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Dissertação de Mestrado
Mestrado de Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação de
Professora Doutora Cristina Maria da Silveira e Silva Pereira Wilson

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RESUMO: Valorização de leguminosas da dieta Mediterrânica como fonte dietética alternativa de proteína

Mediante o crescimento populacional e o resultante aumento global do consumo e produção de alimentos, são necessárias novas fontes nutricionais alternativas e dietas mais sustentáveis que colmatem estes desafios. As leguminosas demonstram grande potencial relativamente ao seu perfil nutricional, sendo benéficas para a saúde, e representam uma alternativa proteica vegetal e uma solução agrícola mais ecológica.

Este trabalho teve como objetivo caracterizar o perfil nutricional de dois tipos de leguminosas, tipicamente consumidas na dieta Mediterrânica, feijão frade (*Vigna unguiculata*) e feijão manteiga (*Phaseolus vulgaris*), com foco no estudo da biomassa obtida a partir dos grãos, sementes germinadas e extratos ricos em proteína e compostos fenólicos. A composição nutricional foi determinada relativamente a cinzas (4,03 % e 5,15 %), humidade (10,93 % e 14,03 %), lípidos (1,20 % e 1,32 %), proteína (38,44 % e 31,90 %) e carbohidratos (42,07 % e 43,92 %), para o feijão frade e manteiga respetivamente. A proteína foi fracionada pelo método sequencial de Osborne, tendo a fração solúvel em água recuperado a maior percentagem de proteína para ambos os feijões. O efeito de diferentes temperaturas de extração (25, 45 e 98 °C) foi estudado aquando da obtenção de extratos bio funcionais de forma a determinar a recuperação de proteína, compostos fenólicos e a capacidade antioxidante dos extratos. A maior recuperação de proteína foi observada a 45 °C em ambos os casos (150,27 mg/g no feijão frade e 125,68 mg/g no feijão manteiga), tal como a recuperação de compostos fenólicos. Relativamente à atividade antioxidante, os extratos de feijão frade obtidos a 45 °C (DPPH e ABTS) demonstraram a maior atividade, no entanto para o feijão manteiga foram os extratos obtidos a 25 °C (DPPH) e a 45 °C (ABTS) que apresentaram maior atividade antioxidante. Os mesmos extratos, de feijão frade e feijão manteiga a 45 °C, apresentaram também maior poder redutor (FRAP). Os extratos obtidos com farinha de sementes germinadas de feijão frade a 45 °C demonstraram a maior recuperação de proteína, compostos fenólicos, maior poder redutor e maior capacidade antioxidante (ABTS). No entanto, a tendência oposta foi observada no feijão manteiga relativamente ao poder redutor. O índex de emulsificação foi calculado a 25 °C (22,78 % e 32,14 %) e 45 °C (31,03 % e 2,41%) para feijão manteiga e frade, respetivamente. A estabilidade da emulsão foi mantida durante 18 dias. Os resultados obtidos sugerem que as leguminosas estudadas representam uma valiosa alternativa vegetal, rica em compostos ativos e nutrientes, essencial para os desafios alimentares globais do futuro. A água de demolha e cozedura do feijão podem também ter propriedades funcionais interessantes, podendo ser usadas como ingredientes alimentares, aumentando a circularidade desta fonte de nutrientes.

Palavras-chave: Leguminosas; alternativas vegetais proteicas; extratos proteico-fenólicos; atividade antioxidante; índex de emulsificação.

ABSTRACT: Valorization of pulses of the Mediterranean diet as alternative dietary protein sources

Sustainable food sources and diets are needed to meet future demands regarding global nutrition, as the population and global food demand for consumption and production increase. Pulses, show great nutritional profile providing benefits for human health, representing an interesting plant protein-based alternative and a greener solution in crop productivity.

The present study aimed at characterizing the nutritional composition profile of two types of dry pulses typically consumed in the Mediterranean diet, cowpea (*Vigna unguiculata*) and kidney bean (*Phaseolus vulgaris*), while focusing on the study of both the biomass obtained from dried seeds, germinated seeds and protein-phenolic rich extracts. The proximate composition regarding ash (4.03 % and 5.15 %), moisture (10.93 % and 14.03 %), lipids (1.20 % and 1.32 %), protein (38.44 % and 31.90 %) and carbohydrates (42.07 % and 43.92 %) was determined for both bean varieties. Protein was fractionated using the Osborne sequential method and the overall highest protein recovery was achieved in the water-soluble fraction, for both bean varieties. The effect of different extraction temperatures (25, 45 and 98 ˚C) used to obtain bio-functional extracts were studied to determine differences in protein and phenolic contents, and antioxidant capacity of extracts. For both cowpea and kidney bean the highest protein recovery was at 45 ˚C (150.27 mg/g and 125.68 mg/g, respectively). About phenolic content, cowpea and kidney bean extracts had the highest value also at 45 °C. Regarding antioxidant activity, for cowpea, higher radical scavenging activity was observed for extracts obtained at 45 °C (DPPH and ABTS). For kidney bean, the extracts obtained at 25 °C (DPPH) and 45 °C (ABTS) showed greater radical scavenging activity. According to FRAP assay, extracts obtained at 45 °C for kidney bean and cowpea showed the greatest reducing power. Extracts obtained at 45 °C with dry sprout flour showed higher protein and phenolic compound recovery, greater reducing power and higher radical scavenging activity (ABTS) when compared to the water-soluble extracts for cowpea. The opposite trend was observed regarding reducing power (FRAP) for kidney bean. Emulsification index was calculated at 25 °C (22.78 % and 32.14 %) and 45 °C (31.03 % and 29.41 %) for kidney bean and cowpea, respectively. The samples' emulsification stability was maintained throughout 18 days. The results suggest that the studied pulses represent an interesting plant-based alternative source of bioactive nutrients essential for future global food demands. Further, bean soaking and cooking waters may have interesting functional properties and may be used as food ingredients, thus increasing circularity of this source of nutrients.

Keywords: Pulses; plant-protein alternatives; protein-phenolic extracts; antioxidant activity; emulsifying activity.

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LIST OF ABBREVIATIONS

ABTS: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)

BSA: Bovine Serum Albumin

$C_6H_{18}O_{24}P_6$: Myo-inositol hexakisphosphate

dH_2O: Deionized water

DPPH: 2,2-diphenyl-1-picryl-hydrazyl

DW: Dry Weight

EI: Emulsification Index

ES: Emulsification Stability

EtOH: Ethanol

FAO - Food an Agricultural Organization

FRAP: Ferric Ion Reducing Antioxidant Power

GAE: Gallic Acid Equivalent

H_2SO_4: Sulfuric Acid

HPLC: High Performance Liquid Chromatography

M: Molar

N: Normality

NaCl: Sodium Chloride

NaOH: Sodium Hydroxide

nm: Nanometers

rpm: Revolutions per minute

TE: Trolox Equivalent

TPC: Total Phenolic Content

TPTZ: 2,4,6-tripyridyl-s-triazine

UV – Ultraviolet

UV/vis – Visible ultraviolet

1. INTRODUCTION

1.1. An Overview on Pulses

Pulse, a term derived from the Latin word "*puls*" (porridge, thick soup or broth) refers to grain legumes – plants that have their seeds enclosed in a pod. For the purpose of this study, the definition used for "pulses" is the one provided by the Food and Agricultural Organization (FAO) of the United Nations in reference to the dry, edible variety of beans, peas and lentils. According to FAO (1994), pulses are a subgroup of legumes, members of the Leguminosae family that produce edible seeds, which are used for human and animal consumption. The classification of pulse is only attributed to the legumes harvested for dry grain. Grain legumes used for oil production, sowing purposes or as vegetables are not considered pulses**.** Whole seed, split grain, dehulled split grain and flour are all different forms of pulse consumption. The demand for pulses as animal feed is also noticeable in some industrialized countries [9]–[11].

The legume family, Leguminosae (Fabaceae), is the third largest plant family having the greatest importance to global agriculture and being by far the most utilized in the diets of many cultures, both in industrialized and developing countries, comprising a significant portion of daily caloric intake. Fabaceae includes 740 genera and 19,400 species and can generically be divided in four subgroups according to their role - food legumes, green vegetable legumes, oilseed legumes and forage legumes. The plants belonging to this family have, not only significant economic value, but are also essential components of many vegetation types around the globe. In one hand, pulse consumption is known to be very nutritious, being associated with various health benefits. In the other hand, legumes have atmospheric nitrogen fixation capacity due to the presence of root nodules, reducing the use of chemical fertilizers in agriculture thus contributing to the soil's fertility and rotating crop production. This characteristic gives them the ability to prosper even in inefficient places. [11]–[13]

In 2016, FAO launched the "International Year of Pulses" campaign, [14], in order to raise public awareness to the diet-related health benefits of pulses as part of sustainable food production. This was one of many attempts to increase vegetable consumption alongside the opportunity to encourage the utilization of pulse-based proteins and increase the global production of pulses. The rising demand for alternative protein sources required for vegan diets as well as whole legumes or isolated fractions as food

ingredients needed for non-dairy and gluten-free foods is an opportunity to introduce pulses in the launch of various unique and innovative products [9], [15].

1.2. *Phaseolus vulgaris* and *Vigna unguiculata*

The morphological similarities between *Vigna* and *Phaseolus* have stirred some confusion regarding systematic and nomenclature [16]. The genus *Phaseolus* comprises more than 50 species. *Phaseolus vulgaris*, one of the five species that are cultivated from this genus, refers to hundreds of varieties and cultivars of the common bean, representing half of the total legumes consumed as grains [17]. The domestication of *Phaseolus vulgaris* (kidney bean) occurred in South America and Central America/Mexico. This separate occurrence led to two different domesticated gene pools, the Andean and Mesoamerican, respectively [18]. The genus *Vigna* comprises approximately 100 species. The genus first evolved in Africa, according to present distribution patterns of *Vigna* diversity. The commonly cultivated cowpea (Figure 1 - a)) belongs to the subspecies *unguiculata* (cowpea), that is divided into five cultivar groups namely Unguiculata, Sesquipedalis, Textilis, Biflora and Melanophthalmus [19].

For the interest of this work, cowpea and kidney bean were the selected bean varieties for the analysis. Cowpea, (*Feijão Frade*) is an herbaceous annual crop mostly grown in the dry agro-ecologies of the tropics in Latin America, Africa and south Asia [20]. Kidney Bean is cultivated in several regions of Indonesia, has diversified different types of kidney beans, being the light red kidney beans popular in the Caribbean region, Portugal and Spain. Light red kidney bean (Figure 1 – b)) is a common dry bean, commonly named butter bean in the Portuguese cuisine (*Feijão Manteiga*) [21], [22].

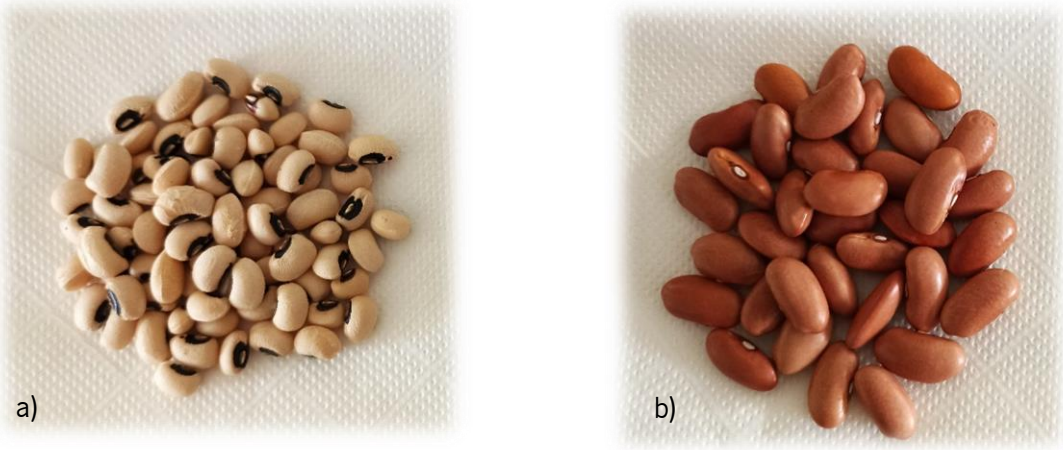

Figure 1. Representation of the bean varieties used for the purpose of this study: a) Cowpea (*Vigna unguiculata*); b) Kidney Bean (*Phaseolus vulgaris*).

1.3. Global Production

Historically, legumes were domesticated during the Neolithic period, along with other crops. Pulses have been a major staple food in the world for several millennia. There are records of cultivation and consumption discovered from Peru and the Egyptian pyramids to small rural areas in Switzerland and Hungary, being a part of the daily energy and nutrient intake of early humans [15].

Nowadays, pulse production occupies 81.8 million hectares on a global scale, with a total production of 77.5 million tons annually (2013-2015) and a projection of production of 95.9 million tons (2016-2025). Regarding the production of beans, which represents the largest percentage of global pulse production (46%), by 2014 dry beans accounted for the production of 24 million tons while cowpea (*Vigna unguiculata L.*) accounted for the production of 7 million tons at a crop level. The "dry beans" category includes common bean (*Phaseolus vulgaris*), lima or butter bean (*Phaseolus lunatus*), scarlet runner bean (*Phaseolus coccineus*), tepary bean (*Phaseolus acutifolius*), adzuki bean (*Vigna angularis*), mung bean (*Vigna radiata*), urd bean (*Vigna mungo*), rice bean (*Vigna umbellata*) and moth bean (*Vigna aconitifolia*). Pulses belonging to the *Phaseolus* and *Vigna* genera account for 41% of the global production. Nigeria, Myanmar, India and Brazil were at the top of global production for both genera, each country producing different types of beans [11], [23].

Although the production of pulses takes place all around the globe, occupying 5.8% of the total arable land, making use of less fertile and marginal land or growing as intercrops with cereals and oilseeds. South Asia and sub-Saharan Africa, where the bulk of production comes from small farm owners, account for half of that production. Meanwhile, industrialized countries account for about a fifth of the global production, being the bulk of production primarily for livestock feed and some for export to developing countries [24]. China, North Africa and some Mediterranean countries are responsible for the production of faba beans; South Asian countries produce mainly chickpeas, lentils and pigeon pea; beans are predominantly grown in Central and South America while cowpea is grown in West African countries [13]. Currently, the largest producer of pulses is India as well as the world's biggest market. The largest exporter of pulses, with a multi-billion-dollar industry, is Canada followed by Australia, Myanmar, the United States and China [25].

1.4. Consumption Trends

When talking about consumption, reports show that the consumption of pulses has remained stagnant (21 grams per capita per day), in the last three decades. At the same time, the preference for animal-based protein has been growing. This reflects the changes in nutritional habits in the food pattern followed today associated with the difficulty of the local and domestic production to accompany the rapid growth of the global population, expected to reach 8.5 billion in 2030 [11], [26]. Socio-economic status, region and traditional cuisine are all factor that also have to be considered when analyzing both the amount and the variety of pulses consumed. The constraints of cooking, taste aversion, uncomfortable gastrointestinal side effects and availability are all reasons that have a negative impact in the intake of pulses [27].

Among the European countries, the highest pulse consumption is observed in the Mediterranean countries [28]. The Portuguese Food Balance Sheet for 2016-2020, [29], disclosed that, despite the increase of 21% (12.7 g/inhabitant/day) in the consumption of dried pulses, this group still had a deficient availability (0.6%) when compared to the recommendation from the Food Wheel guide (4%). The energy contribution of fats in the Portuguese diet was above the maximum limit recommended for consumption (30%), while the contribution of carbohydrates was lower than the recommended range (55-75%). The

estimates made per inhabitant per day are a caloric average availability of 4,075 kcal, when the recommendations are an average of 2,000 kcal ([30]). This trend in pulse consumption is uncharacteristic of the diet followed by the Portuguese population – the Mediterranean diet – a healthy food pattern with a substantial intake of pulses that has been followed for centuries. The low adherence to the Mediterranean diet that has been observed in the last decades, the decrease in pulse availability alongside food consumption being the biggest reason for transgressing the carrying capacity of Earth ecosystems in Portugal, calls for an urgent change in the Portuguese food systems [26].

