT-DUMAS METHOD

Dumas method is an easy and fast technique to measure the nitrogen content in organic samples. This method is divided into 3 phases called combustion, reduction and separation and detection. During combustion, the sample is heated at 1.000°C in the presence of pure oxygen producing different substances, above all nitrogen oxides. The gas mixture is passed through a reduction chamber containing copper heated to around 650 °C. This stage converts nitrogen oxides into elemental nitrogen and collects the oxygen in excess. The measured signal from the thermal conductivity detector for the sample is converted into total nitrogen content.

Dumas method was used to measure the nitrogen content of all fractions produced during the dry protein enrichment. 20 mg of the sample was weighed into small aluminium cup and placed into the dumas analyser (Flashsmart, Thermoscientific). Methionine was used as positive control and as standard curve (1,5, 10, 15, 20, 25, 30 mg). Cellulose was used as blank. 6.25 as conversion factor for food proteins was used as in Kjeldahl method to calculate the total nitrogen of the samples.

5.2.7 PARTICLE SIZE DISTRIBUTION AND SEM PICTURES

The milling of the material is the crucial step to optimize the dry separation, since the aim of the grinding is to release the protein bodies without break them. The size of the protein bodies could vary based on the protein source considered; in case of sunflower and camelina the size is about 10-15 µm.

The particle size distribution and the display of the fractions obtained from the dry fractionation processes at scanning electron microscope were performed to verify the presence of the protein bodies.

The distribution of the particles of the materials was verify using a laser diffraction particle size analyser (Mastersizer 3000, Malvern Instruments). The feed rate and the pressure were varied depending on the material tested. In general, 2 bar and 80% of feed rate were set, but different values as 3/4 bar and 60/70% of feed rate

were adjusted in case of sticky material. For both sunflower and camelina the refractive index was 1.47, and the scattering model assuming a non-spherical shape of the particles. For every sample, a minimum of 3 measurements of 60 seconds were performed.

Pictures using a scanning electron microscope (JEOL JCM-7000, Tabletop SEM) were taken at the magnifications of 240 X and 930 X, in order to verify the size, type and shape of the particles of the obtained material.

5.2.8 STRUCTURING PROCESS- SHEAR CELL TECHNOLOGY

The main process used from food industries to produce plant-based meat analogues is the extrusion of the proteins mixture, obtaining a sort of gelling product. Structured products more similar to meat could be achieved through the extrusion with application of simple-shear and heat in Shear-cell or Couette-cell devices. Indeed, the application of simple-shear and heat produces an anisotropic-fibrous product, similar to the meat (Krintiras et al., 2016).

Protein fractions obtained from the dry separation were tested on their own and mixed with other protein materials, in order to evaluate the visible features of the product obtained after the shear-structuring process.

Firstly, preliminary tests were conducted at very small scale using closed cavity rheometer (figure 24, CCR TA Instruments) in order to evaluate the properties using small amounts of material. The principle of the CCR is the same of the shear-cell technology, heat and rotational shearing is indeed apply to the sample and returns information about the changes in elasticity property varying the temperature. A visual evaluation about gelation of the material allows to understand if the mixture could be applied to the next scale. A total amount of 9 g of mixture were prepared mixing the protein material at 40 % dry matter and NaCl 1 % with water (figure 23).

Figure 23. Mixture of protein ingredients prepared for closed-cavity rehometer. The first two pictures show mixtures of sunflower protein fraction from electrostatic separation and camelina fine fraction from air classification. In the two pictures in the right side, faba bean and lentils commercial flours were used for the mixture, but resulted too liquid and unsuitable for CCR-test

The mixture was then placed between two plastic sheets and placed in the CCR plate avoiding air bubbles. The programme of the CCR started at 40°C and the temperature increased to 150°C with a constant rotation of the plate. After reaching 150°C, the temperature decreased to 40°C and the storage-loss modulus were constantly measured.

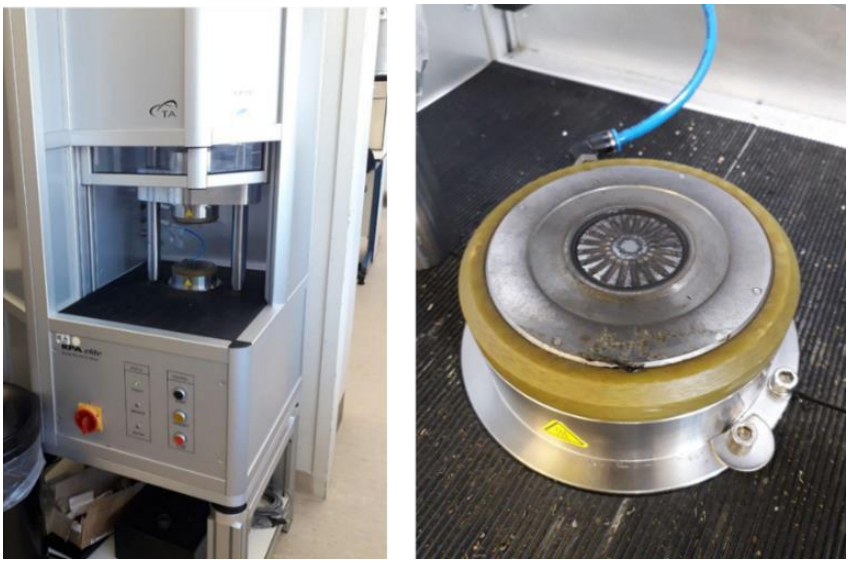


Figure 24. Closed cavity rheometer. In detail, the plate where the protein mixture is placed. The plate above close the material and rotate to produce the shearing

Once identified the mixtures that produced at least a gelled product using CCR, the tests were conducted using the shear-cell device (figure 25, Haake Polylab QC-drive, Thermoscientific). The standard protocol was 15 minutes at 120°C, the

rotation was constant at speed of 30 rpm. The mixture of 90 g was prepared mixing 40 % dry matter of the protein materials and NaCl 1 % with water and allow to rehydrate for 30 minutes before starting the extrusion. Following the mixtures prepared using the fine fraction obtained from air classification:

1) Fine fraction of sunflower flour milled at 7.000 rpm
2) Fine fraction of sunflower flour milled at 7.000 rpm and gluten (1:1)
3) Fine fraction of sunflower flour milled at 7.000 rpm and fine fraction of camelina cake (1:1)
4) Fine fraction of camelina cake and gluten (1:1)
5) Fine fraction of camelina milled at 7.000 rpm
6) Fine fraction of camelina milled at 7.000 rpm, fine fraction of 130 sunflower flour, gluten (1:1:1)
7) Fine fraction of sunflower flour milled at 7.000 rpm, lentils flour, gluten (1:1:1)
8) fine fraction of 130 sunflower flour, lupin flour, gluten (1:1:1)
9) fine fraction of 130 sunflower flour, faba flour, gluten (1:1:1)

Figure 25. Shear-cell device. In detail, the cone where the mixture was placed. When the cone is closed, it rotates to produce the shearing

5.3 Results

One of the key parameters for the efficacy of the dry separation is the particle size of the starting material. In figure 26 and graph 54, a comparison of the sunflower flour (code 30) milled at different conditions. The sample was milled using the rotomiller at 0.5 mm and through impact milling at 4.000, 7.000, 10.000 rpm. The picture at scanning electron microscope (figure 26) shows the diversity of the milling: it is clear that the impact miller grinded the sample at lower particle size, further breaking the fibres and producing as much round particles compared to the rotomiller at 0.5 mm.

