Good Food Institute IN

Plant protein extraction guide

The plant protein extraction guide contains an overview of industrially relevant plant protein extraction technologies and their sustainability aspects, process parameters, advantages, and limitations as a function of the plant source and principle of extraction. This guide has been created as a gateway for startups and companies involved in manufacturing plant-based products to locate plant protein production facilities that are in their immediate vicinity and available to cater to their R&D and commercialisation needs.

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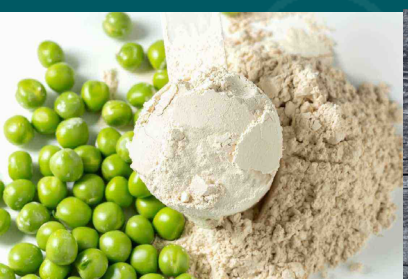

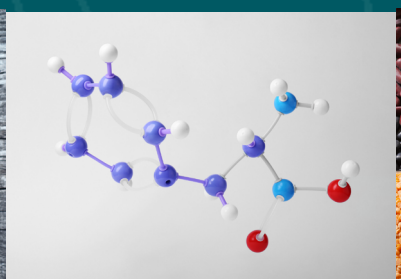


Executive summary

The global market for plant-based foods is experiencing explosive growth. It is projected to triple from USD 11.3 billion in 2023 to a whopping USD 35.9 billion by 2033. This surge is a clear indication of increasing consumer engagement and interest around the globe. Consequently, plant proteins are increasingly important as food producers and consumers shift toward sustainable ingredients. Plant proteins are more sustainable choices for our planet in terms of water, land, and energy use as they have a significantly lower carbon, water, and energy footprint.

This document is an overview of the technologies used to extract plant proteins from their sources. It presents the classification, working principles, advantages, limitations, and applications of three major categories of plant protein extraction: (1) dry fractionation, (2) wet fractionation, and (3) non-thermal or green extraction technologies.

We hope this resource serves as a guidebook to help choose the appropriate protein extraction technology for different plant sources and help ingredient manufacturers locate the appropriate facilities pertaining to plant proteins with the help of our directories (see Appendix). In addition, it will provide insights into the potential of green protein extraction technologies and motivate prospective plant protein ingredient manufacturers to develop sustainable production facilities.

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Background

What are plant-based proteins?

Definition

Plant-based proteins are proteins derived solely from crop sources such as cereals, pulses, legumes, nuts, edible seeds, oilseeds, and tubers (Fig. 1).

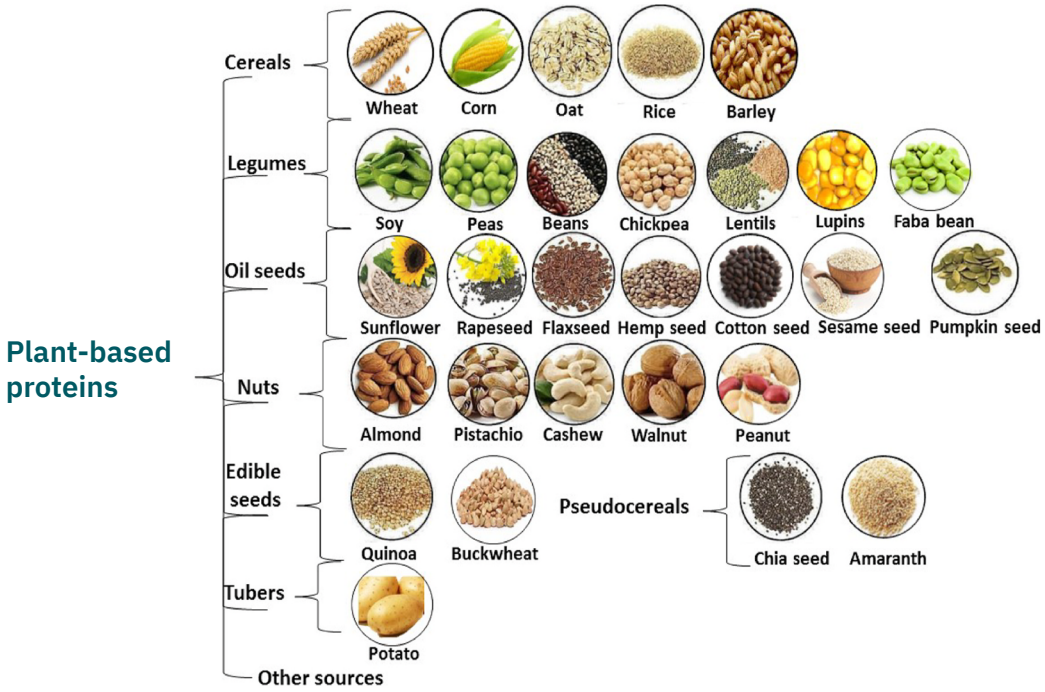

Figure 1. Sources of plant protein (Source: Nikbakht Nasrabadi et al. 2021, CC BY 4.0.)

Building blocks, structure, composition, and classification of plant proteins

Amino acids are the building blocks of proteins derived from any source. These are small organic molecules composed of a hydrogen atom, a carboxyl group (-COOH), an amino group (-NH2) attached to a carbon atom, and a variable part known as a side chain (Fig. 2). Peptide bonds link the amino acids together to form the lengthy chain of proteins. The formation of peptide bonds is a biochemical process that removes a water molecule to link the amino group of one amino acid to the carboxyl group of the neighbouring amino acid. The resultant linear sequence of amino acids within a protein is considered its primary structure (Nature Education, 2014). There are 20 different amino acids, of which nine are designated as ‘essential amino acids’ (EAA) (Fig. 3) because the human body cannot produce them. Hence, the essential amino acids must be obtained from the diet.

Each of the 20 amino acids is composed of a unique side chain.

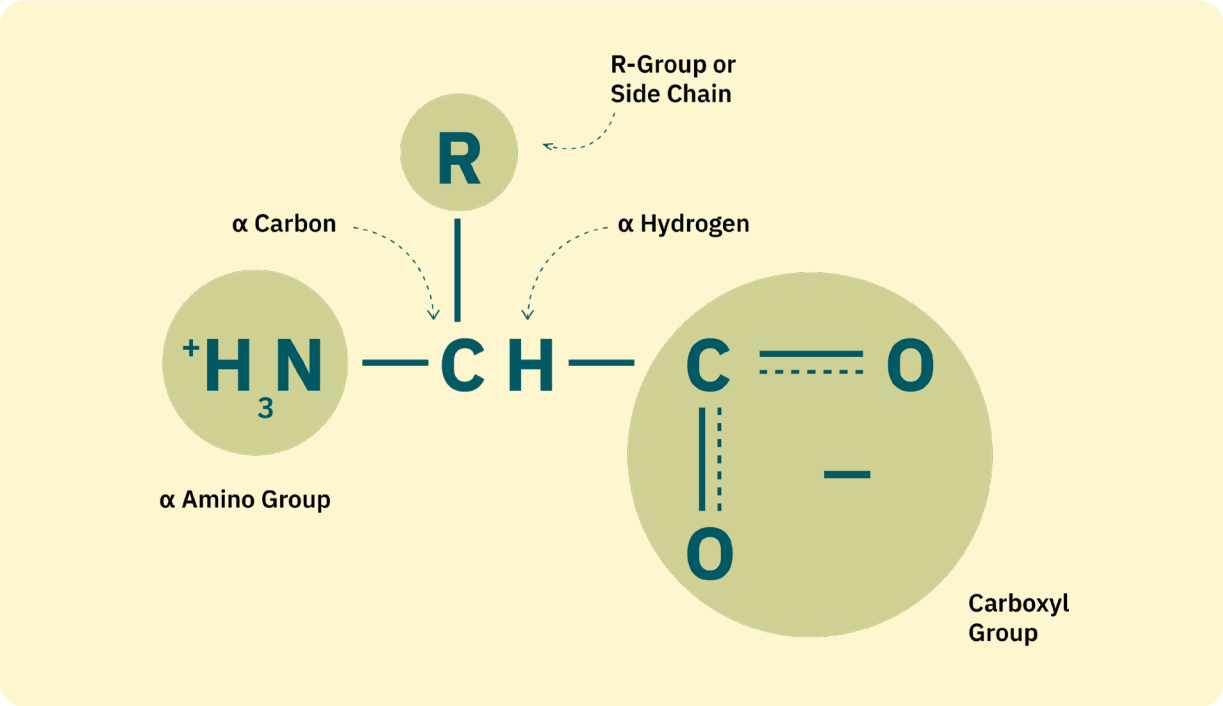

Figure 2. Structure of an amino acid

1 Alanine	2 Arginine	3 Asparagine
4 Aspartic acid	5 Cysteine	6 Glutamic acid
7 Glutamine	8 Glycine	9 Histidine*
10 Isoleucine*	11 Leucine*	12 Lysine*
13 Methionine*	14 Phenylalanine*	15 Proline
16 Serine	17 Threonine*	18 Tryptophan*
19 Tyrosine	20 Valine*	*Essential amino acids