Focusing now on the global perspective, in order to achieve sustainable development solutions, multiple crises must be simultaneously addressed. Reviewing the way resources are produced and consumed is imperative and food plays a central role. This matter pressed the world leaders to adopt an ambitious agenda of sustainable development goals (SDGs) – the United Nations 2030 Agenda for Sustainable Development and the Paris Agreement, a treaty on climate change aiming to achieve a climate neutral world [31]. Scientists of 16 countries also joined forces in the EAT-Lancet Commission to develop scientific targets for healthy diets and sustainable food production, in a first attempt to set scientific targets for the food system that apply to everyone at a global level [32].

Up until now, increases in the yield of the main crops (wheat, maize, rice and soybean) have led the way in meeting food demand. Nevertheless, food security and sustainability cannot depend solely on improvements in production yields and resource processing technologies due to intense climate-related events (early heat waves, drought, heavy rainfall) happening during crucial phenological phases, such as flowering and grain filling, as well as the scarcity of natural resources [33]. Additionally, 32% of the food volume produced for human consumption never reaches the consumer. The global pattern of food wastage shows that in high-income economies, the volume of food wastage is higher in the processing, distribution and consumption phases of the food chain, while the opposite is true in low-income economies and in rural areas where food wastage occurs in production, post-harvest handling and storage [34].

At the same time, the world faces a new public health challenge. The World Health Organization (WHO) estimates that in 2016, more than 1.9 billion adults (39% of men and 40% of women) are overweight (BMI ≥ 25 kg/m^2), while 13% of the world's population (11% of men and 15% of women) are obese (BMI ≥ 30 kg/m^2) [35]. Yet, more than one in seven people do not have access to nutritious food. Malnutrition is the result of a diet with deficiencies in energy, protein or micronutrients, which has an impact not only in health, but also on educational and work achievement that contributes to social unrest,

poverty and hunger [23]. Although the global hunger index (GHI) indicates that world hunger has decreased by 27% in the last decades [36]. Regarding micronutrient malnutrition, also known as "hidden hunger", it is considered a major health challenge in most developing countries, with 30% of the world's population being iron (Fe) deficient, 17.3% zinc (Zn) and iodine (I) deficient, and 15% selenium (Se) deficient [37]. The need to invert the unbalances in dietary patterns, social trends and the unsustainable economy is of utmost importance [23]. Different approaches were implemented, from food fortification and dietary supplementation and diversification to agronomic fortification of staple crops, yet the success rate was limited. [38], [39]. Inclusion of nutritionally superior pulse crops into local food systems has a significant impact by providing essential dietary requirements, especially in terms of protein and micronutrients (Whiting et al., 2019). Biofortification of pulse crops through conventional breeding and modern biotechnology is, not only possible, but also recommended as an effective approach to mitigate malnutrition worldwide [23].

1.5. Nutritional Profile of Pulses

Pulses are an inexpensive and preeminent source of dry vegetable protein. Containing double the amount of protein found in whole-grain cereals, pulses, as a source of protein, can provide the essential nutritional requirements in developing countries suffering with food insecurity. Pulses are also sources of vitamins and minerals (iron, zinc, calcium, folate, magnesium and potassium) with a low sodium content [41] and a low to moderate content of phenolic compounds, important for antioxidant protection. Characterized by a high water-insoluble fiber and carbohydrate content, pulses are placed lower in the glycemic index than any other carbohydrate-rich food (rice, white bread or potatoes) and present a very low lipid content [9].

Therefore, the health benefits provided by pulses are very appealing in industrialized countries, where protein intakes exceed the requirements, but the risk of chronic diseases is high and directly related to dietary habits, regardless of age and gender. Studies show that an improvement in diet could prevent one in every five deaths at a global level, as the non-optimal intake of three dietary factors (whole grains, fruits, and sodium) accounted for 11 million deaths and 255 million disability-adjusted life-years, in 2017 [25], [42].

1.5.1. Major Nutritional Components

Table 1. Proximate Composition (%) ranges obtained from different studies to cowpea (CP) and kidney bean (KB) (dry seed) (Sources: [1]–[8]).

	Lipids	Carbohydrates	Starch	Fiber	Proteins	Moisture	Ash
CP	1.3 – 4.5	52.1 – 63.9	28.3 - 48.3	4.3 - 9.4	19.9 – 28.2	6.6 – 12.4	3.2 – 4.5
KB	1.3 – 15.8	42.6 – 60.7	34.1 - 36.5	3.6 - 22.9	15.8 - 21.8	2.4 – 12.4	3.0 – 4.4

1.5.1.1. Carbohydrates

Pulses have always been looked upon as a protein source even though the carbohydrate content of most pulse crops makes up for over half of their total weight. These chemical compounds consisting of oxygen, hydrogen and carbon atoms can be classified according to the number of constituent sugar units: monosaccharides (glucose and fructose), oligosaccharides and polysaccharides (starch and cellulose) [43]. As stated before, beans have a low glycemic index when compared with other carbohydrate-rich foods. This can be justified by resistant starch and fiber content.

- Starch and Dietary Fiber

The main form of storage carbohydrate is starch. The starch content present in pulses consists of a high amount of resistant starch, that consists of starch and products of starch degradation not digested in the small intestine. This is reflected by the high ratio of amylose and amylopectin. Amylose is a nonbranched, linear polymer of glucose units that is less readily digested than amylopectin [10], [44]. Starches from different pulse types present different physicochemical, pasting and technological properties, until now neglected not being produced commercially due to their high price when compared to the alternatives [45]. Pulse starches show high retrogradation tendency, making paste preparation

difficult to rupture or swell while cooking, as well as resistant to the action of digestive enzymes. A high stability property can also be observed when pulse starches are exposed to mechanical shearing and heat. These characteristics make pulse starches interesting alternatives to cross-linked starches in food application, especially in the formulation of products for diabetic consumers [45], [46].

Dietary fiber is composed by cellulose, hemicelluloses, polysaccharides and lignin. Cellulose is the major fiber component in beans, such as in pinto beans and cowpeas. The pulses of smaller grain size show a higher total dietary fiber content, due to the greater surface to volume ratio (greater proportion of seed coat to cotyledon), and whole grains also have higher dietary fiber content when compared to flour. Dietary fiber can be classified as either soluble or insoluble fiber fractions, which are constituted by a variety of sugars (arabinose, glucose, uronic acid, rhamnose, mannose, galactose) [43]. Both fiber fractions are responsible for improving glycemic and blood pressure control and support a healthy gut bacterial environment, by reducing the incidence of constipation, diarrhea, and gastrointestinal infections while improving gut barrier functions resulting in enhanced fecal transit and the development of a more robust gastrointestinal tract [23], [47].

1.5.1.2. Protein Content

The nutritional quality of food proteins depends on the amino acid composition, which have bioactive roles and can be precursors of biologically active peptides with physiological functions. Amino acids can be divided into essential, not synthesized in the human body and thus need to be supplied through food, or non-essential, synthesized in the human body. Protein content is focused on the cotyledon, as it is the major portion of the seed. Agronomic, genetic and environmental factors all give their contribution when talking about protein content variation among different types of pulse grains or cultivars. Even so, the average protein content is comparable to that of meat, and double than that found in cereals [43]. According to Osborne [48], the proteins found in pulses fall into five different categories/classes, based on their solubility. Globulins are the primary/principal storage proteins in pulse grains and constitute 35–72% of the total protein content, the remaining 15-25% of protein fraction is constituted by albumins. Together, albumin and globulin are classified as soluble proteins since they are soluble in water or dilute salt solutions. Prolamins and glutelins, soluble in alcohol and soluble in dilute acid/base, constitute a small portion, generally less than 5% [49].

Nutritional quality of food proteins is also dependent on the level of digestibility, a nutritional constraint towards the utilization of pulse protein in food formulations. Digestibility is measured by studying the susceptibility of proteins to proteolysis and, thus their availability. These characteristics, digestibility and protein availability, are especially important when addressing the functional properties needed for food formulation. The application of heat, for example, enables proteins to be denatured and hydrolyzed. Soaking and cooking beans increases protein and starch digestibility, improving bioavailability and nutritional quality [10]. Recent studies also show that starch plays a role in the digestibility of proteins, increasing the digestibility of albumins and globulins. In the presence of starch, the protein structure opens and binds to the surface of starch granules, resulting into easier access to the proteolytic enzymes [9], [50].

1.5.1.3. Lipid Content

Lipids are highly diverse molecules that are insoluble in water. Humans consume lipids in foods or oils, and their composition has important effects on our health. The simplest lipids, fatty acids, are also the most abundant. Fatty acids are the simplest lipids, and are chemically defined as: a linear, non-branched, nonpolar hydrocarbon chain that can be saturated or unsaturated, with a single carboxyl group at one end. Unsaturated fatty acids can be further classified as monounsaturated fatty acids (MUFAs), or as polyunsaturated fatty acids (PUFAs) [51].

Pulses contain approximately 1–21% lipidic content, with beneficial composition of exogenic unsaturated fatty acids: linoleic (21–53%) and linolenic acid (4–22%) [52]. Lipids in pulses can be found mostly in the cotyledons and seed coat are only present in very small amounts. Beans are an important source of free unsaturated fatty acids accounting for 61.1% of total fatty acids. The major fatty acids are palmitic (16:0), oleic (18:1), and linoleic (18:2). The major acid among the unsaturated fatty acids is linolenic (18:3) acid, there is 43.1% in fatty acids of the common bean. The main fatty acid present in legumes is linoleic (18:2), followed by linolenic (18:3) [43], [53]. Linoleic acid (18:2) or linolenic acid (18:3) cannot be produced in our bodies. These are essential fatty acids that must be consumed through food sources, to maintain our health. Pulse lipids can also be a source of other micronutrients, such as phytosterols, that can contribute to cognitive, cardiovascular, and overall wellbeing [51]. A study analyzing the content of the lipid fraction of different grains, seeds and legumes concluded that butter bean (*Phaseolus lunatus*) has the highest saturated fatty acid content (28.7 g/100 g) and kidney beans

(*Phaseolus vulgaris*) have the highest content of PUFA (71.1 g/100 g). PUFA (n-6) have numerous beneficial effects on cardio-vascular disease including improved blood lipid profile, improved insulin sensitivity, lower incidence of type-2 diabetes and anti-arrhythmic effects [54].

1.5.2. Minor Nutritional Components

1.5.2.1. Minerals

Minerals are vital components of our food, as they are important as building materials for our bones, influencing muscle and nerve function. They also take part in the composition of various biologically active compounds, such as hormones and enzymes, maintaining various physiological functions in the human body. These minerals are divided into macro-minerals and trace minerals, according to the body's requirement [55]. Minerals also have an important role in the functioning of the immune system. This concerns both the innate defense system and the adaptive immune response, as the supply of minerals can influence both the susceptibility to infections, but also has the development of chronic diseases [56]. Calcium (Ca), phosphorus (P), magnesium (Mg), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn) and chromium (Cr) are all essential micronutrients that play important roles in the human metabolism. The determination of minerals and trace elements in food is an important part of nutritional analyses. In the study of [57] regarding mineral content in legumes, the level of minerals was found to range between 1.5–5.0 μg/g for copper, 0.05–0.60 μg/g for chromium, 18.8–82.4 μg/g for iron and 32.6–70.2 μg/g for zinc. Nevertheless, the variety and quantity of minerals found in pulses is dependent on the quality and type of the soil in which they grow, as well as the application of fertilizers. Regardless of the high content of minerals observed in pulses, especially in beans, compounds that lower the digestibility and bioavailability of nutrients are also present [53].

1.5.2.2. Bioactive Components

Phenolic compounds, phytates, enzyme inhibitors, lectins and oligosaccharides are all part of the group of bioactive substances present in pulses. Amidst these compounds, some have a positive impact in the metabolism of the consumer, while others have a negative effect limiting the bioavailability of other nutritional components, as proteins and carbohydrates. These have been described as antinutritional factors, such as phytic acid, tannins, trypsin inhibitors, chymotrypsin inhibitors and oligosaccharides [43]. The chemical composition of pulses varies based on their genotype, environmental conditions and soil content and quality, influencing not only the nutrient composition but the antinutrient composition as well [40].

Phenolic compounds are grouped into phenolic acids, tannins and flavonoids and represent the second most abundant group of organic compounds in plants, playing different roles since structural support to protection against different threats (ultraviolet solar radiation, biotic or abiotic stress, herbivores). They can also affect the bitterness, color, and flavor qualities of plants [58]. The phenolic compounds are concentrated in the hull portion of pulses and its content can vary between types of pulses. The presence of phenolic compounds in the hull also affects the color of grain, therefore darker and pigmented grains have a higher content of phenolic compounds. Phenolic compounds are known for their anti-tumoral, anti-inflammatory and anti-allergic properties, as they demonstrate high antioxidant activity – preventing lipid peroxidation and scavenging free oxygen radicals, protecting against oxidative damage while regulating different cellular processes. Thus, phenolic compounds can improve the stability of food formulations and are an indispensable component in a variety of nutraceutical, medicinal and cosmetic applications [59]. Many studies have suggested that antioxidant capacity was positively correlated with phenolic content of pulses [60]. The ferulic acid was the most abundant phenolic compound followed by p-coumaric acid and sinapic acid in common beans [61]. The major role of tannins in nature lies in the defense mechanism of plants. They have the capability to chelate with metal ions and form hydrogen bonds with proteins. Thus, these compounds reduce mineral absorption and digestibility of proteins, have the ability to form bonds with starch and are capable of binding cations (Fe) compromising their bioavailability, characteristics that reduce the nutritional value of pulses [46].

The molecule myo-inositol hexakisphosphate (InsP6), is usually referred to as phytate and has the chemical formula $C_6H_{18}O_{24}P_6$ [62]. InsP6 is the main storage unit for phosphate and inositol in plants, and in pulses it accumulates in the cotyledon, as it is essential for seed germination and plant growth.

Phytate is known for forming irreversible complexes with proteins and minerals, having the capacity to chelate divalent cations, such as Ca, Mg, Zn and Fe, which translates once more in a negative impact in the bioavailability of these nutritional compounds [63].

Overall, it is important to remove or inactivate the antinutritional components from pulses, to make them more appealing to consumers, since many bioactive compounds are also present in pulses which have health promoting properties. There is a growing interest around pulses as food ingredients, the different nutritional fractions from pulses are receiving increased attention as their complex properties are being uncovered to solve the problems encountered in food formulations [64], [65].

1.6. Pulse Processing and Utilization on Food

Pulses, such as cowpea and kidney bean, are mainly sold as whole or split dried seeds. Yet, there is a unique opportunity in expanding the use of beans into processed foods, making use of flours, isolates and fractions [66]. It was the growing trend of convenient, fast-cooking and ready-to-eat foods that has stirred the interest in the processing of pulses into bakery products, pasta, soups, canned products and meat products [67]. Germination, drying, dehulling, splitting, fermentation, flour milling, fractionation, roasting, puffing and extrusion are all primary and secondary techniques described in the processing of the different pulses, which will be briefly explained below [66].

- Germination

When talking about beans, the germination process begins with soaking the whole seed in water for, at least, 12-24 h. The soaked seed is then allowed to germinate until sprouts of 1–2 cm appear (can go up to 6 cm in length, depending on the end-product). The sprouting process in pulses can reduce levels of phytic acid, trypsin inhibitors and oligosaccharides, at the same time increasing levels of proteins [66].

- Drying

The process that succeeds germination and harvest is the drying process. Drying ensures the safe storage, processing and grain quality preservation of pulses. The objective of grain drying is to remove the excess moisture that, when combined with temperature leads to microbial growth and enzymatic activity, and, as a consequence, to grain degradation. Moisture content at the time of harvesting accounts for 18–25%, while the optimum moisture content ranges 9–12%. The drying and conditioning processes of pulses are usually done by carefully controlled artificial methods, in temperate regions. In tropical regions, open sun drying at farm field level is performed, a low cost yet time consuming and labor-intensive process dependent of environmental and seasonal factors. However, sun-dried grains do not fulfil quality standards and cannot be sold on some more demanding international market [68]. There are different methods of artificial drying with hot air: fixed/moving-bed drying, fluidized-bed drying, spouted-bed drying, thin-layer drying [69], [70].

