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# Nutritional Quality and Food Safety of Food Sources and Food Processing By-products

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# INDEX

# Aim of the thesis

# Motivations and outlines of the PhD thesis

In the area of functional foods and dietary supplements, peptides that confer health benefits, also called bioactive peptides, are increasingly recognized as useful tools for improving health and preventing chronic diseases (Udenigwe and Aluko 2012). In fact, food proteins do not only supply nutrients, but also provide numerous health benefits through their impact on specific biochemical pathways. The importance of plant proteins has been underlined by numerous clinical studies performed on grain protein. Most of these activities are due to peptides encrypted in the parent protein sequences, which are delivered by digestion, absorbed by intestinal cells, and transported to the target organs, where they can exert their biological activity (Rutherfurd-Markwick 2012). Actually, bioactive peptides have become a hot research topic, since they provide a large spectrum of health benefits with minimal side effects compared to other chemicals. These health benefits are particularly in the area of hypercholesterolemia and hypertension prevention.

Plant-based diet and plant proteins are becoming more and more important to meet nutritional requirements of the growing human population and simultaneously to reduce the negative impacts of food production on the environment. With the aim to boost the environmental sustainability, the valorization of byproducts is certainly one of the main challenges. Some food by-products, such as those deriving from oat, rice, almond, soy okara and coconut processing, are currently among the most interesting by-products of food industries due to their high nutritional potential.

Another issue I have addressed in my thesis is the valorization of underutilized species of the genus *Lupinus*. belonging to the *Fabaceae* family, whose seeds are characterized by a high protein content. In particular, I have focused the attention on some wild Mexican lupin beans. In fact, a recent review collects data on the nutritional and bioactive compounds of these plants presenting data on proteins, lipids, minerals, dietary fiber, and other bioactive compounds (Ruiz-Lopez, Barrientos-Ramirez et al. 2019), but a characterization of the protein profile is still pending. The information of the protein profile of wild Lupinus species will be beneficial for fundamental and applied studies concerning the storage proteins of the seeds.

Based on these considerations, the main aims of my PhD thesis were focused on the discovery, quantification and functional analysis of plant proteins or peptides. These objectives were reached by use of different tools, comprising advanced analytical techniques, such as mass spectrometry (MS) coupled to biochemical and

bioinformatics tools, in order to improve the understanding of their mechanism of action at cellular level, their intestinal absorption profile as well as to forecast their potential bioactivities.

Briefly, the main aims of the present study were:

- To evaluate whether the treatment with some food-grade enzymes, such as amylase, cellulase/xylanase, protease, and their combination, may be useful to achieve this goal.

- To valorizing the press cakes deriving from almond and coconut drinks production by using ultrasound-assisted extraction (UAE) to obtain protein ingredients for human use.

- To characterize the potential pleotropic activity of two commercially available soybean and pea protein hydrolysates, respectively.

- To investigate the composition of the seed proteins of four lupin species: *Lupinus aschenbornii*, *Lupinus campestris*, *Lupinus hintonii*, and *Lupinus montanus.*

The thesis contains three parts:

Part I introduces the state of art of proteins and peptides from food and food by-product. Chapter 1 discusses Recovering and obtaining bioactive peptides based on ultrasound-assisted extraction as well as enzymatic methods.

Part II presents my scientific contributions in the PhD period.

Chapter 2 is the investigation of the use of multiple pre-enzymatic treatments for obtaining valuable protein concentrates and polyphenols extracts from an oat press cake, since these seeds are considered highly nutritional. The food protein hydrolysates are attracting particular interests because of their various functional and biological properties. In spite of their valuable composition, the press cakes of these foods have been only rarely investigated and mainly used in low-value applications, such as feeds, or considered wastes. The recovery and reuse, instead, may allow to obtain additional economic benefits and reduce the waste of natural resources. Besides the proteins and peptides, also phenolic profile and phytic acid, a main antinutritional factor, were quantified.

Chapter 3 presents the valorization of the press cakes deriving from almond and coconut drinks production by using ultra sound assisted extraction (UAE) to obtain protein ingredients for human use. The quality of the extracted protein ingredients were assessed in term of phytic acid content, protein profile, techno-functional features, and antioxidant properties. The effect on digestibility of sonication was also evaluated.

Chapter 4 is the integrated evaluation of the multifunctional DPP-IV and ACE inhibitory effect of soybean and pea protein hydrolysates.

Finally, Part III presents my contributions to another project related to an investigation of the proteome of four wild lupin species with the goal of providing useful information to integrate other chemical, molecular, and morphological data to improve the taxonomic identification of these species. The investigation was performed by integrating classical analysis methods, such as SDS-PAGE and 2D-electrophoresis, with chromatographic purification and mass spectrometry analysis by comparing the results with proteomic data of domesticated species.

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# Part I.

# State of the art

# Chapter 1

# Introduction

## Food and food by-product as a promising source of proteins and peptides

Plant-based protein products are currently gaining much interest as a more sustainable alternative to animal-based protein products. Recent scientific evidence suggests that plant-based protein do not only serve as nutrients but can also modulate some physiological functions of the body. These physiological functions are primarily regulated by peptides that are encrypted in the native protein sequences: these bioactive peptides can exert health beneficial properties and are thus considered as lead compounds for the development of nutraceuticals or functional foods (Chakrabarti, Guha, & Majumder, 2018).

Food wastes are produced by a variety of sources, ranging from agricultural operations to household consumption. It is important to underline that about 38% occurs during food processing: however, many plant residues from food industries are good candidates as low cost materials for plant proteins. Some examples are coconut, rice, oat, almond, soy okara, and so on.

Compared with other cereals, oat (*Avena sativa*) can tolerate harsher growing conditions, such as a wet climate and acidic soil, and is, therefore, more resilient than other crops (Gangopadhyay, Hossain, Rai, & Brunton, 2015). The health benefits of oat are attributed to its multifunctional characteristics and nutritional profile, it being an important source of nutrients and phytochemicals, i.e. well-balanced proteins, essential amino acids, fatty acids, dietary soluble fibre, such as β-glucan, and phenolic compounds (Gangopadhyay et al., 2015). Among phenolics, a predominant position is occupied by avenanthramides (AVNs) and avenalumic acids, which are unique to oat among cereals. These compounds have been shown to possess antioxidant activity in vitro and in vivo and are believed to be present also in the by-products deriving from the production of oat beverages (Meydani, 2009).

Coconut and almond are more and more used for producing vegetal drinks and their press cakes are among the by-products that would require a better valorisation (Chambal, Bergenståhl, & Dejmek, 2012; Zhang et al., 2014). Efforts are therefore needed to identify new strategies for an added-value use of these materials in order to achieve the final goal of a more sustainable process for the production of these plant-based beverages. It is

useful to underline that almonds contain 20-22% (w/dry w) protein that provides all essential amino acids (mainly leucine, valine, and phenylalanine) and a particularly high intake of arginine (Barreca et al., 2020), whereas coconut contains 5-6% (w/dry w) protein with a relatively favourable amino acid profile, containing all essential amino acids (predominantly leucine, lysine, and valine) (Thaiphanit & Anprung, 2016). Since part of these proteins remain in the press cake, a main priority is certainly to develop new methods for the recovery of protein-rich ingredients.

Legumes, pseudocereals, and hempseed are among the plant foods that can be considered good sources of bioactive peptides (Aguchem, Okagu, Okagu, Ndefo, & Udenigwe, 2022; Cruz-Chamorro et al., 2022; Fukui et al., 2002; Udenigwe & Aluko, 2012).

In this panorama, legumes, which stand out thanks to their high protein content, are cheap, sustainable, and healthy sources of nutrients. For example, soybean (*Glycine max*) is on average composed of ~35 – 40% protein (Arnoldi, Zanoni, Lammi, & Boschin, 2014). Clinical studies have linked the consumption of soy-based food with a reduced risk of developing a number of chronic diseases, such as obesity, hypercholesterolemia, and insulin-resistance/type II diabetes. As for the active substance in soy foods, protein plays a role in cardiovascular disease prevention (Fukui et al., 2002; Liu et al., 2014), and some cholesterol-lowering and anti-diabetic peptides have been already singled out in glycinin and β-conglycinin sequences (Lammi, Zanoni, & Arnoldi, 2015).

In addition to soybean, pea (*Pisum sativum* L.) represents one of the major legumes in the world and it is composed of ~26% protein (Arnoldi et al., 2014). Thanks to its excellent yields, availability, and its low production costs, pea is most widely used as a commercial source of proteins for different purposes (Sun & Arntfield, 2011). Many studies have highlighted the health benefits associated with the consumption of pea protein. In particular, pea protein and its hydrolysates exert antioxidant, antihypertensive, and hypocholesterolemic activities (Aluko et al., 2015; Ge, Sun, Corke, Gul, Gan, & Fang, 2020; H. Li & Aluko, 2010; H. Li et al., 2011).

# Recovering and obtaining bioactive peptides based on ultrasound-assisted extraction as well as enzymatic methods

The European Union legislation encourages the exploitation of the food industry by-products to decrease as much as possible wastes. This valorisation can be achieved through the extraction of high-value components such as proteins, polysaccharides, fibres, flavour compounds, and phytochemicals, which can be used as nutritional and techno-functional ingredients. Extraction can proceed according to solid-liquid extraction, Soxhlet extraction, pressurized fluid extraction, supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction, pulsed electric field extraction, and enzyme-assisted extraction (Baiano, 2014). Ultrasound-assisted extraction (UAE) is an interesting process to obtain high valuable compounds and could contribute to the value increase of some food by-products when used as sources of natural compounds (Esclapez, García-Pérez, Mulet, & Cárcel, 2011). The main benefits will be a more effective extraction. The important parameters of UAE have impact on protein extraction yield. For a successful application of the UAE, it is necessary to consider the influence of several process variables, the main ones being the applied ultrasonic power, the frequency, the extraction temperature, the reactor characteristics.

The enzymatic hydrolysis of food by-product proteins may be another potential method to use food by-product and an alternative way of recovering and obtaining added-value bioactive peptides. According to (Elias, Kellerby, & Decker, 2008), the antioxidant activity of proteins is related to their amino acid composition. However, such property is limited by the tertiary structure, because many amino acids with antioxidant potential can be buried within the protein core where they are inaccessible to pro-oxidants. Therefore, enzymatic hydrolysis favours the exposure of antioxidant amino acids, increasing antioxidant activity of the hydrolysates. Several factors affect the antioxidant properties of protein hydrolysates, and depend on the type of protein and enzyme, the degree of hydrolysis, and the pre-treatment of the substrate.

# 1.Ultrasound extraction

## 1.1 Effects on protein yield

Ultrasound is a key-technology in achieving the objective of sustainable ‘‘green” chemistry and extraction. Ultrasound is well known to have a significant effect on the rate of various processes in the chemical and food industry. Using ultrasound, full extractions can be now completed in minutes with high reproducibility, reducing the consumption of solvent, simplifying manipulation and work-up, giving higher purity of the final product, eliminating post-treatment of waste water and consuming only a fraction of the fossil energy normally needed for a conventional extraction method such as Soxhlet extraction, maceration or Clevenger distillation. Several classes of food components, such as aromas, pigments, antioxidants, and other organic and mineral compounds have been extracted, analysed and formulated efficiently from a variety of matrices (mainly animal tissues, microalgae, yeasts, food and plant materials) (Chemat, Rombaut, Sicaire, Meullemiestre, Fabiano-Tixier, & Abert-Vian, 2017). As reported (Preece & University of, 2017), ultrasound provides intensification via cavitational effects: imploding vapour cavities in a liquid result in increased mass transfer and cell disruption. During soybean processing at lab-scale, ultrasound has improved oil, protein and solids extraction yields during slurry and okara treatment (Preece et al., 2017). Particle size data and confocal laser scanning microscopy (CLSM) have confirmed that the ultrasonic treatment disrupts aggregated protein outside of intact cells. Not only solubility was affected by ultrasound, since separation efficiency was also slightly improved and effects were observed after 30 seconds of treatment. After 1 min treatment time, protein extraction yields had improved by approximately 10% versus the zero-time point. Ultrasound increased significantly the extraction yield of protein over the conventional method ($P < 0.05$) by at least one time on the extraction from rice dreg flour . A comparable yield was obtained with the ultrasonic assisted extraction at a temperature of 50 °C and an ultrasonic intensity of 5.7 $W \cdot cm^2$. The use of ultrasound assisted extraction enhanced the yield by 24.6% compared to the conventional extraction.

The maximum yield (26.4%) and protein content (86.1%) were reached at the optimized extraction conditions of ultrasound-assisted alkaline extraction and enzymatic deamidation by protein-glutaminase (PG) on evening primrose seed cake protein (Hadidi, Ibarz, & Pouramin, 2021).

Spent coffee grounds, the residue from coffee brewing, are still underutilized even though they contain several

useful organic compounds including proteins, pre-treatments involved different ultrasound amplitudes (40%, 60% and 80%) and extraction times (10, 20 and 30 min) and their effects on the physicochemical and functional properties including antioxidant activity of protein extract. It was found that the protein content extracted was increased approximately by 2 times (Samsalee & Sothornvit, 2021).

As for the consequence on protein integrity, some studies have investigated the structural changes after ultrasound treatment. In general, the increased intra-molecular mobility causes changes in the free sulfhydryl groups, particle size, surface hydrophobicity, and secondary structure organization (Gulseren, Guzey, Bruce, & Weiss, 2007), although minimal structural variations are reported for bovine serum albumin after ultrasonication (Krešić, Lelas, Jambrak, Herceg, & Brnčić, 2008). It is, however, important to underline that several literature reports are focused on long-term or continuous sonication processes, whereas the effects of short-time or intermittent sonication have been so far mainly neglected.

**Table 1.** Applications of ultrasound in the extraction of compounds from food by-product.

| Food | improvement | Ultrasound-assisted extraction conditions | Ultrasonic power / frequency/ time | solvent | reference |
|---|---|---|---|---|---|
| rice bran | 220% | from 0 to 15 W/g | | de-ionized water | (Ly, Tran, Tran, Ton, & Le, 2018)(Ly, Tran, Tran, Ton, & Le, 2018) |
| okara | 250% | high intensities (10 µm-pp and 15 µm-pp) | | Alkaline phosphate buffer | (Eze, Chatzifragkou, & Charalampopoulos, 2022) |
| rice dreg | 204% | 448w | 40 min | NaOH | (K. Li, Ma, Li, Zhang, & Dai, |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 2017) |
| rapeseed cake | 124.6% | 50 ˚C , 5.7 W·cm$^2$ | | NaOH | (Boukroufa et al., 2017) |
| evening primrose seed cake | | 30.8 °C | 35.5 min | NaOH | (Hadidi et al., 2021) |
| spent coffee grounds | 200% | ultrasound amplitudes 60% | 30 min | distilled water and Na3PO4 | (Samsalee et al., 2021) |
| sesame bran | | 836 W ultrasound power, 43 °C | 98 min | NaOH | (Gorguc, Bircan, & Yilmaz, 2019) |

## 1.2 Effects on other nutrients

In the ultrasound-assisted extraction, the ultrasound waves accelerate mass transfer during the process resulting in the release of proteins and polyphenols from the parenchyma cell wall via cavitation effect (Roselló-Soto et al., 2014).

Ultrasonication technology has recently attracted much interest as a technique to assist extraction processes from a variety of raw materials; examples represent the extraction of phenolic compounds from coconut shell powder (Rodrigues, Pinto, & Fernandes, 2008).

The use of cellulase for extraction of phytochemicals in black currant pomace was found to increase cell wall polysaccharide deterioration as well as the availability of phenols in methanol extracts (Kapasakalidis, Rastall, & Gordon, 2009), protein extract using ultrasonic-assisted extraction resulted in higher total phenolic content and antioxidant activity without changes in the protein structure as confirmed by changes in FT-IR spectra and SDS-PAGE profiles. The ultrasound-assisted extraction at the elevated ultrasound power facilitated the

recovery of protein and polyphenols from sesame bran. This could be explained by the mechanical vibration effect of the ultrasound waves, which provide wider surface area between solid matrix and liquid solvent to contact. Likewise, high temperature and shock waves generated by ultrasound are reported to break the cell wall and molecular bonds, increase mass transfer through the cavitation and thus, increase the efficiency of protein and phenolic extraction.

Approximately 1 million tons of agave plants are processed annually by the Mexican tequila and mezcal industry, generating vast amounts of agro industrial solid waste. This type of lignocellulosic biomass is a agro-industrial residue, which can be used to produce enzymes, giving it added value. However, the structure of lignocellulosic biomass makes it highly recalcitrant, and results in relatively low yield when used in its native form. Ultrasound and high temperature as pre-treatments result in modification of the lignocellulosic structure and composition; the ultrasound pre-treatment improved the production of inulinase by 4 U/mg and cellulase by 0.297 U/mg (Contreras-Hernandez et al., 2018).

## 1.3 Effects on the biological function

Ultrasound pre-treatment has attracted great consideration in food science as a non-thermal novel and delicate procedure to enhance the safety and quality of processed foods (Chandrapala & Leong, 2015; Dujmic´ et al., 2013). Its use in food processing can cause major changes in the physical, chemical, and functional characteristics of food ingredients that might be of concern as well as a technical advantage, particularly with 20 and 100 kHz power treatments. The cavitation phenomena, dynamic stirring, heating, turbulent flow, and shear stress could explain the changes in protein molecules triggered by ultrasound energy (Knorr, Zenker, Heinz, & Lee, 2004), which may produce the cleavage of covalent bonds, generating low molecular weight peptides, and breaking large polymer fragments into tinier particles altering the proteins functional properties. Highland barley brewer's spent grain (BSG), being China's brewing industry's major by-product, is the focus of current research. The ultrasound pre-treatment of highland barley BSG protein at 40 and 50 kHz has significantly ($P < 0.05$) enhanced about 57 and 67% the oxygen radical absorption capacity of obtained hydrolysate versus the untreated substrate. The 1,1-diphenyl-2-picrylhdrazl (DPPH) radical scavenging activity (DRSA) 28%, metal chelating activity (MCA) 54%, superoxide radical scavenging activity (SRSA)

18%, and hydroxyl radical scavenging activity (HRSA) 25% of HBSGPH at 50 kHz were also enhanced ($P < 0.05$) significantly. HBSGPH from heat treatment at 100 °C showed no SRSA and HRSA scavenging activities but around 27% ferric reducing antioxidant power (FRAP) assay values improved ($P < 0.05$) significantly . In this work, the resultant highland barley BSG protein hydrolysates (HBSGPH) had stronger antioxidant properties with ultrasound pre-treatment at 50 kHz and the enzymatic hydrolysis after 4 hr was facilitating the enzymatic release of antioxidant peptides from HBSGPH (Ikram, Zhang, Ahmed, & Wang, 2020).

# Enzymatic treatment

## 2.1 Effects on protein yield and degree of hydrolysis (DH)

Enzyme-assisted extraction is another method used for the protein extraction from plants. It has been reported that carbohydrases (cellulase, pectinase, viscozyme L) increase the extraction yields by releasing the proteins attached to the polysaccharide matrix in plant materials (Kim & Lim, 2016). Besides carbohydrases, proteases such as alcalase (Hanmoungjai, Pyle, & Niranjan, 2001; Demirhan, Apar, & Özbek, 2011), flavourzyme (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Kanu et al., 2009) and protizyme (Sharma, Khare, & Gupta, 2002) have also been used for improving protein extraction.

As reported (Montilha, Sbroggio, Figueiredo, Ida, & Kurozawa, 2017), protein hydrolysis of okara by protease presents a potential process to recovery protein as hydrolysate form. Protein hydrolysates can be used for protein supplementation, antioxidant component in food systems and diets due to the high biological value peptides. Temperature of 55 °C, enzyme:substrate ratio of 8.8% and pH of 9.0 were considered as the optimum enzymatic protein hydrolysis condition of okara, on the basis of the degree of hydrolysis DH. Under this condition, the DH could reach 37.3%. Oat pre-treated with amyloglucosidase (8 units/g) had the higher protein content (82%) while celluclast (5–60 endoglucanase units (EGU)/g) pre-treated samples had protein content similar to that of control (54%) (Table 2).

Table 2. Applications of enzymatic treatment in the extraction of compounds from food by-products.

| Food | Improvement | Enzyme | Conditions | reference |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Okara | 33.6% | Alcalase | Temperature 55 °C, pH 9.0 | (Sbroggio, Montilha, Figueiredo, Georgetti, & Kurozawa, 2016) |
| | 5.8% | Flavourzyme | Temperature 50°C, pH 7.0 | |
| Oat bran | 16% improvement | Amylase | Temperature 45 °C, pH 6.5 | (Jodayree, Smith, & Tsopmo, 2012) |
| | No improvement | Celluclast | Temperature 45°C, pH 5.5 | |
| | 16% improvement | Viscozyme | Temperature 45°C, pH 4.5 | |
| | 18% improvement | Amyloglucosidase | Temperature 45°C, pH 5.5 | |
| Coconut cake | 20.94 % | Alcalase | Temperature 45 C, pH 8.5, 2 h | (Zheng, Li, & Li, 2019) |
| | 16.29 % | Flavourzyme | Temperature 50 C, pH 7.0, 2 h | |
| | 3.15 % | Pepsin | Temperature 37 C, pH 2.0, 2 h | |
| | 10.67 % | Trypsin | Temperature 37 C, pH 7.0, 2 h | |
| | 27.62 % | Alcalase, flavourzyme, pepsin and trypsin | | |
| Almond cake | 3.3% improvement | - | Temperature 50 C, pH 9.0, 1 h | (de Souza, Dias, Koblitz, & de Moura Bell, 2020) |
| Sesame bran | | Viscozyme L, alcalase | pH (2.5 to 6.0 for viscozyme L and 6.0 to 11.0 for alcalase) | (Gorguc et al., 2019) |

## 2.2 Effects on the biological function

Proteins hydrolysis is used to improve or modify the chemical, functional and sensory properties of proteins without affecting their nutritional value (González- Tello et al., 1994). Protein hydrolysates, especially di- and tripeptides, have greater nutritional value and more efficient gastrointestinal absorption compared to intact protein and free amino acids (Bhaskar et al., 2007). In addition, the protein hydrolysis can release bioactive

peptides, defined as short sequences of amino acids (from 2 to 20 units) that exert physiological benefits on the organism. The bioactivity of peptides can be described by their antimicrobial, anticancer, immuno-modulating, antithrombotic, antioxidant, or antihypertensive propertie (Clare & Swaisgood, 2000).

The enzyme-assisted treatment of citrus by-products has already been proven to provide some advantages, such as a change in the phenolic profile and enhanced antioxidant activity (Barcelos et al., 2020; B. B. Li, Smith, & Hossain, 2006; Ruviaro, Barbosa, & Macedo, 2019), whereas the carbohydrase-assisted protein extraction has been applied to rapeseed press cake (Rommi et al., 2015), oat bran (Jodayree et al., 2012), and defatted soybean flour (Rosset, Acquaro, & Beléia, 2014). The treatment of oat bran with commercial carbohydrases (i.e. Viscozyme, Celluclast, alpha-amylase, and amyloglucosidase) greatly increases the content of soluble phenolic compounds and the antioxidant activity (Alrahmany & Tsopmo, 2012). Carbohydrases may, in fact, effectively hydrolyse the plant cell wall polysaccharide matrix and release valuable compounds, such as protein and phenols (Gligor, Mocan, Moldovan, Locatelli, Crișan, & Ferreira, 2019). Typically, enzyme formulations include cellulases, hemicellulases, and pectinases (Puri, Sharma, & Barrow, 2012). However, whilst the technological processing facilitates the digestibility and bioavailability of nutrients by disrupting the food matrix, it may impair the technological and nutritional functionalities by altering the structure of its components (e.g., depolymerization of β-glucan and/or protein denaturation) and/or the interaction between them.

Enzymatic protein hydrolysis consists of the cleavage of protein molecules: various sizes small peptides. Peptides present biological activity, such as antioxidant, antimicrobial and antihypertensive properties, they have positive influence on human health (Cumby, Zhong, Naczk, & Shahidi, 2008);(Pazinatto, Malta, Pastore, & Maria Netto, 2013). The enzymatic hydrolysis may favor the exposure of antioxidant amino acids in proteins, increasing the antioxidant activity of peptides. Several factors affect the antioxidant properties of protein hydrolysates and depend on: type of protein and enzyme, degree of hydrolysis, and pretreatment of the substrate (Elias et al., 2008);(Pazinatto et al., 2013); (Polanco-Lugo, Dávila-Ortiz, Betancur-Ancona, & Chel-Guerrero, 2014).

Peroxyl radical scavenging activity (ORAC assay) of protein isolates from at least one concentration of each enzyme was significantly higher than the control sample (no enzyme pretreatment). Analysis of alcalase protein hydrolysates demonstrated that viscozyme pretreated samples had good antioxidative activity in both

peroxyl radical and hydroxyl radical assays (Jodayree et al., 2012). Tandem mass spectrometric analysis of the hydrolysates (control and viscozyme) allowed the identification of four novel peptides using this preparative strategy. The Authors concluded that cell wall degrading enzymes, especially viscozyme and amyloglucosidase show potential for being used in the preparation of protein isolates and hydrolysates with improved antioxidative properties. Future studies will evaluate the biological activities (e.g. anti-oxidant, anti-inflammatory) of the isolated peptides. Fractionation of control and viscozyme pretreated digests will also allow identification of more peptides with potential biological functions (Jodayree et al., 2012).

To the best of our knowledge, no research has evaluated the protein/peptide and phenol profile changes induced by the treatment of the solid by-products from the production of oat-based drinks with food-grade enzymes, such as amylase, cellulase/xylanase, and proteases. Therefore, an investigation of the effects of multiple enzymatic treatments on the composition of protein concentrates and phenol extracts from an oat press cake is meaningful.

Many studies have highlighted the health benefits associated with the consumption of pea protein. In particular, pea protein and its hydrolysates exert antioxidant, antihypertensive, and hypocholesterolemic activities. In general, most of the previous studies in this area have focused on the consumption of pea and soybean proteins, but the health-promoting activity does not lie in the protein but in the peptides, which are encrypted within the protein and released upon digestion/hydrolysis. Hence, with a more mature perception of the phenomenon, owing to the presence of numerous bioactive peptides, these protein hydrolysates may provide more than one biological activity, therefore eliciting multiple health benefits (Bollati et al., 2022; Lammi et al., 2020; Lammi, Arnoldi, & Aiello, 2019; Lammi, Zanoni, Ferruzza, Ranaldi, Sambuy, & Arnoldi, 2016). For this reason, the production of hydrolysates with multifunctional behaviour represents a valid strategy in the development of new generations of functional foods and nutraceuticals (Chakrabarti et al., 2018).

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# Part II.

# Scientific contributions

# Chapter 2

## Quality Assessment of the Protein Ingredients recovered by Ultrasound-assisted Extraction from the Press Cakes of Coconut and Almond Beverage Preparation

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**Abstract:** The manufacture of vegetal beverages has the drawback of producing large amounts of press cakes that are generally used as feed components. This work had the objective of valorising the press cakes deriving from almond and coconut drinks production by using ultra sound assisted extraction (UAE) to obtain protein ingredients for human use. Starting from coconut and almond press cakes, whose initial protein contents were 19.7% and 18.6%, respectively, the UAE treatment permitted to obtain liquid fractions that were then freeze-dried in a 24.4% yield in case of coconut and a 49.3% yield in case of almond, whose protein contents were 30.10% and 22.88%, respectively. The quality of the extracted protein ingredients were assessed in term of phytic acid content, protein profile, techno-functional features, and antioxidant properties. The sonication had also a favourable effect on digestibility.

## 1. Introduction

The interest for a plant-based diet is continuously increasing owing to the consumer demand caused by health concerns and vegetarianism trends as well as by sustainability reasons [1, 2]. In particular, the market of vegetal beverages is becoming larger and larger, because these products are attractive alternatives to cow milk for vegans, vegetarians, and flexitarians as well as for people with lactose intolerance or cardiovascular disease. Their manufacturing procedure foresees pressing of the grinded starting material in the presence of water: the liquid phase is the final beverage, whereas the solid phase constitutes a residual press cake that still contains a certain protein amount. These by-products are mainly used as feed components, although the use in human nutrition would be much more desirable and profitable. Coconut and almond are more and more used for producing vegetal drinks and their press cakes are among the by-products that would require a better valorisation [3]. Efforts are therefore needed to identify new strategies for an added-value use of these materials in order to achieve the final goal of a more sustainable process for their production. In this contest, it is useful to underline that almonds contain 20-22% (w/dry w) protein providing all essential amino acids (mainly leucine, valine, and phenylalanine) and a very high intake of arginine [4], whereas coconut contains 5-6% (w/dry w) protein with a relatively favourable amino acid profile, containing all essential amino acids (mainly leucine, lysine, and valine) [5]. Since part of these proteins remain in the press cake, a main priority is certainly the development of new methods for the recovery of protein-rich ingredients from these by-products.

Among the numerous strategies used to improve the extraction yields of proteins and other bioactive components from food by-products, ultrasound-assisted extraction (UAE) appears to offer a useful solution because this technology can accelerate mass transfer and enhance the extraction kinetics [6]. The UAE is cheaper and easier to operate than other novel extraction techniques, such as pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE) [7]. Moreover, it permits to extract a wide variety of natural compounds from food matrices, including proteins, due to the cavitation effect that enhances mass transport by disrupting the plant cell walls [8] and its application is not restricted by the solvent or type of matrix used. The ultrasonic power is one of the key factors leading to an efficient extraction [9].

As for the consequence on protein integrity, some studies have investigated the structural changes after ultrasound treatment. In general, the increased intra-molecular mobility causes changes in the free sulfhydryl

groups, particle size, surface hydrophobicity, and secondary structure organization [10], although minimal structural variations are reported for whey proteins after ultrasonication [11]. It is, however, important to underline that several literature reports are focused on long-term or continuous sonication processes, whereas the effects of short-time or intermittent sonication are so far scarce. In this context, this work had the objective of evaluating the effects of UAE on the quality of the proteins extracted from almond and coconut press cakes. To work in an energy-efficient manner, the ultrasonication was performed at high intensity and for a short time, in operational conditions (time, frequency, and temperature) that had been optimized in preceding experimentations performed on soy press cake (soy-okara) [12]. The protein integrity was evaluated using different analytical methods, including also high-performance liquid chromatography coupled with tandem mass spectrometry using either an untargeted or a targeted method to investigate the variations of the primary structures of extracted proteins. Finally, the techno-functional properties were evaluated as well as the antioxidant properties.

## 2. Materials and Methods

### *2.1 Chemicals*

All reagents were of analytical grade. Acetonitrile (ACN), tris(hydroxymethyl)aminomethane (Tris-HCl), 2-iodoacetamide (IAM), 1,4-dithiothreitol (DTT), trypsin from bovine pancreas (T1426, lyophilized powder, ≥ 10,000 units/mg protein), diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), azo 2,2′ -azobis(2-methylpropionamidine) dihydrochloride (AAPH), Trolox, fluorescein were from Sigma-Aldrich (St. Louis, MO, USA). Mini-Protean apparatus, precision plus protein standards, Bradford reagent, and Coomassie Blue G-250 were purchased from Bio-Rad (Hercules, CA, USA).

### *2.2 Press Cake Samples and UAE Treatment*

The almond and coconut press cakes were supplied by a German company as frozen materials in 3 kg units directly from the production process. The protein content of the coconut press cake was 19.67% and that of the almond press cake was 18.56%. The ultrasound-assisted protein extraction experiments were performed

at Fraunhofer UMSICHT, Germany, using an ultrasonic continuous flow system (TC 10, BSONIC GmbH, Germany). The reactor volume was 4.6 L and the probe tip had a diameter of 41.75 mm. The defined ratio of 1:2.5 of press cake and tap water was heated up to a temperature of 60 ° C under continuous stirring. Then, the mixture was pumped into the ultrasound reactor at a flow rate of 140 L/h (membrane pump): the ultrasound parameters were fixed as follows: power input 4.5 kW, frequency 18 kHz, oscillation amplitude 45-60 µm, and the residence time 2 min. Hence, the total energy input for each sample was 117 kJ / L. The regulation of power input and recording of process parameters were performed via PC. The raw and ultrasonicated press cakes were analysed by scanning microscopy to investigate their morphologies. Afterwards, the processed samples were separated mechanically via a sieving press (150 µm) receiving a solid and a liquid fraction that were separately freeze-dried (Alpha 2-4 LSC plus, Christ). The liquid fraction was then submitted to a series of analyses, whereas the solid fraction was discarded. The extraction yields of the liquid phases were 24.4 ± 3.16% for coconut and 49.3 ± 0.91% for almond and their protein contents were 30.10% and 22.88%, respectively.

*2.3 Scanning Electron Microscopy (SEM) of raw and treated Press Cakes*

Scanning electron microscopy (SEM) was performed with a Vega-3 microscope (TESCAN GmbH, Dortmund, Germany), at 20 kV acceleration voltage under high vacuum. To guarantee a high resolution, the images were taken with a secondary-electron detector (SE). Freeze-dried samples (raw and treated press cakes) were fixed on a special two-sided adhesive tape and coated by a 10 nm gold surface to prevent electrostatic charging.