Figure 3. List of amino acids

Plant proteins found in nuts, legumes, oilseeds, and cereals are storage proteins. These proteins could be designated as complete or incomplete, depending on whether they include all the essential amino acids. While most plant proteins lack one or more EAAs, certain exceptions contain all of the EAAs. These include the protein derived from soybeans, amaranth, finger millet, and quinoa. The native globular structure of these storage proteins is typically incapable of forming fibrous structures found in animal muscles. Hence, the plant proteins need to be structured in a way to replicate the texture of animal protein, which requires top-down (transforming plant proteins through the application of thermal, chemical, and mechanical techniques; e.g., extrusion, freeze-structuring, shear cell technology) or bottom-up (assembling proteins and non-protein components by 3D printing, electrospinning, and wet spinning) approaches.

Depending on their solubility, plant proteins can be classified as:

- Albumins: Soluble in water
- Globulins: Soluble in dilute salt solutions
- Prolamins: Soluble in aqueous ethanol solutions
- Glutelins: Soluble in dilute acid/alkaline solutions or insoluble in water

While prolamins (found in wheat, maize, barley, and rye) and glutelins (found in wheat) account for 85% of the total protein in the cereal and pseudocereal family, albumin, and globulins are primarily found in all pulses (>50%) and some pseudocereals (quinoa and amaranth).

Fundamentals of plant protein extraction

Protein extraction is the first step of plant protein production. It involves the separation of protein from other macromolecules, such as fibre, starch, and fat present in the plant material. The general scheme of plant protein extraction can be divided into four steps: (1) defatting of the plant material in its whole or ground/milled form, (2) extraction, (3) precipitation of protein, and (4) purification of protein. The defatting step removes the oily phase that interferes with protein extraction. This is achieved either by solvent extraction (using solvents like petroleum ether, n-hexane, and n-pentane) or cold pressing. Further, proteins are extracted using suitable solvents, which could be hot or cold water, salt solution (e.g., sodium chloride), alcohols (e.g., ethanol, methanol), alkaline solution, acidic solution, or organic solvents.

Non-thermal approaches such as microwave and ultrasound treatments and enzymatic hydrolysis can enhance protein extraction efficiency. Finally, the isolated protein is precipitated, separated, enriched, and concentrated using a series of chemical (e.g., isoelectric precipitation) and physical (e.g., centrifugation, microfiltration, ultrafiltration) methods.

Depending on the water requirement, the techniques for plant protein extraction can be categorised into two types: (1) dry fractionation and (2) wet fractionation. Air classification and alkaline extraction followed by isoelectric precipitation are the well-known methods of dry and wet fractionation, respectively (Fig. 4). While dry fractionation is governed by differences in the size, density, and tribocharging properties of particles, wet fractionation is based on the variations in protein solubility at different pHs of the extraction medium.

Figure 4. General schemes of plant protein extraction by dry fractionation via air classification and wet fractionation via alkaline extraction followed by isoelectric precipitation (Source: GFI, n.d.)

Industrially relevant plant protein extraction techniques

The wet methods of protein extraction are known for their high protein yield, economic sustainability, and continuous operation.

Water-based extraction

Working principle: This method involves water as the solvent to extract protein from the starting material, which could be whole grains, whole grain flour, or dehulled grain flour. The starting material is soaked in water and adjusted to acidic or basic pH (preferably at pH 7) at a defined temperature for a set duration. Then, the mixture is drained or filtered to remove water, ground up, and mixed with water again under stirred conditions for a defined time period. The resulting components are subjected to a series of separation and purification steps involving unit operations such as ultracentrifugation, filtration, microfiltration, ultrafiltration, and chromatographic techniques, including affinity, membrane, or column chromatography. The resultant product is then spray-dried or freeze-dried to obtain the protein concentrate or isolate (Prabhakar, 2022) (Fig. 5).

Processing parameters: pH, temperature, and quality of water (soft water, hard water, distilled water), plant material-to-water ratio, extraction time

Advantages: Water-extracted protein is known for its high solubility and stability. Since this process does not involve the use of chemicals, the resultant ingredient can be claimed as a clean-label product.

Limitations: The requirement for water and a drying step at the end of the process curtails the sustainability of plant protein production using this method.

Alkaline extraction followed by isoelectric precipitation

Working principle: Alkaline extraction (AE) followed by isoelectric precipitation (IEP) is an established method for the extraction of plant proteins. This method is based on the principle that the solubility of the protein in an extraction solvent and its extraction yield would increase with the ionisation of acidic and neutral amino acids at high (alkaline) pH in the range of 8.5-9 (Contreras et al., 2019). Besides, the alkaline or basic pH can disrupt the disulphide bonds linking the thiol groups of cysteine residues, promote unfolding, and thereby increase the hydrophobicity, all of which can collectively improve the extraction yield. Particularly, the solubility of

legume proteins is maximum at alkaline pH (>7) and minimum at pH values close to their isoelectric point (pI) (pH 4–5). At pH values greater or lesser than pI, the proteins gain a net negative or positive surface charge, and their solubility increases (Karaca, Low & Nickerson, 2011). Pulse proteins are typically extracted under mild alkaline conditions, after which they are recovered by IEP. The extraction of plant protein by the AE-IEP method follows the below steps (Fig. 5):

- Ground pulse flour (with or without hulls) or defatted millet flour is dispersed in water at a flour-to-water ratio ranging from 1:5 to 1:20.
- The pH of the flour-water mixture is adjusted to be in the alkaline range (pH 8–11) using sodium hydroxide (NaOH).
- The mixture is held for 30-180 min to achieve maximum solubility of proteins. During this phase, the temperature may be increased to 55–65°C to further improve the solubility and extractability of protein.
- The mixture is filtered to remove insoluble material, if any.
- The pH of the extract is set to the isoelectric point (pH 4–5) using hydrochloric acid (HCl) to facilitate protein precipitation.
- The extract is centrifuged, filtered, or sieved to recover the protein and separate it from insoluble seed materials such as starch and insoluble fibres, washed with water or acid solution to remove salts, and neutralised, followed by freeze-drying or spray-drying (Burger & Zhang, 2019; Chen et al., 2019) to obtain the protein-rich fraction.

Factors influencing the extraction efficiency: Alkali such as sodium hydroxide (NaOH) and potassium hydroxide (KOH) are commonly used to maintain the basic pH and achieve a higher extraction yield than organic extraction.

Advantages: Extraction of protein in an alkaline environment gives higher protein yields (Kumar et al., 2021).

Limitations: The major disadvantage of this extraction method is that it is not environment-friendly due to solvent usage and the generation of waste water and toxic reactive species such as lysinoalanine that can form at high pH. The use of acids to attain the pH or pI required to insolubilise the protein can damage it irreversibly. Consequently, the proteins can be denatured and exhibit poor functional properties.

