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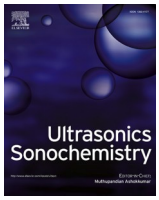

Review

Ultrasound-assisted processing: Science, technology and challenges for the plant-based protein industry

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ABSTRACT

The present-day consumer is not only conscious of the relationship between food consumption and positive health, but also keen on environmental sustainability. Thus, the demand for plant-based proteins, which are associated with nutrition and environmental sustainability. However, the plant-based protein industry still demands urgent innovation due to the low yield and long extraction time linked with traditional extraction methods. Although ultrasound is an eco-innovative technique, there exist limited data regarding its impact with plant-based protein. In this paper, the scientific principles of ultrasonication with regards to its application in plant-based protein research were reviewed. After comparing the cavitational and shearing impacts of different ultrasonic parameters, the paper further reviewed its effects on extracted protein characteristics and techno-functional properties. Additionally, current technological challenges and future perspectives of ultrasonication for the plant-based protein industry were also discussed. In summary, this review does not only present the novelty and environmental sustainability of ultrasound as a plant-based protein assisted-extraction method, but also highlights on the correlation between protein source, structure and subsequent functional properties which are important crucial factors for maximum application of ultrasound in the growing plant-based protein market.

1. Introduction

Globally, the demand for protein has been predicted to grow from $25.62 billion to $48.77 billion between 2016 and 225 [1]. Proteins are macromolecules required for optimal growth and development. Notwithstanding their basic nutritional value, research has attributed protein consumption to positive health benefits such as antioxidative, anti-diabetic, antihypertension and many more [2]. Dietary proteins are either sourced from animal (e.g., meat, egg and dairy) or plant (e.g., legumes, cereals, grains and microalgae) sources. However, in recent times consumer demand for plant proteins has gained massive prominence over animal proteins due to (i) increased knowledge of the relationship between plant proteins and positive health compared to animal sources (ii) high cost of animal-based proteins (iii) negative environmental effects from animal protein production (iv) high demand for kosher and halal foods [3]. With the global population predicted to reach about 9.22 billion by 207 [4]. there is the urgent need to sustainably ensure nutrition security without sacrificing the next generations environmental sustainability. In pursuit of this, plant-based proteins are promising sources because they are healthy diets, eco-friendly and sustainable.

Although it can be argued that the plant protein industry has reached its maturation stage, there is no doubt that there still exists a compelling need for innovation. One of the most critical stages needing innovation is the extraction process, where eco-innovative techniques are urgently needed to optimize protein extraction yield, without compromise on environmental sustainability and the protein's nutritional and techno-functional properties. Because proteins are structurally bounded to other biomolecules such as carbohydrates, fats, fiber and polyphenols, an efficient extraction method should be able to break these structural bonds, disintegrate structural membranes and cell walls for effective release of proteins. In pursuance of this, in-depth literature has been reported on traditional extraction methods such as alkali, salt and solvent based extraction methods [3]. However, the challenge with these traditional extraction methods is their inability to efficiently disrupt structural molecules, thus, extracting about half of the available proteins in a plant system [5]. To bridge this challenge, several pre-treatment methods including enzymatic and physical techniques (e.g., pulsed electric field, ultrasound, microwave and high pressure) are currently being employed to maximize structural disintegration and optimize

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protein extraction yield.

However, due to the high-cost, complexity and possible racemization of amino acids associated with enzymatic pre-treatment, physical technologies are gaining the attention of researchers. Among these physical techniques, although ultrasound require less energy use, installation and maintenance cost, it is the least applied in the plant protein industry [6]. The technology of ultrasound works by acoustic cavitation, where there is a sequential formation and asymmetric implosion of low-pressure bubbles with mechanical and chemical effects [7]. Cavitational and mechanical stresses created by ultrasound can improve extraction yield and functionalities of plant proteins through its creation of cellular wounds and disintegration of structural bonds and molecules bounded to proteins [8]. Although ultrasound has been proven to effectively disintegrate cells walls and membranes, the food industry has largely exploited its potential with the extraction of bioactive compounds such as polyphenols. Extremely scanty literature has been documented on the application of ultrasound for plant protein extraction. Considering the eco-innovativeness of ultrasonication and its disruptive cavitational effect, in-depth information on its influence in protein research will guide the food industry in making strategic decisions towards their extraction and processing of sustainable plant proteins/formulated products. Therefore, the focus of this review is to discuss and bring to light a deeper understanding of the conceptual principles of ultrasonication and related critical factors (such as technological, molecular and biochemical factors) to be considered when exploiting its full potential for optimized plant protein extraction and functionalities.

2. Plant-based protein extraction techniques

In a plant system, proteins are bounded to structural and phyto molecules such as polysaccharides, starch, sugars, fiber and secondary metabolites [9]. Thus, to maximize isolation of proteins from a plant matrix, it is crucial to break intramolecular bonds, weaken and disintegrate intact cell structures for efficient diffusion of extraction solvent, separation of proteins and subsequent liberation of proteins into extraction medium. Irrespective of the choice of extraction technique, protein extraction involves three main steps, including sample defatting, protein extraction and protein precipitation. This section of the review will highlight on traditional plant-based protein extraction techniques, and their limitations, as well as discuss the potential of ultrasound as a pre-treatment to leverage these challenges compared to other physical pre-treatment techniques.

2.1. Traditional plant-based protein extraction methods

2.1.1. Alkali-based extraction

Alkali extraction is the most widely applied technique in the plant protein industry. This method involves pH modulation within ranges of 8.5–9, based on the principle that, ionization of acidic and neutral amino acids occurs at high pH, thus, increasing protein solubility in an extraction solvent for maximum yield [10]. Another explanation for alkaline extraction is that basic pH can break disulphide bonds linking thiol groups of cysteine residues, increasing unfolding, and hydrophobicity for improved extraction yield. Briefly, alkali method include extraction at slightly basic pH; protein precipitation at isoelectric pH; resolubilization at neutral pH [11]. Factors such as temperature, choice of alkali (e.g., NaOH and KOH), alkali concentration, plant substrate to solvent ratio, extraction time and plant substrate molecular size are key for maximum protein extraction yield [12].