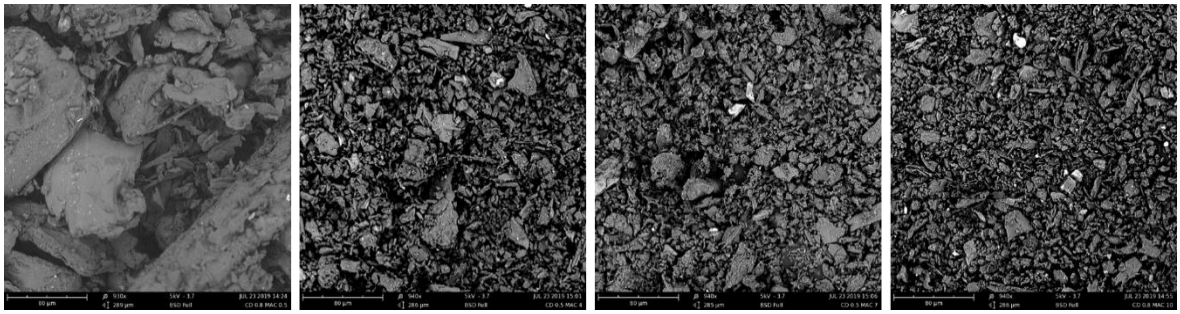
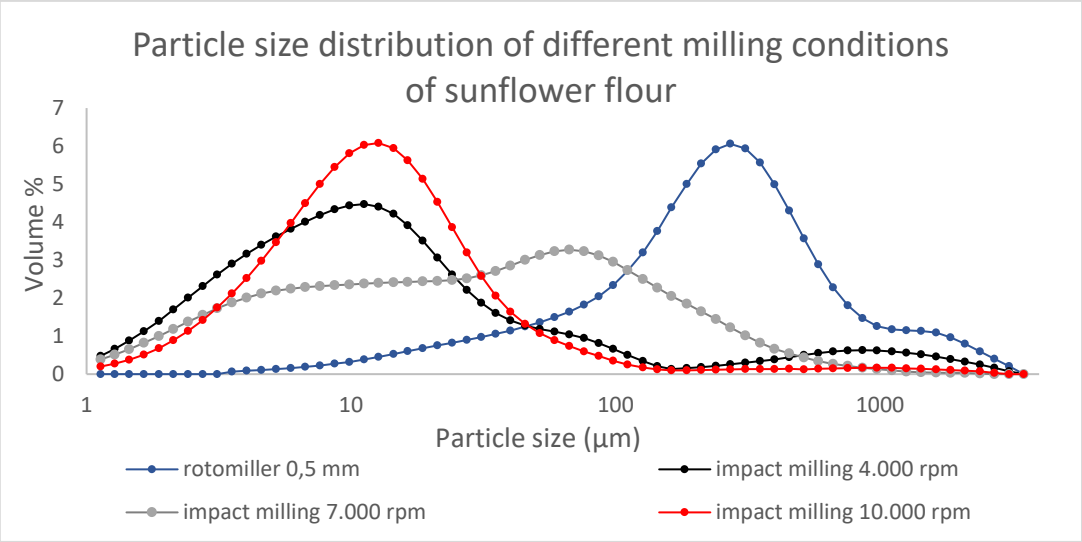


Figure 26. Sunflower flour (code 30) milled, from left: 0,5 mm with rotomiller, 4.000 rpm though impact milling, 7.000 rpm and 10.000 rpm. Pictures taken using SEM at the same magnification (940 X)

Graph 54 reported the particle size distribution: the most quantity of the particles of the sample milled with rotomiller are comprised between 100 and 1.000 µm (peak around 300). Sunflower milled through impact milling at 4.000 rpm highlight the main peak at 10 µm, comprising particles between 1 and 100 µm. The sample has also a small number of particles around 1.000 µm. Sunflower milled at 7.000 rpm does not show a unique peak, but the particles seems to be divided into two population: one lower than 10 µm and the other around 70. The sample milled at 10.000 rpm presents a narrower peak at about 12 µm, partially overlapped with the samples milled at 4.000 and 7.000 rpm.



Graph 54. Particle size distribution of sunflower flour code 30 milled at different conditions

5.3.1 AIR CLASSIFICATION ENRICHMENT

Camelina meal (code 27) and sunflower flours (codes 30 and 130) were used to test the dry protein enrichment using air classification method.

In each test performed, the fine fraction (usually richer in proteins) and coarse fraction output of the air classifier were visually distinguishable (figure 27), since in the fine fraction the particle size was lower and the colour lighter compared to the coarse fraction.

Figure 27. Fine and coarse fractions after air classification process of sunflower flour (left) and camelina meal (right)

In some experiments of sunflower, in the fine fraction, was distinguishable also a "lighter layer" (figure 28) that was also analysed to determine the protein content.

The lighter layer showed a protein content slightly higher than the fine fraction, and a lower particle size (graph 56).

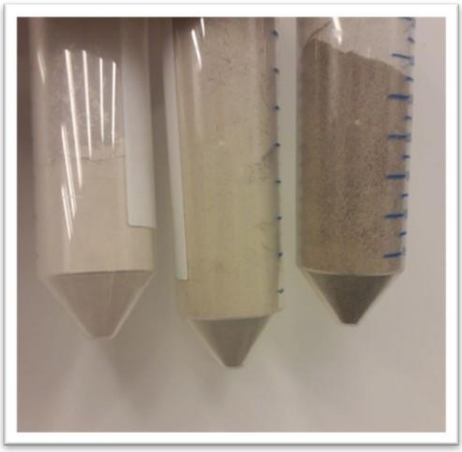


Figure 28. Fine and coarse fractions after air classification process of sunflower flour (code 30). In some tests, the fine fraction had a lighter layer (first falcon from left) richer in proteins

Images taken using scanning electron microscope showed the differences about size and shape of the particles of the fine and coarse fractions produced during air classification experiments. The particle size depends obviously on the degree of milling of the starting material, by the way in each experiment the differences between the FF and CF were clear. In figure 29, an example of FF and CF of sunflower at 930 X of magnification: in the fine fraction the protein bodies (<10 µm) may be visible.

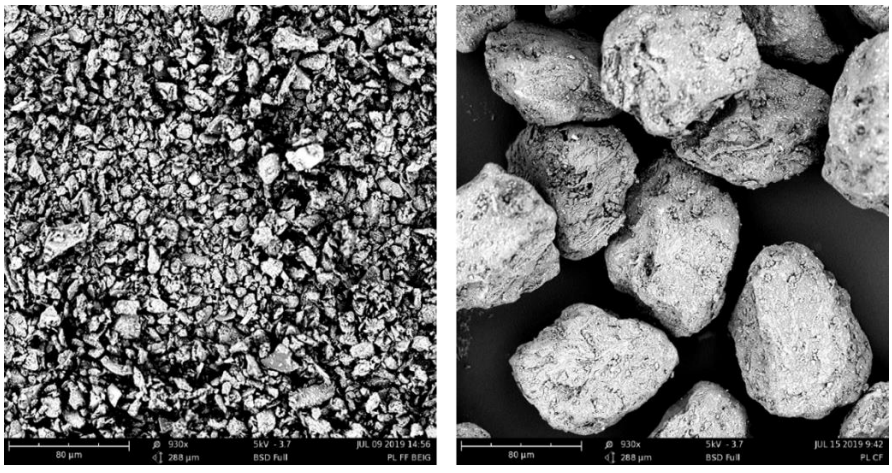


Figure 29. Sunflower fine and coarse fractions derived from air classification process, at the same magnification of 930 X. In the fine fraction, protein bodies could be distinguishable

In table 18, the main experiments performed using air classification process. Protein content, mass and protein yields are reported for both FF (fine fraction) and CF (coarse fraction) obtained in each test.

Concerning sunflower tests, the experiments conducted using sunflower flour not milled or milled at 0.5 mm led to a minimum enrichment (1-3 %) or a zero-protein enrichment in the fine fractions. In these cases, the coarse fraction often had about the same amount of proteins %, with a protein yield higher compared to the FF. Interestingly, sunflower only sieved at 0.4 mm resulted in a protein increasing in the coarse fraction (+ 4 %) and a decreasing in the fine fraction (- 6 %). Sunflower flour milled using impact miller, showed a slightly higher protein increasing (2-3 %), and the protein yield of FF is higher than the CF. The mass yield followed the same trend of the protein yield: in the experiment in which sunflower flour not milled and milled at 0.5 mm with rotomiller were used, the mass yield was higher in the CF. In the experiments in which were used samples milled with impact miller, the mass yield was higher in the fine fraction.

Considering camelina meal, three tests were conducted, one with camelina coarsely milled after oil extraction, and two with camelina milled using impact miller at 7.000 rpm. The camelina coarsely milled led to a protein enrichment of 4.5 %. By the way, protein and mass yields are clearly higher in the coarse fraction compare to the fine fraction. The two tests in which camelina milled with impact miller was used, differed for the wheel speed and the results are different. The experiment with the wheel speed settled at 3.000 rpm, the protein enrichment is about 3.5 %, and mass and protein yields are much higher in the fine fraction compared to the CF. The experiment with the wheel speed settled at 5.000 rpm led to a minimal enrichment (1 %). However, also in this case, mass and protein yield are higher in the FF compared to the CF.