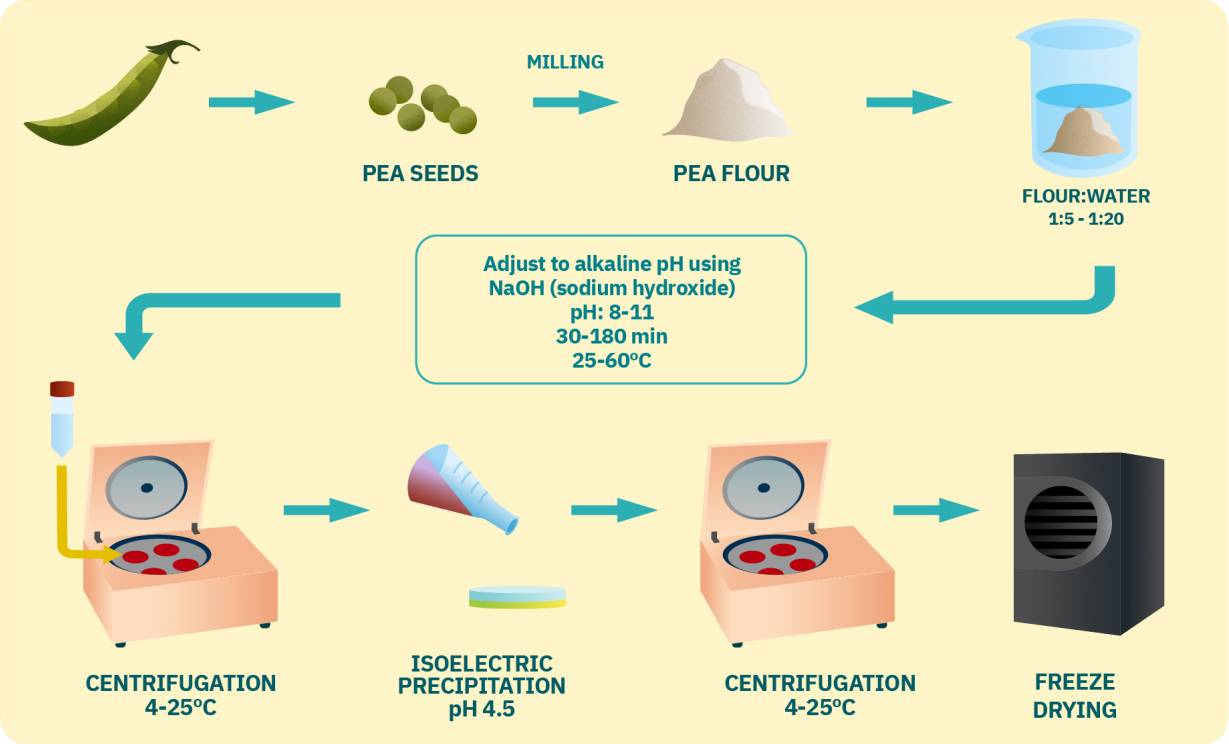

Figure 5. Schematic representation of the extraction of plant protein by alkali extraction followed by isoelectric precipitation (Reproduced with permission from Shanthakumar et al., 2022).

Air classification

Working principle: This method of protein extraction works on the principle of classifying particles into coarse and fine fractions based on their aerodynamic properties such as particle size, particle density, shape, or powder dispersibility (Adamčík, Svěrák & Peciar, 2017). Specifically, with millets and pulses, air classification entails a milling process that fractionates grains into high starch and high protein flours. During this process, whole or dehulled grains are ground into very fine flour. Resultant particles of two distinct sizes and densities are differentiated through a classifier (usually a rotor-type classifier), wherein the flour is dispersed in a spiral air stream and then through a rotating classifier wheel. The small and large particles are separated by the centrifugal force, wherein the light fine fraction is composed of protein, and the heavy coarse fraction comprises starch (Fig. 6). The coarse fraction may be re-milled and re-classified into coarse and fine streams for the purpose of component enrichment (Schutyser & van der Goot, 2011). The protein-rich final fraction with a high degree of purity can be obtained at the end of several such cycles of milling and air classification as described above (Gueguen, Vu & Schaeffer, 1984).

Factors governing the efficiency of air classification

The key factors that govern the process efficiency and the yield and purity of protein obtained from air classification are:

- Prior dehulling of seeds: Besides enhancing the protein content of seeds, dehulling reduces the anti-nutritional factors, removes the bitter/astringent components, and improves colour (Carmo et al., 2020).
- Milling method: Choice of a milling method that is capable of resulting in a very fine grind whilst being selective enough to break up cells and cell fragments without severely damaging the starch granules (Jones, Taylor & Senti, 1959). Finely ground cotyledons of the pulse seeds would allow the disruption of cells and separate starch granules from protein bodies. This allows the starch granules to be released with minimal damage and protein to be ground to finer particles (Boye, Zare & Pletch, 2010; Schutyser et al., 2015; Tyler & Panchuk, 1982). Impact milling or jet milling can be used to achieve finely ground flour (Pelgrom et al., 2013).
- Seed moisture content: This is an additional factor that determines the purity of high protein fractions (HPF) obtained after air classification. Depending on the moisture content of seeds (3.8–14.3%), the protein content of air-classified pea and faba bean HPF varied from 46–52% and 69–74%, respectively. Low seed moisture content is associated with a drop in starch fraction yield, protein content of the starch and protein fractions, and starch separation efficiency. On the other hand, protein yield, starch contents of the starch and protein fractions, and protein separation efficiency improved with a reduction in seed moisture. Higher seed hardness at lower moisture contents and its positive correlation with impact milling efficiency were stated as reasons for the above observations (Tyler & Panchuk, 1982).
- Characteristics of the cell wall: The impact milling efficiency is governed by variations in the thickness and structural rigidity of the cell wall and the degree of adhesion between the cell contents and the cell wall and between proteinaceous material and starch granules (Pelgrom et al., 2013).

Regardless of the above, a portion of the protein may remain adhered to the starch, as protein is derived from the membranes and stroma of chloroplasts that are the sites of starch granule development (Tyler, 1984; Reichert & Young, 1978). This reduces the purity of both protein and starch fractions obtained from air classification relative to that achievable with aqueous extraction processes. Nevertheless, several

researchers have reported protein content ranging from 40–70% of dry matter in the pulse protein concentrates produced by air classification (Schutyser et al., 2015; Aguilera et al., 1984; Patel, Bedford & Youngs, 1980).

Processing parameters: The efficiency of air classification in fractionating proteins can be measured by a parameter called the protein separation efficiency (PSE). PSE is calculated as the percentage of total flour protein recovered in the fine fraction. Cowpea and mung bean demonstrated a PSE of 78.2% and 88.9%, respectively (Tyler, 1984). Generally, based on the values of PSE, mung bean (PSE: 88.9%), and lentil (PSE: 87.2%) are the most suitable legumes for air classification, but lima bean (PSE: 80.2%) and cowpea (PSE: 78.2%) are the least suitable (Tyler, Youngs & Sosulski, 1981).

Advantages: The advantages of air classification over wet fractionation include (Pelgrom et al., 2013; Vogelsang-O'Dwyer et al., 2020):

- Greater sustainability;
- Significantly lower demand for energy and water to enrich proteins;
- Drying process not being a mandate;
- The addition of chemicals not being required;
- Less need for harsh processing conditions, allowing air-classified protein concentrates to maintain a structure closer to their natural form, which results in improved functionality. For instance, protein from faba beans processed in this way has shown to be more soluble at neutral pH and possesses better foaming and gelling qualities.

Limitations: Air classification is greatly dependent on particle size and hence not suitable for crops with high starch concentrations or small starch granules with similarly-sized protein bodies (e.g., cowpeas) (Cloutt, Walker & Pike, 1987). In addition, lower protein content and poorer in vitro digestibility than its acid-extracted or isoelectric precipitated counterparts are the other limitations of the air classification approach (Vogelsang-O'Dwyer et al., 2020).

Many pulses such as cowpea, lentil, mung bean, navy bean, faba bean, and pea have been subjected to air classification for protein extraction. The yield and purity of protein varied with the pulse type, and the maximum and minimum protein purities were observed for faba bean and lentil, respectively. After the first air classification step, 71% to 75% of protein was obtained from faba beans. The second protein-rich fraction derived after re-milling of the high starch fraction (HSF) contained 64–68%

of protein. In the case of lentils, the protein concentration in fractions obtained after the first and second steps of air classification was 49–65% and 38–54%, respectively. The corresponding starch content in the protein fractions after the first and second milling was 0–4.6% and 4–10.4% (Tyler, Youngs & Sosulski, 1981).