*2.4 Phytic Acid Determination*

Phytic acid was analysed in the freeze-dried liquid fractions and raw press cakes, following a modified colorimetric method [13]. The concentration range of 0 – 100 µg/mL of aqueous phytic acid was used as the standard for quantification. Aliquots of samples and standards (100 µL) were diluted 25 times with 2.4 mL of $H_2O$; 600 µL of the diluted samples and standards were combined with 200 µL of modified Wade reagent (0.03% of $FeCl_3$ $6H_2O$ and 0.3% of sulfosalicylic acid), and the absorbance was measured at 500 nm.

*2.5 Protein Profiling by SDS-PAGE*

To compare the effects of UAE on the protein profiles, either the raw press cakes and the freeze-dried liquid fractions were extracted with 0.1 M Tris-HCl buffer (pH 8.2, 0.5 M NaCl) by using a 1:15 (w/v) ratio, stirring for 2 h at 4 ℃. The mixtures were then centrifuged at 8,000 *g* for 15 min, the supernatants were collected and the protein concentrations were determined by the colorimetric Bradford test, carrying out the analyses at a wavelength of 595 nm and employing a standard curve based on BSA. These samples were analyzed by SDS-PAGE, using a 12% of polyacrylamide gel and a Tris-glycine buffer (pH 8.3, 0.1% SDS), and then submitted to the following analyses as described beneath.

*2.6 Discovery Proteomics by HPLC-Chip ESI-MS/MS*

The protein profile was investigated in detail by untargeted MS analysis after in-solution tryptic digestion. An equivalent volume of protein extract and 50 mM of $NH_4HCO_3$ were mixed. The reduction and subsequently alkylation was carried out according to a previously published procedure [14]. Then 4 mg/mL of trypsin solution were added to the processed samples with a ratio of 1:50 (w/w, E/S) and the reaction mixture was incubated for 16 h at 37 ℃. Formic acid (FA, 0.1 %) was used to block the reaction by adjusting the pH to 3-4. Digested samples were purified using Sep-Pak C18 cartridges (Thermo Fisher Scientific, Life Technology, Milan, Italy), and analyzed by LC-MS according to Aiello et al. [12]. The Spectrum Mill Proteomics Workbench (Rev B.04.00, Agilent) was used for the automated peptide identification from tandem mass spectra, consulting the *Cocos nucifera* (599) and *Prunus dulcis* (41767) databases. Trypsin was selected as cutting enzyme with two allowed missed cleavages and carbamidomethylation was chosen as a fixed modification. The mass tolerance was set at 1.0 Da and 0.8 Da for MS1 and MS2, respectively. Auto-validation strategy for both peptide and protein polishing mode were performed using FDR cut-off ⩽1.2%.

*2.7 Quantitative Evaluation of the UAE Effects by MRM Analysis*

A more precise evaluation of the effects of the UAE process on the protein quality was carried out by a quantitative MRM assay. Specifically, unique peptides belonging to some main coconut and almond proteins (a legumin and a vicilin for coconut and a legumin for almond) were selected using the Skyline software

(version 20.1, 64-bit, Seattle, WA, USA) with the setting described in a published paper [15]. In silico digestion was achieved by using the following parameters: enzyme, trypsin; peptide length, 7–25 amino acids long missed cleavages, none. Peptides containing cysteine and methionine were omitted from the analysis due to their possible modifications. For MRM analysis the following parameters were used "transition settings" (settings > transition settings): precursor charges, 2; fragment ion charges, 1; ion type, y and b; product ions, 3.

*2.8 Free Sulfhydryl Content*

The free sulfhydryl content was determined by a literature method [16] with appropriate adjustments. Protein extracts (20 μL) were dissolved in 180 μL Tris-glycine buffer solution (0.086 mol/L Tris, 0.09 mol/L glycine, 4 mmol/L ethylenediaminetetraacetic acid, 8 mol/L urea, pH 8.0). Then 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 5 μL) was added to the mixed solution, the mixture was reacted for 15 min at room temperature and the absorbance was measured at a wavelength of 412 nm. Glutathione (0-0.16 μmol/mL) was used as a standard to prepare the calibration curve. The mixture solution without DTNB was used as a control. The free sulfhydryl contents (-SH) were calculated as follows:

$$\text{Free SH } (\mu\text{mol/g}) = [(A-b)/(a \times C)] \times 10 \times 1000$$

Where a and b terms are from the equation of the standard curve y = ax + b; A is the absorbance of the samples; C is the protein concentration (mg/mL); and 10 is the dilution factor.

*2.9 Circular Dichroism*

CD spectra were recorded in a continuous scanning mode (190-250 nm) at 25 °C using a Jasco J-810 (Jasco Corp., Tokyo, Japan) spectropolarimeter. The experimental conditions used were previously described [12]. The estimation of the peptide secondary structure was achieved by using the DichroWeb site (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [17].

*2.10 Intrinsic Fluorescence*

The intrinsic fluorescence spectra were obtained using a fluorescence spectrophotometer (Synergy H1, Biotek). The samples were diluted in phosphate-buffered saline (PBS, 10 mM, pH 7.0) in order to reach the equal concentration of 0.05 mg/mL and transferred in Greiner UV-Star® 96 well plates with flat bottom clear cyclic-olefin copolymer (COC) wells. The excitation wavelength was set as 280 nm and the excitation and emission slit widths were set as 5 nm. The emission wavelength range was set up from 300 nm to 450 nm and the scanning speed was 10 nm/s.

*2.11 Micro Near InfraRed (NIR) Spectroscopy*

NIR spectra were recorded on a MicroNIR device (Viavi Solutions, JDSU Corporation, Milpitas, CA) operating in the spectral region 900 – 1700 nm. This is an ultra-compact device consisting of a linear variable filter (LVF), as dispersing element, directly connected to a 128-pixel linear indium gallium arsenide (InGaAs) array detector and two tungsten light bulbs as radiation source. Collection of spectra was performed with a nominal spectral resolution of 6.25 nm, as the most performing condition, using a special tool designed to get the optimal focal point in order to improve the chip sensitivity. Spectralon was used as the NIR standard reference with a 99% diffuses reflectance. All collected spectra were recorded with an integration time of 10 ms, resulting in a total measurement time of 2.5 s per sample.

*2.12 Determination of Protein Solubility, Water binding Capacity (WBC) and Oil binding Capacity (OBC)*

The protein solubility (PS) was determined according to a method previously described [18] with small modifications. Each sample (0.1 g) was dispersed into 2 mL of 0.1 M phosphate buffer solutions (at pH values from 2.0 to 10.0) and stirred for 20 min at RT. After the pH adjustment, the samples were stirred 30 min at RT and then centrifuged at 14,000 rpm for 30 min. The protein concentration in the liquid phase was determined according to the Bradford assay using BSA as a standard. The PS was expressed as percentage ratio of supernatant protein content to the total protein content. All determinations were conducted in triplicate. The WBC was determined according to a method previously described [12]. In details, 0.05 g of samples was dispersed in 500 µL distilled $H_2O_2$ and vortexed for 1 min. The mixture was incubated at room temperature

for 30 min and then centrifuged at 7000 g for 25 min at RT. The resulting supernatant was carefully decanted, and the tube containing the precipitation weighed. The OBC was determined according to a literature method [18]. In details, 0.05 g of samples was dispersed in 500 μL sunflower oil and vortexed for 1 min. The mixture was incubated at room temperature for 30 min and then centrifuged at 5000 g for 20 min at RT. The resulting supernatant was carefully decanted, and the tube containing the precipite weighed.

*2.13 Foaming Properties*

The foaming properties were determined in triplicate using a literature method [19] with slight modifications. Solutions containing 1% protein were prepared in distilled water. Aliquots of 50 mL (V1) were blended for 5 min using a magnetic stirrer (VELP Scientifica Srl, Italy) at the highest speed, poured into 250 mL graduated cylinders, and the volume of foam (V2) were immediately recorded at 0, 5, 30, and 60 min. The foaming was calculated using the following equation: Foaming = (V2 − V1) × 100 / V1. The foaming capacity was determined at 0 min and the foam stability (FS) after 5, 30, and 60 min.

*2.14 In vitro Protein Digestibility (IVPD)*

With a two-stages digestion procedure, two enzymes (pepsin and pancreatin) were applied to simulate the in-vivo protein digestion in the gastrointestinal tract [20]. During the first digestion stage, the pH of the protein extract (500 μL) was adjusted to 2-3 using 1 M HCl and then 5 μL of pepsin solution (10 mg pepsin/mL in 0.01 M HCl) were added. After 30-min incubation at 37 ℃, 5 μL of 1.0 M NaOH solution were added to stop the hydrolysis and adjust the pH to 7.8. Then 15 μL of pancreatin solution (10 mg/mL in $H_2O$, pH 7.0) were added to continue the digestion incubating the mixture for 1 h at 40 ℃. Finally, 5 μL of $Na_2CO_3$ solution (150 mM) were added to stop the reaction. During this procedure, 20 μL of the initial protein extract solution, pepsin digested protein solution, and pancreatin digested protein solution were collected. These samples were centrifuged at 5000g for 10 min, and the supernatants were collected for peptide and protein measurement using Bradford assay. The in-vitro protein digestibility was calculated using the following equation:

$$IVPD\,(\%) = \frac{P_0 - P_1}{P_0} \times 100$$

where $P_0$ is initial protein content, $P_1$ is the final undigested protein content.

*2.15 Evaluation of the Antioxidant Properties*

Three different assays were applied. The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay was performed by a standard method with slight modifications [21]. The TEAC assay, based on the reduction of the 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical was performed as previously described [21] as well as the ferric reducing ability (FRAP) assay [21].

*2.16 Statistical Analysis*

All experiments were performed in triplicate and the results were presented as the mean ± standard deviation. The collected data were subjected to analysis of variance (ANOVA) and Duncan's multiple range test was used to analyze differences between treatments ($p < 0.0001$).

**3. Results**

*3.1 Ultrasound Assisted Extraction*

As already indicated in the introduction of this chapter, the almond and coconut press cakes were treated with ultrasounds in conditions (time, frequency, and temperature) that had been optimized in a preceding investigation with the scope of improving the protein extraction yields while preserving as much as possible the protein integrity (Aiello et al, 2021). The UAE was thus performed at high intensity (power input 4.5 kW, frequency 18 kHz, oscillation amplitude 45-60 μm), at 60 ° C and for a short residence time (2 min). Starting from coconut and almond press cakes, whose initial protein contents were 19.7% and 18.6%, respectively, the UAE treatment, followed by a simple sieve filtration, permitted to obtain liquid fractions that were then freeze-dried in a 24.4% yield in case of coconut and a 49.3% yield in case of almond, whose protein contents were 30.10% and 22.88%, respectively. The increase of the protein content of both materials underlines the efficiency of the UAE for recovering protein rich materials from food by-products. This was particularly true in the case of coconut.

*3.2 Effects on the Morphology of the Coconut and Almond Press Cakes*

Scanning electron microscope analysis was used to compare the morphology of the press cakes before and after the ultrasonication process (Figure 1). Whereas the structures of the raw coconut (Figure 1A) and almond (Figure 1C) press cakes were regular and compact, those of the sonicated materials showed a slight degree of disintegration, mostly visible in the coconut sample (Figure 1B), while the almond sample appeared to be less changed (Figure 1D). Possibly, this may explain the higher protein content of the coconut liquid phase.

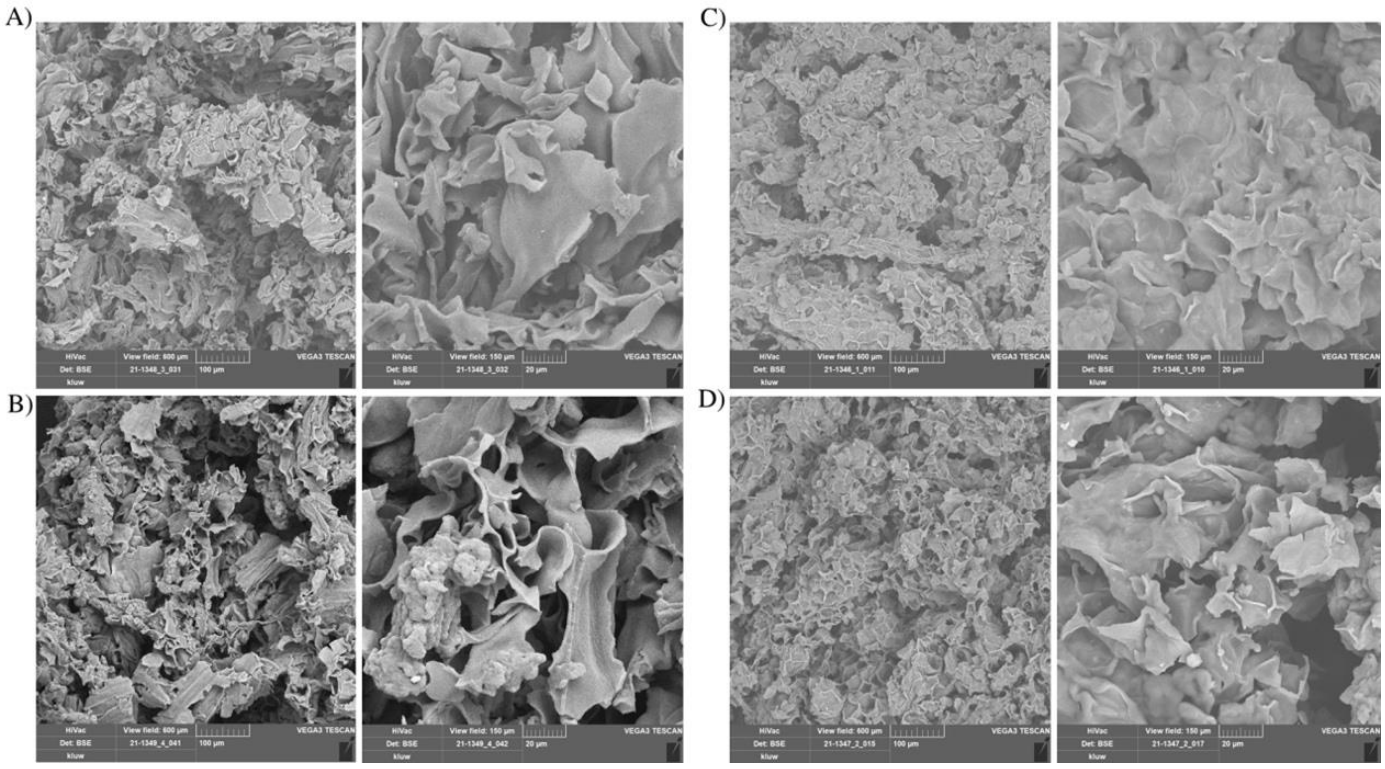


Figure 1. Comparison by the SEM of raw and ultrasound treated press cakes at two different magnifications (left 300x and right 1500x). A) Raw coconut press cake; B) Ultrasound treated coconut press cake; C) Raw almond press cake; D) Ultrasound treated almond press cake.

*3.3 Phytic Acid Content*

Phytic acid (PA) is a well-known undesirable antinutritional factor. Literature indicates that several processing methods, such as soaking, malting, fermenting and heat treatments, may be useful for reducing its content [22], although none of these techniques results in the complete removal of PA. Since some evidence also supports a favorable effect induced by ultrasound treatments [23], it was decided to measure the residual PA contents of the protein-rich liquid fractions. Indeed, our findings demonstrate that the process induced a 44.4 ± 1.0% reduction (****p <0.0001) of the PA content of the almond sample (from 2.16 ± 0.04 mg/g of protein in raw press cake to 1.21 ± 0.01 mg/g of protein in the UAE liquid fraction), and a 12 ± 0.5 % reduction in the case of coconut (from 1.05 ± 0.01 mg/g of protein in the raw press cake to 0.92 ± 0.03 mg/g of protein in the UAE liquid fraction). The smaller reduction observed with coconut may depend on the lower content of this antinutrient in the raw press cake. The PA reduction may be explained by a disruption of the phosphate bonds with the liberation of inositol induced by the cavitation as well by the dissolution of water-soluble PA salts and the activation of endogenous phytases during soaking and ultrasonication [24].

*3.4 Protein Profile*

The following experiments were dedicated to compare the protein quality in the raw press cakes and in the freeze-dried liquid fractions. In order to do so, the proteins were extracted from both materials with a standard laboratory procedure [0.1 M of Tris-HCl buffer, pH 8.2, 0.5 M NaCl, using an 1:15 (w/v) ratio] and then submitted to a series of analyses. In the Figures, the protein extracted from the coconut and almond press cakes are indicated as CtrlC and CtrlA, whereas those extracted from the coconut and almond freeze-dried liquid fractions are indicated as UltraSC and UltraSA, respectively. The protein concentrations of these materials provide a rough comparison of the amount of “soluble proteins” in the raw press cakes and the liquid samples. Figure 2A shows that these parameters were increased by the UAE in both cases. With coconut, a 132% increase was observed (from 0.65 ± 0.03 mg/mL in the raw sample to 1.51 ± 0.16 mg/mL in the treated sample), whereas with almond a smaller 53% increase was observed (from 1.30 ± 0.02 mg/mL in the raw sample to 1.89 ± 0.27 mg/mL in the treated sample). These results agree with the higher protein contents of the liquid phases versus the raw press cakes measured by Kjeldhal method.

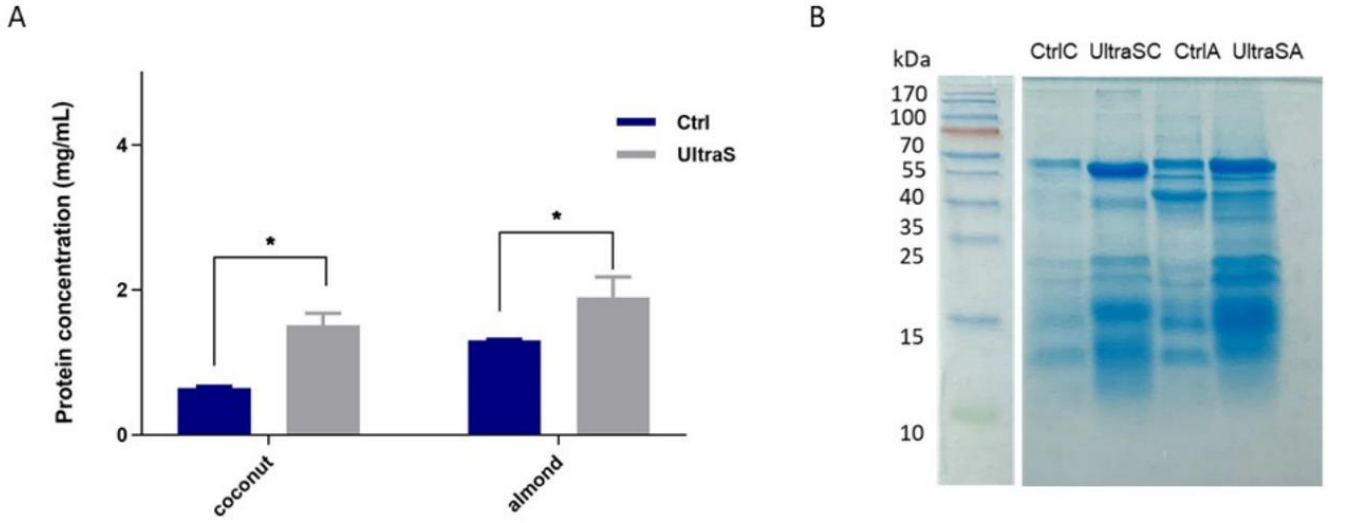


Figure 2. A) Comparison of the "soluble protein" contents of coconut and almond samples: raw press cakes (Ctrl) and UAE treated freeze-dried liquid fractions (UltraS), * significantly different ($p < 0.05$) versus the raw press cake. B) Reduced SDS-PAGE protein profile of raw press cakes (CtrlC and CtrlA) and the treated liquid fractions (UltraSC, UltraSA); M, pre-stained molecular marker. Each sample (10 μL) was added to 10 μL of loading buffer, loading 20 μL for each well.

These findings are comparable to those reported on other food materials, such as soy, whey, and egg white treated by UAE [25]. In particular, different Authors have observed that an increase in sonication time and intensity (W/cm2) or power (W) up to an optimal value may improve the solubility of the 7S fraction of a soy protein isolate (SPI). The best conditions were 400 W/cm2 for 40 min [26]; 131 – 138 W/cm$^2$ for 30 min (Hu et al., 2013c); or 200 W for 30 min and 251.16 W for 60 min [27].

However, the UAE may produce changes in the primary structures due to the cleavage of peptide bonds or, on the contrary, due to aggregation. For elucidating this aspect, the protein profile was investigated by SDS-PAGE as shown in Figure 2B. In detail, the absence of bands at the top of the separation gel and loading port confirms that no protein aggregates were formed upon the ultrasonication. Moreover, no clear signs of the cleavage of the protein primary structure were observed in the ultrasonicated samples versus the raw materials. Instead, more intense bands along the entire range of molecular weights were observed in the lanes of the ultrasonicated samples: this evidence is stronger in the coconut sample in agreement with the sharp rise of the protein content after ultrasound treatment. This suggests that the UAE disrupts the food matrix thus permitting

the release of otherwise less accessible proteins. Our results agree with a published paper where the soy protein yields were improved by 20, 23, 30, and 46%, respectively, versus the control, after sonication times of 15, 30, 60, and 120 sec at a frequency of 20 kHz [28]. However, the different treatment conditions used in that paper suggest that the total energy/energy input was much lower than in our case. A recent paper [29] has shown no difference in the primary structure of the protein profile of untreated and ultrasound-treated egg white, with a treatment conducted at 55 kHz, 45.33 $Wcm^{-2}$ for 12 min.

*3.5 Proteomic Analysis*

The proteins of the UAE liquid fractions and raw press cakes were then subjected to discovery proteomics by LC-MS analysis to fully characterize the proteome compositions. The results of the data-dependent acquisition (DDA) analysis are reported in Table 1S. The most abundant storage proteins, i.e. vicilin and legumin, were easily detected either in the raw almond press cakes or the ultrasonicated liquid sample, whereas in both coconut samples the most abundant protein was cocosin, the main component of legumin.

In addition to storage proteins, some membrane proteins such as Non-specific lipid-transfer protein and Putative lipid transfer protein (Fragment) were detected in CtrlA, whereas proteins involved in the response to water stimuli, i.e. Dehydrin xero 1 (Fragment) and Abscisic acid response protein. were identified in UltraSA. Besides storage proteins, i.e. 11S globulin and Cocosin, in UltraSC and CtrlC cytoplasmatic proteins such as Ribosomal protein L2, Non-specific serine/threonine protein kinase, Acyl-[acyl-carrier-protein] hydrolase and nuclear proteins, such as DNA-directed RNA polymerase subunit, were detected. These results indicate the presence of a subset of proteins involved in metabolic processes, such as binding with regulatory enzymes.

*3.6 Label-free Multiple Reaction Monitoring for the Quantification of Main Storage Proteins*

In order to get a further insight in the changes induced by the UAE treatment, it was decided to develop a method for the quantification of the most abundant storage proteins detected by DDA MS analysis. The ideal choice of peptides and their transitions is critical for the sensitivity and selectivity of a MRM experiment. The proteotypic peptides and their transitions were forecast by the Skyline software ver. 20.1, according to the

following criteria: (i) peptides containing 7 to 25 residues, (ii) peptides with double or triple charged m/z values within the mass range of the instrument, (iii) peptides without modification sites or amino acids susceptible to to variable changing during the sample processing. Three peptides per each protein and the relative transitions calculated with the Skyline software are listed in Table 1. The generated transition list was then exported, and the MRM method was set up on the IT instrument. Two transitions per peptide were monitored simultaneously for quantitative and qualitative analysis of the target proteins in coconut and almond samples.

**Table 1**. Transition list of selected peptides for relative protein quantification of most abundant protein identified in coconut and almond samples.

| Protein Name Accession N. | Precursor | Precursor ion $[M+2H]^{2+}$ | Transition | Ratio (UltraS/Ctrl) |
|---|---|---|---|---|
| Coconut | | | | |
| Legumin A0A5E4FFS0 (11S globulin) | (R)ALPDEVLQNAFR(I) | 687.0 | 687.0à 847.47<br>687.0à 397.20 | 2.5 |
| | (R)VQVVNENGDPILDDEVR(E) | 637.8 | 637.8à 859.45<br>637.8à 540.31 | 6.8 |
| | (R)NLQGQDDNRNEIVR(V) | 836.1 | 836.1à 630.35<br>836.1à 541.27 | 1.9 |
| Vicilin A0A5E4EZP4 (7S globulin) | (R)QLAFGPEMEQIFSK(Q) | 812.9 | 812.9 à 494.29<br>812.9 à 743.37 | 1.6 |
| | (R)EQLQALSQAASSR(R) | 695.0 | 695.0 à 499.25<br>695.0 à 819.43 | 1.9 |
| | (R)FEEFFPAGSR(N) | 594.0 | 594.0 à 634.33<br>594.0 à 553.22 | 2.0 |
| Almond | | | | |

| | | | | |
|---|---|---|---|---|
| 11S globulin isoform 2 A0A0R7UCT6 (legumin) | (K)QNIGDPRR(A) | 478.2 | 478.2 à 413.21<br>478.2 à 543.29 | 4.6 |
| | (K)QNIGDPRRADVFNPR(G) | 877.8 | 877.8 à 818.41<br>877.8 à 528.24 | 5.2 |
| | (R)GGRITTLNSEK(L) | 588.32 | 392.5 à 792.40<br>392. 5à 384.23 | 2.6 |

Nominal peak areas in the extracted ion chromatogram (XIC) were adopted for a relative quantification of each peptide. No calibration curve was used and the ratio between the areas of each peptide in the UltrS and Ctrl samples was adopted for quantification. The average ratios of each peptide per proteins (Table 1) showed that the intensity of the main proteins in coconut and almond increased after the ultrasound treatment. These results will be discussed here using the average of the ratios of the three peptides monitored for each protein. In coconut the legumin was 3.7-fold more abundant and the vicilin 1.8-fold more abundant in the UAE treated sample than in the raw press cake, whereas in almond the 11S globulin isoform 2 was 4.1-fold more abundant in the UAE treated sample than in the raw press cake. These outcomes confirm more accurately what had been highlighted by SDS-PAGE.

*3.7 Secondary and Tertiary Structure of Coconut and Almond Proteins*

Although the protein primary structures were not changed very much, low-frequency and high-intensity ultrasonication may induce changes in the secondary and tertiary protein structures due to the cavitation-induced shear leading to protein aggregation and cross-linking due to oxidation.

Hence, CD spectroscopy was used to quantify the changes of the protein secondary structure induced by the ultrasound processing. The results are summarized in Table 2 and Figure 3A. Globally, it may be affirmed that these kinds of proteins have a low content (around of 2-3%) of α-helix, about 25-30% of β-sheet substructures, 20-25% turns, and over 40% of random coils. After ultrasound processing, the CD spectra indicated a small decrease of α-helix percentage (larger in almond proteins), an evident decrease of the β-sheet in both samples, an increment of turn only in the case of almond, and a general increase of random coils. Remembering that these CD spectra reflect the situation of very complex protein mixtures, it seems possible to affirm that the UAE produced only a moderate destructuring of the protein conformation. This is very important, since the α-helix and β-sheets are the major substructures responsible for the maintenance of the tertiary protein structure, which affects important techno-functional properties, such as the solubility, swelling ability, and viscosity.

The changes in the protein secondary structures might be due to shear forces, shock waves, and turbulence caused by ultrasonication. In fact, the cavitation disrupts the interactions between the local sequences of amino acids and between different parts of the protein molecule, resulting in changes in their secondary structures [30]. Furthermore, ultrasound application may lead to selective disruption of hydrogen bonds resulting in secondary structure changes depending on the local protein conditions and amino acid sequence [31]. However, it is well documented that the specific conditions of ultrasonication, i.e. the time and the power, may affect the secondary structures rearrangement in terms of changing the α-helix and β-turn content indicating that over-processing with ultrasonic waves might increase the extensions of certain types of hydrogen bonds as verified for the β-lactoglobulin [32].

**Table 2**. Percentages of secondary structures in the samples

| Sample | α-Helix (%) | β-Sheet (%) | Turn (%) | Random coils (%) |
|---|---|---|---|---|
| CtrlC | 3.2 | 29.4 | 19.7 | 47.8 |
| UltraSC | 3.4 | 16.7 | 19.1 | 60.8 |
| CtrlA | 2.6 | 33.9 | 20.3 | 43.1 |
| UltraSA | 1.7 | 25.2 | 24.9 | 48.3 |

The measurement of the content of free-SH groups located on the surface of coconut and almond proteins as well as the detection of their intrinsic fluorescence spectra were used to provide further insight into the ability of sonication to cause changes in the protein tertiary structure. Literature evidence indicates that ultrasonication may change the number of free-SH groups, by exposing buried SH groups initially located inside the protein [27]. Our findings, instead, demonstrate a significant reduction of the number of free-SH group either in the coconut or almond ultrasonicated proteins (Figure 3B). This is particularly evident in coconut, where the free SH content of 125.15 ± 9.0 μmol/g of the raw press cake was reduced to 52.25 ± 0.58 μmol/g in the proteins submitted to the UAE.

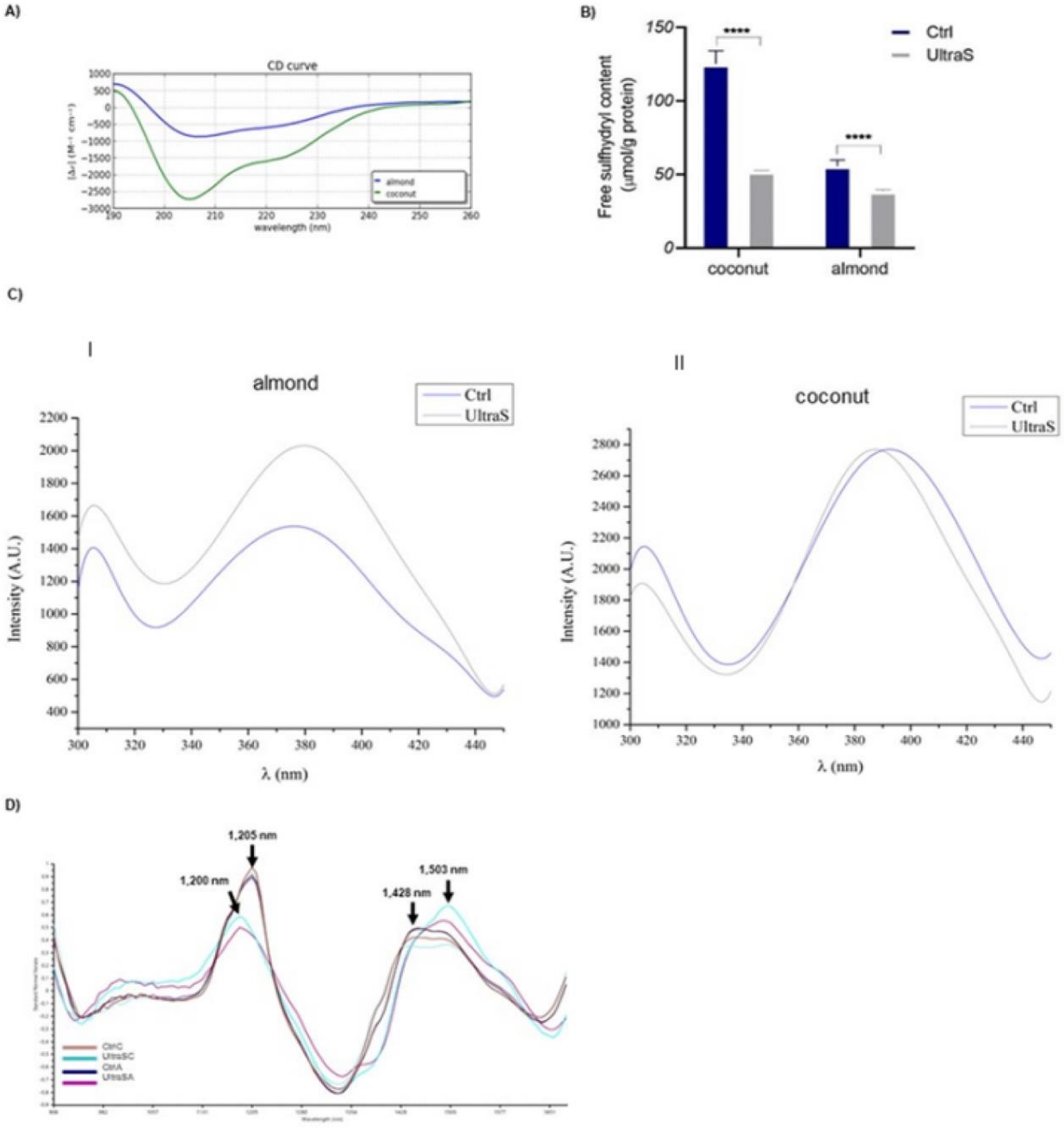


**Figure 3**. A) CD spectra of almond and coconut UltraS; B) Free-SH content in UltraSC and UltraSA, CtrlC and CtrlA; C) Intrinsic fluorescence signal detection; D) Micro-NIR Spectra expressed as standard normal variate (SNV).