- Dehulling and Splitting

Dried beans can be consumed as whole seed or can undergo dehulling, the removal of the seed coat, to improve digestibility and remove astringency and palatability and reduce cooking time. There are two methods that can be used for dehulling. The wet method, which consists in softening the seed coat or hull to ease removal, and the dry method, with oil/water application followed by sun-drying. The second method has the disadvantage of high dehulling losses due to breakage and powdering [71]. Red lentils, desi chickpea and yellow peas are usually processed into splits after dehulling. This process consists in the cleavage of two cotyledons to obtain a product that takes less time to cook, splits or "*dhal*". [66].

- Flour Milling

The milling process of pulses involves grinding whole, dehulled seeds or splits into flour. The properties and functionality of the resulting flour depend on prior removal of the seed coat (dehulling).

Dehulling is known to reduce antinutritional factors (tannins and insoluble fiber), improving nutritional quality, protein digestibility, texture and taste [72]. At the same time, whole meal flours present a higher content of fiber. Research has shown that the milling process used has an effect in properties such as starch damage, pasting properties and water holding capacity. Pre-treatment of pulses prior to the milling process, either using infrared heating [73], cooking or roasting [74] also have impact in flour functionality [66].

- Fractionation

Pulses can be fractionated to obtain protein and starch concentrates and isolates, and a fiber fraction as a by-product of the process. The principal pulse fractions commercially available are extracted from yellow peas. However, other pulses have been fractionated for commercial purposes lately, including lupins, faba beans, lentils, chickpeas, cowpeas, lima beans and navy beans, primarily to obtain the protein fraction. The fractionation process can be divided into dry or wet fractionation. The dry fractionation process separates pulse flour according to differences in the size and density of particles using a stream of air, since protein bodies tend to be smaller and lighter. Thus, the fraction obtained with lower density is a “protein concentrate” or a “protein-rich fraction”, which contains up to 50–60% protein, and the fraction with higher density is referred to as “starch concentrate” or a “starch-rich fraction” and contains 70–80% starch and 15–20% protein [66].

Protein concentrates are used in both the food and pet food industries, whereas starch concentrates have been used mainly in animal feed or in the pet food industry because of their relatively high protein content (15–20%) and low price. On the other hand, the aqueous fractionation process is more complex. Different wet protein extraction processes can be used according to the material of interest to isolate, sometimes even a combination of processes is needed in order to obtain a higher purity fraction from a heterogeneous mixture. The aqueous fractionation method has been reviewed in different studies by Hood-Niefer & Tyler, 2010, Naguleswaran & Vasanthan, 2010 and K. Tiwari & Singh, 2012. The starch and protein fractions obtained in this extraction process are of higher purity compared to those obtained from a dry fractionation process [66]. The processes involving protein extraction and recovery can have influence in the structure (secondary, tertiary or quaternary) of the extracted protein molecules, therefore affecting protein functionality depending on the method of preparation [77]

1.7. Pulse Protein Flours, Concentrates and Isolates

Proteins have a fundamental role in the functional properties of food formulation contributing to the solubility, foaming, emulsification, gelling and oil absorption properties. Either individually or together with other ingredients, proteins can cause interactions that result in varied functional properties in the end-products. The prevalent high protein-containing pulse flours that are commercialized nowadays are from pea protein concentrates and isolates. Protein flours contain up to 65 % of protein, protein concentrates contain 65 % - 90 % of protein and protein isolates have more than 90 % of protein. The addition of protein flours, concentrates and isolates results in increased nutritional value and can ensure specific functional properties desired in the food formulation industry. Pulse proteins are rich in lysine, leucine, aspartic acid, glutamic acid and arginine, but lack methionine, cysteine and tryptophan, essential amino acids. Thus, blending of pulse proteins with cereal flours, for example, significantly increases their nutritional value [67], [78].

1.8. Nutraceutical Properties of Pulses

The word “nutraceutical”, a combination of nutrition and pharmaceutical, was coined in 1989 in the United States. Nutraceutical indicates “a food, or components of a food, that provides health benefits, including the prevention and treatment of diseases” [79]. Nutraceutical is used to describe an isolated molecular extract, whereas functional food describes whole foods or their concentrates, even though both terms are generally used interchangeably. Many food components, particularly of plant origin, have been described as beneficial for human health, such as dietary fiber, phenolic compounds, minerals and vitamins, proteins and peptides. Nevertheless, the molecular mechanisms responsible are not yet fully understood and further research needs to be done in that field [80].

According to epidemiological studies, a correlation between regular intake of legume seeds and maintenance of good health in humans has been noted. The health benefits related to the consumption of legume seeds have been reported in a wide spectrum of conditions. Small peptides, partially digested proteins and intact proteins that can be found in common beans, perform hormone-like activities that

have a beneficial effect in cancer, cardiovascular disease, diabetes, obesity, the immune response and aging process as well as in mental health [81]–[83]. α-Amylase inhibitors have demonstrated an antidiabetic activity and potential applications regarding the control of obesity. Lectins, blood grouping substances, immunomodulators and tissue markers, that have the ability to combine with sugars and glycoconjugates. These substances have a role in the prevention of cancers, in the activation of immune system, in antimicrobial mechanisms and may have an application in the control of obesity. Clinical and *in vitro* studies have also demonstrated that both proteins and peptides have hypocholesterolemia, glucose, and blood pressure-lowering properties [84]. Celiac disease is accompanied by high risk of bone disease. Measurement of bone density, serum calcium, alkaline phosphate and parathyroid hormone levels all need to be checked in order to adjust dietary intake of Ca, vitamin D and fiber, thus a gluten-free source of these bioactive compounds is crucial, and the answer may be in pulse food formulation with gluten free applications [85].

Ongoing research of several groups at a global level will continue to uncover the biological mechanisms by which pulses (flours, concentrates and isolates) carry out all the properties mentioned above and more. Pulses show great potential in being a source of nutraceuticals, in order to fortify nutritional content for human supplementation, mainly compounds that disappear with cooking conditions but have important biological activity [86].

1.9. Objectives

There is evidence that pulses have great potential concerning the formulation of functional food. Research directed to the development of plant-based alternative sources of bioactive essential nutrients is crucial to face future global food demand. The main objective of this work is the assessment of the nutritional profile, functional and bioactive properties in kidney bean (*Phaseolus vulgaris L.*) and cowpea (*Vigna unguiculata L.*), in the biomass, in co-products and in protein-phenolic extracts obtained from the dried seeds' flour. The following objectives were addressed for this study:

- Characterization of the nutritional composition profile regarding both types of dry pulses;
- Characterization of the main bean protein-fractions obtained with the Osborne sequential protein fractionation method;
- Selection of the extraction conditions to obtain aqueous protein-phenolic rich extracts;
- Germination and characterization of the resulting biomasses;
- Extracts' antioxidant activity and emulsifying activity.

2. MATERIALS AND METHODS

2.1. Material Preparation

Two different species of beans were used in this study, kidney beans (*Phaseolus vulgaris L.*) and cowpea (*Vigna unguiculata L.*), obtained dried from the Portuguese market. 500 g of each variety were milled in a thermomixer (Bimby™) for 30 seconds at velocity 9. Three fractions of different granulometries were obtained (< 0.45mm; 0.45-0.71mm; > 0.71mm). The fractions were used separately for an Osborne type fractionation method and Kjeldahl analysis, and later the fractions were used together, for the protein extraction and characterization methods. All the chemicals used in the assays performed were of analytical grade.

2.2. Extraction Conditions and Sample Preparation

2.2.1. Osborne Sequential Protein Extraction Method

Proteins were extracted from the seed flour samples of kidney bean and cowpea, according to the Osborne fractionation procedure. For this method, the seed flour samples from both bean varieties were used and different solution were prepared for the fractionation. The seed flour samples were added to deionized water (dH_2O) in a ratio of 1:10. The solution was mixed through magnetic stirring for 1 hour at 200 rpm, followed by a 30-minute centrifugation at 5900 rpm (Figure 2 – 1). The supernatant was recovered. The seed flour sample used in the previous procedure was washed, at least three times with dH_2O. The 0.5M NaCl solvent was added to the previous sample in a 10:1 ratio. Magnetic stirring was applied to the solution for 1 hour at 200 rpm, followed by a centrifugation for 30-minutes at 5900 rpm (Figure 2 – 2). The supernatant was recovered, and the sample residue was again washed three times with dH_2O and used in the following fractionation step with EtOH 70 % as solvent in a ratio of 10:1. Magnetic stirring was applied to the solution for 1 hour at 200 rpm, followed by a centrifugation for 30-minutes at 5900 rpm (Figure 2 – 3). The supernatant was recovered. The sample was, once more, washed and used for the final fractionation step, using NaOH 0.1N in a ratio 1:10. The solution was mixed through magnetic stirring for 1 hour at 200 rpm, followed by a 30-minute centrifugation at 5900

rpm (Figure 2 – 4). The supernatant was recovered. Each fractionation step was repeated twice for maximization of protein recovery and the extracts lyophilized to measure crude protein content of each protein-rich fraction through the Kjeldahl's method.

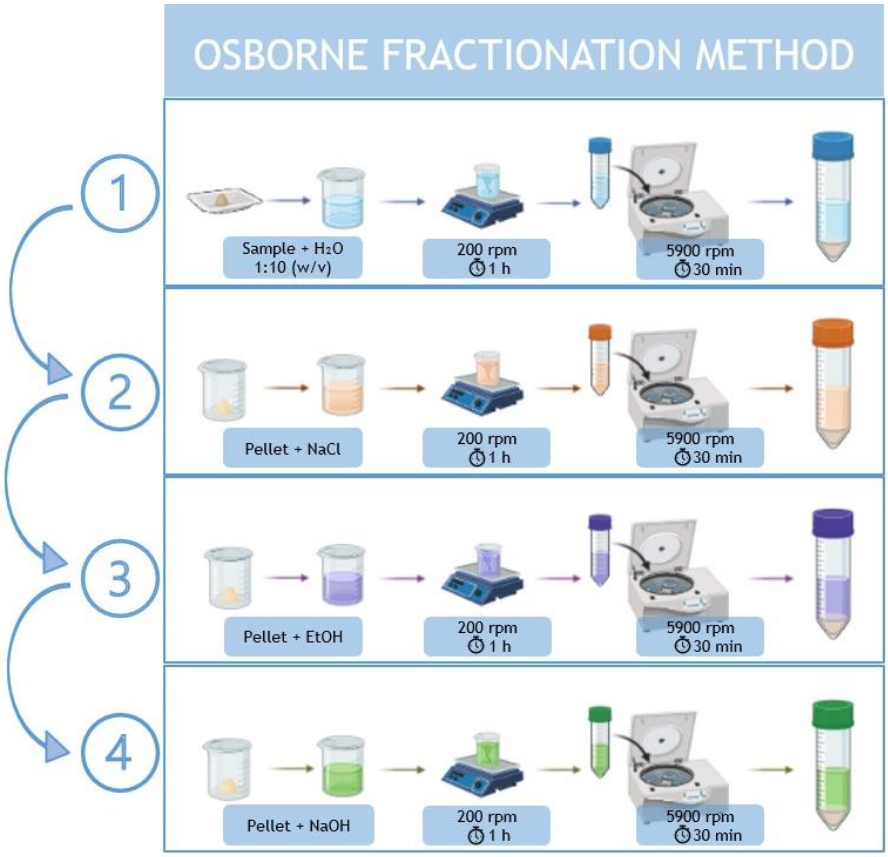

Figure 2. Schematic overview of the four steps involved in the Osborne sequential protein extraction method used. (1: fractionation of an albumin-rich protein extract soluble in dH_2O; 2: fractionation of a globulin-rich protein extract soluble in NaCl; 3: fractionation of a prolamin-rich extract soluble in EtOH; 4: fractionation of a glutelin-rich protein extract soluble in NaOH).

2.2.2. Protein Extraction

Protein rich extracts were obtained from the seed flour samples of both bean varieties. The extraction procedure was based on the results from the Osborne fractionation method (Figure 3). The

seed flour samples were added to dH_2O in a ratio of 1:10. For this extraction, duplicates of each condition were produced for the purpose of analysis. The solutions were stirred at 140 rpm in a water bath for 1 hour. The extracts were then filtered with a flat filter, with 10-12 μm pore size and 70 mm diameter. The filtered extracts were collected and frozen at - 20 °C until analysis. The flour sample residues were also collected to measure the extract yield.

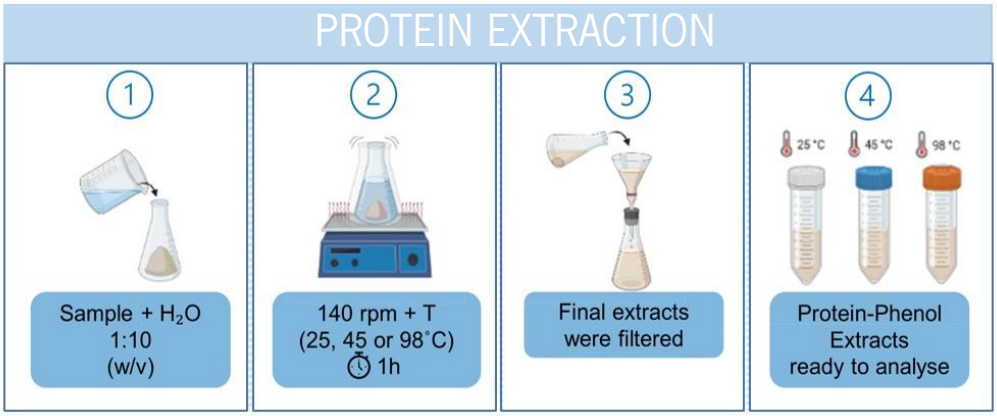

Figure 3. Schematic overview of the extraction procedure used to obtain the protein rich soluble in dH_2O, at different temperature conditions (25 °C, 45 °C and 98 °C).

As phenolic compounds are also an important constituent of legumes and may represent an interesting bioactive fraction, an extra extraction with EtOH (70 %) was also performed to obtain a phenolic compounds-rich fraction. Nevertheless, phenolic compounds are frequently solubilized together with the protein fraction and may also represent an important constituent of the aqueous fractions.

2.2.3. Bean Germination

Regarding the seed germination protocol for both varieties, cowpea and kidney bean, a three-step approach was used: seed cleaning, soaking and germination. The dry seeds were selected and soaked in a sodium hypochlorite (0.07 %) solution for 30 minutes. The seeds were then washed with dH_2O, a minimum of three times, to eliminate all the sodium hypochlorite traces. The bean seeds were then

soaked in bottled water (pH = 5.8 ± 0.15), for 14-16 h prior to germination. In preparation for the germination, all the material required was sterilized under UV light in a laminar flow chamber. After soaking, the seeds were watered and incubated at 26 °C with 90 % humidity for 5 to 7 days and checked daily watering when needed, until the sprouts developed cotyledons. The sprouts were collected followed by lyophilization of the samples and milling.

2.3. Raw Material Characterization

2.3.1. Protein – Kjeldahl Method

Initially developed by Johan Kjeldahl in 1883, the Kjeldahl method is an AOAC official method for crude protein content and involves three stages to quantify protein: digestion, distillation and titration. The digestion of organic material is necessary to convert the nitrogen content in the sample into ammonium sulphate. The digestion is achieved using concentrated sulfuric acid (H_2SO_4), potassium sulphate (K_2SO_4), a catalyst to speed the reaction and heat. The digestate is neutralized by addition of sodium hydroxide (NaOH), converting the ammonium sulphate to ammonia. The resulting ammonia is then distilled and collected in a receiving flask of excess boric acid, forming ammonium borate. The residual boric acid is then titrated with a standard acid and a suitable end-point indicator is used to measure the total nitrogen content of each sample. The total nitrogen content is used together with a specific conversion factor to convert the measured nitrogen content to crude protein content. [87].

For the purpose of this study, 0.5 g of dried flour sample were added to each of the digestion tubes, followed by 10 mL of H_2SO_4 (95-97 %) and 1 selenium tab (reaction catalyst). The digestion unit block performed a 50-minute digestion at 420°C. After digestion, the tubes cooled down for approximately 15 minutes and the nitrogen content (N) was measured by the Kjeldahl distillation unit (Kjeltec 8400 Analyzer, FOSS, Hilleroed, Denmark). The nitrogen-to-protein conversion factor of 5.28, proposed by Mariotti et al. (2008), was used to convert the nitrogen content measured into crude protein content (N x 5.18). The protein content was expressed as percentage per gram of dry biomass.

