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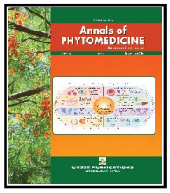

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Physicochemical, structural and functional properties of freeze-dried green gram protein isolate (*Vigna radiata* (R.) Wilczek) using ultrasonication-assisted extraction method

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Abstract

Vigna radiata (R.) Wilczek was a new plant protein source, which was highly rated on its good nutritional value, digestibility, and functional characteristics in food systems. In this study, we examined the effects of ultrasonication-assisted extraction of protein on the physicochemical, structural and functional characteristics of freeze dried (–40°C) green gram protein isolates by following the ultrasonic specifications, *viz*., pulsation time: 2 sec on, resting time: 4 sec off, total work time: 20 min, temperature: 25-30°C and power and amplitude: 30-35% compared to conventional extraction method. The results showed that there is an increase in the protein yield (10.23%) and protein recovery (13.01%) significantly. SEM images indicated a disruptive effect caused by cavitation, enhanced roughness on the surface of the ultrasonic *V. radiata* isolate. The FT-IR spectra showed exhibited nitro and cyclic alkane (1634.38 cm-1) groups stretching due to sonication, indicating that the secondary structure was altered. Functional characteristics of UAE *V. radiata* protein isolate had improved (p<0.5) as compared to conventional protein extraction method allows use in plant-based meat analogues and other functional food product formulations.

1. Introduction

The *V. radiata*, which is a very important pulse cum legume, used widely in Asia and is slowly gaining popularity in the world as one of the best nutritionally rich foods. It can be used in both traditional diets and contemporary functional food formulations since it is regarded as a rich, readily digested, and hypoallergenic plant-based protein source. Also, the *V. radiata* proteins are low in incidences of oligosaccharides that cause flatulence and good digestibility as compared to other legumes, which aid in enhancing gastrointestinal tolerance.

Leucine, proline, arginine, glutamic acid, and phenylalanine are concentrated in high levels in *V. radiata* protein isolate making it an effective source of protein. Different researchers revealed that *V. radiata* protein has most of the same amino acids with that of soybean protein as reported by Seetapan *et al.* (2023) that can meet the recommended amino acid levels as stipulated by FAO/WHO in adults. On the other hand, *V. radiata* protein has good solubility, foaming, emulsification, and gelation qualities (Huang *et al*., 2024), which makes it appropriate for a range of food applications (such as meat substitutes and emulsified foods).

In recent years, *V. radiata* protein isolate can be utilized in a native or modified form in different food sectors, such as biodegradable/edible films, colloidal systems, and plant-based alternative products (Tarahi *et al.*, 2024) because of its high nutritional value and peculiar functional properties. In this regard, various modification technologies have been applied to further broaden its application (Feng *et al*., 2024). At present scenario, *V. radiata* protein is a chief ingredient to substitute whole-egg (Wang *et al.,* 2022, 2024) and is recognized as a protein source for manufacturing high-moisture meat analogues (Brishti *et al.,* 2021).

Ultrasonication is a non-thermal technique for removing or altering different chemicals from plant matrices. It describes sound waves with frequencies of 20-100kHz, 100 kHz-1 MHz, and 1-10 MHz, respectively, which fall into three categories: diagnostic, power, and high-frequency ultrasound (Ahmed *et al.,* 2023).

Power ultrasound is used in the majority of food processing processes for a variety of tasks, including extraction, homogenization, filtration,

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enzyme inactivation, microbial decontamination, and more (Kumar *et al.*, 2023). In a solid-liquid biphasic medium, it involves the creation, expansion and violent collapse of air bubbles that result due to temperature, pressure, and shear energy waves during sonication. Together, these impacts cause a variety of alterations in a treated system that result in extraction and/or modification. Additionally, it has been shown that *V. radiata* protein isolate functional characteristics are comparable to those of soy protein isolate, making it a high-quality natural plant-based protein (Branch *et al.*, 2017). About half of the available proteins in substrates may be extracted using the traditional alkali solubilisation approach. Numerous research has tried to improve the extractability of proteins, such as by using physical and enzymatic pretreatments. The use of ultrasonic technology to improve the extraction of plant-based proteins and its effects on the protein structure and subsequent constituent functions have not yet been thoroughly reviewed by scientists (Rahman *et al.,* 2021).

Although, there are already a lot of industrial-scale ultrasound extraction devices for the food industry on the market, such as those made by Branson (USA), Hielscher (Germany), and REUS (France), they are primarily used for the extraction of high-value bioactive compounds (Chemat *et al.,* 2017); plant protein extraction and modification applications are still restricted to laboratory or pilot plants.

Thus, the primary goal of this work was to examine how ultrasonication affected the physicochemical, structural, and functional characteristics of *V. radiata* protein, as a contemporary green extraction method of protein. Specific objectives of this research work in: (i) comparison of protein extraction from *V. radiata* using conventional and modern extraction technologies, (ii) processing parameters for ultrasound-assisted extraction, (iii) physical modifications of cell matrix and protein during ultrasonication, and (iv) nutritional, and functional modifications in protein due to ultrasonication.

2. Materials and Methods

Procured *V. radiata* var. VBN 6 from National Pulses Research Centre (NPRC), Vamban, Pudukkottai, Tamil Nadu, India. This variety was released by NPRC in 2023. It is popular for its suitability for cultivation under rice fallow condition. The *V. radiata* was subjected to removing foreign matter, dehulling, milling, sieving and defatting the flour. All chemicals and reagents procured from Sigma Aldrich Private Limited and used during the study period.

The protein was extracted by two methods: i) Ultrasonic (U) treatment followed by alkaline extraction-isoelectric precipitation method, and ii) Alkaline extraction isoelectric precipitation. The protein content of the defatted. Flour was estimated using the Kjeldahl method**.**

2.1 Extraction of protein from green gram

Ultra-assisted extraction followed by alkaline extraction isoelectric precipitation

The defatted *V. radiata* flour and distilled water were used in the ratio of 1:10 for flour dispersion by adjusting to pH 9.0 with 1N NaOH for 1 h at room temperature. The solution was subjected to ultrasonication by following specifications, *viz.,* pulsation time: 2 sec on, resting time: 4 sec off, total work time: 20 min, temperature : 25-30°C and power and amplitude:30-35% (Quintero *et al.*, 2022). Flour suspension was centrifuged at 8000 rpm for 20 min (Centrifuge-compressor model No-412, Lag- remi instruments Ltd., Vasai, India), and then the supernatant and residue precipitates were obtained. The collected supernatant was subjected to isoelectric precipitation by adjusting the pH to 4.5 using 1N HCl, centrifuged at 3000 rpm for 10 min, extracted the protein, washed with distilled water and then stored at 4°C before subjected to freeze drying (–40°C).