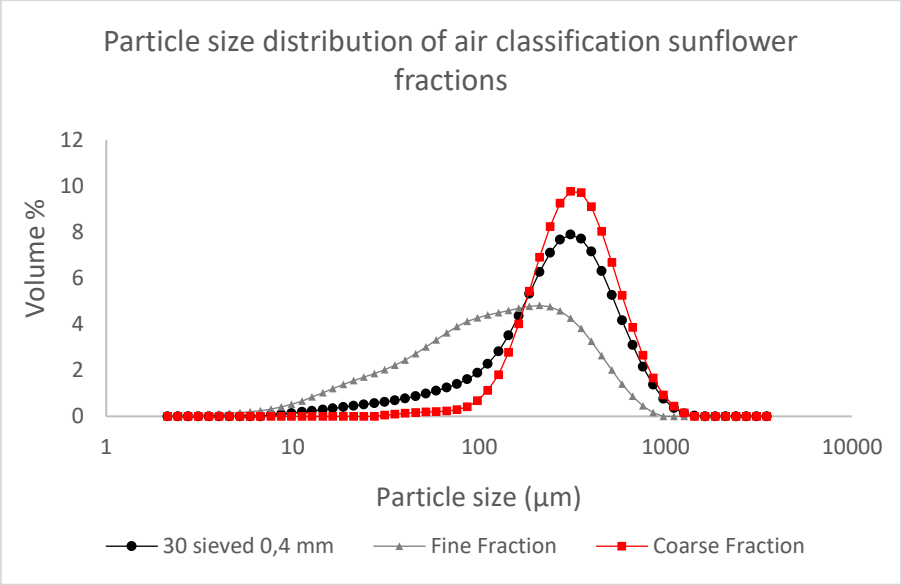
Table 18. Protein content and mass-protein yields of the fine and coarse fractions after air classification protein enrichment

CODE	SIEVING/ MILLING	WHEEL SPEED (rpm)	% PROTEIN FF	% PROTEIN CF	% MASS YIELD FF	% PROTEIN YIELD FF	% MASS YIELD CF	% PROTEIN YIELD CF
30	sieving 0.8 mm	2.500	33.20	35.00	22.08	23.95	65.38	71.15
30	sieving 0.4 mm	2.500	31.00	41.00	28.33	23.72	66.67	73.07
30	milling 0.5 mm (rotomiller)	5.000	26.6	23.3	9.4	9.4	77.8	67.6
30	sieving 0.8 mm and milling 0.5 mm (rotomiller)	5.000	31.5	32.2	12.8	12.0	73.3	70.3
30	sieving 0.5 mm and milling 4.000 rpm (impact miller)	5.000	35.4	32.2	62.8	58.5	20.0	16.9
30	sieving 0.5 mm and milling 7.000 rpm (impact miller)	5.000	44	39.6	59.5	68.1	28.0	29.2
130	(previously sieved-extruded and milled)	2.500	42.8	30.2	73.3	82.6	20.0	15.9
27	camelina meal	3.000	45.4	38.5	11.4	12.7	86.2	81.0
27	milling 7.000 rpm (impact miller)	3.000	44.3	39.4	84.4	91.2	6.7	6.4
27	milling 7.000 rpm (impact miller)	5.000	41.4	39.7	58.9	59.4	7.8	7.5

In the following graphs (55, 56, 57, 58), data about particle size distribution of some air-classification fractions are reported.

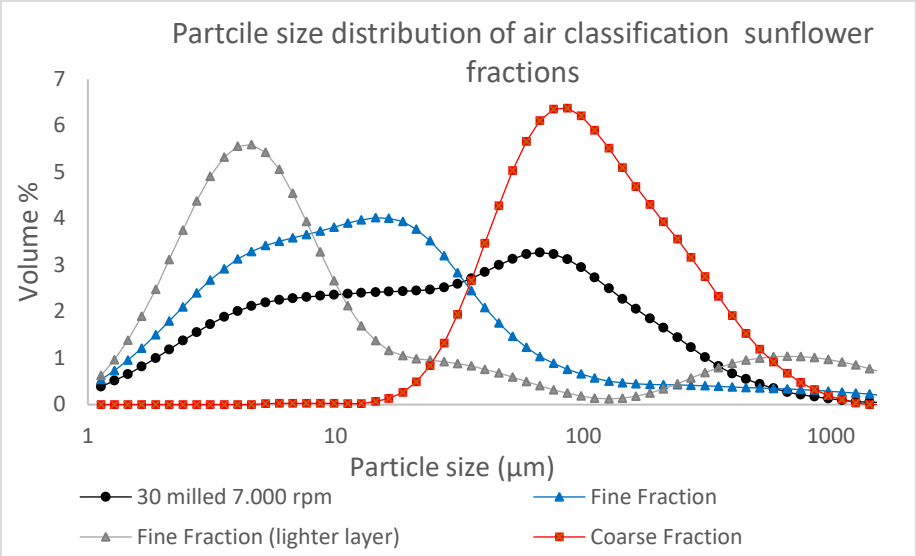
Graph 55 reports the data about sunflower flour sieved at 0.4 mm and not milled, and the related fine and coarse fractions obtained from air classification. The peaks of the sieved flour and the coarse fraction are overlapped and most

particles had a size of about 350 µm. The area of the fine fraction is larger and partially shifted toward lower particle size (70-310 µm).



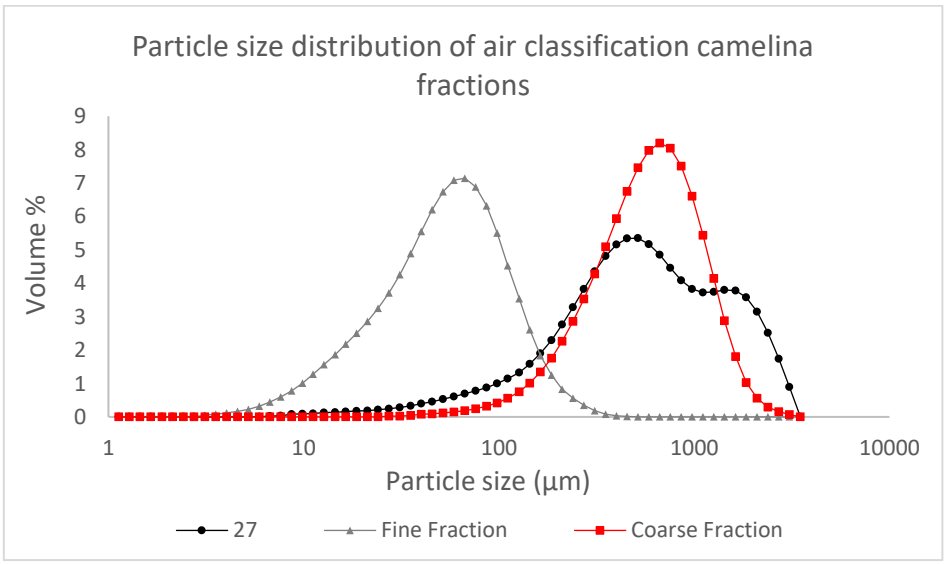
Graph 55. Particle size distribution of sunflower flour (code 30) sieved at 0,4 mm and fine and coarse fractions derived from air classification. The peaks of the sieved flour and the coarse fraction match each other, whereas the peak of the fine fraction is partially shifted to lower particle size

The graph 56 showed the data about particle size of sunflower milled at 7.000 rpm through impact milling, and the related FF and CF. In this case, the lighter layer was visible within the fine fraction, and was comprised in the particle size analysis. The range of the particle size of sunflower milled at 7.000 rpm is wide (most particles are comprised between 5 and 80 µm) with a not-well defined peak. The fine and the coarse fractions are almost completely separated, the FF has the most volume of particles around 14 µm, whereas CF has a peak around 90 µm. Interestingly, the lighter layer is partially overlapped with FF and the most part of particles are comprised between 1 and 10 µm (peak at 4.5 µm).