The wide use of alkali technique for plant protein extraction can be attributed to its low cost, enhanced digestibility and bioavailability of extracted proteins. However, this technique is limited by (i) its reduced yield of extracted protein (about 50%) (ii) reduced protein quality through denaturation, loss of amino acids and formation of lysinoalanine (iii) time consuming (iv) energy intensive (v) non-ecofriendly due to the use of organic solvents [13]. Nevertheless, these limitations can be resolved by introducing green and novel physical pre-treatments capable of (i) breaking the complex bonding of proteins to other macromolecules (e.g., starch, pectin and cellulose) in the plant matrix to enhance protein extraction yield (ii) reducing the use of high alkali concentrations and volumes for protein quality and environmental sustainability (iii) operating at reduced temperatures and extraction time to avoid the loss of protein quality [12].

2.1.2. Aqueous and organic solvents extraction

Aqueous protein extraction is one of the commonest methods of protein isolation. Its advantage is due to its high solubility and stability of isolated proteins [14]. Depending on the type of plant substrate and its matrix characteristics (i.e., presence of proteins with polar/and or non-polar side chains, aromatic amino acids and lipid binding capacity), different protein substrates can easily be dissolved in organic solvents such as isopropanol, ethanol, acetone and butanol [15]. Combination of two aqueous phase systems such as polymers (e.g., polyethylene glycol and dextran) and water/salt-like mediums (e.g., citrate, sulphate and phosphate) are also efficient for plant protein extraction. Parameters that are crucial for efficient aqueous protein extraction yield and functionality include protein bioaffinity, molecular size, electrical potential between composites of the phase system and hydrophobicity of the developed extraction phase [16]. Besides aqueous phase systems, plant proteins can also be extracted by subcritical water extraction, where a plant sample is exposed to high pressure at temperatures within 100–380 °C, and then cooled at ambient temperature. The advantage of subcritical water extraction is its capacity to dissolve proteins without the need of a catalyst, due to the high dissociation constant associated with the conversion of water to hydroxyl and hydrogen ions compared to water at room temperature [3].

2.2. Advanced plant-based protein extraction techniques

To increase the release of proteins from plant matrices, it is recommended to employ various techniques capable of degrading complex structural membranes and molecules bounded to proteins. Currently, different techniques are being applied as pre-treatment methods to assist alkali extraction of plant-based proteins. For environmental, economic and technological sustainability, pre-treatment techniques that are novel and eco-friendly will be discussed in the next section of the text.

2.2.1. Non-physical pre-treatments

The most widely exploited non-physical pre-treatment method is enzyme assisted protein extraction. Basically, enzyme assisted extraction functions by using enzymes to degrade cell wall structural molecules such as hemicellulose, cellulose and pectin [17]. Proteases and carbohydrases are the key enzyme groups applied by the protein industry for extraction. Activity of carbohydrases (e.g., cellulase, pectinase and xylanase) enhance protein extraction by breaking structural cell wall carbohydrates into smaller sizes, thus, enhancing the diffusion of alkali solution into cellular matrices for improved protein solubility and yield [18].

For protease (e.g., papain) assisted extraction, protein yield is enhanced by their capacity to hydrolyse proteins into smaller size for increased surface area and solubility into the alkali extraction solution [18]. However, literature has reported protease assisted extraction as more effective, compared to carbohydrase, as observed from the work of Hanmoungjai, Pyle, and Niranjan [19]. Some previous authors also reported on bi and tri- enzyme assisted protein extractions, with the authors observing high protein yield compared to their independent catalytic activities [20]. For example, cocktail enzyme treatment of soy flour before alkaline extraction resulted in a 21% increased protein yield, compared to alkaline extraction alone [21].

However, although enzyme assisted protein extraction has limited environmental drawbacks, it is presented with technological challenges

such as (1) high enzyme and processing cost (2) formation of complex interactions between enzymes and native polysaccharides, pigments and nucleic acids, leading to reduced enzyme activity, protein degradation, reduced yield and instability (3) stimulation of microbial growth due to prolonged enzyme incubation period (4) energy and time intensive (5) limited industrial scale-up [3].

2.2.2. Physical mediated protein extraction techniques

Overall, the primary principles for physical pre-treatment extraction techniques are the breakdown of structural cell walls bounded to proteins; increased diffusion of extraction medium into cellular fragments; improved solubilization of proteins; increased liberation of proteins into extraction medium. Selection of a physical method for plant protein extraction should be based on factors such as (i) environmental sustainability (ii) installation and maintenance cost (iii) impact on protein yield and protein techno-functional properties (iv) technological sustainability [22]. Currently, eco-innovative physical methods such as high hydrostatic pressure processing (HHP), pulsed electric field (PEF), ultrasound (US) and microwave assisted processing (MAP) are being exploited by the plant protein industry for plant protein purposes. For example, rice bran treated with HHP (500 MPa, 5 min, 25 °C) enhanced protein yield by 66.3% compared to traditional extraction [23].

In another study, watermelon seeds treated with ultrasound (35 kHz, 836 W, 98 min, 30 °C) showed increased protein yield by 87%, compared to those subjected to only alkaline extraction [24]. Similarly, soybean treated with MAP (2450 MHz, 30 min, 60.1 °C) showed improved protein yield by 58%, compared to its conventional extraction [25]. In another study by Scherer et al. [26], protein extraction efficiency from *Chlorella vulgaris* was improved by about 18% upon treatment with PEF (40 kV/cm, 150 J/g), compared to their control form. However, comparing ultrasound to HHP, PEF and MAP, ultrasound is more efficient for the food industry because (a) require less time and energy (b) limited installation and maintenance cost (c) reduced processing temperature [27]. Therefore, ultrasound is a more promising eco-innovative technique that can help leverage nutritional and functional demands of the growing plant-based protein industry.

3. Ultrasound technology (US)

3.1. Principles of ultrasound generation and applications in plant protein research

Presently, the food industry makes major use of high-power sonication (HPS), which is characterised by low frequency (16–100 kHz) and power intensity (10–1000 W/cm^3). In an aqueous extraction medium, HPS functions by producing high shear energy and macroturbulence through the initial creation of cavitation bubbles and their violent subsequent collapsing at elevated temperatures (5000 K) and pressures (1000 atm), as displayed in Fig. 1 [28].

