DEPARTMENT OF AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES

DEGREE COURSE: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

INFLUENCE OF INGREDIENT QUALITY ON THE RHEOLOGICAL PROPERTIES AND EXTRUDABILITY OF HEMP PROTEINS

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ACRONYMS AND ABBREVIATIONS

ACE	Angiotensin converting enzymes
CE	Circular equivalent
CVD	Cardiovascular disease
D[3,2]	Surface-weighted mean particle diameter
D[4,3]	Volume-weighted mean particle diameter
D_N[0.1]	Particle size below which 10% of number particles is found
D_N[0.5]	Particle size below which 50% of number particles is found
D_N[0.9]	Particle size below which 90% of number particles is found
DTNB	5,5'-Dithiobis 2-nitrobenzoic acid
DTT	Dithiothreitol
D_V[0.1]	Particle size below which 10% of sample volume is found
D_V[0.5]	Particle size below which 50% of sample volume is found
D_V[0.9]	Particle size below which 90% of sample volume is found
EAA	Essential amino acid
G*	Complex modulus
G'	Storage modulus
G''	Loss modulus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HME	High moisture extrusion
HPC	Hemp protein concentrate
HPHT	High pressure high temperature
HPI	Hemp protein isolate
LME	Low moisture extrusion
MW	Molecular weight
MS	Mass spectrometry
OAC	Oil absorption capacity

OMH	Optimal matching hydrophobicity
PAGE	Polyacrylamide gel electrophoresis
PB	Phosphate buffer
PDA	Photo diode array
PDCAAS	Protein digestibility corrected amino acid score
PDS	Particle size distribution
P_L	Longitudinal peak force
PPC	Pea protein concentrate
PS	Protein solubility
P_T	Transversal peak force
PUFA	Polyunsaturated fatty acid
RP-UPLC	Reverse phase ultra performance liquid chromatography
RVA	Rapid visco analyzer
SDS	Sodium dodecyl sulphate
SDR	Short chain dehydrogenase/reductase
SPC	Soy protein concentrate
TFA	Trifluoroacetic acid
T_G	Glass transition temperature
THC	Tetrahydrocannabinol
TVP	Texturized vegetable protein
WAC	Water absorption capacity
WSI	Water solubility index

1. Introduction

During the last decade, the rapid growth of the world population has raised the issues of food security and environmental sustainability (Aiking and de Boer 2020). The Food and Agriculture Organization has stated that in the future proteins will be the first limiting macronutrient (Arrutia et al. 2020) and since they are essential in human nutrition, it is fundamental to ensure enough dietary proteins for everyone and to produce them in a sustainable way (Schweiggert-Weisz et al. 2020).

Among dietary proteins, those derived from animals are considered of high-quality, and their demand is still growing especially in developed countries. However, from a nutritional point of view, a large intake of meat and animal-based products is often related to health problems, e.g., cardiovascular diseases, cancer (Schweiggert-Weisz et al. 2020). Moreover, the intensive meat production is not sustainable due to the large amount of land and water required, and CO_2 emissions (Day 2013; Fiorentini, Kinchla, and Nolden 2020).

On the other hand, plant proteins are able to satisfy the same human nutrition needs with less environmental impact (Nadathur, Wanasundara, and Scanlin 2016). Therefore, a switch from animal to alternative protein sources is necessary, which is why food companies have started an in-depth research on the characterization of the physicochemical properties of plant proteins and their potential to be used in a variety of food products (Nadathur, Wanasundara, and Scanlin 2016). This has been paralleled by the commercialization of a wide variety of plant protein-enriched ingredients, including, but not limited to, those derived from cereals (e.g., wheat, corn, rice, oat, barley), pseudo-cereals (e.g., chia, amaranth, quinoa, buckwheat), legumes (e.g., pea, fava bean, lentils, lupins), tubers (e.g., potato) and oilseeds (e.g., soybean, canola, sunflower, hempseeds) (Loveday 2019). These ingredients are obtained using different extraction methods (e.g., alkaline extraction (Momen, Alavi, and Aider 2021), oil pressing (Arrutia et al. 2020), air classification (Schutyser et al. 2015)) and processing conditions, and thus display differences in terms of purity (concentrates *vs* isolates), physical state (native, denatured, hydrolysed) and composition, which ultimately lead to differences in terms of functional properties. Regarding the composition of plant proteins, they may be classified according to their solubility in various solvents (Osborne 1924) and to their sedimentation coefficient in:

- **Albumins**: water-soluble proteins with a sedimentation coefficient of 2S; they consist of two cross-linked polypeptide chains with a molecular weight lower than 10 kDa.
- **Globulins**: salt-soluble proteins, they comprise two main fractions with a sedimentation coefficient of 7S and 11S. The former is a trimer with a MW of about 180 kDa, while the latter is a hexamer of about 350 kDa; the monomers constituting the 7S and 11S proteins

are held together through non-covalent interactions, while the acidic (~35 kDa) and basic (~20 kDa) subunits that compose each 11S monomer are linked covalently.

- **Glutelins**: alkali/acid-soluble proteins, they have a sedimentation coefficient of 11S; they consist of two polypeptide chains (α and β) with a MW of 34-40 kDa and 20-23 kDa, respectively, which are characterized by extensive aggregation through disulphide bonds and glycosylation that make them difficult to extract.
- **Prolamins**: alcohol-soluble proteins, they present a large variation in their molecular weight that can range from 10 to 90 kDa depending on the different species of grains in which they are mainly found.

The differences in term of protein composition strongly affect the functionality of the protein ingredients. It should be noted that protein composition is not only influenced by the extraction process, but also by cultivar, farming practices and environmental conditions. In **Table *1***, some examples of plant protein sources with relative protein concentration and composition are reported (Arrutia et al. 2020; Day 2013).

Table 1. Protein concentration and composition of different plant sources (adapted from Day (2013)).

Plant source	Protein concentration (%)	Albumins (%)	Globulins (%)	Prolamins (%)	Glutelins (%)
Wheat	8-15	6-10	5-8	35-40	40
Rice	7-9	2-6	12	4	80
Soybean	35-40		90		
Pea	20-30	15-25	50-60		
Chickpea	20-25	8-12	53-60	3-7	19-25
Canola	17-26	20	60	2-5	15-20
Hempseed	25-30	20-40	60-80		

Nowadays, a vast range of plant protein-based food products are commercially available, including liquid (e.g., plant-based milk analogues, smoothies, toppings), semi-solid (e.g., yoghurt, culinary creams, ice cream) and solid ones (e.g., breakfast cereals, bread, crackers, energy bars, meat analogues). Meat analogues have gained particular interest over the last few years due to the ever-increasing proportion of flexitarian, vegetarian and vegan consumers (Beniwal et al. 2021). As a result, companies have shifted their attention towards finding novel protein sources, new formulations, technologies, and cooking methods in order to mimic as much as possible the color, taste, texture and juiciness of real meat (Fiorentini, Kinchla, and Nolden 2020). Most of the commercially available meat analogues are based on texturized vegetable proteins (TVPs) obtained *via* low moisture extrusion of flours or protein concentrates, which food companies buy directly from food ingredient suppliers and which need to be rehydrated before being mixed with

the other ingredients to produce the desired product. However, the use of TVPs limits the range of textures which can be achieved. In order to be able to modulate the texture of the extrudates, and thus expand their range of applications, several food companies have recently switched to the use of high moisture extrusion, which allows them to produce in-house the starting material for the development of various types of meat analogues (Riaz 2011). This process has been widely investigated with plant sources like gluten, soy, pea, and combinations thereof. However, there is still a lack of knowledge in relation to high moisture extrusion of novel plant protein ingredients such as those derived from hemp.

In order to fill this knowledge gap, the current project aims at investigating the influence of ingredient quality on the rheological properties and extrudability of commercially available hemp proteins.

2. Literature review

2.1. Hemp, hempseed composition and nutritional properties

Hemp (*Cannabis sativa L.*) is an annual herbaceous plant that belongs to the Cannabaceae family. Traditionally, it has been cultivated as a source of fibers used in the textile industry, and oil in the medical and cosmetic fields. Unfortunately, the presence of the psychoactive compound Δ^9-tetrahydrocannabinol (THC) has been limited its application in the food sector for a long time (Aluko 2017). Over the last decades, new species with a low THC level (<0.3%), commonly named industrial hemp, have been developed and their cultivation has been legalized by many countries. The production of hemp is still increasing, thanks also to the good adaptation of the plant to a variety of climates and soil types, and nowadays, the largest producing countries are China, Australia, Canada and USA, while in Europe are mainly France, Romania and Hungary (Shen et al. 2021). The most common cultivated variety is *Finola* and a proper cultivation can lead to 1-2.2 t of seeds per hectare (Aluko 2017).

From a botanical point of view, hempseeds are fruits, with a round shape (3-5 mm in diameter) and a dark brownish color. Each seed has a thin pericarp that surrounds the endosperm and the two cotyledons (**Figure *1***). Hempseeds composition can vary depending on varieties and farming practices. Generally, they contain about 25-30% oil, 25-30% protein, 30-40% fiber, 6-7% moisture, vitamins and minerals (Leonard et al. 2020b).

Figure 1. Whole hempseed and hempseed cut vertically.

Most of the fibers are present on the outer shell of the seed, therefore during dehulling their concentration is reduced, and this also facilitates oil extraction. Hempseed oil, obtained mainly through pressing or solvent extraction, is rich in polyunsaturated fatty acids (PUFAs), e.g., linoleic and α-linolenic acid. From a nutritional perspective, the intake of PUFAs has been related to the reduction of cardiovascular diseases (CVDs), cancer, hypertension, inflammation, and autoimmune diseases (Abedi and Sahari 2014).

The by-product remaining from oil extraction usually called “press-cake” if coming from the mechanical pressing, or “meal” if from solvent-based defatting process, is an enriched-protein ingredient containing around 40-70% proteins (S. A. Malomo, He, and Aluko 2014; Q. Wang and Xiong 2019; Arrutia et al. 2020).

Hempseed proteins have gained attention especially for their high digestibility (~95%) and desirable amino acid composition. The PDCAAS of these proteins, estimated around 0.6, is similar to those of pulses (e.g., chickpea, pea; ~0.7) and considerably higher than those of cereals (wheat, rice, maize, barley; <0.5), but lower than those of proteins derived from animal sources (e.g., beef, casein, egg white; ~0.9-1.0) as well as other oilseeds (e.g., lupin, canola, soy; ~0.9-1.0) (House, Neufeld, and Leson 2010; Day 2013). Therefore, hemp proteins could be combined with other plant protein sources in order to develop food products with a nutritional value similar to that of animal-based ones.

On the negative side, hempseeds often contain antinutritional compounds such as trypsin inhibitors and phytic acid (Leonard et al. 2020b). The latter was largely investigated due to its conflicting effects. While it is able to chelate cations (e.g., minerals), thus reducing their absorption, it exhibits antimicrobial activity as well as antioxidant capacity protecting human cells from oxidative damage (R. Wang and Guo 2021).

Another interesting aspect is the bioactivity of peptides which are produced by enzymatic digestion of hempseed proteins. These peptides are able to block disease-related enzymes, leading to several beneficial effects on human health, e.g., ACE inhibition, antioxidant activities, antihypertensive and hypocholesterolemia effects (Q. Wang and Xiong 2019).

2.2 Protein composition

Hempseed proteins are mainly accounted for by two major storage proteins, i.e., globulins (60-80%) and albumins (20-40%). The former, also called edestin, comprises two main fractions which are 11S and 7S proteins. Crystallographic techniques revealed that 11S fraction has a hexametric structure made up of six identical subunits (Patel, Cudney, and McPherson 1994) that are held together through non-covalent interactions, while each monomer is composed by an acid subunit of ~35 kDa and a basic one of ~20 kDa linked covalently (Q. Wang and Xiong 2019). On the other hand, the 7S is a trimer composed of polypeptide chains with a molecular weight of around 50-70 kDa and held together by non-covalent interactions. The two fractions have been isolated by X.-S. Wang et al. (2008) by using pH adjustments taking advantage of their different isoelectric point, i.e., 6.4 for 11S and 4.6 for 7S, and differences in terms of amino acid composition have been highlighted, namely a higher concentration of essential amino acids and sulfur-containing amino acids was found in the 11S.

The minor fraction of hempseed proteins is represented by the water-soluble albumins, which are characterized by a sedimentation coefficient of 2S. They have a flexible structure consisting of two polypeptide chains with a low molecular weight (<15 kDa) covalently linked (Q. Wang and Xiong 2019).

2.3 Extraction processes

Over the last 20 years, since the cultivation of industrial hemp have been legalized by many countries, the willingness to use hempseed proteins in the food sector increased considerably, especially due to their nutritional and health benefits, as well as the development of methods aimed at extracting, purifying and improve the functionality of these proteins (Leonard et al. 2020b).

Protein extraction from hempseeds can be achieved by removing the oil fraction and by conventional alkaline extraction (Shen et al. 2021). The de-oiling process allows to obtain an enriched-protein ingredient with a protein concentration that can vary from about 40% to 70% (S. A. Malomo, He, and Aluko 2014; Q. Wang and Xiong 2019) and the two most common industrially employed methods are mechanical pressing or solvent extraction (Arrutia et al. 2020). The latter usually makes use of hexane as solvent due to its excellent solubilizing ability that allows for a good oil recovery, and its low boiling temperature (~65°C) that facilitates its separation (through distillation) from the oil fraction. Nevertheless, the use of solvents such as hexane has led to issues such as toxicity and environmental issues (Kumar et al. 2017). In this regard, the removal of oil through mechanical pressing is often preferred, even though the

recovery yield may be lower. This method is simply based on a solid-liquid phase separation and depending on whether or not a heat treatment is employed, it is called hot or cold press extraction, respectively (Çakaloğlu, Özyurt, and Ötleş 2018).

Alkaline extraction remains the most widespread method to obtain relatively pure plant protein ingredients (>70% protein). It consists of an alkaline solubilization of proteins (pH around 8.0-12.0), followed by a separation of the remaining insoluble fraction. Afterwards, the adjustment of pH of the soluble fraction to the isoelectric point of proteins (around 4.5-5.0 for hempseed) allows for their precipitation and collection. This is sometimes followed by a neutralization step to improve the solubility and thus the overall functionality of the protein. (Momen, Alavi, and Aider 2021).

The conditions used during the extraction processes (e.g., pH, heat treatment) have a significant influence on the physicochemical and functional properties of the protein, which is why ingredients derived from different suppliers often display significant differences in their performance in the final application (Q. Wang and Xiong 2019; Hadnađev et al. 2018; Schutyser et al. 2015).

2.4 Functional properties

The use of protein as a food ingredient in liquid, semi-solid or solid formulations depends on its functional properties, which determine its behavior and performance during preparation, processing, storage, and consumption. These properties are influenced by the nature and extent of interactions among proteins and with other components present in the food system (Q. Wang and Xiong 2019).

For the development of meat analogues, the most relevant functional properties are briefly introduced below.

Solubility. Protein solubility is the thermodynamic manifestation of the equilibrium between protein-protein and protein-solvent interactions under a given set of conditions (Hettiarachchy and Ziegler 1994). It is considered the first essential functional property because it gives useful information about the potential application of proteins and their functionality. For example, while a high solubility is typically required for the development of liquid products, for the solid ones this could be even detrimental, e.g., it might hinder proper texturization during high moisture extrusion.