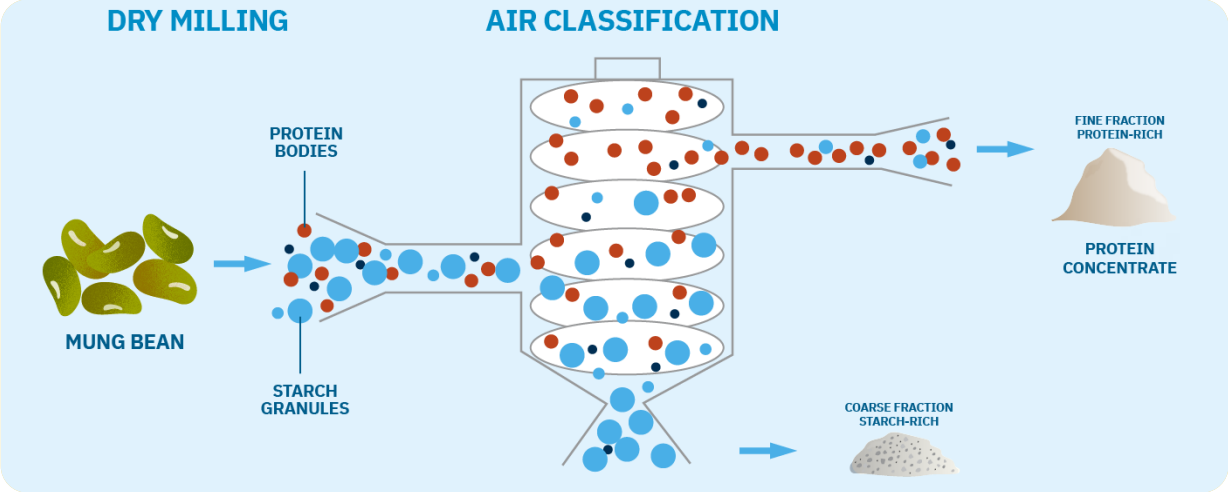

Figure 6. Schematic representation of the extraction of protein by air classification (Modified and redrawn from Zhu et al., 2020)

Triboelectric separation is another dry fractionation technique to extract protein from flours and their air-classified fine and coarse fractions (Pelgrom et al., 2015; Tabtabaei et al., 2016 & 2017). An external electric field is applied to particles entrained in a gas flow through a channel, which turn charged as they collide with the walls of the channel. Subsequently, the particles are separated based on the difference in charge (Wang et al., 2015). 42% of protein was extracted from navy bean flour using triboelectric separation (Tabtabaei et al., 2016). Future research should focus on improving the protein content resulting from this method.

Green technologies for plant protein extraction

Enzymatic extraction

Working principle: This method of plant protein extraction is based on the hydrolytic action of enzymes on the major components of the cell wall, such as cellulose, hemicellulose, and pectins, to release the cellular proteins. Food-grade enzyme preparations, including carbohydrase (a group of enzymes from the large family of glycosidases that catalyse reactions converting carbohydrates into simple sugars) and protease (enzymes that break down protein) can facilitate the extraction of protein from different plant sources (Fig. 7).

Factors influencing the extraction yield of protein:

- Enzyme-to-substrate ratio
- pH
- Incubation temperature
- Hydrolysis time

Advantages: Compared to physical and chemical extraction processes, the use of enzymatic method for the extraction of plant proteins offers the following advantages:

- Mild operating conditions
- Low waste generation
- Reduced energy consumption
- Enhanced nutritional digestibility and techno and bio-functional properties of the extracted proteins, mainly while using protease-assisted extraction

Limitations:

- Enzymes are relatively expensive for industrial-scale production
- Available enzymes cannot break down the plant cell walls completely
- Enzyme-assisted extraction is not always feasible to be applied on an industrial scale because enzyme action is limited by environmental conditions.

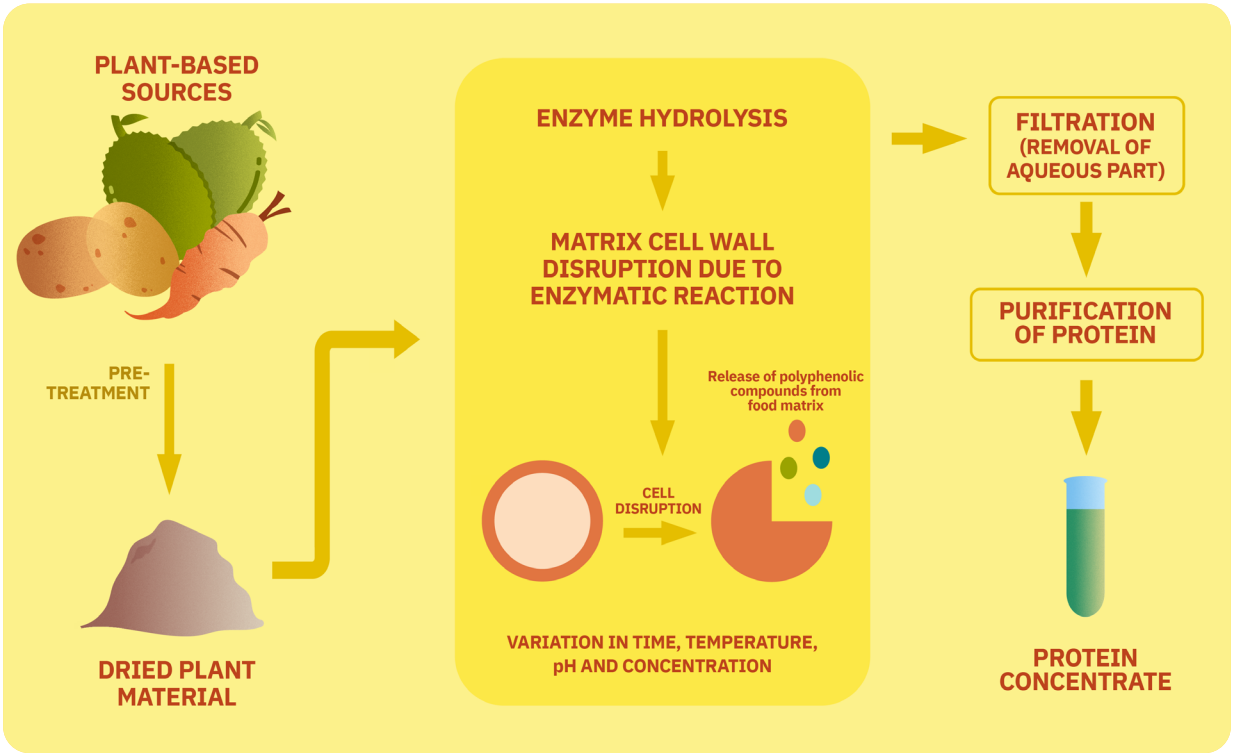

Figure 7. Enzyme-assisted plant protein extraction

Ultrasound-assisted extraction

Working principle: This unconventional and non-thermal technique for plant protein extraction subjects the plant material to high-intensity and low-frequency sound waves ranging from 20 to 100 kHz (Pojic, Misan & Tiwari, 2018). This method works based on the principle of the 'cavitation' phenomenon that comprises a cascade of events: (1) formation of air bubbles in the liquid phase; (2) volume expansion of air bubbles, and (3) explosion of air bubbles. This creates high shear forces in the extraction medium, improves the solubility of compounds to be extracted by imposing high stress and deformation on the cellular structure (Gençdag, Görgüç & Yilmaz, 2020), and enhances the mass transfer of compounds into the extraction medium (Görgüç, Bircan & Yilmaz, 2019). Further, the bubble collapse improves the mass transfer by the creation of microchannels (Fig. 8).

Factors influencing the extraction yield of protein: Extraction temperature and time, intensity and energy density of ultrasound, sample size, solvent-to-sample ratio, turbulence, mixing effects by cavitation, sonoreactor, and sonotrode characteristics

Advantages: Ultrasonication is a favourable approach for plant protein extraction owing to its environmental sustainability. Moreover, high-intensity ultrasound is a quick and cost-effective technology to modify the structural and functional properties of globular proteins (Xiong et al., 2018). Ultrasonication can modify and functionalise proteins by altering the H-bonds, obtaining high protein yield using short extraction time, and reducing the degree of protein aggregation (Contreras et al., 2019). In addition, the cavitation bubbles on the protein surface cause micro-jetting and particle breakdown, which improve the permeability of solvent into the food matrix and change the protein allergen conformation and reactivity (Contreras et al., 2019; Pojic, Misan & Tiwari, 2018).

Limitations: Ultrasound can be used at low, medium, high, or extreme power/intensity levels. Generally, the food industry uses high-power/intensity ultrasonication. However, the use of ultrasound at certain levels for a long treatment time can cause protein denaturation due to elevated temperatures and the generation of reactive oxygen species. A rise in temperature over an extended sonication time can break the hydrogen and hydrophobic bonds and lead to protein unfolding, denaturation, and consequent alterations in the protein's structure and functionalities. Nevertheless, these changes may be favourable or unfavourable depending on the end-use of the protein for targeted food applications. Further, the cavitational forces generated in the ultrasound extraction medium (i.e., water) can decompose the water molecules

to their constituent radicals (i.e., hydroxyl radicals and hydrogen atoms), the concentration of which depends on the degree of ultrasound power/intensity (Weiss, Kristbergsson & Kjartansson, 2011; Rahman et al., 2020). These free radicals can oxidise the free SH groups to SS bonds, modify the secondary and tertiary structures of protein and bring about aromatic hydroxylation and the formation of carbonyl groups (Zhu et al., 2018). The consequent protein oxidation causes structural changes that can change the nutritional and functional properties of the isolated protein.