2.2 Estimation of physicochemical properties

2.2.1 Proximate analysis

The moisture content was estimated by using the hot air oven method, subjecting the sample to 105°C until it reached a constant weight (AOAC, 2005). The protein content was estimated using the Kjeldahl method by multiplying the results by the nitrogen conversion factor 6.38 (AOAC, 2005). Ash was created by burning at 550°C in a muffle furnace (AOAC, 2000). The Soxhlet extraction method, which uses petroleum ether or hexane as the extracting solvent, was employed to determine fat (AOAC, 2000). The fibre content was determined by using the automated Fibra-Plus apparatus (AOAC, 2000). The carbohydrate content was determined by using the anthrone method (AOAC, 2005).

2.2.2 Calculation of protein yield, protein recovery, and protein purity

An extraction efficiency of extraction methods was estimated by using standard procedure of Boye and Barbana (2012):

$$\text{Protein purity (\%)} = \frac{\text{Mass of protein in extract (g)}}{\text{Total mass of recovered extract (g)}} \times 100$$

$$\text{Protein recovery (\%)} = \frac{\text{Mass of protein in extract (g)}}{\text{Total mass of protein in starting material (g)}} \times 100$$

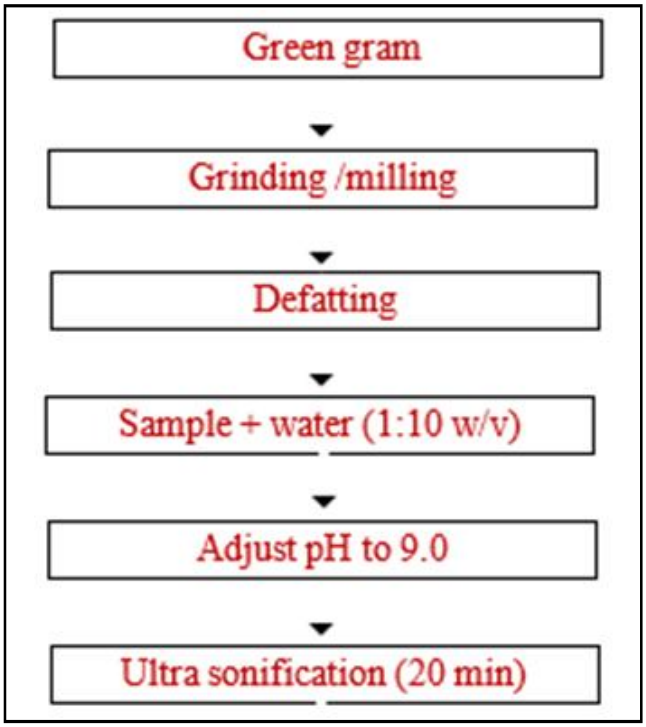
$$\text{Protein yield (\%)} = \frac{\text{Mass of protein in extract (g)}}{\text{Total mass of starting material (g)}} \times 100$$

Plate 1: *V. radiata* flour (DD).

Plate 2: *V. radiata* isolate (AEP).

Plate 3: *V. radiata* protein isolate (UAE).

Figure 1: Comparative visualization of *V. radiata* protein isolates produced by AEP and UAE.



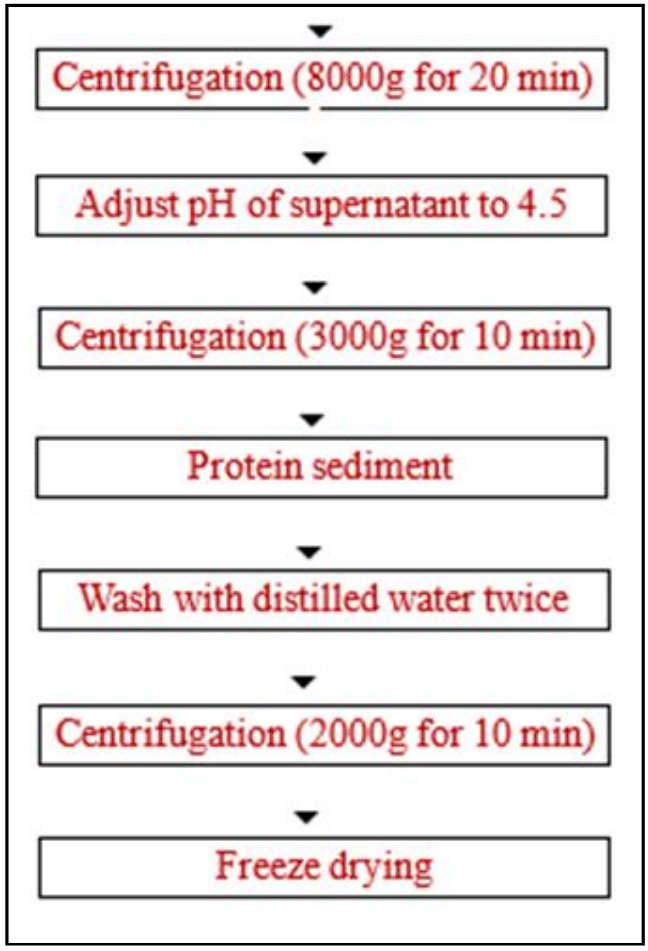


Figure 2: Flow chart of protein extraction using ultrasonication.

2.3 Structural properties

2.3.1 Scanning electron microscopy (SEM)

Morphological structure of ultrasonicated *V. radiata* protein isolate was done using scanning electron microscopy (SEM) (Manufacturer: FEI, Czech republic, Model: Quanta 250). The powdered sample was spread on a double-sided conductive carbon tape fixed on the stub and placed in the sample chamber of Environmental scanning electron microscope (ESEM). After attaining high vacuum, the filament was turned on and adjusted various parameters like electron beam, intensity, spot size, voltage, and emission. The images were captured and depicted.

2.3.2 Structural analysis

The structural analysis of protein samples was evaluated using Fourier transform infrared spectroscopy (JASCO FTIR-6800). IR wavelength range 4000 to 400 cm^{-1} mode-ATR mode.

2.3.3 Particle size analysis

The sample was diluted in the ratio of 1:10 for sonication time:60 seconds in particle size analyser (Name- Horiba scientific, Model-nano partica SZ-100, Japan).