Protein solubility is influenced not only by intrinsic factors (e.g., amino acid sequence and composition, molecular weight) but also extrinsic ones (e.g., ionic strength, pH, type of solvent, temperature, processing conditions) (Zayas 1997). Generally, plant proteins are characterized by

poor solubility in water as they are derived from solid matrices and thus are not designed by nature to be soluble in aqueous environments (Amagliani et al. 2017). Regarding hempseed proteins, many authors report a typical U-shaped solubility curve with a minimum at pH 4-5, corresponding to their isoelectric point. By shifting the pH towards more acid or more basic conditions, the net charge becomes more positive (protonated groups) or more negative (deprotonated groups), respectively, and therefore solubility increases because of the higher degree of electrostatic repulsion between protein molecules (Tang et al. 2006; X.-S. Wang et al. 2008; Q. Wang and Xiong 2019). However, Sunday A. Malomo and Aluko (2015b) demonstrated that protein solubility is strongly affected by the extraction process, showing across all pH range investigated (3.0-9.0) high values (>75%) for hemp protein concentrate obtained via membrane ultrafiltration, while low values (<20%) for that one produced via mechanical pressing.

Looking more in detail at the two main hempseed protein fractions, namely globulins and albumins, Sunday A. Malomo and Aluko (2015a) found that, in the pH range 3.0-9.0, the latter had a solubility ranging from 50% to 90% with a minimum at pH 3, whereas the former displayed lower values (~5-50%) with a minimum at pH 5.

Water absorption capacity. For the formulation of processed foods, the water absorption capacity (WAC) of proteins is another important property because it provides information on their ability to interact with water molecules (Haque, Timilsena, and Adhikari 2016). High WAC is usually required in various food systems because it contributes to provide optimal mouthfeel and texture (Amagliani et al. 2017; Sunday A. Malomo and Aluko 2015b). For extrusion, and thus for meat analogues production, the WAC of gluten is typically used as reference as this is the most widely employed protein ingredient for this purpose due to its peculiar functional properties.

Hempseed press-cake and alkali extracted protein isolate have been reported to have a WAC of 7.0 and 8.7 mL/g respectively, suggesting that alkali extraction led to conformational changes and exposure of additional hydrophilic groups (Teh et al. 2013). However, Sunday A. Malomo and Aluko (2015b) found much higher WAC values, e.g., 12.3, 13.2, 12.0, and 12.1 g/g, respectively, for a hempseed protein meal, a protein concentrate obtained by membrane ultrafiltration, a protein isolate obtained by isoelectric precipitation and a commercial hempseed protein concentrate. By contrast, very low WAC (1.59 g/g) was measured for the hemp protein isolate obtained *via* alkaline extraction/isoelectric precipitation by Hadnađev et al. (2018). Discrepancies in the water absorption capacity of the ingredients may be due to differences in the extraction methods and processing conditions (pH, ionic strength, heat treatment) used to obtain the protein ingredients (S. A. Malomo, He, and Aluko 2014). Therefore, by adjusting these

parameters, it is possible to obtain hempseed protein ingredients with desirable water absorption capacities for meat analogues production.

Oil absorption capacity. Oil absorption capacity, that depends on the ability of hydrophobic groups of amino acids to interact with lipids, is another important property required to improve mouthfeel and flavor of the final food product (Khan et al. 2011). In fact, oil is often added to improve the sensory attributes of plant-based meat products (e.g., juiciness, tenderness, flavor) (Beniwal et al. 2021). In the study of Sunday A. Malomo and Aluko (2015b) hempseed protein isolate produced through alkaline extraction (pH 8) followed by isoelectric precipitation was reported to have higher oil absorption capacity (13.7 g/g) compared to a hempseed protein meal (12.5 g/g), probably due to the higher concentration of proteins that enhance the interaction with lipids rather than non-proteinaceous material. However, comparing the results obtained by Sunday A. Malomo and Aluko (2015b) and Hadnađev et al. (2018), the ingredient whose alkaline extraction was performed at a lower pH displayed a higher OAC (13.7 g/g), while that one extracted at higher pH showed a lower value (1.79 g/g), suggesting the former as functional ingredient in high-fat foods.

Emulsifying property. Emulsifiers play an important role in the physicochemical properties of processed food by affecting their viscosity, texture, and mouthfeel. The amphiphilic nature of food proteins allows them to act as natural emulsifier by absorbing at the interface of water/oil emulsions and stabilizing dispersions (W. Kim, Wang, and Selomulya 2020). Emulsifying activity, emulsifying capacity, emulsion stability and oil droplet size distribution are parameters commonly used to evaluate the emulsifying properties of proteins (Q. Wang and Xiong 2019). In the study of Sunday A. Malomo and Aluko (2015b), a hempseed protein meal and a protein isolate obtained by isoelectric precipitation were found to promote the formation of emulsions (50% canola oil, 10, 25 and 50 mg/ml protein concentration, pH 3.0, 5.0, 7.0 and 9.0) with oil droplet size <1 μm, and can thus be considered excellent emulsifiers, whereas higher values (6-15 μm) were reported for the hemp protein concentrate obtained by membrane ultrafiltration and the commercial one.

Gelling capacity. Protein gelation is a complex phenomenon that involves denaturation and cross-linking. Protein gelation can be influenced by factors like heat treatment, pH, presence of solvents and salts, and mechanical forces (i.e., pressure and shear) (Q. Wang and Xiong 2019; Moure et al. 2006). Therefore, this property has a significant impact on the structure and properties of many food products. The gelation capacity of proteins is traditionally measured using the least gelation concentration method. S. A. Malomo, He, and Aluko (2014) found that the gelation capacity of hempseed protein meal (12%) was significantly lower than that of the

isolate (22%). Comparable values were reported also for lupin (12%) (Lqari et al. 2002) and soybean (16%) (Moure, Domínguez, and Parajó 2005). However, different methodologies can be employed to measure the same functional property, making difficult the comparison among different studies. For example, Dapčević-Hadnađev et al. (2018) investigated the gelation properties through confocal microscopy of two hempseed protein isolates, that is obtained through isoelectric precipitation and micellization, as induced by heat treatment and salt addition. Thermally induced hemp protein isoelectric-precipitated gels were characterized by larger pore size than micellized ones, whereas the latter gels were more susceptible to heat and NaCl.

Rheological properties. Rheological properties are fundamental for the design of flow processes, to predict the texture, and to learn about molecular and conformational changes in food products (Davis 1973). However, the understanding of these aspects becomes far more challenging when macromolecules (like proteins) are subjected to high moisture extrusion conditions (i.e., high moisture, high temperature and high shear) (Emin and Schuchmann 2017). In this study, the rheological properties of protein-based doughs (i.e., protein-enriched ingredients mixed with water) were measured with small amplitude oscillatory tests performed with a high pressure high temperature (HPHT) rheometer. These measurements consist in the application of a sinusoidal oscillating strain (or stress) with a certain frequency applied to the material, and the oscillating stress (or strain) response is measured along with the phase difference between the two sinusoids (i.e., $0°<\delta<90°$ for viscoelastic materials such as food ingredients, as shown in **Figure *2***) (Ronald 1976). Moreover, HPHT rheometers allow to investigate dynamic rheological properties (i.e., complex modulus (G*), storage modulus (G'), loss modulus (G'') and loss factor (tan δ)) under high pressure (~50 bar) and high temperature (up to 180°C) conditions, being therefore ideal to predict the behaviour of protein ingredients during high moisture extrusion.

To better understand the meaning of these parameters, it is possible to imagine a food material like a sponge drenched in water (**Figure *3***A). The system is composed by two elements, namely the sponge and the water, and thus both contribute to the overall resistance to deformation denoting the complex modulus, G*. However, the sponge is an elastic solid, and it contributes to the resistance to deformation of the elastic fraction, called storage (elastic) modulus (G'). On the other hand, the water is a viscous/inelastic fluid, and therefore it represents the contribution of the viscous fraction to the overall resistance to deformation, namely loss (viscous) modulus (G''). Hence, depending on G' and G'', the complex modulus as well as the phase angle δ (**Figure *3***B) can vary (Dogan and Kokini 2006).

The tangent of the phase angle (tan δ) is equal to G''/G' and it gives information about the structure of the material. When a food material is subjected to a thermal treatment, the maximum

point of tan δ is often used to identify the glass transition temperature (T_g), that is the temperature at which the material undergoes a phase transition from a glassy (solid) to a rubbery state (Cocero and Kokini 1991). Every protein displays a different T_g at which changes in the molecular structure occur, involving protein denaturation as well as formation of new covalent and non-covalent interactions (Pietsch et al. 2019). However, when low molecular weight compounds such as water are added, they act as plasticizers by lowering the T_g of protein ingredients (Dogan and Kokini 2006). In this project, rheological tests were performed at a water concentration (50% moisture) similar to the one used for high moisture extrusion in order to investigate the behavior of protein-enriched ingredients (i.e., two commercial hemp proteins individually and in combination with soy and pea protein concentrates) and to predict the temperature range to achieve the desired texture.

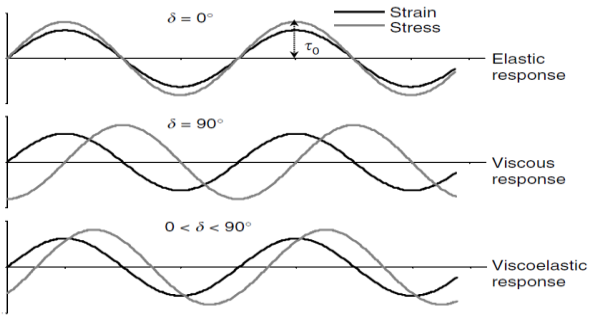

Figure 2. Small amplitude oscillatory tests measurements.

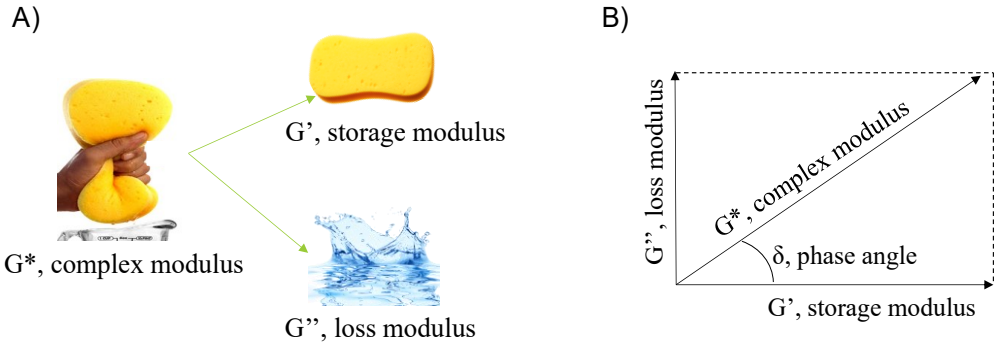

Figure 3. Interpretation of G*, G' and G'' through the example of the sponge (A) and through vectors (B).

2.5 High moisture extrusion

The increasing number of consumers willing to reduce or eliminate meat from their diet has led to the development of various meat analogues (Beniwal et al. 2021). By definition, meat analogues are products that mimic the appearance, flavour and fibrous structure of animal meat, starting from plant proteins. In order to obtain high quality products with the desired characteristics, the traditional method used is extrusion, during which plant proteins undergo a series of transformations such as denaturation, alignment and cross-linking, as shown in **Figure *4*** (Boukid 2020). Two types of extrusion processes can be distinguished: low moisture extrusion (LME) and high moisture extrusion (HME) (Zhang et al. 2019). In LME, flours or protein concentrates are processed at a moisture concentration of about 10-30%. The products are texturized vegetable proteins (TVPs) that need to be rehydrated before use, thus providing a spongy and fibrous structure to the final matrix (Dekkers, Boom, and van der Goot 2018). For many years, TVPs have been a commercial success not only for the nutritional benefits, but also for the economic feasibility of their production, and for their easy handling and long shelf-life due to the low water activity (Riaz 2011). Food companies are used to buy TVPs directly from food ingredient suppliers, and then to rehydrate and mix them with other ingredients to obtain the desired product. However, this limits the range of applications because the texture of TVPs cannot be modulated according to food companies' requirements. To overcome this challenge, many food companies have switched to in-house HME, which allows them to produce their own starting material for the development of meat analogues as well as to broaden the range of applications of the resulting extrudates by modulating the processing parameters.

HME is the most promising technology for obtaining anisotropic meat-like structures from plant protein sources (Osen et al. 2014). During this process, powdered proteins (concentrates or isolates) and water (40-60% moisture) are fed to the extruder (**Figure *5***), in which two co-rotating screws push the material through a cylindrical barrel with different heating zones whose temperatures range from about 20°C to 200°C (Leonard et al. 2020a; Beniwal et al. 2021). In the screw section, the thermal and mechanical stresses induce physicochemical changes on the proteinaceous material (e.g., gelatinization, melting, denaturation, flavour formation, etc.), which play a decisive role on the resulting structure and properties of the plasticized material pushed through the die section (Camire, Camire, and Krumhar 1990). For the purpose of meat analogues production, long cooling dies are usually preferred and placed to the end of the extruder, with a temperate set between 50°C and 80°C. The role of the die section is to prevent the expansion of the material, creating a specific structure and giving a certain form to the final product (K. Liu and Hsieh 2008).

Extrusion is a very complex process, and it is very challenging to find correlations between processing parameters and protein structuring mechanisms. It should thus come as no surprise that extruders are defined "black boxes" (Emin and Schuchmann 2017). For this reason, extrusion process and design of new products are currently mostly based on empirical knowledge, and thus linking independent process variables (e.g., screw speed and configuration, die geometry, temperature profile, ingredients, etc.) to final product structure.

Nowadays, most of the products obtained *via* high moisture extrusion are based on protein ingredients like gluten, soy, pea and combination thereof, and thus there is a related in-depth research aimed at investigating the relationship between extrusion processing parameters and fibrous structure formation (Osen et al. 2014; Fang, Zhang, and Wei 2014; Emin et al. 2017), as well as understanding protein-protein interactions (e.g. H-bonds, covalent bonds, hydrophobic interactions) that can affect the texture of the final products (K. Liu and Hsieh 2008; Chiang et al. 2019; Chen, Wei, and Zhang 2011).

However, there is still a lack of knowledge about the functional and rheological properties of novel plant proteins like those derived from hempseeds, their correlation with the behavior of these ingredients during high moisture extrusion as well as the properties of the resulting extrudates (protein-protein interactions, texture, microstructure).

To the best of our knowledge, only Zahari et al. (2020) investigated the rheological properties of hempseed proteins and their potential to replace soy proteins in high moisture meat analogues using a laboratory twin-screw extruder. The authors measured the rheological properties (i.e., viscosity) of blends of soy and hemp protein concentrates (at 0-20-40-60% HPC) using a Rapid Visco Analyzer (RVA). Nevertheless, the water concentration (14%) was much lower than those used during their extrusion tests (65-75%), which makes difficult to identify any possible correlation.