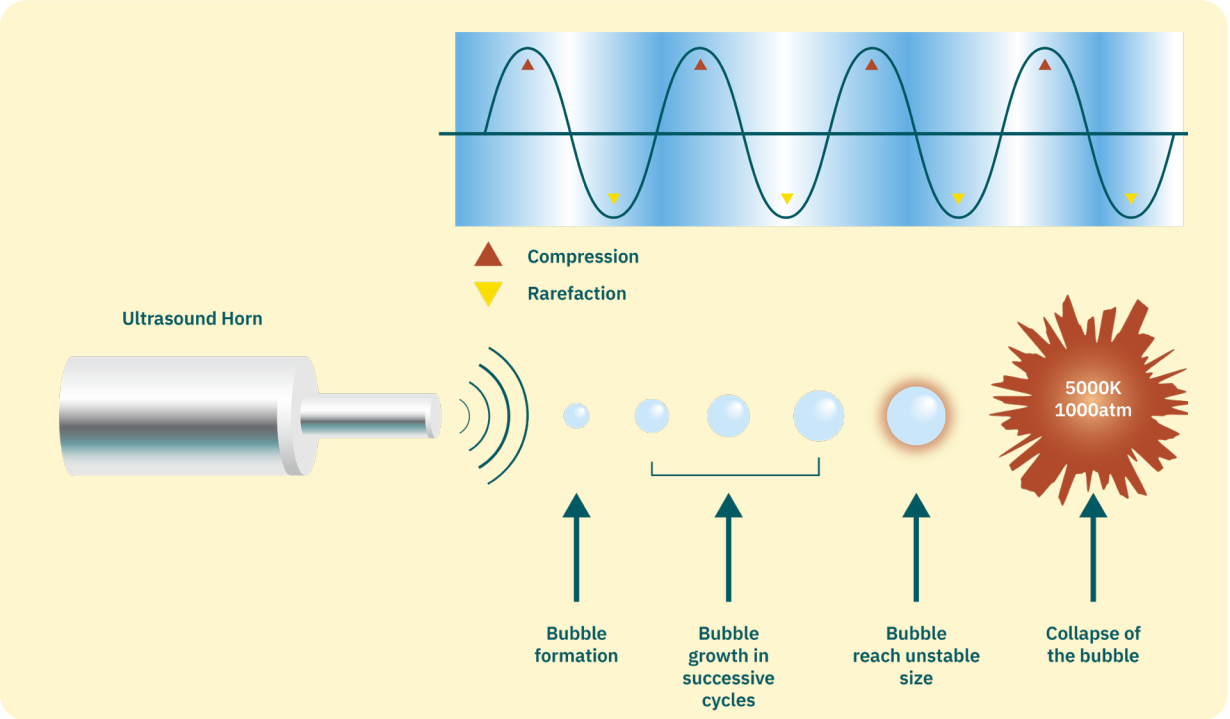

Figure 8. Mechanism of ultrasound-assisted protein extraction

Pulsed electric field-assisted extraction

Working principle: Pulsed electric field (PEF) treatment applies ultra-short electric pulses (10^{-4} to 10^{-2}s) and relatively high amplitude (0.1–80 kV/cm). This method facilitates the protein extraction process by generating a critical electrical potential across cell membranes (Contreras et al., 2019; Pojic, Misan & Tiwari, 2018). Application of PEF to plant cell tissues positively alters the membrane transport characteristics and promotes the extractability of protein.

Factors influencing the extraction yield of protein: Amplitude, number, shape, and pulses duration), characteristics of the solvent and sample composition (i.e., shape, size, pH level, and conductivity), characteristics of the tissues and cells under extraction, choice of solvent, electric field strength, temperature, and time of PEF extraction

Advantages: Enhanced mass transfer, high extraction yield, short processing time, minimal degradation of proteins, and reduced energy costs (Melchior et al., 2020).

Limitations: A major factor that limits the commercial applications of PEF processing is that the effectiveness of treatment can be affected by the device parameters and external factors such as conductivity, pH, and the concentration of the starting solution. The abovementioned consequences are mainly due to the inevitable electrochemical reactions that take place at the electrode–food interface of a PEF treatment chamber under the typical conditions for PEF processing. Future research efforts should focus on minimising these reactions leading to corrosion and fouling of the electrodes, electrolysis of water, migration of electrode material components, and chemical changes of food products. Resolving these challenges can improve the commercialisation scope of PEF technology and improve its safety, quality, process efficiency, equipment reliability, and cost aspects.

Microwave-assisted extraction

Generally, microwave-assisted extraction (MAE) is recommended for the extraction of proteins from biological materials with hard structures that are difficult to digest by enzymes and ultrasound (Kumar et al., 2021).

Working principle: Microwave processing employs non-ionizing electromagnetic waves with frequencies ranging from 300 MHz to 300 GHz (Datta & Anastheswaran, 2001). Microwave-assisted extraction works on the principle of direct heat generation within the solvent by ionic conduction of the polar solvent's dipole rotation and dissolved ions (He et al., 2014). Therefore, constituents with low polarity are not heated due to microwave exposure. However, the heat generated disrupts the cell wall of plant material, breaks the H-bonds, increases the food matrix porosity, and facilitates the extraction of protein with enhanced functionality (Pojić, Misan & Tiwari, 2018).

Factors influencing the extraction yield of protein: Microwave power level, solvent nature, solvent-to-feed ratio, extraction time, temperature, sample size and geometry, effect of stirring/system agitation, solubility, dielectric constant, and dissipation factor

Advantages: The key advantages of microwave-assisted extraction are its efficiency in inactivating the antinutritional factors present in plant proteins (Vagadia, Vanga & Raghavan, 2017) and improving protein digestibility (Sá, Moreno & Carciofi,

2019). Compared to conventional/thermal protein extraction approaches, MAE demonstrates uniform heat distribution within the raw material for extraction, rapid extraction rate, lower solvent consumption, and shorter extraction time (Bußler et al., 2015). Further, combining MAE with other physical or biochemical methods can synergistically improve the efficiency of protein extraction.

Limitations: The high amount of heat energy that is generated during microwave extraction can degrade the heat-labile bioactive constituents, thus limiting its applications for protein extraction.

High pressure-assisted extraction

Working principle: High pressure-assisted extraction (HPAE) is a non-thermal approach for protein production that subjects a feed material to hydrostatic pressures up to 1000 MPa under controlled temperature and time conditions (Júnior et al., 2017). After mixing the starting material with the extraction media and placing it inside the pressure vessel, the pressure is increased from ambient to a predefined level ranging between 100 to 1000 MPa within a short duration. As the pressure increases, the differential pressure between the intracellular and extracellular environments increases, which leads to cell deformation and cell wall damage. The solvent penetrates through the damaged cell wall and cell membrane into the cell, increasing the mass transfer of soluble compounds (Kumar et al., 2021).

Factors influencing the extraction yield of protein: Extraction pressure, operating time, nature and concentration of extraction solvent, and solid-liquid ratio

Advantages: HPAE is effective in improving protein functionality and digestibility, besides inactivating their antinutritional factors (Vagadia, Vanga & Raghavan, 2017). This has been proven in the case of proteins derived from cereals and legumes (Belmiro et al., 2018). In addition, HPAE exhibits a faster extraction rate and results in pure protein at a high yield. As this process is carried out at ambient temperature, thermal degradation of proteins is avoided. Also, HPAE is an eco-friendly extraction method since it does not require the use of solvents.

Limitations: A major challenge associated with HPAE is the high cost of the plant mainly incurred by the safety assurance of the process due to its high-pressure operation. However, the high operational cost is justified and counterbalanced by the high purity of the resultant protein that is obtained with minimal requirement for post-processing operations.

Deep eutectic solvent extraction

Working principle: Deep eutectic solvent (DES) is a newly developed protein extraction solvent that has opened up new research avenues because of its high sustainability, cheap cost, biodegradability, and nontoxicity (Patra, Prasath & Pandiselvam, 2023). A mixture of two or more ionic and non-ionic compounds in a particular molar ratio is known as a deep eutectic solvent, wherein an external force—such as heating, stirring, mechanical forces, sonication, or microwave—is needed to bring the individual melting points of the compounds down to a common eutectic point.