2.4 GC-MS analysis

V. radiata isolate was subjected to GC-MS analysis to identify the bioactive compounds. It was conducted using a Shimadzu GC-MS-TQ8040 triple-quadrupole system equipped with Smart MRM technology, enabling high sensitivity targeted analysis and simultaneous acquisition of qualitative and quantitative data.

2.5 Functional properties

The water and oil absorption capacities of ultrasonicated *V. radiata* protein isolate and *V. radiata* protein isolate using conventional method were measured using the method (Deng *et al.*, 2019). The foaming capacity and stability were measured following the method (Branch *et al.*, 2017). Emulsion activity and stability properties assessed by the method (Branch *et al.*, 2017). Determination of bulk density was carried out by the method of (Penchalraju and John Don Bosco, 2022).

2.6 Statistical analysis

All analyses were carried out in triplicate. SPSS software was used to statistically analyse the data using Two-way analysis of variance (ANOVA), and the results were displayed as mean ± standard deviation, Post-hoc test results at a 5% significance level ($p<0.05$), the differences were compared.

3. Results

3.1 Chemical properties of *V. radiata* isolates

The proximate composition of *V. radiata* flour and its isolates showed significant variation among treatments. The results are depicted in Table 1. Moisture decreased significantly from 11.05% in raw flour to 4.02% in AEP isolate and further to1.84% in UAE isolate. Smriti Shrestha *et al.* (2023) also reported that there is a decrease in moisture content in the *V. radiata* protein isolates than when compared to moisture content in the green gram flour. Protein increased drastically from 22.85% (flour) to 81.60% (AEP isolate) and 83.70% (UAE isolate). This indicates that UAE is more effective in concentrating protein compared to AEP alone. A study conducted by Banura *et al.* (2023) showed there was an increase in protein content with an increase in ultrasonication duration for both *V. radiata* and lentils. A sharp reduction in fat (from 1.54% to 0.12%), fibre (from 1.09% to 0.40%), and carbohydrate content (from 62.05% to 3.89%) was observed in the UAE isolate, demonstrating effective removal of non-protein components during processing.

Table 1: Proximate composition of *V. radiata* isolates

Nutritional parameters	*V. radiata* flour	*V. radiata* isolate (AEP)	*V. radiata* isolate (UAE)
Moisture (%)	11.05 ± 0.44^{f}	4.02 ± 0.11^{d}	1.84 ± 0.07^{bc}
Protein (%)	22.85 ± 0.61^{g}	81.6 ± 0.95^{i}	83.70 ± 2.41
Fat (%)	1.54 ± 0.03^{bc}	0.16 ± 0.00^{a}	0.12 ± 0.00^{a}
Ash (%)	3.60 ± 0.01^{d}	2.80 ± 0.11^{cd}	1.33 ± 0.00^{bc}
Fiber (%)	1.09 ± 0.02^{ab}	0.73 ± 0.02^{ab}	0.40 ± 0.01^{ab}
Carbohydrate (%)	62.05 ± 1.11^{h}	08.9 ± 0.36^{e}	03.89 ± 0.05^{d}
Source	**S S**	**F-value**	**Sig.**
Extraction methods	30.380	19.731	.000
Nutrient parameters	27176.614	7060.338	.000
Extraction methods* Nutrient parameters (Interaction)	13511.742	1755.139	.000

Different superscripts within the same column are statistically significantly different at $p<0.05$.
AEP-Alkaline extraction followed by iso electric precipitation; UAE Ultra-assisted extraction followed by AEP.

Two-way ANOVA revealed that extraction methods had a significant effect ($p<0.05$) on the proximate composition of *V. radiata* samples. Nutrient parameters also showed a highly significant effect ($p<0.01$), indicating substantial variation among compositional components. Furthermore, a highly significant interaction between extraction methods and nutrient parameters ($p<0.01$) was observed. This interaction suggests that the effect of extraction techniques varied depending on the specific nutrient component. The percentage of protein recovery (13.01%) and protein yield (10.23%) were significantly higher in UAE compared to AEP ($p<0.05$).

Tukeys HSD post hoc test (Table 2) revealed that the UAE treatment differed significantly ($p<0.05$) from both AEP and flour. However, no significant difference was observed between AEP and flour ($p>0.05$) and homogeneous subset analysis (Table 3) revealed that UAE formed a separate subset, indicating a significant difference ($p<0.05$) compared to AEP and flour. This indicates that ultrasonication-assisted extraction significantly influenced the measured parameter.

3.2 Structural characterization of *V. radiata* protein isolates

3.2.1 Scanning electron microscopy of *V. radiata* protein isolate.

In Figure 3, the SEM micrographs of the ultrasonicated *V. radiata* protein isolate revealed substantial morphological modifications indicative of cavitation-induced structural disruption. At lower ((1000x and 2000x) magnification, the untreated-like dense, smooth, and compact granules were replaced by irregular, fragmented particles with visibly roughened surfaces. Extensive deformation, rupture,

and peeling of the granules were also observed as a result of ultrasonication as was between the appearance of cracks, fissures and porous areas around the entire structure. Greater magnification (3000x and 5000x) images revealed that collapsed granules, internal fibrillar network exposures as well as layered sheet-like fragments were present proving the outer penetration of ultrasound deep into the protein-starch matrix. The general morphology with structural loosening, surface roughness and the development of microchannels are sure that high-intensity ultrasonication plays a critical role in disrupting the native structure of *V. radiata* protein isolate. The granules breakdown and the formation of microchannels are indicative of the detachment of the protein-starch structure, which is commonly reported to happen in *V. radiata* , pea, chickpea, and soy proteins after ultrasonic treatment (Hu *et al.,* 2013: Arzeni *et al.,* 2012: Tari *et al.,* 2018: Zhang *et al.,* 2020). Earlier research likewise found the enhancement of hydration characteristics and functional features of plant proteins when the structural disruption was done using ultrasound (Loushigam *et al.,* 2023).

3.2.2 Fourier transform infrared spectroscopy (FTIR).

The FTIR spectrum (Figure 4) indicates typical absorption bands that relate to the key functional groups in proteins mainly amide, hydroxyl, aliphatic, and carbohydrate-related vibrations. The freeze-dried *V. radiata* protein isolate has characteristic amide I (1634 cm^{-1}), amide II (1532 cm^{-1}) and amide III (1331 cm^{-1}) bands, which proves the existence of the protein-rich structure. The fact that the amide I peak is located at approximately 1634 cm^{-1} means that the protein isolate is mostly in the β-sheet conformation. The broad band at 3263 cm^{-1} corresponds to O-H and N-H stretching vibrations, signifying hydrogen bonding and the presence of hydrophilic amino acid residues. Peaks at 2931 cm^{-1} and 1075-1148 cm^{-1} represent aliphatic C-H stretching and carbohydrate-associated C-O stretching, respectively, suggesting minimal carbohydrate residues in the isolate.