These findings clearly indicate that the UAE significantly impacts on the structure of the protein with a reduction of the exposition of the cysteine residues, probably due to the formation of intermolecular disulphide bonds S-S, which modulate the folding of the extracted proteins. This trend has been also observed on soy okara proteins [12] after a UAE treatment performed in the same conditions. A similar decrease has been also observed in 7S and 11S soy proteins treated at 400 W for 5, 20, and 40 min [33]. This phenomenon may possibly depend on the generation of radical species during the sonication process, where water molecules may dissociate to generate substantial amounts of hydroxyl radicals that can oxidize the free SH groups to S-S bonds [34]. However, the divergences in the content of SH groups and S-S bonds may depend also on the sonication parameters and time as well as on the presence of impurities.

Further information on the modification of the tertiary structure was obtained applying intrinsic fluorescence spectroscopy that allows to detect the fluorescence due to tryptophan (Trp) [35]. The fluorescence spectra were recorded in the range 300-460 nm. The spectra of the almond samples (Figure 3C-I) showed a main peak at 380 nm, whose intensity increases by 27.2% after the UAE. Similar trends have been previously observed in soybean [12, 36] and chicken plasma proteins submitted to UAE [16]. Instead, in the coconut samples the modifications of the intensities were only marginal (Figure 3C-II).

Finally, Figure 3D shows the micro-NIR spectra in the range of 900 – 1,700 nm represented as standard normal variate (SNV). All samples exhibited three fundamental absorption peaks at 1200-1205, 1428, and 1503 nm. The band with maximum at 1200 (untreated samples) or 1205 nm (treated samples) may be assigned to the first harmonic stretching vibrations of the –CH groups of the protein side chains, the peak at 1428 nm to a C=C symmetric stretching, whereas the peak at 1503 nm to the first overtone of the stretching of the –OH groups [37]. The small differences observed in the UAE samples indicate only marginal modifications induced by this treatment.

*3.8 Modifications of Techno-Functional Properties*

The solubility of coconut and almond samples before and after UAE as a function of pH is shown in Figure 4 A-B. The lowest solubility values of untreated (7.8%) and treated (2.9%) almond were obtained at pH 4.0, whereas in alkaline conditions the samples were very soluble. At low pH the UAE sample was less soluble than the untreated sample. These results agree with a recent study on wild almond [38], which showed low

solubility levels around the pH 4-5 and high ones in alkaline conditions. With coconut (Figure 4B), the lowest solubility was observed at pH 6.0 either in the untreated (7.3%) or treated (9.2%) sample. Above pH 7 the solubility increased reaching the maximum value at pH 8 for the untreated sample and at pH 10 for the UAE one. These data agree with literature data [5].

The WBC and OBC are useful pieces of information for the application of these ingredients in foods. Figure 4C-D shows that in the case of almond the WBC was only slightly modified by the UAE (from 7.86 ± 0.11 g water/g sample in CtrlA to 6.89 ± 0.11 g water/g sample in UltraSA), whereas the OBC was significantly decreased (from 3.16 ± 0.10 in CtrlA to 0.59 ± 0.01 g water/g sample in UltraSA). In coconut samples, the ultrasonication decreased either the WBC or the OBC: also in this case the most significant reduction was observed on OBC (from 2.54 ± 0.01 g water/g sample in CtrlC to 0.53 ± 0.01 g water/g sample in UltraSC (Figure 4C-D).

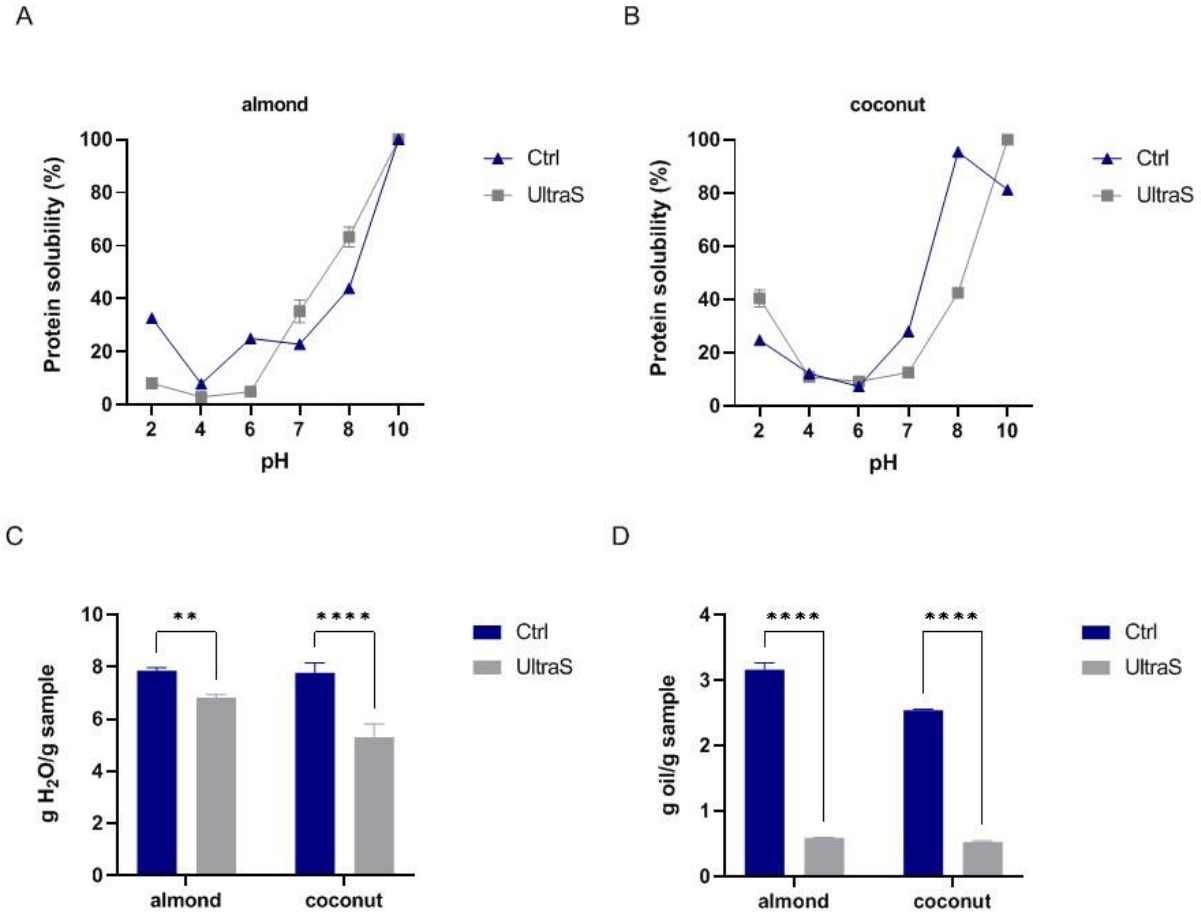


**Figure 4**. Protein solubility before and after the UAE for (A) almond and (B) coconut samples. (C) Water Binding Capacity and (D) Oil Binding Capacity of untreated and treated samples. The data are represented as

the means ± s.d. of three independent experiments. All data sets were analyzed by Two-way ANOVA followed by Šídák's multiple comparisons test. CTRL: control sample. (**) $p < 0.01$, (****) $p < 0.0001$.

The solubility and water/oil binding capacity are important properties strictly correlated to other technical-functional aspects of proteins: i.e. their foaming capacity (FC) and stability (FS). Proteins in dispersion cause a lowering of the surface tension at the water / air interface thus generating FC. The foaming properties of the proteins extracted from almond and coconut are shown in Table 3. In almond samples, which have modest foaming properties, FC remained practically unchanged after the UAE, whereas the FS was increased. This behavior agrees with literature reports indicating that the UAE displays a positive effect on foaming properties of food derived proteins [39, 40]. In the case of coconut, which has much better foaming properties, the treatment caused important decrease either in the FC or in the FS. This reduction is probably due to extensive alterations of the physicochemical properties induced by the UAE.

**Table 3**. Foaming capacity (FC) and foam stability (FS) of almond and coconut proteins

| Samples | FC (%) | FS, 5 min (%) | FS, 30 min (%) | FS, 60 min (%) |
|---|---|---|---|---|
| CtrlA | 1.7 ± 0.8 | 0.5 ± 1.2 | / | / |
| UltrSA | 1.9 ± 0.6 | 1.1 ± 0.8 | / | / |
| CtrlC | 21.6 ± 1.9 | 14.1 ± 0.6 | 10.7 ± 1.3 | 3.7 ± 1.4 |
| UltrSC | 14.1 ± 0.5 | 12.0 ± 1.8 | 6.7 ± 0.4 | 4.6 ± 1.6 |

*3.9. In vitro Protein Digestibility (IVPD)*

The intense shear stress and pressure in a short time caused by the cavitation may improve the protein digestibility, owing to changes in the secondary structures as well as to disruption of the protein sequences [41]. It was thus decided to evaluate the IVPD% using a two-stage digestion with pepsin and pancreatin. The IVPD% of the raw coconut and almond samples were 35.0% and 59.2 %, respectively, whereas after UAE, the values became 40.8% and 62.6%, respectively (Figure 5). The sonication had therefore a favorable effect on digestibility.

The most prominent factors affecting the in-vitro digestibility after processing are the protein solubility and

secondary structure changes [42, 43]. Digestibility is often positively affected by protein solubility: for example, an increased solubility of potato protein facilitates the interaction with digestive enzymes, leading to higher digestion rates [44]. This might happen because the ultrasound treatment exposes more hydrophilic groups by enhancing the interaction between the proteins and the water molecules. In addition, there is a negative correlation between the IVPD% and the β-sheet content [42]. Indeed, the digestibility process does not involve protein regions with a β-sheet structure and a high negative linear correlation coefficient (r = -0.980) has been found between the β-sheet contents of all proteins and the food digestibility values [45]. In our case, the increases of IVPD% induced by sonication may depend mainly on the variations of the protein secondary structures, in particular on the reduction of β-sheet substructures.

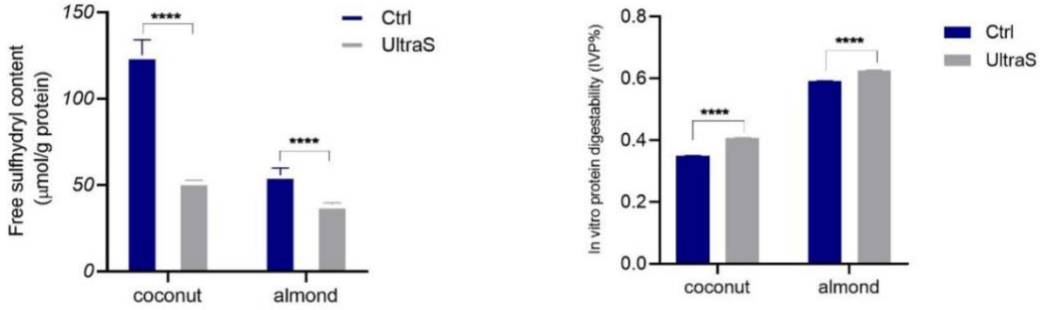


Figure 5. In-vitro digestibility (IVPD%) of the same samples.

*3.10 Antioxidant Properties*

The antioxidant properties were assessed using different methods, such as the ABTS, DPPH, and FRAP assays. As shown in Figure 6, the CtrlA and UltraSA samples scavenged the ABTS radical by 21.24 ± 2.02% and 14.85 ± 3.34% at 0.075 mg/mL, whereas CtrlC and UltraSC by 21.72 ± 2.18% and 25.06 ± 1.65% at 0.03 mg/mL, respectively (Figure 6A).

In the DPPH test, CtrlC and UltraSC reduced the DPPH radical by 8.06 ± 1.59% and 7.09 ± 0.94% at 0.1 mg/mL, respectively, whereas CtrlA and UltraSA by 7.99 ± 0.66% and 11.77 ± 0.96% at 0.25 mg/mL, respectively (Figure 6B). Thus only in almond the UAE improved the ability to scavenge the DPPH radical: a similar behavior had been observed in a previous investigation on proteins extracted from soybean okara by UAE [12]. The increase may be due to the exposure of hidden amino acid residues and/or side chains with antioxidant capacities, before hidden within the three-dimensional structure of proteins.

Finally, the UAE treatment decreased in a significant way the FRAP activity either in almond samples (from 2446 ± 23.08% in CtrlA to 1362 ± 23.08% in UltraSA at 0.075 mg/mL), or in coconut samples (from 2400 ± 23.08% in CtrlC to 1823 ± 18.31% in UltraSC at 0.03 mg/mL) (Figure 6C). The reduction is probably related to the presence of chelating amino acids in the protein sequence (Cis, His, Glu) [46]. Previously (Figure 3B), we showed that the UAE reduced the content of free-SH groups positioned on the surface of coconut and almond proteins. Since these groups are critical for the interaction with free radicals, their reduction in both treated samples has certainly a main role in the observed reduced FRAP ability.

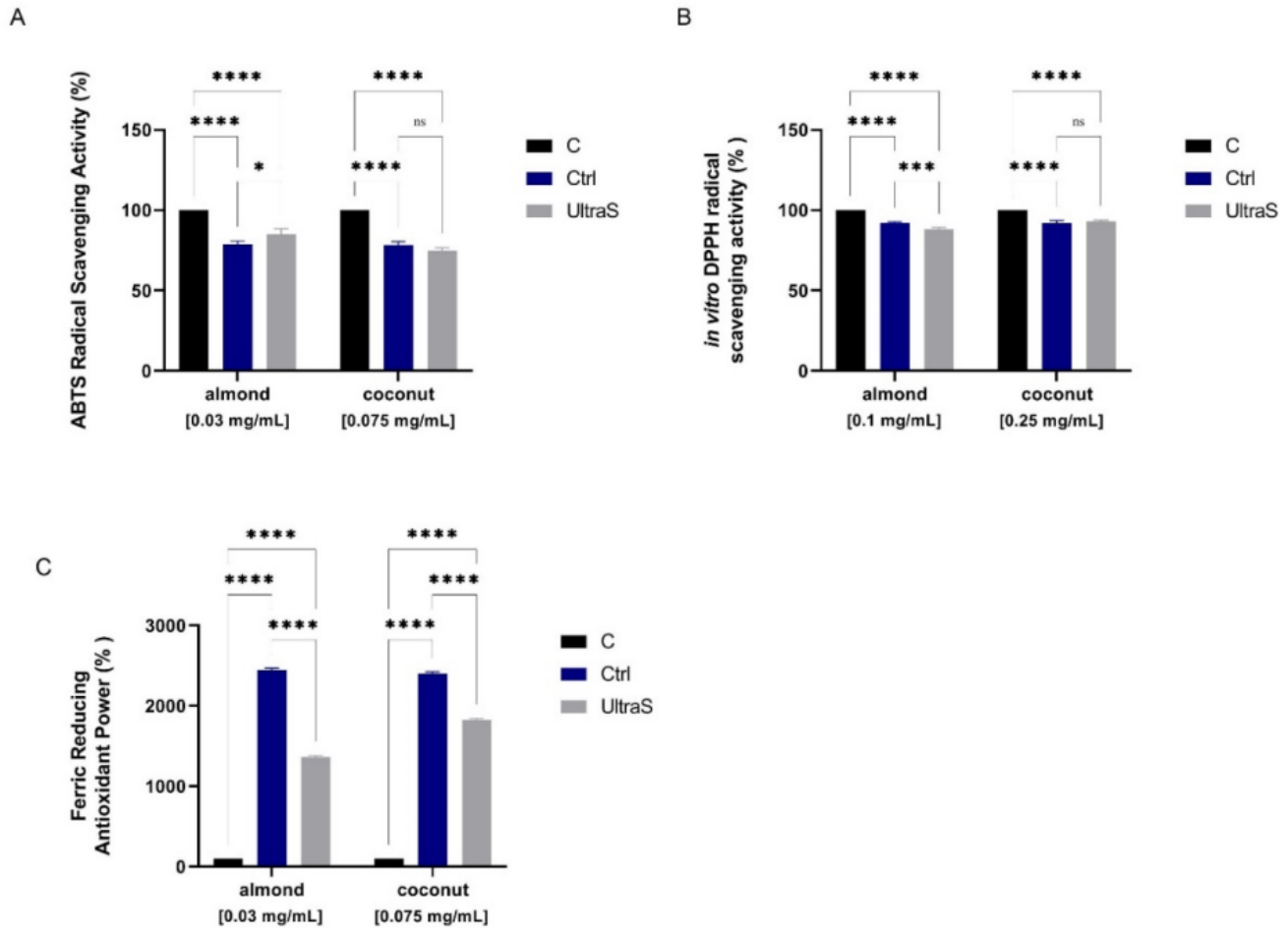


Figure 6. Antioxidant power evaluation of almond and coconut before and after UAE treatment: A) 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS); B), 2,2-diphenyl-1-picryl-hydrazyl (DPPH); C) ferric reducing antioxidant power (FRAP) assays. The data points represent the averages ± s.d. of four independent experiments performed in duplicate. All data sets were analyzed by Two-way ANOVA followed by Šídák's multiple comparisons test. ns: no significance. (*) $p < 0.05$; (***) $p < 0.001$; (****) $p < 0.0001$.

## 4. Conclusions

This paper reports data on the application of UAE for improving the recovery of protein ingredients from press cakes deriving from vegetal drinks manufacturing. Indeed, the protein contents of the recovered ingredients were increased by the treatment with a parallel decrement of phytic acid. The mass spectrometry analysis indicated that the UAE improved mainly the content of storage proteins. The investigation on the secondary and tertiary protein structures pointed out a decrease of β-sheet content that had certainly a role in the observed improved digestibility. As for the techno-functional properties, the WBC of the protein ingredients were only slightly lower than those of the raw press cakes, whereas the OBC were much damaged by the treatment. Different effects were observed as far as the foaming properties are involved: the modest foaming properties of almond samples were scarcely modified by the UAE treatment, whereas the good foaming properties of the coconut samples were much more sensitive. Interestingly, however, the foaming capacity was highly decreased, while the foaming stability remained practically unchanged. These materials had also moderate antioxidant properties that were only slightly modified by the UAE, with the exception of the poor results of the FRAP assay: this is probably linked to the observed reduction of free SH groups. In conclusion, in this work we have collected numerous complementary pieces of information on the protein ingredients extracted from press cakes using UAE. These results confirm that this technique is a promising strategy to be implemented at industrial level to improve the recovery and enhance the quality of protein ingredients from the by-products deriving from vegetal beverage production.

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# Chapter 3

## Composition of the Protein Ingredients from Insoluble Oat Byproducts Treated with Food-Grade Enzymes, Such as Amylase, Cellulose/Xylanase, and Protease

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**Abstract:** The manufacture of plant-based drinks has the drawback of a huge production of underexploited press cakes. In particular, the oat press cake is mainly used in feed formulation, whereas added-value applications in human nutrition are scarce. Considering that enzymatic treatments may be useful to improve the nutritional quality of these insoluble byproducts, this study aimed to evaluate whether the treatment with some food-grade enzymes, such as amylase, cellulase/xylanase, protease, and their combination, may be useful to achieve this goal. Proteomic and peptidomic studies showed that the enzymatic treatments improved the protein extraction yields and induced a release of low molecular weight (LMW) peptides that were demonstrated to provide a useful antioxidant activity. In the treated oat press cake proteins, the concentration of the bound phenolic compounds was decreased, with the exception of caffeic acid, which was increased, and avenanthramides, which remained unchanged. Finally, the enzymatic treatment decreased the concentration of phytic acid. All these results indicate that the enzymatic treatments may be useful to ameliorate the nutritional profile of these protein ingredients, before their inclusion in different food products.

## 1. Introduction

A plant-based diet and plant proteins are becoming more and more important to meet the nutritional requirements of the growing human population, as well as to reduce the negative impact of food production on the environment. In this context,

the valorization of sidestreams is certainly one of the main challenges for boosting the environmental and economic sustainability. Some food byproducts, such as those derived from the production of plant-based drinks, are currently among the most interesting waste materials owing to their high nutritional potential.

Compared with other cereals, oat (*Avena sativa*) can tolerate harsher growing conditions, such as a wet climate and acidic soil, and is, therefore, more resilient than other crops [1]. The health benefits of oat are attributed to its multifunctional characteristics and nutritional profile, it being an important source of nutrients and phytochemicals, i.e., well-balanced proteins, essential amino acids, fatty acids, dietary soluble fiber such as β-glucan, and phenolic compounds [1]. Among phenolics, a predominant position is occupied by avenanthramides (AVNs) and avenalumic acids, which are unique to oat among cereals. These compounds have been shown to possess an antioxidant activity in vitro and in vivo and are believed to be present also in the byproducts deriving from the production of oat beverages [2].

In view of making the agro-industry more sustainable and competitive by the valorization of the sidestreams, enzymatic treatments are nowadays widely employed. They aim to reduce the cell wall rigidity and to improve the extraction yields of protein and small molecules, such as phenols, and represent a promising method for improving the nutritional quality of these byproducts. The use of carbohydrate-degrading enzymes may improve the release of proteins from the matrix; in fact, plant proteins are partially linked to the lignocellulosic fraction, as reported in leaves, kernels, and other residues [3].

The enzyme-assisted treatment of citrus byproducts has already been proven to provide some advantages, such as a change in the phenolic profile and enhanced antioxidant activity [4–6], whereas the carbohydrase-assisted protein extraction has been applied to rapeseed press cake [7], oat bran [8], and defatted soybean flour [9]. The treatment of oat bran with commercial carbohydrases (i.e., Viscozyme, Celluclast, alpha-amylase, and amyloglucosidase) greatly increases the content of soluble phenolic compounds and the antioxidant activity [10]. Carbohydrases may, in fact, effectively hydrolyze the plant cell wall polysaccharide matrix and release valuable compounds, such as protein and phenols [11]. Typically, enzyme formulations include cellulases,

hemicellulases, and pectinases [12]. However, whilst the technological processing facilitates the digestibility and bioavailability of nutrients by disrupting the food matrix, it may impair the technological and nutritional functionalities by altering the structure of its components (e.g., depolymerization of β-glucan and/or protein denaturation) and/or the interaction between them.

To the best of our knowledge, no research has evaluated the protein/peptide and phenol profile changes induced by the treatment of the solid byproducts from the production of oat-based drinks with food-grade enzymes, such as amylase, cellulase/xylanase, and proteases. This study, therefore, aimed to investigate the effects of multiple enzymatic treatments on the composition of protein concentrates and phenol extracts from an oat press cake. High-performance liquid chromatography coupled with mass spectrometry (HPLC–MS/MS) and data analysis were used to investigate the proteins, peptides, and phenolic profiles. Finally, the antioxidant activities of 3 kDa fractionated peptides were measured and the antinutritional factor phytic acid was quantified.

## 2. Materials and Methods

### *2.1. Materials and Reagents*

All chemicals and reagents were of analytical grade and from commercial sources. Acetonitrile (ACN), Tris(hydroxymethyl)aminomethane (Tris–HCl), hydrochloric acid (HCl), ammonium bicarbonate, 2-iodoacetamide (IAM), 1,4-dithiothreitol (DTT), and trypsin from bovine pancreas (T1426, lyophilized powder, ⩾10,000 units/mg protein) were from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), Mini-Protean apparatus, precision plus protein standards, Bradford reagent, and Coomassie Blue G-250 were purchased from Bio-Rad (Hercules, CA, USA). The liquid bacterial α-amylase used for starch hydrolysis and the liquid preparation Cellustar XL (containing cellulase and xylanase) used for the hydrolysis of non-starch polysaccharides were obtained from AB Baltic Enzymes (Vilnius, Lithuania), whereas the neutral protease SQzyme PS-NL used for protein hydrolysis was from SUNTAQ (Guangzhou, China).

### *2.2. Enzymatic Treatments of the Oat Press Cake and Protein Extraction*

The oat press cake (moisture content 64.17 ± 0.08%) from an industrial oat drink production was provided by

a German company in a frozen state and stored at −18 °C. Its composition is shown in Table 1. After defrosting at room temperature, grinding by a laboratory grinder, and passage through a 200 µm sieve, the oat press cake meal was dissolved in distilled water in a 1:3 (*w*/*v*) ratio until the formation of a homogenous slurry, and then hydrolyzed with the different enzymes or enzyme combinations. The amounts of enzymes added to 100 g of slurry were the following: amylase 100 AU, cellulase/xylanase 400 AU, and protease 200 AU. The enzymatic activities are those indicated by the producing companies.

The characteristics of these enzymatic treatments are reported in Table 2. Each hydrolysis was performed at 50 °C for 90 min, then each suspension was centrifuged for 15 min at 3000× *g*. The supernatants were collected and the proteins were precipitated by adding 0.1 N hydrochloric acid until pH 5.0. The oat press cake proteins were collected by centrifugation and then freeze dried. The procedure for protein precipitation was performed also on the untreated oat press cake meal to obtain the control sample. The samples were thus the following: untreated oat press cake protein (Oat_Ctrl), protein from the oat press cake treated with amylase (Oat_Amy), protein from the oat press cake treated with cellulase/xylanase (Oat_Cxl), and protein from the oat press cake treated with amylase + cellulase/xylanase + protease (Oat_Mix).

**Table 1.** Proximate analysis of the untreated oat press cake (%, d. m.).

| Component | Percentage |
|---|---|
| Moisture | 64.17 ± 0.08 |
| Protein | 32.42 ± 0.45 * |
| Lipids | 7.79 ± 0.03 * |
| Insoluble dietary fibre | 22.97 ± 0.15 * |
| Soluble dietary fibre | 3.19 ± 0.02 * |
| Starch | 27.32 ± 0.20 * |

* Percentage of dry matter; data are expressed as mean value ($n$ = 3) ± SD; SD—standard deviation.

**Table 2.** Characteristics of used enzymes.

| Enzyme | Activity, U/g | Organism of Origin | Optimal pH | Optimal |
|---|---|---|---|---|

| | | | | Temperature, °C |
|---|---|---|---|---|
| α-Amylase | >1400 | *Bacillus licheniformis* | 5.5–7.5 | 70–85 |
| Cellulase/xylanase mixture | >45,000 cellulase >34,000 xylanase | *Trichoderma reesei* | 5.0–6.5 | 40–60 |
| Protease | 116,350 | *Aspergillus oryzae* | 6.0–7.5 | 30–50 |

*2.3. Analysis by SDS–PAGE and Mass Spectrometry*

Each protein sample was suspended in 10 mL of 100 mM Tris−HCl/0.5 M NaCl buffer at pH 8.0 at 4 °C overnight [13] and any solid residue was eliminated by centrifugation at 10,000× *g* for 30 min at 4 °C. The protein content of each solution was then assessed according to the Bradford method using BSA as standard for the calibration curve. The molecular weight distributions of the proteins from the untreated and treated samples were determined using reducing dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The solutions were prepared by mixing 15 µL of each sample with 10 µL of Laemmli buffer (4% SDS, 20% glycerol, 10%, 0.004% bromophenol blue, and 0.125 M Tris−HCl, pH 6.8). Each solution was boiled for 5 min at 95 °C and then 25 µL were loaded in each lane of the gel, which was composed of a 4% polyacrylamide stacking gel over a 12% resolving polyacrylamide gel. The electrophoresis was conducted at 100 V until the dye front reached the gel bottom. Staining was performed with colloidal Coomassie Blue and destaining with 7% (*v*/*v*) acetic acid in water. The gel image was acquired by using the Bio-Rad GS800 densitometer and analyzed by using the software quantity One 1-D. Gel bands for all samples were sliced, digested with trypsin [14], and analyzed by nano-HPLC-CHIP-ESI Ion Trap using the experimental conditions previously reported [13]. The MS data were analyzed by Spectrum Mill Proteomics Workbench (Rev B.04.00, Agilent, Santa Clara, CA, USA), consulting the *A. sativa* (2508 entries) protein sequences database down-loaded from the National Center for Biotechnology Information (NCBI).

*2.4. Circular Dichroism (CD) Spectroscopy*

CD spectra were recorded in continuous scanning mode (190–300 nm) at 25 °C using a Jasco J-810 (Jasco

Corp., Tokyo, Japan) spectropolarimeter. All spectra were collected using a 1 mm path-length quartz cell and averaged over three accumulations (speed 50 nm $min^{-1}$). A reference spectrum of distilled water was recorded and subtracted from each spectrum. The estimation of the peptide secondary structure was achieved by using the method proposed in the literature [15,16].

*2.5. Degree of Hydrolysis and Free Sulfhydryl Group Determination*

The degree of hydrolysis (DH) of each sample was measured by the o-phthaldialdehyde (OPA) assay [17]. The sulfhydryl groups at the surface of the oat press cake proteins were determined according to a method proposed in the literature [18] with some modifications. Briefly, the Ellman's reagent was prepared as follows: 4 mg of DTNB reagent was added to 1 mL of Tris–glycine buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA, pH 7.0). Each solution was diluted in Tris–glycine buffer (*w*/*v* 0.15%). Then, 5 µL of Ellman's reagent was added to 200 µL of protein suspension. The resulting protein suspensions were incubated at room temperature for 15 min under shaking and then centrifuged at 10,000× *g* for 10 min at room temperature. The absorbance was then recorded at 412 nm. A buffer solution without proteins was used as a reagent blank.

*2.6. 3 kDa Fractionation, Peptide Content, and LC–MS Analysis*

To collect the peptides released by the enzymatic treatment, the untreated and treated oat press cake protein samples (0.1 g) were solubilized in 1 mL of water. The solubilized peptides were fractionated by ultrafiltration, using membranes with a 3 kDa molecular weight cut-off (MWCO) (Millipore, Burlington, MA, USA). The peptide content was determined by o-phthalaldehyde (OPA) assay, following the procedure detailed in the literature [19] with some modifications. This assay is based on the formation of an adduct between the peptide α-amino group and OPA reagent by mixing 200 µL of OPA reagent with 20 µL of sample. After 1.5 min of incubation at 25 °C, the absorbance was measured at 340 nm using the Synergy H1 fluorescent plate reader (Biotek, Bad Friedrichshall, Germany). GSH (0–5 mg/mL) was used to build the calibration curve and the peptide content was obtained by interpolation. The peptides were analyzed by nano LC–MS/MS analysis according to chromatographic and MS condition reported in the Materials and Methods. Figure S1 shows the MSn TIC of the analyzed samples. The MS data were analyzed by Spectrum Mill Proteomics Workbench (Rev

B.04.00, Agilent), consulting the *A. sativa* (22,508 entries) protein sequences database downloaded from the National Center for Biotechnology Information (NCBI). For MS/MS analysis and searching against a polypeptide sequence database, a non-enzyme-specific search considering all of the possible proteolytic cleavages was selected as a criterion. The percentage of amino acid composition was calculated by using the ExPASy-ProtParam tool by inserting as an input data set the sequences of the peptides identified by LC–MS.

### *2.7. Extraction of Bound Phenols*

Briefly, 20 mg of untreated and treated oat press cake proteins were suspended in 400 µL of $H_2O$ and mixed thoroughly by vortex. The pH of solution was adjusted to 2 by adding 1 M HCl, then 10 µL of pepsin (4 mg/mL) was added and the solution was stirred for 1 h at 37 °C. This first digestion step was followed by a second one. The pH of solution was changed to 8 with 1 M NaOH, and 10 µL of trypsin (4 mg/mL), 10 µL of chymotrypsin (4 mg/mL), and 2 µL of pancreatin (4 mg/mL) were added. The digestion was performed at 37 °C for 2 h. After enzymatic digestion, ethanol (1.6 mL) was added to the mixture (to reach an 80% ethanol concentration), to extract the phenolic compounds. The solution was incubated under magnetic stirring overnight, centrifuged at 13,000× *g* for 5 min at room temperature, and the supernatant was collected. For the LC–MS injection, the supernatant was dried by Speed-Vac and then dissolved in 100 µL of 95% $H_2O$, 5% ACN, 0.1% FA.