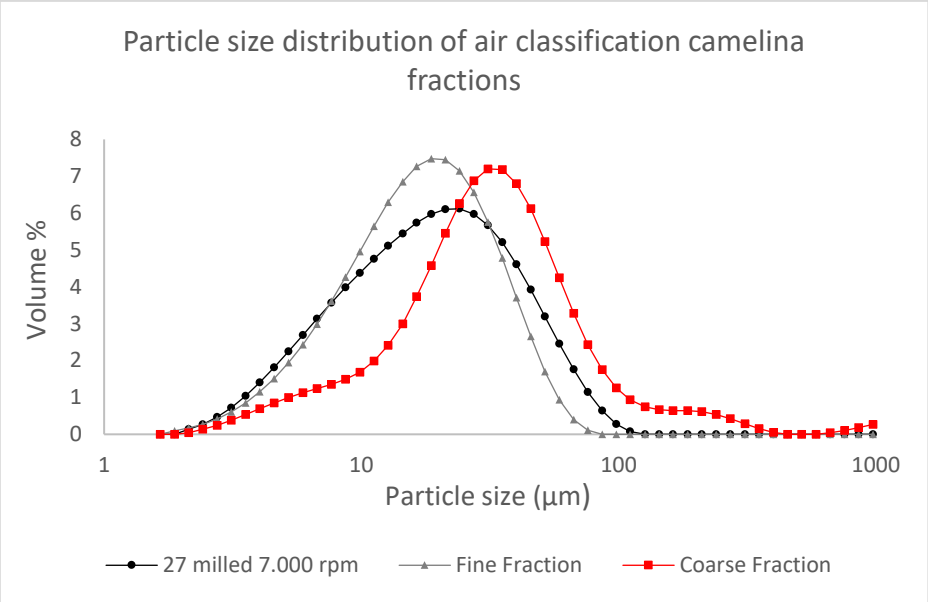
Graph 56. Particle size distribution of sunflower flour (code 30) milled at 7.000 rpm through impact milling and fine and coarse fractions derived from air classification. The peak of the lighter layer of the fine fraction is also showed in the graph and comprises particle of size between 1-10 µm

The graph 57 reports the data about particle size distribution of camelina meal coarsely grinded (code 27) and the related fine and coarse fractions. The camelina meal presented 2 peaks and the size range is wide, the higher peak around 520 µm and the smaller at about 1500 µm. Coarse and fine fractions peaks are almost completely separated, the CF has the peak at around 760 µm, while the peak of FF is around 70 µm.



Graph 57. Particle size distribution of camelina meal (code 27) coarsely grinded and fine and coarse fractions derived from air classification. The peaks of the meal and the coarse fraction match each other, whereas the peak of the fine fraction is partially shifted to lower particle size

Particle size data of camelina meal milled with impact miller at 7.000 rpm and the FF-CF are reported in the graph 58. The three peaks are partially overlapped: the main peak of the FF is at 20 µm, while the peak of CF is around 35 µm.



Graph 58. Particle size distribution of camelina meal (code 27) milled at 7.000 rpm through impact milling and fine and coarse fractions derived from air classification. The peaks of fine and the coarse fractions partially match each other

5.3.2 ELECTROSTATIC SEPARATION ENRICHMENT

Another dry protein enrichment method is the electrostatic separation. In this study, a lab-scale electrostatic device was used, and the output were the “left fraction, LF” richer in proteins, and the “right fraction, RF”. Besides to these 2 fractions, some material that did not adhered to the rotating belts, was collected in a left-bag filter and in a right-bag filter. As for the fine and coarse fractions air classification output, also the left and right fractions are visually distinguishable (figure 30), since the LF colour is lighter compared to the RF.

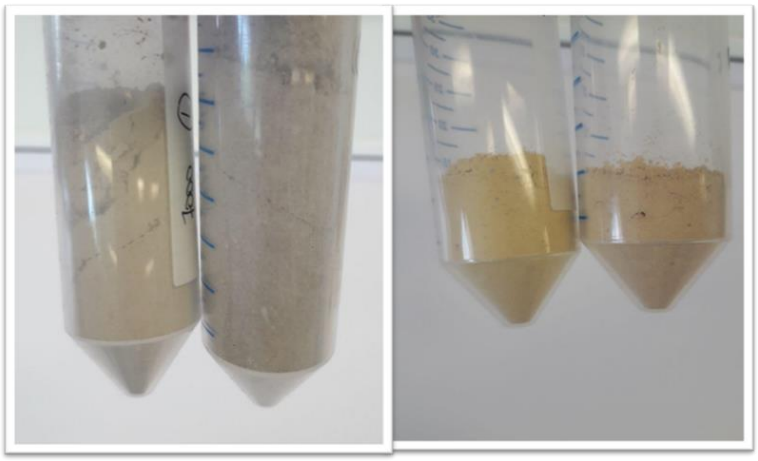


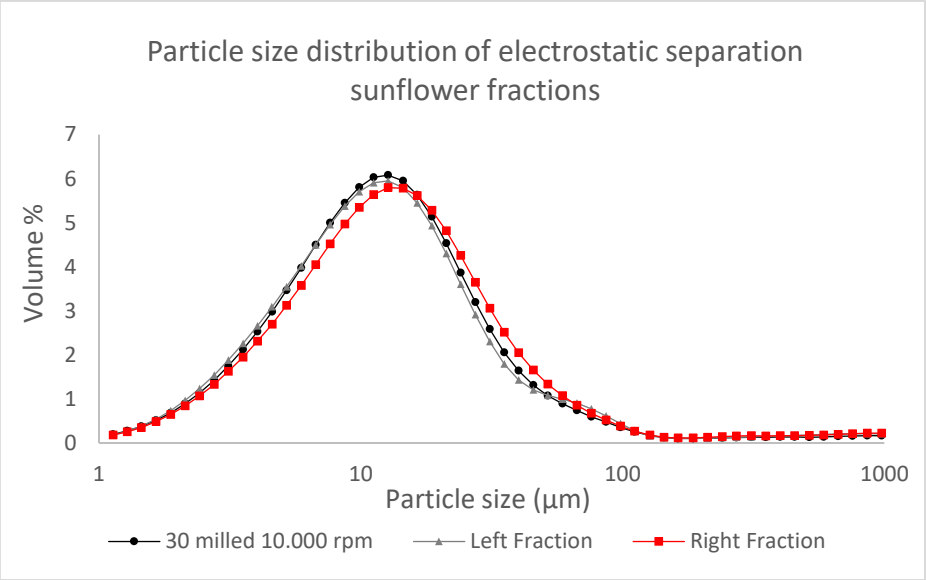
Figure 30. Left and right fractions after electrostatic separation process, in the left picture the sunflower code 30 and in the right picture the camelina meal code 27

Data concerning the protein content, mass and protein yields of LF and RF obtained for each sample tested are reported in table 19. Left fraction of sunflower flour (code 130) had a protein content of 43.1 %, meaning that the process increased the protein purity of 5 %. The mass and protein yields were therefore very low (5.7 and 6.4 %), as the yields of the right fraction. In the right fraction, the protein content is 9 % lower compared to the starting material. In three tests the sunflower flour code 30 was used, at different conditions of sieving and milling. The LF of sunflower sieved at 0.8 mm and milled at 10.000 rpm had a protein content of 34.5 %, increase of 2 % compared to the starting material. The RF has instead a protein content lower of about 2 %. Mass and protein yields of both fractions are very low (< 5 %). The protein content of LF of sunflower sieved at 0.5 mm and milled at 4.000 rpm is 39.8 % (increased of 6.8 %). The RF was decreased of 3 %. Mass and protein yields are higher, 13.6 and 16.3 % respectively, while the yields of RF were 3.2 and 23.8 %. The sunflower sieved at 0.5 and milled at 7.000 rpm produced the best results, with a protein content in LF of 51.9 % (increased of 10.9 % compared to the starting material). The protein content in RF decreased of 9 % compared to starting material. Mass and protein yields were respectively 14.7 and 17.9 % in the left fraction and 23.1 and 17.9 % in the right fraction. One test was performed with the camelina meal milled at 7.000 rpm. The LF increase of 4.6 % and RF decreased of 4.4 %. The yields of both fractions are really low (< 5 %).