Elevated shear energy and macroturbulence created along HPS is the basis for US membrane and cell wall disintegration, resulting in the increased transfer of extraction solvent into broken cells for enhanced extraction yield of molecules bounded to cell wall components and intracellular matrices [29]. Besides their cavitation principle, US can enhance extraction yield by reducing substrate particle size by 10-fold [6]. Together with its extraction efficacy, US can also alter the techno-functional properties of proteins by breaking hydrogen bonds and hydrophobic interactions responsible for stabilizing the functional structures (i.e., secondary and tertiary structures) of proteins [30]. Although the technological and environmental effects of US make it a sustainable and efficient extraction technique, it has very limited applications in the plant-based protein industry. By taking advantage of its positive cavitational and shearing forces, US can be harnessed as a green pre-treatment method with alkaline extraction to enhance the recovery of proteins from plant-based substrates with limited effects on the techno-functional properties of extracted proteins.

To produce ultrasound, electrical pulses are converted into acoustic energy of specific intensity using transducers such as magnetostrictive and piezoelectric transducers [31]. As discussed in the recent review of Bhargava et al. [32] magnetostrictive transducers generate ultrasonic waves on the principle of magnetostriction, where electro-acoustic transducers alter ultrasound waves in length per unit length. Additionally, application of magnetostrictive transducers involve the use of a magnetic field and a magnetostrictive material. With respect to piezoelectric transducers, its operation is based on the principle that a piezoelectric or quartz crystal material can produce electrical charges on its surface for sequential conversion into acoustic energy. Utilization of

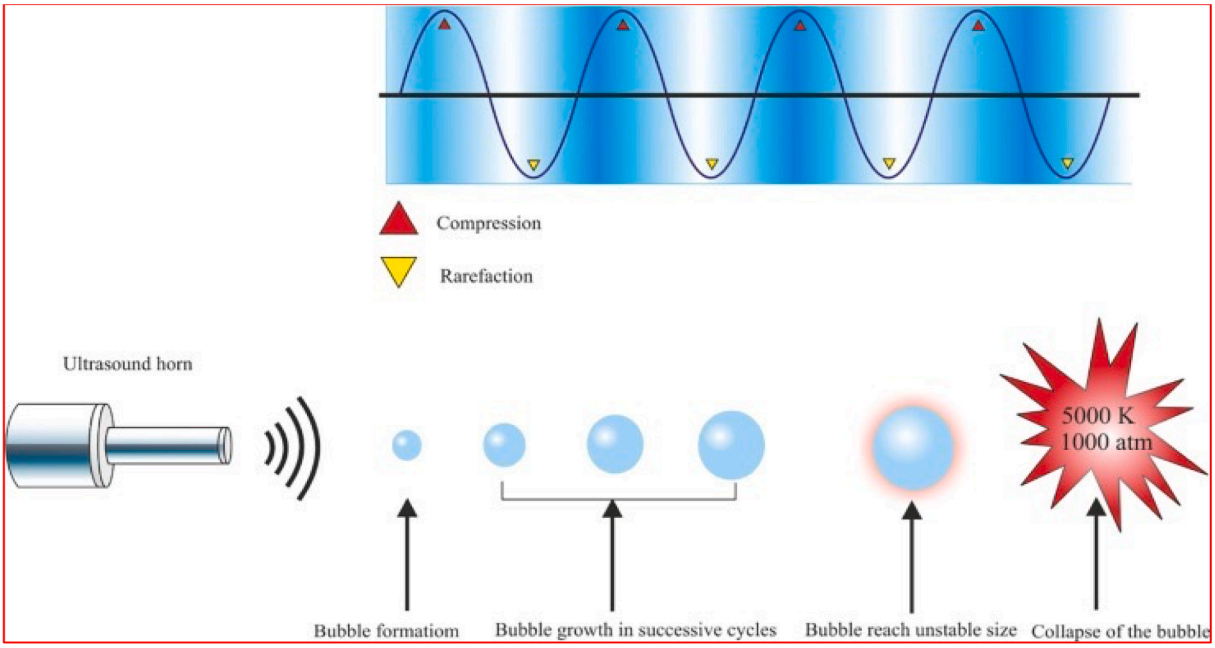

Fig. 1. Schematic illustration of ultrasonic generation of cavitation and collapsing of bubbles [28].

ultrasound in the food industry can be achieved by two approaches including the use of a sonotrode or an ultrasonic water bath (Fig. 2).

A sonotrode consist of a generator component which produce an alternating current with a specific ultrasound frequency. Afterwards, its transducer and amplifier components convert the generated electric currents to mechanical forces and sound waves, respectively [34]. For the ultrasonic water bath, a piezoelectric transducer is attached to the bottom of the water bath to convert low frequency acoustic energy into high frequency sound waves. High intensity sound waves created by the piezoelectric transducer creates a high compression capable of tearing apart the extraction solvent in the water bath to produce macroscopic bubbles, which subsequently collapse violently at high pressure and temperature to disintegrate samples placed in the extraction solvent of the water bath [35].

3.2. Factors influencing ultrasound-assisted plant-based protein extraction

Compared to animal-based proteins, plant-based proteins are sustainable nutritional ingredients for the development of healthy foods for both the vegetarian and nutrition-conscious consumer. However, to increase the competitiveness of the plant-based protein industry to become a long-term viable option, there is the need to exploit and develop optimized conditions critical for novel plant-based protein extraction techniques such as ultrasonication. Thus, to highlight the subject matter, this section of the context will discuss it further.

3.2.1. Extraction temperature and time

In ultrasound assisted plant-based protein applications, the duration for which protein substrate/extracts are exposed is very crucial with respect to yield and functional properties. Ultrasonication time depends on the intensity of the power applied to the sonoreactor and the frequency generated in the ultrasonic system [32]. For the protein and other agri-food industries, high intensity or high power/short time ultrasonication is the most advantaged and desired compared to low intensity/long time ultrasonication. High intensity or high power/short time ultrasonication is more conducive for the plant-based protein industry because it's less expensive and has limited effect on protein denaturation and loss of functional properties, compared to low intensity or low power/long time ultrasound processing which is more expensive and has been reported to induce temperature rise resulting in protein denaturation [36]. For laboratory scale ultrasound assisted plant-based protein extraction, previous studies have reported extraction solvent temperatures above 60 °C as non-desirable because of its negative effect on protein structure [37].

According to Chemat et al. [27] increase in temperature along ultrasonication decreases viscosity and surface tension, as well increases vapour pressure, therefore, limiting the cavitational and sonochemical effects. To control temperature rise, some studies has recommended the use of pulsing or ice baths at different time stages of the ultrasonic-assisted extraction process. Hence, to encourage the utilization of ultrasound for plant-based protein purposes, there is the need for sonotrode/ultrasonic water bath manufacturing companies to develop high intensity/high power ultrasound systems that can be manipulated to save production cost at reduced temperatures via their shorter time operations.