In this study, after HPHT rheology measurements of hempseed proteins (concentrate and isolate) in combination with soy and pea protein concentrates at 50% w/w water, high moisture extrusion trials (~55% moisture) were performed using a bench scale twin-screw extruder, and the texture and protein solubility in different chaotropic agents of the resulting extrudates were analyzed.

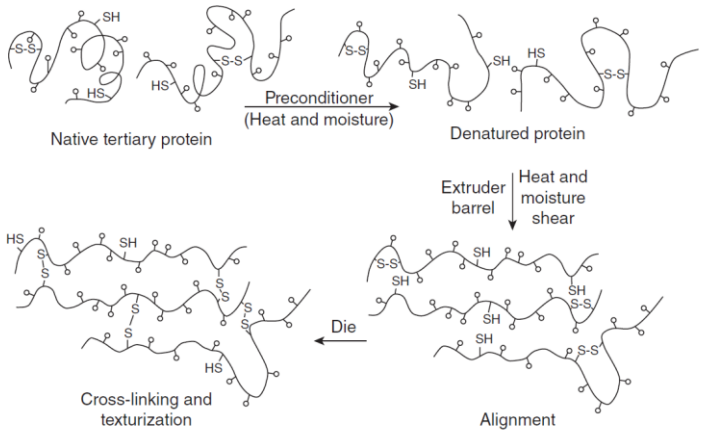

Figure 4. Protein denaturing during extrusion (Riaz 2011).

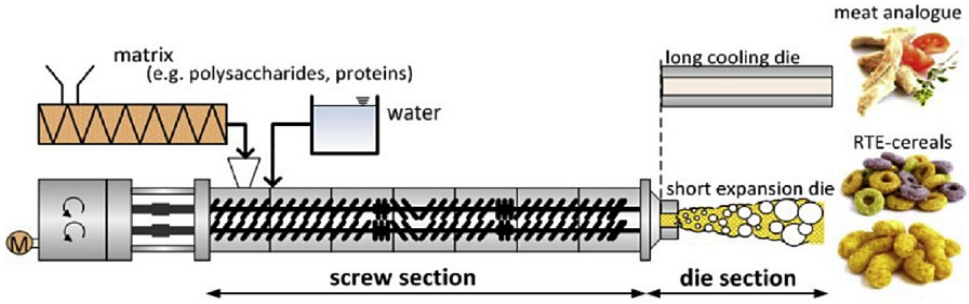

Figure 5. Schematic illustration of a twin-screw extruder (Emin and Schuchmann 2017).

3. Materials and methods

3.1. Materials

The ingredients analyzed included two hemp proteins ingredients, i.e., a hemp protein concentrate (HPC) and a hemp protein isolate (HPI), a soy protein concentrate (SPC) and a pea protein concentrate (PPC). HPC (72.2 wt%), supplied by Vegetein (Estonia), was produced through mechanical hot pressing of hempseeds. HPI (85.9 wt%), provided by EvoHemp (Denver, US), was obtained *via* wet extraction, without the use of any solvent, followed by spray-drying. The process used to obtain HPI is protected by a patent, and thus no further details were provided by the supplier. SPC (65.6 wt%), supplied by Dupont (Solae LLC, Missouri), was obtained through solvent extraction of oil followed by alkaline treatment. PPC (76.8 wt%), supplied by Cargill (France), was obtained through alkaline extraction followed by isoelectric precipitation and neutralization.

The physicochemical and rheological properties as well as the extrudability of the two hemp protein ingredients were analyzed individually and in combination with soy and pea proteins at different ratios.

3.2. Compositional analysis

Total nitrogen of HPC, HPI, SPC and PPC samples was determined using the AOAC 979.09 Official Method ("AOAC, 2005"). Total nitrogen was calculated with the following formula:

$$\mathrm{TN}\left(\frac{\mathrm{g}}{100\mathrm{g}}\right) = \frac{\mathrm{V} * \mathrm{M} * 14 * 100}{\mathrm{m} * 1000}$$

Where V is the volume (ml) of HCl used for titration; M is the molarity of the HCl solution; m is the mass (g) of the sample. A nitrogen-protein conversion factor of 6.25 was used to calculate the total protein concentration of the ingredients.

Macro- and micronutrient compositional analyses were performed by the Nestlé Quality Assurance Centers (NQACs) of York and Nunspeet. The pH was determined at 1, 3.5 and 10 wt% protein using a PHM220 lab pH meter (Radiometer Analytical SAS, France). Quantification of phytic acid was carried out using a Phytic Acid Assay Kit (K-PHYT, Megazyme, Ireland).

3.3. Free thiols analysis

Determination of free thiol groups was performed according to the method of Rade-Kukic, Schmitt, and Rawel (2011) with minor modifications. Briefly, a standard calibration curve was created with L-cys (Sigma-Aldrich) in Tris-SDS-Urea buffer (200 mM Tris aminomethane, 1% SDS, 8M Urea at pH 8) in the concentration range 5-250 µM. Each ingredient (HPC, HPI, SPC and PPC) was dispersed in Milli-Q water at 6 wt% protein. Further dilutions (at 2, 1.5, 1, 0.75 wt% protein) were prepared by adding Tris-SDS-Urea buffer. 50 µl of 10 mM DTNB (5,5' -dithio-bis 2-nitrobenzoic acid) in ethanol was added to 3 ml of each sample and left at room temperature for 20 min. The absorbance of the samples was measured at 412 nm in a Nicolet Evolution 100 spectrophotometer (Thermo Electron Corporation, USA) and the diluted dispersions without DTNB were used as blanks. The quantification of free thiols was determined by comparing the absorbance of the samples to the calibration curve and expressed as nmol/ mg of protein.

3.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The protein profile of HPC, HPI, SPC and PPC was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions.

Each ingredient was dissolved in Tris-SDS-Urea buffer (Tris aminomethane 200 mM, SDS 1%, Urea 6 M at pH 8.5) to obtain dispersions with the same protein concentration (3.08 mg/ml protein). An aliquot of each sample was mixed with Nupage LDS buffer and Nupage reducing agent, containing 500 mM dithiothreitol (DTT, for reducing conditions) or water (for non-reducing conditions). The samples were then vortexed, heated at 70°C for 10 min in an Eppendorf Thermomixer (Eppendorf, Germany) and cooled at room temperature. For each sample, 20 µg proteins were loaded onto Nupage 12% Bis-Tris Gel (ThermoFischer, Waltham, MA). Mark 12 Unstained Standard (ThermoFischer, Waltham, MA), containing proteins with a MW ranging from 6 to 200 kDa, was used as marker. For the electrophoresis, 200 V were applied for about 50 min. The gel was stained with Colloidal Blue Stainer B (Thermo Fischer, Waltham, MA) and washed repeatedly with Milli-Q water to get a clear background. The gels were scanned using an Epson scanner (Epson, US) and saved as a JPEG image.

3.5. Hydrophobicity score by RP-UPLC

For the analysis of the hydrophobicity score, each ingredient (0.6 wt% protein) was dispersed into 4 parts of dissociating buffer (7.5M Guanidine, 6.25mM Trisodium citrate and 23mM DTT) and 1 part of water. After 2 hours of magnetic stirring, the samples were heated at 60°C for 10 min in an Eppendorf Thermomixer (Eppendorf, Germany) and centrifuged at 20°C for 10 min at 16000 g using a Centrifuge 5418 (Eppendorf, Germany). An aliquot of the supernatant was diluted in 3 parts of Milli-Q water. For HPC and HPI ingredients, further conditions were investigated to improve protein solubility and thus the elution quality, including: i) same buffer, pH adjusted to 2.0, 3.0 and 6.0; ii) original pH, buffer containing various DTT concentrations (23 mM, 50 mM and 100 mM), each one tested upon heat treatment at 60 and 100°C. The calibration curve was created by reconstituting a protein standard mix (Waters Corporation, Milford, MA) containing ribonuclease A, cytochrome c, myoglobin, enolase, and phosphorylase b in eluent A (see below).

Reversed phase-ultra performance liquid chromatography (RP-UPLC) analysis was performed using a Acquity System (Waters Corporation, Milford, MA) equipped with a binary solvent manager, a sample manager, a column heater and a photo diode array (PDA) detector. The samples and the standard proteins were injected into a Polyphenyl column (2.1 x 150 mm), 450 Å pores, 2.7 µm particles (Bioresolve, Waters Corporation, Milford, MA). The mobile phase consisted of eluent A, containing 0.1% TFA (Trifluoroacetic acid) in H_2O, and eluent B, containing 0.08% TFA in H_2O: ACN 10: 90 v/v. The samples were eluted with a gradient from 20 to 100% B over 26 min, then with 100% B for other 2 min, and with 20% B for the last 3 min. The flow rate was 0.6 ml/min, the column oven was set at 65°C and the PDA detector at 214 nm.

The results were processed according to the method of Sweet and Eisenberg (1983) based on optimal matching hydrophobicity (OMH). From the chromatogram of each ingredient, all the peaks were integrated and a weighted average of their retention times was calculated. The estimation of hydrophobicity (or OMH) score was performed by comparing the retention time obtained with the external standard calibration curve described above.

3.6. UPLC-MS/MS analysis

Peptide sequence identification was obtained by UPLC-MS/MS analysis on a Thermo Vanquish UPLC system coupled with an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). For each sample, an equivalent amount of 10 and 40 µg of digested hemp proteins was loaded onto the column. Peptides were separated on a Waters Acquity BEH C18 column (1 x 150 mm, 1.7 µm) using a binary solvent system (eluent A: 0.1% v/v formic acid, 2% v/v acetonitrile, in ultrapure LC-MS grade water; eluent B: 0.1% v/v formic acid, 80% v/v acetonitrile, in ultrapure LC-MS grade water). The samples were eluted with a gradient from 2 to 50% B in 45 min at a flow rate of 75 µL/min. Total run time was 60 min. The mass spectrometer operated in a data-dependent Top10 setup to acquire full-scan (MS1) and peptide fragment (MS2) spectra over the entire chromatographic run. The raw data was processed using the PEAKS X+ software (Bioinformatics Solutions Inc., Canada) to identify peptide sequences using a protein database searching strategy. A specific database was generated using "hemp & cannabis" as keywords to select all hemp proteins described in the SwissProt and TrEMBL protein database on www.uniprot.org. Unannotated protein sequences were identified by using the BLAST search tool available on the same website.

3.7. Particle size distribution

The particle size distribution of all samples was determined using a Morphologi 4 equipped with a dry powder dispersion unit (Malvern Instrument, Worcestershire, UK). A 5x magnification was used to detect particles in the range 0.5-50 µm. The parameters reported are the following: circular equivalent (CE) diameter; D[n, 0.1], D[n, 0.5] and D[n, 0.9], which correspond to the particle size below which 10%, 50% and 90%, respectively, of the total number of particles of the sample is detected; D[4,3], volume mean particle diameter; D[3,2], surface mean particle diameter; D[v, 0.1], D[v, 0.5] and D[v, 0.9], which correspond to the particle size below which 10%, 50% and 90% of sample volume is found, respectively. Morphology parameters such as circularity, elongation and convexity are also reported.

3.8. Colour

Colour of the powders was determined by measuring their CIELAB coordinates (L*, a* and b*) with ColorFlex EZ (HunterLab, Virginia). Before conducting the tests, the instrument was calibrated using a white calibration tile. The measurements were conducted in duplicate and the results are expressed as L*, brightness, with values ranging from 0 (black) to 100 (white); a*, degree of redness (positive values) or greenness (negative values); and b*, degree of yellowness (positive values) or blueness (negative values).

3.9. Protein solubility

HPC, HPI, SPC, and PPC samples were dispersed in Milli-Q water (1 wt% protein) and left under stirring for 2 hours, then the pH was adjusted to values in the range 3-10 (at 1 pH unit intervals) with HCl or NaOH 0.1-1 M. Samples were centrifuged at 15000 g for 20 min using a Sorvall evolution centrifuge (Thermo Fischer, Waltham, MA) equipped with a fixed angle rotor SS-34. Protein concentration of the supernatant was determined using the Kjeldahl method, following the same procedure reported above used to determine total protein concentration (see Section 3.2). The solubility was calculated as the protein concentration of each supernatant expressed as percentage of the total protein concentration of the initial dispersion.

3.10. WAC, WSI and OAC

Water absorption capacity (WAC), water solubility index (WSI) and oil absorption capacity (OAC) were determined according to the method of Anderson et al. (1969) with slight modifications. The blends HPC: SPC, HPC: PPC, HPI: SPC and HPI: PPC at the ratios 100:0, 80:20, 50:50, 20:80 and 0:100 were analysed. To measure WAC and WSI, an aliquot (2,5 g) of each combination was dispersed in Milli-Q water, kept under magnetic stirring for 30 min and then centrifuged at 9000 g for 15 min at 25°C using a Sorvall evolution centrifuge (Thermo Fischer) equipped with a SCL 4000 rotor. The supernatant was collected and dried overnight at 100°C and the sediment was weighed. The WAC and WSI were calculated with the following formulae:

$$\text{WAC (g/g)} = \frac{w5-w1}{w2*\left(1-\left(\frac{MC}{100}\right)\right)} \qquad \text{WSI (\%)} = \frac{w6-w3}{w2*\left(1-\left(\frac{MC}{100}\right)\right)}\ \%$$

Where W1 is the weight of the empty centrifuge tube (g); w2 is the weight of the sample (g); w3 is the weight of the stainless-steel container for solid measurements (g); w5 corresponds to w1 + weight of the sediment; w6 corresponds to w3 + weight of the solid (g); MC is the moisture concentration (%).

Oil absorption capacity was measured following the same procedure described to determine WAC, the only difference being the replacement of water with rapeseed oil. The OAC was expressed as g of oil/g of protein using the same formula reported above for WAC.

3.11. HPHT rheology

Dynamic rheological properties of the two hemp protein ingredients blended with soy or pea protein at various ratios, including 100:0, 80:20, 50:50, 20:80 and 0:100, were measured using the oscillatory rheometer MCR502 (Anton Paar, Austria) equipped with a heating-cooling system and a pressure cell. Each ingredient combination was tested at 50 wt% moisture to mimic the conditions used during high moisture extrusion. During the measurements, the temperature was increased from 20°C to 180°C and then decreased back to 20°C at a heating-cooling rate of 2°C/min, the pressure maintained at 50 bar to avoid water evaporation, the frequency set to 1 Hz and the strain to 1%. The Rheocompass software provided storage modulus (G'), loss modulus (G'') and loss factor (tan δ) parameters in real time.

3.12. High moisture extrusion trials

Extrusion trials were carried out using a bench scale twin-screw extruder Eurolab 16 (Thermo Fisher Scientific, Waltham, MA) (**Figure *6***) with a screw diameter of 16 mm and length to diameter ratio of 25:1. The extruder barrel is divided into 5 sections heated separately and the temperature settings were chosen based on the HPHT rheology data obtained for each blend (**Table *2***). A gravimetric and volumetric powder feeder (DDW-MD1-MT-2 from Brabender, Duisburg, Germany) was used to feed protein powders into the first section of the extruder at a rate of 0.7 kg/h. Water was injected into the extruder by a piston-driven pump (series D, Teledyne ISCO, USA) at a constant flow rate to obtain the desired moisture concentrations. The screw configuration consisted of transport elements with different gaps and its speed was set at 100 rpm. A cooling die of 31 x 54 x 210 mm (H x W x L) was installed at the end of the extruder and set at 70°C. The exit of the cooling die was 3.4 x 20 mm (H x W).

