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Extraction, optimization, and functional properties of a novel plant protein from kiwifruit (*Actinidia Chinensis*) seeds using ultrasound; a clean and green extraction procedure

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Abstract

The current trend of protein production in New Zealand is primarily focused on proteins sourced from dairy and meat which are known to generate enormous amounts of greenhouse gases and carbon footprints. Thus, to establish a flexitarian world for future sustainability, novel plant proteins from several sources are being recognized and researched lately so that they could replace the meat proteins. One such source of protein are oleaginous plant seeds which are being imagined and explored as a renewable resource of plant proteins worldwide. The oleaginous seeds and seed meals from oil extraction industries, cosmetic industries, and beverage industries related to kiwifruit, represent a significant waste of protein present in these seeds. Moreover, in New Zealand *Actinidia Chinensis* production, exports and wastage have been increasing manyfold since last decade, as the fruit commodity is shifting from a high-end product to more generalised fruit. Thus, to explore the novel protein in seeds and seed meals of *Actinidia Chinensis* this study was carried out.

Actinidia Chinensis seeds were purchased from Pure ingredients Ltd. New Zealand and were primarily investigated for their physicochemical characteristics to understand the properties of the starting material for the protein extraction process. The seeds were then pulverized, defatted, and analysed for its microstructure and proximate compositions to understand the effect of defatting and grinding on protein contents of defatted seed flour from the *Actinidia Chinensis* seeds. Further, seed protein was extracted using two different extraction technologies. A clean and green extraction technology; ultrasonication and the conventional stirring method of protein extraction. The results show that ultrasound extraction significantly increased the overall protein yield from the defatted seed flour by 12% if compared to the conventional method. The optimized extraction parameters for the ultrasonic extraction of defatted *Actinidia Chinensis* seed meal were pH 11.5, temperature 55^{0}C, ratio of seed flour to solvent (4:100) and 100 mins of ultrasonic extraction. pH was identified as the most significant factor involved in successful extraction of proteins from kiwifruit seeds whereas temperature and ratio were respectively significant factors after pH. SEM was used to analyze the effects of ultrasonication on the seed meal, seed flour and seed protein extracted from the *Actinidia Chinensis* seeds. The results confirmed significant effects of fragmentation and cavitation caused by ultrasound waves on seed meal, flour, and protein respectively and increased the protein extraction yield by 12% in this study.

The amino acid analysis of seed protein in this study defines a typical acidic profile for *Actinidia Chinensis*, where glutamic and aspartic acid were the dominating amino acids which defines an acidic profile. the most abundant essential amino acids were Leucine, Lysine and Valine respectively and the least available EAAs were the sulphur AAs such as Cysteine and Methionine. Moreover, in the final sections of this study a few functional properties of the seed protein concentrates are compared with the functional properties of conventionally extracted protein. The results reveal a significant increase in the WHC and OBC of the protein concentrate, whereas the least gelation capacity of the proteins was also positively affected by ultrasonic assisted extraction of kiwifruit seed proteins.

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List of abbreviations

SPs – Seed storage proteins

PBS – Phosphate buffer saline

PSV – Protein storage vacuoles

TAG – Triacylglycerols

BBD – Box Behnken Design

DOE – Design of experiments

KS – Kiwifruit seeds

WHO – World Health Organization

KSF – Defatted kiwifruit seed flour

KSPC – Kiwifruit seed protein concentrate

ANOVA – Analysis of variance

UAE – Ultrasound assisted extraction

WHC – water holding capacity

OBC – Oil binding capacity

SH – Sulfhydryl

KSP – Kiwifruit seed protein

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Chapter 1: Introduction and background

In the 21st century, protein has emerged as one of the most critical nutrients to support human health and longevity as it serves multiple functions in a human body such as biocatalysts, messengers (hormones), structural elements of a cell (cytoskeleton) and protectors (toxins) etc. which are crucial for maintaining a healthy lifestyle. Today, the global supply of proteins for human consumption is predominantly hinged to the livestock industries, which provide us with meat as a form of dietary protein and milk through our dairy industries (Ertl, Knaus, & Zollitsch, 2016). However, these conventional sources are also partially responsible for the emission of several greenhouse gases and have a high carbon footprint, making them an unsustainable food source in the longer term (Weindl et al., 2020). Thus, a balanced partial switchover in global protein consumption from animal-based to plant-based has been suggested by several environmental pundits as an effective way to reduce greenhouse gas emission and other adverse impacts on the environment (Aschemann-Witzel, Gantriis, Fraga, & Perez-Cueto, 2020; De Boer & Aiking, 2011; He, Evans, Liu, & Shao, 2020; Millward, 1999). Based on food and agriculture data (FAO, 2018), the total production of plant proteins was about 410 Mt in 2016, five times the production of edible protein originating from an animal-based (livestock)/ dairy source. In contrast, the consumption of animal proteins is comparatively reported to be 1.5 times higher than the consumption of plant-based proteins, indicating that a significant amount of edible plant protein is either being used as animal feed or is dumped as a waste (FAO, 2018; Mayer, 1958). Therefore, it is fairly justified that the global expectation of feeding a population of 10 billion by 2050 can be possibly met by revaluation of plant protein waste streams from preexisting food and processing industries, which are currently being ignored.