Table 2: Post-hoc Tukeys HSD analysis showing pairwise comparison of extraction methods for protein extraction

Extraction method (I)	Extraction method (J)	Sig.
AEP	*V. radiata* flour	.075
	UAE	.001
V. radiata flour	AEP	.075
	UAE	.000
UAE	AEP	.001
	V. radiata flour	.000

Table 3: Homogeneous subset grouping of extraction methods for extraction of protein based on Tukey's HSD test.

Extraction	N	Subset	
		1	2
UAE	18	15.21	
AEP	181		6.37
V. radiata flour	18		17.03
Sig.	1.000		.075

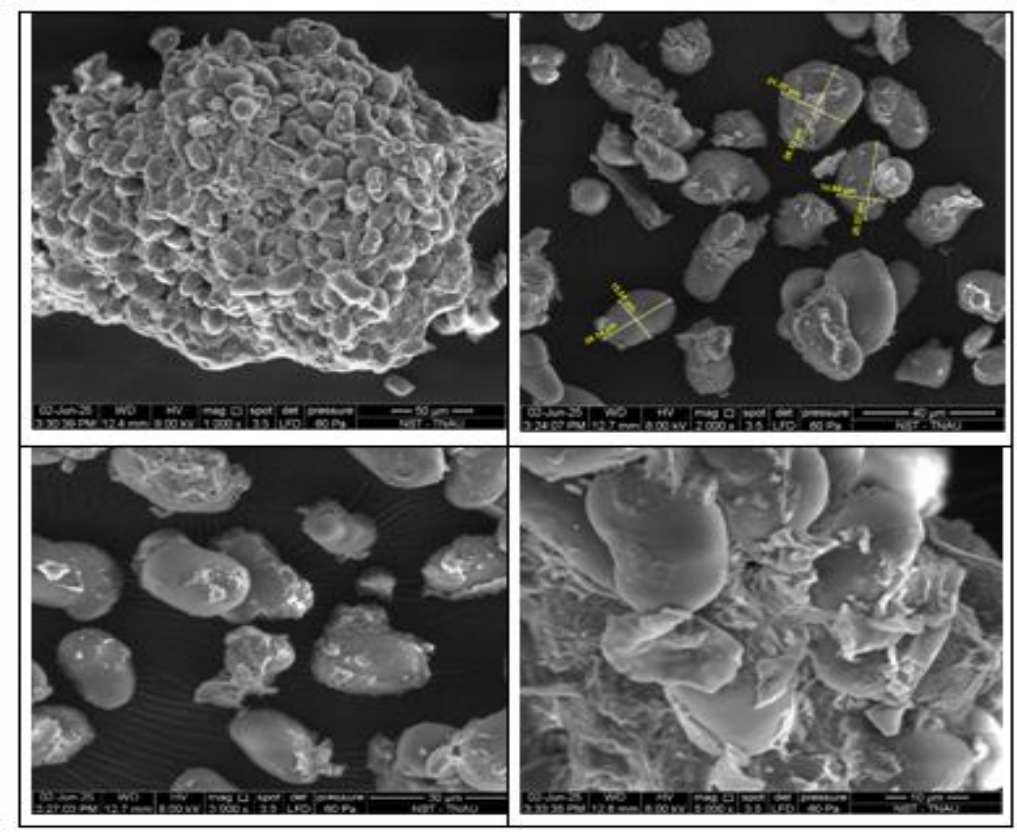


Figure 3: Scanning electron microscopy (SEM) images of ultrasonicated *V. radiata* protein isolate.