A summary of the processing parameters used is reported in **Table *2***.

Table 2. High moisture extrusion processing parameters used for the different ingredient formulations.

Ingredients	Ratios	H_2O (%)	Flow rate (kg/h)	Screw speed (rpm)	Temperature (°C)					
					T1	T2	T3	T4	T5	T long die
HPC:SPC	100:0	54.5	0.84	100	30	80	200	200	200	70
	80:20	54.5	0.84	100	30	80	210	210	210	70
	50:50	54.5	0.84	100	30	80	210	210	210	70
	20:80	54.5	0.84	100	30	80	210	210	210	70
	0:100	56.9	0.92	100	30	80	220	220	220	70
HPC:PPC	100:0	54.5	0.84	100	30	80	200	200	200	70
	80:20	54.5	0.84	100	30	80	210	210	210	70
	50:50	54.5	0.84	100	30	80	210	210	210	70
	20:80	Not processable								
	0:100	Not processable								
HPI:SPC	from 100:0 to 50:50	Not processable								
	20:80	54.5	0.84	100	30	80	240	240	240	70
HPI:PPC	from 100:0 to 0:100	Not processable								

Figure 6. Twin-screw extruder Prism Eurolab 16 (Thermo Fischer).

3.13. Solubility of texturized proteins

The solubility of texturized proteins was measured in different chaotropic agents, including the following: i) phosphate buffer, PB (to extract proteins in their native state), ii) PB + 50 mM DTT (reducing agent), iii) PB + 4M or 8M guanidine (denaturing agent), iv) PB + 4M or 8M guanidine + 50 mM DTT (combination of denaturing and reducing agents). Extraction buffer iv) in the presence of 8M guanidine provided complete protein solubilization and it was thus used as reference to determine protein solubility of the extrudates in the different chaotropic extraction buffers.

Extrudates were then minced with a lab grinder (meat grinder 483CH1, Switzerland) with a grinding size of 8 mm. An aliquot of each minced extrudate (5 wt% protein) was dispersed in the different chaotropic buffers and left under magnetic stirring for 2 hours. The samples were centrifuged at 20°C for 20 min at 20000 g using a Centrifuge 5418 (Eppendorf, Germany). An aliquot of each supernatant was mixed with a dissociating buffer (7.5 M guanidine + 6.25 mM trisodium citrate +23 mM DTT), except the samples solubilized in 8M guanidine and 8M guanidine + DTT that were mixed with the dissociating buffer diluted 4 times in H_2O. The samples were heated at 60°C for 10 min in an Eppendorf Thermomixer (Eppendorf, Germany) and centrifuged at 20°C for 10 min at 16000 g using a Centrifuge 5418 (Eppendorf, Germany), the supernatant was collected, and an aliquot was diluted 3 times with Milli-Q water prior to RP-UPLC analysis.

RP-UPLC was performed applying the same conditions used to measure hydrophobicity (see Section 4.5). From each UPLC chromatogram, all peaks were integrated, and their areas were summed. The results were expressed as percentage of the total area obtained for each chaotropic agent (i.e., PB, DTT, G4M, G8M, G4M + DTT) over the total area obtained in G8M + DTT.

3.14. Texture analysis

Texture of extrudates was measured using a texture analyser (TA.HD Plus, Stable Micro Systems, UK). Extrudates were cut into a rectangular shape (6 x 2 cm) and a knife blade probe (length, 6 cm) was used to cut samples longitudinally (P_L) and transversely (P_T) to the direction of extrudates outflow from the extruder. Ten measurements were performed for each sample for both T and L directions. Results were expressed as average of the peak force (N) required per cm (N/cm). The degree of anisotropy, which is a measure of preferential fibre orientation and fibrous structure formation, was calculated as the ratio of P_T to P_L.

4. Results and discussion

4.1. Macronutrient composition

Macronutrient composition of the four ingredients is reported in **Table *3***. HPI showed a higher protein concentration (85.9 wt%) compared to HPC (72.2 wt%), which is to be expected since wet extraction methods typically allow to obtain ingredients with a relatively high degree of purity, as also demonstrated by other authors (X.-S. Wang et al. 2008; Mamone et al. 2019; S. A. Malomo, He, and Aluko 2014). On the other hand, SPC and PPC displayed protein concentrations of 65.6 wt% and 76.8 wt%, respectively.

Carbohydrates, usually classified as structural (e.g. cellulose, hemicellulose, pectin) and non-structural carbohydrates (e.g., starch, mono-, di- and oligosaccharides), generally account for ~40-50% in raw hempseed and soybean, and ~50-60% in pea (Nikolopoulou et al. 2007; Medic, Atkinson, and Hurburgh 2014; Kotecka-Majchrzak et al. 2020). All the four protein-enriched ingredients displayed very low carbohydrate concentrations (0-0.17 wt%), including starch and sugars, and therefore the extraction methods used led to the removal of these compounds.

By contrast, the four ingredients displayed significant differences in dietary fibre concentration, which includes carbohydrates and lignin that are resistant to digestion and absorption in the human small intestine, but are completely or partially fermented in the large intestine (Committee 2001). HPC had a concentration 8 times higher than HPI (~1 wt%). These results are partially in line with the outcomes of Mamone et al. (2019) who reported much higher values for defatted hemp meal (49%) compared to HPI obtained by alkaline extraction, were no fibre was detected. However, this can also be linked to factors like variety, farming practices, environmental conditions, and different extraction processing conditions. SPC exhibited a considerably higher fibre concentration (~22 wt%) compared to the two hemp proteins, whereas PPC (~6 wt%) had a value closer to HPC. Considering that soybean can have a fibre concentration around 20-30 wt% (depending on extrinsic factors) (Medic, Atkinson, and Hurburgh 2014), it seemed they were not greatly affected by neither alcohol solution nor alkaline treatment. On the other hand, the wet extraction method employed for PPC production seemed to significantly affect fibre concentration, which usually ranges around 15-20 wt% in pea cultivars (Nikolopoulou et al. 2007). It has been demonstrated that a proper intake of dietary fibre is related to various health benefits (e.g., reduction of the risk of cardiovascular diseases, regulation of appetite, improvement of colonic health) (Barber et al. 2020).

Oilseeds have particularly high concentrations of oil (approximately 25-40%), which needs to be removed to facilitate protein extraction. This explains why hemp and soy protein-enriched

ingredients displayed a low concentration of fat between 0.5 and 3.2 wt%, unlike PPC (9.5 wt%) which did not undergo any fat removal step.

Ash represents the total mineral content of an ingredient. HPC contained almost three times more ash compared to HPI (5.2 wt%). While the value obtained for HPI was in line with data reported in the literature (Siano et al. 2018), the ash concentration of HPC (14 wt%) was considerably higher than available data (approximately 4-7 wt%), although previous studies confirm an enrichment in ash as a consequence of oil pressing extraction (Folegatti et al. 2014; Tang et al. 2006). On the other hand, soy and pea displayed values closer to HPI (i.e., 6.5 and 6.2 wt%, respectively) and which are comparable to data reported by other authors (Gao et al. 2020; El-Shemy 2011)

As regards phytic acid, values ranged from 2.39 wt% for SPC to 10 wt% for HPC. The considerably higher concentration of phytic acid in HPC compared to HPI (4.5 wt%) can be related to the extraction process, with oil pressing providing lower purity compared to the wet extraction used to obtain the latter, and thus higher concentration of all the other macro- and micronutrients, including this antinutritional factor (Pojić et al. 2014). When compared to hemp proteins, SPC and PPC exhibited lower values, i.e., 2.39 and 3.23 wt%. Phytic acid is the primary phosphorous reserve in legumes and oilseeds necessary during germination. It is considered an antinutrient compound because it can bind minerals (especially Ca, Mg, Zn and Fe), and it can thus reduce their bioavailability (R. Wang and Guo 2021; Feizollahi et al. 2021).

Among the protein-enriched ingredients investigated, a significant pH difference was detected, being 4.3 for HPI and 7.0 for HPC, SPC and PPC. Although the wet extraction process used to obtain HPI was protected by a patent, it might very likely be that proteins were isoelectric precipitated before being spray dried.

Isoflavones values ranged between 0.04 mg/kg for HPI and 138 mg/kg for SPC. The huge difference in isoflavones concentration found between HPI (0.04 mg/kg) and HPC (1.83 mg/kg) can be related to both the cultivar and the extraction process. This class of phenolic compounds, also defined as phytoestrogens, are not the main ones found in hempseeds, and therefore are less studied. By contrast, the high concentration found in SPC confirms that soybean is the main vegetable source of phytoestrogens (Bustamante-Rangel et al. 2018). Legumes can have considerable concentrations of isoflavones as well, however, PPC displayed a concentration almost 9 times lower than that of HPC. Several studies have already demonstrated the potential health benefits of isoflavones, but the link between their consumption and disease prevention is still controversial (Medic, Atkinson, and Hurburgh 2014).

Analysis of free thiols demonstrated great variability among these protein-enriched ingredients, ranging from 2 to 15.1 nmol/mg protein for HPI and HPC, respectively. The low value displayed by HPI may have been promoted by the extraction process, which may have caused protein denaturation and aggregation. Similar to HPI, SPC displayed free thiols values of 3.8 nmol/mg protein. On the other hand, PPC exhibited free thiols value slightly lower than that of HPC, that is 11.5 nmol/mg protein. This concentration is comparable with the one reported by Gao et al. (2020) for a pea protein isolate (12.2 μmol/g) obtained *via* alkaline extraction.

Table 3. Macronutrient composition (wt%), phytic acid (wt%), pH at 1 wt% protein, free thiols (nmol/mg protein) and isoflavones (mg/kg) concentration of hemp protein concentrate (HPC), hemp protein isolate (HPI), soy protein concentrate (SPC) and pea protein concentrate (PPC).

	HPC	HPI	SPC	PPC
Protein	72.2	85.9	65.6	76.8
Carbohydrates	0.00	0.00	0.17	0.00
Fibre	8.16	0.93	21.9	5.97
Fat	0.53	3.17	0.86	9.54
Ash	14.1	5.17	6.52	6.23
Moisture	4.13	4.76	5.18	4.85
Phytic Acid	10.0	4.44	2.39	3.23
pH	7.04	4.32	7.07	7.07
Isoflavones	1.83	0.04	138	0.20
Free thiols	15.1	2.00	3.80	11.5

4.2. Micronutrient composition

Table *4* shows the mineral composition of the ingredients. These are classified as major (P, Na, K, Ca, Mg, Cl) and trace minerals (Mn, Fe, Cu, Zn), which is based on the recommended daily intake, that is higher or lower than 100 mg/day, respectively.

As regards major minerals, HPC displayed always higher values compared to HPI, especially in P, K and Mg concentration, i.e., 3431, 2623 and 1478 mg/100 g of powder, respectively, against 2028, 151 and 364 mg/100 g of powder, respectively. This discrepancy between the two hemp ingredients may be related to both growing conditions (e.g., fertilization, type of soil, etc), as also reported by other authors (Siano et al. 2018), as well as to the extraction process (i.e., oil pressing), which provided a less pure ingredient than the isolate obtained *via* wet extraction, and thus a higher concentration of minerals. On the other hand, the soy protein ingredient had higher amounts of Na and Ca (96.5 and 435 mg/100 g powder) compared to the hemp proteins, and a K concentration (2100 mg/100 g powder) almost 14 times higher than HPI. These results might be due to the use of alkaline salts such as sodium hydroxide (NaOH) and potassium hydroxide

(KOH) for the alkaline treatment. In PPC, the level of Na (300 mg/100 g powder), Ca (842 mg/100 g powder) and Cl (113 mg/100 g powder) were the highest among the ingredients tested. This might again be related to the extraction process, which consisted of alkaline extraction followed by isoelectric precipitation, and thus to the use of both alkali (NaOH) and acids (HCl).

Among the trace minerals, HPC and HPI had considerably higher amounts of Mn (16.7 and 10.3 mg/100 g powder), Fe (33.3 and 40.8 mg/100 g powder), Cu (3.5 and 1.0 mg/100 g powder) and Zn (20.1 and 25.7 mg/100 g powder) compared to soy and pea protein concentrates.

Table 4. Mineral composition (mg/100 g powder) of hemp protein concentrate (HPC), hemp protein isolate (HPI), soy protein concentrate (SPC) and pea protein concentrate (PPC).

		HPC	HPI	SPC	PPC
Major minerals	P	3431	2028	707	1550
	Na	< 20.0	< 20.0	96.5	300
	K	2623	151	2100	308
	Ca	152	104	435	842
	Mg	1478	364	346	299
	Cl	47.1	14.3	14.8	113
Trace minerals	Mn	16.7	10.3	4.20	5.90
	Fe	33.3	40.8	10.3	18.9
	Cu	3.50	1.00	1.00	0.70
	Zn	20.1	25.7	2.30	10.7

4.3. Amino acid composition

The amino acid profile of the ingredients is presented in **Table *5***. As regards non-essential amino acids, no significant differences were found among the two hemp protein ingredients, however they displayed a higher amount of arginine when compared to SPC and PPC (12.9-13.5 *vs* 7.24-8.30 g/100 g protein) in agreement with the findings of other authors (Hadnađev et al. 2018; Tang et al. 2006).

Furthermore, HPC showed a higher concentration of sulphur-containing amino acids (Methionine + Cysteine) (3.86 g/100 g protein) than all the other ingredients in which it ranged from 2.03 g/100 g protein for PPC to 2.81 g/100 g protein for SPC.

On the other hand, both hemp protein ingredients exhibited a lower amount of total essential amino acids than SPC and PPC (31.4-32.5 *vs* 37.8-39.2 g/100 g protein). Twofold lower concentration of lysine in hemp protein ingredients compared to SPC and PPC highlighted it as the first limiting amino acid, in agreement with the outcomes of House, Neufeld, and Leson (2010).

Therefore, from a nutritional perspective, the combination of hemp proteins with other plant proteins like soy and pea proteins might be a good strategy to achieve a well-balance amino acid composition as well as a higher nutritional value in the derived meat analogues.

Table 5. Non-essential and essential amino acid composition (g/100 g protein) of hemp protein concentrate (HPC), hemp protein isolate (HPI), soy protein concentrate (SPC) and pea protein concentrate (PPC).

		HPC	HPI	SPC	PPC
Non-EAAs	Ala	4.09	4.32	4.42	4.22
	Arg	12.9	13.5	7.24	8.30
	Asp	10.1	11.0	11.5	11.6
	Cys	1.61	1.02	1.37	0.98
	Glu	17.7	17.5	18.5	16.2
	Gly	4.18	4.14	4.29	3.97
	Pro	3.81	3.89	4.87	4.19
	Ser	5.03	5.17	5.19	5.21
	Tyr	3.55	3.76	3.68	3.95
EAAs	His	2.69	2.65	2.61	2.36
	Ile	3.43	3.99	4.64	4.73
	Leu	6.26	6.62	7.58	8.27
	Lys	3.45	3.08	6.36	7.37
	Met	2.26	1.58	1.43	1.06
	Phe	4.29	4.73	4.97	5.51
	Thr	3.30	3.47	4.01	3.87
	Trp	1.36	1.37	1.36	0.98
	Val	4.35	4.97	4.85	5.00
Tot EAAs		31.4	32.5	37.8	39.2

4.4. Protein profile

Electrophoretic profiles of the four protein ingredients both under reducing and non-reducing conditions are shown in **Figure *7***. Hemp, soy and pea proteins consist mainly of globulins (11S and 7S) and albumins in different ratios.