### *2.8. Phenolic Compound Identification and Quantification by MS*

The quantification of gallic acid, vanillic acid, ferulic acid, caffeic acid, p-coumaric acid, cinnamic acid, and three AVNs (AVN A, AVN B, and AVN C) in the extracted phenolic fractions was performed by multiple reaction monitoring (MRM) mass spectrometry, monitoring one transition for each phenol. The monitored MRM transition for ferulic acid was *m*/*z* 585.3 → *m*/*z* 178.8, for p-coumaric *m*/*z* 165.2 → *m*/*z* 120.0, for caffeic acid *m*/*z* 181.2 → *m*/*z* 162.9, for gallic acid *m*/*z* 171.1 → *m*/*z* 142.9, for cinnamic acid *m*/*z* 149.3 → *m*/*z* 130.9, and for vanillic acid *m*/*z* 169.1 → *m*/*z* 142.9. The transitions monitored for the quantification of AVNs were: *m*/*z* 300.1 → *m*/*z* 147.0, *m*/*z* 330.1 → *m*/*z* 177.0, and *m*/*z* 316.1→ *m*/*z* 163.0 for AVN A, AVN B, and AVN C, respectively. The LC separation was performed applying the following gradient: 0% solvent

B (0 min), 40% solvent B (0–10 min), 95% solvent B (10–20 min), and back to 0% in 15 min. The drying gas temperature was set at 300 °C, flow rate 3 L/min (nitrogen). Data acquisition was carried out in positive ionization mode. Capillary voltage was −63.0 for AVN A, AVN B, and AVN C, respectively. ass spectra were acquired in the mass range from m/z 50 to 600 Da. Three technical replicates (LC–MS/MS runs) were run for each sample. Analytical parameters, i.e., LOQ and LOD, were measured to ensure the appropriate performance of the developed method. The accuracy of the assay was assessed by spiking the untreated oat press cake protein sample with 25 µg/mL of each standard phenolic acid and 20 ppb of each AVN. The sensitivity of the method was calculated by the LOQ (signal-to-noise (S/N) = 10) and LOD (S/N = 3). The analytical validation study evaluated the assay accuracy, the intra-day precision linearity, and the recovery.

### *2.9. Radical Scavenging Activity of the 3 kDa Peptide Extracts measured by Ferric Reducing Ability (FRAP) Assay*

The FRAP assay was carried out as described by Benzie and Strain [20], with minor modifications for working on a 96-well microplate. The FRAP reagent was prepared by mixing 25 mL of 300 mmol/L sodium acetate buffer, 2.5 mL of 10 mmol/L TPTZ solution, and 2.5 mL of 20 mmol/L $FeCl_3$ solution in a 10:1:1 ratio. Each sample (20 mL) was mixed with 200 µL of FRAP reagent, mixed vigorously, and incubated at 37 °C for 10 min. The ferric tripyridyltriazine ($Fe^{III}$-TPTZ) complex is reduced to ferrous tripyridyltriazine ($Fe^{II}$-TPTZ) form in the presence of antioxidants and develops an intense blue color, with maximum absorption at 593 nm. Concentrations of 0 to 1000 µM $FeSO_4 \bullet 7H_2O$ were used for the calibration curve. The results are expressed as µmol/L of $Fe^{2+}$ equivalents.

### *2.10. Phytic Acid (PA) Quantification*

The untreated and treated oat press cake protein samples were lyophilized before phytic acid determination, which was performed following a colorimetric method [21]. Aqueous phytic acid solutions at the concentrations of 0–100 µg/mL were used for the quantification. Samples (100 µL) and standard solutions were diluted 25 times with 2.4 mL of $ddH_2O$; then, 600 µL of the diluted samples and standards were combined with 200 µL of modified Wade reagent (0.03% of $FeCl_3$ $6H_2O$ and 0.3% of sulfosalicylic acid), and the absorbance was measured at 500 nm.

*2.11. Total Carbohydrate Quantification*

The total carbohydrate content of each sample was measured by the phenol sulfuric acid assay [22]. Untreated and treated oat press cake proteins (10 mg) were suspended in 1 mL of 1 M HCl, and then the solution was heated at 100 °C for 2 h. The solution was separated by centrifugation at 5000× *g* for 10 min at room temperature, and the supernatant was collected. An aliquot of 10 µL of supernatant was mixed with 100 µL of 5% (*w*/*w*) phenol solution and then 500 µL of concentrated sulfuric acid. The mixture was shaken for 25 min at 28 °C and the absorbance was measured at 490 nm.

*2.12. Statistical Analysis*

All experiments were performed in triplicate, and the data are presented as the mean ± standard deviation. The collected data were subjected to analysis of variance (ANOVA). Duncan's multiple range test was used to analyze differences between treatments.

## 3. Results and Discussion

*3.1. Effects of the Enzymatic Treatments on Bound Carbohydrates*

To investigate how the total carbohydrates of the oat press cake proteins had been affected by the enzymatic treatments, the phenol sulfuric acid assay was employed. Figure 1 shows that the enzymatic treatments induced the release of bound carbohydrates. In detail, increases of 54%, 12%, and 63% in the carbohydrate content were observed in Oat_Amy, Oat_Cxl, and Oat_Mix, respectively, versus Oat_Ctrl. It is not surprising that amylase was more efficient than the other enzymes in releasing bound carbohydrates, since starch is very abundant in the oat press cake (Table 1). However, the combination of cellulases and xylanase produced a moderate release of bound carbohydrates (with a low statistical significance) by a partial hydrolysis of the oat fiber polysaccharides.

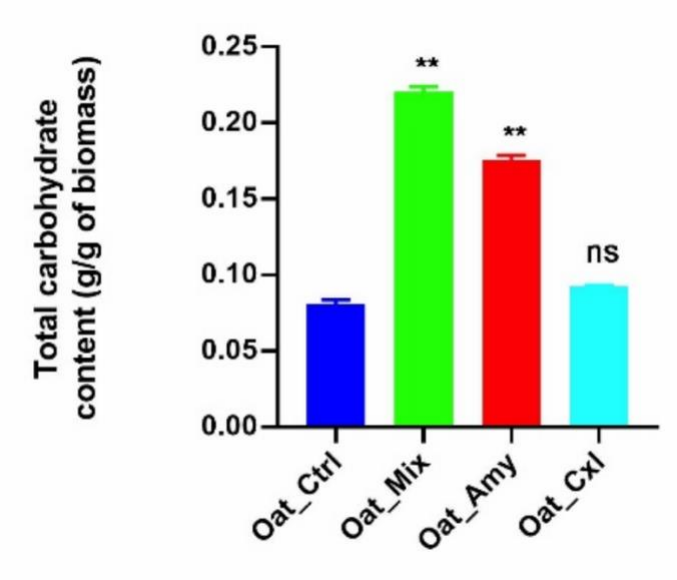



**Figure 1.** Total carbohydrate content determination. Statistical analysis was performed by one-way ANOVA. The data are presented as the means ± s.d. of three independent experiments, ** $p < 0.001$.

*3.2. Effects of the Enzymatic Treatments on the Primary Structure of the Oat Press Cake Proteins*

Proteins are largely responsible for the main characteristics of most foods, since their composition influences the nutritional, rheological, and sensory properties. Enzymatic treatments may induce chemical and structural modifications, impacting the nutritional features of the final products. The effects of the enzymatic treatments on the oat press cake proteins were initially explored by evaluating their molecular weight profile using electrophoresis in reducing conditions. Figure 2 shows the SDS–PAGE of the untreated and treated oat press cake protein samples. Gel bands were then sliced, digested with trypsin, and analyzed by nano-HPLC–ESI–MS/MS. The proteins identified in each sample are listed in Table 3.

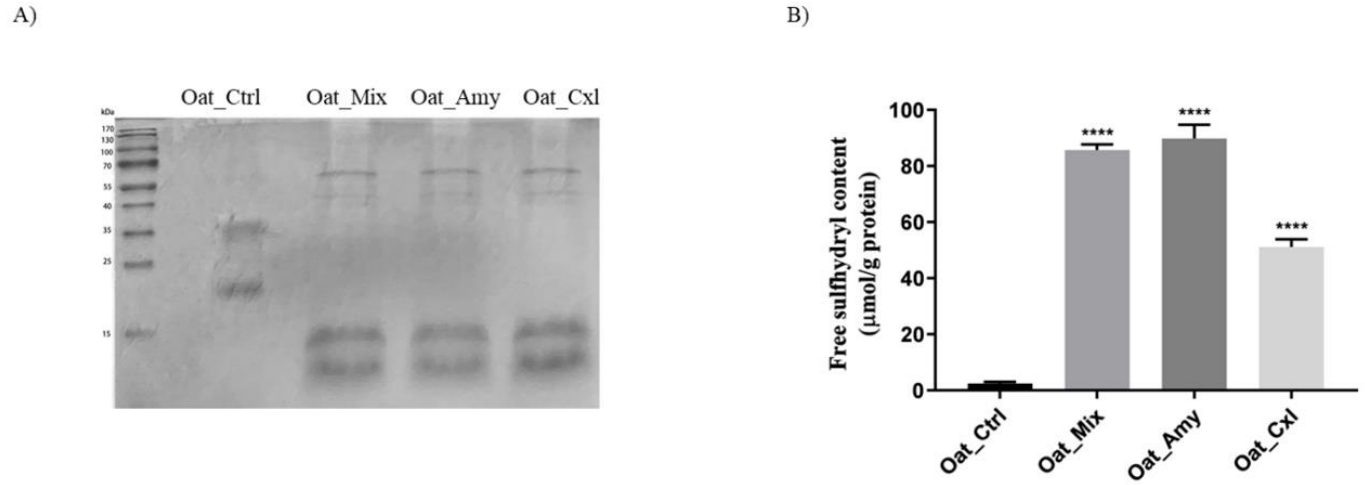



**Figure 2.** Reduced SDS–PAGE protein profile of untreated and enzymatically treated oat press cake proteins: M, pre-stained molecular marker; Oat_Ctrl, Oat_Amy, Oat_Mix, Oat_Cxl. Each sample (20 µL) was added to 10 µL of loading buffer, loading 30 µL for each well.

The SDS–PAGE of Oat_Ctrl (Figure 2) shows two very intense bands, at 32–35 kDa and 22–24 kDa, that may be attributed to the 12S α-polypeptide and the 12S β-polypeptide, respectively. This profile is in line with the protein extract from the seed [23]. The protein profiles of treated oat press cake protein samples are completely different, suggesting that the enzymes had induced a cleavage of the peptide bonds, i.e., partial hydrolysis of the proteins, indicated by the loss of intensity in the bands at 32–35 kDa and 22–24 kDa and the appearance of two intense bands at 10 kDa and 17 kDa. The nano-HPLC–ESI–MS/MS analysis (Table 3) indicated that these bands correspond to Avenins, Avena amylase trypsin inhibitors, Vromindolines, and Tryptophanin. Specifically, Vromindolines are starch-bound proteins that can contribute up to a 50% reduction in the oat grain hardness [24], whereas tryptophanins also contribute to oat grain softness, because they are bound to lipids [25]. It is thus possible to affirm that the enzymatic treatments efficiently disrupted the flour matrix to release these proteins, which were undetected in Oat_Ctrl, favoring in the meanwhile the degradation of the 11S and 12S storage proteins, as confirmed by the total spectrum intensity, which showed higher values in Oat_Ctrl compared to those detected in Oat_Amy, Oat_Cxl, and Oat_Mix. Interestingly, it was possible to identify hydroxyanthranilate-hydroxy-cinnamoyltransferase, which plays a pivotal role in the biosynthesis of the avenanthramides.

The proteolytic activity of all enzymatic treatments was confirmed by the degree of hydrolysis, which in

Oat_Amy, Oat_Cxl, and Oat_Mix was higher by 86.5%, 84.5%, and 87.4%, respectively, than in Oat_Ctrl.

**Table 3.** Proteins identified in each sample, i.e., Oat_Ctrl, Oat_Amy, Oat_Mix, and Oat_Cxl, with their main MS/MS features.

| Distinct Summed MS/MS Search Score | % AA Coverage | Total Spectrum Intensity | Protein MW | Protein pI | Accession (#)[a] | Protein Name |
|---|---|---|---|---|---|---|
| | | | **Oat_Ctrl** | | | |
| 27.86 | 4.8 | 6.61 × 108 | 58,999.9 | 9.22 | P12615 | 12S seed storage globulin 1 |
| 27.86 | 7.9 | $6.61\times 10^{8}$ | 35,779.4 | 10.2 | Q38781 | Oat storage protein 12S globulin (Fragment) |
| 18.96 | 2.3 | $6.55\times 10^{8}$ | 58,566.4 | 8.8 | O49258 | 12S globulin |
| 18.96 | 5.6 | $6.55\times 10^{8}$ | 24,685.5 | 7.94 | P27919 | Avenin |
| 28.39 | 4.7 | $1.27\times 10^{8}$ | 59,804 | 9.52 | Q38780 | 11S globulin |
| 5.73 | 4.1 | $1.19\times 10^{8}$ | 59,773.6 | 5.38 | A0A4Y5UJ50 | 4-coumarate:CoA ligase |
| 6.26 | 9.4 | $6.95\times 10^{8}$ | 23,329.1 | 11.17 | A0A3G1AXD2 | Ribosomal protein S4 |
| 7.26 | 4.2 | $6.11\times 10^{7}$ | 53,845.5 | 5.16 | A0A3G1AWG0 | ATP synthase subunit beta |
| 5.58 | 8.2 | $2.79\times 10^{7}$ | 28,805.3 | 5.98 | I4IY74 | Pollen allergen Ave s 5 (Isoallergen A) |
| 6.65 | 1.4 | $2.17\times 10^{7}$ | 1,221,607 | 9.18 | A0A3G1AUJ8 | DNA-directed RNA polymerase subunit beta |
| 5.77 | 17.7 | $1.55\times 10^{7}$ | 10,773.6 | 11.07 | A0A3G1AU31 | 30S ribosomal protein S15 |
| 6.05 | 4.3 | $1.30\times 10^{7}$ | 50,203.8 | 8.69 | Q941N4 | Receptor kinase |
| | | | **Oat_Mix, Amy, Cxl** | | | |
| 5.55 | 14.6 | $1.15\times 10^{9}$ | 16,544 | 8.3 | A0A1B2LQF1 | Avena alpha amylase trypsin inhibitor |
| 10.1 | 5.5 | $1.05\times 10^{9}$ | 78,923 | 9.03 | A0A3G1ATL7 | DNA-directed RNA polymerase subunit gamma |
| 9.8 | 12.2 | $9.76\times 10^{8}$ | 30,425 | 5.28 | I4IY75 | Pollen allergen Ave s 5 (Isoallergen B) |
| 15.5 | 4.2 | $6.07\times 10^{8}$ | 100,456 | 7.61 | A0A482JYP4 | Phototropin-like protein |
| 11.78 | 6.1 | $5.38\times 10^{8}$ | 59,614 | 5.39 | P54411 | T-complex protein 1 subunit epsilon |
| 4.57 | 3.9 | $4.67\times 10^{8}$ | 58,836 | 8.14 | F5B4I6 | Non-specific serine/threonine protein kinase |
| 6.22 | 7.6 | $2.74\times 10^{8}$ | 32,980 | 8.86 | Q7XXP0 | Hydroxyanthranilate hydroxycinnamoyltransferase 4 (Fragm) |
| 5.31 | 1.8 | $1.75\times 10^{8}$ | 96,836 | 5.66 | G1JSL5 | Lipoxygenase |
| 15.08 | 16.6 | $1.66\times 10^{8}$ | 41260 | 6.6 | D5L0B2 | Putative 2-oxoglutarate dependent dioxygenase |
| 21.65 | 5.2 | $1.59\times 10^{8}$ | 170,146 | 7.26 | A0A3G1AXC3 | RNA polymerase beta subunit |
| 12.49 | 8.6 | $1.31\times 10^{8}$ | 53,847 | 5.16 | A0A3G1AWG0 | ATP synthase subunit beta |
| 15.26 | 4.2 | $1.26\times 10^{8}$ | 126,405 | 5.75 | P06594 | Phytochrome A type 4 |
| 11.29 | 9.6 | $1.14\times 10^{8}$ | 49,110 | 5.66 | A0A481SVJ0 | Phenylalanine ammonia lyase II (Fragm) |
| 5.82 | 11.9 | $1.10\times 10^{8}$ | 16,508 | 8.71 | R4I3I8 | Vromindoline 3 |
| 5.82 | 11.9 | $1.10\times 10^{8}$ | 16,482 | 8.34 | A7U440 | Tryptophanin |
| 13.9 | 12.5 | $9.08\times 10^{7}$ | 53,822 | 6.17 | Q43380 | Non-specific serine/threonine protein kinase |
| 7.36 | 5.9 | $8.75\times 10^{7}$ | 67,169 | 6.06 | P22220 | Arginine decarboxylase |
| 5.33 | 6.3 | $8.38\times 10^{7}$ | 35,780 | 10.13 | Q38781 | Oat storage protein 12S globulin (Fragm) |
| 13.55 | 14.9 | $7.25\times 10^{7}$ | 22,640 | 5.5 | A0A2P0ZEN0 | Ribulose bisphosphate carboxylase |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | large chain (Fragm) |
| 13.08 | 5.9 | 3.89× $10^7$ | 112,385 | 6.48 | Q38766 | Glycine cleavage system P protein |
| 6.91 | 10.8 | 3.73× $10^7$ | 20,706 | 6.13 | A0A0R6HRG0 | Ribulose bisphosphate carboxylase large chain (Fragm) |
| 5.29 | 3.8 | 2.01× $10^7$ | 42,153 | 9.01 | Q9LLD7 | Fructose-bisphosphate aldolase |
| 6.83 | 3.4 | 1.82× $10^7$ | 65,678 | 6.03 | Q38786 | Avenacosidase 1 |
| 3.71 | 1.2 | 1.69× $10^7$ | 105,789 | 8.63 | A0A3Q8R3E1 | Cellulose synthase-like CslF6 |
| 3.68 | 13.9 | 1.39× $10^7$ | 18,766 | 7.84 | Q071L4 | Aluminum-activated malate transporter (Fragm) |
| 3.46 | 20.9 | 1.05× $10^7$ | 9356 | 8.8 | A0A2L0U0E7 | Defensin 16 |
| 7.28 | 4.6 | 4.29× $10^6$ | 53,776 | 9.36 | O49257 | 12S globulin |
| 7.28 | 4.1 | 4.69× $10^6$ | 59,811 | 9.14 | Q38780 | 11S globulin |

(*a*) *#: Accession number reported in UniprotKB.* Protein identified as belonging to *A. sativa*.

### *3.3. Effects of the Enzymatic Treatments on the Secondary and Tertiary Structure of the Proteins*

To investigate how the secondary and tertiary structure of the proteins had been affected by the enzymatic treatments, circular dichroism spectroscopy was employed and the content of free SH groups was measured. The CD spectra in the far UV region (190–230 nm) are shown in Figure 3A. One positive Cotton effect at 190 nm was observed for Oat_Ctrl, suggesting an α-helix-rich conformation. The enzymatic treatments induced a secondary negative Cotton effect with a minimum peak at 200 nm, indicating the predominance of β-sheet structures and random coils.

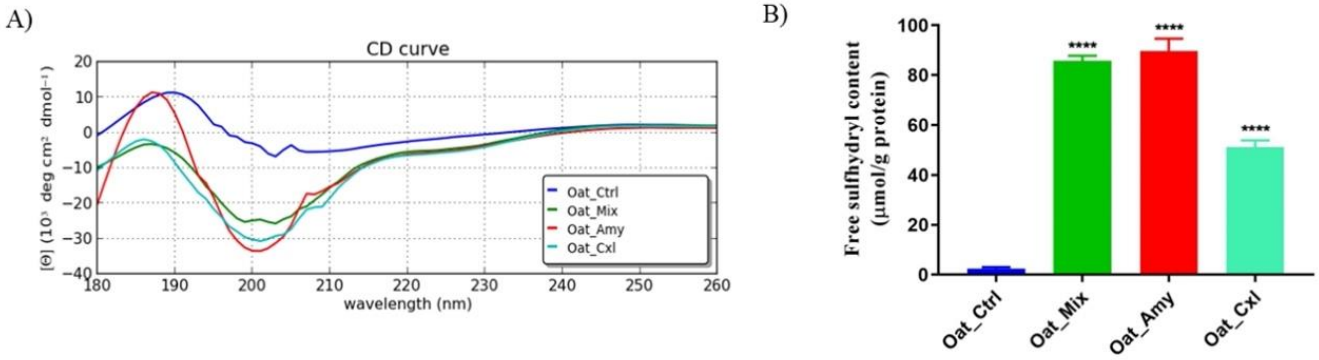



**Figure 3.** (**A**) CD spectra and (**B**) free SH group determination of Oat_Ctrl, Oat_Amy, Oat_Cxl, and Oat_Mix. The statistical analysis was performed by one-way ANOVA; **** $p < 0.0001$.

To obtain further information about the secondary structure of the oat press cake proteins, the BestSel tool was applied [26]. The results (Table 4) suggest that a reduction in the percentage of α-helices and an increase in β-sheet had been induced by the enzymatic treatment versus the untreated sample (Oat_Ctrl). Reductions

in the α-helices up to 8.9%, 3.7%, and 5.9% for Oat_Mix, Oat_Amy, and Oat_Cxl, respectively, were observed versus the Oat_Ctrl (22.2%). In addition, increases in the β-sheet up to 20.3%, 28.0%, and 28.1% for Oat_Mix, Oat_Amy, and Oat_Cxl, respectively, were detected versus Oat_Ctrl (8.5%). In the meanwhile, parallel increases in random coils were observed.

**Table 4.** Percentage of secondary structure composition of the oat press cake extracted proteins.

| | **α-Helix (%)** | **β-Sheet (%)** | **Turn (%)** | **Others (%)** |
|---|---|---|---|---|
| Oat_Ctrl | 22.2 | 8.5 | 32.7 | 36.6 |
| Oat_Mix | 8.9 | 20.3 | 13.7 | 57.1 |
| Oat_Amy | 3.7 | 28.0 | 7.2 | 61.2 |
| Oat_Cxl | 5.9 | 28.1 | 7.4 | 58.6 |

The measurement of the content of free SH groups located on the protein surface was used to provide further insights into changes in protein tertiary structure caused by the enzymatic treatments. Figure 3B shows a significant increase in free SH groups after the enzymatic treatments. In detail, the free SH contents of Oat_Mix, Oat_Amy, and Oat_Cxl were 85.7 ± 2.0, 89.9 ± 4.9, and 51.09 ± 2.8 μmol/g, respectively. All these values are much larger than the value of untreated oat press cake protein (Oat_Ctrl), equal to 2.5 ± 0.6 μmol/g ($p < 0.00015$). The SH content increase might be mainly attributed to the conversion of disulfide bonds into sulfhydryl groups, leading to protein unfolding and dissociation [27]. The proteolysis has been reported to be involved in the increment in the SH group by releasing free amino acids and other water-soluble components [28]. The alteration of the structure could reflect the change in the protein internal hydrogen bonds, hydrophobic bonds, and tightness of intermolecular binding [29]. These structural changes could expose more functional groups inside the protein molecule, thereby changing the antioxidant activity and the functional properties of the proteins. In addition, phenolic compounds, such as chlorogenic acid, avenanthramides, quercetin, and gentianic acid, could preferentially react with free radicals, leading to a protective effect on the SH groups [30].

*3.4. Analysis of the 3 kDa Peptide Fractions by Liquid Chromatography–Mass Spectrometry (LC–MS)*

Since LMW peptides (cut-off 3 kDa) are known to provide useful health benefits [13,31,32], it was decided to evaluate whether the enzymatic treatments influenced their formation. The quantification of the peptides, performed by the OPA assay, indicated that Oat_Mix contained the highest concentration of peptides (0.49 ± 0.02 mg/mL), whereas the concentrations in Oat_Amy and Oat_Cxl were 0.45 ± 0.01 mg/mL and 0.44 ± 0.02 mg/mL, respectively. All these values are much higher than the value of Oat_Ctrl (0.010 ± 0.001 mg/mL). The enzymatic treatment therefore highly incremented the concentration of these potentially bioactive compounds. The samples were then submitted to LC–MS analysis, whose results are reported in Table S1 in the Supplementary Materials (complete list) and in Table 5 (only peptides belonging to storage proteins). In agreement with the OPA results, the total number of identified peptides were 160 in Oat_Mix, 124 in Oat_Amy, 36 in Oat_Cxl, and only 11 in Oat_Ctrl (Table S1 in the Supplementary Materials). Focusing the attention only on the storage proteins, which are the most abundant proteins in oat seeds (Table 5), it is not surprising that the treatment with the enzyme combination including a protease (Oat_Mix) released nine peptides belonging to the 11S and 12S globulins, Avenacosidase, Vromindoline, and Gliadin-like avenin, whereas the treatment with amylase (Oat_Amy) released six peptides belonging to 11S globulin, avenin, Gliadin-like avenin, and Avenacosidase 1, and the treatment with cellulase/xylanase released only one peptide belonging to Gliadin-like avenin. Only one peptide belonging to a storage protein was identified in the control sample.

**Table 5.** Peptides from storage proteins identified in 3 kDa fractions of Oat_Ctrl, Oat_Amy, Oat_Mix, and Oat_Cxl.

| Spectrum Intensity | Peptide Sequence | *m*/*z* (Da) | % Coverage | AA | $MH^+$ (Da) | Peptide pI | Protein MW (Da) | Accession # | Protein Name |
|---|---|---|---|---|---|---|---|---|---|
| Mix | | | | | | | | | |
| $1.40\times 10^8$ | SQQGPVEHQAYQPIQS | 599.57 | 1.2 | | 1796.9 | 5.22 | 58,674.5 | P14812 | 12S seed storage globulin 2 |
| $4.13\times 10^7$ | ALGISQQAAQRIQSQNDQRGEI | 804.27 | 1.3 | | 2411.2 | 6.12 | 59,404.6 | Q38780 | 11S globulin |
| $4.09\times 10^7$ | DLGADVR | 746.06 | 3.0 | | 745.4 | 4.21 | 65,692.1 | Q9ZP27 | Avenacosidase 2 |
| $3.48\times 10^7$ | YQPIQSQEGQSTQYQVGQSTQ | 795.80 | 3.1 | | 2385.1 | 4 | 58,224.1 | O49258 | 12Sglobulin |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $1.44\times 10^7$ | QQSEIMKQVHVAQTLPSK | 684.75 | 2.5 | 2052.1 | 8.6 | 15,927.3 | R4I3I8 | Vromindoline 3 |
| $5.96\times 10^6$ | TNPNSMVSHIAGKSSILRALPVDVLAN | 935.66 | 1.5 | 2804.5 | 8.44 | 58,224.1 | O49258 | 12S globulin |
| $5.93\times 10^6$ | KGTLDGGINHEGIQYYNDL | 703.26 | 1.8 | 2107.0 | 4.54 | 65,039.4 | Q38786 | Avenacosidase 1 |
| $5.50\times 10^6$ | FLVQQCSPVAAVSFLRSQILQQSSCQ | 956.79 | 1.9 | 2867.5 | 8.07 | 24,076.7 | L0L845 | Gliadin-like avenin |
| $2.58\times 10^6$ | NNRGEEFGAFTPKFAQTGSQSYRTRE | 993.74 | 2.3 | 2978.4 | 8.59 | 35,722.3 | Q38781 | Oat storage protein 12S globulin (Fragm) |
| Amy | | | | | | | | |
| $1.82\times 10^7$ | LQQVTQGIFQPQMQGQIEGMRAFA | 903.08 | 3.2 | 2706.4 | 6 | 25,275.1 | P80356 | Avenin-3 |
| $1.61\times 10^7$ | MAQLFGQSSTPWQSSRQGG | 685.03 | 1.0 | 2053.0 | 9.5 | 61,861.4 | Q38779 | 11S globulin |
| $1.17\times 10^7$ | QQQQQQQPFVQQQQMF | 683.35 | 1.1 | 2049.0 | 5.52 | 24,012.3 | L0L6K1 | Gliadin-like avenin |
| $9.98\times 10^6$ | LQLQQQVFQPQLQQQVFQPQL | 855.81 | 2.8 | 2566.4 | 5.52 | 25,471.1 | Q09072 | Avenin |
| $8.33\times 10^6$ | TFNEPHSFCGLGYGTGLHAPGAR | 796.91 | 2.1 | 2389.1 | 6.61 | 65,039.4 | Q38786 | Avenacosidase 1 |
| $7.93\times 10^5$ | YFDEQNEQFRCTG | 546.07 | 3.5 | 1636.7 | 4.14 | 61,861.4 | Q38779 | 11S globulin |
| Cxl | | | | | | | | |
| $1.38\times 10^7$ | LQALPAMCDVYVPPHCPVATTPXGF | 918.14 | 2.0 | 2753.4 | 5.08 | 24,076.7 | L0L845 | Gliadin-like avenin |
| Ctrl $6.73\times 10^6$ | KIQSQNDQRGEIIRV | 595.10 | 1.0 | 1783.9 | 8.75 | 58,674.5 | O49258 | 12S globulin |

*3.5. Evaluation of the Antioxidant Activity of the Oat Press Cake Proteins and Low Molecular Weight Peptides*

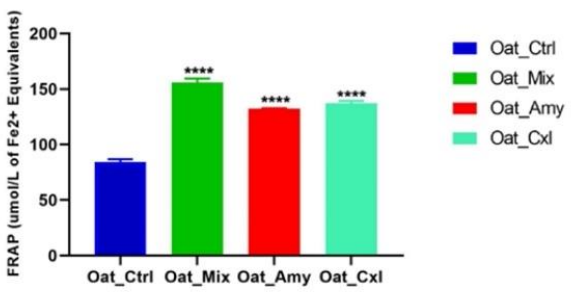
It was decided to assess the potential antioxidant activity of the oat press cake proteins using the FRAP assay, which showed that all of the samples were endowed with an antioxidant activity that was, however, higher in the enzymatically treated samples. Specifically, FRAP levels were significantly increased by 40.2% for Oat_Mix (220.7 µmol/L of $Fe^{2+}$ equivalents), 41.1% for Oat_Amy (224.07 µmol/L of $Fe^{2+}$ equivalents), and 33.6% for Oat_Cxl (198.87 µmol/L of $Fe^{2+}$ equivalents), respectively, versus Oat_Ctrl (131.9 µmol/L of $Fe^{2+}$

equivalents).

The fact that oligopeptides derived from hydrolyzed oat proteins have been reported to have biological activities, including antioxidant properties [33], encouraged us to investigate the contribution of 3 kDa peptides to the total antioxidant activity. The results are shown in Figure 4A. The treatments increased the FRAP value by 85%, 57%, and 64%, for Oat_Mix, Oat_Amy, and Oat_Cxl, respectively, compared to Oat_Ctrl ($p < 0.0001$), highlighting a significant enhancement of the antioxidant activity of the released peptides.

The literature indicates that antioxidant peptides are characterized by hydrophobic amino acids, such as Leu or Val, in their N-terminal regions, aromatic amino acid residues (Phe, Trp, Tyr, and His), and nucleophilic sulfur-containing amino acid residues (Cys and Met) [34,35]. Another amino acid that may also contribute to the antioxidant activity is Lys [36]. In addition, amino acids with aromatic side chains can also donate protons to the electron-deficient radicals, further improving the radical scavenging property. Figure 4B compares the amino acid compositions of the analyzed samples; indeed, some specific amino acids (such as Val, Phe, Tyr, Ser, Thr, and Lys) are more represented in the treated oat press cake LMW peptides. For example, the content of Phe is 2.2-fold higher and that of Tyr 2-fold higher in Oat_Cxl peptides than in the Oat_Ctrl ones, and the content of valine in Oat_Amy peptides is 1.5-fold higher than in Oat_Ctrl ones. According to previous studies, some di- and tripeptides with aromatic amino acid residues (Tyr or Trp) and valine are highly likely to have strong antioxidant activity [37]. Particularly, the peptides LVYIL and YHNAPGLVYIL have been reported to be associated with increased activities of antioxidant enzymes, with a 29% increase in cell viability [33].

A)




B)

| AA | Oat_Ctrl (%) | Oat_Mix (%) | Oat_Amy(%) | Oat_Cxl(%) | Ctrl, Mix, Amy, Cxl |
|---|---|---|---|---|---|
| Ala | 10.4 | 10.1 | 8.8 | 8.7 | |
| Arg | 6.6 | 4.8 | 4.2 | 3.5 | |
| Asn | 3.3 | 3.9 | 3.8 | 4.7 | |
| Asp | 7.1 | 4.9 | 4.9 | 4.7 | |
| Cys | 1.4 | 1.8 | 2 | 2.3 | |
| Gln | 5.2 | 4.1 | 4.5 | 4.1 | |
| Glu | 5.7 | 5.3 | 6 | 6.7 | |
| Gly | 9.5 | 8.4 | 8.3 | 10.8 | |
| His | 2.8 | 2.5 | 2.3 | 2.6 | |
| Ile | 7.1 | 5.1 | 5.2 | 4.7 | |
| Leu | 9.5 | 8.6 | 10.4 | 8.8 | |
| Lys | 2.8 | 4.6 | 4.7 | 4.1 | |
| Met | 3.3 | 2.1 | 1.9 | 2 | |
| Phe | 1.9 | 3.5 | 3.3 | 4.3 | |
| Pro | 5.2 | 6.3 | 5.8 | 5 | |
| Ser | 5.7 | 9.2 | 8.1 | 8.1 | |
| Thr | 3.3 | 5 | 5.1 | 5.2 | |
| Trp | 2.8 | 1.1 | 0.9 | 0.8 | |
| Tyr | 1.4 | 2.4 | 2.9 | 3 | |
| Val | 4.7 | 6.2 | 6.9 | 5.8 | |

**Figure 4.** Antioxidant activity of the low molecular weight peptide fractions. (**A**) Results of the FRAP assay. Bars represent

the average ± SD of 3 independent experiments in duplicate. **** $p < 0.0001$ versus untreated sample. (**B**) Amino acid (AA) compositions of the samples.