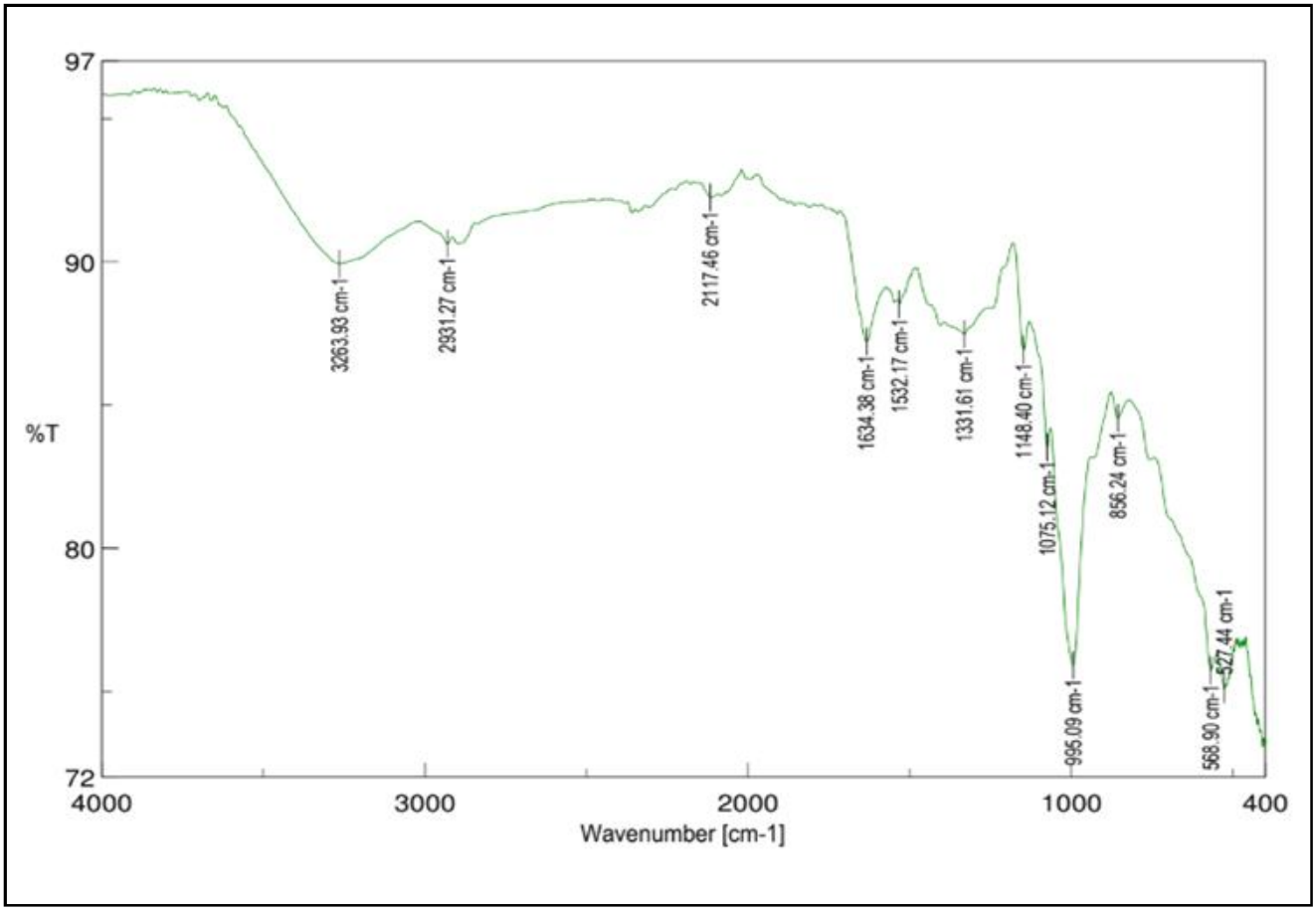


Figure 4: Structural analysis using FTIR for analysing the structural changes of *V. radiata* protein isolate upon ultrasonication.

Table 4: Presence of structural and functional groups in ultrasonicated *V. radiata* isolate

Frequency, cm^{-1}	Bond	Functional groups
O-H and N-H stretching	3263.93 cm^{-1}	Carboxylic acids, protein N-H (amide A)
C-H stretching	2931.27 cm^{-1}	Alkanes indicate aliphatic chains (lipids)
C (triple bond) C-stretch	2117.46 cm^{-1}	Alkynes
N-H bend	1634.38 cm^{-1}	Confirms protein secondary structure, primary amines
N-O asymmetric stretch	1532.17 cm^{-1}	Amide II
C-N stretch aromatic amines	1331.61 cm^{-1}	Aromatic amines
C-O stretching	1148.40 cm^{-1}	Presence of carbohydrates
=C-H bend	1075.12 cm^{-1}	Alkenes
N-H wag, C-H “oop”	996.09 cm^{-1}	Primary, secondary amines, aromatics

The main components of the nitro compound (1600-1500 cm^{-1}) of proteins are the N-O and C=C stretching vibrations of the nitro group and the cyclic alkane group, which can be used to investigate the structure. The ultrasonicated *V. radiata* protein isolate showed nitro and cyclic alkane groups stretching during sonication, as seen in Figure 4, suggesting that the secondary structure of the chickpea was changed. The α-helix structure was stabilised after ultrasonic treatment because ultrasound cavitation disrupted the links between the nitro, hydrogen, and alkane groups (Kang *et al.,* 2022b). Similar results were noticed in the study conducted by Sidiq *et al.* (2024), the ultrasonicated *V. radiata* protein showed increased intensity for the amide II region (Peak range:1500-1580) in comparison to the native *V. radiata*. A similar increase was reported by Gani *et al.* (2022) and Sun *et al.* (2022) for ultrasonication of apple seed protein.

3.2.3 Particle size analysis (PSA).

In our results, the *V. radiata* isolate showed a unimodal distribution (Figure 5), meaning most particles are around one main size range.

D_{50} (median particle size) appears to be in the slightly smaller range, likely in the 15-40 ìm range. This decrease might be because of the protein unfolding, which is being stimulated by ultrasonication treatment, or it may also be because of the cavitation generated from the breakdown of microbubbles propagating as sound waves (Jiang *et al.*, 2017). The distribution curve is narrow, which means that there are fewer oversized or very fine particles. Smaller particles imply more surface area which may increase properties of hydration and emulsification, increase water-binding and oil-binding. The size is the most suitable to apply in plant-based meat analogues, as it contributes to the creation of smooth, cohesive structures with the assistance of binders such as wheat gluten.

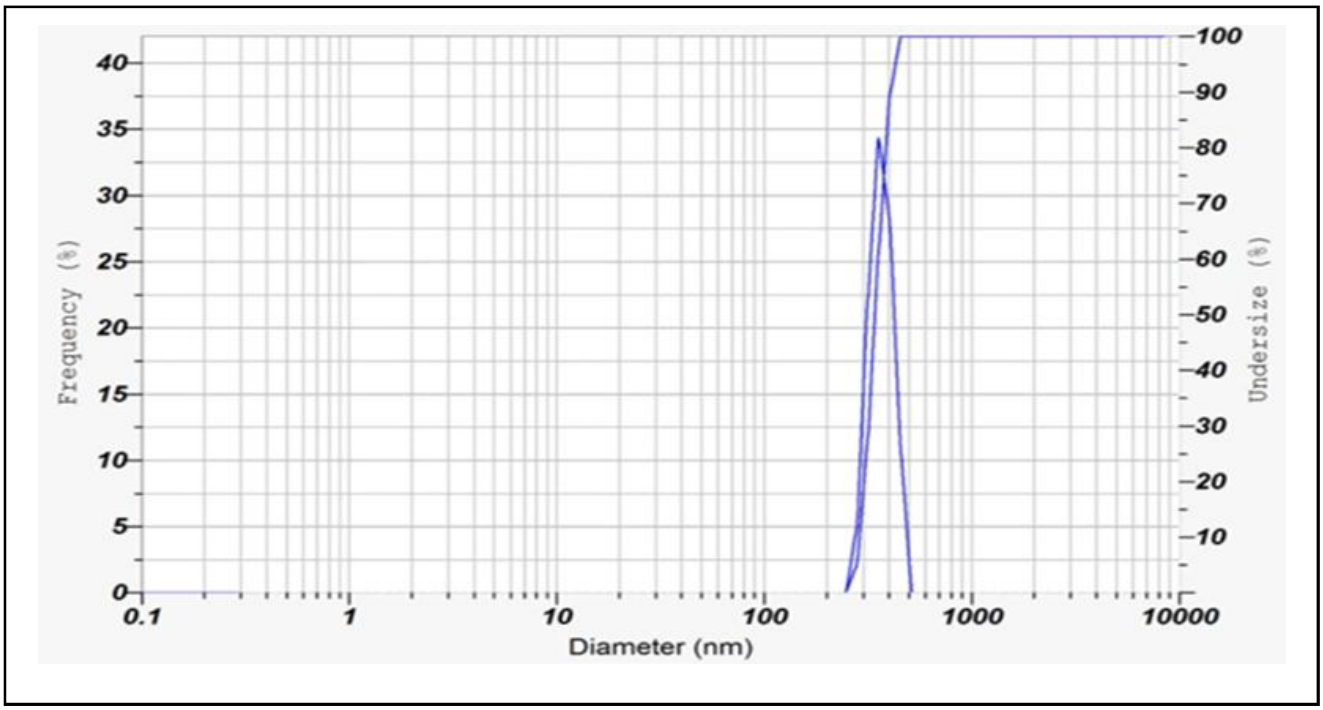


Figure 5: Particle size analysis of *V. radiata* protein isolate upon ultrasonication.