Under non-reducing conditions, the two hemp protein ingredients (HPI, lane 1, and HPC, lane 2) displayed completely different protein profiles, suggesting that the extraction process had a huge impact on their physical state. Regarding the electrophoretic profile of HPC, it seemed that the mechanical pressing did not greatly affect the physical state of proteins, which were probably mostly kept in their native state. In fact, in agreement with previous studies (Sunday A. Malomo and Aluko 2015b; Tang et al. 2006; Patel, Cudney, and McPherson 1994), HPC displayed an intense band of about 55 kDa corresponding to the 11S edestin monomer, its acid and basic

subunits (~33 and 22 kDa, respectively), and the 7S monomer of about 48 kDa. The albumin fraction appeared as a low MW polypeptide of about 10 kDa.

On the other hand, the same bands were completely absent (under non-reducing conditions) or faint (under reducing conditions) in HPI. Moreover, the electrophoretic profile of HPI did not show any band corresponding to albumin fraction, probably removed by the wet extraction method. However, other low molecular weight (MW) proteins (<18 kDa) appeared which were not affected by the treatment with reducing agents, indicating the lack of covalent interactions. Furthermore, the presence of unresolved protein material (>200 kDa) in the gel loading wells implied that the extraction process used to obtain HPI affected the physical state of the protein by creating large macromolecular complexes which neither denaturing nor reducing agents could break down.

The electrophoretic profile of SPC (lane 3) under reducing and non-reducing conditions displayed the same proteins, even though more bands (ranging from about 8 to 80 kDa) appeared in the presence of the reducing agent DTT. The bands corresponding to the acid and basic subunits of 11S glycinin appeared at around 36 and 21 kDa, respectively. The α', α and β monomers of 7S β-conglycinin were detected approximately at 80, 66 and 48 kDa. These findings are in line with those reported by Medic, Atkinson, and Hurburgh (2014) and Nishinari et al. (2014).

A better protein profile of PPC (lane 4) was obtained under reducing condition, even though high MW aggregates (>200 kDa) did not enter the gel, suggesting that the extraction process might have promoted protein denaturation and aggregation. The majority of the polypeptides had a MW ranging from about 12 to 80 kDa, belonging to legumin (11S), vicilin and convicilin (7S). The acid and basic subunits of 11S showed bands at approximately 40 and 22 kDa, respectively. Different bands corresponding to the heterogeneous vicilin fraction were detected at around 66, 48, 36, 28, 25 and 12 kDa, whereas convicilin displayed a MW of ~80 kDa.

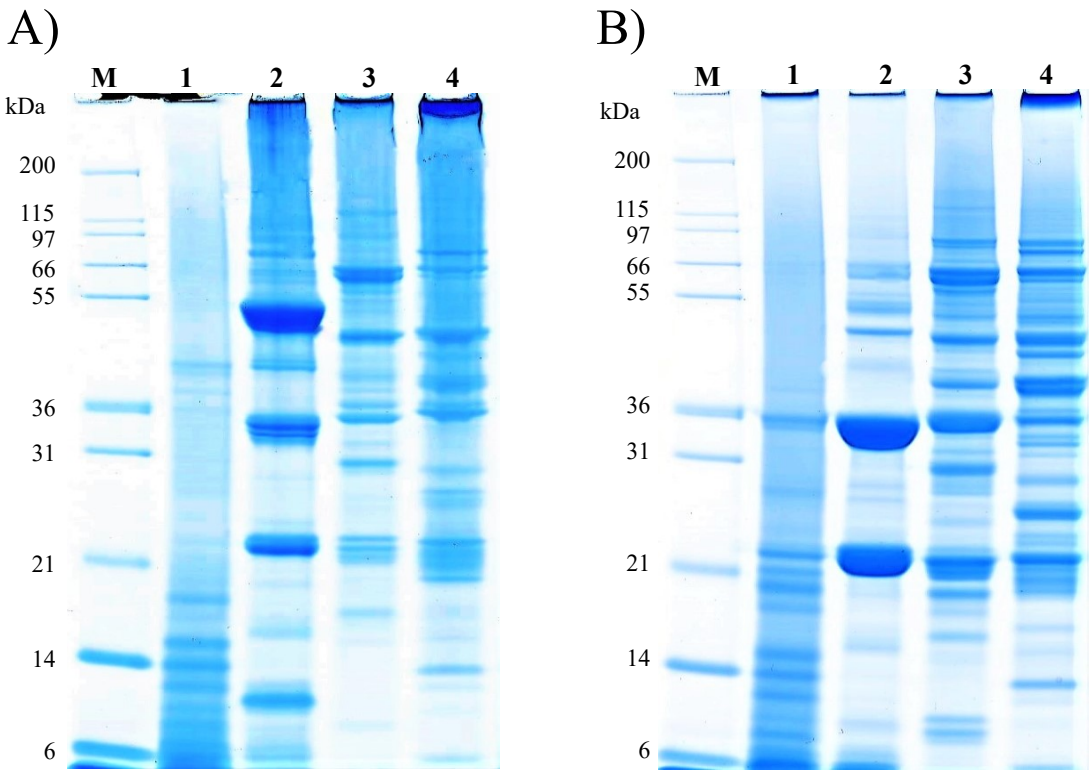

Figure 7. SDS-PAGE profiles under non-reducing (A) and reducing (B) conditions. M: molecular weight marker;1: hemp protein isolate (HPI); 2: hemp protein concentrate (HPC); 3: soy protein concentrate (SPC); 4: pea protein concentrate (PPC).

4.5. Hydrophobicity score by RP-UPLC

The overall hydrophobicity of protein ingredients was measured by dissolving powders with denaturing and reducing agents (urea and DTT) prior to UPLC analysis, and their profiles are presented in **Figure *8***. According to the OMH method, HPC displayed a hydrophobicity score of 0.43, against ~0.51 of pea and soy. In fact, the UPLC chromatograms show that HPC had the lowest retention time (i.e., lower hydrophobicity) compared to the other ingredients, with all the peaks appearing within 8 min. By contrast, it was not possible to estimate the hydrophobicity score for HPI, since no peaks appeared during UPLC analysis, which is somehow comparable with the results obtained by SDS-PAGE (section 4.4).

As regards soy and pea, peaks were detected within 13 min, these ingredients being indeed slightly more hydrophobic compared to HPC. The presence of poorly resolved peaks suggested that the extraction process may have promoted protein aggregation.

Different sample preparation conditions for HPC and HPI were also tested to check if a higher concentration of proteins could be solubilized, and chromatographic profiles are presented in

Figure *9*. The influence of temperature (60 and 100°C) and concentration of reducing agent (23, 50 and 100 mM DDT) were investigated.

Figure *9*A shows the influence of temperature (i.e., 60°C and 100°C) on HPC. It was evident that at 100°C, peak resolution decreased and two protein families (green circles) were lost, suggesting that denaturation, aggregation and precipitation phenomena occurred. As regards HPI, the influence of temperature (**Figure *9***B at 60°C; **Figure *9***C at 100°C) as well as that of reducing agent concentration (red, 23 mM; green, 50 mM (hidden); blue, 100 mM) were investigated. Nevertheless, no significant variation in the quality of the chromatograms was detected with any of these sample preparation conditions. These findings strengthen the hypothesis of the presence of large macromolecular complexes difficult to be dissolved even with relatively high concentration of dissociating and denaturing agents.

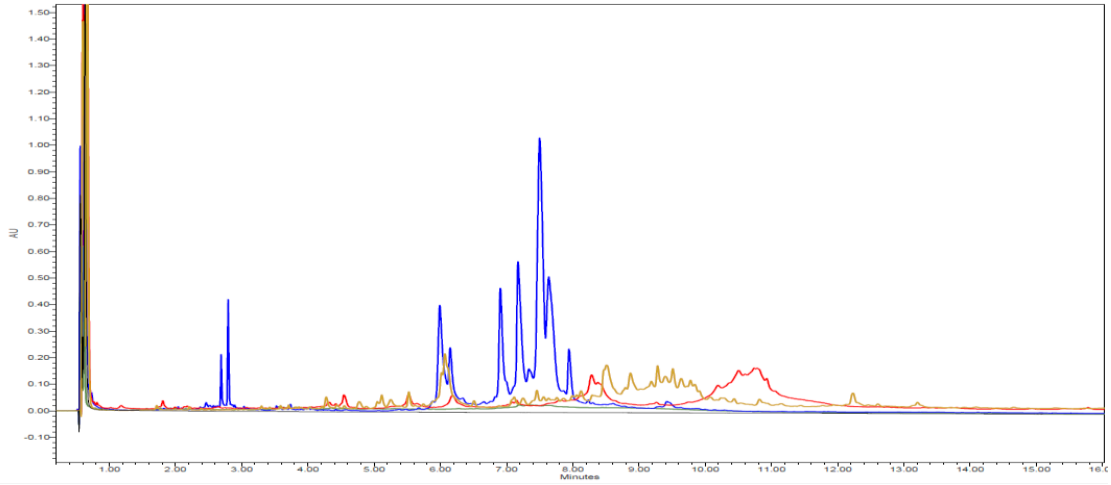

Figure 8. RP-UPLC chromatograms. A) hemp protein concentrate (HPC, blue), hemp protein isolate (HPI, green), soy protein concentrate (SPC, red) and pea protein concentrate (PPC, yellow).

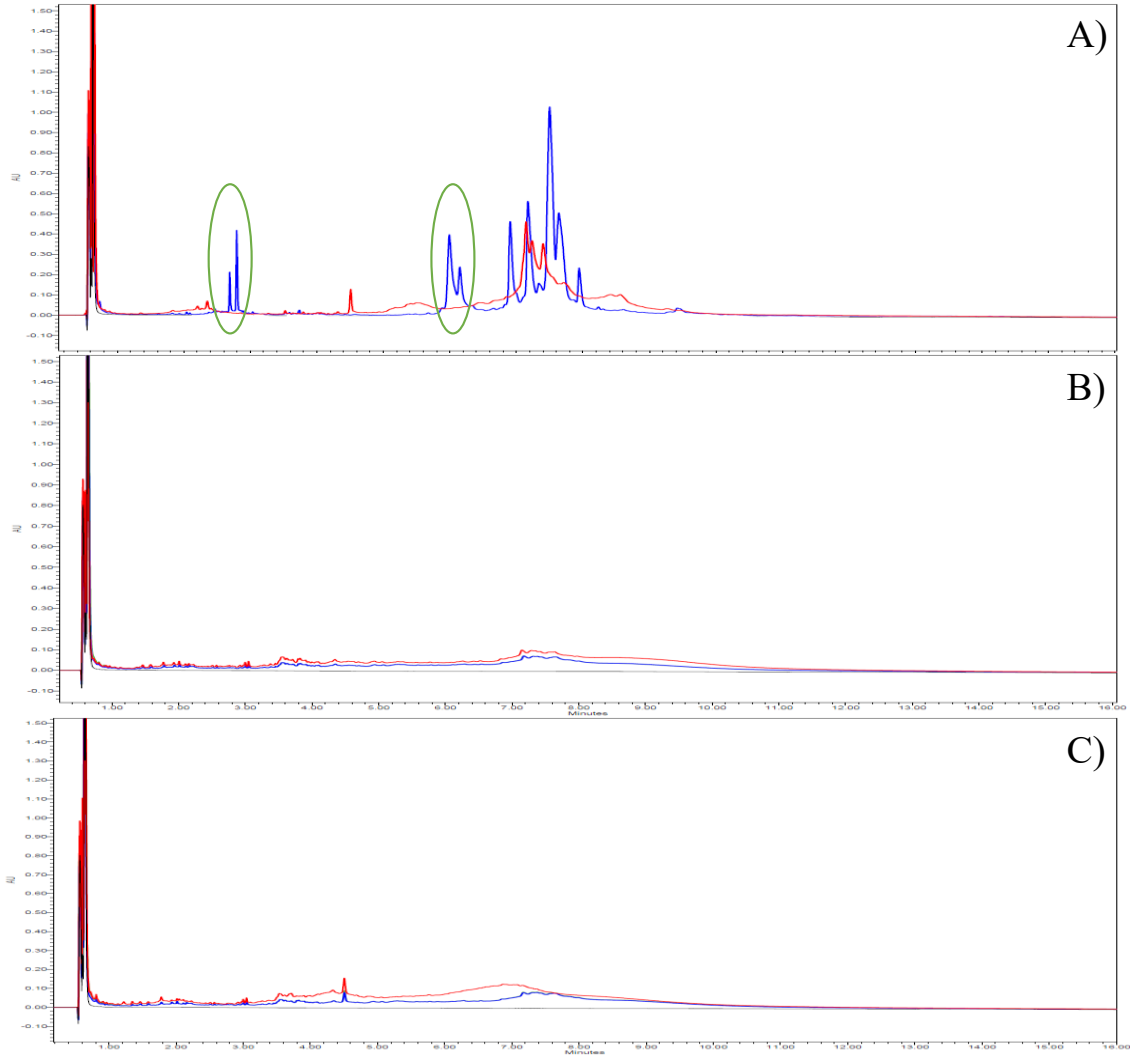

Figure 9. A) hemp protein concentrate (HPC) in dissociating buffer (7.5 M Guanidine, 6.25 mM Trisodium citrate and 23 mM DTT) incubated at 60°C (blue) and 100°C (red); B) hemp protein isolate (HPI) incubated at 60°C in dissociating buffer in the presence of 23 mM DTT (red) and 100 mM DTT (blue); C) hemp protein isolate (HPI) incubated at 100°C in dissociating buffer in the presence of 23 mM DTT (red) and 100 mM DTT (blue).

4.6. UPLC-MS/MS analysis

The protein composition of both hemp ingredients was investigated more in depth through trypsin digestion followed by UPLC-MS/MS analysis.

Trypsin is a proteolytic enzyme, also produced by the human pancreas, able to hydrolyse bonds in proteins and peptides involving the carboxyl group of lysine or arginine (Rick 1974). Therefore, the following UPLC-MS/MS analysis allowed to investigate the presence of protein modifications that could explain the differences between the electrophoretic and chromatographic profiles of HPC and HPI described above (Sections 4.4 and 4.5).

In **Figure *10***, the top 10 most abundant proteins found in HPC and HPI are reported. The analysis revealed that both HPC and HPI can be digested by trypsin, however the protein profiles highlighted some differences. Globulins resulted the main storage protein in both ingredients, in agreement with available literature (Aluko 2017; Q. Wang and Xiong 2019; Mamone et al. 2019). Nevertheless, HPI displayed a concentration of peptides belonging to the globulin fraction more than two-fold higher compared to HPC (~400 peptides).