*3.6. Identification and Quantitation of the Main Phenolic Compounds by HPLC–ESI–MS/MS*

It is well documented that phenolics are concentrated in the outer seed coat/pericarp of the grain, that these compounds are often bound within the walls of plant cells, and that the antioxidant activity of the bran is higher than that of the refined endosperm [38]. It is also known that phenolic acids mostly occur as compounds bound to proteins, causing very low bioavailability [39], and that these interactions take place either via non-covalent (hydrophobic, ionic, and hydrogen bonds) or covalent bonds [40]. These facts suggest that the oat press cake may represent a potential source of these important phytochemicals, and it seemed feasible that the enzymatic treatments may improve the bioavailability of these phytochemicals.

After having verified that the phenols could not be directly extracted from the samples (data not shown), the analysis of the bound species was performed by HPLC–MS/MS after two sequential digestion steps with pepsin and trypsin. The chromatograms and MS/MS spectra are shown in Figure S1. It was possible to identify and quantify six phenolic acids (gallic acid, vanillic acid, ferulic acid, caffeic acid, p-coumaric acid, and cinnamic acid), and three AVNs (AVN A, AVN B, and AVN C). The identification was performed by comparing the retention times and the fragmentation ions with those of authentic standards, whereas the quantification was based on standard curves, which showed high correlation values ($R^2$~0.998).

Table 6 reports the concentrations of phenolic acids and the content of AVNsin the oat press cake protein samples. The quantitative analysis revealed that the oat press cake is an interesting added-value material, since Oat_Ctrl contains a much higher amount of vanillic acid (407.56 ± 60.73 μg/g DW) than eight cultivars of husked oat, among which the richest was the Peppi cultivar (7.05 ± 0.37 μg/g DW). In addition, the contents of p-coumaric acid, vanillic acid, gallic acid, and caffeic acid are also much higher (63.06, 407.56, 2894.26, and 4.72 μg/g, respectively) than those reported in oat bran (12 ± 0.22 μg/g of p-coumaric acid, 24 ± 2.4 μg/g of vanillic acid, 5.4 ± 0.15 μg/g of caffeic acid) as well as in oat grain (113.3 ± 2.6 μg/g of gallic acid) [41,42]. Instead, the amount of cinnamic acid (12.07 ± 0.38 μg/g) is comparable to that quantified in a Finnish husked oat [43], and ferulic acid is much lower (1.98 ± 0.07 μg/g) than the value detected in the Akseli variety (829 ± 73.8 μg/g) [43]. A comparison of the main phenolic acid profiles in Oat_Ctrl with those of some commercial

oat products shows that the amount of p-coumaric acid is comparable, whereas the amount of vanillic acid is much higher [44]. In different oat cultivars, bound phenolic acids represent 89.6–97.3% of the total phenolic compounds and p-coumaric acid is mostly present in the bound fraction, accounting for 59% of total bound compounds [15]. Similarly, in different oat products, bound phenolics were from two to ten times more concentrated than the free species, with vanillic acid, caffeic acid, and p-coumaric acid mostly present in the bound form [44].

There are, however, significant changes in the phenolic compound profiles induced by the enzymatic treatments. The results in Table 6 clearly indicate that most phenolic acids, in particular cinnamic acid, p-coumaric acid, vanillic acid, gallic acid, and ferulic acid, decrease in the treated oat press cake protein versus Oat_Ctrl. The effects are more evident in Oat_Mix and Oat_Cxl. The fact that enzymes such as cellulase and xylanase greatly influence the polyphenol content underlines the role of cellulose and xylans in the binding of these phytochemicals to proteins. On the contrary, the only phenolic acid that was increased was caffeic acid, again, especially in Oat_Mix and Oat_Cxl. Another paper observed a significant increase in caffeic acid after treatment with cellulase [45].

**Table 6.** Contents of phenolic acid (µg/g DW) and AVNs (ppb) in different oat samples (mean ± SD, n = 3).

| **Polyphenols (*m*/*z*)** | **Oat_Ctrl (Mean ± SD) (µg/g DW)** | **Oat_Mix (Mean ± SD) (µg/g DW)** | **Oat_Amy (Mean ± SD) (µg/g DW)** | **Oat_Cxl (Mean ± SD) (µg/g DW)** |
|---|---|---|---|---|
| Cinnamic acid (149.2) | 12.1 ± 0.4 | 4.7 ± 0.7 $^{(a)}$ | 5.1 ± 0.4 $^{(a)}$ | 6.6 ± 0.2 $^{(a)}$ |
| p-Coumaric acid (165.2) | 63.1 ± 4.1 | 20.1 ± 8.9 $^{(c)}$ | 60.4 ± 0.1 $^{ns}$ | 16.4 ± 1.2 $^{(a)}$ |
| Vanillic acid (169.1) | 407.6 ± 60.7 | 94.3 ± 23.8 $^{c)}$ | 228.5 ± 78.1 $^{(d)}$ | 178.0 ± 21.7 $^{(c)}$ |
| Gallic acid (171.1) | 2894.3 ± 435.5 | 806.4 ± 4.5 $^{(c)}$ | 1241.1 ± 114.6 $^{(c)}$ | 579.7 ± 60.9 $^{(b)}$ |
| Caffeic acid (181.2) | 4.7 ± 0.8 | 262.2 ± 30.9 $^{(b)}$ | 18.5 ± 2.0 $^{(b)}$ | 265.3 ± 37.0 $^{(b)}$ |
| Ferulic acid (195.2) | 1.9 ± 0.1 | 0.7 ± 0.1 $^{(b)}$ | 0.7 ± 0.1 $^{(b)}$ | 0.99 ± 0.03 $^{(b)}$ |
| **AVN (*m*/*z*)** | **Oat_Ctrl (Mean ± SD) (ppb)** | **Oat_Mix $^{(b)}$ (Mean ± SD) (ppb)** | **Oat_Amy $^{(a)}$ (Mean ± SD) (ppb)** | **Oat_Cxl $^{(c)}$ (Mean ± SD) (ppb)** |

| | | | | |
|---|---|---|---|---|
| AVN A (300.1) | 6.4 ± 0.8 | 14.8 ± 2.8 [(c)] | 9.6 ± 0.9 [(d)] | 11.1 ± 4 [ns] |
| AVN B (330.1) | 45.6 ± 8.8 | 12.8 ± 1.2 [(d)] | 4.3 ± 0.3 [(d)] | 6.0 ± 0.8 [(d)] |
| AVN C (316.1) | 7.9 ± 2.2 | 10.9 ± 3.5 [ns] | 5.6 ± 0.8 [ns] | 26.4 ± 0.5 [(c)] |

The statistical analysis was performed by T-parametric test. [(a)] **** $p < 0.0001$, [(b)] *** $p < 0.006$, [(c)] ** $p < 0.004$, [(d)] * $p < 0.05$, ns: not significant versus Oat_Ctrl.

AVNs are a group of unique phenolic acid derivatives typical of oats. The Oat_Ctrl sample contains an amount of AVNs lower than that reported in the literature for oat grain (40–130 μg/g) [46]. However, the various levels of AVNs analyzed in oat varieties from Finland and China depend on the cultivar, geographic location, environment, and genetics, which play crucial roles in the generation of secondary metabolites such as AVNs [46]. The total amount of these phytochemicals is smaller in the enzymatically treated samples. This depends mostly on the relevant decrease in ANV B, whereas the contents of AVN A are slightly higher in Oat_Amy, Oat_Cxl, and Oat_Mix, as well as those of AVN C in Oat_Mix and Oat_Cxl. While most phenolic compounds are often covalently bound to proteins, AVNs belong to the free phenolic acid fraction [44], a fact that can explain why their residues are very small in the byproducts and in these samples [15].

### *3.7. Effects of Enzymatic Treatments on the Phytic Acid Content*

In order to check the potential release of anti-nutritional factors induced by the enzymatic pre-treatment, phytic acid was quantified. Figure 5 shows that the enzymatic treatments and protein precipitation reduced the phytic acid. In detail, a reduction in the PA content by 46 ± 1%, 42 ± 1%, and 46 ± 1% was observed in Oat_Mix (14.08 ± 0.02 mg/g), Oat_Amy (15.11 ± 0.02 mg/g), and Oat_Cxl (13.43 ± 0.05 mg/g), respectively, versus Oat_Ctrl (26.38 ± 0.5 mg/g). This may be explained by considering that phytate is mainly present in the form of water-soluble salts, such as sodium and potassium phytate. By improving the protein solubility, the enzymatic treatment by cellulase, xylanase, and proteases increased the passive diffusion of water-soluble phytates during the exposure to water [47]. Successful applications of enzymes for treating raw material have been already described as efficient in reducing the phytic acid content [48].

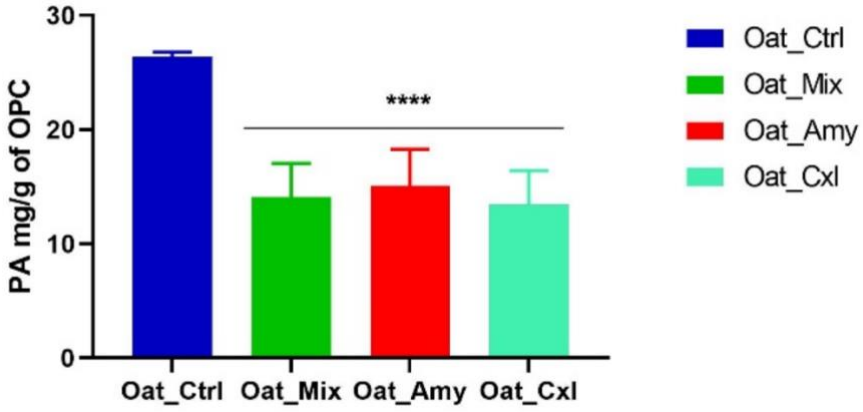



**Figure 5.** PA content determination. Statistical analysis was performed by one-way ANOVA (****) $p < 0.0001$. The data are represented as the means ± s.d. of three independent experiments.

## 4. Conclusions

Here, we provide evidence that the treatment of the oat press cake, a by-product which is generally discarded after oat drink preparation, with enzymes, such as amylase, cellulase/xylanase, and protease, may improve its nutritional value. This is particularly relevant considering the increasing market for these kinds of products that are more and more appreciated by vegans, vegetarians, and flexitarians. The protein extraction was greatly facilitated by the enzymatic treatments, as well as the release of LMW peptides, with useful technological and nutritional consequences, such as improved water solubility. In addition, it is well known that LMW peptides provide useful health benefits, mainly in the area of metabolic syndrome prevention [12,32]. Here, in particular, we evaluated the antioxidant activity of these peptides, which indeed was superior after enzymatic treatment. These results underline the importance of peptides in the global antioxidant activity of these food ingredients; in fact, the higher antioxidant activity of treated oat press cake proteins cannot be explained by the phenols, whose concentrations are decreased by the enzymatic treatments, but only by the presence of antioxidant peptides. Finally, it seems possible to affirm that the application of amylase, cellulase/xylanase, and proteases (alone or in combination) represents a new strategy to recover nutritional ingredients otherwise inaccessible from industrial byproducts. The recovery and reuse of these materials may obtain economic benefit by reducing the waste of natural sources.

**Supplementary Materials:** The following Supplementary Materials are available online at www.mdpi.com/xxx/s1, Table S1: Peptide sequences of 3 kDa identified in oat press cake proteins. Figure S1: (A) TIC of six polyphenols and the AVNs. MS/MS spectra of (B) ferulic acid, (C) gallic acid, (D) p-coumaric acid, (E) caffeic acid, (F) cinnamic acid, (G) vanillic acid, (H) AVN A, (I) AVN B, and (J) AVN C.

**Abbreviations**

| | |
|---|---|
| AA | amino acid |
| ACN | acetonitrile |
| AMY | amylase |
| AVNs | avenanthramides |
| CD | circular dichroism |
| DTNB | (5,5-dithio-bis-(2-nitrobenzoic acid) |
| FA | formic acid |
| LMW | low molecular weight |
| MIX | amylase/cellulase/protease |
| FeIII-TPTZ | ferric tripyridyltriazine |
| FRAP | ferric reducing ability |
| HPLC–MS/MS | high-performance liquid chromatography–tandem mass spectrometry |
| OPA | o-phthalaldehyde |
| PA | phytic acid |
| SH | free sulfhydryl group |
| Tris–HCl | Tris(hydroxymethyl)aminomethane hydrochloride |

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# Chapter 4

## Integrated Evaluation of the Multifunctional DPP-IV and ACE Inhibitory Effect of Soybean and Pea Protein Hydrolysates

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**Abstract:** Nowadays, food bioactive peptides from plant sources garner increasing attention for their ability to impart one or more beneficial effect on human health. Legumes, which stand out thanks to their high protein content, represent valuable sources of bioactive peptides. In this context, this study is focused on the characterization of the potential pleotropic activity of two commercially available soybean (SH) and pea (PH) protein hydrolysates, respectively. Since the biological activity of a specific protein hydrolysate is strictly correlated with its chemical composition, the first aim of the study was to identify the compositions of the SH and PH peptides. Peptidomic analysis revealed that most of the identified peptides within both mixtures belong to storage proteins. Interestingly, according to the BIOPEP-UWM database, all the peptides contain more than one active motive with known inhibitory angiotensin converting enzyme (ACE) and dipeptidyl-dipeptidases (DPP)-IV sequences. Indeed, the results indicated that both SH and PH inhibit DPP-IV and ACE activity with a dose-response trend and IC50 values equal to 1.15 ± 0.004 and 1.33 ± 0.004 mg/mL, and 0.33 ± 0.01 and 0.61 ± 0.05 mg/mL, respectively. In addition, both hydrolysates reduced the activity of DPP-IV and ACE enzymes which are expressed on the surface of human intestinal Caco-2 cells. These findings clearly support that notion that SH and PH may represent new ingredients with anti-diabetic and hypotensive effects for the development of innovative multifunctional foods and/or nutraceuticals for the prevention of metabolic syndrome.

## 1. Introduction

Food bioactive peptides are short protein fragments (2–20 amino acid residues in length) that, in addition to their known nutritional value, are able to modulate physiological pathways, thereby exerting a positive impact on human health [1]. Hence, both animal and plant foods or by-products with high protein content represent valuable sources of functional bioactive peptides, which can be produced either by enzymatic hydrolysis (using proteolytic enzymes from either plants or microbes), hydrolysis with digestive enzymes (simulated gastrointestinal digestion), or by fermentation. Some studies also used a combination of these methods to produce peptides with a biological activity [2].

Legumes, pseudocereals, and hempseed are among the plant foods which can be considered good sources of bioactive peptides [3–6]. In this panorama, legumes, which stand out thanks to their high protein content, are a cheap, sustainable, and a healthy source of nutrition. Notably, soybeans (*Glycine max*) are on average composed of ~35–40% protein [7]. Clinical studies have linked the consumption of soy-based food with a reduced risk of developing a number of chronic diseases, such as obesity, hypercholesterolemia, and insulin-resistance/type II diabetes. As for the active substance in soy foods, protein plays a role in cardiovascular disease prevention [3,8], and some cholesterol-lowering and anti-diabetic peptides have already been singled out in glycinin and β-conglycinin sequences, respectively [9].

In addition to soybean, pea (*Pisum sativum* L.) represents one of the major legumes in the world and it is composed of ~26% protein [7]. Thanks to its excellent yields, availability, and its low production costs, pea is most widely used as a source of commercial proteins for different purpose [10]. Many studies have highlighted the health benefits associated with the consumption of pea protein. In particular, pea protein and its hydrolysates exert antioxidant, antihypertensive, and hypocholesterolemic activities [11–14].

In general, most previous studies in this area have focused on the consumption of pea and soybean proteins, but the health-promoting activity does not lie in the protein but in the peptides, which are encrypted within the protein and released upon digestion/hydrolysis. Hence, with a more mature perception of the phenomenon, owing to the presence of numerous bioactive peptides, these protein hydrolysates may provide more than one biological activity, therefore eliciting multiple health benefits [15–18]. For this reason, the production of

hydrolysates with multifunctional behavior represents a valid strategy in the development of new generations of functional foods and nutraceuticals [19].

Indeed, this study focused on the deep characterization of the potential pleotropic health-promoting behavior of two commercially available soybean (SH) and pea (PH) protein hydrolysates, respectively. Both SH and PH are obtained by industrial processes, beginning with the selection of soybean and pea protein sources (isolated proteins or enriched protein flours) in combination with a hydrolysis process which is able to deliver an optimized performance in term of structure and, thanks to the resulting taste being almost neutral, allows for a nutritional contribution to food and beverage formulations. SH and PH are then filtered and concentrated before spray drying to obtain a purified product for different food applications.

In addition to, and in part thanks to, their nutritional and technological properties (as a smoothing and whipping agent), SH and PH are successfully used in every formulation to replace nutritional content of animal origin, and they may also be exploited as new valuable and pleotropic ingredients for making innovative multifunctional foods and/or nutraceuticals.

Thus, since the biological activity of a specific protein hydrolysate is strictly correlated with its chemical composition, the first aim of this study was to identify the compositions of SH and PH peptides by peptidomic analysis. Then, their potential hypotensive and anti- diabetic activities were evaluated by initially measuring the ability of both hydrolysates to inhibit, in vitro, angiotensin converting enzyme (ACE) and dipeptidyl-dipeptidases (DPP)-IV activity and, afterwards, by carrying out experiments using Caco-2 cells that express both enzymes on their membrane surfaces.

## 2. Materials and Methods

### *2.1. Chemicals*

All the commercial chemicals which were used are listed in the Supplementary Materials. A. Costantino & C. S.p.a (Italy), supplied the soybean and pea hydrolysates as spray dried samples (Soy Peptone FM batch 221/00351, 100 g; Pea Protein Hydrolysate GT Plus batch 221F0001, 100 g) directly from the production process. See the Supplementary Materials for the Technical data sheet of both samples.

*2.2. SH and PH Ultrafiltration*

Before proceeding to the assessment of biological activity, SH and PH were passed through ultrafiltration (UF) membranes with a 3 kDa cut-off, using a Millipore UF system (Millipore, Bedford, MA, USA). The recovered peptides solutions (SH (F3) and PH (F3)) were lyophilized and stored −80 ◦C until use.

*2.3. Mass Spectrometry Analysis (HPLC Chip ESI-MS/MS)*

SH and PH samples were analyzed by HPLC CHIP-ESI-MS/MS. All further details and experimental conditions are described in the Supplementary Materials.

*2.4. Biochemical Investigation of DPP-IV and ACE Inhibitory Activity of SH and PH Peptides*

*2.4.1. In Vitro DPP-IV Activity Assay*

The experiments were carried out in a half-volume 96-well solid plate (white) with SH and PH at final concentration range of 0.01–2.0 mg/mL and using conditions previously optimized [20]. For further details, see the Supplementary Materials.

*2.4.2. In Vitro Measurement of ACE Inhibitory Activity*

In order to assess their ACE-inhibitory activity, the SH and PH hydrolysates were tested as previously reported [21,22]. The detailed procedures are available in the Supple- mentary Materials.

*2.5. Cellular Measurement of SH and PH Inhibitory Effect of DPP-IV and ACE Activities*

*2.5.1. Cell Culture Conditions*

Caco-2 cells, obtained from INSERM (Paris, France), were routinely sub-cultured following conditions which have been already optimized [16] and detailed reported in the Supplementary Materials.

*2.5.2. Evaluation of Caco-2 Cell Viability by MTT Experiments*

In order to assess the safe range of concentrations of the SH and PH hydrolysates on Caco-2 cells, preliminary cell viability experiments were carried out using MTT assay. The detailed procedure is shown in the Supplementary Materials.

*2.5.3. Evaluation of the Inhibitory Effect of SH and PH on Cellular DPP-IV Activity*

A total of 5 × 104 Caco-2 cells/well were seeded in black 96-well plates with a clear bottom. The second day after seeding, the spent medium was discarded and cells were treated with 1.0, 2.5, and 5.0 mg/mL of SH and PH for 1, 3, and 6 h at 37 ◦C. Experiments were carried out following previously optimized conditions [23]. More details are available in the Supplementary Materials.

*2.5.4. Evaluation of the Inhibitory Effect of SH and PH on Cellular ACE1 Activity*

Caco-2 cells were seeded on 96-well plates at a density of 5 × 104 cells/well and treated with SH and PH (from 0.1 to 5.0 mg/mL) or vehicle in growth medium for 6 h at 37 ◦C. The ACE inhibitory activity was measured using the ACE1 Activity Assay Kit (Biovision, Milpitas Blvd., Milpitas, CA, USA). The experimental method is detailed in the Supplementary Materials.

*2.6. Statistical Analysis*

All the data sets were checked for normal distribution by the D'Agostino and Pearson test. Since they are all normally disturbed with $p$-values < 0.05, statistical analyses were carried out by one-way ANOVA (Graphpad Prism 9, GraphPad Software, La Jolla, CA, USA) followed by Tukey's multiple comparison test. For each assay, at least four independent experiments were performed, and each experiment were performed in triplicate. Values were expressed as means ± SD; $p$-values < 0.05 were considered to be significant.

## 3. Results

*3.1. Peptidomic Characterization of SH and PH*

The compositions of the peptides of SH and PH hydrolysates were analyzed by HPLC-ESI-MS/MS. Figure 1

reports the total ion current (TIC) of the MS/MS of eluted peptides, while Table 1 shows the peptide molecular weight distribution of each sample, and the overall peptides, which were identified, are reported in Table S1.

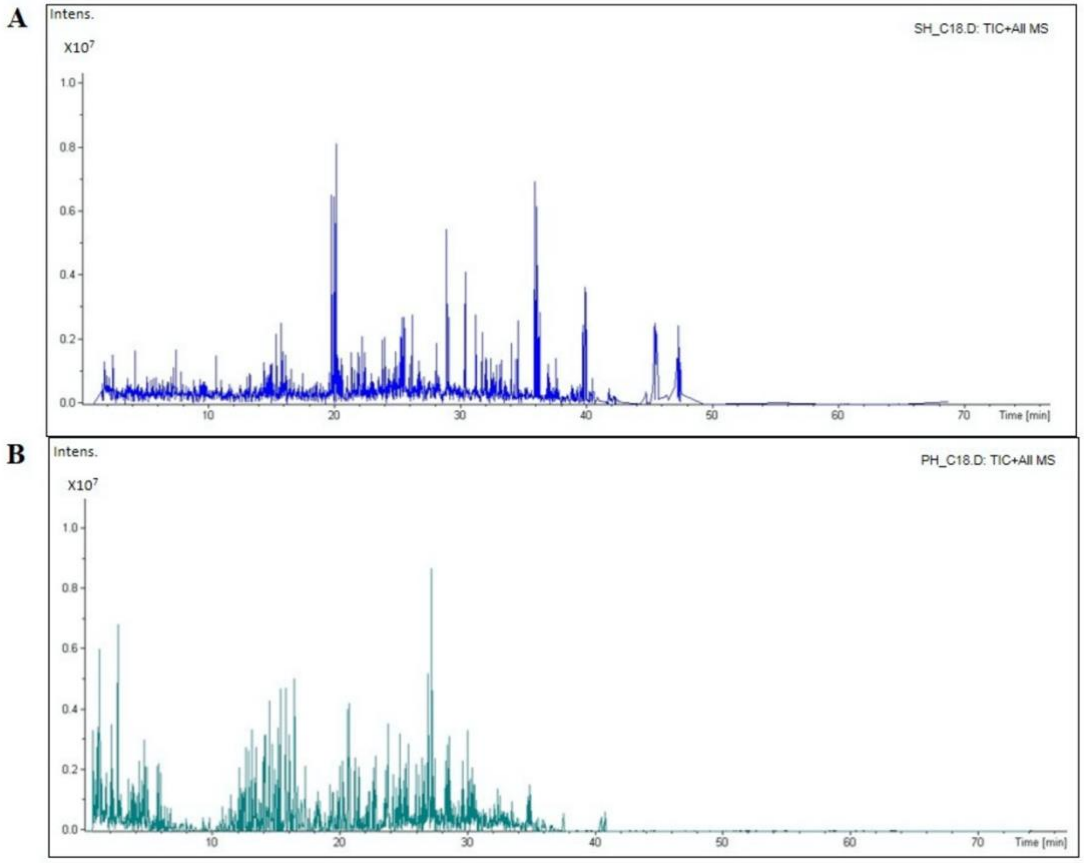


**Figure 1.** The total ion chromatogram (TIC) of the soybean (SH, (**A**)) and pea (PH, (**B**)) hydrolysates, respectively. The mass spectrometer ran for 70 min. Most of the SH sample peptides were eluted between 20 and 40 min, while the PH peptides were mostly eluted in 30–40 min.

**Table 1.** Molecular weight distribution of SH and PH peptides.

| Hydrolysate | MW > 3 kDa (%) | MW < 3 kDa (%) |
|---|---|---|
| SH | 53.6 | 46.4 |
| PH | 57.15 | 42.85 |

Table 2 lists the identified peptides from the most abundant proteins. Among these, six and nine peptides were identified in the SH and PH samples, respectively. The length of those peptides ranged from 9 to 25 amino acids with a molecular weight in the range 1055 –2422.3 KDa, and in both samples, most of the identified peptides belong to storage proteins. Indeed, 50% of the identified soybean peptides belong to Glycinin G1, whereas in the case of the pea derived peptides, 55% and 33% belong to the Vicilin and Legumin A2 proteins, respectively. Interestingly, according to BIOPEP-UWM database (https:// biochemia.uwm.edu.pl/biopep-uwm/, accessed on 20 May 2022), all the peptides contain more than one active motive with both known inhibitory ACE and DPP-IV sequences.

**Table 2.** SH and PH peptides from the most abundant protein, with ACE and DPP-IV inhibitory activity.

| Hydrolysate | Protein Name | Peptide Sequence | Intensity | ACE Inhibitor Sequence [a] | DPP-IV Inhibitor Sequence [a] |
|---|---|---|---|---|---|
| SH | Ankyrin repeat domain-containing protein 52 | IRSWIVQVMS | $5.11 \times 10^7$ | IVQ, VQV, VM | WI, IR, QV, SW, VM, VQ |
| | Glycinin G1 | VSIIDTNSLENQLDQ | $4.56 \times 10^7$ | | SL, DQ, II, NQ, QL, SI, TN, VS |
| | | IIDTNSLENQLDQMPR | $2.07 \times 10^7$ | PR | MP, SL, DQ, II, NQ, QL TN |
| | | ANSLLNALPEEVIQ | $1.75 \times 10^7$ | EV, LN, ALP, LP | LP, LL, AL, SL, EV, IQ, LN, NA, VI |
| | Hydrolase_4 domain-containing protein | AAEGGGFSDPAPAPPRLAIPEV | $1.45 \times 10^7$ | PR, AIP, IP, AP, LA, AA, GF, GG, AI, EG, PAP, EV, PP | PP, LA, AP PA, IP, AA AE, DP, EG EV, GF, GG, RL |
| | DNA-directed RNA polymerase (fragment) | FDIYRVMRPGEPPTMDSAEAMFNA | $1.48 \times 10^7$ | IY, MF, GEP, RP, GE, EA, PG, PT, PP, VM | PP, RP, EP, AE, FN, GE, MF, MR, NA, PG, PT, TM, VM, YR |
| PH | Vicilin 47k | EITPEKNQQLQDLDIFVN | $2.26 \times 10^7$ | IF, EI, LQ EK, TP | TP, EK, EI NQ, QD, QL, QQ, VN |
| | | NQQLQDLDIFVN | $2.80 \times 10^7$ | IF, LQ | NQ, QD, QL, QQ, VN |
| | | KNQQLQDLDIFVN | $7.09 \times 10^7$ | IF, LQ | NQ, QD, QL, QQ VN |
| | Vicilin | ITPEKNPQLQDLDIFVN | $1.58 \times 10^7$ | IF, LQ PQ, EK, TP | TP, NP, EK PQ, QD, QL, VN |
| | | KNPQLQDLDIFVN | $5.13 \times 10^7$ | IF, LQ PQ | NP, PQ, QD QL, VN |
| | AsmA family protein | GGLSFDRKAAKTTASGGLTLSKADA | $2.73 \times 10^7$ | AA, GL, DA, GG, SG, SF, KA, TLS, DR | KA, TA, TT GL, AA, AD AS, DR, GG KT, LT, RK SF, SK, TL |
| | Legumin A2 | LFGQAGLDPLPVDVGA-NGRL | $1.80 \times 10^7$ | PLP, RL LF, PL, VG GA, GL, AG GR, FG, GQ NG, LP | LP, GA, GL PL, AG, DP NG, PV, QA RL, VD, VG |
| | | ALEPDNRIE | $1.53 \times 10^7$ | IE, ALEP | EP, AL, DN NR, RI |
| | | SVINNLPLDVVA | $4.96 \times 10^7$ | PL, LPL, LP | VA, LP, VV LPL, PL, IN NL, NN, SV, VI |

[a] According to the BIOPEP-UWM database; https://biochemia.uwm.edu.pl/biopep-uwm/ accessed on 20 May 2022.

*3.2. SH and PH Peptides: Biochemical Investigation of DPP-IV and ACE Inhibitory Activities*

*3.2.1. SH and PH Inhibit In Vitro DPP-IV Activity*

In order to assess the ability of SH and PH to modulate DPP-IV activity, preliminary in vitro experiments were performed using the purified recombinant DPP-IV enzyme. Figure 2A shows that SH drops in vitro DPP-IV activity by 10.18 ± 7.0%, 40.0 ± 1.6%, 58.22 ± 2.57%, and 82.48 ± 1.41% at 0.1, 0.5, 1.0, and 2.5 mg/mL, respectively. Figure 2B indicates that PH decreases DPP-IV activity in vitro by 0.11 ± 0.21%, 31.0 ± 3.9%, 52.05 ± 2.31%, and 79.1 ± 1.04% at 0.1, 0.5, 1.0, and 2.5 mg/mL, respectively. Both SH and PH inhibit the enzyme with a dose-response trend and calculated IC50 values equal to 1.15 ± 0.004 and 1.33 ± 0.004, respectively.

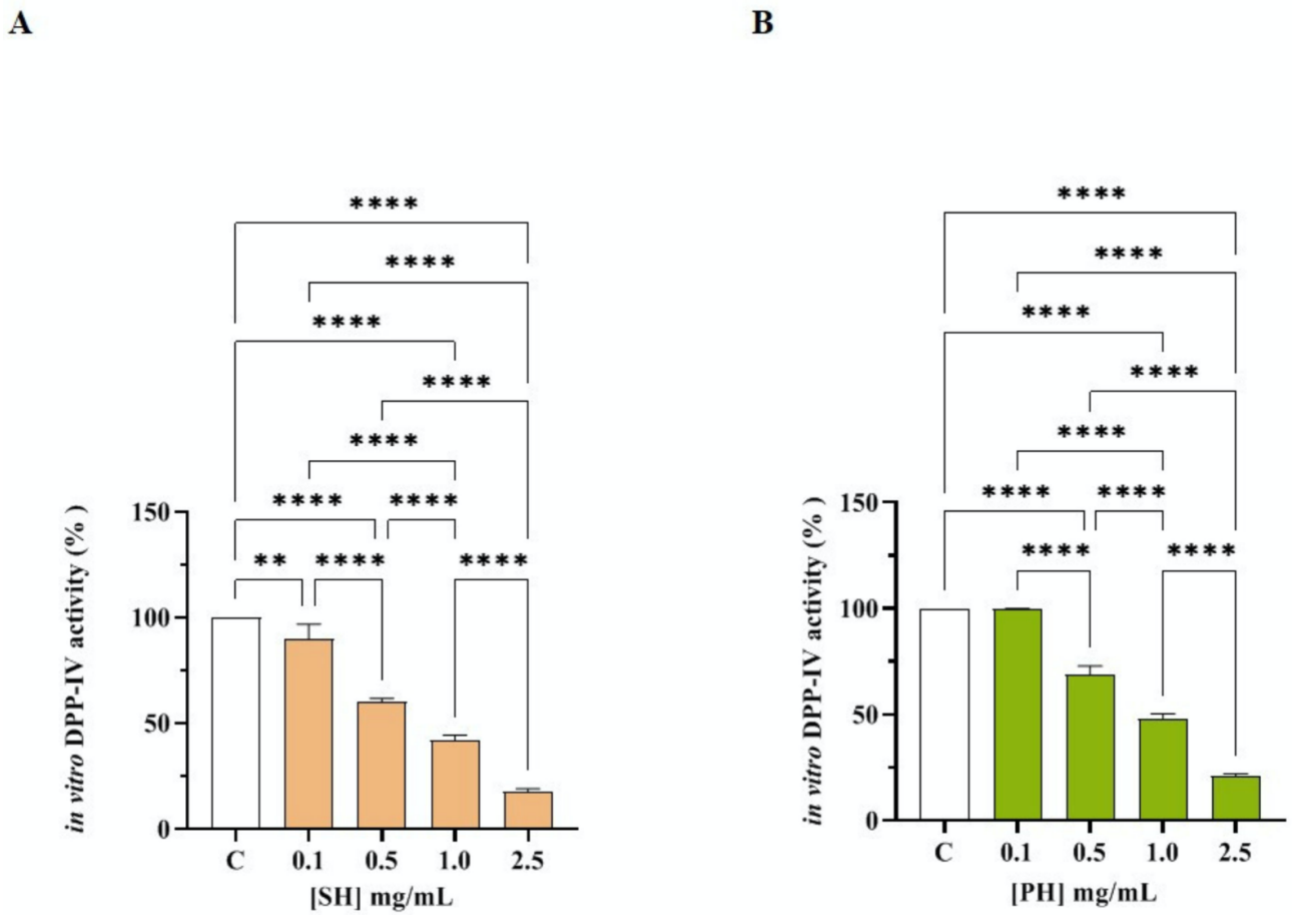


**Figure 2.** Evaluation of the in vitro inhibitory effects of SH (**A**) and PH (**B**) hydrolysates on human recombinant DPP-IV. Bars represent the average ± SD of three independent experiments in duplicates. **** $p < 0.0001$, ** $p < 0.01$, versus control

(C) sample (activity), non-significant (ns) is not shown.