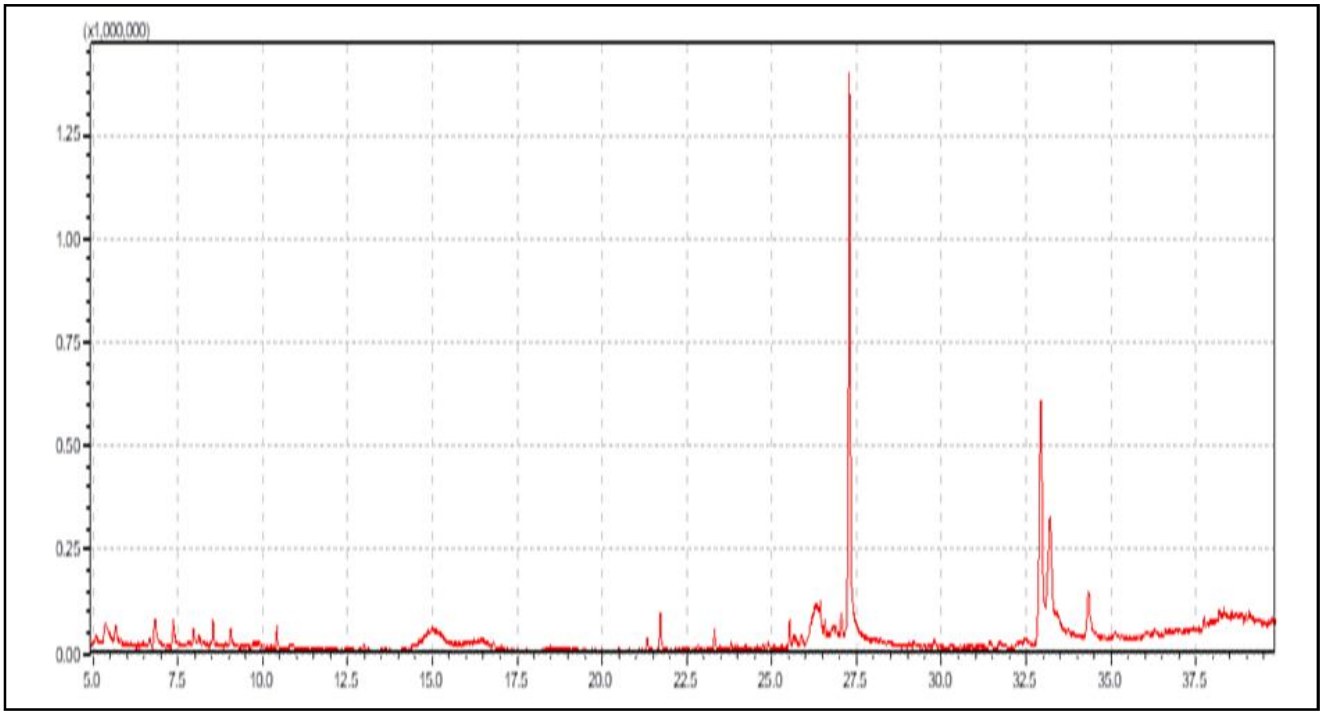


Figure 6: GC-MS chromatogram of *V. radiata* protein isolate.

Sidiq *et al.*(2024) was discovered that the average particle size of native and ultrasonicated *V. radiata* protein is 10.37 µm ± 0.9 and 484 nm ± 0.89, respectively. There is a decrease in average particle size due to ultrasonication. Similar results found by Shanshan Jiang *et al.* (2017) in pea protein isolate at different pH levels treated by power ultrasound for 5 min, increased the solubility than the control (untreated with ultrasonication), reaching an average particle size of 45.2 nm. Thus, ultrasonication treatment improved protein properties notably.

3.4 GC-MS analysis

GC-MS analysis was employed indirectly to assess the purity of the *V. radiata* protein isolate by identifying residual non-protein constituents, as intact proteins are not detectable by this technique.

Peak#	Retention time (Min)	Area%	Name	Formula	Molecular weight	Biological activity	References
1	8.540	1.69	Undecane, 4,7-dimethyl-	$C_{13}H_{28}$	184.36 g/mol	Helps in retention of aroma in food systems	Huang *et al.*, 2024.
2	12.648	0.22	Thiazole	$C_{14}H_{12}N_2O_2S$	272.32 g/mol	Antibacterial, anti-inflammatory, antifungal, antimalarial, antidiabetic, antitubercular, antioxidant, anticonvulsant, anticancer, and cardiovascular activities	Farghaly *et al.*, 2024.
3	14.395	0.32	D-Serine	$C_3H_7NO_3$	105.09 g/mol	Acts as a gliotransmitter regulating excitatory neurotransmission	Ito *et al.*, 2018.
4	14.421	0.32	Hexanoic acid, 2-methyl-	$C_7H_{14}O$	130.18 g/mol	Flavor enhancer, flavoring agent or adjuvant	Shilov, *et al.*, 2022.
5	14.894	1.06	Ribitol	$C_5H_{12}O_5$	153.14 g/mol	Helps in macro nutrient metabolism, as well as iron and vitamin B6 absorption plays a role in energy formation and cell respiration in the cell	Ahmet Gürses *et al.*, 2024.
6	15.130	1.31	l-Aspartic acid, beta.-methyl ester	$C_5H_8NO_4$	146.12 g/mol	Antimicrobial and antitubercularactivities, immune suppressive and anticancer activities	Lemrova *et al.*, 2022.
7	15.211	0.88	Heptanal	$C_7H_{14}O$	114.19 g/mol	Acts as antifungal agent	Li *et al.*, 2022.
8	16.068	0.65	5-Hexen-1-ol	$C_6H_{12}O$	100.16 g/mol	Utilized as a flavoring agent, aroma compound	NCBI, 2026.
9	16.930	0.22	dl-Ornithine	$C_5H_{12}N_2O_2$	32.16 g/mol	Acts as a "kokumi" (taste-enhancing) substance that improves flavor,	NCBI, 2026.
10	27.046	3.78	11,14-Eicosadienoic acid	$C_{20}H_{36}O$	308.49 g/mol	Exhibits anti-inflammatory properties, antimicrobial activity	Taha *et al.*, 2019.
11	27.291	83.46	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43 g/mol	Multifunctional bioactive compound	Ghfil *et al.*, 2025.