On the other hand, the amount of peptide sequences identified as albumins was low, i.e., 8 for HPI and 14 for HPC. When compared to HPI, HPC showed a higher concentration of other peptides belonging to different proteins (e.g., caleosin, oleosin) and enzymes (e.g., short chain dehydrogenase/reductase (SDR), glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) (data only partially reported). These results suggested that the wet extraction process employed to obtain HPI removed most of the water-soluble proteins including albumins, whereas the mechanical pressing used for HPC allowed to retain the vast majority of proteins.

From the analysis of peptide sequences, only few protein modifications such as oxidations and deamidations were detected (data not shown) in both hemp protein ingredients. Summarizing the results of UPLC-MS/MS analysis together with those obtained by SDS-PAGE (Section 4.4 and 4.5), it seems that the macromolecular complexes of HPI were composed mainly by globulins which were so tightly packed that could not be solubilized by neither denaturing nor reducing agents, but they could be easily hydrolysed *via* enzymatic treatment.

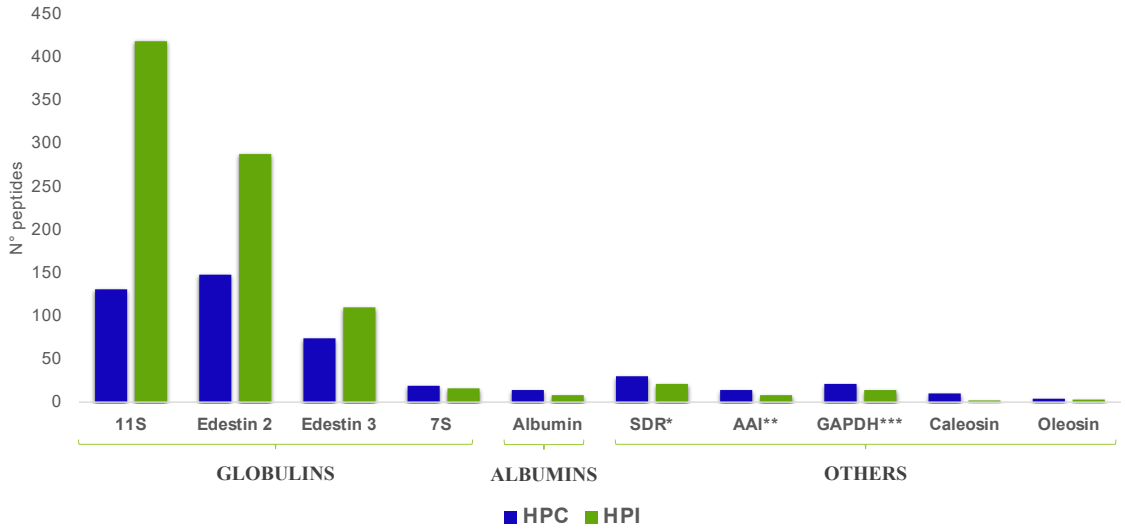

Figure 10. Protein composition of hemp protein concentrate (HPC) and isolate (HPI) after trypsin digestion and UPLC-MS/MS analysis.

4.7. Particle size distribution

Physical properties of powders such as particle size and shape affect their flow properties and are thus of paramount importance during extrusion, particularly if the powder is to be fed into the extruder through a powder feeder (i.e. not mixed with water beforehand) (Amagliani et al. 2016). As particle size decreases, the relative surface area increases and, consequently, there are more contact and frictional forces between particles that reduce the flowability of powders. However, the particle shape influences the surface contacts between particles, as well (E.H.-J. Kim, Chen, and Pearce 2005).

The particle size distribution parameters of the powders are presented in **Table *6***. Volume-weighted mean particle diameter (D[4,3]) values of HPC and HPI were 65.3 and 76.9 μm, respectively. The corresponding value for SPC (70.0 μm) was in between the values obtained hemp proteins, whereas PPC value (28.8 μm) was more than twofold lower compared to the other protein ingredients analyzed. Surface-weighted mean particle diameter (D[3,2]) and median particle diameter (D[v, 0.5]) followed the same trend as D[4,3], being considerably higher for hemp and soy protein ingredients compared to PPC.

When compared to the volume-based parameters (the contribution of each particle in the distribution related to the volume of the particle, i.e., the relative contribution will be proportional to $(size)^3$), no major differences in terms of number-based parameters (each particle is given equal weighting irrespective of its size) were observed between the different samples. This indicates the presence of small amount of relatively large particles in hemp and soy protein ingredients, as can be inferred from the considerably higher D[4,3] values (most sensitive to the presence of large particulates in the size distribution) compared to the D[3,2] ones (most sensitive to the presence of fine particulates, as well as their high Dv(90) values (132-196 μm).

Particle shape parameters did not reveal any significant difference among all the investigated powders, with circularity, elongation and convexity being in the range 0.93-0.96, 0.17-0.21, and 0.99-1, respectively.

Table 6. Particle size distribution and particle shape parameters of hemp protein concentrate (HPC), hemp protein isolate (HPI), soy protein concentrate (SPC) and pea protein concentrate (PPC).

	D[4,3]	D[3,2]	Dv (10)	Dv (50)	Dv (90)	CE	Dn (10)	Dn (50)	Dn (90)	Circularity Mean	Elongation Mean	Convexity Mean
HPC	65.3	23.2	7.83	56.7	132	6.08	3.56	5.15	8.78	0.96	0.19	1.00
HPI	76.9	33.9	16.4	59.6	186	6.59	3.62	5.00	9.76	0.96	0.17	1.00
SPC	70.0	27.3	11.7	46.4	196	6.69	3.34	5.16	10.9	0.93	0.20	0.99
PPC	28.8	17.4	8.06	25.1	54.7	6.99	3.59	5.55	11.5	0.93	0.21	0.99

4.8. Colour

Colour values of the powders are presented in **Table *7***. HPC powder had a higher L* value (80.9 *vs* 70.2) and a lower redness (a*, 0.66 *vs* 3.81) and yellowness (b*, 9.80 *vs* 15.3) compared to HPI. The L* and a* values displayed by HPI were the highest among the ingredients analysed. The degree of redness found in HPI (15.3) was much higher than the values reported by Hadnađev et al. (2018), who investigated differences in the colour of hemp protein isolates obtained through different isolation techniques (i.e., a* values of 8.6 and 10.5 for hemp protein isolate produced *via* micellization and isoelectric precipitation, respectively).

SPC and PPC showed L*, a* and b* values in the range 87.3-83.0, 0.38-2.38 and 12.3-19.1, respectively. The low degree of yellowness found in SPC and HPC could be due to the use of alcohol solutions during processing that might have led to the removal of yellow pigments such as carotenoids.

The overall appearance of a product is important for driving consumer choices and creating expectations prior to consumption. In order to develop meat analogues, the choice of certain ingredients that have colour parameters similar to those of meat products is important, even though it should be highlighted that colour might be significantly affected by the extrusion process (Fiorentini, Kinchla, and Nolden 2020).

Table 7. Colour space values of hemp protein concentrate (HPC), hemp protein isolate (HPI), soy protein concentrate (SPC) and pea protein concentrate (PPC).

	L*	a*	b*
HPC	80.9 ± 0.21	0.66 ± 0.01	9.80 ± 0.29
HPI	70.2 ± 0.26	3.81 ± 0.03	15.3 ± 0.08
SPC	87.4 ± 0.04	0.38 ± 0.01	12.3 ± 0.07
PPC	83.0 ± 0.22	2.38 ± 0.01	19.1 ± 0.06

4.9. Protein solubility

Protein solubility (PS) of HPC, HPI, SPC and PPC were investigated in the pH range 3-10 (**Figure *11***). The hemp protein ingredients showed poor solubility across all pH values. HPC displayed PS ranging from around 5 to 18%, with an increase only above pH 9, which might be due to the heat treatment (~80°C) used during mechanical oil pressing. However, similar results were reported by S. A. Malomo, He, and Aluko (2014) even though their ingredient (i.e., hemp protein meal, 37 wt% protein) was obtained *via* cold mechanical oil pressing, thus suggesting that hemp proteins have an inherent poor solubility.

The PS of HPI at the various pH values was always <10%, confirming the hypothesis that the extraction process might have promoted extensive protein-protein interactions leading to the formation of macromolecular complexes difficult to break down by means of both an increase in the degree of electrostatic repulsion between proteins (i.e., pH adjustment to values far away from the isoelectric point) and the use of denaturing and reducing agents, as it was shown by SDS-PAGE and RP-UPLC analysis (Section 4.4 and 4.5, respectively). Even though the exact process used to produce HPI was not disclosed by the supplier, similar PS values were reported by S. A. Malomo, He, and Aluko (2014) for a hemp protein isolate obtained *via* isoelectric precipitation.

Unlike the hemp protein ingredients, SPC and PPC displayed a typical U-shaped PS curve with a minimum at pH 4-5, corresponding to their isoelectric point, and a maximum (~35% for SPC and ~17% for PPC) at pH 10. The same behaviour was described by other authors (Tang et al. 2006; Burger and Zhang 2019; F. Wang et al. 2020), although PS values (especially at alkaline pH) were much higher compared to those reported in this study. The differences in PS may be attributed to differences in the raw material as well as in the processing conditions used (e.g., alkaline treatment, extraction temperature, spray-drying, etc.) (W. Wang, Nema, and Teagarden 2010). It should be pointed out here that a high PS is not required to obtain a fibrous structure during high moisture extrusion, and it could indeed be detrimental, suggesting that all the four protein ingredients could be suitable for that purpose.

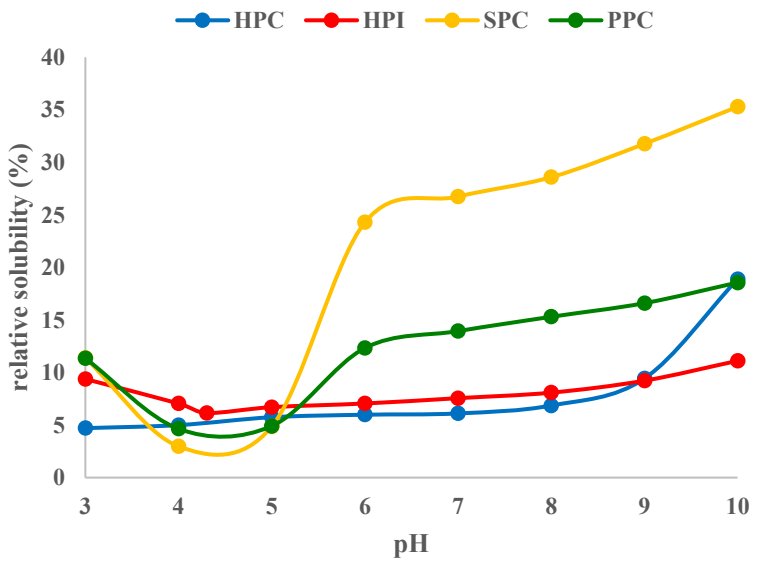

Figure 11. Solubility of hemp protein concentrate (HPC), hemp protein isolate (HPI), soy protein concentrate (SPC) and pea protein concentrate (PPC), dispersions (1 wt% protein) as a function of pH.

4.10. Water absorption capacity and water solubility index

Water absorption capacity (WAC) and water solubility index (WSI) were measured in order to determine the hydration capacity of the protein powders as this influences their behaviour during high moisture extrusion. Polar groups of amino acid side chains are able to interact with water molecules by forming H bonds, which affects both the texture and flavour of foods (F. Liu et al. 2013). WAC of the blends HPC:SPC, HPC:PPC, HPI:SPC and HPI:PPC are shown in **Figure *12***A. Hemp protein ingredients displayed lower WAC values (2.5 and 3.4 g/g, respectively for HPC and HPI) compared to SPC (4.7 g/g) and PPC (5.1 g/g). This is in agreement with the findings of Teh et al. (2013) who reported that ingredients subjected to alkaline treatments (in this case SPC and PPC) showed higher values of WAC. As expected, WAC decreased by replacing larger portions of soy and pea ingredients with hemp. The decrease of WAC was quite linear with the blends HPC:SPC, HPC:PPC and HPI:SPC, whereas the replacement of 20% PPC with HPI caused a steep decrease of WAC (3.5 g/g), i.e., the blend displayed a hemp-like behaviour even at low concentrations of HPI.

In **Figure *12***B, the values of WAC and WSI are plotted against each other. For the sake of simplicity, the blends were not included, and gluten was added for comparison. Gluten is often used as a reference as it is typically the plant protein source with the best performance in terms of fibrous structure formation upon extrusion (Riaz 2011; Beniwal et al. 2021). Interestingly, both HPC and HPI displayed WSI (~10%) and WAC values comparable to gluten, which suggests that the hemp protein ingredients could potentially represent promising substrates for high moisture extrusion.

As regards SPC and PPC, the former showed the highest WSI (19%), which was almost two times higher than that of gluten and hemp, whereas PPC displayed a WSI close to the latter (7%) but a considerably higher WAC.

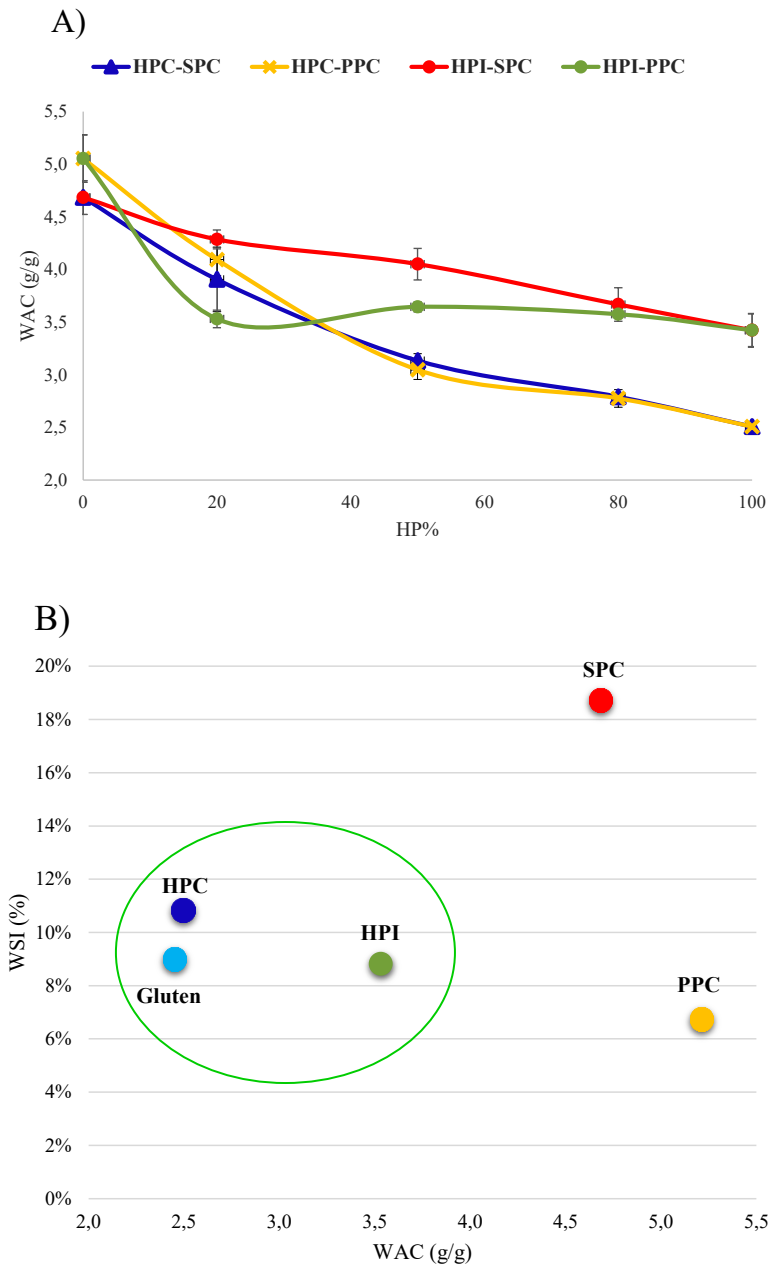

Figure 12. A) water absorption capacity of hemp protein concentrate (HPC) and isolate (HPI) in combination with soy protein concentrate (SPC) and pea protein concentrate (PPC); B) water absorption capacity plotted against water solubility index of HPC, HPI, SPC, PPC and gluten (reported as reference).