In parallel, the in vitro DPP-IV activity inhibition of both hydrolysates was confirmed by the experiments conducted on the low molecular weight fractions (<3 kDa) of SH (F3) and PH (F3), as reported in Figure S1. Notably, SH (F3) inhibits DPP-IV activity in vitro by 12.7 ± 4.9%, 23.9 ± 4.56%, 41.9 ± 2.56%, 57.3 ± 2.3%, and 79.5 ± 2.5% at 0.01, 0.1, 0.5, 1.0, and 2.0 mg/mL, respectively (Figure S1A), and PH (S3) reduced the enzymatic activity by 8.5 ± 2.01%, 15.1 ± 4.21%, 41.6 ± 3.4%, 56.8 ± 4.4%, and 79.1 ± 1.70% at the same concentrations (Figure S1B). Indeed, both SH (F3) and PH (F3) decreased DPP-IV activity with a dose-response trend and IC50 values equal to 0.82 ± 0.01 and 1.0 ± 0.01 mg/mL, respectively. Comparing the IC50 values obtained by analyzing the total SH and PH hydrolysates with the those obtained by testing the <3 kDa fractions of SH and PH, it is evident that the short peptides which are contained within SH and PH hydrolysates, respectively, are those responsible of DPP-IV inhibitory activity.

*3.2.2. SH and PH Peptides Inhibit In Vitro ACE Activity*

Both SH and PH efficiently inhibited ACE activity with a dose-response trend and calculated $IC_{50}$ values that were 0.33± 0.01 and 0.61 ± 0.05 mg/mL, respectively (Figure 3A,B). More specifically, SH inhibited ACE activity by 19.88 ± 1.21%, 32.74 ± 0.57%, 53.01 ± 0.43%, 72.35 ± 0.16%, and 83.38 ± 0.06% at 0.08, 0.17, 0.35, 0.7, and 1.0 mg/mL, respectively (Figure 2A), whereas PH reduced ACE activity by 3.55 ± 0.07%, 12.92 ± 0.11%, 35.40 ± 0.01%, 53.51 ± 0.11%, and 62.23 ± 0.15% at the same concentrations (Figure 3B).

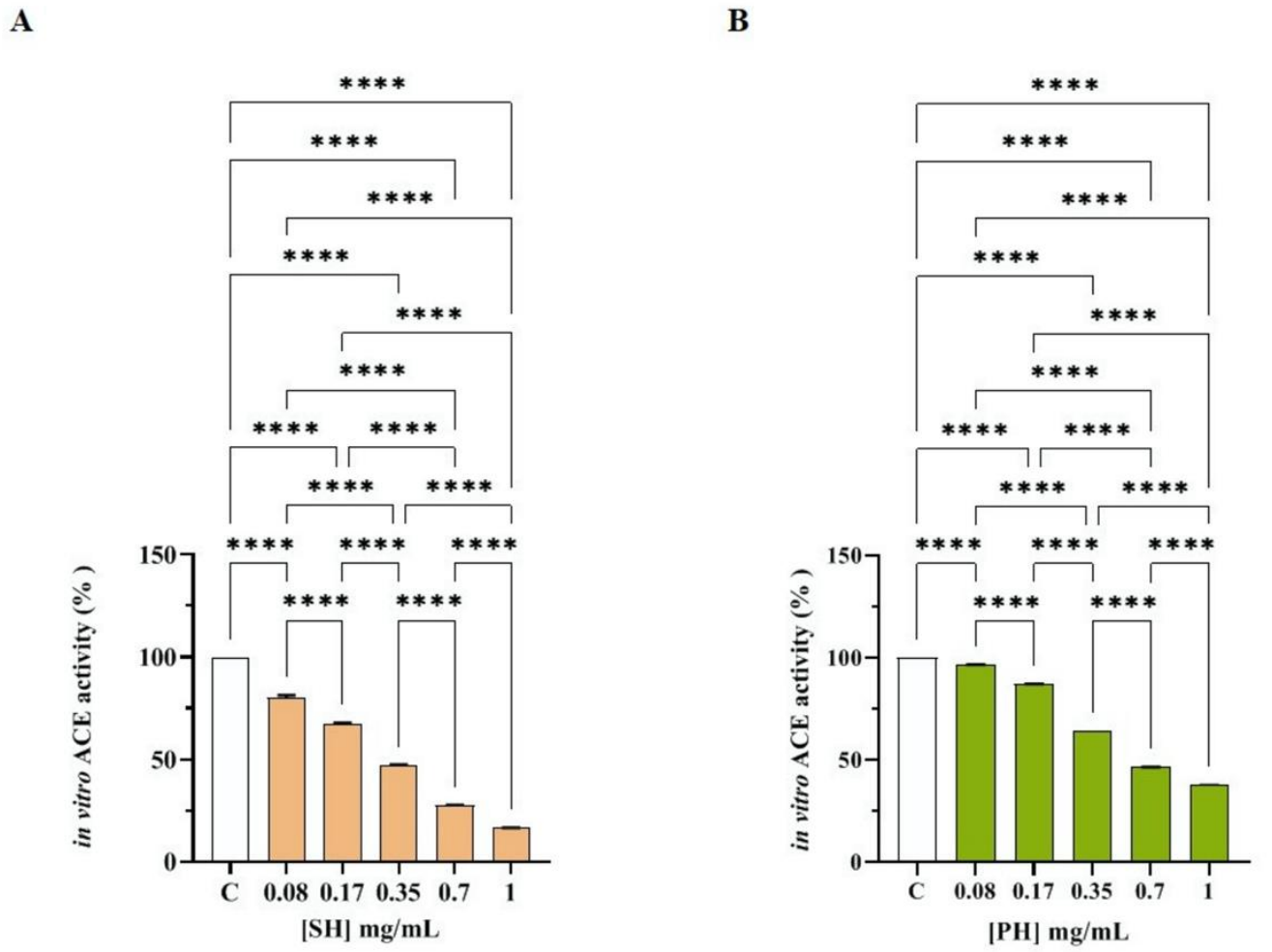


**Figure 3.** Assessment of the in vitro ACE-inhibitory effects of SH (**A**) and PH (**B**) hydrolysates. Bars represent the sd of three independent experiments in duplicate. **** $p < 0.0001$ versus control sample (C).

In parallel, the in vitro ACE activity inhibition of both hydrolysates was confirmed by the experiments conducted on the low molecular weight fractions (<3 kDa) of both SH (F3) and PH (F3), as shown in Figure S2. In particular, SH (F3) inhibits ACE activity in vitro by 9.15 ± 0.07%, 24.62 ± 0.13%, 48.39 ± 0.02%, 63.29 ± 0.02%, and 74.09 ± 0.08% at 0.08, 0.17, 0.35, 0.7, and 1.0 mg/mL, respectively (Figure S2A), whereas PH (F3) reduced the enzymatic activity by 17.21 ± 0.10%, 26.74 ± 0.25%, 44.26 ± 0.02%, 63.96 ± 0.01%, and 74.64 ± 0.08% at the same concentrations (Figure S2B).

Indeed, both SH (F3) and PH (F3) reduced the ACE activity with a dose-response trend and $IC_{50}$ values equal to 0.40 ± 0.01 and 0.43 ± 0.01 mg/mL, respectively, clearly suggesting that the short peptides which are contained within SH and PH hydrolysates are those responsible for ACE inhibitory activity.

Finally, Table 3 summarizes the comparison of IC50 values of both samples against DPP-IV and ACE.

**Table 3.** IC50 values obtained testing SH, PH and their corresponding low molecular fractions (<3 kDa) against DPP-IV and ACE targets.

| | $IC_{50}$ (mg/mL) DPP-IV | $IC_{50}$ (mg/mL) ACE |
|---|---|---|
| SH | 1.15 ± 0.004 | 0.33 ± 0.01 |
| PH | 1.33 ± 0.004 | 0.61 ± 0.05 |
| SH <3 kDa (F3) | 0.82 ± 0.01 | 0.40 ± 0.01 |
| PH <3 kDa (F3) | 1.0 ± 0.003 | 0.43 ± 0.01 |

### *3.3. Cellular Assessment of DPP-IV and ACE Inhibition by SH and PH Peptides*

#### *3.3.1. Effect of SH and PH Peptides on Caco-2 Cell Viability*

The MTT assay was used for assessing the safe range of concentrations of the SH and PH hydrolysates on Caco-2 cells. After a 48 h treatment, any effect on the Caco-2 cell viability was observed in the range of 1.0–5.0 mg/mL versus untreated cells (C) (Figure 4).

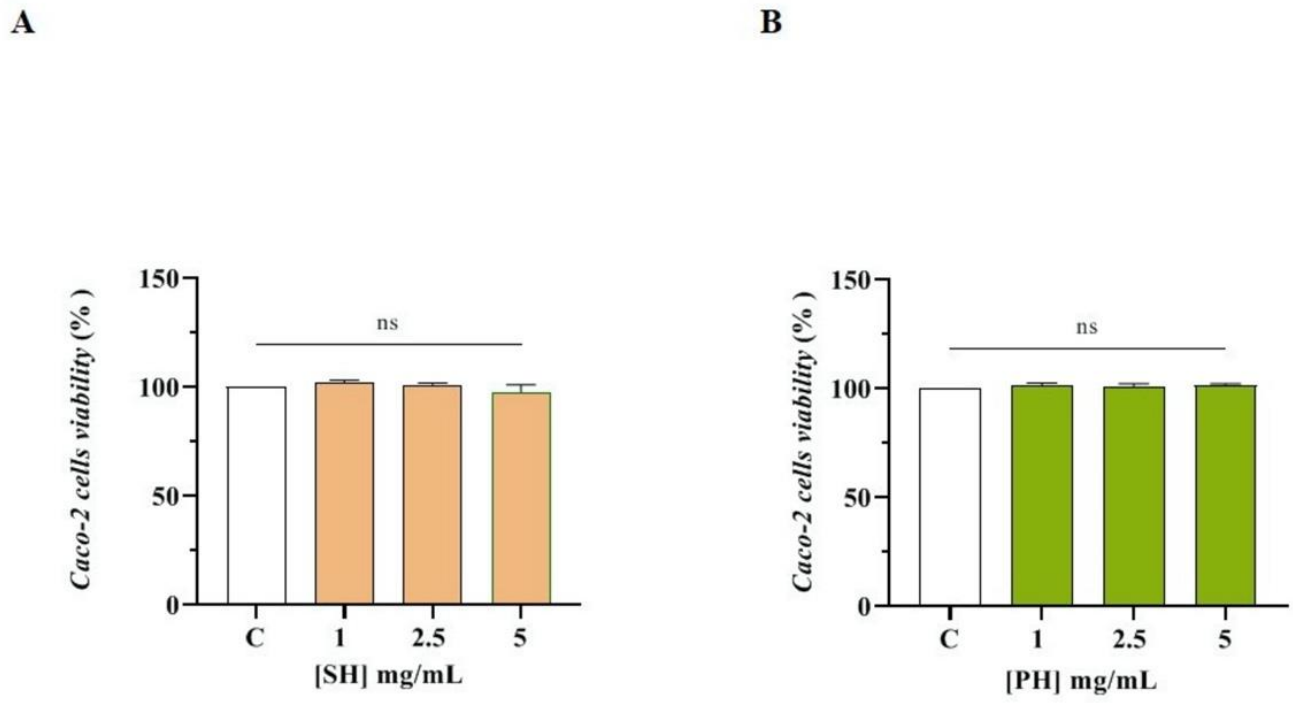


**Figure 4.** MTT assay. Effect of SH (**A**) and PH (**B**) hydrolysates on Caco-2 cells viability. Data represent the averages ± SD

of four independent experiments performed in triplicate.

The ability of SH and PH to inhibit DPP-IV was then investigated in cell-based conditions using Caco-2 cells, which express high levels of this protease on their membranes. Briefly, these cells were treated with SH and PH (1.0- 5.0 mg/mL) and their DPP-IV inhibitory effects were assessed in a kinetic mode after 1, 3, and 6 h (Figure S3A,C). Figure S3A shows that, after 1 h, SH inhibited cellular DPP-IV activity by 25.07 ± 8.07%, 36.91 ± 2.37%, and 40.32 ± 2.46 at 1.0, 2.5, and 5 mg/mL, respectively, and by 25.07 ± 6.78%, 42.46 ± 6.49%, and 48.69 ± 7.29% at the same concentrations after 3 h. The maximum reductions in DPP-IV activity were observed at 6 h (Figure S3A and Figure 5A), where SH inhibited cellular DPP-IV activity by 37.9 ± 10.0%, 56.6 ± 3.6%, and 59.4 ± 2.4% at 1.0, 2.5, and 5 mg/mL, respectively, versus untreated cells, following a dose-response trend, confirming the in vitro results.

As indicated in Figure S3C, after 1 h, the PH sample inhibited cellular DPP-IV activity by 19.9 ± 8.2%, 40.9 ± 3.6%, and 47.4 ± 3.3% at 1.0, 2.5, and 5 mg/mL, respectively, and by 22.6 ± 5.4%, 47.9 ± 2.79%, and 53.6 ± 2.3% at the same concentrations after 3 h (Figure S3C). The maximum inhibition of DPP-IV activity was observed after 6 h (Figure S3C, Figure 5B), where PH inhibited cellular DPP-IV activity by 27.9 ± 5.3%, 52.8 ± 2.2%, and 60.7 ± 1.6% at 1.0, 2.5 and 5 mg/mL, respectively, versus untreated cells, supporting the in vitro results.

In parallel, the in situ DPP-IV activity inhibition of both hydrolysates was confirmed by the experiments carried out on the low molecular weight fractions (<3 kDa) of both SH (F3) and PH (F3), as shown in Figure S3B,D. In particular, Figure S3B shows that, after 1 h, SH (F3) inhibited cellular DPP-IV activity by 10.4 ± 8.3%, 37.2 ± 4.1%, and 42.3 ± 3.10% at 1.0, 2.5, and 5 mg/mL, respectively, and by 13.62 ± 6.88%, 43.1 ± 5.91%, and 47.9 ± 4.4% at the same concentrations after 3 h. The highest decreases in DPP-IV activity were observed at 6 h (Figure S3B), where SH (F3) inhibited cellular DPP-IV activity by 17.7 ± 4.9%, 54 ± 1.6%, and 61.5 ± 3.9% at 1.0, 2.5, and 5 mg/mL, respectively, versus untreated cells, in a dose-dependent manner, supporting the in vitro results.

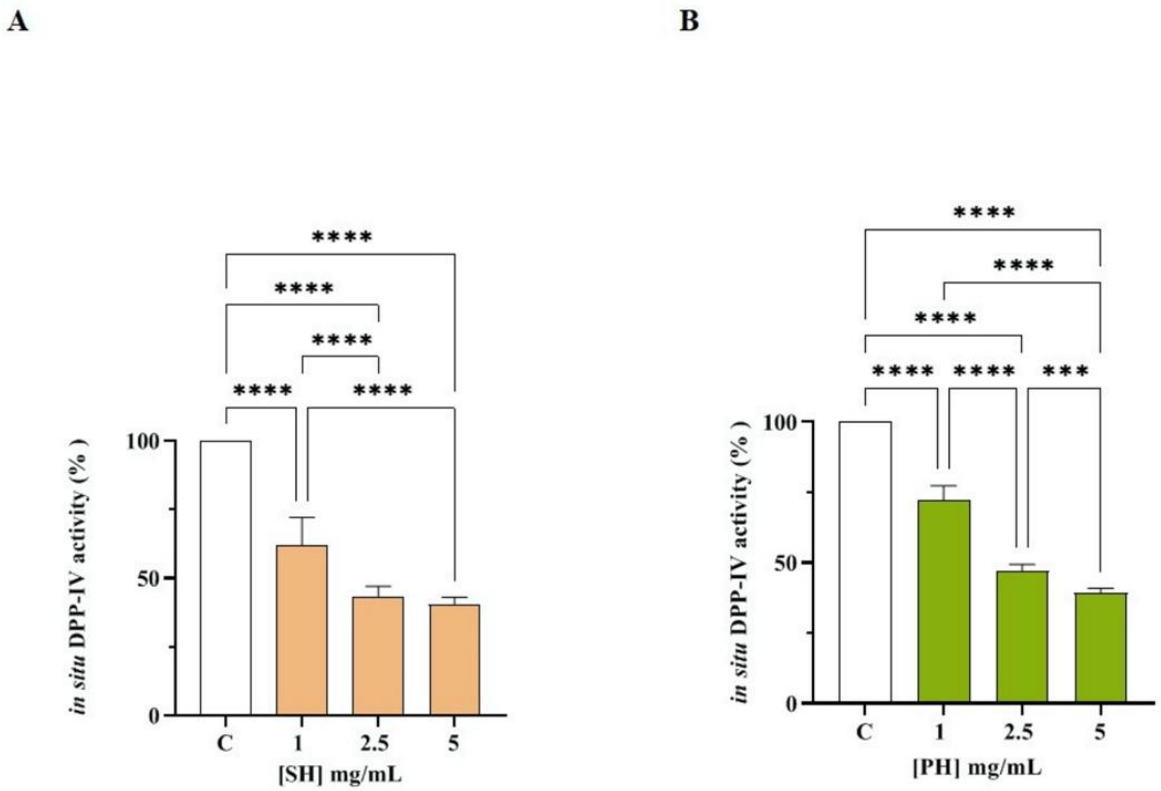


**Figure 5.** Effect of SH (**A**) and PH (**B**) on the cellular DPP-IV activity. The data points represent the averages ± SD of four independent experiments performed in triplicate. All data sets were analyzed by one-way ANOVA followed by Tukey's post-hoc test; C: control sample (H2O), **** $p < 0.0001$, *** $p < 0.001$, non-significant (ns) is not shown.

Similarly, as shown in Figure S3D, PH (F3) inhibited cellular DPP-IV activity by 6.8 ± 6.5%, 31.6 ± 5.5%, and 45.5 ± 4.9% at 1.0, 2.5, and 5 mg/mL, respectively, after 1 h, and by 7.25 ± 4.03%, 48.67 ± 2.42%, and 54.11 ± 3.09% at the same concentrations after 3 h. The highest inhibition of DPP-IV activity was observed at 6 h (Figure S3D), where PH (F3) inhibited cellular enzymatic activity by 14.4 ± 3.9%, 60.2 ± 1.5%, and 61.8 ± 2.7% at 1.0, 2.5, and 5 mg/mL, respectively, versus untreated cells, confirming the in vitro results.

### *3.3.2. SH and PH Inhibit ACE Activity Expressed on Human Intestinal Caco-2 Cells*

Human intestinal Caco-2 cells were treated with SH and PH (1.0–5.0 mg/mL) for 6 h. The ACE activity was measured in the presence of a fluorescent substrate using cell lysates. The results indicated that both hydrolysates reduced cellular ACE activity with a dose-response trend. In more detail, SH reduced the enzyme activity by 29.9 ± 1.8%, 57.9 ± 12.6%, 67.5 ± 8.1%, and 74.6 ± 4.9% at 0.1, 0.5, 1.0, and 5.0 mg/mL,

respectively (Figure 6A), whereas PH hydrolysate reduced it by 39.6 ± 14.6%, 52.2 ± 10.6%, 64.7 ± 5.2, and 82.3 ± 4.2%, respectively, at the same concentrations (Figure 6B). In parallel, the cellular ACE activity inhibition of both hydrolysates was confirmed by the experiments performed on the low molecular fractions (3 kDa) of both SH and PH (Figure S4). Notably, SH (F3) inhibited cellular ACE activity by 10.1 ± 4.5%, 47.6 ± 2.7%, 61.0 ± 7.5%, and 76.2 ± 2.3% at 0.1, 0.5, 1.0, and 5.0 mg/mL, respectively (Figure S4A), whereas PH (F3) reduced the cellular enzymatic activity by 32.0 ± 13.7%, 42.7 ± 11.3%, 60.5 ± 13.5%, and 69.5 ± 11.3% at the same concentrations (Figure S4B).

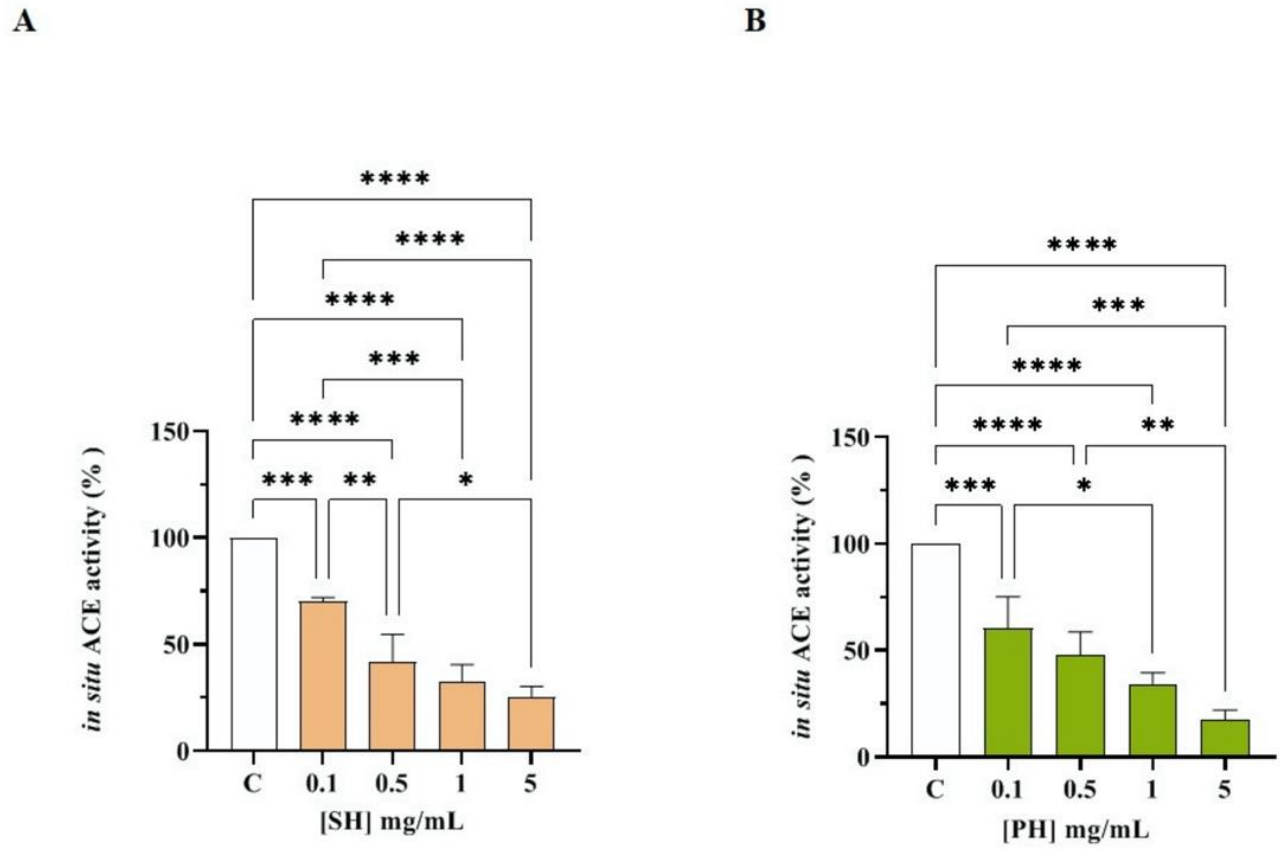


**Figure 6.** ACE inhibitory effects of SH (**A**) and PH (**B**) hydrolysates in cell-based conditions. Bars represent the SD of three independent experiments in triplicate. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ versus control sample (C), non-significant (ns) is not shown.

## 4. Discussion

In recent decades, many studies have clearly demonstrated the ability of food protein hydrolysates to modulate

ACE or DPP-IV activity [20,24,25]. In particular, hydrophobic medium-length and/or shorter peptides from different food sources are considered to be mostly responsible for the inhibition of both enzymes [24,25]. In this panorama, the main limitation of most of previous studies lies in the fact that the characterization of the DPP-IV or ACE inhibitory property of food hydrolysates was performed by analyzing each bioactivity without taking into account that the same hydrolysate may be endowed with both activities. In addition, most works available in the literature rely exclusively on in vitro tests for assessing the biological activity: this is particularly true in the case of the inhibition of DPP-IV and ACE activity. In light of these observations, a standout feature of this study is that the multifunctional DPP-IV and ACE inhibitory activities of two commercially available soybean (SH) and pea (PH) protein hydrolysates were evaluated together using in vitro tests which were implemented by cellular assays that permitted deeper insights into the mechanism of action and, contextually, a consideration of other relevant issues, such as metabolism (this is particularly true when Caco-2 cells were employed).

Overall, the present study demonstrates that SH and PH are effective at reducing DPP-IV and ACE activity in both cell-free and cell-based conditions. More specifically, as indicated in the Table 3, when comparing the calculated IC50 values, it is clear that SH and PH display the same ability to reduce DPP-IV activity, with SH being about 2-fold more potent than PH in terms of ACE inhibition (***, $p < 0.001$). In addition, the results indicated that SH and PH are 3- and 2-fold more potent as ACE than DPP-IV inhibitors, respectively (****, $p < 0.0001$). Moreover, comparing the IC50 values of each total hydrolysate with those corresponding to each low molecular weight fraction (<3 kDa) (Table 3), it is clear that the medium-length and shorter peptides, which are abundant in each total hydrolysate (Table 1), are responsible for the biological activities, even though, in the case of SH and SH (F3), the same IC50 values were observed against ACE enzyme. Interestingly, Table 2 indicates that all the most abundant peptides identified within both SH and PH contained at least one motive with DPP-IV and ACE inhibitory activity that has already been demonstrated, explaining why these hydrolysates are active on two different targets, such as ACE and DPP-IV.

As reported in the Table 3, in the case of PH, the low molecular weight fractions (<3 kDa) are about 0.8- and 1,5-fold more potent than total PH as DPP-IV and ACE inhibitors, respectively (****, $p < 0.0001$). This result might be explained considering that the bioactivity of total hydrolysate depends strictly on its total composition, including the inactive and active species and possible synergistic or antagonist effects [26,27].

Therefore, it is reasonable to conclude that longer peptides of PH (57.2%) may affect the activity exerted by the medium-length and shorter ones (42.8%) when present in PH hydrolysate. A similar trend was observed for SH. In fact, the SH (F3) is more active than the total hydrolysate against DPP-IV (***, $p < 0.001$), whereas a similar IC50 value against the ACE target was calculated (Table 3).

Recently, it was demonstrated that soybean hydrolysates obtained using pepsin and trypsin reduced in vitro DPP-IV activity by 16.3% and 31.4%, and by 15.3% and 11.0%, respectively, at 1.0 and 2.5 mg/mL [18]. Other recent studies demonstrated that the protein hydrolysates from germinated and non-germinated soybean, obtained after simulated gastrointestinal digestion, show a modest ability to inhibit the DPP-IV [28,29]. In addition, soybean hydrolysates obtained using Corolase L10, Promod 144 MG, or Protamex reduced the enzyme, with IC50 values of 2.5, 0.86, and 0.96 mg/mL, respectively [28]. Indeed, SH is more active than hydrolysates obtained using pepsin, trypsin, and Corolase, whereas its activity is very similar to those of peptide mixtures obtained using both Promod and Protamex.

Pea proteins digested with Corolase L10 and Promod 144 MG inhibited DPP-IV activity with IC50 values higher than 2.5 mg/mL, whereas the pea hydrolysate obtained using Protamex dropped the enzyme activity with an IC50 value of 0.96 mg/mL [28]. In this case, PH displays a DPP-IV activity totally in line with those of hydrolysates obtained using Protamex, whereas it is more active than the peptide mixtures obtained using Corolase and Promod.

Soybean proteins extracted with microwave-assisted technology and hydrolysate using Alcalase inhibited the ACE enzyme, and peptides belonging to the low molecular weight fraction were responsible for the biological activities. Moreover, it was also demonstrated that pea proteins digested using a thermolysin-generated peptide mixture exerted ACE inhibitory properties in a spontaneous hypertensive rat (SHR) model and in a clinical study [12,14].

Interestingly, among the peptides belonging to the low molecular weight fraction (<3 kDa) of pea hydrolysate, peptide LTFPG was isolated, whose hypotensive activity has been demonstrated both in vitro and in vivo in the SHR model [12]. Notably, LTFPG is a conserved active peptide that has also been identified and characterized in a lupin sample [15].

DPP-IV and ACE are important membrane peptidases which are physiological expressed by many tissues; i.e., intestine [30,31]. Indeed, human intestinal Caco-2 cells represent a reliable model which has been already

developed and validated for the study of peptides with DPP-IV or ACE inhibitory properties [23,32,33]. In this study, it was clearly demonstrated that both SH and PH maintain their ability to reduce the activity of both DPP-IV and ACE on Caco-2 cells, even though both hydrolysates are active at a concentration ranging between 0.1 and 5 mg/mL, indicating that SH and PH are less active in cell-based than in cell-free conditions, respectively. Similar results have been previously obtained on peptic and tryptic hydrolysates of spirulina and chlorella proteins, respectively [32,34,35]. Also, in those cases, it was observed that all the tested hydrolysates were more active in cell-free than in cell-based assays, respectively. The reduced activity in the cellular assays may be explained considering the metabolic ability of Caco-2 cells [32]. Indeed, the intestinal brush border expresses many active proteases and peptidases that might actively hydrolyze food peptides modulating their bioactivity through the produc- tion of new breakdown fragments. Therefore, the intestine plays an important role not only in the process of valuable nutrient absorption, but also in actively modulating the physico-chemical and biological profiles of food protein hydrolysates. All these biochemi cal and cellular results represent an important starting point for future investigations of SH and PH through in vivo and clinical studies on suitable animal models and human volunteers in order to obtain a “proof of concept” with respect to their multifunctional and pleotropic behavior.

## 5. Conclusions

In conclusion, our results indicate that SH and PH are multifunctional hydrolysates endowed with both anti-diabetic and hypotensive activity. It is doubtless that they are among the most potent DPP-IV and ACE inhibitor hydrolysates reported in the literature, suggest- ing that they may be successfully used as new valuable ingredients for the development of innovative functional foods and or dietary supplements for the prevention of cardiovascu- lar disease and metabolic syndrome. Therefore, future in vivo and clinical studies need to be undertaken for their benefits to be better exploited in the nutraceutical sector.

**Supplementary Materials:** The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/nu14122379/s1, Materials and Methods; Table S1: LC-MS/MS- based identification of SH and PH peptides; Figure S1: Evaluation of the in vitro inhibitory effects of SH (F3) (A)

and PH (F3) (B) hydrolysates on human recombinant DPP-IV; Figure S2: Evaluation of the in vitro inhibitory effects of SH (F3) (A) and PH (F3) (B) hydrolysates on ACE; Figure S3: The kinetics of the inhibition of cellular DPP-IV activity after incubating Caco-2 cells with the SH (A), SH (F3) (B), PH (C), and PH (F3) (D) hydrolysates for 1, 3, and 6 h at different concentrations; Figure S4: Evaluation of the inhibitory effects of SH (F3) (A) and PH (F3) (B) hydrolysates on ACE expressed on Caco-2 cell membranes; Technical data sheet S1: technical data sheet of SH hydrolysate; Technical
data sheet S2: technical data sheet of PH hydrolysate.