Factors influencing the extraction yield of protein: Temperature, molecular structure and composition of the DES (polarity of DES), toxicity of DES, viscosity, extraction time, water content in the DES system, additives to DES, solvent-to-sample ratio, pH, and separation techniques

Advantages: The use of DES for plant protein extraction offers several advantages such as environmental sustainability, low processing cost, and favourable solvent characteristics such as wide range of polarity, low volatility, vapour pressure and toxicity, high thermal and chemical stability, inflammability, biodegradability, and so on (Silva et al., 2019). In addition, as their constituents react through intermolecular forces rather than covalent or ionic interactions, deep eutectic solvents are effective alternatives to ionic liquids and other conventional/corrosive solvents (e.g. sodium hydroxide, hydrochloric acid, sulfuric acid) (Liu et al., 2018). Thus, DES extraction is a promising green approach for plant protein extraction.

Limitations: Despite the abovementioned merits, not every DES is suitable for protein extraction. Currently, a universal method is not available to choose a DES based on the intended application, which demands screening of deep eutectic solvents before conducting the protein extraction trials (Smith et al., 2014). On the other hand, the viscosity and conductivity of DESs are temperature-dependent. At ambient temperature, the viscosity of a DES is generally higher than that of water, but it decreases with rise in temperature. Contrarily, its conductivity increases with temperature (Lores et al., 2016). This temperature dependency poses serious limitations during the recovery and isolation of proteins from the DES extraction medium ('back extraction') and impedes the scalability and industrial applications of this extraction method. The high interfacial mass transfer resistance decelerates the separation of protein from DES (Kaijia et al., 2015). Therefore, future efforts should be focused on improving the protein back extraction methods and the recovery of deep eutectic solvents in order to make this process industry-friendly (Li et al., 2016).

Subcritical water extraction

Working principle: This method uses subcritical water as the medium to extract less polar components within a short extraction time of 30 min (Ko, Cheigh & Chung, 2014). Subcritical water (SCW) is defined as water that is maintained in a liquid state under temperature (100–374°C) and pressure (less than 22.064 MPa). Higher temperatures reduce the dielectric constant of water and weaken its hydrogen bonding to bring the subcritical water closer to less-polar organic solvents such as ethanol and methanol. Consequently, water in the subcritical state presents unique traits, such as a shift in the structure of its hydrogen bonds and an enhanced ionic product, K_w, which is three-fold higher than that of water under ambient conditions. The increased concentration of ionic products accelerates the production of hydronium (H_3O+) and hydroxide (OH^-) ions, due to which SCW can act as a base or an acid catalyst (Kumar et al., 2021).

The subcritical water extraction occurs comprises the following six steps (Haghighi & Khajenoori, 2013) (Fig. 9):

1. Rapid entry of the fluid;
2. Desorption of solutes from the active sites of the feed material;
3. Diffusion of solutes through organic materials;
4. Diffusion of solutes through static fluid in porous materials;
5. Diffusion of solutes through a layer of stagnant fluid outside particles; and
6. Elution of solutes by the flowing bulk of fluid.

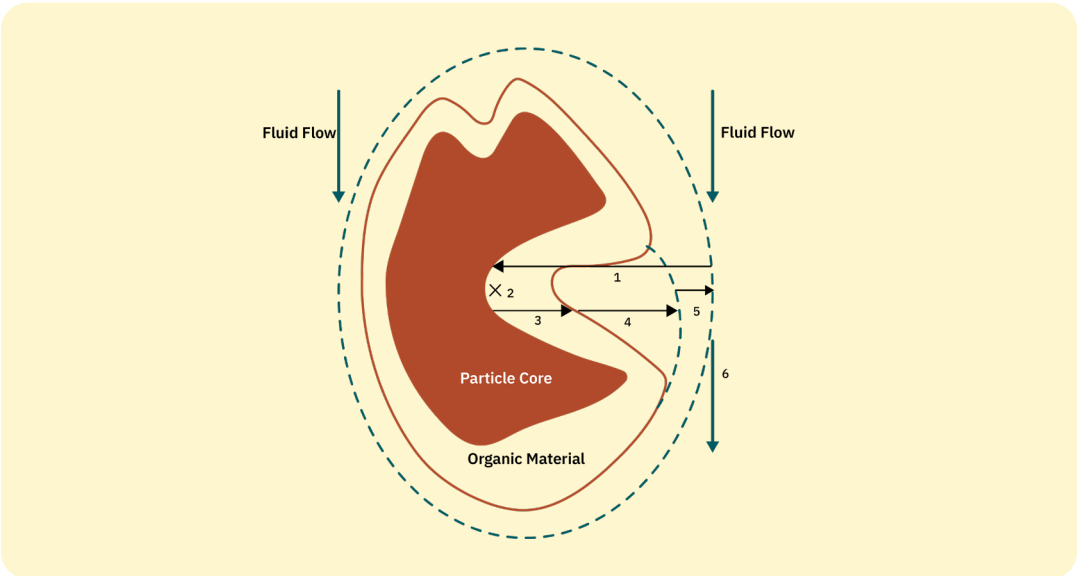

Figure 9. Different steps of the subcritical water extraction process (Redrawn from Haghighi & Khajenoori, 2013)

Factors influencing the extraction yield of protein: Critical factors that influence SWE are extraction temperature, reaction time, water-to-solid ratio, physical state of the feed material (dry powder or slurry forms), particle size of the feed material, water flow rate, type, and amount of catalyst used. For analytes with poor SBWE efficiency, a small number of organic modifiers such as ethanol, surfactants, or ionic liquids may be added.

Advantages: Due to the non-toxicity of water and the absence of liquid waste for disposal, subcritical water extraction is a green technology for the extraction of plant proteins. SWE is rapid, clean, and less expensive than its conventional counterparts. Relative to organic solvents, subcritical water is advantageous in terms of its temperature-tunable density, concentration of ionic product, and dielectric constant, which facilitate selective extraction of polar compounds at lower temperatures and less polar components at higher temperatures (Kumar et al., 2021). Specifically, SCW extraction by the principle of hydrolysis has been identified as an effective technique to extract proteins from agro-food industrial side streams such as sunflower cake/ meal and rice bran.

Limitations: The safety of the SWE process is critical as it involves a high-temperature and high-pressure operation. The temperature stability of protein to be extracted must be evaluated prior, as high temperature can potentially lead to its degradation. Further, frequent pluming blockage may occur during the SWE process.

The plant protein-related applications of the different extraction methods described above are summarised in Table 1.

Table 1. Extraction of plant proteins using different extraction techniques: Summary of processing conditions & salient findings

Protein extraction techniques	Type of plant protein	Processing conditions	Salient findings	Reference
Water-based extraction	• Mung bean (Vigna radiata)	• Liquid ratio: 10%, Extraction temperature: 31.74°C, Extraction pH: 8.97, Settlement pH: 4.4, Extraction time: 33.24 min	• Maximum protein yield: 78.33%	• Wang et al. (2011)
	• Cowpea (Vigna unguiculata)	• Ground whole cow pea/pulse flour/cow pea pulse dehulled flour to water: 1-10% w/v; Stirring RPM: 1000 to 5000 RPM, Stirring time: 1-6 h (preferably 6 h), pH: 4-8 (preferably 7), Temperature: 2-65°C (preferably 65°C)	• The end product is cowpea protein concentrate with a maximum protein content of 70%	• Prabhakar (2022)