4.11. Oil absorption capacity

Oil absorption capacity (OAC) is another important parameter to consider in food production. Generally, ingredients with high OAC are better suited for high-fat preparations (e.g., cake,

mayonnaise, sausages) (F. Liu et al. 2013). Even though oils are not the main ingredients in meat analogues, they are typically added to improve flavour, juiciness and tenderness (Bohrer 2019).

In **Figure *13***, OAC of the blends of HPC and HPI with SPC and PPC are reported. HPC and HPI displayed OAC values of 2.3 and 2.9 g/g, respectively. These results are significantly lower than those reported by S. A. Malomo, He, and Aluko (2014) for hempseed protein meal and isolate, i.e., 12.5 and 13.7 g/g, respectively.

Since SPC and PPC showed OAC values similar to those of hemp proteins, also the combinations HPC:SPC, HPC:PPC, HPI:SPC and HPI:PPC exhibited values in a narrow range (2.3-2.9 g/g).

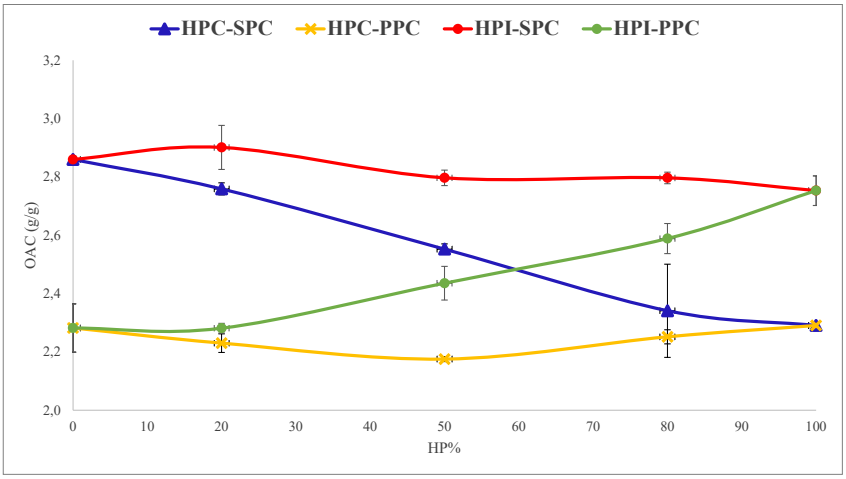

Figure 13. Oil absorption capacity of hemp protein concentrate (HPC) and isolate (HPI) in combination with soy protein concentrate (SPC) and pea protein concentrate (PPC).

4.12. Pasting properties

The pasting properties of the blends HPC:SPC, HPC:PPC, HPI:SPC and HPI:PPC at different ratios (100:0, 80:20, 50:50, 20:80 and 0:100) were determined at 50 wt% moisture and the results are presented in **Figure *14***. The same water concentration was also used to perform HPHT rheology analysis on the same blends, and it was chosen because it is close to that used for high moisture extrusion.

Hemp proteins displayed different pasting properties, namely HPC was a crumbly dough while HPI displayed a wet sand-like behaviour. SPC and PPC were also sand-like, but they appeared drier compared to HPI. The blends of HPC and HPI with soy and pea at different ratios were all sand-like, with an increasing degree of dryness as the concentration of hemp decreased. These results can be explained by the differences observed in terms of WAC. In fact, the higher the WAC the more sand-like and the drier the dough was.

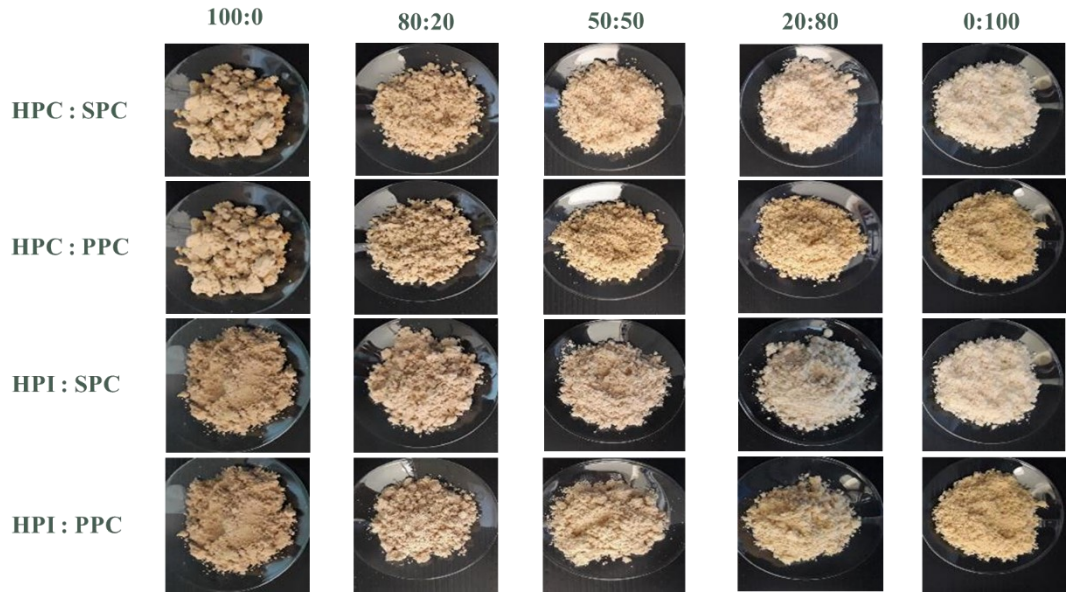

Figure 14. Pasting properties of hemp protein concentrate (HPC) and hemp protein isolate (HPI) mixed with soy protein concentrate (SPC) and pea protein concentrate (PPC) at different ratios at 50 wt% moisture.

4.13. Rheological properties

The rheological properties of hemp, soy and pea protein ingredients and combinations thereof were analyzed at 50 wt% moisture using a HPHT rheometer, which allowed to measure the dynamic rheological properties of the protein ingredients under extrusion-like conditions (i.e., high temperature and high moisture).

As regards storage modulus (***Figure 15***A), all the protein ingredients displayed a decrease in G' with increasing temperature, even though at different rates. HPC and HPI had a more pronounced softening (i.e., softening of the material upon heat treatment) around 110-120°C, and SPC at above 130°C, while PPC showed an almost linear decrease in G' as temperature increased. A decrease in the storage modulus indicates that covalent bonds and non-covalent interactions are breaking, and the material is becoming softer. Conversely, an increase in G', corresponding to fibrous structure formation, was observed for all the ingredients above 150°C.

In ***Figure 15***B, the variation of tan δ of the four ingredients analyzed alone as a function of temperature is reported. Tan δ is equal to G''/G' and provides information about the structure of the material. The closer to 0 the tan δ the higher the solid-like behavior of the material and vice versa. In this regard, the highest initial tan δ of HPC was in line with its crumbly dough structure (see Section 4.12).

The increase of tan δ upon heat treatment suggested a change in the material structure ascribed to protein denaturation and formation of new covalent bonds and non-covalent interactions.

Furthermore, the maximum tan δ corresponded to the temperature at which the material undergoes a phase transition from a glassy to a rubbery state, usually referred to as glass transition temperature (T_g). Knowledge of T_g is useful to understand the optimal heating protocol to be used during high moisture extrusion in order to get a final product with the desired texture and fibrous structure. In this regard, the glass transition temperatures resulting from all the blends of HPC and HPI in combination with SPC and PPC are presented in ***Figure 16***. The four ingredients analysed alone displayed glass transition temperatures ranging from 150°C for HPC to 162°C for HPI. Therefore, the difference of T_g among the ingredients cannot be ascribed to their protein concentration (e.g., HPC had 72 wt% protein and T_g lower thn that of SPC (66 wt% protein)), but rather to their protein profile and/or protein state (i.e., extent of denaturation/aggregation). Consequently, it is reasonable to assume that the higher temperature necessary for the phase transition of HPI might be related to the presence of globulin macromolecular complexes, which required more energy in order to be broken down.

Interestingly, when the hemp protein ingredients were mixed with soy or pea, which showed similar T_g (157°C), they exhibited completely different behaviors. In fact, the replacement of SPC or PPC with HPC did not affect T_g regardless of the concentration of the latter (except when HPC was tested individually). Conversely, a significant increase in T_g was observed upon replacement of only 20% SPC or PPC with HPI, the different blends showing a hemp protein-like behavior across all the ratios investigated.

From an industrial perspective, these findings suggested that for the processing of HPC in combination with SPC or PPC it would be possible to maintain the same settings employed to perform high moisture extrusion of soy and pea protein powders individually, whereas higher temperatures would need to be applied to process the blends HPI:SPC and HPI:PPC.

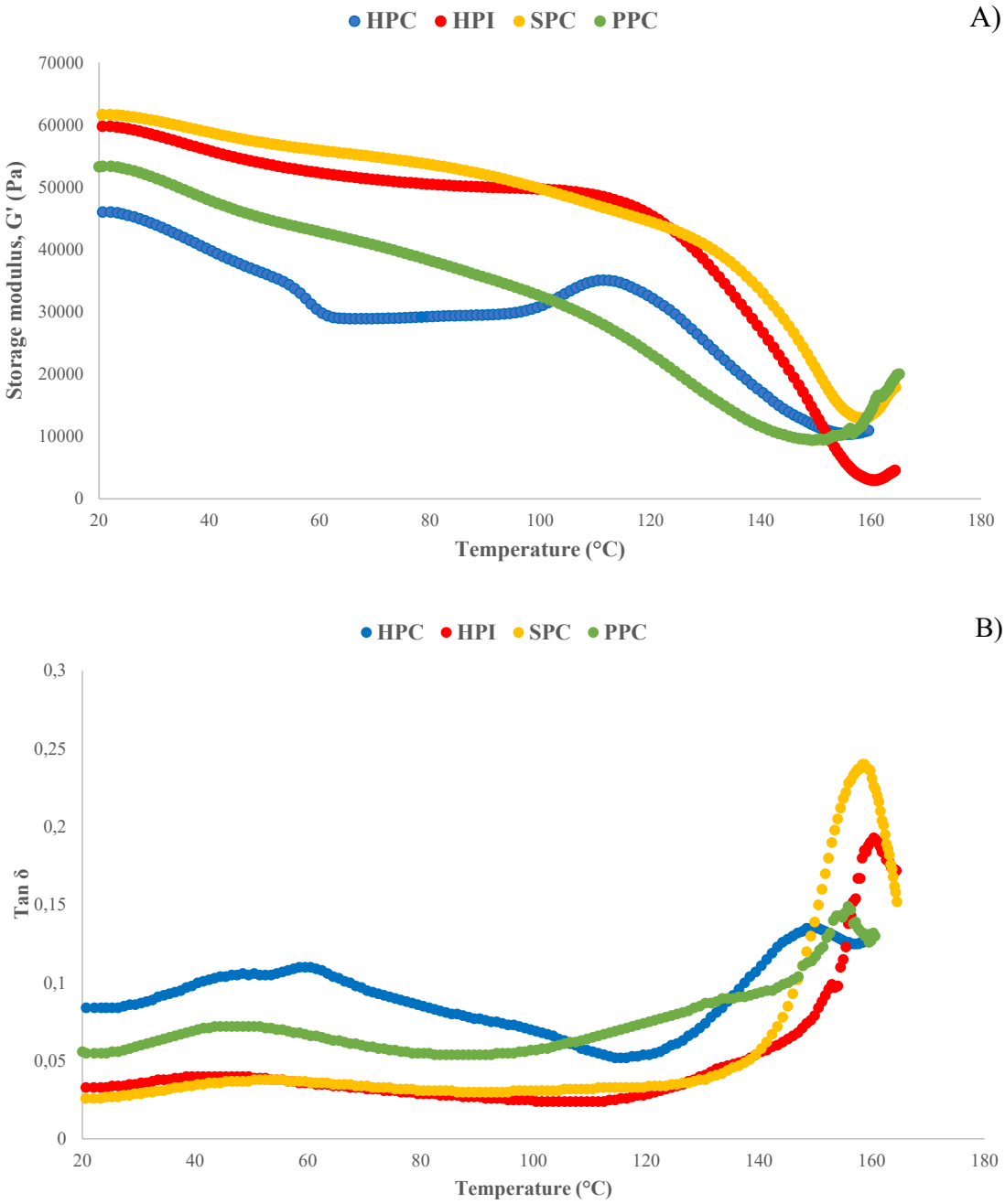

Figure 15. G' (A) and Tan δ (B) of hemp protein concentrate (HPC), hemp protein isolate (HPI), soy protein concentrate (SPC) and pea protein concentrate (PPC) doughs at 50 wt% moisture as a function of temperature.

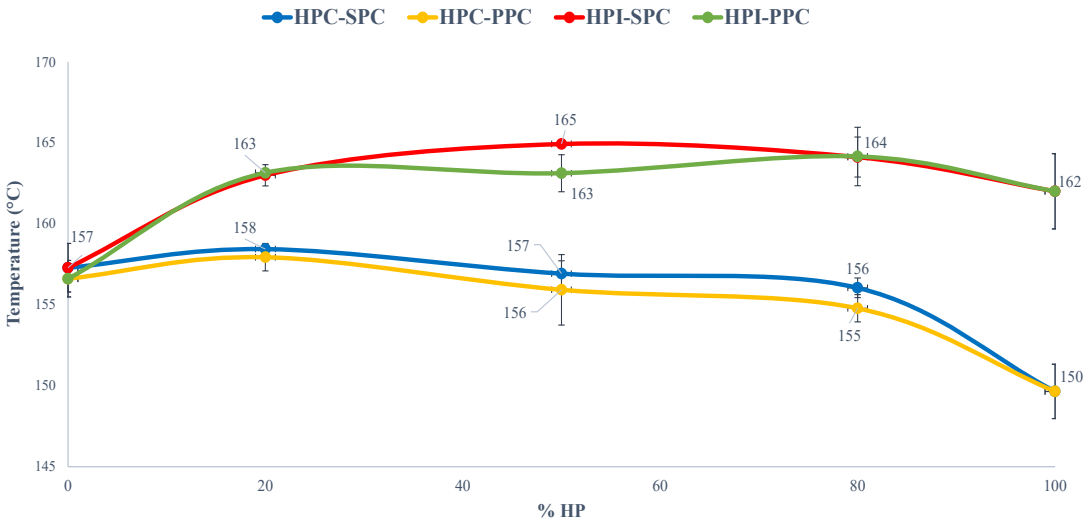

Figure 16. Glass transition temperatures of hemp protein concentrate (HPC) and isolate (HPI) in combination with soy protein concentrate (SPC) and pea protein concentrate (PPC) at different relative ratios.