2.3.2. Carbohydrates – NREL PSA

The procedure provided by the National Renewable Energy Laboratory (NREL) for determination of structural carbohydrates in biomass uses a two-step acid hydrolysis, fractionating the biomass into forms that can be easier to quantify. During the hydrolysis process, the polymeric carbohydrates are hydrolyzed into the monomeric forms, soluble in the hydrolysis liquid. The polymeric carbohydrates obtained are then measured by HPLC (High-Performance Liquid Chromatography) [89].

For this method, 0.3 g of each sample were added into a tared pressure tube, in duplicate. Then, 3.00 mL of 72% H_2SO_4 were added to each pressure tube and a Teflon stir rod was used until the sample was completely mixed. The pressure tubes were placed in a water bath at 30°C for 60 minutes. Using the stir rod, the samples were stirred every 5 to 10 min to ensure a uniform hydrolysis, without removing the sample from the bath. Upon completion of the 60-minute hydrolysis, the tubes were removed from the water bath and the acid was diluted to a 4 % concentration by adding 84.00 mL dH_2O. The samples were mixed thoroughly to eliminate phase separation between low and high concentration acid layers. The tubes were then placed in the autoclave for one hour at 121 °C. Finally, the samples were analyzed by HPLC, and the polymeric carbohydrate content was expressed as percentage (based in mass) of dry biomass.

2.3.3. Lipids

2.3.3.1. Bligh-Dyer Method

The Bligh-Dyer method is a widely practiced lipid extraction method introduced in 1959 by E.G. Bligh and W.J. Dyer [90]. The basic principle for the extraction is based on Folch's method [91], mainly using different solvent/solvent and solvent/tissue ratios. A biological sample is homogenized in a monophasic 1:2 (v/v) chloroform/methanol mixture. A phase separation is induced by addition of chloroform and water and the lipids from the chloroform phase are extracted and processed [92].

The Bligh and Dyer method used for this study had some modifications. 0.05 g of the dried flour sample were added to 1 mL of a mixture of chloroform/methanol (2:1 v/v) and the mixture was

homogenized for 2 minutes by vortexing. The samples were exposed to ultrasounds for 10 to 15 minutes and incubated on a water bath at 30 °C for 30 minutes. After incubation, the mixture was centrifuged at 2000 rpm for 10 minutes and the organic phase was collected to a pre-weighted glass tube. The procedure was repeated, and the biomass residue was re-extracted three times, with 1 mL solvent. The organic phase was dried under a stream of nitrogen gas and the initial extract weight was recorded, as the tube was weighted. Non-lipid contaminants were removed by re-dissolving the initial extract in 2 mL chloroform and 1 mL methanol, the extract was solubilized by vortexing for 1 minute. 0.75 mL of water were added to the organic phase and vortexed for 2 minutes. The mixture was centrifuged at 2000 rpm for 10 minutes, to promote phase separation, and the organic phase (at the bottom) was collected to a new pre-weighted tube. The aqueous phase was re-extracted with 2 mL chloroform. The combined organic phases were then dried under a stream of nitrogen gas and weighted. The lipid content was expressed as the percentage (in mass) of dry biomass.

2.3.3.2. Modified Soxhlet Extraction - Soxtec

The classical Soxhlet method, devised in 1879 by Franz Von Soxhlet, provides the fundamental basis for a modern-day solvent extraction system, the Soxtec™. The submersion method (Randall modification) is used in Soxtec to provide a faster solvent to solvent extraction for quantification of fat and oil, requiring only around 20 % of the time of a traditional Soxhlet extraction [93]. In brief, cellulose thimbles and aluminium extraction cups with glass beads were previously oven-dried at 105 °C for 12 h. 5 g of each bean flour, oven-dried at 105 °C for 2 h, were weighted in triplicates, and added to the cellulose thimbles which were then weighted again containing the sample and covered with defatted medical grade cotton. The thimbles and the aluminium extraction cups were inserted into the Soxtec 8000 Extraction Unit (FOSS), where each extraction cup was filled with 50 mL of petroleum ether and the extraction cycle took place. A three-step program (boiling, rising and recovery) ran for approximately 14 h. After the extraction cycle the thimbles and extraction cups were recovered. The extraction cups were oven-dried at 105 °C for 12 h and the samples contained in the thimbles were transferred to aluminium containers which were then oven-dried at 105 °C for 12 h. The aluminium extraction cups and containers were weighted three times. The total lipid content was expressed as percentage (in mass) of dry biomass.

2.3.4. Ash

For the purpose of determining ash content, 1 g of seed flour samples was added, in duplicates, to a container and air-oven dried at 105 °C for 3 h, cooled in a desiccator and weighted. The containers with the samples were then incinerated in a muffle furnace at 550 °C for about 14 to 16 hours, cooled in a desiccator and reweighted. The total ash was calculated according to the following Equation (1) and was expressed as percentage per gram of dry weight (DW).

$$\boldsymbol{Ash}\ (\%) = \frac{\boldsymbol{W1}}{\boldsymbol{W2}} \times\ 100 \qquad \textbf{(1)}$$

W1: Weight of sample after 105 °C.

W2: Weight of the dried sample.

2.3.5. Moisture

The moisture content of the samples was measured by adding 1 g of seed flour, in duplicate, to a previously air-oven dried at 105 °C for 3 h and weighted aluminium container. The samples dried overnight in the air-oven at 105 °C, cooled in a desiccator and reweighted. Total moisture content was calculated according to Equation (2) and expressed as percentage per gram of dry weight (DW).

$$\boldsymbol{Moisture}\ (\%) = \frac{(\boldsymbol{W1}-\boldsymbol{W2})}{\boldsymbol{W1}} \times\ 100 \qquad \textbf{(2)}$$

W1: Weight of sample.

W2: Weight of the dried sample.

2.4. Extracts Characterization

2.4.1. Lowry Method

The Lowry protein assay is based on the biuret reaction combined with the reduction of the Folin-Ciocalteu phenol reagent (phosphomolybdic-phosphotungstic acid). In the biuret reaction, copper interacts with four nitrogen atoms of peptides to form a cuprous complex. The Folin-Ciocalteu reagent interacts with the cuprous ions and the side chains of tyrosine, tryptophan, and cysteine to produce a blue-greenish color that can be detected at 750 nm. [94].

For the purpose of this study, two different solutions were prepared, solution A (4 mg/mL NaOH; 20 mg/mL Na_2CO_3) and B (10 mg/mL Potassium Sodium Tartrate; 5 mg/mL $CuSO_4$). A BSA 10 % standard was prepared for the calibration curve (R^2=0.957). The solutions A and B were mixed (Lowry's solution) in a ratio 50:1 and stored at 4 °C. In a 96-well plate, 20 µL of extract or standard were added to each well plate together with 200 µL of Lowry's solution and 20 µL of Folin-Ciocalteu. The 96-well plate was then incubated at 42 °C at 180 rpm for 4 hours. The absorbance was measured at 750 nm (high sensitivity for low protein concentrations) by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). Lowry values were expressed as milligrams of protein per gram of dry biomass.

2.4.2. Total Phenolic Content (TPC)

The total phenolic content (TPC) was measured according to the Folin–Ciocalteu method, based on the colorimetric reduction/oxidation reaction of phenols [95]. The method was performed for a 96-well plate, adding 20 µL of extract or standard to each plate well, followed by 100 µL of Folin–Ciocalteau:H_20 (1:10) and 80 µL of sodium carbonate (7.5 %). The microplate is then incubated at 42 °C for 30 minutes in the dark. The absorbance was measured at 750 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). Gallic acid (0-200 mg/L) was used as standard to perform the calibration curve (R^2=0.988). The results were expressed as milligram of gallic acid equivalents (GAE) per gram of dry biomass (mg GAE/g).

2.4.3. DPPH and ABTS

The DPPH method, or free radical scavenging assay, consists in the reduction of the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH•) radical in the presence of a hydrogen-donating antioxidant, resulting in the formation of a non-radical DPPH-H form. For the purpose of this study, 10 μL of protein-phenolic extract were added to 190 μL of DPPH working solution (prepared in ethanol with an absorbance of 0.700 ± 0.01 at 515 nm) in a microplate and incubated at room temperature for 30 minutes in the dark. The absorbance was measured at 515 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). Trolox (6-500 mg/L) was used as standard to prepare the calibration curve (R^2=0.984), to determine the extracts' radical scavenging activity. Inhibition of free radical DPPH in percent (%) was calculated according to Equation (3). DPPH radical scavenging activity of the samples was determined by interpolation of the calibration curve and the results were expressed in μmol Trolox equivalent per gram of dry biomass (μmol TE/g).

In the ABTS assay, the scavenging activity of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•+) is based on the interaction between antioxidant and ABTS radical. To calculate ABTS radical inhibition, 10 μL of extract were added to 200 μL of ABTS working solution in a microplate and incubated at room temperature for 30 minutes in the dark. The absorbance was measured at 734 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). Trolox (6-800 mg/L) was used as standard to prepare the calibration curve (R^2=0.995), to determine the extracts' radical scavenging activity. The results were calculated as previously described for the DPPH assay and expressed in μmol Trolox equivalent per gram of dry biomass (μmol TE/g).

$$\%\ \boldsymbol{Inhibition} = \mathbf{1} - \frac{\boldsymbol{As}}{\boldsymbol{Ac}} \times \mathbf{100} \qquad \textbf{(3)}$$

As: sample absorbance

Ac: control sample absorbance

2.4.4. FRAP

The ferric reducing antioxidant power method, or FRAP assay, consists in the ability of the extracts in study to reduce ferric ions (Fe^{3+} to Fe^{2+}), in the form of ferric 2,4,6-tripyridyl-s-triazine (TPTZ), and was performed as described by [96]. In a 96-well microplate, 10 μL of sample were added to 290 μL of FRAP working solution and incubated at 37°C for 15 minutes in the dark. The absorbance was measured at 593 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). An aqueous solution of ferrous sulphate $FeSO_4 \cdot 7H_2O$ (1000-100 μM) was used as standard for the preparation of a standard curve (R^2=0.973). FRAP values are expressed as micromoles of ferrous equivalent per g of dry biomass (μmol Fe^{2+}/g).

2.4.5. Emulsification Activity and Stability

The emulsification index (EI) was assessed in the protein-phenolic extracts obtained. Sunflower oil was used as a hydrocarbon. For each extraction condition, 3 mL of 0.1% extract solutions were added to 1 mL of sunflower oil, mixing for 5 min at 8000 rpm followed by 4 min at 13600 rpm with a homogenizer (T 25 digital ULTRA-TURRAX, IKA). The mixture was allowed to stand for 15 hours, and the emulsification index was measured. Over the course of 18 days the EI was measured to assess the emulsification stability (ES) of each sample. A white phase is indicative of emulsion formation. EI was calculated according to the following Equation 4:

$$\boldsymbol{EI}\ (\%) = \frac{\boldsymbol{El}}{\boldsymbol{T}} \times \mathbf{100} \qquad \textbf{(4)}$$

El: emulsion layer (cm)

T: total mixture (cm)

2.4.6. Statistical Analysis

The data regarding the extraction analysis is presented as mean ± standard deviation (SD) values. GraphPad Prism software (version 8.0.2; GraphPad Software, Inc., San Diego, CA, U.S.A.) was used to perform the statistical analysis. The analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to determine statistically different values at a significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

Having the purpose of assessing the potential of both kidney bean and cowpea as food or food ingredient, a proximate composition characterization of seed flour samples from both beans was firstly performed. Secondly, an Osborne type fractionation method was used to obtain the beans' protein profile and select the most effective solvents to obtain protein-rich extracts. Water was then selected as solvent and the extraction parameters were tuned to obtain new extracts rich in protein and eventually in phenolic compounds, with bioactive potential or technologic features. Finally, the bioactive potential was preliminarily assessed by measuring the antioxidant activity of liquid extracts and the technological potential was preliminarily assessed by measuring the emulsification capacity (index and stability). Fresh samples were prepared for forthcoming characterization and comparison with the results obtained so far, thus, providing insight in the properties and possible food applications regarding the use of the dry biomass and liquid extracts obtained from pulses.

3.1. Proximate Composition Analysis

The proximate composition obtained from the dried kidney bean and cowpea flour mixture of all grain sizes, is presented in Table 2. The ash content was superior in the kidney bean flour (5.15 %) than in the cowpea flour (4.03 %). These results are similar to recent results reported by Siddiq et al. (2010), in a study to flours obtained from different common bean cultivars (4.60 % to 5.00 %). The ash content elucidates on the total quantity of the mineral elements present in the sample, as it indicates the inorganic composition after the proteins, carbohydrates, lipids and moisture have been removed by incineration. The minerals are essential nutrients, with a role in different metabolic functions [98], [99].

Moisture content is also greater in the kidney bean flour, with a total of 14.03 %, and 10.93 % in the cowpea flour. Previous studies which corroborate the results obtained, also suggest that the initial moisture content of flours can play a role in the bulk density of flours reflecting, not only in the capacity of the packaging material of the product, but also in the suitability of the flour for application in food formulation [99], [100].

Table 2. Physicochemical composition (%/g sample) of cowpea and kidney bean seed flour samples. Values are expressed as mean ± SD.

	KIDNEY BEAN	COWPEA
Ash	5.15 ± 0.05	4.03 ± 0.18
Moisture	14.03 ± 0.06	10.93 ± 0.23
Protein	31.90 ± 0.27	38.44 ± 0.25
Carbohydrates	43.92 ± 1.93	42.07 ± 0.96
Lipids	1.32 ± 0.09	1.20 ± 0.02

The samples show high protein content of 31.90 % and 38.44 % for kidney bean and cowpea seed flour samples, respectively. These results are higher than the previously results, 28.25 % for cowpea and 21.83 % for kidney bean, presented by Sasanam et al., 2011. The conversion factor used in this study, upon Kjeldahl method analysis, was 5.28 according to Mariotti et al. (2008), a specific conversion factor for dry bean proteins calculated from amino acid analysis, contrary to the standard 6.25, which dates back to the 19th century and is commonly used since. Nevertheless, protein content in beans differs depending on many factors, such as variety, germination, development and environmental conditions and even type, quantity and frequency of fertilizer application. The values obtained in the present work were within the limits supported by the literature, 20 % - 40 % for cowpea and 20 % - 30 % for kidney bean [49], [101], [102]. Protein content is important as it will play a crucial role upon evaluation of the functional performance (water-holding capacity, oil-absorption capacity, gelling and emulsification capacity) of both the biomass and liquid extracts in search for possible applications in food formulation.

The total carbohydrate contents are very similar in both flour samples and are the main macronutrient family present in the physicochemical composition, with 43.92 % for kidney bean and 42.07 % for cowpea (Table 2). These results are consistent with previous results obtained regarding kidney bean (42.6 %), but lower than those observed for cowpea (55.3 %) [8]. A high carbohydrate content can impact the thermodynamic affinity of proteins for aqueous solutions. At the same time, the presence of carbohydrates increases the interactions between protein molecules and, consequently, the gelling

capacity of the extract. Even though, this study focusses mainly on protein content and the possible functionalities present in the biomass and the extracts obtained, having a high carbohydrate concentration is a plus as it is a good source of energy. This characteristic is important and desirable in the formulation of weaning formulas and breakfast formulations [103].

Results for the monosaccharide content measured by HPLC are presented in Table 3. The major monosaccharide found in both bean flour samples was glucose, thus confirming that the main polysaccharides present are starch and cellulose.

Table 3. Monosaccharide content (g/100 g sample) of kidney bean and cowpea seed flour samples, measured by HPLC (*n.d. – not detected).

	GLUCOSE	GALACTOSE	RAMNOSE
KIDNEY BEAN	43.92	0.08	n.d*
COWPEA	41.90	0.01	0.16

Regarding quantification of the lipidic content in the flour samples, two different methods were used, Bligh-Dyer and Sohxlet extraction. The results for the Bligh-Dyer method (Table 4) were 0.53 % for kidney bean and 0.52 % for cowpea. While the lipid content, measured with this method, is similar among both samples, it differs from the results obtained with the Soxhlet extraction method and those found in the literature: 1.4 % and 1.3 %, respectively [8]. The lipid content obtained with Sohxlet extraction presented a higher percentage for kidney bean (1.32 %) than for cowpea (1.20 %). The contrasting results between methods can be due to the selection of an appropriate solvent, the most critical factor concerning an efficient lipid extraction. Soxhlet extraction is known to provide a high yield of lipids and is commonly used to extract the crude lipids from dehydrated biomass due to its efficiency [104].