The global production of fruits was increased by 8.9% over the last decade (2011-2019), as there has been a colossal rise in fruit consumption across the globe (Viktorivna, 2020). Moreover, as fruit consumption is being encouraged by international organizations such as the WHO for better mental health and fitness, the entire fruit industry is experiencing an exponential growth (Abadi, Mahdavian, & Fattahi, 2021; Brookie, Best, & Conner, 2018). However, this exponential growth and expansion of fruit consumption and processing has also generated enormous quantities of fruit wastes, which is proved to have striking negative impacts at environmental, social, and economic levels (Abadi et al., 2021; C. Chen, Chaudhary, & Mathys, 2020). In the fruit

sector, this issue of wastes represents a critical point for products that belong to specialty foods, for instance kiwifruit, berries etc. These commodities generally have such high standards of quality and consumer acceptance, that even a minor aesthetic defect is enough to compromise the compactivity of these products in the market chain. These wastes, which until a few decades ago were considered to be of no benefit as a commodity and were used as animal feed, have turned out to contain high value components such as proteins, fats, polysaccharides, fibers, flavor compounds, and phytochemicals (Baiano, 2014). These nutritionally and pharmacologically-functional ingredients have also proved to be a vast source of natural food ingredients, additives and supplements with high economical value (Sagar, Pareek, Sharma, Yahia, & Lobo, 2018; Wadhwa & Bakshi, 2013). Several detailed evaluation studies on bioactive compounds contained in different parts of a fruit have found that the fruit seeds in particular, which are by and large considered to be the nonedible portion of fruits have higher quantities of bioactive components than other by product components such as flesh, peel, rind etc. (Augustin, Sanguansri, Fox, Cobiac, & Cole, 2020). Moreover, utilization of such bioactive constituents of these fruit residues in the production of food, cosmetic and nutraceuticals industries are the most efficacious, economical, and nature-friendly way to reduce fruit wastes. (Babbar, Oberoi, Uppal, & Patil, 2011).

1.1 kiwifruit seed protein: a potential plant protein source wasted

Kiwifruit, a perennial woody vine fruit is of considerable importance for New Zealand's economy. New Zealand developed its first commercially viable kiwifruit in 1937, and since then the country is amongst the leading kiwifruit producing countries worldwide (Ferguson, 2010). New Zealand also accounts for about one-third of the total volume of kiwifruit exported in the world today and captures around two-third of the total net market returns (Belrose, 2012; Müller, Holmes, Deurer, & Clothier, 2015). Moreover, the country is also by far the largest consumer of kiwifruit, with per capita annual consumption estimated to be over 5 kg of fruit, while other major countries have per capita annual kiwifruit consumption levels between 2 and 3 kg (Belrose, 2012). However, with a steep increase in production, as has been seen with other fruits, the NZ Kiwifruit Industry has suffered from huge increase in fruit rejection lately due to the postharvest handling of significantly higher volumes of fruit (Müller et al., 2015). Thus, as this fruit is transitioning from a high value item to a more common commodity, more efforts are being put into recovering value from its waste stream in the form of potential

co-products such as, oil, distilled kiwifruit liquor, kiwifruit wine, vinegar, pollen, and mushroom compost etc. (Kennedy et al., 1999).