3.2.2. Intensity and energy density

In ultrasonication, the amount of energy applied per unit volume of sample slurry is described as energy density. High intensity ultrasound (ranges from 10 to 1000 W/cm^2) can create high power ultrasound characterized with high energy density from 1 to 10 W/mL [38]. When a sample with a smaller surface area is exposed to a high intensity ultrasound, the amount of energy density generated is very limited, thus leading to less cavitation effects [39]. Hence, energy density and ultrasound intensity are critical factors for efficient ultrasonication because they are directly linked to how robust the generated cavitational effect will be. Majority of the literature available on ultrasonication has been reported with frequency ranges of 20 – 40 kHz, with most of these studies concluding that 20 kHz is the best condition to generate high intensity and cavitational effects with minimal protein denaturation, compared to extending frequencies (i.e., 40 kHz) [40].

In general, 40 kHz will be expected to generate high intensity and cavitation, but this trend has been shown to be the leading cause of the negative consequences of foaming and protein unfolding during ultrasonication [41]. According to Qsonica [41] most results on ultrasonic treatment of biological samples are reported in terms of intensity (W/cm^3) or power density (W/mL), with a total ignore of the effect of wave amplitudes at the tip, especially with sonotrode ultrasonication. Therefore, for vivid research report, parameters such as tip diameter, sonoreactor tip amplitude, intensity (W/cm^2), power density (W/mL) and the volume of liquid/sample slurry should also be evaluated with their impacts reflected in the observed results.

3.2.3. Sample size and solvent to sample ratio

To optimize ultrasonic extraction of plant-based proteins, particle size of substrates from which the proteins are to be extracted is very crucial. The particle size of protein substrate is crucial because it contributes to the substrate surface area available for extraction. Substrates with lower molecular sizes will provide a bigger surface area for

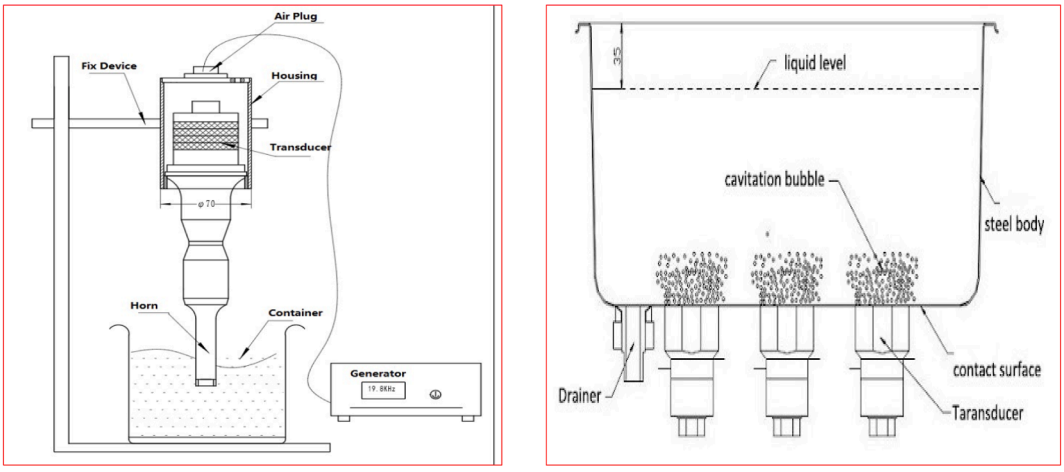

Fig. 2. (a-b). Schematic diagram of a sonotrode (a) and water bath (b) mechanism of ultrasonication (a- [33]; b- literature).

enhanced solvent diffusion, cavitation effects and subsequent protein yield, compared to samples with high molecular sizes. As further discussed by Karki et al. [6] exposure of protein substrates to acoustic cavitation increases the exposure of the cell surface area to extraction solvent for increased yield, by breaking the cells native particle size into smaller sizes. However, it is also important to note that, most of these reports are postulated with very scanty research on how different molecular sizes of a plant substrate influence the yield of proteins when treated with ultrasonic-assisted extraction. Also, literature has reported different results from the same plant substrates when their nature before ultrasonic extraction were different. Most of these reports show higher protein yield when plant substrates were extracted in their flake form, compared to those treated in their flour form. In general, research on plant substrate particle size and substrate-to-solvent ratio for different plant substrates in their varied forms (i.e., whether flour or flakes) should be investigated to increase the efficiency of ultrasonic-assisted plant-based protein extraction.

3.2.4. Sonoreactor and sonotrode characteristics

Inappropriate submersion of a sonotrode tip into an extraction medium can result in unfavourable consequences such as foaming, and uneven distribution of acoustic energy and cavitational effects. For efficient plant-based protein extraction, it is recommended to submerge the sonotrode tip at the center of the sonoreactor containing the extraction medium and plant substrate. From the works of Chittapalo and Noomhorm [42] and Annandarajah et al. [43] ultrasonic extraction with a sonotrode tip submerged within length ranges of 2–3 cm sample depth is the most efficient. Besides sonotrode, another important factor is the shape of the sonoreactor containing the extraction medium and the plant-based protein substrate. According to Qsonica [41] for uniform distribution of cavitation and shear forces, a sonoreactor should be characterised by a narrow, flat bottomed cylindrical shape. However, studies on how sonotrode tips and sonoreactor shapes influence ultrasonic-assisted extraction of plant macromolecules is very dearth. There is therefore the need for deeper studies into this subject matter in order to optimize the yield of plant-based proteins via ultrasonic-assisted extraction. With this information, manufacturing companies will be able to design and supply ultrasonic equipment with specific dimensions tailored for optimized plant-based protein extraction, without sacrificing the protein's techno-functional properties.

4. Impact of ultrasound on plant-based protein extraction and structural modifications

4.1. Extraction yield

As discussed in previous sections of the text, protein yield from a chosen extraction technique largely depends on the ability of the extraction method to induce structural disintegration, enhance extraction solvent diffusion and dissolution of proteins from structural matrices into the extraction medium. Data from previous authors has shown positive results between ultrasound-assisted extraction and the yield of proteins from various plant substrates (Table 1).

It should be noted that, most of these studies reported ultrasound as a pre-treatment method before alkali extraction, and not as an independent plant-based protein extraction method. For instance, defatted soy flakes and un-defatted soy flour were treated with ultrasound (20 kHz, 2.2 kW, 720 W/mL and 4 min for flakes; 20 kHz, 2.2 kW, 360 W/mL and 2 min for flour) before water and alkali plant protein extractions from the work of Rahman, Dutta and Lamsal [36].