Table 6: Functional properties of *V. radiata* isolate with different protein extraction methods

Functional property	*V. radiata* isolate (AEP)	Ultrasonicated *V. radiata* isolate (UAE)
WAC (g/g)	3.64 ± 0.08^{b}	2.30 ± 0.07^{o}
OAC (g/g)	8.85 ± 0.03^{ab}	11.25 ± 0.01^{j}
WAI (g/g)	3.46 ± 0.14^{c}	4.15 ± 0.00^{k}
WSI (%)	16.28 ± 0.71^{d}	23.28 ± 0.20^{e}
EC(%)	28.5 ± 0.10^{b}	45.14 ± 1.40^{h}
ES (%)	74.80 ± 0.77^{b}	82.94 ± 2.99^{l}
EAI (m2 /g)	$47 .0 \pm 0.06^{f}$	51.0 ± 0.18^{n}
ESI (min)	13.0 ± 0.4^{g}	28.0 ± 0.15^{m}
FC (%)	65.07 ± 0.26^{h}	75.04 ± 1.72^{p}
FS (%)	72.99 ± 0.53^{i}	96.22 ± 0.75^{a}
BD (g/ml)	0.52 ± 0.02^{mn}	0.71 ± 0.03^{a}
Source	**F-value**	**Sig.**
Extraction method	10392.7421	0.000 **
Functional property	1065.0699	0.000 **
Extraction method* Functional property	2768.6112	0.000 **

Different superscripts within the same column are statistically significantly different at $p<0.05$.

WHC-Water holding capacity; OHC-Oil holding capacity; WAI-Water activity index; WSI-Water solubility index; EC-Emulsion capacity; ES-Emulsion stability, EAI-Emulsifying activity index, ESI-Emulsifying stability index, FC- Foaming capacity, FS-Foaming stability, BD-Bulk density.

3.5 Functional properties of *V. radiata* isolate

The functional properties of *V.radiata* isolates showed significant variation among treatments. The results are depicted in Table 6.

3.5.1 Water and oil absorption capacities.

Significant differences were observed in the functional properties of *V. radiata* isolates obtained through UAE and AEP extraction. Since the protein has both hydrophilic and hydrophobic nature, it absorbs both water and oil. It depends on the amino acids present in the isolate, based on the treatments given. In this case, the WAC of AEP isolate (3.64 g/g) was greater than that of UAE isolate (2.30 g/g), which showed that ultrasonication decreased the capacity of proteins to bind water, probably because, ultrasonication caused the structure to unfold exposing lesser number of hydrophilic groups. In contrast, OAC increased substantially following ultrasonication, rising from 8.85 g/g in the AEP isolate to 11.25 g/g in the UAE isolate, suggesting enhanced hydrophobic interactions resulting from cavitation-induced modification of protein surfaces. Increasing the OHC results in flavor retention and a good mouth feel (Gopika Sudarsanan *et al.,* 2023). The same results were noticed by Sidiq *et al.* (2024) in ultrasonicated *V. radiata* nanoparticles.

3.5.2 Emulsion activity index and emulsion stability index.

EC and ES determine the emulsifying property. Since they adsorb at the oil-water interface and create a tightly packed layer, proteins are utilized as emulsifiers. The amount of oil that can be emulsified by protein is indicated by the emulsifying activity, and the stability of the emulsion over time is determined by its stability. These two properties are affected by the configuration on the surface of a protein of hydrophobic and hydrophilic residues and the folding and unfolding process of a protein molecule at the oil-water interface (Sidiq *et al.,* 2024). Ultrasonication also significantly improved the emulsion activity level, increasing the respective EC levels of 28.5% to 45.14% and the ES of 82.94% as compared to AEP isolate (74.80%), showing that ultrasonication improved the interfaces behaviour of proteins.

Our results are in the range of the EA of 23.56% described in the work of Brishti *et al.* (2017) in the study of the impact of different drying processes on the different properties of the *V. radiata* isolate powder. The EA and ES were found to be increased in ultrasonicated *V. radiata* protein isolate, which can be attributed to effect of ultrasonication procedure that improves physicochemical properties of protein isolate. Additionally, the outcomes show a positive correlation with the protein isolates surface hydrophobicity (Branch *et al.*, 2017).

3.5.3 Foaming capacity and foaming stability.

The FC and FS of AEP compared to UAE protein have been represented in (Table 6). FC and FS also showed slight improvements in the UAE treated *V. radiata* isolate (75.04% and 96.22%) relative to the AEP isolate (65.07% and 75.04%), likely due to improved protein flexibility and reduced particle size. A similar increase was reported by Xiong *et al.,* (2018) in the foaming capacity of pea protein upon ultrasonication and by Brishti *et al.* (2017) for the foam stability of *V. radiata* protein isolate.

3.5.4 Bulk density

Ultrasonication creates intense shear forces and cavitation bubbles. These break down large protein aggregates into smaller, more compact particles. Smaller particles pack more efficiently, leaving fewer air spaces leads to higher bulk density. In our results, the UAE assisted *V. radiata* protein isolate showed higher bulk density (0.71 ± 0.03 g/ml) when compared to the AEP (0.52 ± 0.02 g/ml) extraction method. Our results coincide with those of Sidiq *et al.* (2024) in ultrasonicated *V. radiata* nanoparticles.

Statistical analysis ($p<0.05$) showed significant effects of treatment and interaction for all functional parameters, confirming that ultrasonication altered the structural and interfacial properties of the *V. radiata* protein isolate, thereby improving its performance in emulsification and foaming applications.

4. Discussion

The current experiment indicated that UAE, as well as AEP, were both effective in changing the proximate composition of *V. radiata* isolates leading to a very potent protein-containing ingredient. The significant difference in protein content in the AEP isolate (81.60%) and raw flour (22.85%) is in agreement with previous studies that alkaline solubilization followed by isoelectric precipitation is selective in recovering proteins in legumes by excluding starch, fiber, and other non-protein compounds (Chandra and Shamasundar, 2015:Stone *et al.,* 2019). The use of ultrasonication before extraction additionally enhanced protein yield (83.70%), which could be explained by the fact that acoustic cavitation breaks the cell walls, facilitates the mass transfer, and increases solubility of proteins (Jambrak *et al.,* 2008). Protein recovery improvement has also been seen to be enhanced in a similar way as is found in chickpea, soybean, and lentil proteins following ultrasonication (Gorguc *et al.,* 2020; Hu *et al.,* 2013).