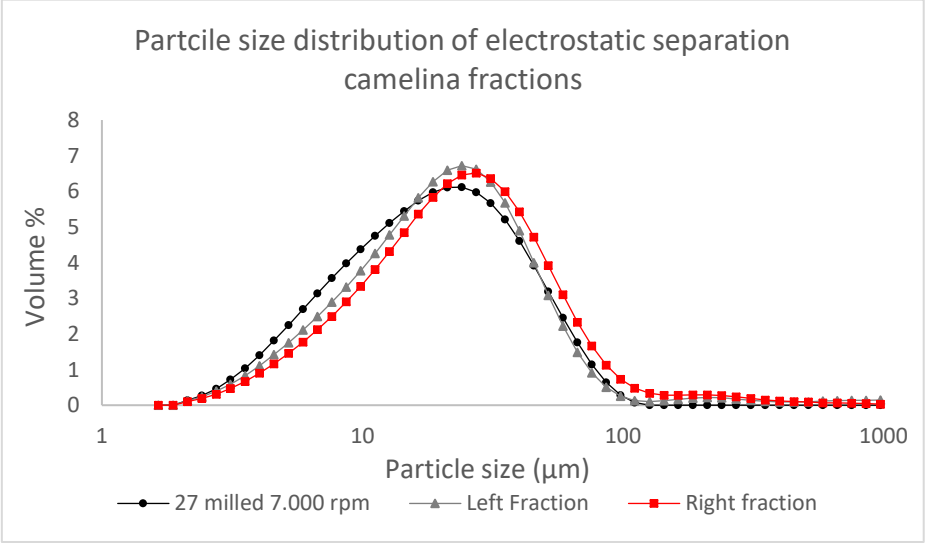
Table 19. Protein content and mass-protein yields of the left and right fractions after electrostatic protein enrichment

CODE	SIEVING MILLING	% PROTEIN LF	% PROTEIN RF	% MASS YIELD LF	% PROTEIN YIELD LF	% MASS YIELD RF	% PROTEIN YIELD RF
130	sieved-extruded and milled	43.1	29.5	5.7	6.4	4.8	3.7
30	sieving 0.8 mm milling 10.000 rpm	34.5	29.9	3.9	4.1	3.7	3.3
30	sieving 0.5 mm and milling 4.000 rpm	39.8	30.1	13.6	16.3	26.2	23.8
30	sieving 0.5 mm and milling 7.000 rpm	51.9	32.1	14.7	17.9	23.1	17.9
27	milling 7.000 rpm	45.0	37.0	4.6	5.0	4.2	3.8

In the graphs 59 and 60, the particle size distribution of sunflower flour and camelina meal and their related LF and RF is reported. Concerning the sunflower sieved at 0.8 mm and milled at 10.000 rpm through impact milling, the most of particles had a size of about 12 µm. The curves of left and right fractions are overlapped to those of starting material, as expected since electrostatic process do not separate the particles based on their size as air classification. The trend is exactly the same in the case of camelina (graph 60), in which the most particles had a size of 24 µm.



Graph 59. Particle size distribution of sunflower flour (code 30) milled at 10.000 rpm through impact milling and left and right fractions derived from electrostatic protein separation. The three peaks completely match each other



Graph 60. Particle size distribution of camelina meal (code 27) milled at 7.000 rpm through impact milling, and left and right fractions derived from electrostatic protein separation. The three peaks completely match each other

5.3.3 SHEAR-CELL TECHNOLOGY TEXTURIZATION

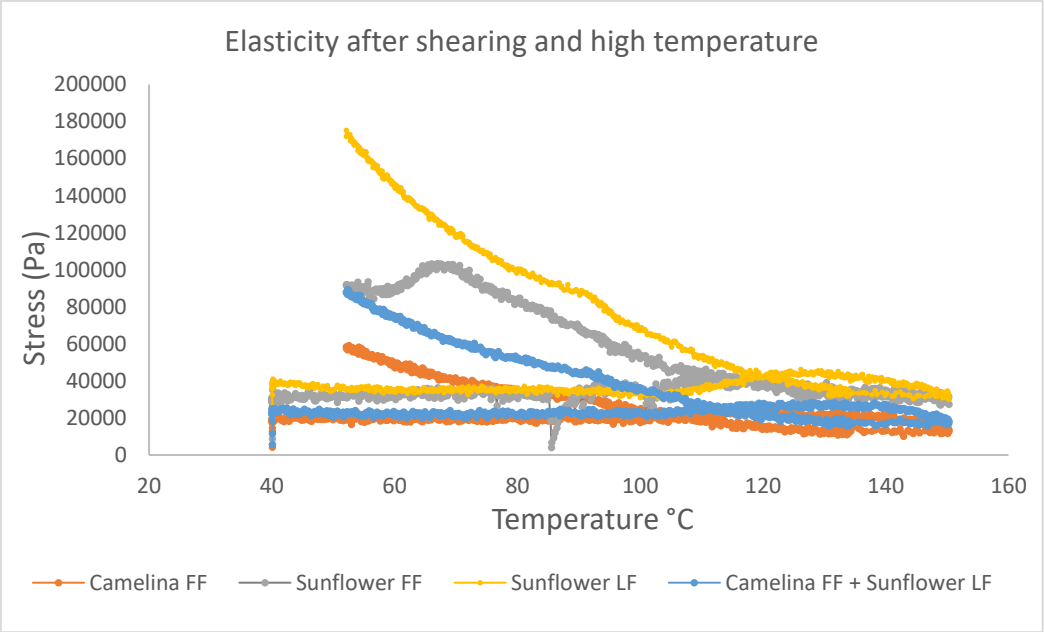
Protein extracts obtained from the dry separation were tested in structuring process, in order to preliminary investigate the behaviour of the dry protein ingredients in the plant-based food production. The process used is the shear-cell technology, and the sunflower-camelina extracts were tested alone and with

other commercial flours. Moreover, the gluten was added in order to highlight the differences in fibres formation.

The protein extracts were firstly testes at closed-cavity rheometer, that works similarly to the shear-cell device. In figure 31, the solid disks obtained after CCR test are showed. The mix in which sunflower LF, camelina FF and both together, lead to a completely gelled disk, that was the correct output to allow to test them in higher scale.

Figure 31. Protein gelled disks obtained after closed-cavity rheometer test. The first disk is obtained using the fine fraction of camelina, the second using the left fraction of sunflower and the last disk is obtained mixing sunflower and camelina fractions

The graph 61 reports data about the elasticity of the materials used during CCR tests, at firstly increasing the temperature until 150 °C and then to let them cooling down to 50 °C. As showed in the graph, in each test the elasticity increased during the drop-in temperature: the sunflower LF obtained from electrostatic separation with the highest protein content (51.9 %) is the extracts with the most elastic increasing. Camelina showed a lower elasticity increasing compared to sunflower.



Graph 61. Elasticity increasing after high temperature and shearing obtained during CCR test

In the following pictures (figures 32-40), the products obtained after structuring using shear-cell device.

Figure 32 shows the product obtained mixing only sunflower fine fraction (44 % proteins), water and salt. The product is compact and completely gelled, but no fibres formation occurred. The colour was very dark.

Figure 32. Product obtained using shear cell technology. Sunflower fine fraction (code 30) from air classification was used in the mix

In figure 33, the product obtained mixing sunflower FF (44 % proteins) and gluten 1:1 with water and salt. Compared to the product obtained using only sunflower, the colour is brown, but slightly, and the material more elastic. As showed in the right picture, some fibres were formed during the process.

Figure 33. Product obtained using shear cell technology. Sunflower fine fraction (code 30) from air classification and gluten were used in the mix

In figure 34, the product obtained mixing only camelina FF (45 % proteins) with water and salt. Compared to the products obtained using sunflower, the material was soft, sticky and not completely gelled. The colour was light brown tending to yellow, with some darker spots due to the oil residual.

Figure 34. Product obtained using shear cell technology. Camelina fine fraction from air classification was used in the mix

Mixing camelina FF (45 % proteins) with gluten 1:1, the product is completely gelled, elastic and come fibres occurred (figure 35). The colour is lighter than the product with only camelina, and is tending to yellow-ochre.

Figure 35. Product obtained using shear cell technology. Camelina fine fraction from air classification and gluten were used in the mix

Figure 36 shows the product obtained mixing camelina FF (45% proteins) and sunflower FF (44 % proteins) with ratio 1:1. The product was completely gelled but without elasticity and completely broke. No fibres occurred but the aspect in cross-section was grainy. The colour was brown.