Nevertheless, whatever the method used, the lipid content found in both beans was very low and a low-lipidic content is favorable since fat is known to have a negative impact in the functional properties of bean flours, when correlated with protein content, as it was shown by Siddiq et al., (2010).

Table 4. Lipid content (%/g sample) of cowpea and kidney bean seed flour samples, measured according two different methods, Bligh-Dyer and Sohxlet extraction.

	KIDNEY BEAN	COWPEA
BLIGH-DYER	0.53 ± 0.01	0.52 ± 0.20
SOHXLET EXTRACTION	1.33 ± 0.09	1.20 ± 0.02

3.2. Protein profile Analysis

The Osborne sequential fractionation method was applied to the different flour granulometries obtained from the dried seeds of kidney bean and cowpea for protein characterization purposes. The overall yield of fractionation for each flour granulometry was 47.42 % and 63.97 % in the smaller grain flour, 39.11 % and 37.90 % in the medium grain flour and 27.46 % and 31.67 % in the largest grain flour, for kidney bean and cowpea respectively. Extracts obtained with this method, albumin, globulin, prolamin and glutelin - rich protein fractions (soluble in H_2O, NaCl, EtOH and NaOH, respectively, were analyzed through the Kjeldahl method and the results can be found in Table 5. Pulse proteins are constituted mostly by albumins and globulins (soluble in water and salt solutions), followed by prolamins (soluble in EtOH) and glutelins (soluble in dilute acid/base) [49].

After application of the Osborne fractionation method both matrices showed an overall higher protein content when H_2O was used as solvent (albumin-rich protein extracts), 74.69 % and 90.17 % in the smaller grain flour (< 0.45mm), 37.69 % and 50.59 % in the medium grain flour (0.45mm - 0.71mm) and 53.23 % and ≈100 % in the largest grain flour (> 0.71mm), for cowpea and kidney bean respectively, being the smaller grain flour the one that allowed for the highest protein content. Albumins are constituted by metabolic proteins and include enzymatic and non-enzymatic proteins. Pulse albumins are known to have low molecular weight (5–80 kDa) and a high cysteine and methionine content when compared to globulins. For kidney bean, the extracts soluble in EtOH had the highest protein content after H_2O, with 27.62 % in the smaller grain flour, 72.00 % in the medium grain flour, a highest content than in the water-soluble extracts for this case, and 5.28 % in the largest grain flour. The cowpea extracts showed a different trend, as the extracts soluble in EtOH had a highest content than H_2O in the largest grain flour with 61.34

%, and the extracts soluble in NaOH had the highest content (30.26 %) after H_2O in the smallest grain flour. The extracts soluble in NaCl had the lowest protein content in the three grain sizes for both bean varieties.

The results obtained suggest that it is advantageous to work with water as solvent regarding protein extraction. The water-soluble proteins are the most nutritive in pulse seeds due to their amino acid composition, yet they may also contain some anti nutritional compounds such as trypsin and chymotrypsin inhibitors, hemagglutinins, lectins and amylase inhibitors [49], [105].

Table 5. Protein contents (%/g fraction) obtained by Kjeldahl method for the kidney bean and cowpea protein extracts obtained in with Osborne fractionation method (*n.a. - not analized, due to insufficient biomass).

		KIDNEY BEAN		**COWPEA**	
	Protein Extract	% N	Protein (%)	% N	Protein (%)
Flour Grain < 0.45 mm	H_2O	8.60	90.17	6.95	74.69
	NaCl	0.53	5.61	0.83	8.73
	EtOH	0.86	27.62	1.16	16.03
	NaOH	1.96	23.75	2.07	30.26
Flour Grain 0.45mm - 0.71 mm	H_2O	3.88	50.59	3.72	37.69
	NaCl	0.46	4.87	0.64	6.74
	EtOH	1.65	72.00	1.43	32.89
	NaOH	2.50	40.33	1.41	18.10
Flour Grain > 0.71 mm	H_2O	4.94	≈100	4.18	53.23
	NaCl	0.27	2.80	0.45	4.67
	EtOH	0.16	5.28	1.53	61.34
	NaOH	0.40	5.28	n.a.*	n.a.*

3.3. Aqueous Extraction

The previous analysis to the extracts obtained with the Osborne fractionation led to the selection of H_2O as the most appropriate solvent to obtain protein-rich extracts. Three different extraction temperatures were tested (25 °C, 45 °C and 98 °C) to study temperature influence in the yield and protein recovery regarding the new extraction method. The extraction method was further optimized by adding a filtration step to reduce sample complexity and to improve clarity of viscous samples, especially in the case of samples obtained at 98 °C that showed an especially viscous nature. The overall yield results for the extraction can be found in Table 6. A higher yield was obtained when the extraction was performed at the highest temperature (98 °C) and decreased as the temperature of extractions decreased. Though the yield of solubilization for both bean samples in all different extraction temperatures was not high, the yields achieved indicate that a possible circular economy approach may be implemented to valorize beans and their by-products. In fact, beans are usually soaked at lower temperatures, prior to cooking, and that soaking water may be already rich in nutrients. Furthermore, around 30 % of the biomass is solubilized at 98 °C, thus suggesting that cooking water will also be a rich source of nutrients.

Table 6. Overall extraction yield (%), for cowpea and kidney bean protein-rich extracts obtained at different extraction temperatures (25˚C, 45˚C and 98˚C). Values are expressed as mean ± SD.

	KIDNEY BEAN	COWPEA
25 ˚C	10.36 ± 2.33	13.38 ± 2.48
45 ˚C	16.61 ± 2.03	14.23 ± 1.92
98 ˚C	35.93 ± 1.99	29.47 ± 1.02

The protein recovery was measured in the new protein extracts using the Lowry method and the results can be found in figure 4. Different from Kjeldahl method which, as previously discussed, measures the nitrogen content of the samples, the Lowry method measures a combination of peptide bonds and

specific amino acids. The popularity of the Lowry method lies in its simplicity, being widely used to determine proteins obtained through extraction methods from food systems [106].

For cowpea, the highest protein recovery was at 45 °C followed by 25 °C (150.27 ± 15.33 mg/g and 117.43 ± 12.83 mg/g, respectively) and the lowest recovery at 98 °C (25.52 ± 4.08 mg/g) (figure 4), while the kidney bean samples had the highest protein recovery also at 45 °C (125.68 ± 6.25 mg/g) followed by the 98 °C extraction (76.52 ± 9.42 mg/g) with the lowest being at 25 °C (56.77 ± 8.67 mg/g). The strong decrease in protein extraction from 45 °C to 98 °C was expected, as strong protein denaturation is expected to occur at higher temperatures, thus decreasing its solubility.

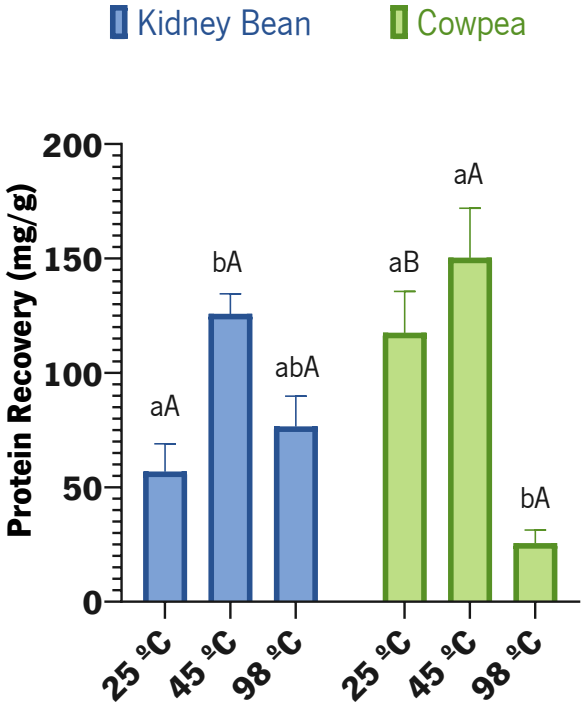

Figure 4. Protein recovery (mg/g sample) obtained by Lowry method for cowpea and kidney bean protein-rich extracts at different extraction temperatures (25 °C, 45 °C and 98 °C). Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between extraction temperatures for the same bean flour sample. Different capital letters (A, B) indicate significant differences ($p < 0.05$) between different bean variety at the same extraction temperature.

Legumes, and in particular beans, are also known for its content in phenolic compounds. Though they are usually more soluble in hydroethanolic solvents, they are frequently bound to proteins and may be co-extracted. Therefore, the total phenolic content recovery was also measured for the protein extracts obtained at different temperatures. The results concerning kidney bean flour samples and cowpea flour samples can be found in figure 5.

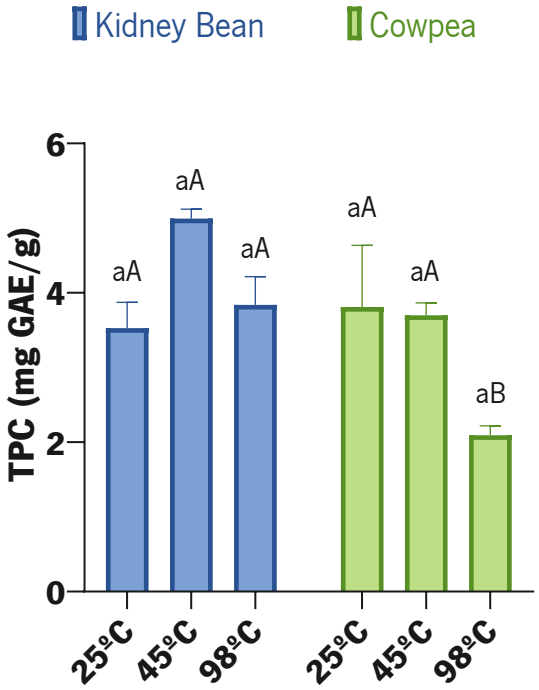

Figure 5. Total Phenolic Content (TPC) (mg GAE/g sample) obtained by Folin-Ciocalteu method for cowpea and kidney bean protein-phenols rich extracts at different extraction temperatures. Values are expressed as mean ± SD. Different capital letters (A, B) indicate significant differences ($p < 0.05$) between different bean variety at the same extraction temperature.

The cowpea extracts had the highest value of total phenolic content at 25 ˚C (figure 5) with 3.81 ± 0.58 mg GAE/g and 45 °C with 3.70 ± 0.12 mg GAE/g followed by the 98 °C with 2.09 ± 0.09 mg GAE/g. The kidney bean extracts showed the highest value at 45 ˚C with 4.99 ± 0.09 mg GAE/g, 3.84 ± 0.27 mg GAE/g at 98 °C and 3.53 ± 0.24 mg GAE/g at 25 °C. These results are within the limits obtained in previous results (0.325–6.378 mg GAE/g) by Marathe et al. (2011), in an analysis to several

bean flour samples from the Phaseolus and Vigna genera. Furthermore, except for the cowpea at 25 °C, the trend is similar to the one found for protein extraction (Figure 4).

Fabaceae plant seeds are a source of antioxidants. Though protein fractions may also exhibit antioxidant activity (particularly when hydrolyzed), these antioxidant compounds derive mainly from the secondary metabolism, the majority being phenolic compounds, and have a crucial role against oxidative stress. These substances are known for their beneficial effects upon removal of reactive oxygen species (ROS) from the blood stream and prevention of their formation. Therefore, these compounds are believed to protect against diseases such as cardiovascular disease and cancer having a protective effect when incorporated into human nutrition, as their antioxidant capacity is effective at low concentrations in plant extracts [108]. The determination of antioxidant activity in the extracts obtained could contribute to establish their value as source of antioxidant compounds. Thus, the antioxidant activity of the protein-phenolic rich extracts was analyzed by three different methods, DPPH, ABTS and FRAP, in the extracts obtained at 25 °C, 45 °C and 98 °C (figure 6).

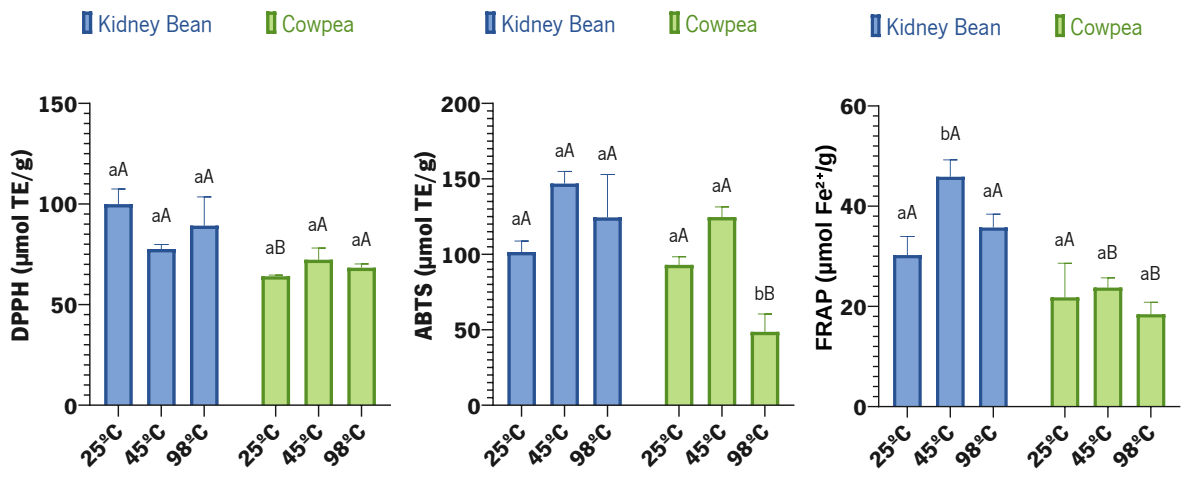

Figure 6. Antioxidant activity obtained by DPPH, ABTS (µmol TE/g sample) and FRAP (µmol Fe^{2+}/g sample) assays for the kidney bean and cowpea protein-phenolic extracts obtained at different extraction temperatures. Values are expressed as mean ± SD. Different letters (a,b) indicate significant differences ($p < 0.05$) between extraction temperatures for the same bean flour sample. Different capital letters (A, B) indicate significant differences ($p < 0.05$) between different bean variety at the same extraction temperature.

For the kidney bean samples (figure 6), the extracts obtained at 25 °C showed greater radical scavenging activity for the DPPH assay, 99.91 ± 5.33 μmol TE/g, while the extracts obtained at 45 °C showed greater radical scavenging activity for the ABTS assay, 146.86 ± 5.69 μmol TE/g. The lowest radical scavenging activity was observed in the extracts obtained at 45 °C with 77.61 ± 1.58 μmol TE/g for DPPH and at 25 °C with 101.54 ± 5.12 μmol TE/g for the ABTS assay. The differences observed between temperature conditions were not significant. According to the FRAP assay, the extracts that showed greatest reducing power were the ones obtained at 45 ˚C with 45.88 ± 2.37 μmol Fe^{2+}/g, followed by the extracts obtained at 98 °C (35.75 ± 1.88 μmol Fe^{2+}/g) and finally, at 25 °C (30.25 ± 2.63 μmol Fe^{2+}/g) with the lowest reducing power.

Regarding antioxidant activity for the cowpea samples, higher radical scavenging activity (figure 6) was observed for the extracts obtained at 45 ˚C with 72.32 ± 4.09 μmol TE/g for the DPPH assay and 124.67 ± 4.74 μmol TE/g for ABTS. The lowest radical scavenging activity was observed in the extracts obtained at 25 °C with 64.05 ± 0.44 μmol TE/g for DPPH and at 98 °C with 48.64 ± 8.34 μmol TE/g for the ABTS assay. The FRAP assay showed that the extracts obtained at 45 ˚C (23.75 ± 1.38 μmol Fe^{2+}/g) showed the greatest reducing power for the cowpea samples, followed by the extracts obtained at 25 °C and 98 °C (21.81 ± 4.81 μmol Fe^{2+}/g and 18.44 ± 1.69 μmol Fe^{2+}/g, respectively).