Alkaline extraction followed by isoelectric precipitation	• Winged bean • Tea • Mung bean • Ground nut • Tomato • Peanut • Grass, barley straw and oolong tea residue		Protein yield: • 94.8% • 89.7% • 77.32 • 86% • 80.37% • 85.2% • 95% from grass, 95% from barley straw and 92% from oolong tea residue	• Riyawati et al., 2015 • Cui et al. (2017) • Du et al. (2018) • Jain, Prakash, and Radha (2015) • Mechmeche et al. (2017) • Shafiqur et al. (2018) • Zhang et al. (2014)
Air classification	• Corn fiber • Soybean hulls • Yellow pea • Lupine (defatted)	• Pin milled • Pin milled • Pin milled/impact milled • Impact milled	Maximum protein content (% dry matter) • 17.1% • 43.31% • 42.9% • 56.9%	• Wu and Norton (2001) • Wolf et al. (2002) • Pelgrom, Boom & Schutyser (2015a) • Pelgrom et al. (2015b)
Enzyme-assisted extraction	• Soybean • Peanut • Sesame bran • Moringa oleifera seed • Rapeseed meal	• Protease M®, pH 4.5, 50-100°C, 10-120 min • Alcalase® 1.5%, 60°C, pH 9.5, 5 h • Viscozyme L®, Alcalase®, 25-55°C, 10-120min • Protex 7L®, 45°C, 15 min • Viscozyme®, Alcalase®, 80 min	• Protein yield: 59.3% • Protein yield: 71.4% • Protein yield: 88.8% • Protein yield: 75.4% • Protein yield: 82.1%	• Lu et al. (2016) • Jiang et al. (2010) • Görgüç et al.(2019) • Latif et al.(2011) • Niu et al. (2012)
Ultrasound-assisted extraction	• Soybean • Soybean okara (by-product) • Millet protein concentrate • Pea protein concentrate • Cowpea	• 20 kHz, 20min • 20 kHz, 65W • 20–100W, 18.4-73.9 W/cm², 5-20 min • 412.5–712.5W, 336–582 s • 100 and 200 W, 5 to 20 min	• Inactivation of trypsin inhibitor by 55% • Protein yield: 70% • Improvement of solubility and emulsifying capacity • Improved emulsifying properties • Increase in protein yield (58.96%), solubility (68.85%), water- holding capacity (3.68 g/g), foaming capacity and stability (83.74%, 60.01%) emulsion activity and stability (64.26%, 87.71%) , zeta-potential (44.2 mV), and in-vitro protein digestibility (89.99%)	• Huang, Kwok & Liang (2008) • Preece et al. (2016) • Nazari et al. (2018) • Oliveira et al. (2020) • Loushigam & Shanmugam (2023)
Pulsed electric field-assisted extraction	• Rapeseed stems and leaves • Pea, rice, and gluten protein concentrates	• 0.2–20 kV/cm • 60,000 pulses, 1.65 kV/cm	• Protein yield: 80% • Modified protein structure by inducing unfolding, intramolecular rearrangement, and formation of aggregates.	• Yu et al. (2015) • Melchior et al. (2020)

Microwave-assisted extraction	• Soybean • Rapeseed meal • Pumpkin protein	• 2450 MHz, 500 W, 2-4min • 800 W, 2-6min	• Inactivation of trypsin inhibitor • 2 and 4 min increased in vitro protein digestibility (IVPD) and for 6 min decreased IVPD • Extraction yield: 93.95%	• Esaka et al. (1986) • Arvanitoyannis & Tziatzios (2010); Sadeghi & Shawrang (2006) • Chao, Jung & Aluko (2018)
High pressure assisted extraction	Soy protein	• 350 MPa/20°C/16 min • 200–700 MPa/20°C/ 20 min • 400–600 MPa/20°C/20min • 50–125 MPa	• Reduced allergenicity by 46.6% • Efficient to eliminate phytates • Increased IVPD 68% • High extraction yield of 82% at 100 MPa	• Li et al. (2016) • Torrezan, Frazier &, Cristianini (2010) • Su et al. (2010) • Preece et al. (2017)
Deep eutectic solvent extraction	Pea and rice protein isolate	107°C, pH 9–11	Enhanced solubility, emulsifying, foaming, and gelling	Pietrysiak et al. (2018)
Subcritical water extraction	• Sunn hemp protein • Rice bran protein isolate	• Temperature: 160-240°C, Reaction time: 30 min and 60 min, Different biomass feeding methods: Dry powder versus slurry feeding, Different catalysts: NaOH and Na2CO3), Different catalyst amount: 2.5-28% (w/w); Optimum conditions: Temperature: 240°C, catalyst amount: 28% • Optimum conditions: Solid/water ratio: (0.12), bran-to-rice ratio: 8:92, time: 45 min, particle size: 420 µm; temperature: 120°C	• Maximum protein extraction yield: 74% • Enhanced protein yield and functional properties such as solubility, emulsifying activity index	• Nyankson et al. (2013) • Ardali et al. (2023)

Environmental and energy sustainability of plant protein extraction techniques

Food producers and consumers are shifting to plant proteins mainly for sustainability and health reasons. Plant proteins are expected to impart similar techno-functionalities as animal-derived proteins in plant-based meat, egg, and dairy products to achieve sensory parity. It is equally important to ascertain the sustainability metrics of plant proteins. Therefore, it is necessary to understand the environmental impact of the different protein fractionation techniques. From the above sections, it is evident that the production of plant protein isolates and concentrates involves using energy or chemicals, or both. In general, the environmental and energy sustainability of the protein fractionation processes is inversely related to the degree of refining. 'Low degree of refining' refers to the omission of chemicals during the protein extraction process and the ability of the fractionation method to valorise the whole crop. For instance, dry fractionation and mild aqueous fractionation techniques do not require the same amount of chemicals and energy as the other methods. Moreover, these methods avoid water, which dilutes the feed material, demands a drying step at the end, and emanates wastewater that contains a fraction of the proteins and other valuable macromolecules.

A Life Cycle Analysis (LCA) conducted in 2021 compared the environmental impact of protein-rich fractions derived from starch and oil-bearing crops using conventional and milder fractionation. The study hypothesised that due to the lower use of resources, a lesser degree of refining reduces all the sustainability indicators such as the global warming potential (kg CO eq), human carcinogenic toxicity (kg 1,4-DCB), land use (m^2a crop eq), mineral resource scarcity (kg Cu eq), dearth of fossil resources (kg oil eq), and water consumption (m^3) (Lie-Piang et al., 2021). This study demonstrated that using dry instead of conventional fractionation reduces the impacts by up to 99% with case studies involving yellow pea and lupine and concluded that the environmental impact of plant-based protein production methods can be significantly reduced by decreasing the degree of refining (Fig. 10). This is because processing is a major contributor to the total environmental impact, which can be larger than that of crop cultivation in certain cases. For example, avoiding the protein precipitation step in mild aqueous fractionation reduces the impact on the abovementioned sustainability indicators by 30–40%. Besides, eliminating the oil extraction step in the fractionation process of oil-rich seeds reduces the impact parameters by 20–30%. The drying step is the most impactful step, avoiding which

can lead to a substantial reduction (up to 93%) in the global warming potential compared to the conventional way of fractionation. Choosing between conventional wet extraction methods and dry fractionation comes down to a compromise: the former provides high yield and purity of protein but at a greater environmental cost, while the latter is more sustainable and uses less energy but results in protein ingredients that are less refined (Lie-Piang et al., 2021). Compared to conventional fractionation, mild aqueous fractionation and dry fractionation exhibit higher exergy efficiency (35% versus 54% and 99–100%, respectively), mainly due to the loss of immaterial exergy and limited usage of electricity (Geerts et al., 2018). Thus, a combination of dry and mild aqueous fractionation can be a promising solution towards obtaining sustainable plant protein ingredients with lower environmental impact and reduced water and energy consumption.

		Yellow pea				Lupine			
Impact category	**Unit**	Conventional fractionation	Mild aqueous fractionation	Dry fractionation	Hybrid fractionation	Mild aqueous fractionation	Conventional fractionation	Dry fractionation	Hybrid fractionation
Global warming	**kg CO eq**	**940**	**541**	**107**	**251**	**1464**	**1156**	**107**	**564**
Stratospheric ozone depletion	kg CFC11 eq	0.0002	0.0001	0.00003	0.00006	0.00034	0.0003	0.00003	0.00014
Ionizing radiation	kBq Co-60 eq	16.8	9	3.8	6	24.5	24.6	3.8	12.1
Ozone formation, Human health	kg NOx eq	0.012	0.0042	0.0008	0.0019	0.0979	0.0613	0.0008	0.0563
Fine particulate matter formation	kg PM2.5 eq	0.26	0.13	0.03	0.07	0.48	0.41	0.03	0.19
Ozone formation, Terrestrial ecosystems	kg NOxeq	0.0168	0.0068	0.0013	0.0031	0.1277	0.0688	0.0013	0.0607
Terrestrial acidification	kg SO_2 eq	0.83	0.41	0.1	0.21	1.57	1.33	0.1	0.61
Freshwater eutrophication	kg P eq	0.00006	0.00003	0.00001	0.00001	0.00013	0.00012	0.00001	0.00006
Marine eutrophication	kg N eq	0.004	0.0016	0.0002	0.0006	0.0091	0.0067	0.0002	0.0027
Terrestrial ecotoxicity	kg 1,4-DCB	39.1	18.3	6.8	11.5	81.1	77.7	6.8	37.2
Freshwater ecotoxicity	kg 1,4-DCB	0.017	0.009	0.003	0.005	0.028	0.026	0.003	0.013
Marine ecotoxicity	kg 1,4-DCB	0.084	0.045	0.013	0.025	0.157	0.143	0.013	0.082
Human carcinogenic toxicity	**kg 1,4-DCB**	**0.28**	**0.17**	**0.02**	**0.07**	**0.43**	**0.31**	**0.02**	**0.15**
Human non-carcinogenic toxicity	kg 1,4-DCB	2.04	1.07	0.35	0.62	4.11	3.32	0.35	1.69
Land use	m^2a crop eq	0.11	0.07	0	0.02	0.2	0.14	0	0.08
Mineral resource scarcity	kg Cu eq	0.03	0.017	0.004	0.009	0.048	0.041	0.004	0.021
Fossil resource scarcity	**kg oil eq**	**248+**	**153**	**25**	**67**	**370**	**283**	**25**	**146**
Water consumption	**m^3**	**15.2**	**10.4**	**0.2**	**3.1**	**27**	**18.3**	**0.2**	**9.6**

Green *Yellow* *Peach* *Rust*

Figure 10. Environmental impacts of the fractionation of yellow pea and lupine to process 1000 kg crops, excluding cultivation (Green-yellow-peach-rust boxes indicate the fractions with the lowest to highest impact among all fractions from both lupine and yellow pea) (Redrawn from Lie-Piang et al., 2021)

Appendix

Glossary of terms

Protein: Proteins are macromolecules that are polymer chains of amino acids linked together by peptide bonds.