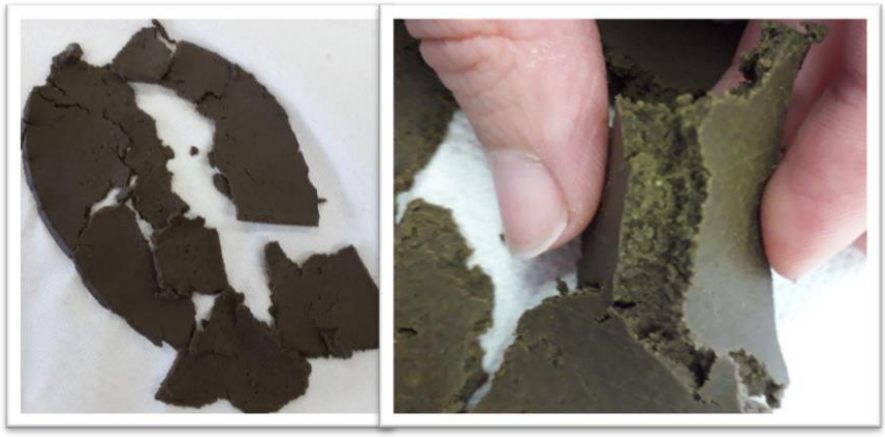


Figure 36. Product obtained using shear cell technology. Camelina and sunflower (code 30) fine fractions from air classification were used in the mix

The mixing of camelina, sunflower and gluten 1:1:1 is showed in figure 37. The product was compact and lighter compared to sunflower and camelina without gluten, and some fibres occurred during shearing.

Figure 37. Product obtained using shear cell technology. Camelina and sunflower (code 30) fine fractions from air classification and gluten were used in the mix

A mixing of sunflower code 130 FF (43 % proteins), faba flour (26 % proteins) and gluten 1:1:1 was tested. As figure 38 shows, the product obtained is compact and highly elastic and fibrous. The colour is brown.

Figure 38. Product obtained using shear cell technology. Sunflower (code 130) fine fractions from air classification, commercial faba flour and gluten were used in the mix

Another mix tested was composed of sunflower FF code 130, lentils flour (25 % proteins) and gluten 1:1:1 (figure 39). As for the mix with faba flour just shown, the product was compact, elastic and fibrous. The colour was also similar.

Figure 39. Product obtained using shear cell technology. Sunflower (code 130) fine fractions from air classification, commercial lentils flour and gluten were used in the mix

In figure 40, the product obtained mixing sunflower code 130, lupin flour (39 % proteins) and gluten 1:1:1. Compared to the mix with faba and lentils, the colour is lighter and less elastic. The aspect was higly fibrous.

Figure 40. Product obtained using shear cell technology. Sunflower (code 130) fine fractions from air classification, commercial lupin flour and gluten were used in the mix

Chapter 6 Discussion

The consumption of plant-based food as alternatives to meat and animal derivatives is constantly growing since years, generating specific requests from both food producers and protein-ingredients producers. The desired characteristics of protein ingredients are different based on the application of proteins. For example, the problem to solve in vegetable-milks is the lack of protein content, that often is < 2.5 %. The protein-ingredients suitable for milk-analogues must be first of all completely water-soluble at pH comprises between 6.8 and 7.5. The main problem of most plant proteins commercially available is indeed the poor solubility, determining an unpleasant “sandy effect” to the palate. Other functional characteristics searched for this application are a high foaming capacity, needed for the production of cappuccino foam or the whipped cream, and high emulsification capacity to produce cream in pastry sector. Plant proteins used to replace eggs need to have a very high emulsification and gelation capacities. Concerning meat substitutes, the required properties are high emulsification and good water and oil holding capacities, since to reproduce the meat both proteins and fats are required. Another important feature is high gelling capacity that must be stable at high temperature, in order to preserve the texture during the cooking. The protein ingredients commercially available at industrial quantities to date are mostly soy and pea proteins, that do not completely satisfy the market. The emerging trends indeed are to find alternatives to soy, high functional properties and lack of off-flavours, problem mainly attributed to the pea proteins (Pam Ismail et al., 2020). Protein ingredients producers, besides to satisfy the mentioned functional properties required from food manufacturers, must guarantee first of all a stable production of the adequate industrial quantities. Furthermore, they must consider the seasonality and the price of the protein sources: indeed, if a protein source has the correct functionalities but it is too costly or difficult to farm, it is not convenient for producers. For all these reasons, the key point is the process used to produce the protein ingredient. At the moment, the process mostly used to produce protein concentrates and isolates is the wet alkaline extraction (also called isoelectric precipitation). This

process, besides to require huge quantities of water (and energy to dry the product), uses hydrochloric acid and sodium hydroxide to shift the pH firstly to 8-9 and then towards acidic conditions (isoelectric point). The process is thus very costly and environmentally low sustainable (Van Der Goot et al., 2016). In addition, often the proteins obtained with this process show a decrease of functional properties due to denaturation caused by the pH changes (Hadnadjev et al., 2017). The phase of isoelectric precipitation in some processes is substituted by an ultracentrifugation, reducing the denaturation caused by pH shifting. However, the drawback of the water consumption remains. Furthermore, the cut-off of the membranes used determines the range of recovered proteins (Hadnadjev et al., 2017). Other wet processes, as the use of eco-friendly solvents, enzymatic extraction or subcritical water are being tested, but are not ready for the industrial scale (Pojić et al., 2018 Hadnadjev et al., 2017). The main drawbacks are related with the low yield, the cost of the enzymes or still the utilisation of water. Another approach is the protein enrichment without the use of water, called dry protein fractionation. There are two dry processes, one is now used also at industrial scale and is called air classification, the second is at pilot experimental stage and is called electrostatic separation. The air classification produces two or three fractions, of which one is the fine fraction rich in proteins, and divide the particles based on their size and density. The electrostatic separation divides the particles based on their charge: proteins are positively charged and in presence of electric field move towards the negative electrode. The advantages of the dry enrichments are clearly the total absence of water utilisation and thus also the drying step is not needed, and the preservation of protein functionalities. Moreover, the low-protein fractions could be used in other field without drying process as needed for the co-products of wet extraction. The main drawback is the protein content of the product obtained, indeed this processes lead to protein flours (30-50 % proteins) or protein concentrates (50-85 % proteins), but protein isolates could not be obtained (Pelgrom, Wang, et al., 2015).

The aim of this study was a comparison of two different wet protein-enrichment processes, in order to evaluate both the yields of the process, and thus their industrial feasibility, and the characteristics of the product obtained. The

traditional wet process (alkaline solubilisation and isoelectric precipitation) was compared with a simpler process using phosphate saline buffer (50 mM, pH 7.8) as extraction solvent. The PBS method included a solubilisation step and a centrifugation to remove the insoluble material. The two processes were compared using the same samples as starting material: sunflower, canola, camelina, chickpea and lentils. For sunflower and canola, meal from mechanical oil extraction and flour from chemical oil removing were both tested, to investigate how the oil residual in meals influences the protein fractionation. In the graph 5 the protein content of the extracts obtained from the two processes for each protein source tested was plotted with the protein yield. The difference in the protein content % was strong, all the alkaline extracts had a % > of 60 and the PBS extracts showed a protein content below to 40 %. Alkaline extracts produced therefore protein concentrates (sunflower meal 75 %, canola flour 75 %, canola meal 66 %, camelina meal 63 % and chickpea 70 %) and protein isolates (sunflower flour 90 % and dehulled lentils 87 %). PBS process, in 4 samples did not led to a protein enrichment: sunflower flour had a protein content of 25 % and the extract 22 %, sunflower meal similarly had 26 % of proteins and the extract 22 %, camelina meal 33 % and its extract 29 %. Interestingly, there was a huge difference between canola flour and canola meal: canola flour had 33 % of proteins and PBS extract only 20 %, whereas canola meal, that had 31 % of protein content, its extract increased in protein content of 4 %. PBS process thus lead to protein flours production in canola meal, chickpea (32 %, with an increasing of 10 %) and in dehulled lentils (38 %, with an increase of 15 %). The range of protein recovery was really wide between the protein sources considered. The protein recovery of alkaline extracts ranged from 9 % to 65 %, in which the oleaginous seeds as sunflower and canola showed lower protein recovery, whereas the pulses chickpea and lentils showed higher values. Similarly, in PBS extracts the range was from 21 % to 72 %, slightly higher than the yields of alkaline extracts. Concerning the mass yield, there was a huge difference between the processes (graph 6): in alkaline enrichment the mass yield was very low, ranging from 4 % to 22 %. PBS process showed a higher mass yield, that ranged from 24 % to 47 %. The difference in mass yield of the two processes confirm the assumption that increasing protein purity, the mass yield

decreases. As found in protein yield, also considering the mass yield results the lower values were those referred to the oleaginous seeds, whereas the higher are related to the pulsed (chickpea and lentils).