Considering all the previous results, the 45 °C temperature of extraction was selected for the following assays, considering all factors analyzed so far (yield of extraction, protein recovery, total phenolic content and antioxidant activity) to test the potential of the extracts as functional ingredients. Extraction yield was considerably higher at 98 °C, yet, the fact that protein stability is compromised at high temperatures, due to protein denaturation and possible protein aggregation, was also a crucial factor to consider. Even though controlled heat-induced denaturation is important in order to express some technological properties, it should occur during the food formulation step and not during the extraction step. Pulse protein denaturation occurs between 80 °C and 100 °C, exposing reactive groups, especially the hydrophobic amino acid residues enclosed in the core of the native protein and consequently compromise the functional properties of the extracts with a reduction in quality regarding sensorial attributes, such as flavor and color, and loss in nutritional value [109].

Thus, the analysis proceeded with a new extraction process at 45 °C, now using dry sprout flour samples for comparison with the previous results. Furthermore, a new extraction with EtOH 70 % as solvent was also tested for the dry bean flour samples to assess if another interesting fraction richer in

phenolic compounds could be obtained. The results for both bean varieties concerning extraction yield can be found in table 7.

Table 7. Overall extraction yield (%), of different extraction conditions for cowpea and kidney bean protein-phenolic rich extracts obtained at 45 °C (1 - extracts obtained with dry sprout flour (dH_2O); 2 – extracts obtained with dry bean flour (EtOH 70 %). Values are expressed as mean ± SD.

	KIDNEY BEAN	**COWPEA**
1	39.81 ± 0.49	31.53 ± 0.12
2	10.04 ± 0.38	12.40 ± 0.40

A higher yield was obtained for the extractions using dry sprout flour (dH_2O) for both cowpea (31.53 %) and kidney bean (39.81 %) compared to the yield obtained for the extractions using dry bean flour with water and ethanol (14.23 % and 12.40 % for cowpea and 16.61 % and 10.04 % for kidney bean, respectively), meaning a higher solubilization of biomass is achieved with dry sprout flour suggesting a richer source of nutrients.

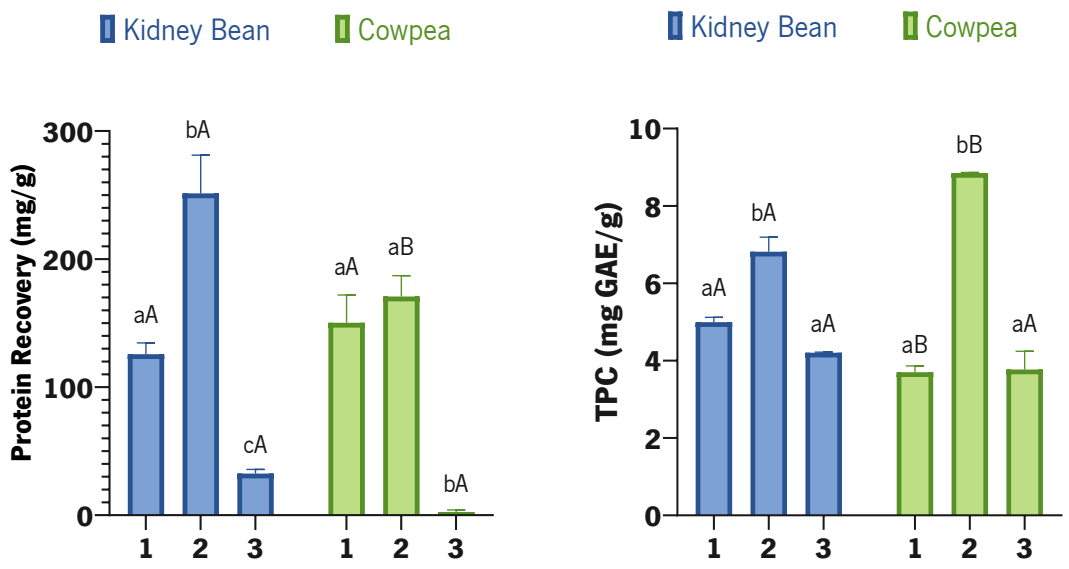

Figure 7. Protein recovery (mg/g dry biomass) and Total Phenolic Content (TPC) (mg GAE/g dry biomass) obtained by Lowry method and Folin-Ciocalteu method, respectively, for cowpea and kidney bean protein-phenolic rich extracts obtained at 45 °C (1 - extracts obtained with dry bean flour (dH_2O); 2 – extracts obtained with dry sprout flour (dH_2O); 3 – extracts obtained with dry bean flour (EtOH 70 %). Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between different extraction condition at 45 °C. Different capital letters (A, B) indicate significant differences ($p < 0.05$) between different bean variety at the same extraction condition.

The protein recovery results, measured for the protein-phenolic rich extracts obtained at 45 °C for the three different conditions, showed that both the kidney bean and cowpea extracts had the highest protein recovery in the extracts obtained with dry sprout flour (dH_2O) with 251.35 ± 21.08 mg/g and 170.77 ± 11.50 mg/g, respectively. Kidney bean and cowpea extracts had the lowest protein recovery in the extracts obtained with dry bean flour (EtOH) with 32.52 ± 2.25 mg/g and 2.52 ± 1.08 mg/g, respectively. TPC assay results, followed the same trend observed for the protein recovery with the extracts obtained with dry sprout flour (dH_2O) having the highest total phenolic content, 6.82 ± 0.27 mg GAE/g for kidney bean and 8.85 ± 0.01 mg GAE/g. Kidney bean extracts also show the lowest total phenolic content in the extracts obtained with dry bean flour (EtOH) with 4.20 ± 0.01 mg GAE/g while cowpea show the lowest results for both dry bean flour conditions with 3.70 ± 0.27 mg GAE/g for the extracts soluble in dH_2O and 3.77 ± 0.33 mg GAE/g. Yet, the results observed in both conditions using

dry bean flour are not significantly different. These results indicate that sprouting may be used to increase protein and phenolic compounds recovery, thus potentially increasing the nutritional value of beans and the corresponding extracts. Furthermore, an extra extraction step with ethanol will not be advantageous.

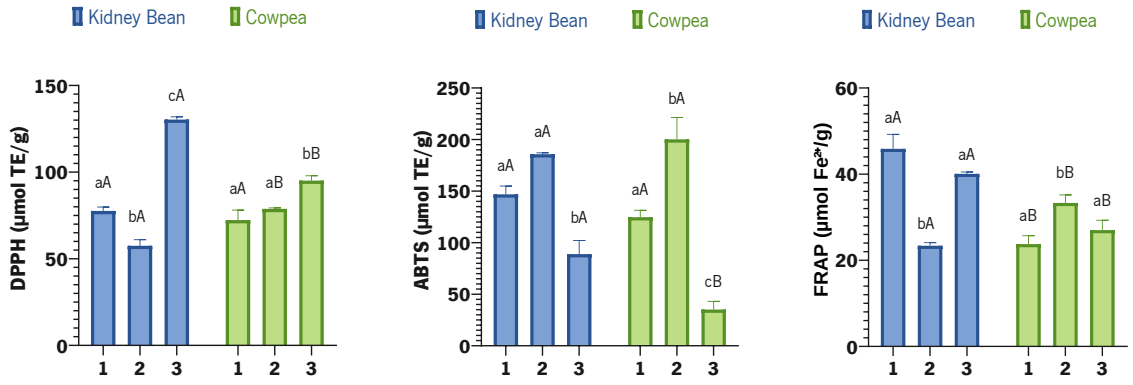

Figure 8. Antioxidant activity obtained by DPPH, ABTS (µmol TE/g sample) and FRAP (µmol Fe^{2+}/g dry biomass) assays for the kidney bean and cowpea protein-phenols rich extracts obtained at 45 °C (1 - extracts obtained with dry bean flour (dH_2O); 2 – extracts obtained with dry sprout flour (dH_2O); 3 – extracts obtained with dry bean flour (EtOH 70 %). Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between different extraction condition at 45 °C. Different capital letters (A, B) indicate significant differences ($p < 0.05$) between different bean variety at the same extraction condition.

Regarding antioxidant activity, extracts obtained with dry bean flour (EtOH 70 %) have the highest radical scavenging activity in the DPPH assay for both bean varieties (130.33 ± 1.11 µmol TE/g for kidney bean and 95.10 ± 1.96 µmol TE/g for cowpea) while the ABTS assay shows that extracts obtained with dry sprout flour (dH_2O) have the highest radical scavenging activity (185.73 ± 0.95 µmol TE/g for kidney bean and 200.14 ± 14.98 µmol TE/g for cowpea). The greatest reducing power for the kidney bean samples can be observed in the extracts obtained with dry bean flour (H_2O) (45.88 ± 2.37 µmol Fe^{2+}/g), measured with the FRAP assay. For the cowpea sample, the protein-phenolic rich extracts obtained with dry sprout flour (H_2O) show a greater reducing power (33.31 ± 1.31 µmol Fe^{2+}/g) than the extracts obtained with dry bean flour.

3.4. Emulsification activity and stability

The emulsifying properties observed in pulse flours are linked to the protein content present in the samples. Proteins have the ability of unfolding to form a densely packed layer around the surface of oil droplets in the medium, thus acting as emulsifiers while preventing structural changes (creaming, sedimentation, coalescence). The hydrophobicity/hydrophilicity ratio of the proteins and their solubility are important factors that affect the sample's emulsifying properties [102], [110].

These properties can be evaluated by two indices, the emulsification index (EI) and the emulsification stability (ES). EI measures the amount of oil that can be emulsified per unit of protein at the initial stage, whereas the ES measures the ability of the emulsion to maintain its structure over time.

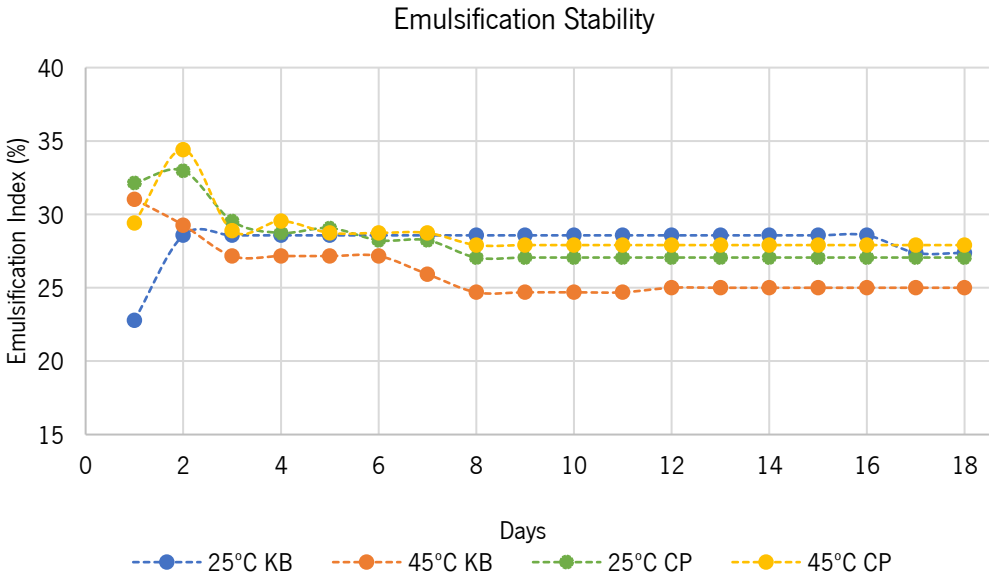

Figure 9. Representation of Emulsification Stability (ES), measured over 18 days, in emulsions of the protein-phenolic rich extracts obtained at different temperature conditions (25 °C and 45 °C) for both bean varieties, kidney bean (KB) and cowpea (CP).

The results obtained showed that, concerning kidney bean samples, the protein-phenolic rich extracts obtained at 45 °C presented a higher emulsification activity (31.03 %) after 15h and decreased slightly over the 18 days, when compared to the extracts obtained at 25 °C, which had a lower EI (22.78

%) after 15h with an increase in the second day (28.57 %) that remained stable over time. For cowpea, the protein-phenolic rich extracts obtained at 45 °C also presented a higher emulsification activity (29.41 %) after 15h with an increase in the second day (34.41 %) reaching the highest EI observed in all samples followed by a slight decrease in the third day. The extracts obtained at 25 °C, showed the highest initial emulsification activity (32.14 %) after 15h with an increase in the second day (32.97 %). All samples were able to maintain the ES over the course of the experiment.

These results are lower than those obtained previously, where the emulsification activity of samples from pulse cooking water ranged between 46-54 % [111]. Nevertheless, in pulses, variety and protein fractions are factors that contribute to great variability when discussing emulsifying properties, different techniques and even sample processing conditions have impact in the functional properties of extracts. For example, reports show that higher vicilin protein content relates to better emulsifying properties, and, in some varieties, albumin proteins are better emulsifiers than globulin proteins [49]. A change to the pH of the environment, altering the net charge of the proteins and their conformation, as well as enzymatic hydrolysis of proteins, which promotes a change in their globular structures and is known to improve solubility, could be strategies used to increase the emulsification capacity of the extracts [112], [113].

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The non-stopping increase in global population, predicted to reach 9.8 billion of people by 2050 and 11.2 billion by 2100, associated with climate change, increase in global temperature, limited and declining water resources, decreasing soil fertility and limited arable land are but a few factors in the multitude of challenges the future holds. The pressing situation regarding diet related health problems that millions of people face nowadays, ranging from malnutrition and "hidden hunger" to chronic diseases such as obesity, cardiovascular disease, diabetes and cancer result in millions of deaths around the world each year. Therefore, providing novel alternatives for a sustainable and nutritionally valuable food production is the right strategy to assume when combating global micronutrient and calorie malnutrition [11], [14], [114].

Pulses are considered a suitable candidate for this fight, due to their nutritional richness. As crops, pulses are able to grow in less favourable soil conditions, with limited access to water and exposed to higher temperatures and dry climate conditions. At the same time, pulses provide high carbohydrate and protein content, being important not only in human nutrition but also as livestock feed and have increased interest in the food industry as they present an appealing option concerning plant-protein alternatives for food formulation of a vast range of products [9]. This could be helpful to decrease the environmental impact associated to the excessive exploration of animal-protein production, with the meat industry being the number one contributor in GHG emissions, land deforestation and water consumption. The positive health impact attributed to a pulse-rich diet also plays a major role in their importance, having reported antioxidant, antimicrobial, anti-inflammatory, hypoglycaemic effects important when addressing several diseases. thus, the need to study and understand how to uncover the potential of pulse biomass and extracts while minimizing the negative effects observed so far [115].

Overall, the proximate composition of both bean varieties analyzed confirmed the high content of protein and carbohydrates expected, as well as a very low lipidic content. Cowpea proved to have a higher protein content than kidney bean. The Osborne fractionation allowed the selection of the best solvent concerning protein extraction. Water was the solvent which allowed for highest protein recovery in both dry bean flour varieties, especially in the kidney bean dry bean flour extracts. The use of water and ethanol as solvents is a "green" approach, when discussing (protein) extraction methods. They are considered natural, nontoxic and environmentally friendly solvents, and, more importantly, food grade, as the extracts in question are being studied with the perspective of food formulation for human consumption.

The different conditions applied to the protein-phenolic extraction suggest that the best temperature of extraction is 45 °C, showing the highest protein content recovery alongside a high total phenolic content, though the extraction yield is considerably higher at 98 °C. Regarding antioxidant activity, ABTS was the methodology exhibiting the highest radical scavenging activity for both kidney bean and cowpea extracts at 45 °C (146.86 ± 5.69 μmol TE/g and 124.67 ± 4.74 μmol TE/g, respectively). FRAP method showed that the greatest reducing power was also observed in the extracts obtained at 45 °C, for both bean varieties. Nevertheless, room temperature and 98 °C are very relevant when considering circular economy approaches where the soaking and cooking waters of cowpea and kidney bean may be used as co-products from the legume processing industries.

The extraction at 45 °C was the condition chosen to compare the dry bean flour sample used for water to the dry sprout flour used to obtain protein-phenolic extracts soluble in water. The results for this analysis suggest that dry sprout flour extracts have the highest protein recovery and also the highest total phenolic content. The antioxidant activity analysis shows that, again, ABTS was the method exhibiting highest antioxidant activity for both kidney bean and cowpea extracts, with dry sprout flour extracts showing the highest results (185.73 ± 0.95 μmol TE/g and 200.14 ± 14.98 μmol TE/g, respectively). The emulsification activity showed promising results regarding stability, which could be useful in a variety of functional food products where emulsions are utilized.