After the extraction period, ultrasound improved protein yield from flakes and flour by 16.58 and 9.76 %, respectively. However, although ultrasound improved protein yield with both flakes and flour treatments, the authors attributed their observed variations in protein yield to the negative interference of fat in the whole soy flour. Thereby, making it critical for plant-based substrates to be defatted before initiating protein extraction. Furthermore, in the recent research of Byanju et al. [51] protein recoveries from defatted soy flake and flour were significantly higher compared to undefatted chickpea flour. Ultrasonication (20 kHz, 363 W, 24 min, pulse mode: 2.4 and 2 s off) improved wheat germ protein extract yield from 37 to 57 %, according to the study of Zhu, Sun and Zhou [52]. In another study, ultrasonication (450 W, 80 min) of defatted rapeseed meal showed improved protein yield by 8.31%, compared to their control form [53].

Table 1
Impact of ultrasound-assisted alkaline extraction on some selected plant-based protein yield.

Plant substrate	Ultrasound conditions	Extraction conditions	% Yield	References
Sesame	35 kHz; 836 W	1:1 (w/v); 45C; 98 min; pH- 9.5	+ 59.8%	[17]
Sunflower	220 W	0.5:10 (w/v); 45C; 15 min, pH-8	+ 54.26%	[40]
Rice bran	20 kHz	1:10 (w/v); 40C; 2 min; pH- 7	+ 64.5%	[44]
Defatted soy flakes	20 kHz; 2.2 kW	0.30:2.56 (w/v); 2 min; pH-8.5	+ 34%	[6]
Defatted peanut	20 kHz;	30 W/g; 15 min; pH- 6.8	+ 10.6%	[45]
Spirulina	55 W/cm^2, 2 bar	1:20 (g/g); 24C; 15 s;	+ 28.42%	[46]
Rice bran	26 kHz; 300 W;	1:10 (w/v); 55C; 30 min;	+ 37%	[47]
Rapeseed	0.228 W/cm3; power- 40%	1:30 (w/v); 41.48 min; pH-11.71	+ 38.09%	[48]
Wampee seed	40 kHz; 240 W	1:29 (w/v); 64 min; pH- 12	+ 15.06%	[49]
Cauliflower	175 W	4 ml/g; 15 min; pH- 11	+ 53.07%	[50]
Watermelon seeds	90 W; 25 kHz; Duty cycle- 75%	1:50 (w/v); 30C; 9 min; pH- 9	+ 87%	[24]

4.2. Protein molecular structure

Structural characteristics of proteins can undergo unfolding, denaturation and reaggregation when exposed to ultrasonic cavitation. Low and high- power ultrasonication usually leads to the breakdown of hydrophobic and hydrogen bonds through the effects of cavitation and shearing forces [54]. Thereby, resulting in conformational changes (i.e., secondary and tertiary structural changes), which can subsequently alter the protein's techno-functional and nutritional properties in food systems. Black bean protein isolate exposed to different ultrasound conditions (power – 150, 300 and 450 W; time – 12 and 24 min) showed that, protein unfolding increased significantly from 300 W up to 450 W, with a decreasing trend in structure and particle sizes with increasing treatment time [9]. From this study, the researchers concluded that, cavitational and microstreaming effects produced under low-power ultrasonication led to their formation of unstable protein aggregates with increased particle sizes, compared to high ultrasound powers where their formed protein aggregates were broken into smaller sizes. In another study, isolated proteins from jackfruit seeds were treated with ultrasound (0, 200, 400 and 600 W for 15 min) [55]. According to the authors, increasing ultrasonication power decreased jackfruit protein microstructure, with the authors attributing their observation to the increased turbulence and microstreaming of ultrasound. From these previous studies, it was explained in the review of Rahman and Lamsal [5] that high power ultrasonication with increasing treatment time creates smaller protein microstructure with more SH groups, capable of reacting with themselves or oxidizing into larger aggregates.

Besides structural changes, exposure of plant proteins to cavitation and microstreaming leads to changes in their electrical conductivity and zeta potential. Ultrasonication of proteins has been shown to decrease/

increase electrical conductivity depending on the applied ultrasound power/intensity, treatment time and substrate type [56]. High ultrasound intensity/power with increasing time has been shown in protein studies to increase electrical conductivity through the formation of hydroxyl radicals or protein aggregates, with subsequent decrease in electrical conductivity [5]. Supporting this literature, is the work of Jambrak et al. [57] where ultrasound (20 kHz) treated soy protein isolates showed increased electrical conductivity after 15 min of treatment, compared to their unsonicated form. Another important attribute that determines some techno-functional properties of plant-based proteins in food systems is zeta potential, which is the electrical potential difference between a particle's stationary layer and its dispersion medium [58]. A protein's zeta potential is important in a food system because of its crucial function in helping stabilize the protein in a dispersion medium, in connection to the presence of amino acids on the protein surface [54]. Jiang et al. [9] investigated the influence of ultrasonication on protein zeta potential. After their study, the authors observed a decreasing trend with absolute zeta potential and attributed their report to the presence of high levels of negatively charged amino acids on the protein surface, compared to their positively charged counterparts upon ultrasound exposure.

4.3. Changes in conformational structures

Exposure of proteins to low-intensity/power ultrasound leads to the breakdown of weak covalent bonds to form partially unfolded proteins, whereas exposure to high-intensity/power ultrasound breaks down proteins into smaller peptides [38]. Therefore, causing alterations in the primary, secondary and tertiary structures of the treated protein, with sequential changes in its techno-functional and nutritional properties (Fig. 3).