Amino acid: Amino acids are small organic molecules functioning as the building blocks of proteins. Each amino acid molecule has a carboxylic acid group and an amine group that are each attached to a carbon atom called the α carbon.

Albumins: A class of proteins found in both plant and animal tissues. Albumins are water-soluble and form solid or semi-solid masses upon heating.

Globulins: Globulins are a major type of seed storage protein, which are widely distributed among higher plants. These proteins are soluble in dilute salt solutions.

Prolamins: Prolamins are a category of seed storage proteins rich in the amino acids proline and glutamine and deficient in lysine. These are the main storage proteins in cereals and other members of the grass family. Due to their rich cysteine content, prolamins are stable to thermal processing and enzyme proteolysis. These proteins are insoluble in water and dilute salt solutions but soluble in 60–80% alcoholic solutions.

Glutelins: Glutelins are a class of water-insoluble plant proteins found in cereals. They form a major component of the protein composite known as gluten. These proteins are soluble in dilute acids and alkalis. Glutelins coagulate when heated. Examples are glutenin from wheat and oryzenin from rice.

Plant protein extraction: The process of isolating and purifying the protein from the plant matrix which is a composite of polysaccharides (starch), fat (oil) and fibre.

Mass transfer: Mass transfer is the transportation of a substance or constituent (mass) in liquid and gaseous media from a region of higher concentration to that of a lower concentration.

Extraction yield of protein: Extraction yield of protein is defined as the ratio of protein mass contained in the aqueous extracts or dry fraction to the protein masses estimated in the starting material (whole grains or seeds, grain or seed flour, leaf powder, or spent materials).

Enzymes: Enzymes are proteins that help speed up metabolism, or the chemical reactions in our bodies.

Enzymatic hydrolysis: Enzymatic hydrolysis is a biochemical process in which enzymes cause the cleavage of bonds in molecules with the addition of the elements of water. It plays an important role in the digestion of food.

Plant-based foods: Refers to products made from plants that are alternatives to animal-based products. This includes plant-based meat, eggs, dairy and seafood that are produced directly from plants. Like animal products, they are composed of protein, fat, vitamins, minerals, and water. Next-generation plant-based options look, taste, and cook like conventional meat, and offer complex carbohydrates and fibre.

Life Cycle Analysis (LCA): An LCA is a systematic analysis of environmental impact throughout the entire life cycle of a product, material, process, or other measurable activity.

Directory of plant protein ingredient suppliers

IFF

IFF provides end-to-end services and has a diverse product portfolio, including soy & pea proteins, probiotics, enzymes, and other plant-based solutions.
Specialisation: Soy and Pea based Ingredients

Proeon

Proeon is a Pune-based startup which specialises in extraction of plant based proteins.
Specialisation: Mung and peanut-based protein isolates

Devigere Biosolutions

Founded in 2020, Devigere Biosolutions produces novel, sustainable plant-based protein concentrates for usage in multiple industries. Their products are clean-label

and GMO-free.
Specialisation: Mung and Cowpea-based protein concentrates, egg replacement, and plant based peptides

Relsus
Relsus is a Singapore-based company with its R&D and manufacturing located in India. Relsus has developed an integrated solution for plant-based protein powders and specialty starches.
Specialisation: Plant-based protein concentrates, isolates and specialty starches from sources like Mung, Chick pea, Pea, and Rapeseed

Sun Nutrafoods
Sun Nutrafoods (SNF) is a division of Agro Solvent Products Pvt Ltd established with a vision to manufacture and market premium quality non-GMO plant-based ingredients for applications in food, nutraceutical, pharmaceutical, and feed industries.
Specialisation: Soy and Pea based TVP and ingredients

Kerry
Kerry is a large ingredient supplier that has ventured into plant-based proteins with a unique portfolio of plant-based ingredients and solutions for the development of sustainable products that are nutritionally optimised with cleaner labels, authentic taste, and appealing texture.
Specialisation: Plant-based meat and dairy alternatives solutions

Ingredion India Private Limited
Ingredion is a global leader in ingredient solutions, specialising in the transformation of grains, fruits, vegetables, and other plant materials into essential ingredients for a wide range of products, including foods, beverages, paper, and pharmaceuticals.
Specialisation: Ingredient solutions for plant-based meat and dairy alternatives

Barentz India
Barentz India is a life science ingredients distributor specialising in human nutrition, pharmaceuticals, personal care, performance materials, and animal nutrition, creating unique synergies across all fields of expertise.
Specialisation: Plant-based protein ingredients

Agrocorp

Agrocorp India Trade Services Private Limited is a prominent agri-commodity trading company, established in 2004 with a strong focus on sourcing, processing, and exporting agricultural products.

Specialisation: Plant-based protein ingredients

AGT Foods

AGT Food and Ingredients Inc. ("AGT Foods") is a manufacturer of plant-based products, protein concentrates/isolates, pulse, grains, staple food and food ingredient processing and distribution.

Specialisation: Plant-based ingredients

ADM

ADM is a US based international ingredient supplier which specialises in human as well as animal nutrition.

Specialisation: Soy, wheat, and pea-based ingredients

Directory of manufacturers for protein extraction equipment

Alfa laval

Alfa Laval is a leading global provider of first-rate products in the areas of heat transfer, separation and fluid handling.

Specialisation: Separation solutions

Pall corporation

Pall offers innovative purification and filtration technologies for new and expanding markets, leading the way with consistent, reliable performance for state-of-the-art cleaning methods and manufacturing processes.

Specialisation: Filtration, separation, and purification solutions

Steer

Steer is a Bangalore-based company that makes twin screw extruders. They also undertake process development and provide support for trials and developmental studies across various areas within the food processing segment.

Specialisation: Twin-screw high moisture extruder

Steller

Stellar stands out as a premier global design-build firm specialising in food processing, offering a comprehensive range of services executed by a cross-trained expert team. This team brings insights and best practices from various industries, focusing on critical aspects like food safety, innovation, and production rates.

Specialisation: Turnkey service provider

Coperion

Coperion is a global industrial and technological company in the areas of compounding and extrusion systems, sorting, shredding and washing equipment, including conveying, mixing and feeding technology.

Specialisation: Extruders and process equipment

Clextral

Clextral provides integrated turnkey extruder production lines, dryers and ancillary equipment.

Specialisation: Lab instruments

Anton Paar

Anton Paar develops, produces and distributes highly accurate laboratory instruments and process measuring systems, and provides custom-tailored automation and robotic solutions.

Specialisation: Lab instruments

GEA India

GEA is one of the largest suppliers for food processing technology and of related industries. The global group specializes in machinery, plants, as well as process technology and components.

Specialisation: Process equipment

Hosokawa Micron India PVT LTD

The Hosokawa Alpine Group is the manufacturer of machines and systems for processing and handling of powders, granulates and bulk materials, as well as systems for the extrusion of blown films.

Specialisation: Dry separation methods for plant protein extraction

Buhler

Bühler Group is a leading Swiss multinational company specialising in plant equipment manufacturing. Bühler serves the Grains & Food industry by ensuring safe and healthy food and feed production.

Specialisation: High moisture extrusion, Grain processing

Directory of pilot-scale facilities for plant protein extraction

APIC

APIC is a one-of-a-kind integrated research and pilot facility, providing services on contract or rental basis for development of alternative proteins and ingredients.

Specialisation: Plant protein extraction, plant based product development

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