During alkaline process, a co-product is generated after the second centrifugation, the supernatant. In this study, supernatants derived from the alkaline extraction of the different protein sources were collected, dried and analysed for their protein content and functionalities. The protein content of this co-product generated was significant: about 16 % in sunflower supernatants, 20 and 21 % respectively in canola flour and camelina meal supernatants, 30 and 33 % in lentils and canola meal supernatants, and 22 % in chickpea supernatants.

Considering the oil content in the meal compared to the flour of the same protein-source (graph 3), after alkaline extraction emerged that the oil content negatively influenced the protein content in the extracts. Both sunflower and canola meal extracts indeed showed a lower protein content compared to the related flour extracts, in particular 15 % less in sunflower and 9 % less in canola. However, the protein content of the extracts is not the only parameter to consider to evaluate the oil content influence, as for example, the protein yield of canola flour is lower than the one of canola meal, meaning that more protein were “extracted” from the starting material. Even the mass yield of canola meal extract is the double of that of canola flour, meaning the double quantity of extract from the same amount of starting material. The same trend could not be observed in the PBS extracts: sunflower meal and sunflower flour had indeed the same protein content and yields, whereas in canola the opposite trend emerged, as the meal extract had a protein content and yields higher than the related flour.

Concerning the alkaline process, a second extraction of the same material (sunflower flour and lentils) was repeated, in order to evaluate if a double extraction could increase the protein recovery. The results (data not reported) showed that the second extraction led to a very low yields, suggesting that a second extraction does not change significantly the protein enrichment results.

PBS protein enrichment was tested in this study as a previously observation in a sample of defatted soy flour: the process indeed produced a soy protein water-

soluble concentrate. The results found confirmed that observation, as in pulses (lentils and chickpea) showed a protein increasing that could be improved varying the extraction parameters as the pre-treatments of the starting material (for example the milling), variation of incubation time, temperature and pH/solvent concentration. The variation of the mentioned parameters should be carried out tailored to the different protein sources of interest. Considering the oleaginous sources as canola, sunflower and camelina, the process seems to not lead to a protein increasing, but also in this case, further experiments varying the parameters would be necessaries to confirm the outcomes. Moreover, it could be interesting to repeat the extraction of the same material as tested for alkaline process, in order to evaluate if a second-extraction could improve the protein recovery.

Summarizing, alkaline process results confirm literature data, producing protein concentrates and isolates with mass and protein yields quite low, even if in literature are reported also higher protein yields (J. I. Boye et al., 2010). PBS process is not suitable to obtain ingredients with very high protein content, but it produces completely water-soluble protein ingredients/concentrates that could be useful in all those applications that require soluble proteins, as the plant milk-analogues. Alkaline extraction of camelina meal, a “new” plant protein source, generated a protein concentrate (63% proteins) with discrete yields. Both processes results need to be confirmed and could be improved varying the process parameters tailored to each protein source. The mass and protein yields are index crucial to understand the industrial feasibility of the process used to enrich in proteins. Indeed, a process could produce extracts with a very high protein content, but if mass and protein yields are too low, the industrial feasibility decrease, as the price of the ingredient produced would be too high for the market. It is therefore necessary to find the correct compromise between the protein content, the yields and the functionalities of the ingredients. Moreover, it is also crucial to evaluate the co-products and by-products generated during this kind of processes. In alkaline extraction, a co-product with an interesting protein content is produced, but it is necessary determine the use and thus the adequate treatment to apply, as for example the drying process. The by-product generated is the insoluble material discarded at the first centrifugation: it is wet material that

need also to be dried to be used. The same kind of by-product is generated during PBS process: the insoluble material is still rich in proteins and to be used for example in feed sector need to be dried. The drying of the by-product is an additional cost that protein producers must include in the process charge.

The protein extracts obtained from the two enrichment processes were tested for functional properties as water and oil holding capacity, foaming capacity and foam stability, and the gelling capacity. PBS extracts, as completely water-soluble, had no water holding capacity: this process therefore determines a decrease of WHC. Alkaline extracts derived from oleaginous seeds (sunflower, canola and camelina) showed a WHC value lower than the value of the related starting material, meaning that the protein extraction decreased this property. WHC values of the alkaline extracts derived from pulses had, in the case of chickpea, the same value, and in lentils higher value compared to the related starting material. The water holding capacity of supernatants (co-product of alkaline extracts) was zero, as the components, as in PBS extracts, are completely water-soluble. Concerning the oil holding capacity, each extract derived from both processes, showed a higher value compared to the related flour/material, with the only exception of the PBS extract derived from canola flour. The oil holding capacity values of the alkaline supernatants were similar or lower than those of the related protein extracts. In general, these two protein enrichment processes increased the oil holding property. Gelling capacity of each protein source after PBS extraction drastically decreased, with the exception of camelina that is maintained (LGC 20 %). The alkaline process, in only one case determined an increase of the gelation property (canola flour extract). The other alkaline extracts instead had a lower gelation compared to the initial flour, with the exception of canola meal and chickpea that the LGC is equal to the related starting material. Gel formation did not occur in any alkaline supernatant tested (data not reported). To notice, dehulled lentils flour showed a very good gelling capacity (LGC 10 %) compared to the other protein sources, and after PBS and alkaline extraction completely lose this property, meaning that probably the gelation capacity is not mainly related to the proteins. Protein extracts derived from the two processes in general showed a higher foaming capacity compared to the initial related materials, with the only exceptions of the alkaline extracts of

camelina and chickpea. In particular, alkaline extract of camelina completely lose the capacity to form foam, whereas the PBS extract had a very high FC (160 %). To notice, the foaming capacity of PBS extracts of camelina, canola meal and chickpea considerably increased compared to starting material. The stability of the produced foam, in general, is similar between the flours and the extracts. The protein enrichment processes therefore increased the foaming capacity, the entity of this increasing depends on the process and protein source considered. The interesting characteristic of almost each alkaline supernatant is the elevate foaming capacity: lentils supernatants for example, at 2 % had a foaming capacity of 420 %. Probably in the supernatants and PBS extracts the major protein component is represented by albumins (water-soluble), that have a high capacity to foam (J. Boye et al., 2010; Shevkani et al., 2019).

Comparing functionalities of sunflower and canola flours with the meals, emerged that the values are generally similar, with the exception of oil holding capacity, that is slightly higher in meals extracts. Moreover, some differences in the foaming capacity emerged. However, the differences observed do not appear linked to the presence of oil into the meals compared to the flours.

In this study, functional properties of commercial samples of protein concentrates/isolates were also analysed to compare them with the extracts of chickpea and sunflower found in this thesis. Moreover, samples of faba concentrate obtained from dry processes and faba isolate from wet processes were compared to investigate the influence of the protein-enrichment processes on the functionalities. A sample of soy isolate was also included, as the soy is currently the “gold standard” of the protein ingredients used to produce plant-based food.

Functional properties of commercial sunflower isolate and chickpea concentrate were generally higher than those of the extracts produced within this project, with the exception of the oil holding capacity. OHC values of sunflower alkaline extract indeed is slightly higher (2.6 ml/g in the flour and 3.2 ml/g in the meal extract) than the commercial sunflower isolate (2.4 ml/g), and chickpea extract value is 2.8 ml/g versus the 1.5 ml/g of commercial chickpea concentrate. WHC values of the commercials sunflower and chickpea were about three-times compared to the

alkaline extracts. Foaming capacity at 2 % of concentration was also higher in commercial proteins (320 % the sunflower isolate and 25 % the chickpea concentrate). The FC values of the alkaline extracts were 120 % for the sunflower and only 4 % for the chickpea. Concerning gelling property, the two chickpea concentrates showed the same values (LGC 20 %), whereas there was a huge difference between the commercial sunflower isolate, that showed a good gelation (LGC 15 %) and sunflower alkaline isolate that was not gelled at 20 %, with a very low score. The differences between the chickpea concentrate obtained in this thesis and the commercial one could be due to different cultivars used, whereas the strong differences of the sunflower isolates seems to suggest that they derived from different wet processes. Faba concentrate and isolate showed different functionalities values, confirming the possibility that wet and dry protein enrichment processes produce protein ingredients with different characteristics. WHC of faba isolate was significantly higher (4.6 ml/g) than the faba concentrate (0.9 ml/g). LGC of the faba concentrate was 20 %, whereas the isolate did not gel at 20 %. The foaming capacity at 2 % was also higher in the faba concentrate (88 %) compared to the isolate (52 %). Soy isolate showed good functionalities: the WHC value was 3.7 ml/g and 1.9 ml/g of OHC, the lower gelation concentration was 15 % and the foaming capacity at 2 % was 98 %.