However, it should be discussed that, a proteins conformational change induced by ultrasound will depend on the sonication conditions (i.e., power, time and temperature levels) and the type/biochemical nature of the plant substrate. A protein's primary structure is made up of an amino acid sequence, whereas the secondary structure (i.e., alpha-helix, beta-sheet and beta-turn) is characterized by C-O and NH hydrogen bonds between different amino acids. Protein secondary structure is measured by its ratio of alpha-helix, beta-sheet, beta-turn, random coil and unordered groups. In a previous study by Hu et al. [59], ultrasound treated soy protein isolate showed a reduced trend of alpha-helix and random coil at 200 W, compared to their observed treatments at 600 W. The same authors exposed 7S and 11S soy protein fractions to ultrasonication and observed no obstruction in the hydrogen bonds between C = O and H-N groups of the polypeptide backbone [60]. To prove that protein substrate types respond differently to ultrasonication during extraction, Byanju et al. [51] investigated secondary structural changes in ultrasound-assisted chickpea, soy and kidney bean protein extraction. Their research concluded that, proteins extracted from soy and chickpea under high-power ultrasound showed no significant changes in secondary structure, compared to the protein extract from kidney bean which showed significant changes in its secondary structure. According to Rahman et al. [39] these observed variations could be attributed to the capacity of a protein substrate to form complexes with hydroxyl radicals (generated during ultrasonication), get oxidized and induce rearrangement of the protein molecular structure. The tertiary structure of protein is made up of a single protein molecule with the presence of hydrophobic nucleus, hydrogen bonds, disulfide bonds, and salt bridges, whereas a protein's quaternary structure consists of interactions between different subunits to form a functional complex structure [58]. With respect to changes in protein tertiary structure, Jiang et al. [9] found that ultrasonication of black bean protein isolate disrupted internal hydrophobic interactions and exposed them to the surface.

5. Effect of ultrasound on plant-based protein techno-functional applications

5.1. Surface hydrophobicity (H_0)

Protein hydrophobicity is characterized by the number of hydrophobic groups present on the protein's surface. Compared to total hydrophobicity, a protein's functionality in a food system is largely influenced by its surface hydrophobicity [61]. Hydrophobicity (H_0) is an important attribute because it contributes to a protein's conformation, stability and functionality in a food system. Flores-Jimenez et al. [61] investigated the impact of ultrasonication (power- 130 W; intensity- 1 W/cm^2; time – 15 and 30 min; temperature 15 °C) on surface hydrophobicity of canola protein isolates. According to their results, increasing ultrasonication time led to an increase in the surface hydrophobicity of treated canola protein isolate, with 30 min ultrasound treatment exhibiting H_0 of 17.6 % higher than the control (Fig. 4).

Jiang et al. [9] investigated the impact of ultrasonication (power- 150, 300 and 450 W; time- 12 and 24 min) on surface hydrophobicity of soy protein isolate. From their study, although ultrasonication increased H_0 of the soy protein isolate at all treated power and time levels, the

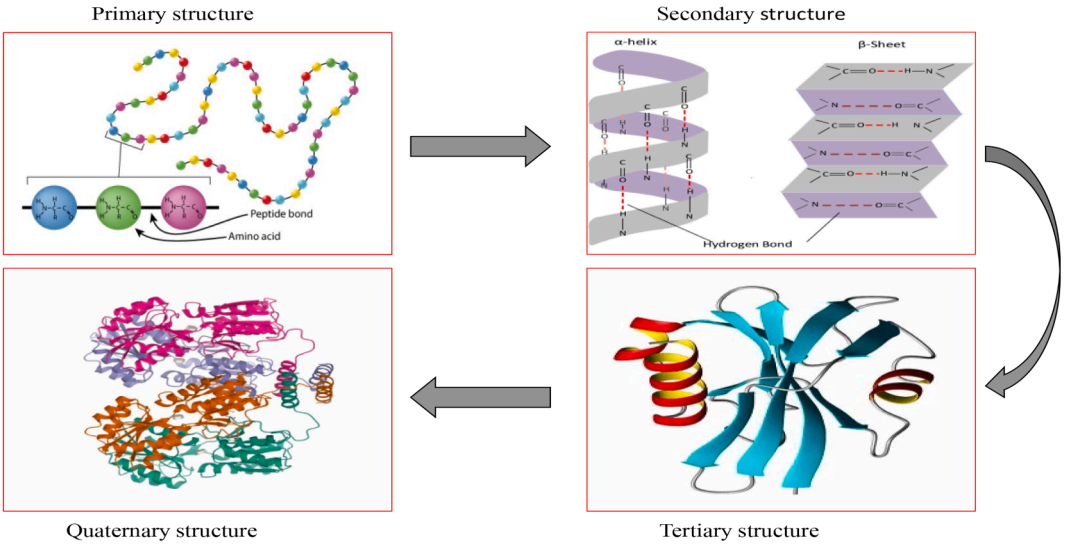

Fig. 3. Schematic illustration of protein conformational levels.

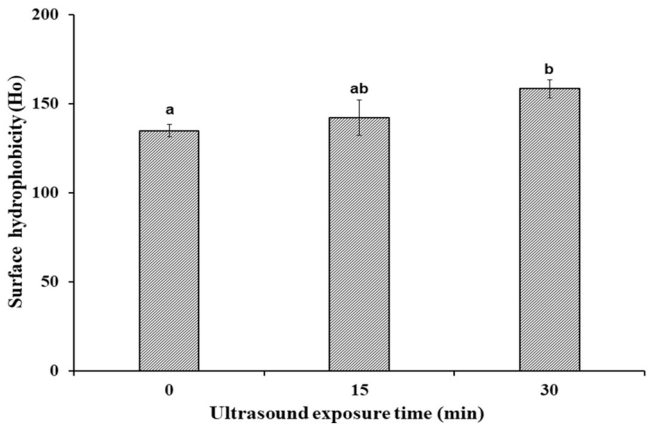

Fig. 4. Surface hydrophobicity of canola protein isolates treated with ultrasound [61].

highest H_0 level was observed with 300 W, irrespective of treatment time. A similar trend was also observed by Chen et al. [62], who also reported increased H_0 with ultrasound treated soy protein isolate. According to Jiang et al. [9] improved H_0 of proteins upon sonication is a proof of a certain level of molecular unfolding during ultrasonication, thus increasing the release and availability of otherwise internal hydrophobic groups and regions to an external polar surrounding environment. From this same study, H_0 decreased when ultrasonication power increased beyond 300 W, which is similar to the conclusions of other researchers such Malik, Sharma and Saini [63]. This alternative trend associated with extended high-power ultrasonication can be attributed to (a) induction of partial protein denaturation, with sequential increase in intramolecular bonding and reduction of H_0 (b) formation of protein aggregates through disulphide network, which sequentially protect the hydrophobic regions and reduce H_0 [64].