Collective waste from kiwifruit and kiwifruit processing industries can be divided into two basic forms. The first is a whole fruit that has not made the grade for direct sale and the second is kiwifruit pomace which is the press cake residue left after manufacturing and processing activities. Kiwifruit juicing in large-scale results in the generation of a vast quantity of leftovers such as seeds, skin, and pomaces (Kheirkhah, Baroutian, & Quek, 2019). These so-called reject kiwifruits collectively comprise up to 30% of the total kiwifruit crop wasted on an average each year (Kennedy et al., 1999). Land care research New Zealand reports that average kiwifruit rejection rate at the pack houses is around 15% of the total quantity received from the orchards in the country (Mithraratne, Barber, & McLaren, 2010) out of which 4% are usually recovered and sent to the regional markets for repacking, while the remaining (10%) are sold to dairy farms as stock feed or dumped in landfills (0.9%). Previous research has suggested that these waste streams of kiwifruit such as pomace, peel and seeds have high phenolic content, proteins and antioxidant activity (Park et al., 2015). Until now, kiwifruit seed oil has been the most thoroughly researched part of the kiwifruit pomace and has been successfully extracted as an ingredient, serving as a new generation health ingredient for food, and cosmetic applications (Yongkang, 2001) (Li, Chen, Ma, & Wang, 2005). In contrast, kiwifruit seed protein (KSP), (150–200 g/kg seeds), also one of the major components of defatted meals remains underutilized (Deng et al., 2014; Kennedy et al., 1999). So far, to the best of our knowledge, there is a clear scarcity of reliable scientific data on functional, structural, and nutritional properties of kiwi seed proteins thus, this research tries to present a detailed study on kiwifruit seed proteins (KSP) which could be helpful for kiwifruit seed researchers in the future.

New Zealand has always led the global reasoning on "total utilization" of foods since early 1970s, when whey was produced as a value-add from dairy waste streams. In the wake of this approach, it was also recognized that isolation of value add from a source is quite economical when the extracted component is one of several products isolated and ignored during a manufacturing process. Thus, the current trend of consumer inclination towards plant-based foods and modern "flexitarian" lifestyle opens significant opportunities for New Zealand to expand and develop its plant-based protein sources. The kiwifruit waste data presented above indicates a potential source of protein

being wasted and underutilized in the country and justifies the need to transform these waste streams into added value products by applying adequate techniques. This would not only reduce the carbon footprints of the kiwi fruit waste produced in the country but also might hint a gateway to a novel plant protein source which can be further looked upon by scientists and give a new direction to develop plant-based products, ultimately achieving economic growth for the nation.

1.2 Hypothesis

1. Protein extracted from Kiwifruit seeds can provide a valuable source of plant protein.
2. Ultrasonic assisted extraction can be used as a clean and green method to extract protein from kiwi fruit seed meal.
3. Ultrasonication also affects the functionality of the extracted kiwifruit seed protein

1.3 Specific objectives

1. To extract the protein fraction from kiwifruit seed meal using an ultrasound probe.
2. To optimize the extraction process using response surface methodology.
3. To analyze the physicochemical and functional properties of extracted kiwi seed protein and compare with conventionally extracted protein from kiwifruit seeds