In the future, it would be interesting to analyse the phenolic profile in the extracts soluble in water obtained at different temperatures, as well as in the extracts obtained with dry sprout flour (dH_2O) and the extracts obtained with dry bean flour (EtOH 70 %). Uncovering the phenolic profile could contribute not only to establish the extracts' value as source of antioxidant compounds but also for better understanding the presence and influence that the anti nutritional and toxic compounds have in the extracts, since pulse interest and usefulness decrease with their presence, associated with their large content of protein. There is also a need to perform further analysis to the functionality of the extracts obtained. Water absorption capacity, oil absorption capacity, foaming capacity and stability are some functional properties that could contribute to a more adequate commercial processing regarding food alternatives. Finally, the plant proteins are less digestible than animal proteins which can lead to the gastro-intestinal discomfort reported for pulse consumption that associated with undesirable flavours might represent a significant limitation in the nutritional value of the extracts. Thus, digestibility assays are crucial regarding extracts which are being studied with a future perspective for food formulation.

This work is a valid contribution to unravelling the potential of dry biomass from bean's seeds and sprouts and the respective soaking and cooking waters as functional ingredient source of nutrient.

4. REFERENCES

[1] S. Sasanam, A. Moongngarm, S. Sasanam, T. Paseephol, and A. Moongngarm, 'Comparison of proximate compositions, resistant starch content, and pasting properties of different colored cowpeas (Vigna unguiculata) and red kidney bean (Phaseolus vulgaris)', 2011, Accessed: Oct. 29, 2022. [Online]. Available: https://www.researchgate.net/publication/288579782

[2] K. Preet and D. Punia, 'Proximate composition, phytic acid, polyphenols and digestibility (inï¿¿vitro) of four brown cowpea varieties', *Int J Food Sci Nutr*, vol. 51, no. 3, pp. 189–193, 2000, doi: 10.1080/09637480050029692.

[3] A. S. Gerrano *et al.*, 'Acta Agriculturae Scandinavica, Section B-Soil & Plant Science Selection of cowpea genotypes based on grain mineral and total protein content Selection of cowpea genotypes based on grain mineral and total protein content', 2018, doi: 10.1080/09064710.2018.1520290.

[4] G. Ddamulira, C. A. F. Santos, P. Obuo, M. Alanyo, and C. K. Lwanga, 'Grain Yield and Protein Content of Brazilian Cowpea Genotypes under Diverse Ugandan Environments', *Am J Plant Sci*, vol. 06, no. 13, pp. 2074–2084, 2015, doi: 10.4236/AJPS.2015.613208.

[5] F. Olayinka Henshaw and F. Henshaw, 'Varietal Differences in Physical Characteristics and Proximate Composition of Cowpea (Vigna unguiculata)', *World Journal of Agricultural Sciences*, vol. 4, no. 3, pp. 302–306, 2008, Accessed: Oct. 29, 2022. [Online]. Available: https://www.researchgate.net/publication/237835320

[6] O. A. Owolabi, U. S. Ndidi, A. O. Owolabi, U. S. Ndidi, B. D. James, and F. A. Amune, 'Antinutrient and Mineral Composition of Five Varieties (Improved and Local) of Cowpea, Vigna unguiculata, Commonly Consumed in Samaru Community', *Asian Journal of Food Science and Technology*, vol. 4, no. 2, pp. 70–72, 2012, Accessed: Oct. 29, 2022. [Online]. Available: https://www.researchgate.net/publication/272149446

[7] O. Emmanuel *et al.*, 'Effect of boiling and roasting on the nutrient composition of kidney beans seed flour Nut rit ional Composit ion and Ant inut rient s Cont ent of Raw and Processed Effect of boiling and roasting on the nutrient composition of kidney beans seed flour', *Sky Journal of Food Science*, vol. 4, no. 2, pp. 24–029, 2015, Accessed: Oct. 29, 2022. [Online]. Available: http://www.skyjournals.org/SJFS

[8] Instituto Nacional de Saúde Doutor Ricardo Jorge, 'Tabela da Composição de Alimentos', 2021. https://portfir-insa.min-saude.pt/ (accessed Sep. 09, 2022).

[9] N. Singh, 'Pulses: an overview', *J Food Sci Technol*, vol. 54, no. 4, pp. 853–857, Mar. 2017, doi: 10.1007/s13197-017-2537-4.

[10] A. P. Mullins and B. H. Arjmandi, 'Health Benefits of Plant-Based Nutrition: Focus on Beans in Cardiometabolic Diseases', *Nutrients*, vol. 13, no. 2, p. 519, Feb. 2021, doi: 10.3390/nu13020519.

[11] FAO, *The Global Economy of Pulses*. 2019.

[12] D. Tekdal, 'Plant genes for abiotic stress in legumes', in *Abiotic Stress and Legumes*, Elsevier, 2021, pp. 291–301. doi: 10.1016/B978-0-12-815355-0.00015-1.

[13] S. Gupta and S. Pandey, 'Exploiting the potential of plant growth-promoting rhizobacteria in legume production', in *Abiotic Stress and Legumes*, Elsevier, 2021, pp. 1–32. doi: 10.1016/B978-0-12-815355-0.00001-1.

[14] FAO, 'International Year of Pulses 2016', 2016. https://www.fao.org/pulses-2016/en/ (accessed Sep. 08, 2022).

[15] P. Huebbe and G. Rimbach, 'Historical Reflection of Food Processing and the Role of Legumes as Part of a Healthy Balanced Diet', 2020, doi: 10.3390/foods9081056.

[16] W. Nagl, S. Ignacimuthu, and J. Becker, 'Genetic engineering and regeneration of Phaseolus and Vigna. State of the art and new attempts', *J Plant Physiol*, vol. 150, no. 6, pp. 625–644, Jan. 1997, doi: 10.1016/S0176-1617(97)80277-5.

[17] E. Santos, G. Marques, and T. Lino-Neto, 'Phaseolus vulgaris L. as a functional food for aging protection', in *Aging*, Elsevier, 2020, pp. 289–295. doi: 10.1016/b978-0-12-818698-5.00029-8.

[18] N. Petry, E. Boy, J. P. Wirth, and R. F. Hurrell, 'Review: The potential of the common bean (phaseolus vulgaris) as a vehicle for iron biofortification', *Nutrients*, vol. 7, no. 2, pp. 1144–1173, 2015, doi: 10.3390/nu7021144.

[19] R. S. Pasquet, 'Allozyme diversity of cultivated cowpea Vigna unguiculata (L.) Walp.', *Theoretical and Applied Genetics*, vol. 101, no. 1–2, pp. 211–219, 2000, doi: 10.1007/s001220051471.

[20] N. Tomooka, A. Kaga, T. Isemura, and D. Vaughan, 'Vigna', in *Wild Crop Relatives: Genomic and Breeding Resources*, Berlin, Heidelberg: Springer Berlin Heidelberg, 2011, pp. 291–311. doi: 10.1007/978-3-642-14387-8_15.

[21] J. Garden-Robinson and K. Mcneal, 'All About Beans NUTRITION, HEALTH BENEFITS, PREPARATION AND USE IN MENUS Food and Nutrition Specialist', 2019. Accessed: Sep. 09, 2022. [Online]. Available: www.ag.ndsu.edu/food

[22] S. Y. Chalid, A. Muawanah, S. Nurbayti, and W. M. Utami, 'Characteristics and antioxidant activity of kidney bean (Phaseolus vulgaris L.) tempeh as functional food', in *AIP Conference Proceedings*, Apr. 2021, vol. 2331, p. 40003. doi: 10.1063/5.0041748.

[23] D. Thavarajah, M. McSwain, C. R. Johnson, S. Kumar, and P. Thavarajah, 'Pulses, Global Health, and Sustainability: Future Trends', in *Health Benefits of Pulses*, Cham: Springer International Publishing, 2019, pp. 1–17. doi: 10.1007/978-3-030-12763-3_1.

[24] P. K. Joshi and P. P. Rao, 'Global pulses scenario: status and outlook', *Ann N Y Acad Sci*, vol. 1392, no. 1, pp. 6–17, Mar. 2017, doi: 10.1111/nyas.13298.

[25] A. N. Mudryj, 'Pulse Consumption: A Global Perspective', in *Health Benefits of Pulses*, Cham: Springer International Publishing, 2019, pp. 19–33. doi: 10.1007/978-3-030-12763-3_2.

[26] A. Galli *et al.*, 'Sustainable food transition in Portugal: Assessing the Footprint of dietary choices and gaps in national and local food policies', *Science of The Total Environment*, vol. 749, p. 141307, Dec. 2020, doi: 10.1016/j.scitotenv.2020.141307.

[27] J. Eihusen and J. A. Albrecht, 'Dry Bean Intake of Women Ages 19-45', 2007.

[28] G. Caprioli *et al.*, 'Lipid nutritional value of legumes: Evaluation of different extraction methods and determination of fatty acid composition', *Food Chem*, vol. 192, pp. 965–971, Jul. 2016, doi: 10.1016/j.foodchem.2015.07.102.

[29] Instituto Nacional de Estatística, 'Balança Alimentar Portuguesa 2012–2016', Lisboa, Portugal, 2017.

[30] M. Duarte, M. Vasconcelos, and E. Pinto, 'Pulse Consumption among Portuguese Adults: Potential Drivers and Barriers towards a Sustainable Diet', *Nutrients*, vol. 12, no. 11, p. 3336, Oct. 2020, doi: 10.3390/nu12113336.

[31] B. Soergel *et al.*, 'A sustainable development pathway for climate action within the UN 2030 Agenda', *Nat Clim Chang*, vol. 11, no. 8, pp. 656–664, Aug. 2021, doi: 10.1038/s41558-021-01098-3.

[32] EAT-Lancet Commission, 'Food Planet Health - Healthy Diets From Sustainable Food Systems', 2019.

[33] A. Toreti, S. Bassu, A. Ceglar, and M. Zampieri, 'Climate Change and Crop Yields', in *Encyclopedia of Food Security and Sustainability*, Elsevier, 2019, pp. 223–227. doi: 10.1016/B978-0-08-100596-5.22010-6.

[34] N. E.-H. Scialabba, 'The Food Wastage Challenge', in *Encyclopedia of Food Security and Sustainability*, Elsevier, 2019, pp. 178–186. doi: 10.1016/B978-0-08-100596-5.21989-6.

[35] WHO, 'Obesity and overweight', 2021. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (accessed Sep. 05, 2022).

[36] K. von Grebmer *et al.*, 'Global Hunger Index: The Inequalities of Hunger', 2017.

[37] CDC, 'CDC Center for Global Health - Annual Report', 2017.

[38] R. M. Welch and R. D. Graham, 'Agriculture: The real nexus for enhancing bioavailable micronutrients in food crops', in *Journal of Trace Elements in Medicine and Biology*, Jun. 2005, vol. 18, no. 4, pp. 299–307. doi: 10.1016/j.jtemb.2005.03.001.

[39] G. F. Combs, 'Selenium in global food systems', *British Journal of Nutrition*, vol. 85, no. 5, pp. 517–547, May 2001, doi: 10.1079/bjn2000280.

[40] S. J. Whiting, G. Berhanu, H. A. Haileslassie, and C. J. Henry, 'Pulses and Mineral Bioavailability in Low Income Countries', in *Health Benefits of Pulses*, Cham: Springer International Publishing, 2019, pp. 43–53. doi: 10.1007/978-3-030-12763-3_4.

[41] M. Kazemi *et al.*, 'Pulses and Prevention and Management of Chronic Disease', in *Health Benefits of Pulses*, Cham: Springer International Publishing, 2019, pp. 55–72. doi: 10.1007/978-3-030-12763-3_5.

[42] A. Afshin *et al.*, 'Health effects of dietary risks in 195 countries, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017', *The Lancet*, vol. 393, no. 10184, pp. 1958–1972, May 2019, doi: 10.1016/S0140-6736(19)30041-8.

[43] B. K. Tiwari and N. Singh, 'Pulse Chemistry and Technology', 2012.

[44] V. Messina, 'Nutritional and health benefits of dried beans', *Am J Clin Nutr*, vol. 100, no. suppl_1, pp. 437S-442S, Jul. 2014, doi: 10.3945/ajcn.113.071472.

[45] N. Singh, Y. Nakaura, N. Inouchi, and K. Nishinari, 'Structure and Viscoelastic Properties of Starches Separated from Different Legumes', *Starch - Stärke*, vol. 60, no. 7, pp. 349–357, Jul. 2008, doi: 10.1002/star.200800689.

[46] N. Singh, 'Functional and physicochemical properties of pulse starch', in *Pulse Foods*, Elsevier, 2011, pp. 91–119. doi: 10.1016/B978-0-12-382018-1.00004-6.

[47] W. A. Voderholzer, W. Schatke, B. E. Mühldorfer, A. G. Klauser, B. Birkner, and S. A. Müller-Lissner, 'Clinical response to dietary fiber treatment of chronic constipation.', *Am J Gastroenterol*, vol. 92, no. 1, pp. 95–8, Jan. 1997.

[48] T. B. Osborne, *The proteins of the wheat kernel, by Thomas B. Osborne.* Washington, D.C. : Carnegie Institution of Washington, 1907. doi: 10.5962/bhl.title.22763.

[49] K. Shevkani, • Narpinder Singh, • Ying Chen, A. Kaur, and • Long Yu, 'Pulse proteins: secondary structure, functionality and applications', 2019, doi: 10.1007/s13197-019-03723-8.

[50] A. Ghumman, A. Kaur, and N. Singh, 'Functionality and digestibility of albumins and globulins from lentil and horse gram and their effect on starch rheology', *Food Hydrocoll*, vol. 61, pp. 843–850, Dec. 2016, doi: 10.1016/j.foodhyd.2016.07.013.

[51] J. A. Domínguez-Avila and G. A. González-Aguilar, 'Lipids', in *Postharvest Physiology and Biochemistry of Fruits and Vegetables*, Elsevier, 2019, pp. 273–292. doi: 10.1016/B978-0-12-813278-4.00013-0.

[52] B. D. Oomah, A. Patras, A. Rawson, N. Singh, and R. Compos-Vega, 'Chemistry of pulses', in *Pulse Foods*, Elsevier, 2011, pp. 9–55. doi: 10.1016/B978-0-12-382018-1.00002-2.

[53] R. Campos-Vega, G. Loarca-Piña, and B. D. Oomah, 'Minor components of pulses and their potential impact on human health', *Food Research International*, vol. 43, no. 2, pp. 461–482, Mar. 2010, doi: 10.1016/j.foodres.2009.09.004.

[54] E. Ryan, K. Galvin, T. P. O'connor, A. R. Maguire, and N. M. O'brien, 'Phytosterol, Squalene, Tocopherol Content and Fatty Acid Profile of Selected Seeds, Grains, and Legumes', 2007, doi: 10.1007/s11130-007-0046-8.

[55] M.-H. Kim and M.-K. Choi, 'Seven Dietary Minerals (Ca, P, Mg, Fe, Zn, Cu, and Mn) and Their Relationship with Blood Pressure and Blood Lipids in Healthy Adults with Self-Selected Diet', 2013, doi: 10.1007/s12011-013-9656-1.

[56] C. Weyh, K. Krüger, P. Peeling, and L. Castell, 'The Role of Minerals in the Optimal Functioning of theImmune System', 2022, doi: 10.3390/nu14030644.

[57] C. Cabrera, F. Lloris, R. Giménez, M. Olalla, and M. C. López, 'Mineral content in legumes and nuts: contribution to the Spanish dietary intake', *Science of The Total Environment*, vol. 308, no. 1–3, pp. 1–14, Jun. 2003, doi: 10.1016/S0048-9697(02)00611-3.

[58] L. A. de la Rosa, J. O. Moreno-Escamilla, J. Rodrigo-García, and E. Alvarez-Parrilla, 'Phenolic Compounds', in *Postharvest Physiology and Biochemistry of Fruits and Vegetables*, Elsevier, 2019, pp. 253–271. doi: 10.1016/B978-0-12-813278-4.00012-9.