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# Appendix

## Chapter 5

## Proteomic analysis of the seeds of four wild Mexican *Lupinus* species: focus on storage proteins

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**Abstract:** *Lupinus* is a wide genus, comprising between 300 and 500 species, most of them represented in America. Mexico is a secondary distribution center with more than 100 species growing along the highlands. Due to morphological similarities, the taxonomy of wild *Lupinus* species is still incomplete. It is, therefore, useful to collect morphological, chemical, and molecular data for the correct differentiation of these plants. In the present work, the composition of the seed proteins of four species: *Lupinus aschenbornii* Schauer, *Lupinus campestris* Cham and Schlecht, *Lupinus hintonii* C.P. Smith, and *Lupinus montanus* Kunth were analyzed. Seeds were collected at Iztaccihuatl-Popocatepetl National Park. Both total proteins and single protein families, purified by chromatographic procedures, were analyzed by SDS-PAGE and 2D-electrophoresis and by LC-MS/MS analysis. Data were compared with those of domesticated species whose proteomes had been already described in the literature. The protein profile may be useful for species identification, since they have specific characteristics in each single species.

### 1. Introduction

*Lupinus* is a genus of the Fabaceae family whose seeds are characterized by a high protein content. The number

of species belonging to this genus is still uncertain ranging from 300 to 500 and more [1] and it comprises either annual and perennial plants. Despite this great diversity, only four species have been domesticated and cultivated so far: *Lupinus albus* (white lupin), *Lupinus angustifolius* (narrow-leafed lupin), *Lupinus luteus* (yellow lupin), and *Lupinus mutabilis* (Andean lupin). Owing to their commercial interest, the chemical characterization of these species has been sufficiently investigated, whereas this is not true for wild lupin species.

Mexico is a region of outmost importance for the biodiversity of this genus, because it represents a main center of diversification [2-5]. However, owing to historical reasons, the duplicity of names and synonyms makes the taxonomy of Mexican *Lupinus* species still very problematic: any new chemical characterization may thus be useful for a better understanding of the phylogenetic relationship between species.

We have concentrated our attention on four wild Mexican lupin species: *Lupinus aschenbornii* Schauer, *Lupinus campestris* Cham and Schlecht, *Lupinus hintonii* C.P. Smith, and *Lupinus montanus* Kunth. They are characteristic of the Mexican Iztaccihuatl-Popocatepetl National Park. *L. aschenbornii* is part of the subalpine vegetation of the Central Mexican highlands and grows at altitudes between 3500 and 4000 m a.s.l., *L. campestris* grows in maize fields at altitudes between 2500 and 3000 m a.s.l., *L. hintonii* grows in the Pinus-Quercus forests at altitudes of 2800-3200 m a.s.l., and *L. montanus* has the widest distribution, since it grows in the Pinus-Quercus forests as well as in the subalpine vegetation at altitudes between 2800 and 4200 m a.s.l.

A molecular phylogenic study based on barcode nucleotide sequences of internal transcribed spacer (ITS) indicates that these species belong to the sub-clade of taxa from Mexico included in the first clade gathering West American species [5]. This classification shows that *L. aschenbornii* and *L. campestris* have a higher affinity, whereas *L. montanus* and *L. hintonii* fall at the opposite borders of this sub-clade.

A recent review collects data on the nutritional and bioactive compounds of some wild Mexican lupin beans focusing the attention on proteins, lipids, minerals, dietary fiber, and other bioactive compounds [2], but a characterization of the protein profile is still pending. In the present work we have thus investigated the proteome of these lupin species with the goal of providing useful information to integrate other chemical, molecular, and morphological data to improve the taxonomic identification of these species [5]. The investigation was performed by integrating classical analysis methods, such as SDS-PAGE and 2D-electrophoresis, with chromatographic purification and mass spectrometry analysis by comparing the results

with proteomic data of domesticated species.

Available information on lupin seed proteins derives mainly from studies on domesticated species that have been analyzed in detail using proteomic techniques [6-10]. Seed proteins are mainly represented by albumins and globulins in an approximate 1 to 9 ratio [11-13]. The major protein families are named a, b, g, and d-conglutins. α-Conglutin is a legumin-like globulin, belonging to the 11S family with a storage function. It has an oligomeric structure consisting of hexamers: each monomeric unit is composed of acidic and basic subunits deriving from the cleavage of a pro-polypeptide precursor and linked by a disulfide bridge [13]. β-Conglutin is a vicilin-like protein, belonging to the 7S family, that is often the most abundant protein and has a storage function [11]. It is a trimeric protein, in which monomers consist of several polypeptides deriving from the hydrolysis of a common glycosylated precursor [11]. Its high heterogeneity is due to the expression of multigene families, whose individual genes are very closely related [6, 14]. γ-Conglutin is an unusual basic 7S protein, equally soluble in water and salt solutions, that in *L. albus* and *L. angustifolius* corresponds to 4-5% of the total proteins [11]. It is particularly stable to hydrolysis and gives strong interactions with metal ions [15]. Depending on the pH, it may be a tetramer, hexamer, or a monomer: each monomer is composed of two disulfide linked subunits (17 kDa and 29 kDa), deriving from the post-translational proteolytic cleavage of a pro-polypeptide [16]. There are evidences indicating that it is not a storage protein, although its role in the seed is still elusive [11].

## 2. Materials and Methods

### *2.1 Sampling.*

The seeds of the four Mexican lupin species were collected between June 2009 and June 2010 in the Popocatepetl-Iztaccihuatl National Park (Mexico). Herbarium vouchers were collected from three flowering individuals per species and deposited at the Herbario Nacional MEXU: *L. aschenbornii* (voucher No. 1297311) and *L. montanus* (voucher No. 1297279) at altitude of 3889 m a.s.l., *L. campestris* (voucher No. 1297299) at 2781 m a.s.l., and *L. hintonii* (voucher No. 1344434) at 2960 m a.s.l. The identification was based on morphological characteristics [17-19].

*2.2 Separation of total protein extracts (TPEs) from lupin seeds.*

The soluble proteins (albumins and globulins) were extracted from defatted lupin flour, obtained from the seeds by using a lab mill, with 100 mM Tris-HCl/0.5 M NaCl, pH 8.0, for 2 h at room temperature (RT), with gentle stirring. The slurry was centrifuged at 6000 *g* for 30 min, 4 °C, and the supernatant was dialyzed against 100 mM Tris-HCl pH 8.0, for 24 h at 4 °C. Dialyzed TPEs were immediately analyzed or aliquoted and stored at -20 °C. The protein content was assessed by the colorimetric method of Bradford [20].

*2.3 Purification of globulins.*

The globulins were purified according to a published method [21], using a preparative HPLC 1200 with a diode-array detector (DAD) (Agilent Technologies, Palo Alto, USA). In brief, the TPE was filtered through sterile membrane Econofilter filters, 0.2 µm (Agilent Technologies), and loaded onto a DEAE-Sepharose Fast Flow column (1.6 x 2.5 cm, 15-70 µm, 5 ml column volume) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Bound proteins were eluted with a linear salt gradient (0-100% NaCl over 16 column volumes) in 100 mM Tris-HCl pH 8.0, and the fractions were collected every 20 sec. The total DEAE elution was further fractionated onto a MonoQ HR 5/5 anion-exchange column (Amersham Biosciences Europe GmbH, Freiburg, Germany) with a linear salt gradient (0-100% NaCl) in 100 mM Tris-HCl pH 8.0, and the fractions were collected every 15 sec. Samples were dialyzed against 100 mM Tris-HCl, pH 8.0, using a dialysis tubing made of regenerated cellulose with a cut-off of 7 kDa (Millipore, Billerica, MA, US), under gentle stirring, for 24 h at 4 °C. Protein samples were immediately analyzed or aliquoted and kept frozen at -20 °C until use. The protein content was assessed according to the Bradford procedure [20].

*2.4 SDS-PAGE analysis.*

Desalted column fractions from the MonoQ column were separated via a classical reducing SDS-PAGE: samples were mixed 1:1 with 2X SDS-loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 0.1% bromophenol blue, 5% β-mercaptoethanol to add immediately before use) and heated for 7 min in Eppendorf tubes in boiling water. Samples were run on 13% linear gradient SDS-gels, and 2-10 µg of protein

were applied to each well. A precision plus protein standard (Biorad Laboratories Inc., Hercules, CA), ranging from 250 to 10 kDa, was used as a reference. The gels were stained with Bio-Safe Coomassie (Bio-Rad), scanned in a VersaDoc 3000 Imaging System (Bio-Rad), and analyzed using Quantity One software (Bio-Rad).

*2.5 2D-electrophoresis.*

TPEs or desalted column fractions (20 µL, about 7 µg/µL) were diluted in IEF sample buffer (7 M urea, 2 M thiourea, 3% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1% ampholyte pH 3-10 and pH 4-8). The proteins were reduced with 65 mM dithiothreitol (DTT) and alkylated with 200 mM 2-iodoacetamide (IAM), both steps for 1 h at RT in the dark [10]. Isoelectric focusing was performed on 7 cm, pH 3-10 non-linear IPG strips (Bio-Rad), and the second dimension was done on 13% SDS-PAGE. The gels were stained with Bio-Safe Coomassie (Bio-Rad), which detects proteins without the use of methanol or acetic acid. This staining produces a white background on the gel, with greater spot intensity, allowing a good qualitative/quantitative analysis by the software. Gels were scanned in a VersaDoc 3000 Imaging System (Bio-Rad). The software used to compare the 2D-maps was PDQuest Basic 2-D Analysis V7.0 (Bio-Rad). Each sample was run in duplicate.

*2.6 LC-MS/MS analysis.*

The proteins separated on the SDS-PAGE were sliced and digested with trypsin (E:S ratio equal to 1:20), and then identified by LC-MS/MS. Aliquots of 5 µL of tryptic peptides were injected into a nano-chromatographic system, HPLC-Chip (Agilent Palo Alto, CA, USA). The analysis was conducted on an SL IT mass spectrometer. LC-MS/MS analyses were performed in data-dependent acquisition AutoMS(n)mode. To increase the number of identified peptides, three technical replicates (LC-MS/MS runs) were run for each hydrolyzed band. The Spectrum Mill Proteomics Workbench (Rev B.04.00, Agilent), consulting *Lupinus* (205,529 entries) database, was used for the automated peptide identification from tandem mass spectra. Trypsin was selected as cutting enzyme with two allowed missed cleavages; carbamidomethylation was chosen as a fixed modification. The mass tolerance was set at 1.0 Da and 0.8 Da for MS1 and MS2, respectively.

Protein identification was performed by applying filters with Score＞8, SPI (Score Peak Intensity)>60%. Proteins identified with at least two different peptide sequences were studied in more detail. The characteristics of these proteins, such as the score, molecular weight, pI, number and sequence of peptides, are summarized in Supporting Information Table S1.

## 3. Results and Discussion

### *3.1 Technical approach*

The investigation of the proteomes of the four wild lupin species was performed using a bottom-up proteomic approach, including: the separation of the total protein extracts (TPEs) from the dry seeds, the purification of the main storage proteins by a chromatographic procedure (ion-exchange chromatography on a DEAE-FF column combined with a MonoQ column) and the analysis by 2D-electrophoresis in reducing conditions. In parallel, the proteins were submitted to tryptic hydrolysis and analysis by LC-MS/MS. The identification of the spots by LC-ESI-MS/MS was, however, impaired by the absence of the protein sequences of these wild lupin species in the international protein databases. The MS/MS data analysis was thus accomplished by a comparison with the data of the two domesticated species *L. albus* and *L. angustifolius*, previously investigated in our laboratory and for which numerous sequences are reported in international databases, such as SWISS-PROT.

For clarity, we report here some relevant features of the proteins of these domesticated species. The HPLC chromatograms of the TPEs are characterized by the presence of the following main peaks that are found also in the wild species: one small peak of γ-conglutin with retention time (RT) 1.5 min, a jagged large peak of β-conglutin with RT between 25 and 32 min that derives from the superimposition of the many peaks of this heterogeneous class of proteins, and an intense simple peak of α-conglutin with RT 35-43 min. The β-conglutin / α-conglutin ratios depend on the species: in *L. angustifolius* this value is approximately equal to 1:2, whereas in *L. albus* is equal to 1:0.6. The 2D-electrophoresis of the total protein extracts and of the fractions separated by preparative HPLC as well as the mass spectrometry data are reported in published papers [8, 10, 22]. These data were the basis for the interpretation of the results obtained by analyzing the wild lupin proteins.

In the wild lupins, the LC-MS/MS approach allowed the identification of seed storage proteins as well as of

some cytosolic proteins. From this point on the discussion will be limited to storage proteins alone which will be described in the following paragraphs, whereas the information concerning the proteins identified as a whole are reported in the supplementary materials (Table S1).

*3.2 Protein profile of L. hintonii.*

Figure 1A shows the HPLC chromatogram of the TPE from *L. hintonii* and Figure 1B the SDS-PAGE of the collected fractions. The first eluting peak (peak 1, RT 5 min) may be assigned to γ-conglutin, the three major peaks (peaks 2-4) between 25 and 35 min to β-conglutin, and the largest peak (peak 5, 37-40 min) to α-conglutin. The following peaks (peaks 6 and 7) remained unidentified, but the SDS-page indicates that they do not contain proteins. These peaks were detected in all species and will not be further discussed. Both the TPE and purified fractions were further characterized by 2D-electrophoresis. Figure 1C shows the 2D-map of the reduced TPE, whereas panels D, E and F, respectively, show the 2D-maps of γ-conglutin, β-conglutin, and α-conglutin. Immobilized pH gradient (IPG) runs were performed in the presence of 7 M urea, hence non-covalently bound oligomers were dissociated in all cases and the total spots, detected by the PDQuest Software, were 116. In most cases rows rather than single spots were detected, in particular series of polypeptides with similar molecular weights (MW), but varying pI values, with different degrees of post-translational phosphorylation and glycosylation. In fact, all the lupin globulins derive from a unique common ancestor polypeptide, which undergoes proteolytic cleavage, giving a complex mixture of polypeptides, which aggregate to form globulins [11]. Owing to these phenomena, globulins are an extremely heterogeneous class of proteins. Since neither gene nor protein sequences are available in the literature and in the recent database for *L. hintonii* so far, the protein identification by LC-MS/MS was based on the hypothesis of existing homologies with other well-known lupin species, i.e. *L. albus* and *L. angustifolius* [6, 8, 10, 23].

Figure 1D shows the 2D-maps of γ-conglutin, where three faint spots at 30 kDa and some other spots at 16-18 kDa appear, corresponding to the two disulfide linked subunits of the monomer. Unfortunately, the identification by LC-MS/MS analysis failed probably owing to a low sequence homology in respect to known species. In addition, γ-conglutin is resistant to tryptic hydrolysis therefore difficult to be observed in this data set.

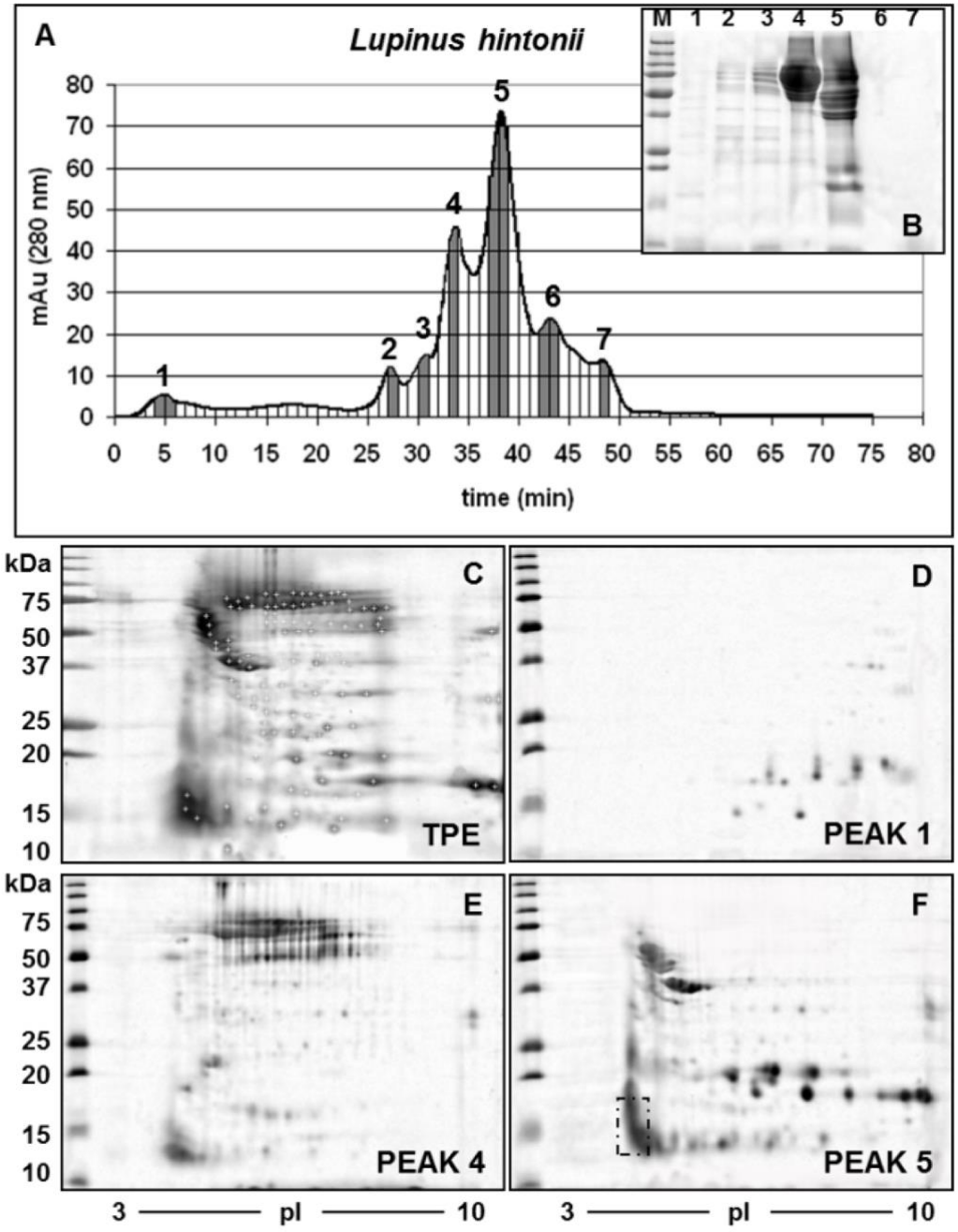


**Figure 1. Analyses on the proteins of *L. hintonii***. A) HPLC chromatogram of the total protein extract (TPE) on a MonoQ column. B) SDS-PAGE of preparative HPLC collected fractions. C) Proteome reference map of reduced TPE (proteins were run in the first dimension on 7 cm, pH 3–10 non-linear IPG strips; in the second dimension on 13% denaturing SDS–PAGE gel), where in total 116 spots were detected. D) 2D-map of purified γ-conglutin. E) 2D-map of purified β-conglutin. F) 2D-map of purified α-conglutin. The standard marker (Precision Plus Protein) is indicated in kDa on the left.

Numerous spots correspond to β-conglutin (Figure 1E), the major seed storage protein in the genus *Lupinus*: 154 spots were identified, ranging from MW 72 to 13 kDa and from pI 5.2 to 8.5. A group of spots around 50-75 kDa corresponds to mature β-conglutin subunits, whereas all other spots from MW 15 to 40 kDa correspond

to different proteolytic forms. Often these spots are further arranged into clusters of rows with closely spaced MW and pI values, due to their different phosphorylation degrees. In detail, by mass spectrometry the following β-conglutin isoforms were detected: conglutin beta (B8Q5G0), conglutin beta (Fragment) (B0YJF7), conglutin beta-1 (F5B8V9), conglutin beta 5 (F5B8W3), and conglutin beta 7 (F5B8W5) of *L. angustifolius* as well as conglutin beta 2 (Q6EBC1) of *L. albus*, with molecular weights ranging from 72 to 61 kDa and from pI 5.3 to 6.4. Table 1 lists the protein and relative identified peptides for each isoform. Among conglutin beta isoforms, the %A.A. coverage ranged from 4 to 14.4%, with the highest one observed for conglutin beta 2 (Q6EBC1). These isoforms show a high degree of identity with conservation often occurring in hydrophilic domains that are enriched in the amino acids Glu, Gln, and Arg. Such a high variety of isoforms among family members is justified by the fact that often insertions/deletions of repeated amino acid stretches of predominantly glutamic acid (E), glutamine (Q), serine (S), glycine (G), and arginine (R) are involved in the sequences. Despite the high similarity between the amino acid sequences across the isoforms of β-conglutin, our study confirmed the high micro-heterogeneities due to their polymorphism which ranges from 1% to 26% [24], mainly due to their multigenic origin. Moreover, there are obvious differences between β-conglutin across the species. In the case of *L. albus*, the 2D analysis of β-conglutin revealed that the mature protein is composed of 10 to 12 major types of subunits, with MW ranging from 15 to 20 kDa with acidic pI, from 17 to 30 kDa with basic pI, a big group of phosphorylated spots from 30 to 50 kDa and from 50 to 70 kDa, as well as a considerable number of minor constituents. In contrast, the protein from *L. angustifolius* contains two groups of polypeptides: a heavier and more abundant group (50 to 72 kDa) and a lighter group (15 to 40 kDa), whereas β-conglutins from *L. luteus* and *L. mutabilis* are essentially composed by heavy polypeptides (50 to 70 kDa) [25]. Considering these different aspects and the data collected so far, in our experimental conditions, the *L. hintonii* β-conglutin profile appears to be more similar to that of *L. angustifolius*, a fact confirmed by the protein identified by mass spectrometry.

The other main storage protein is α-conglutin deriving from a precursor (pre-pro-polypeptides), comprising a N-terminal acidic alpha-chain (with greater MW) and a C-terminal basic beta-chain (with lower MW) [26]. Several spots belonging to α-conglutin were identified in the 2D-electrophoresis (Figure 1F). They are resolved at distinct positions in the 2D-gel under the denaturating conditions used. In particular, the acidic subunit (40-52 kDa) has a pI value of 4.6-5.0, while the basic subunit (17-22 kDa) has a pI value of 6.2-8.6.

Although a high heterogeneity was observed by the 2D-gel, the LC-MS analysis allowed the identification of peptides belonging only to the acidic subunit at 58 kDa and pI 5.3 of conglutin alpha-1 (F5B8V6) of *L. angustifolius* and legumin-like protein (Q53I54) of *L. albus* with % A.A of 6.9 and 6.6, respectively. In the seed of *L. albus* the proteolytic cleavage of the 72 and 67 kDa protomers gives origin to processed precursor polypeptides of either 50-52 or 37-44 kDa, linked through disulfide bonds to the β-polypeptide of 20-22 kDa, typical of the mature legumin. We hypothesized a similar maturation cascade also for *L. hintonii*, which contributes to the significant micro-heterogeneity of α-conglutin. Instead, a major acidic spot at 65-67 kDa appears in the *L. angustifolius* 2D-map, completely absent in all the other protein profiles described here.

*3.3 Protein profile of L. campestris.*

Figure 2A shows the chromatogram of the TPE from *L. campestris*. The first eluting peak (peak 1) corresponds to γ-conglutin, the five partially overlapped peaks (peaks 2-6) between 25 and 38 min to β-conglutin, and the highest peak (peak 7) to α-conglutin (RT 39-41 min). The collected peaks were loaded on a SDS-PAGE after reduction (Figure 2B). Also, in this case, 2D-electrophoresis analyses were performed on the TPE and the main eluted peaks. In the TPE (Figure 2C), a very complex mixture of polypeptides derived from these heterogeneous classes of storage proteins are visible and in total about 100 spots were detected, the major ones belonging either to β-conglutin or α-conglutin. The 2D-electrophoresis of peak 1 (panel 3D) shows spots related to γ-conglutin: a spot triplet at 30 kDa (large subunit) and single spots at 15 and 20 kDa (small subunits), reflecting the SDS-PAGE profile. Again, however, the identification of this protein by mass spectrometry failed.

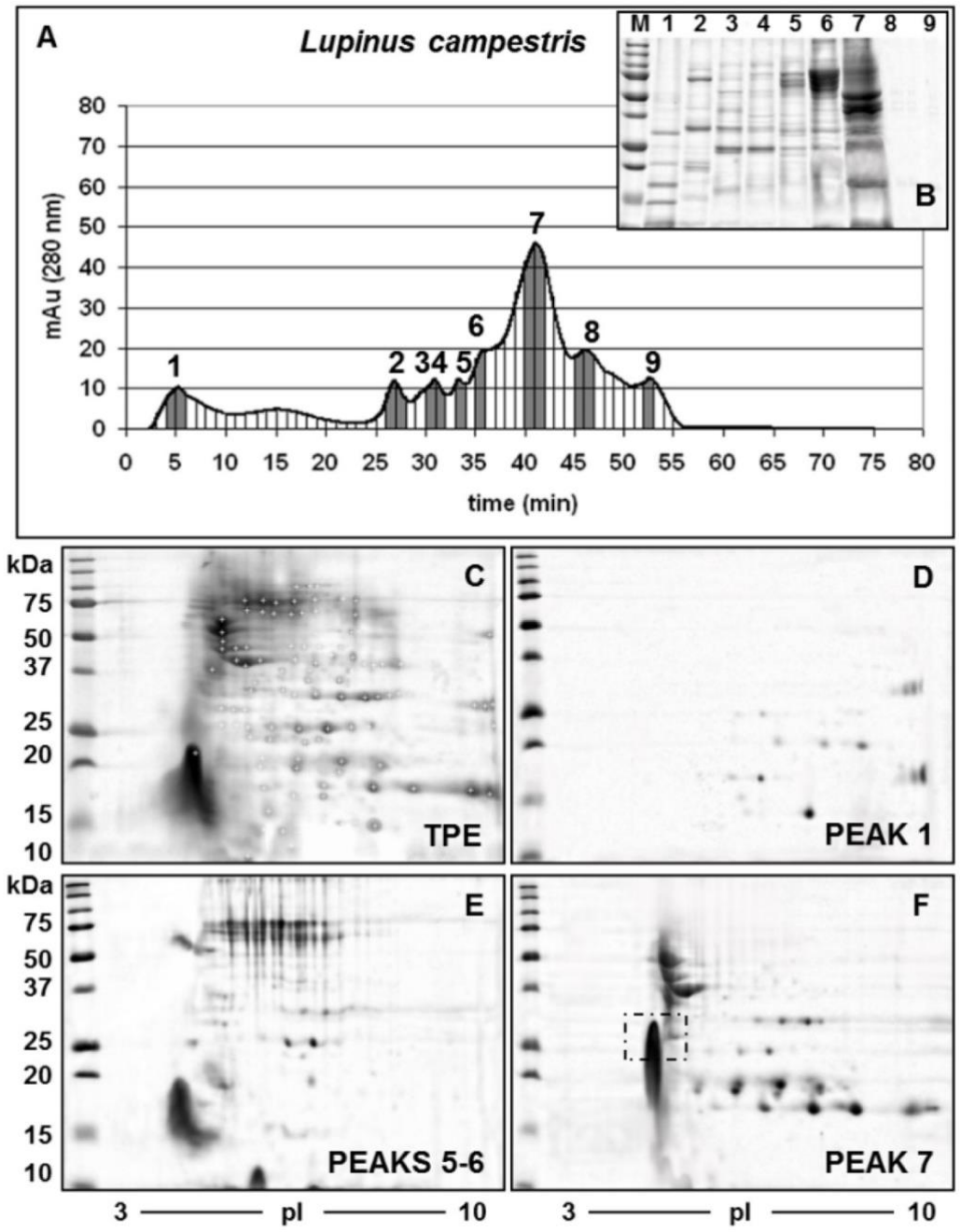


**Figure 2. Analyses on the proteins of *L. campestris*.** A) HPLC chromatogram of the total protein extract (TPE) on a MonoQ column. B) SDS-PAGE of the preparative HPLC collected fractions. C) Proteome reference map of reduced TPE where in total 100 spots were detected. D) 2D-map of purified γ-conglutin. E) 2D-map of purified β-conglutin. F) 2D-map of purified α-conglutin. The standard marker (Precision Plus Protein) is indicated in kDa on the left.

The 2D-electrophoresis analysis of β-conglutin allowed the detection of numerous distinct polypeptides (Figure 2E), deriving from the proteolysis of the protein precursor. The majority of these polypeptides may be grouped into distinct classes, which differ in their mass to charge ratio and may be designated by increasing order of pI: acidic polypeptides characterized by pI between 4.5 and 5.5 and MW of 15 to 20 kDa; moderately

acidic polypeptides, with pI between 5 and 6 and MW between 45 and 70 kDa; neutral to basic polypeptides, with pI between 6 and 8 and MW in the range 25 to 37 kDa. Unfortunately, the last class of spots is not visualized on the 2D-gel, because peaks 2-4 were not loaded on the IPG strip due to their low protein concentration. Anyway, a clear distribution of basic spots is shown in Figure 2C. The MS analysis permitted the identification of numerous β-conglutin isoforms: conglutin beta (fragment) (B0YJF7) with 14%, conglutin beta-1 (F5B8V9) with 8.8%, conglutin beta-2 (F5B8W0) with 11.4%, conglutin beta-3 (F5B8W1) with 14.1%, conglutin beta-4 (F5B8W2) with 9.3%, conglutin beta-5 (F5B8W3) with 12.2%, conglutin beta-7 (F5B8W5) with 8.9% of A.A coverage, respectively from *L. angustifolius* and conglutin beta-6 (A0A6A4QEF0) with 13.7 % of A.A coverage from *L. albus*.

Finally, in panel 2F peak 7 was resolved in several spots belonging to α-conglutin, with the typical distribution already observed in other lupin species: a big cluster of spots including acidic polypeptides characterized by pI between 5 and 5.5 and MW of 37 to 55 kDa, and a row of basic spots comprising the basic polypeptides with pI from 6.5 to 9 and MW in the range 17 to 20 kDa. Also in this case, the α-conglutin precursor undergoes proteolytic cleavage that produces a α-polypeptides of either 52 or 44 kDa linked through disulfide bonds to a β-polypeptide of 21 kDa typical of the mature legumin. The MS analysis allowed the identification of conglutin alpha 3 (F5B8V8) from *L. angustifolius* with a low % A.A. coverage of about 1.7 %.

*3.4 Protein profile of L. aschenbornii.*

The chromatogram of the TPE from *L. aschenbornii* (Figure 3A) is slightly different since the peaks between 26 and 35 min corresponding to β-conglutin are much less resolved than in the just described species. Thus, only peak 2 was considered for further analysis. Another particularity is that the peak corresponding to α-conglutin (peak 3) is here particularly high (RT 39-40 min). The SDS-PAGE after reduction of these fractions is shown in Figure 4B. After this preliminary analysis, each protein fraction was visualized on 2D-gels. Figure 4C shows that the 2D-map of the TPE contains a very large number of distinct polypeptides, deriving from the proteolytic process of the main storage proteins. The spots detected were in total 126. The analysis of peak 1 (panel 4D) shows a triplet of spots at 30 kDa together with single intense spots at lower MW (17 kDa) a basic pI, corresponding to the large and small γ-conglutin subunits, respectively. On panel 3E, several isoforms of β-conglutin polypeptides are evident, divided in two separate clusters: the first with MW in the range 50 to

75 kDa and pI values 5 to 7, the second at lower MW (around 15 kDa) and acidic pI. Since in this case only one eluted peak (peak 2) was collected and analyzed, other β-conglutin isoforms with intermediate MW and pI values may have been possibly missed and consequently not visualized on the 2D-ge*L.* The proteins identified by mass spectrometry were: conglutin beta (B8Q5G0) with 9.4 %, conglutin beta (Fragment) (B0YJF7) and conglutin beta-1 (F5B8V9) with 14.5%, conglutin beta-5 (F5B8W3) with 8.3%, conglutin beta-7 (F5B8W5) with 9.4% of A.A coverage respectively from *L. angustifolius* as well as conglutin beta-2 (Q6EBC1) with 14.4% and conglutin beta-6 (A0A6A4QEF0) from *L. albus* with 8.8% of A.A coverage.