5.2. Solubility

A protein's degree of solubility is an indicator of its level of denaturation and aggregation upon processing [65]. The solubility of a protein at a given pH is characterized by its hydrophobicity, particle size, size of formed aggregates and degree of denaturation [60]. Although there exists scanty literature on the influence of ultrasound as an extraction pre-treatment on protein solubility, there seem to be more literature on alterations of isolated protein solubility when subjected to ultrasonication. The cavitational effect created during sonication can break down hydrogen and hydrophobic bonds to decrease protein molecular weight. A decrease in molecular weight leads to particle size reduction and exposure of hydrophilic amino acids groups, therefore, increasing the protein surface area in a solvent at a specific pH for increased solubility [65]. Lv et al. [2] observed increased solubility with walnut protein isolate treated with ultrasound, compared to the control. Similarly, Arzeni et al. [65] reported increased solubility with ultrasound treated soy protein isolate, compared to its untreated forms, with the authors attributing their observation to the capacity of ultrasound to reduce particle size of the treated soy protein isolate, thus increasing the protein-water interactions for enhanced solubility. In another study, solubility of ultrasound treated black bean protein isolate showed an increasing trend from 150 W up to 300 W at 24 min of treatment time [9]. However, the authors further observed a decreasing solubility trend when ultrasound power and treatment time were increased up to 400 W and 24 min. Similarly, from the study of Hu et al. [60] ultrasonication of 7S and 11S protein fractions increased the solubility of 7S compared to 11S, with this trend in 11S related to the formation of larger molecular and particle sizes. Another explanation for the decreased solubility trend is the initiation of protein unfolding and formation of larger aggregates under extended high ultrasonication power and treatment time, resulting in the exposure of buried hydrophobic amino acid groups, and non-covalent interactions with reactive oxygen species, thus decreasing solubility.

5.3. Emulsifying properties

The amount of oil a protein can adsorb to stabilize a two-phase system is described as it's emulsifying capacity. Emulsifying capacity is affected by the ratio of hydrophobic/hydrophilic amino acid groups, as well as the structural conformation of the protein (i.e., ability of the protein to unfold and create a film around dispersed oil droplets). As emulsifiers, proteins help arrest negative structural changes such as coalescence, creaming, flocculation and sedimentation [66]. As described by Boye, Aksay and Roufik [66] the emulsifying capacity of a protein is measured by two indices, including emulsifying activity (EA) and emulsifying stability (ES), where EA describes the quantity of oil that can be emulsified per unit of protein and ES measures the capacity of the formed emulsion to repel structural changes over a specific time. Researchers have established a link between ultrasonication and potential changes in plant-based protein EA and ES. In the study of Zhu et al. [67] EA and ES of ultrasound treated walnut protein isolates were significantly enhanced by more 26 and 41 %, respectively, compared to the unsonicated form. The authors associated this observation to the ability of high intensity ultrasound to destroy hydrogen bonds and hydrophobic interactions between and within walnut protein molecules, thereby initiating a certain level of unfolding and dissociation. In another study, Nazari et al. [54] reported enhanced ES of millet protein concentrates after ultrasound treatments, compared to the control. Simultaneous to these observations, an interesting study by Resendiz-Vazquez et al. [55] also showed improved emulsion capacity, with jaca protein isolate after ultrasonication at 200 and 400 W. Despite this trend, the authors interestingly observed improved EA and ES of the formed emulsion under high extended ultrasonic power levels of 400 and 600 W. Additionally, Zhang et al. [29] reported improved EA and ES with ultrasound treated wheat gluten, with this trend optimized at the highest sonication power of 900 W. With these positive reports, there exist great opportunities for the food industry to exploit ultrasound as a green innovative technology to create diverse emulsifying food systems with better characteristics.

5.4. Water holding capacity (WHC)

As defined by Boye, Aksay and Roufik [66] WHC is the amount of water that can be absorbed by 1 g of protein. In food systems, a protein's WHC contributes to important characteristics such as thickening, viscosity and juiciness [40]. Although literature has been reported on how ultrasonication influence WHC in diverse food products, it is important to note that majority of these applications are with muscle and dairy foods, compared to plant-based proteins [67,68]. From the few studies available on ultrasonication and WHC of plant-based proteins, Nazari et al. [54] observed that ultrasound treatment of millet protein concentrates showed improved WHC, than observed with the unsonicated millet protein concentrates. Hu et al. [56] also mentioned that ultrasound treatment of soy protein isolates significantly enhanced WHC, attributing their observation to ultrasound cavitational effects which induced structural and functional changes.

5.5. Oil absorption capacity (OAC)

Oil absorption capacity (OAC) is defined as the amount of oil absorbed per gram of protein [66]. Similar to WHC, very limited studies have been reported on the impact of sonication with respect to OAC of plant-based proteins. In food systems, OAC is involved with flavour retention and textural properties [69]. Rasendiz-Vazquez et al. [55] treated jaca protein isolate with ultrasonication (power- 0, 200, 400 and 600 W), and observed increased OAC from 1.99 to 3.60 g/g, with this

trend increasing with increasing sonication power. Likewise, Chittapalo and Noomhorm [42] observed enhanced OAC with ultrasound treated rice bran protein isolate at 1.52 g/g, compared to its untreated form (1.31 g/g). According to these researchers, ultrasonication created protein unfolding which led to the exposure of more hydrophobic amino acid groups, thus, enhancing oil absorption of the rice bran protein isolates.

5.6. Foaming properties

The creation of foams is key in food systems such as mousses, beverages, whipped toppings and meringue cakes. Formation of foams is characterized by protein unfolding into an interfacial skin to (a) form air bubbles in a suspension (b) prevent collapse of the developed air bubbles [66]. The foaming property of a protein is characterized by two indices, namely foaming capacity (FC) and foaming stability (FS). Foaming capacity (FC) refers to a protein's ability to form a foam under processing conditions (i.e., pH, temperature and protein concentration), whereas FS evaluates a protein's capacity to maintain a developed foam volume over a specific period [2]. Overall, the foaming property of a protein is dependent on factors such as protein orientation, surface hydrophobicity, protein size, degree of denaturation and homogenization [70]. Lv et al. [2] investigated the impact of ultrasonication on foaming properties of globulin, albumin and glutelin protein fractions from walnut protein isolates. According to these researchers, ultrasound significantly enhanced FC and FS for the albumin fraction by 20.03 and 9.54 %, respectively, whereas for the glutelin fraction FC and FS were improved by 14.1 and 10.58 %, respectively, compared to the control. Notwithstanding, Lv et al. [2] observed the highest FC and FS for the globulin fraction which reported enhanced levels by 49.39 and 11.21 %, respectively, compared to the control. After their analyses, the researchers attributed their observation to the cavitational and shearing forces of ultrasonication, which reduced the protein particle sizes and dispersed them evenly for improved bubble formation. Also, they suggested that ultrasonication could have induced partial protein unfolding, thus increasing their hydrophobic groups to facilitate protein absorption at the oil–water interface for subsequent increased foaming properties.