[59] A. N. Panche, A. D. Diwan, and S. R. Chandra, 'Flavonoids: an overview', *J Nutr Sci*, vol. 5, pp. 1–15, 2016, doi: 10.1017/jns.2016.41.

[60] B. Xu and S. K. C. Chang, 'Effect of soaking, boiling, and steaming on total phenolic contentand antioxidant activities of cool season food legumes', *Food Chem*, vol. 110, no. 1, pp. 1–13, Sep. 2008, doi: 10.1016/j.foodchem.2008.01.045.

[61] D. L. Luthria and M. A. Pastor-Corrales, 'Phenolic acids content of fifteen dry edible bean (Phaseolus vulgaris L.) varieties', *Journal of Food Composition and Analysis*, vol. 19, no. 2–3, pp. 205–211, Mar. 2006, doi: 10.1016/j.jfca.2005.09.003.

[62] F. Grases and A. Costa-Bauza, 'Key Aspects of Myo-Inositol Hexaphosphate (Phytate) and Pathological Calcifications', *Molecules*, vol. 24, no. 24, p. 4434, Dec. 2019, doi: 10.3390/molecules24244434.

[63] L. Bohn, A. S. Meyer, and S. K. Rasmussen, 'Phytate: Impact on environment and human nutrition. A challenge for molecular breeding', *Journal of Zhejiang University: Science B*, vol. 9, no. 3. Zhejiang University Press, pp. 165–191, Mar. 2008. doi: 10.1631/jzus.B0710640.

[64] J. Ahmed, 'Emerging technologies for pulse processing', in *Pulse Foods*, Elsevier, 2011, pp. 223–247. doi: 10.1016/B978-0-12-382018-1.00009-5.

[65] Y. Yagiz and L. Gu, 'Potential Health Promoting Properties of Isoflavones, Saponins, Proanthocyanidins, and Other Phytonutrients in Pulses', in *Health Benefits of Pulses*, Cham: Springer International Publishing, 2019, pp. 109–127. doi: 10.1007/978-3-030-12763-3_8.

[66] L. Malcolmson and J. Han, 'Pulse Processing and Utilization of Pulse Ingredients in Foods', in *Health Benefits of Pulses*, Cham: Springer International Publishing, 2019, pp. 129–149. doi: 10.1007/978-3-030-12763-3_9.

[67] Z. Farooq and J. I. Boye, 'Novel food and industrial applications of pulse flours and fractions', in *Pulse Foods*, Elsevier, 2011, pp. 283–323. doi: 10.1016/B978-0-12-382018-1.00011-3.

[68] A. Esper and W. Mühlbauer, 'Solar drying - an effective means of food preservation', *Renew Energy*, vol. 15, no. 1–4, pp. 95–100, Sep. 1998, doi: 10.1016/S0960-1481(98)00143-8.

[69] R. Jagan Mohan, A. Sangeetha, H. v. Narasimha, and B. K. Tiwari, 'Post-harvest technology of pulses', in *Pulse Foods*, Elsevier, 2011, pp. 171–192. doi: 10.1016/B978-0-12-382018-1.00007-1.

[70] K. M. Kundu, R. Das, A. B. Datta, and P. K. Chatterjee, 'On the Analysis of Drying Process', *Drying Technology*, vol. 23, no. 5, pp. 1093–1105, May 2005, doi: 10.1081/DRT-200059140.

[71] U. Singh, 'Methods for dehulling of pulses: A critical appraisal', *J Food Sci Technol*, vol. 32, no. 2, pp. 81–93, 1995.

[72] J. A. Wood and L. J. Malcolmson, 'Pulse milling technologies', in *Pulse Foods*, Elsevier, 2011, pp. 193–221. doi: 10.1016/B978-0-12-382018-1.00008-3.

[73] O. Fasina, B. Tyler, M. Pickard, G.-H. Zheng, and N. Wang, 'Effect of infrared heating on the properties of legume seeds *', *Int J Food Sci Technol*, vol. 36, no. 1, pp. 79–90, Jan. 2001, doi: 10.1046/j.1365-2621.2001.00420.x.

[74] Z. Ma, J. I. Boye, B. K. Simpson, S. O. Prasher, D. Monpetit, and L. Malcolmson, 'Thermal processing effects on the functional properties and microstructure of lentil, chickpea, and pea flours', *Food Research International*, vol. 44, no. 8, pp. 2534–2544, Oct. 2011, doi: 10.1016/j.foodres.2010.12.017.

[75] S. D. Hood-Niefer and R. T. Tyler, 'Effect of protein, moisture content and barrel temperature on the physicochemical characteristics of pea flour extrudates', *Food Research International*, vol. 43, no. 2, pp. 659–663, Mar. 2010, doi: 10.1016/j.foodres.2009.09.033.

[76] S. Naguleswaran and T. Vasanthan, 'Dry milling of field pea (Pisum sativum L.) groats prior to wet fractionation influences the starch yield and purity', *Food Chem*, vol. 118, no. 3, pp. 627–633, Feb. 2010, doi: 10.1016/j.foodchem.2009.05.045.

[77] V. Kiosseoglou and A. Paraskevopoulou, 'Functional and physicochemical properties of pulse proteins', in *Pulse Foods*, Elsevier, 2011, pp. 57–90. doi: 10.1016/B978-0-12-382018-1.00003-4.

[78] J. Boye, F. Zare, and A. Pletch, 'Pulse proteins: Processing, characterization, functional properties and applications in food and feed', *Food Research International*, vol. 43, no. 2, pp. 414–431, Mar. 2010, doi: 10.1016/J.FOODRES.2009.09.003.

[79] A. T. Diplock, P. J. Aggett, M. Ashwell, F. Bornet, E. B. Fern, and M. B. Roberfroid, 'Scientific Concepts in Functional Foods in Europe: Consensus Document', *British Journal of Nutrition (United Kingdom)*, 1999, Accessed: Sep. 09, 2022. [Online]. Available: https://agris.fao.org/agris-search/search.do?recordID=GB1999010468

[80] M. Carbonaro, 'Role of pulses in nutraceuticals', in *Pulse Foods*, Elsevier, 2011, pp. 385–418. doi: 10.1016/B978-0-12-382018-1.00014-9.

[81] I. Hernandez-Velazquez, M. Sanchez-Tapia, G. Ordaz-Nava, N. Torres, A. R. Tovar, and A. Galvez, 'Black bean protein concentrate ameliorates hepatic steatosis by decreasing lipogenesis and increasing fatty acid oxidation in rats fed a high fat-sucrose diet', *Food Funct*, vol. 11, no. 12, pp. 10341–10350, Dec. 2020, doi: 10.1039/d0fo02258f.

[82] C. A. Moreno-Valdespino, D. Luna-Vital, R. M. Camacho-Ruiz, and L. Mojica, 'Bioactive proteins and phytochemicals from legumes: Mechanisms of action preventing obesity and type-2 diabetes', *Food Research International*, vol. 130, p. 108905, Apr. 2020, doi: 10.1016/j.foodres.2019.108905.

[83] M. A. McCrory, B. R. Hamaker, J. C. Lovejoy, and P. E. Eichelsdoerfer, 'Pulse Consumption, Satiety, and Weight Management', *Advances in Nutrition*, vol. 1, no. 1, pp. 17–30, Nov. 2010, doi: 10.3945/an.110.1006.

[84] S. Johnson, R. Bhattarai, M. Carbonaro, and A. Nucara, 'Legume Proteins and Peptides as Compounds in Nutraceuticals: A Structural Basis for Dietary Health Effects', 2022, doi: 10.3390/nu14061188.

[85] M. M. Niewinski, 'Advances in Celiac Disease and Gluten-Free Diet', *J Am Diet Assoc*, vol. 108, no. 4, pp. 661–672, Apr. 2008, doi: 10.1016/j.jada.2008.01.011.

[86] S. E. Suárez-Martínez, R. A. Ferriz-Martínez, R. Campos-Vega, J. E. Elton-Puente, K. de la Torre Carbot, and T. García-Gasca, 'Bean seeds: leading nutraceutical source for human health', *CyTA - Journal of Food*, vol. 14, no. 1, pp. 131–137, Jan. 2016, doi: 10.1080/19476337.2015.1063548.

[87] D. A. Goulding, P. F. Fox, and J. A. O'Mahony, 'Milk proteins: An overview', *Milk Proteins: From Expression to Food*, pp. 21–98, Jan. 2020, doi: 10.1016/B978-0-12-815251-5.00002-5.

[88] F. Mariotti, D. Tomé, and P. P. Mirand, 'Converting Nitrogen into Protein – Beyond 6.25 and Jones' Factors', *Crit Rev Food Sci Nutr*, vol. 48, no. 2, pp. 177–184, Jan. 2008, doi: 10.1080/10408390701279749.

[89] A. Sluiter *et al.*, 'Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP) (Revised July 2011)', 2008, Accessed: Sep. 18, 2022. [Online]. Available: http://www.nrel.gov/biomass/analytical_procedures.html

[90] E. G. Bligh and W. J. Dyer, 'A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION', *Can J Biochem Physiol*, vol. 37, no. 8, pp. 911–917, Aug. 1959, doi: 10.1139/o59-099.

[91] J. FOLCH, M. LEES, and G. H. SLOANE STANLEY, 'A simple method for the isolation and purification of total lipides from animal tissues.', *J Biol Chem*, vol. 226, no. 1, pp. 497–509, May 1957.

[92] A. Sündermann, L. F. Eggers, and D. Schwudke, 'Liquid Extraction: Bligh and Dyer', in *Encyclopedia of Lipidomics*, Dordrecht: Springer Netherlands, 2016, pp. 1–4. doi: 10.1007/978-94-007-7864-1_88-1.

[93] S. Anderson, 'Soxtec: Its Principles and Applications', *Oil Extraction and Analysis*, pp. 11–24, Jun. 2019, doi: 10.1201/9780429104527-2.

[94] C.-H. Shen, 'Quantification and Analysis of Proteins', *Diagnostic Molecular Biology*, pp. 187–214, Jan. 2019, doi: 10.1016/B978-0-12-802823-0.00008-0.

[95] C. I. Teixeira-Guedes, D. Oppolzer, A. I. Barros, and C. Pereira-Wilson, 'Impact of cooking method on phenolic composition and antioxidant potential of four varieties of Phaseolus vulgaris L. and Glycine max L.', *LWT*, vol. 103, pp. 238–246, Apr. 2019, doi: 10.1016/J.LWT.2019.01.010.

[96] P. Ferreira-Santos, Z. Genisheva, R. N. Pereira, J. A. Teixeira, and C. M. R. Rocha, 'Moderate Electric Fields as a Potential Tool for Sustainable Recovery of Phenolic Compounds from Pinus pinaster Bark', *ACS Sustain Chem Eng*, vol. 7, no. 9, pp. 8816–8826, May 2019, doi: 10.1021/ACSSUSCHEMENG.9B00780/ASSET/IMAGES/MEDIUM/SC-2019-007809_0006.GIF.

[97] M. Siddiq, R. Ravi, J. B. Harte, and K. D. Dolan, 'Physical and functional characteristics of selected dry bean (Phaseolus vulgaris L.) flours', *LWT - Food Science and Technology*, vol. 43, no. 2, pp. 232–237, Mar. 2010, doi: 10.1016/J.LWT.2009.07.009.

[98] A. C. Godswill, 'Proximate composition and functional properties of different grain flour composites for industrial applications', *International Journal of Food Sciences*, vol. 2, no. 1, pp. 43–64, Nov. 2019, doi: 10.47604/ijf.1010.

[99] M. O. Iwe, U. Onyeukwu, and A. N. Agiriga, 'Proximate, functional and pasting properties of FARO 44 rice, African yam bean and brown cowpea seeds composite flour', *Cogent Food Agric*, vol. 2, no. 1, Feb. 2016, doi: 10.1080/23311932.2016.1142409.

[100] S. Chandra, 'African Journal of Agricultural Research Assessment of functional properties of different flours', vol. 8, no. 38, pp. 4849–4852, 2013, doi: 10.5897/AJAR2013.6905.

[101] F. D. Dakora and A. K. Belane, 'Evaluation of Protein and Micronutrient Levels in Edible Cowpea (Vigna Unguiculata L. Walp.) Leaves and Seeds', *Front Sustain Food Syst*, vol. 3, Sep. 2019, doi: 10.3389/fsufs.2019.00070.

[102] K. Shevkani, N. Singh, A. Kaur, and J. C. Rana, 'Structural and functional characterization of kidney bean and field pea protein isolates: A comparative study', *Food Hydrocoll*, vol. 43, pp. 679–689, Jan. 2015, doi: 10.1016/J.FOODHYD.2014.07.024.

[103] C. G. Awuchi, 'The Functional Properties of Foods and Flours', *International Journal of Advanced Academic Research*, Jan. 2019, Accessed: Sep. 20, 2022. [Online]. Available: https://www.academia.edu/41000810/The_Functional_Properties_of_Foods_and_Flours

[104] R. K. Saini, P. Prasad, X. Shang, Y.-S. Keum, and L. Parinandi, 'Molecular Sciences Advances in Lipid Extraction Methods-A Review', *Int. J. Mol. Sci*, vol. 22, p. 13643, 2021, doi: 10.3390/ijms222413643.

[105] J. Boye, F. Zare, and A. Pletch, 'Pulse proteins: Processing, characterization, functional properties and applications in food and feed', *Food Research International*, vol. 43, no. 2, pp. 414–431, Mar. 2010, doi: 10.1016/j.foodres.2009.09.003.

[106] S. K. C. Chang and Y. Zhang, 'Protein Analysis', 2017, pp. 315–331. doi: 10.1007/978-3-319-45776-5_18.

[107] S. A. Marathe, V. Rajalakshmi, S. N. Jamdar, and A. Sharma, 'Comparative study on antioxidant activity of different varieties of commonly consumed legumes in India', *Food and Chemical Toxicology*, vol. 49, no. 9, pp. 2005–2012, Sep. 2011, doi: 10.1016/J.FCT.2011.04.039.

[108] N. Chaves, A. Santiago, and J. C. Alías, 'Quantification of the Antioxidant Activity of Plant Extracts: Analysis of Sensitivity and Hierarchization Based on the Method Used', *Antioxidants*, vol. 9, no. 1, p. 76, Jan. 2020, doi: 10.3390/antiox9010076.

[109] C. Lefè, P. Bohuon, V. Lullien-Pellerin, and C. Mestres, 'Modeling the Thermal Denaturation of the Protein–Water System in Pulses (Lentils, Beans, and Chickpeas)', *Cite This: J. Agric. Food Chem*, vol. 2022, pp. 9980–9989, 2022, doi: 10.1021/acs.jafc.2c03553.

[110] Z. Farooq and J. I. Boye, 'Novel food and industrial applications of pulse flours and fractions', *Pulse Foods*, pp. 283–323, Jan. 2011, doi: 10.1016/B978-0-12-382018-1.00011-3.

[111] J. J. Damian, S. Huo, and L. Serventi, 'Phytochemical content and emulsifying ability of pulses cooking water', *European Food Research and Technology*, vol. 244, no. 9, pp. 1647–1655, Sep. 2018, doi: 10.1007/S00217-018-3077-5/FIGURES/3.

[112] I. Kralova and J. Sjöblom, 'Surfactants Used in Food Industry: A Review', *https://doi.org/10.1080/01932690902735561*, vol. 30, no. 9, pp. 1363–1383, Oct. 2009, doi: 10.1080/01932690902735561.

[113] R. K. C. Jeewanthi, N. K. Lee, and H. D. Paik, 'Improved Functional Characteristics of Whey Protein Hydrolysates in Food Industry', *Korean J Food Sci Anim Resour*, vol. 35, no. 3, pp. 350–359, Jun. 2015, doi: 10.5851/KOSFA.2015.35.3.350.

[114] L. Day, 'Proteins from land plants - Potential resources for human nutrition and food security', *Trends Food Sci Technol*, vol. 32, no. 1, pp. 25–42, Jul. 2013, doi: 10.1016/J.TIFS.2013.05.005.

[115] S. M. F. Bessada, J. C. M. Barreira, and M. B. P. P. Oliveira, 'Pulses and food security: Dietary protein, digestibility, bioactive and functional properties', *Trends Food Sci Technol*, vol. 93, pp. 53–68, Nov. 2019, doi: 10.1016/J.TIFS.2019.08.022.