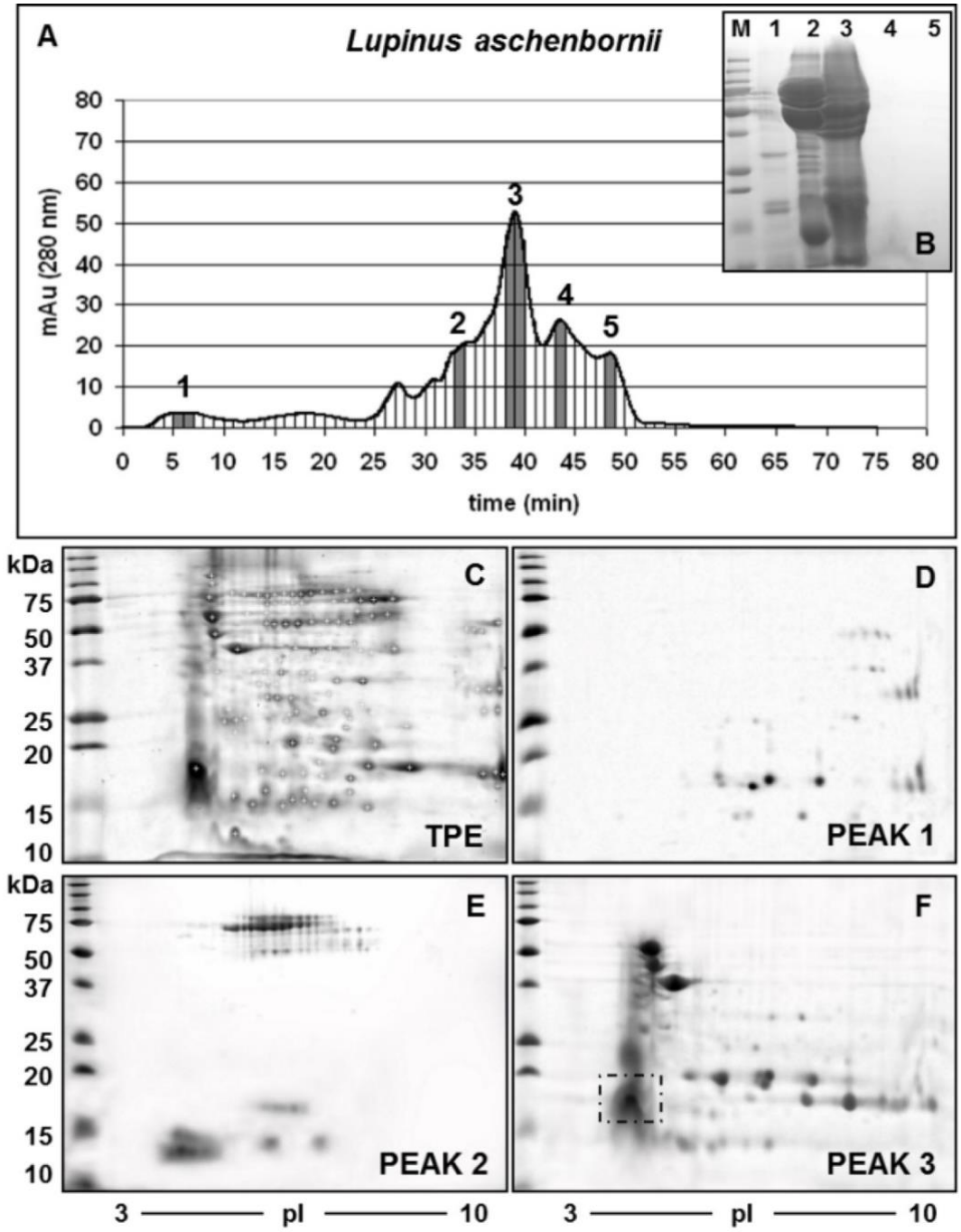


**Figure 3. Analyses on the proteins of *L. aschenbornii.*** A) HPLC chromatogram of the total protein extract (TPE) on a MonoQ column. B) SDS-PAGE of the preparative HPLC collected fractions. C) Proteome reference map of reduced TPE, where in total 126 spots were detected. D) 2D-map of purified γ-conglutin. E) 2D-map of purified β-conglutin. F) 2D-map of purified α-conglutin. The standard marker (Precision Plus Protein) is indicated in kDa on the left.

Panel 3F shows the distribution of the α-conglutin polypeptides resolved at distinct positions in 2D-gel: major spots belong to acidic subunit with MW from 37 to 55 and pI of 5-5.5, whereas the basic subunit displays MW in a range 15 to 20 kDa and pI of 6-8.5. The proteins identified by mass spectrometry were: conglutin alpha-1 (F5B8V6) covered by % A.A of 5.5%, conglutin alpha-2 (F5B8V7) covered by % A.A of 3.2%, and

conglutin alpha-3 (F5B8V8) covered by % A.A of 4.9% from *L. angustifolius* as well as legumin-like protein (Q53I54) covered by % A.A of 3.5% of *L. albus*. *L. aschenbornii* showed a wide heterogeneity in alpha isoforms covered by MS/MS analysis respect to the other wild species.

*3.5 Protein profile of L. montanus.* Figure 4A shows the preparative HPLC chromatogram of the TPE from *L. montanus*. In analogy with the chromatograms of the domesticated species, peak 1 may be attributed to γ-conglutin, the four partially overlapped peaks between 25 and 37 min (peaks 2-5) to β-conglutin and the highest peak (peak 6) corresponds to α-conglutin. Each peak was collected during the elution and loaded on a SDS-PAGE gel in reducing conditions (Figure 4B). Panels 1C, 1D, 1E, and 1F show the 2D-electrophoresis (in dissociating conditions) of the TPE, peak 1, peak 5, and peak 6, respectively. The spots detected in the TPE (Figure 4C) were in total 134. The 2D-map of γ-conglutin (Figure 4D) shows two series of spots around 30 kDa and between 15 and 20 kDa, belonging to the large and small subunits of this tetrameric protein. The proteolytic trimming of the terminal regions is likely a cause of the heterogeneity of the subunits [6, 11]. It is important to note that in other lupin species, while the C-terminus of the protein is a unique peptide [27], three major N-terminus variants in the native protein small subunit are present. This implies that the small subunit variants should have different theoretical monoisotopic molecular masses [16] and could be submitted to many different post-translational modifications. Possibly a similar phenomenon is responsible of the “triplets” of spots clearly visible in Figure 4D. However, although these interpretations may be reasonable in analogy to domesticated species, they remain tentative, since the identification by mass spectrometry failed, possibly owing to the lack of the correct sequences in the available databases.

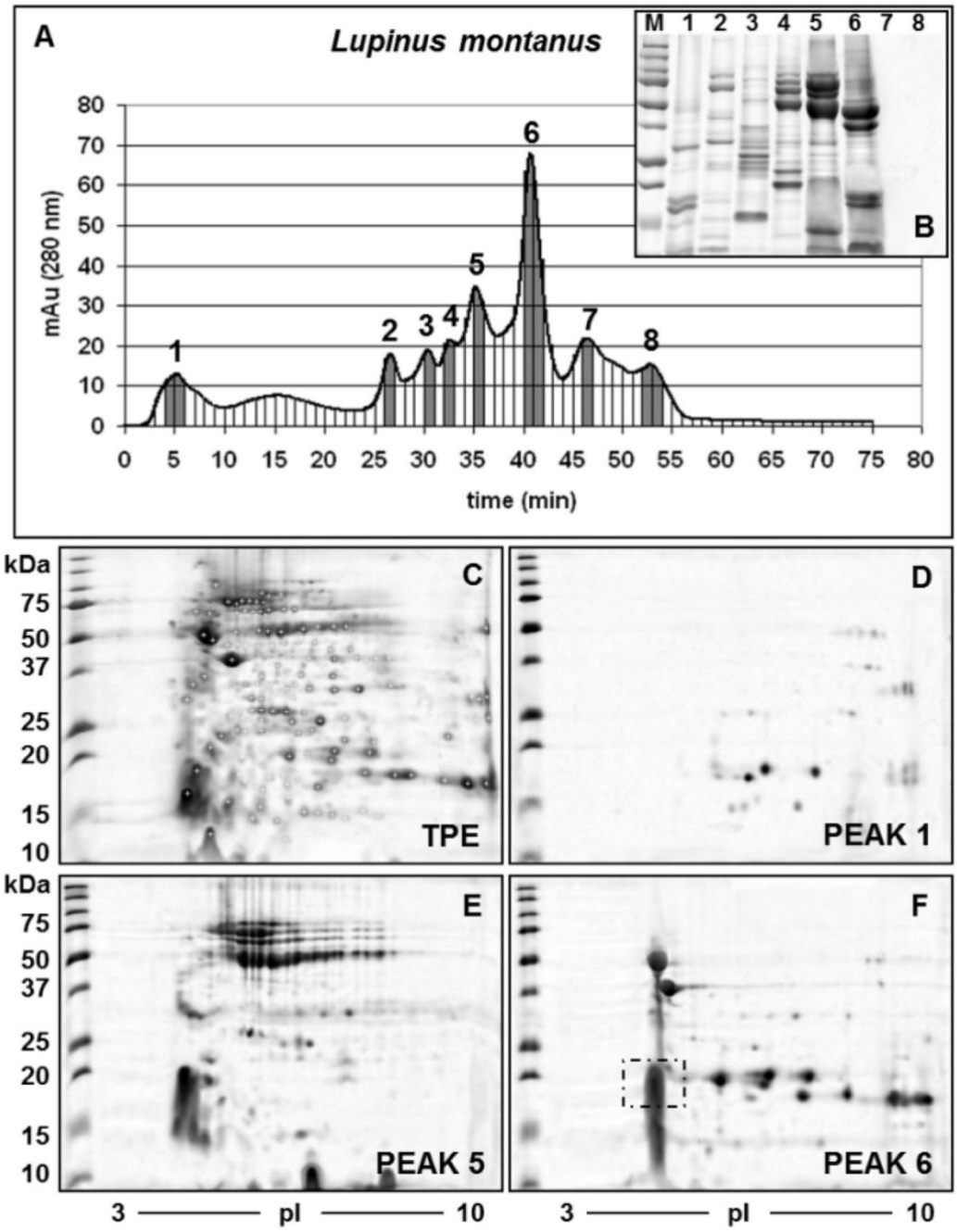


**Figure 4. Analyses on the proteins of *L. montanus*.** A) HPLC chromatogram of the total protein extract (TPE) on a MonoQ column. B) SDS-PAGE of the preparative HPLC collected fractions. C) Proteome reference map of reduced TPE, where in total 134 spots were detected. D) 2D-map of purified γ-conglutin. E) 2D-map of purified β-conglutin. F) 2D-map of purified α-conglutin. The standard marker (Precision Plus Protein) is indicated in kDa on the left.

Several spots corresponding to β-conglutin (Figure 4E) were identified, mostly arranged into clusters and defined as "trains of spot", with closely spaced molecular weight (MW) and pI values. Prominent spots of the mature β-conglutin subunits span from 75 to 50 kDa, whereas a group of spots around 15-25 kDa corresponds to proteolytic forms. This β-conglutin profile is similar to that of *L. angustifolius*. The heterogeneity was

confirmed by LC-MS analysis, since numerous isoforms were identified with a sequence coverage of: conglutin beta (B8Q5G0) with 9.4%, conglutin beta fragment (B0YJF7) with 14.5%, conglutin beta-1 (F5B8V9) with 7%, conglutin beta-3 (F5B8W1) of 6.7%, conglutin beta-4 (F5B8W2) with 9.8%, conglutin beta-5 (F5B8W3) with 8.3%, conglutin beta-6 (F5B8W4) with 9.7%, conglutin beta-7 (F5B8W5) with 9.4% of A.A coverage, respectively, based on *L. angustifolius* sequences as well as conglutin beta-2 (Q6EBC1) with 9.9% of A.A. coverage based on *L. albus* sequences.

The α-conglutin 2D-map (panel F) shows the different subunits of this hexameric protein that are resolved at distinct positions under denaturating conditions. In particular, the acidic subunit (37-50 kDa) has a pI value of 4.6-5.0 and the basic subunit (17-20 kDa) a pI values of 6.0-9.0. The fact that few isoforms of the acidic subunit are present in *L. montanus* may explain the different HPLC profile of this lupin species (see for example *L. hintonii*). The peptides detected by mass spectrometry belong either to conglutin alpha-1 (F5B8V6), whose % A.A coverage was 9.1% and conglutin alpha-3 (F5B8V8) whose % A.A coverage was 2.2% of *L. angustifolius* or legumin-like protein (Q53I54) of *L. albus*, confirming the correct protein identification with the % A.A. coverage of 3.5%.

**Table 1.** Identified storage proteins in the seeds of the four wild lupin species by LC-MS/MS.

| **Protein Name *(Accession N.)*** | **Species** | **% AA coverage** | **Protein pI** | **Peptide Sequences** | **m/z Measured (Da)** | **$MH^+$ Matched (Da)** | **Peptide pI** |
|---|---|---|---|---|---|---|---|
| ***L. hintonii*** | | | | | | | |
| Conglutin beta (Fragment) ***(B0YJF7)*** | *L. angustifolius* | 8.7 | 5.3 | (R)**LPAGTTSYILNPDDNQNLR**(V) * | 701.67 | 2102.051 | 4.21 |
| Conglutin beta ***(B8Q5G0)*** | | 11 | 5.8 | (R)**LLGFGINANENQR**(N) | 723.85 | 1445.750 | 6.00 |
| | | | | (R)NFLAGSEDNVISQLDREVK(E) | 712.34 | 2134.077 | 4.32 |
| | | | | (R)NFLAGSEDNVISQLDR(E) | 593.58 | 1777.872 | 4.03 |
| | | | | (R)TNRLENLQNYR(I) | 711.20 | 1420.729 | 8.41 |
| Conglutin beta 7 ***(F5B8W5)*** | | 8.9 | 5.6 | (R)LENLQNYR(I) | 525.41 | 1049.537 | 6.00 |
| Conglutin beta 1 ***(F5B8V9)*** | *L. angustifolius* | 4 | 5.8 | (R)**NPYHFSSNR**(P) | 560.75 | 1121.512 | 6.5 |
| | | | | (R)LENLQNYR(I) | 525.41 | 1049.537 | 6.00 |
| Conglutin beta 2 ***(Q6EBC1)*** | *L. albus* | 14.4 | 6.4 | (R)**LLGFGINADENQR**(N) | 482.72 | 1446.734 | 4.37 |
| | | | | (K)**INEGALLLPHYNSK**(A) | 523.92 | 1568.843 | 6.75 |
| | | | | (R)TNRLENLQNYR(I) | 711.20 | 1420.729 | 8.41 |
| | | | | (R)IVEFQSKPNTLILPK(H) | 576.55 | 1727.010 | 8.59 |
| | | | | (R)LENLQNYR(I) | 525.41 | 1049.537 | 6.00 |
| Conglutin beta 5 ***(F5B8W3)*** | *L. angustifolius* | 11.2 | 5.8 | (K)HSDADYILVVLNGR(A) | 524.75 | 1571.818 | 5.21 |
| | | | | (R)LLGFGINADENQR(N) | 482.72 | 1446.734 | 4.37 |
| | | | | (R)**LPAGTTSYILNPDDNQDLR**(V) | 701.99 | 2103.035 | 3.93 |
| | | | | (R)**ILLGYEDEQEDEEQR**(R) | 933.90 | 1865.840 | 3.71 |
| | | | | (R)TNRLENLQNYR(I) | 711.20 | 1420.729 | 8.41 |
| | | | | (R)LENLQNYR(I) | 525.41 | 1049.537 | 6.00 |
| Conglutin alfa 1 ***(F5B8V6)*** | *L. angustifolius* | 6.9 | 5.3 | (R)**RFYLSGNQEQEFLQYQQK**(E) | 769.66 | 2306.120 | 6.14 |
| | | | | (R)**FYLSGNQEQEFLQYQQK**(E) | 717.62 | 2150.019 | 5.43 |
| | | | | (R)RPFYTNAPQEIYIQQGR(G) | 694.39 | 2081.056 | 8.59 |
| Legumin like-protein | *L. albus* | 6.6 | 5.5 | (R)**FYLSGNQEQEFLQYQEK**(E) | 717.85 | 2151.003 | 4.25 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***(Q53I54)*** | | | | (R)RPFYTNAPQEIYIQQGR(G) | 694.39 | 2081.056 | 8.45 |
| | | | | (R)**RFYLSGNQEQEFLQYQEK**(E) | 769.73 | 2307.104 | 4.79 |
| ***L. campestris*** | | | | | | | |
| Conglutin beta 1 ***(F5B8V9)*** | *L. angustifolius* | 8.8 | 5.8 | (R)ILLGYEDEQEDEEQR(R) | 622.90 | 1865.840 | 3.71 |
| | | | | (R)TNRLENLQNYR(I) | 474.46 | 1420.729 | 8.41 |
| | | | | (R)LLGFGINADENQR(N) | 724.10 | 1446.734 | 4.37 |
| | | | | (R)SNEPIYSNK(F) | 526.39 | 1051.506 | 5.72 |
| | | | | (R)ATITIVNPDKR(Q) | 409.99 | 1227.706 | 8.79 |
| | | | | (R)LENLQNYR(I) | 524.96 | 1049.537 | 6.00 |
| Conglutin beta 2 ***(F5B8W0)*** | *L. angustifolius* | 11.4 | 5.6 | (R)IVEFQSKPNTLILPK(H) | 576.58 | 1727.010 | 8.59 |
| | | | | (R)LPAGTTSYILNPDDNQNLR(V) | 701.59 | 2102.051 | 4.21 |
| | | | | (R)ATITIVNPDKR(Q) | 409.99 | 1227.706 | 8.79 |
| | | | | (R)LLGFGINADENQR(N) | 723.94 | 1446.734 | 4.37 |
| Conglutin beta 3 ***(F5B8W1)*** | *L. angustifolius* | 14.1 | 5.7 | (R)IVEFQSKPNTLILPK(H) | 576.58 | 1727.010 | 8.59 |
| | | | | (R)TNRLENLQNYR(I) | 474.46 | 1420.729 | 8.41 |
| | | | | (R)LPAGTTSYILNPDDNQNLR(V) | 701.59 | 2102.051 | 4.21 |
| | | | | (R)LLGFGINADENQR(N) | 723.94 | 1446.734 | 4.37 |
| | | | | (R)LENLQNYR(I) | 525.39 | 1049.537 | 6.00 |
| | | | | (K)AIFVVVVDEGEGNYELVGIRDQQR(Q) | 902.40 | 2705.389 | 4.18 |
| Conglutin beta 4 ***(F5B8W2)*** | *L. angustifolius* | 9.3 | 5.9 | (R)ILLGYEDEQEDEEQR(R) | 622.82 | 1865.840 | 3.71 |
| | | | | (R)TNRLENLQNYR(I) | 474.46 | 1420.729 | 8.41 |
| | | | | (R)LLGFGINADENQR(N) | 724.10 | 1446.734 | 4.37 |
| | | | | (R)SNEPIYSNK(F) | 526.39 | 1051.506 | 5.72 |
| Conglutin beta 5 ***(F5B8W3)*** | *L. angustifolius* | 12.2 | 5.8 | (R)TNRLENLQNYR(I) | 474.46 | 1420.729 | 8.41 |
| | | | | (R)ILLGYEDEQEDEEQR(R) | 622.82 | 1865.840 | 3.71 |
| | | | | (R)LLGFGINADENQR(N) | 724.10 | 1446.734 | 4.37 |
| | | | | (R)SNEPIYSNK(F) | 526.39 | 1051.506 | 5.72 |
| | | | | (R)ATITIVNPDKR(Q) | 409.99 | 1227.706 | 8.79 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | (R)LENLQNYR(I) | 525.39 | 1049.537 | 6.00 |
| | | | | (R)**LPAGTTSYILNPDDNQDLR**(V) | 701.71 | 2103.035 | 3.93 |
| Conglutin beta 6 ***(A0A6A4QEF0)*** | *L. albus* | 13.7 | 5.4 | (R)ILLGYEDEQEDEEQR(R) | 622.90 | 1865.840 | 3.71 |
| | | | | (R)TNRLENLQNYR(I) | 474.46 | 1420.729 | 8.41 |
| | | | | (R)LLGFGINADENQR(N) | 724.10 | 1446.734 | 4.37 |
| | | | | (R)SNEPIYSNK(F) | 526.39 | 1051.506 | 5.72 |
| | | | | (R)ATITIVNPDKR(Q) | 613.93 | 1227.706 | 8.79 |
| | | | | (R)LENLQNYR(I) | 524.96 | 1049.537 | 6.00 |
| Conglutin beta (Fragment) ***(B0YJF7)*** | *L. angustifolius* | 6.3 | 5.3 | (R)NFLAGSEDNVISQLDR(E) | 889.93 | 1777.872 | 4.03 |
| Conglutin beta 7 ***(F5B8W5)*** | | 12.5 | 5.6 | (R)LPAGTTSYILNPDDNQNLR(V) | 701.59 | 2102.051 | 4.21 |
| | | | | (R)LLGFGINANENQR(N) | 723.71 | 1445.750 | 6.00 |
| Conglutin beta ***(B8Q5G0)*** | | 5.5 | 5.8 | (R)ATITIVNPDKR(Q) | 613.93 | 1227.706 | 8.79 |
| Conglutin alpha 3 ***(F5B8V8)*** | *L. angustifolius* | 2 | 5.3 | (R)**ADLYNPTAGR**(I) | 539.27 | 1077532 | 5.88 |
| ***L. aschenbornii*** | | | | | | | |
| Conglutin beta ***(B8Q5G0)*** | *L. angustifolius* | 9.4 | 5.8 | (R)LPAGTTSYILNPDDNQNLR(V) | 701.68 | 2102.051 | 4.21 |
| | | | | (R)**IIEFQSKPNTLILPK**(H) | 581.31 | 1741.026 | 8.59 |
| | | | | (R)TNRLENLQNYR(I) | 711.18 | 1420.729 | 8.41 |
| | | | | (R)LLGFGINANENQR(N) | 723.65 | 1445.750 | 6.00 |
| Conglutin beta (Fragment) ***(B0YJF7)*** | *L. angustifolius* | 14.5 | 5.8 | (R)**NFLAGSEDNVISQLDR**(E) | 889.93 | 1777.872 | 4.03 |
| | | | | (R)LPAGTTSYILNPDDNQNLR(V) | 701.68 | 2102.051 | 4.21 |
| | | | | (R)NFLAGSEDNVISQLDREVK(E) | 712.35 | 2134.077 | 4.32 |
| Conglutin beta 1 ***(F5B8V9)*** | | | | (R)TNRLENLQNYR(I) | 711.18 | 1420.729 | 8.41 |
| | | | | (R)LLGFGINANENQR(N) | 723.65 | 1445.750 | 6.00 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Conglutin beta 2 ***(Q6EBC1)*** | *L. albus* | 14.4 | 6.4 | (K)INEGALLLPHYNSK(A) | 523.92 | 1568.843 | 6.75 |
| | | | | (R)LLGFGINADENQR(N) | 724.26 | 1446.734 | 4.37 |
| | | | | (R)TNRLENLQNYR(I) | 711.18 | 1420.729 | 8.41 |
| | | | | (R)IVEFQSKPNTLILPK(H) | 576.54 | 1727.010 | 8.59 |
| | | | | (R)LSEGDIFVIPAGYPISINASSNLR(L) | 845.14 | 2533.330 | 4.37 |
| Conglutin beta 5 ***(F5B8W3)*** | *L. angustifolius* | 8.3 | 5.8 | (R)ILLGYEDEQEDEEQR(R) | 933.88 | 1865.840 | 3.71 |
| | | | | (R)LLGFGINADENQR(N) | 724.26 | 1446.734 | 4.37 |
| | | | | (R)TNRLENLQNYR(I) | 711.18 | 1420.729 | 8.41 |
| | | | | (R)**LPAGTTSYILNPDDNQDLR**(V) | 701.83 | 2103.035 | 3.93 |
| Conglutin beta 6 ***(A0A6A4QEF0)*** | *L. albus* | 10.8 | 5.8 | (R)IVEFQSKPNTLILPK(H) | 575.65 | 1727.010 | 8.59 |
| | | | | (R)TNRLENLQNYR(I) | 711.18 | 1420.729 | 8.41 |
| | | | | (R)SNEPIYSNK(Y) | 526.39 | 1051.506 | 5.72 |
| | | | | (R)LENLQNYR(I) | 525.40 | 1049.537 | 6.00 |
| | | | | (R)**LIENQQQSYFANALPQQQQQSEK**(E) | 907.49 | 2720.327 | 4.53 |
| Conglutin beta 7 ***(F5B8W5)*** | *L. angustifolius* | 9.4 | 5.6 | (R)**NFLAGSEDNVISQLDR**(E) | 889.93 | 1777.872 | 4.03 |
| | | | | (R)LPAGTTSYILNPDDNQNLR(V) | 701.68 | 2102.051 | 4.21 |
| | | | | (R)NFLAGSEDNVISQLDREVK(E) | 712.35 | 2134.077 | 4.32 |
| | | | | (R)TNRLENLQNYR(I) | 711.18 | 1420.729 | 8.41 |
| | | | | (R)LLGFGINANENQR(N) | 723.65 | 1445.750 | 6.00 |
| Conglutin alpha 1 ***(F5B8V6)*** | *L. angustifolius* | 5.5 | 5.3 | (R)**FYLSGNQEQEFLQYQQK**(E) | 717.60 | 2150.019 | 4.53 |
| | | | | (R)**LNALEPDNSVK**(S) | 600.56 | 1199.627 | 4.37 |
| Conglutin alpha 2 ***(F5B8V7)*** | *L. angustifolius* | 3.2 | 5.1 | (K)**TNDLAATSPVK**(Q) | 558.92 | 1116.590 | 5.50 |
| | | | | (R)**LLENIAKPSR**(A) | 570.88 | 1140.674 | 8.75 |
| Conglutin alpha 3 ***(F5B8V8)*** | *L. angustifolius* | 4.9 | 5.3 | (R)**ENIADPSRADLYNPTAGR**(I) | 654.29 | 1959.952 | 4.56 |
| | | | | (R)**ADLYNPTAGR**(I) | 539.39 | 1077.532 | 5.88 |
| Legumin-like protein | *L. albus* | 3.5 | 5.5 | **(R)FYLSGNQEQEFLQYQEK(E)** | 717.95 | 2151003 | 4.25 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***(Q53I54)*** | | | | | (R)RFYLSGNQEQEFLQYQEK(E) | 769.71 | 2307104 | 4.79 |
| ***L. montanus*** | | | | | | | | |
| Conglutin beta ***(B8Q5G0)*** | beta | *L. angustifolius* | 9.4 | 5.8 | (R)NFLAGSEDNVISQLDR(E) | 889.91 | 1777.872 | 4.00 |
| | | | | | (R)LLGFGINANENQR(N) | 723.71 | 1445.750 | 6.00 |
| Conglutin (Fragment) ***(B0YJF7)*** | beta | | 14.5 | 5.3 | (R)NFLAGSEDNVISQLDREVK(E) | 712.25 | 2134.077 | 4.34 |
| | | | | | (R)TNRLENLQNYR(I) | 474.44 | 1420.729 | 8.41 |
| | | | | | (K)HSDADYILVVLNGR(A) | 524.75 | 1571.818 | 5.21 |
| Conglutin beta 1 ***(F5B8V9)*** | | *L. angustifolius* | 7 | 5.8 | (K)**ELTFPGSIEDVER**(N) | 745.86 | 1491.732 | 3.91 |
| | | | | | (R)NFLAGSEDNVISQLDR(E) | 889.91 | 1777.872 | 4.03 |
| | | | | | (R)LLGFGINANENQR(N) | 723.71 | 1445.750 | 6.00 |
| Conglutin beta 2 ***(Q6EBC1)*** | | *L. albus* | 9.9 | 6.4 | (R)IVEFQSKPNTLILPK(H) | 864.48 | 1727.010 | 8.59 |
| | | | | | (K)INEGALLLPHYNSK(A) | 523.89 | 1568.843 | 6.75 |
| | | | | | (R)TNRLENLQNYR(I) | 474.44 | 1420.729 | 8.41 |
| | | | | | (R)LLGFGINADENQR(N) | 482.72 | 1446.734 | 4.37 |
| Conglutin beta 3 ***(F5B8W1)*** | | *L. angustifolius* | 6.7 | 5.7 | (R)IVEFQSKPNTLILPK(H) | 864.48 | 1727.010 | 8.59 |
| | | | | | (K)INEGALLLPHYNSK(A) | 523.89 | 1568.843 | 6.75 |
| | | | | | (R)TNRLENLQNYR(I) | 474.44 | 1420.729 | 8.41 |
| | | | | | (R)LLGFGINADENQR(N) | 482.72 | 1446.734 | 4.37 |
| Conglutin beta 4 ***(F5B8W2)*** | | *L. angustifolius* | 9.8 | 5.9 | (K)**FGNFYEITPNR**(N) | 679.08 | 1358.65 | 5.7 |
| | | | | | (R)TNRLENLQNYR(I) | 474.44 | 1420.729 | 8.41 |
| | | | | | (R)LLGFGINADENQR(N) | 482.72 | 1446.734 | 4.37 |
| Conglutin beta 5 ***(F5B8W3)*** | | *L. angustifolius* | 8.3 | 5.8 | (R)ILLGYEDEQEDEEQR(R) | 933.78 | 1865.840 | 3.71 |
| | | | | | (R)TNRLENLQNYR(I) | 474.44 | 1420.729 | 8.41 |
| | | | | | (K)HSDADYILVVLNGR(A) | 524.75 | 1571818 | 5.21 |
| | | | | | (R)LLGFGINADENQR(N) | 482.72 | 1446.734 | 4.37 |
| Conglutin beta 6 | | *L.* | 9.7 | 6.2 | (R)IVEFQSKPNTLILPK(H) | 864.48 | 1727.010 | 8.59 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***(F5B8W4)*** | *angustifolius* | | | (R)**TDRLENLQNYR**(I) | 474.44 | 1421.713 | 5.73 |
| | | | | (R)LLGFGINADENQR(N) | 482.72 | 1446.734 | 4.30 |
| Conglutin beta 7 ***(F5B8W5)*** | *L. angustifolius* | 9.4 | 5.6 | (R)NFLAGSEDNVISQLDR(E) | 889.91 | 1777.872 | 4.03 |
| | | | | (R)LLGFGINANENQR(N) | 723.71 | 1445.750 | 6.00 |
| | | | | (R)NFLAGSEDNVISQLDREVK(E) | 712.25 | 2134.077 | 4.32 |
| | | | | (R)TNRLENLQNYR(I) | 474.44 | 1420.729 | 8.41 |
| | | | | (K)HSDADYILVVLNGR(A) | 524.75 | 1571.818 | 5.21 |
| Conglutin alpha 1 ***(F5B8V6)*** | *L. angustifolius* | 9.1 | 5.3 | (R)RPFYTNAPQEIYIQQGR(G) | 694.67 | 2081.056 | 8.59 |
| | | | | (R)RFYLSGNQEQEFLQYQQK(E) | 769.68 | 2306.120 | 6.14 |
| | | | | (R)**LNALEPDNSVK**(S) | 600.35 | 1199.627 | 4.37 |
| Conglutin alpha 3 ***(F5B8V8)*** | *L. angustifolius* | 2.2 | 5.3 | (R)**ADLYNPTAGR**(I) | 539.43 | 1077.532 | 5.88 |
| Legumin-like protein ***(Q53I54)*** | *L. albus* | 3.5 | 5.5 | **(R)FYLSGNQEQEFLQYQEK(E)** | 717.95 | 2151003 | 4.25 |
| | | | | (R)RFYLSGNQEQEFLQYQEK(E) | 769.71 | 2307104 | 4.79 |

**peptides marked in bold are unique peptides for each protein isoform.*

## 4. Conclusions

In this report, we provide a detailed characterization of the major storage proteins of *L. hintonii*, *L. campestris, L. aschenbornii,* and *L. montanus* underlining the similarities and differences in respect to the main domesticated species *L. albus* and *L. angustifolius*. In general, it seems possible to affirm that their profiles are more similar to *L. angustifolius* than to *L. albus*.

Despite the large heterogeneity observed among these species in what concerns the polypeptide composition of these storage proteins, they suffer a similar fate, in terms of proteolytic processing and post-translational modifications (phosphorylation and glycosylation). In fact, the SDS-PAGE analyses revealed that the mature proteins are composed of numerous polypeptides differing in terms of MW and pI values, whereas the 2D-electrophoretic analyses further indicate that the individual subunits of each conglutin derive form the assembling of a numerous distinct polypeptides. In particular, β-conglutin is characterized by a rather broad micro-heterogeneity that appears to be very characteristic of this protein, with almost a continuum of polypeptides ranging in molecular mass from 15 to 75 kDa and with pI values from 5 to 9. These data may be certainly useful for a deeper characterization of these wild species.

Finally, it is useful to observe that literature presents scattered information demonstrating some interest in using some of these species in food or animal nutrition. In particular, there is interest in the exploitation of *L. campestris* owing to its adaptation to grow at very high altitudes. One paper [28] has optimized a procedure for protein purification, demonstrating that isoelectric precipitation is more effective than micellization to obtain high yields and effective separation of bitter and toxic quinolizidine alkaloids [29]. Another paper, instead, has investigated the possibility of using the seed of the same species to produce a yogurt-like product [30]. This evidence demonstrates that the interest in these proteins is not only speculative.

Through the integrated approach, proteins and peptide sequences were here identified with a greater degree of confidence for wild lupin species. This information is itself

beneficial for fundamental and applied studies concerning the storage proteins of the seeds. Moreover, to the best of our knowledge, this is the first *L. hintonii*, *L. campestris, L. ascherbornii* and *L. montanus* storage protein catalog, allowing a considerable expansion of the data than previously reported for wild lupin. Having this catalog available as a resource may contribute to fundamental and applied studies of the seeds.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: identified proteins by LC-MS/MS.

**Abbreviations:** a.s.l., above sea level; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate; DAD, diode-array detector; DTT, dithiothreitol; IAM, 2-iodoacetamide; IEF, isoelectric focusing; LC, liquid chromatography; MS, mass spectrometry; MW, molecular weight; IPG strip, immobilized pH gradient strip; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPI, Score Peak Intensity; TPEs, total protein extracts.

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