4.14. High moisture extrusion

Texturization of plant proteins through thermomechanical processing, specifically high moisture extrusion (HME), creates extrudates with a well-defined fibrous structure that closely resemble that one of the real meat, and thus they can be ground and mixed with other ingredients (e.g., fats, flavors) for the development of vegan burgers.

HME was applied to texturize HPC and HPI ingredients alone and in combination with SPC and PPC. However, not all the mixtures were processable with the bench scale twin-screw extruder used in this study, as also shown in **Table *2***.

A good fibrous texture was obtained from HPC:SPC blends at all the tested ratios (100:0, 80:20, 50:50, 20:80 and 0:100). During processing, the temperature of the main barrel sections ranged from up to 200°C for HPC:SPC (100:0) to 220°°C for HPC:SPC (0:100). The significant difference between these temperatures and the T_g obtained by HPHT rheology (***Figure 16***) was attributable to the fact that the extruder was relatively short, and thus higher temperatures were necessary to achieve the same heat load and, in turn, the glass transition of the material during extrusion.

For the blends HPC:PPC, fibrous texture was reached only up to a ratio of 50:50. On the other hand, at relative concentrations of PPC, the powder showed a tendency to stick to the walls of the powder feeder, therefore not allowing for a constant flow rate. Presumably, the high fat concentration (9.54 wt%) together with the relatively small particle size (D[4,3]= 28.8 µm)

negatively affected the flowability of PPC, both being important factors affecting the flow properties of powders (Amagliani et al. 2016; E.H.-J. Kim, Chen, and Pearce 2005).

As regards HPI in combination with SPC, only the mixture 20:80 was processable, although a very high temperature had to be achieved (240°C). However, the extruded product did not display the desired fibrous structure, and it was fragile and brittle. Furthermore, none of the mixtures of HPI with PPC were processable. The issues in relation to the processing of HPI and its combinations with SPC or PPC could be attributed to the fact that, with the equipment used in this study, it was not possible to provide the energy/ heat load necessary to achieve the glass transition, and thus to obtain the desired fibrous structure. This is in line with HPHT rheology data, which showed that, when compared to HPC, much higher glass transition temperatures were obtained for HPI and its mixtures with SPC or PPC.

4.15. Solubility of texturized proteins into chaotropic agents

In order to investigate the relative contribution of non-covalent interactions and covalent bonds in fibrous structure formation, texturized proteins obtained from high moisture extrusion (see Section 4.14) were dissolved in different chaotropic agents (i.e., phosphate buffer (PB), DTT, guanidine 4M and 8M, guanidine 4M + DTT and guanidine 8M + DTT), and their solubility was measured.

The protein solubility of HPC:SPC mixtures is shown in ***Figure 17***A. As regards the solubility in phosphate buffer (PB), which extracts proteins in their native state, values were <5% for all the different ratios, indicating that the vast majority of proteins were involved in the fibrous structure formation of extruded products.

Proteins also showed a high resistance to DTT treatment (reducing agent), with solubility values in the range 5-10%. However, this finding should not mislead into thinking that covalent bonds played a minor role in fibrous structure formation, for the reasons explained below.

When compared to DTT, guanidine 4M (G4M) solubilised a higher amount of proteins (30-34%). Furthermore, while increasing the concentration of guanidine to 8M (G8M) did not affect the solubility of the HPC:SPC mixtures up to a ratio of 80:20, at higher relative concentrations of SPC a marked increase was observed (up to 55%).

The combination of denaturing and reducing agents (G4M-8M + DTT) provided almost complete protein solubilization at all HPC:SPC ratios tested. Therefore, by comparing the protein solubility obtained with the buffers G4M and G4M + DTT (or G8M and G8M + DTT), it could be inferred that the importance of covalent bonds in the fibrous structure formation increased with

an increase in the relative concentration of HPC, and this was also positively correlated with cysteine concentration (1.6 *vs* 1.37 g/100g protein).

As regards HPC:PPC mixtures at the ratios 100:0, 80:20 and 50:50, their protein solubility in different chaotropic buffers is shown in **Figure *17***B. Similarly to combinations of HPC:SPC at the equivalent ratios, HPC:PPC displayed a high resistance to PB and DTT buffers. When G4M and G8M were used, protein solubility increased with an increase in the relative concentration of PPC, from about 30 to 37% and from 33 to 50%, respectively. Moreover, the presence of both denaturing and reducing agents led to an almost complete protein solubilization (>90%) of HPC:PPC blends, and the contribution of covalent bonds in fibrous structure formation increased as the relative concentration of HPC increased, this being again correlated with a higher cysteine concentration in HPC compared to PPC (1.61 vs 0.98 g/100g protein).

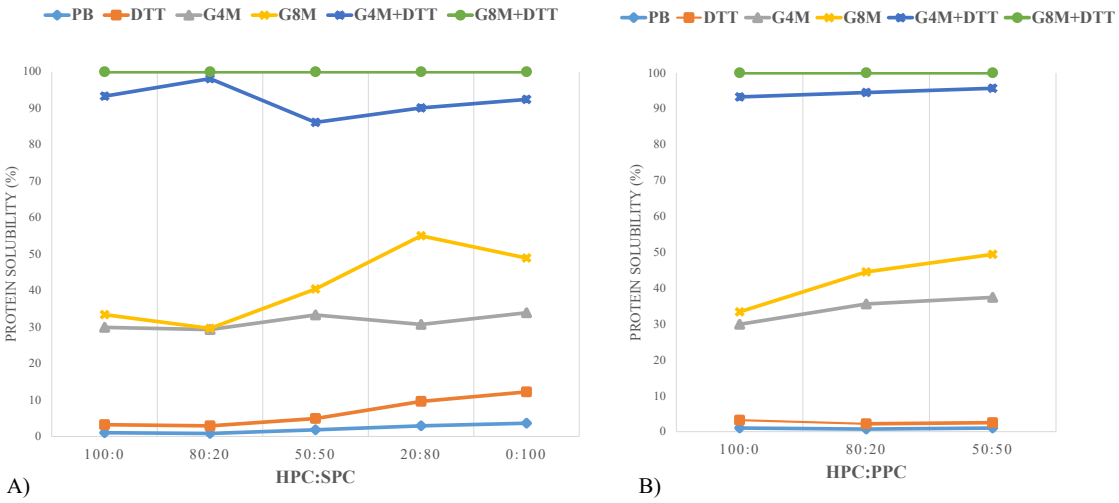

Figure 17. Protein solubility in different chaotropic agents: phosphate buffer (PB), dithiothreitol (DTT), 4M guanidine (G4M), 8M guanidine (G8M), 4M guanidine + dithiothreitol (G4M+DTT) and 8M guanidine +dithiothreitol (G8M+DTT). A) extrudates obtained by mixtures of hemp and soy protein concentrate (HPC:SPC); B) extrudates obtained by mixtures of hemp and pea protein concentrate (HPC:PPC).

4.16. Texture analysis

The textural properties of the extrudates obtained *via* HME have a major influence on those of the derived meat analogues, and therefore on the consumer acceptance of the latter.

The transversal and longitudinal peak force values of extrudates obtained from HME of HPC:SPC mixtures are shown in ***Figure 18***A. The transversal peak force (P_T) was higher than the longitudinal (P_L) one up to a ratio 20:80, suggesting that the preferential fibre orientation was longitudinal to the direction of extrusion flow, while the opposite was observed for SPC processed alone, with the fibres of the latter being preferentially oriented transversely to the direction of

extrusion flow. Moreover, both the transversal and longitudinal peak forces increased with an increase in the relative concentration of HPC, with the only exception of SPC processed alone.

Fibres oriented longitudinally to the direction of extrusion flow (i.e., higher (P_T)) and increasing peak forces with an increase in the relative concentration of HPC were observed also for extrudates obtained from HPC:PPC mixtures (***Figure 18***B).

The outcomes obtained for both the mixtures of HPC with SPC and PPC seemed to be correlated with those of protein solubility in different chaotropic agents (Section 4.15), and thus the peak force increased as the importance of covalent bonds increased.

Interestingly, a statistical analysis of the data obtained within the context of Project MATRIX revealed a positive correlation between the texture of soy protein extrudates as measured analytically and the sensory properties of the derived burgers, with transversal (P_T) and longitudinal (P_L) peak forces being positively correlated to firmness and chewiness. These findings will have to be confirmed for burgers based on HPC and its combinations with SPC and PPC.

The degree of anisotropy (P_T/P_L) of the different combinations of HPC and SPC was then calculated (***Figure 18***C), as it is a measure of the degree of fibre formation in meat analogues, the presence of structural anisotropy being a prerequisite if they are to have muscle-meat like textural characteristics.

All the combinations of HPC with SPC displayed $P_T>P_L$, and therefore degree of anisotropy values higher than 1 (i.e., from 1.1 to 1.4), while SPC processed alone had a degree of anisotropy lower than 1, thus indicating the transversal orientation of the fibres to the direction of extrusion flow. Moreover, the degree of anisotropy increased with an increase in the relative concentration of HPC. These results were positively correlated with the importance of covalent bonds in fibrous structure formation.

HPC:PPC blends (100:0 to 50:50) (***Figure 18***D) showed degree of anisotropy values higher than 1, suggesting the longitudinal orientation of the fibres to the direction of extrusion flow. The values were within a narrow range (i.e., 1.2-1.4) and no specific trend was identified.

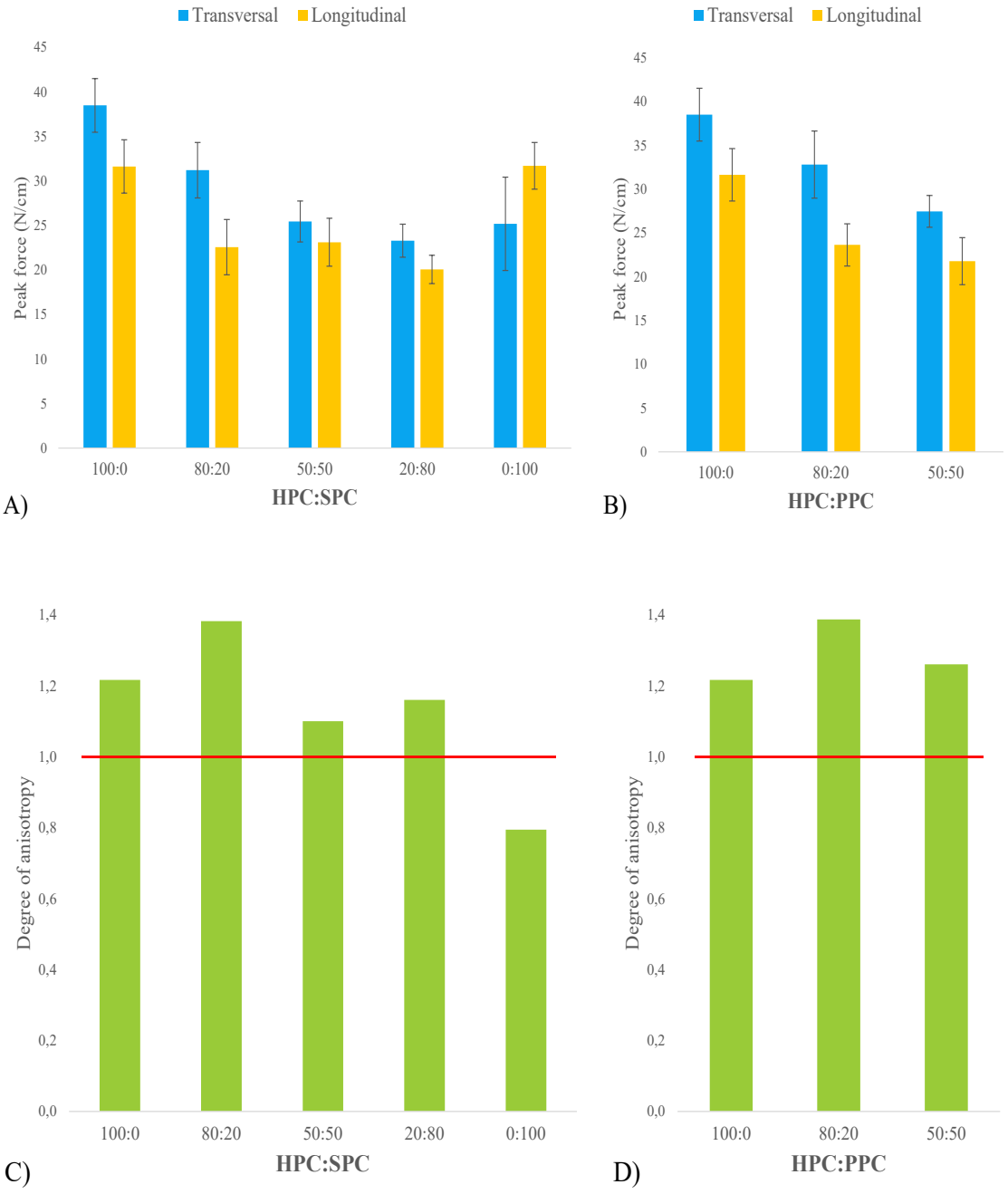

Figure 18. Texture analysis: A) hemp and soy protein concentrate (HPC:SPC) mixtures; B) hemp and pea protein concentrate (HPC:PPC) mixtures. Degree of anisotropy: C) HPC:SPC blends; D) HPC:PPC blends.

5. Conclusions

The extraction methods used to obtain HPC and HPI, that is mechanical oil pressing and wet extraction, respectively, resulted in considerable differences in their composition, protein profile and physicochemical properties, which influenced their rheological behaviour and processability under high moisture extrusion conditions. HPI could not be processed neither alone nor in combination with SPC or PPC, even at the lowest relative concentration tested (20:80). This could be explained by the presence of globulin macromolecular complexes which could not be solubilized under neither denaturing nor reducing conditions. It is reasonable to assume that the relatively low heat load provided by the bench scale extruder used in this study did not allow for a complete breakdown of these aggregates, therefore hindering the formation of the desired fibrous structure. On the other hand, mixtures of HPC with SPC were processable irrespective of the ratio tested (100:0 to 0:100). Furthermore, the presence of HPC made high moisture extrusion of PPC possible up to a ratio of 50:50, with the latter not being processable alone due to its poor flow properties. The degree of anisotropy, which is a measure of the degree of fibre formation in meat analogues, increased with an increase in the relative concentration of HPC for mixtures with both SPC and PPC. These results must be confirmed at larger scale and the sensory properties of the extrudates obtained and derived vegan burgers analysed by a trained panel.

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