In other studies, treatment of protein isolates with ultrasound improved foaming properties up till 25 min, and then showed a decreasing trend when treatment time was increased up till 25 min [63,70]. These studies explained the reducing foaming trend to the formation of larger protein aggregates with increasing ultrasonication time and power, which restricted availability of the protein's hydrophobic groups. Irrespective of these previous studies, Zhang et al. [29] reported increased foaming properties with sonicated wheat gluten proteins under increasing treatment power conditions (i.e., optimized foaming properties at 100 % power level). Thus, confirming previous discussions of the review, that techno-functional attributes of plant-based proteins varies according to the substrate type/source, besides ultrasound parameters and processing conditions.

5.7. Gelation capacity

A protein gel is formed through the aggregation of separate proteins into a 3-dimensional network, resistant to flow when exposed to pressure. According to Resendiz-Vazquez et al. [55], during protein gel formation there is molecular unfolding of the protein, denaturation and sequential formation of aggregates, resulting in a gel when the formed aggregates exceed its critical concentration. In food processing, gelation is an important attribute for food applications such as yoghurts, puddings, jellies, desserts and meat products. Improved gelation reports with ultrasonication and plant-based proteins has been shown in literature. From literature, ultrasound has been applied to form gels with enhanced texture, mechanical and hydrodynamic properties [71]. For instance, Arzeni et al. [65] reported improved gel strength with ultrasound treated soy protein isolate, through hydrophobic interactions.

5.8. Flavour

Legumes are the major food groups from which majority of plant-based proteins are extracted. A key factor that contributes to the purchasing power of plant-based proteins is flavour. However, very few data have been reported on how ultrasonication influence the flavour of plant-based proteins. Off-flavours in plant-based protein foods is a major setback for the industry. It is generated from residual polyunsaturated fatty acids through activity of the enzyme lipoxygenase. From the work of Thakur and Nelson [72] activity of lipoxygenase decreased by 21% in ultrasound treated soy flour, compared to their unsonicated form. In another study, ultrasonication (0, 100, 200 and 300 W) reduced activities of three lipoxygenase isoenzymes (i.e., Lx-I, Lx-II, Lx-III) in soybean sprouted flour by 36.22% (Lx-I) and 55.57% (Lx-II and Lx-III), compared to the control [73]. From these works, it can be postulated that cavitational and shear stresses created by ultrasound induced cell wall and membrane disintegrations of lipoxygenase, thus destroying their structural integrity and subsequent reduced activities. However, these studies were reported with whole flour comprising of proteins and other biochemical molecules. For the plant-based protein industry to develop innovative healthy food products with longer shelf-life and consumer acceptability, it will be interesting for researchers to investigate how ultrasound can mitigate off-flavour development in plant-based proteins both in their isolated forms and as part of complex food systems.

6. Industrial challenges with ultrasound-assisted plant-based protein applications

6.1. Temperature elevation and generation of reactive oxygen species

For food applications, ultrasound can be applied at low, medium, high and extreme power/intensity levels. Although the food industry mostly applies high power/intensity ultrasonication, their use at certain levels of increasing treatment time has been associated with protein denaturation because of elevated temperatures and formation of reactive oxygen species. As discussed in previous sections of this review, temperature rise over prolonged sonication time leads to the breakdown of hydrogen and hydrophobic bonds, thus inducing protein unfolding, denaturation and subsequent modifications in structure and functionalities. It should be noted that, these changes may be positive or negative, depending on the type and preferred characteristics of the final food system being targeted for application. Besides temperature elevation, when water is used as an ultrasound extraction medium, cavitational forces generated can decompose water molecules to its radicals (i.e., hydroxyl radicals and hydrogen atoms). Concentration of free radicals generated will depend on the level of ultrasound power/intensity [38,39]. Free radicals generated during ultrasonication can (i) oxidize free SH groups to SS bonds (ii) alter secondary and tertiary protein structures (iii) induce aromatic hydroxylation and formation of carbonyl groups [67]. Thus, protein oxidation from free radicals generated from cavitational stress can induce structural changes that can alter the nutritional and techno-functional attributes of the isolated protein.

7. Conclusion and future perspectives

In summary, the advent of ultrasound has become an integral part of emerging eco-innovative techniques targeted for sustainable food processing. Certainly, plant-based proteins are potential opportunities to sustainably meet the protein needs of the current population, without sacrificing the environmental sustainability of future generations. As discussed above, combination of the conventional alkali protein extraction method with ultrasound is one of the best eco-innovative approaches to leverage plant protein recovery gaps discussed in this

review. With respect to plant protein extraction yield and their food applications, this review summarized the potential application of ultrasound to induce structural modifications in plant proteins with different physicochemical properties. However, because majority of the works reported with ultrasound are widely reported at the laboratory scale level, it is complicated for the plant protein industry to optimize crucial ultrasound equipment parameters influencing their design and applications such as temperature, time, sonotrode characteristics, frequency/energy intensity and sample-to-solvent ratio. Such information is very critical for industrial optimization and thus need urgent attention. To address these gaps, the future direction of researchers and the plant protein industry should be the provision of a deeper understanding of the relationship between plant protein structural changes, and subsequent functional properties in relation to ultrasound parameters at the micro/macroscopic levels.

Additionally, future innovative research for industrial-scale optimization can be directed in the following advanced ultrasound techniques (a) Barbell Horn Ultrasonic Technology (BHUT) and (b) manothermosonication (MTS). BHUT can generate extreme high ultrasonic amplitudes at limited processing time and temperature, without compromising final plant protein product quality, whereas MTS can be used to modify plant protein quality by combining the trio activities of low pressure, low temperature and ultrasonication. Although these advanced ultrasonic technologies have been applied in other industrial sectors, they have limited applications in the plant protein industry both at the laboratory, pilot or industrial scale. It is important to highlight that, efficient utilization of these advanced ultrasonic techniques will also demand a comprehensive understanding of the relationship between plant protein structural diversity and ultrasound equipment parameters in-order to aid the design of precise ultrasound equipment meeting industrial needs.

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