Further aim of the study was to compare five cultivars of chickpea about their protein content, yields and functional properties after alkaline protein enrichment process. The cultivars were processed using the same parameters (description in material and methods) and results about protein content and yields are statistically different among the cv (graphs 42, 43, 44). Each cultivar led to a protein concentrate similar to those obtained in the previous experiments (code 38), the proteins % was comprised between 71 and 75 %. Anyway, the cultivar 46, that showed the highest protein content, had the lowest mass and protein yields (12 % and 39 % respectively). As previously discussed, a balance of protein content and yields is necessary, since to produce a very small amount of high-protein ingredient is too costly and environmentally unsustainable. The cultivar 48 showed the best compromise, reaching 74 % proteins with higher mass and protein yields (20 % and 70 % respectively). The functional properties of the alkaline extracts of these five cultivars, showed similar values with the

exception of the gelation capacity. Indeed, each extract showed a decrease of gelling capacity compared to the related initial flour (LGC > 20 %), whereas 44 extract maintained the property showing an LGC of 10 %. Concerning water and oil holding capacity and foaming property, the five extracts showed similar results comparable to the previous described code 38. Cultivar 44 could therefore be used in all those applications that need high gelation performance, whereas the cultivar 48 had good extraction results.

Dry protein enrichment separation tests were conducted at Wageningen University & Research, using both air classification and electrostatic separation. Samples of sunflower and camelina sieved and milled at different conditions were used as initial materials, to preliminary test the two technologies. Air classification produced a modest protein enrichment comprised between 1 and 4.5 %. Concerning the sunflower, the samples milled using impact miller produced better outcomes (in terms of protein content and yields) compared to the samples not milled or milled at 0.5 mm using rotomiller. The explanation, as confirmed by the SEM pictures, is that the sunflower material was rich in fibres, and an intense milling detached the protein bodies. The protein bodies, that have different size and density of the fibres and other components, could be better separated. Probably, milling with rotomiller at lower sizes lead to improved results. Anyway, that kind of milling, is really difficult due to the high fibres content, that produce high friction and congestion of the instrument. Impact milling of sunflower at 7.000 rpm gave the highest protein enrichment, anyway further experiments are necessary to confirm these outcomes and improve the protein content and the yields. Camelina coarsely milled and camelina milled at 7.000 rpm led to similar results. Graph 58 suggest that impact milling at 7.000 rpm could be too intense for this protein source, as the fine and coarse fractions are partially overlapped. In general, the crucial step of air classification is to find the combination of milling/ wheel speed that gives the highest separation and yields. The protein content of fractions obtained with electrostatic separation increased between 2 and 10.9 %. In this technique, materials not milled or coarsely milled could not be used as could clog the charge tube. As for air classification, the milling intensity is a crucial step: for example, the sunflower milled at 7.000 rpm led to the best result (protein increase of 10.9 %), whereas the more intense milling 10.000 rpm did not lead to

a satisfactory outcome. This could be explained as too small particles tend to aggregate and the charging phase could be affected. The mass and protein yields are really low, but is partially due to some lab-scale device limitations. Besides to the left and right fractions indeed, other “left and right materials” are collected by two filter bags. These two additional fractions in this thesis were not analysed in protein content, anyway they could be collected and subjected to a second separation run. Results obtained with this technology are promising, anyway they could be improved also in this case varying the milling intensity and testing different charge tubes (different in lengths, diameter and shape). A spiral charge tube for example, increase the time of particles charging, improving the electrostatic separation (J. Wang et al., 2014). The technical drawback of both technologies (air classification and electrostatic separation) is the oil content of the initial material: indeed, in both cases, the oil content of camelina meal negatively affected the separation and the cleaning of the device after the separation. The maximum oil content admitted in these technologies is about 8-10%. Anyway, as emerged in this thesis, also the traditional alkaline extraction could be affected by oil content.

Protein ingredients are subject to texturization processes to produce meat-like plant food. The most used technology to texturize at industrial scale is the extrusion, but other technologies such as the shear-cell are being tested at laboratory and pilot scale from food producers. The peculiarity of shear-cell technology is that temperature and shearing could produce a fibrous texture similar to the muscle fibres of the real meat. In this study, fractions obtained in dry separation processes were texturized to test the shear-cell technology, using always the same recipe and device conditions (time, speed of cone rotation and temperature). Camelina fraction (45 % proteins) did not well texturized, was too soft and the elasticity too low (figure 34). Sunflower fraction (44 % proteins) completely gelled, but no fibres formation meat-like occurred (figure 32). Moreover, the colour of the textured product was very dark. Camelina and sunflower mixed together did not form fibres and a completely gelled product, but the cross-section showed a coarsely effect, that could lead to a meat-like texture varying the parameters (figure 36). In each combination, the addition of gluten improved the texture, fibres meat-like were formed and the colour was lighter

(figures 33, 35 and 37). Furthermore, combination of sunflower fraction, gluten and other commercial flours (lupin, lentils and faba bean) lead to a very good textured products, with fibres similar to meat (figures 38, 39 and 40). Results about texturizing with shear-cell device are really good, and could be further improved varying all the described parameters (% of dry matter used, temperature, time and speed of cone rotation). Moreover, to obtain gluten-free products, additional tests using pectin instead of gluten could be assessed.

Chapter 7 Conclusion and future perspectives

The market of plant-based food is constantly growing for healthy, ethical and environmental reasons. This growth determines some requests to protein ingredients producers: in general, the first aim is to find plant proteins alternative to soy at affordable price. Moreover, each food applications needs protein ingredients with specific functionalities as solubility, gelation, emulsion, foaming and water-oil holding capacity. The functional characteristics and the price of the plant- proteins, as highlighted in this thesis, are related with their production process.

Summarizing:

- currently, the most used process to produce soy/pea protein isolates and concentrates is the wet alkaline extraction. In this thesis, a simpler process was compared to this gold standard using different protein sources: the products obtained have completely different characteristics (low protein purity and higher mass and protein yields). In general, both protein enrichment processes appear to work better using pulses (lentils and chickpea) compared to oleaginous sources. For example, the alkaline extraction of sunflower, originated protein ingredient with high purity but the yields are really low, making this process unsustainable at industrial level.
- Functional characteristics are wide based on the protein source considered and the protein-enrichment process used. It is thus important to investigate them during the development of new protein enrichment processes or the use of innovative protein sources (as camelina, included in this thesis). The protein content indeed is not the only parameter to consider in a protein ingredient, often lower protein purity determines higher functionalities or the presence of other bioactive molecules that contribute to improve the food texture and the nutritional profile. In this project, functional properties of different protein sources and related protein extracts obtained with two processes were investigated,

highlighting how the different protein extraction methods could lead to different products suitable for specific food applications. PBS extracts for example, despite to their low protein purity, showed a very high solubility and foaming capacity, characteristics completely different comparing to the alkaline products.

- dry protein-enrichment processes produce protein ingredients with lower protein content compared to the gold standard (protein concentrates versus isolates). The advantages lie in the costs: water is not consumed and consequently neither the proteins nor the co-product generated need post-extraction drying-processes. Moreover, proteins are not denatured, maintaining their native functionalities. In this thesis, preliminary results obtained from the dry protein enrichment of sunflower using electrostatic separation were promising for future food applications.

Additional experiments varying the process parameters (pre-treatments of the materials, time, temperatures, specific settings) would be interesting for both wet and dry methods used in this thesis, in order to improve the protein content and the mass and protein yields. Moreover, a two-phases extraction could be tested: for example, a first step using a dry method could be followed by a second wet phase. Also, an extraction in which both dry processes are used sequentially could lead to a protein content and yields improvement. It would be also interesting to investigate the functional properties of the protein fractions obtained from dry separation, to compare them with those obtained with wet enrichments. Indeed, the sunflower fraction obtained after electrostatic separation (51.9 % of proteins) tested at CCR, produced a completely gelled-elastic disk, characteristic promising in meat substitute application. The purity of proteins obtained using PBS enrichment method needs to be improved, however the functionalities of the obtained ingredients could make the protein ingredients suitable for the milk-analogues application.

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