The decrease in moisture content under ultrasonic (1.84%) means dehydration and concentration of solids was more favorable because the cavitation facilitated the release of water in cellular matrices (Zhang *et al.,* 2021). The observed reduction of carbohydrates, fat, and fiber in the UAE isolate is also supportive to the influence of ultrasonication in enabling the non-protein component removal. Past research has documented similar changes in the proportion of carbohydrates and fiber in ultrasonicated legume isolates, due to the disturbance of the matrix and the increase in the separation efficiency (Wong *et al.,* 2020). The decrease in the ash content of the ultrasonicated sample due to the good removal of mineral-related cell wall debris, which led to a purer protein isolate.

The enhanced purity of the protein isolate acquired by ultrasonication implies significant functional values. Protein isolates of high purity usually have superior solubility, emulsification and gelation properties because of the reduced number of additional components like starch granules and fiber particles (Shevkani *et al.,* 2015). Thus, the changes in composition, which have been detected in this study, could have applications in food production, including plant-based meat analogues, beverages, and protein-rich formulations.

The extraction method dramatically affected the functional properties of isolated *V. radiata* protein and ultrasonication resulted in significant

enhancements in most of the essential properties. The decrease in the ability of WAC during the cultivation of the UAE isolate in comparison with the AEP isolate indicates that the protein molecules in the UAE isolate, during cavitation, undergo structural changes. Ultrasonication breaks native protein matrices leading to partial unfolding and decreased access to hydrophilic locations to bind water (Zhang *et al.,* 2021). Other comparable reductions in WAC after the ultrasonic treatment of pea, soy, and lentil proteins (Gharibzahedi *et al.,* 2019) show that structural changes lower the water-holding capacity.

Conversely, ultrasonication had a substantial impact on OAC which can be explained by exposing hydrophobic amino acid residues caused by protein unfolding and size reduction (Jahaniaval *et al.,* 2020). Increased OAC is preferable in bakery formulations, meat analogues and in flavor retention. The same previous studies have also shown that the oil-binding of the ultrasound-treated legume proteins is enhanced by the fact that the proteins have enhanced surface hydrophobicity and structural flexibility (Hu *et al.,* 2013).

The marked increase of EC and ES in the UAE isolate suggests that there was an improvement in the interfacial properties. Ultrasonication favors the formation of smaller protein particles that have a larger surface area and are more likely to adsorb quickly to the oil water interface (Gorguc *et al.,* 2020). There is also the additional contribution of cavitation to the solubility and dispersion, which leads to a more stable emulsion as was noted in soybean and chickpea isolates that underwent an ultrasound-assisted extraction (Stone *et al.,* 2019). The greater ES of UAE sample (82.94%) is a sign of greater ability to resist droplet coalescence, which is an important functional property to use in dressings, beverages, and fat-reduced foods.

The ultrasonic isolate also had a slightly better foaming capacity and stability which indicates better protein flexibility and quick diffusion to the air/water interface. The ultrasound-treated legume proteins have a tendency to foam better because the sizes of the particles are reduced, and the solubility is higher (Shevkani *et al.,* 2015). The growth in bulk density of the UAE isolate can be explained by the closer arrangement of protein particles after structural disruption because ultrasonication generally decreases particle sizes and results in smaller aggregates (Wong *et al.,* 2020).

Maintaining secondary structure (based on Amide I/II data) implies that such functional properties as emulsification and gelation could be maintained. Minor carbohydrate peaks are an indication of potential functionality in the water-binding or viscosity. Other peaks of 800-600 cm^{-1} are related to aromatic amino acid and fingerprint region vibrations. Altogether, the FTIR spectrum indicates the successful isolation of a structurally intact protein with typical functional group properties of plant-derived protein isolates.

Table 6 shows the existence of bioactive compounds in the UAE *V. radiata* protein isolate. After lipid-derived compounds, n-hexadecanoic acid (Palmitic acid) was the dominant compound in the chromatographic profile comprising 83.46% of the total peak area with other smaller values. This amount of palmitic acid is characteristic of legumes in terms of protein isolates, since residual lipids are still attached to the protein matrices despite the extraction process, and it also helps formulate plant-based meat products, including its mouthfeel and lubrication properties and fat-like texture. The presence of essential lipid components that facilitate emulsification, thermal stability, flavour formulations in meat analogues or meat alternatives is further proved by other fatty acids such as 11,14-Eicosadienoic acid (3.78%) and a derivative of stearic acid (2.79%). D-serine, L-aspartic acid methyl ester, proline derivatives and ornithine were found in small traces, suggesting that some of the proteins were partially hydrolyzed, denatured, or derivatized as a result of the extraction or GC-MS procedure. These compounds are indicative of the protein rich nature of pulse isolates, and could be precursors to desirable flavour generation in the maillard reaction during cooking plant-based meat. The derivatives of carbohydrates such as ribitol and 1, 6-anhydro- 2-D-glucopyranose indicate the existence of the remnants of oligosaccharides or thermally damaged polysaccharides that are prevalent in legume seed coats and cotyledons. Volatile compounds present in minute amounts include; heptanal, 5-hexen-1-ol, branched hydrocarbons, and linalool methyl ether, which are mostly as a result of the lipid oxidation routes and make up the delicate aroma profile of the isolate. The same findings were achieved by Penchalraju and John Don Bosco (2022).

Limitations

Extended ultrasonication treatment (>25 min) may decrease the protein extractability and functionality potentially due to protein denaturation and aggregation result in the protein re-polymerizing, reducing its solubility (Kang *et al.,* 2022c) and may cause protein aggregation, reduced interfacial adsorption, and heightened droplet coalescence (Alrosan *et al.,* 2026).

5. Conclusion

Based on the research work conducted above, it can be concluded that ultrasonication combined with alkaline extraction and isoelectric precipitation greatly enhanced techno-functional characteristics of *V. radiata* protein isolates by causing microstructural fragmentation, decreasing particle size and rearrangement of protein secondary structure. The drying technique also plays a crucial role in retaining the nutritional compounds which are heat sensitive. The combination of physicochemical and functional benefits recorded in this case shows that ultrasound is potentially a viable green extraction procedure in the production of high-performing plant protein isolates in food industry to develop plant based meat analogues and meat alternatives. Still, there is a need to conduct research for optimization of ultrasonic parameters to extract the protein from *V. radiata* isolate along with other green extraction technologies.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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