1.4 Thesis structure

An outline of the thesis is presented in Figure 1.1

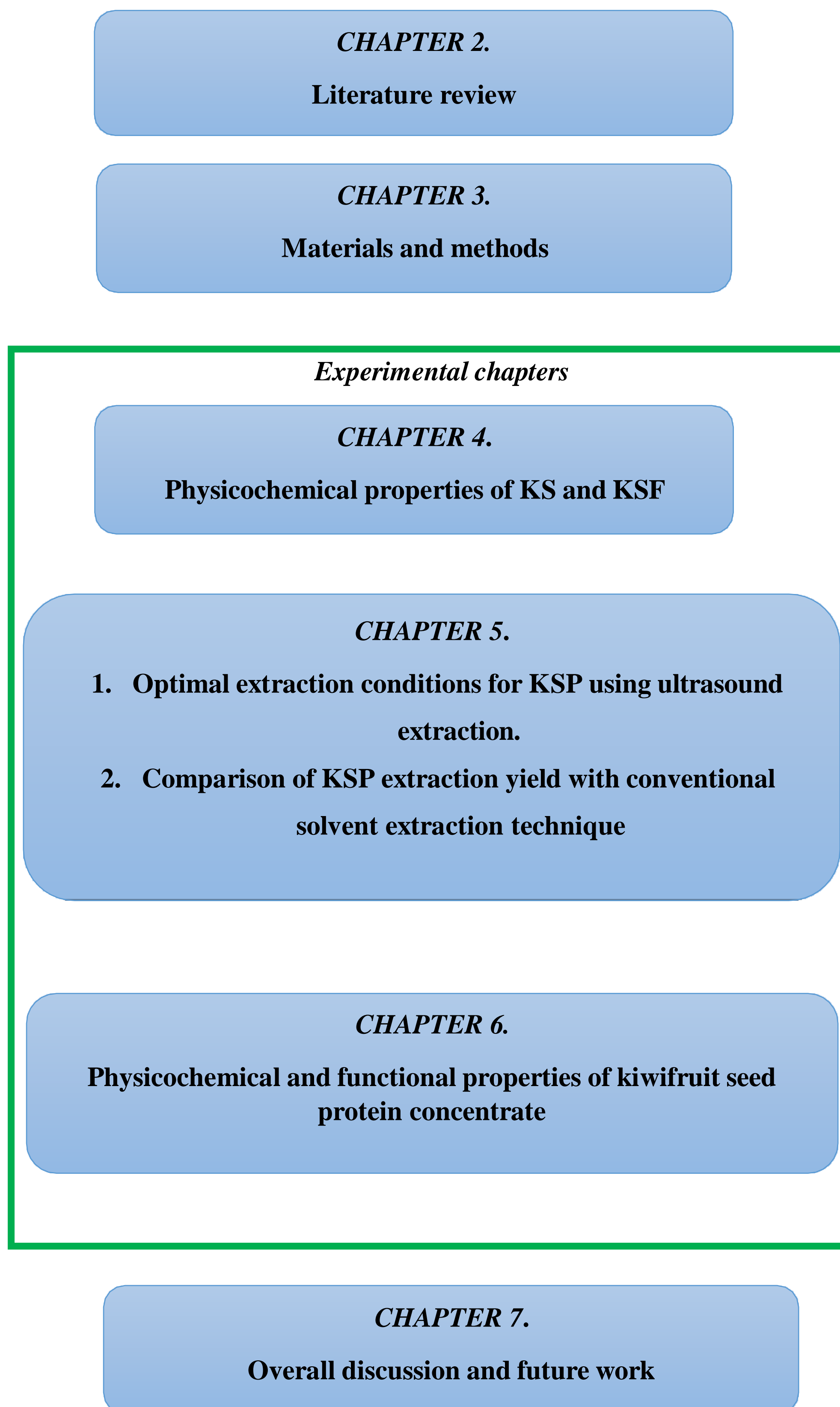

Figure 1.1 Overview of thesis chapters

Chapter 2: Literature review

2.1 Kiwifruit seeds: introduction and background

A kiwifruit plant is a woody and vigorous climbing shrub which botanically falls under the group of angiosperms (flowering plants). With respect to their seed types, angiosperms are further classified into two types namely, monocotyledon and dicotyledon seeds, based on one or two embryonic leaves (cotyledons) contained within their seed structures respectively. The kiwifruit seeds typically fall under the classification of dicotyledon seeds as it consists of two very small cotyledons erected within their embryo**.** Moreover, kiwifruit seeds are also known to have an oblong shape, brown or dark brown in colour, and are extremely small sized seeds (grain weight about 1.2-1.6 g of mass 1000 seeds) (Ferguson, 2010).

Table 2.1 presents a general proximate composition of kiwifruit seeds as published by (Deng et al., 2014). Kiwifruit seeds possess a high (≤ 40%) fat and carbohydrate content due to the fibrous coating on seeds which are structurally like watermelon seeds and red pepper seeds (Krist, 2020; Piombo et al., 2006; Sun, Zhang, Zhao, & Xu, 2008). Moreover, the kiwifruit seeds also represent an approx. 15% edible proteins which is generally considered as an adequate amount for recovery and utilization.

Table 2.1 A proximate analysis of kiwifruit seeds (Actinidia chinensis) sourced from (Deng et al., 2014) Copyright © Taylor & Francis Group, LLC

Composition	Content (%)
Moisture	5.26 ± 0.08
Crude fat	35.54 ± 0.12
Protein (N — 6.25)	15.67 ± 0.05
Ash	2.65 ± 0.11
Carbohydrate	40.77 ± 0.27

In chapter 2 specific examples or references drawn from the dicotyledon seeds will be used to illustrate salient aspects of kiwifruit seeds since the overall literature on kiwifruit seed anatomy and morphology is scarce. The goal is to provide an overview

of the major storage components of kiwifruit seed for a reader unfamiliar with the subject while helping to provide a conceptual framework for the seed protein extraction processes explained in the later chapters.

In a simplified view (like every dicotyledon seed), a kiwifruit seed consists of three major components: a protective structure (i.e. the seed coat), newly formed individual sporophyte (i.e. the embryo), and a compartment of nutrients (i.e. the cotyledons) (Agrawal & Rakwal, 2012). Development of these components (within a seed) is complex, and a highly coordinated process known as 'seed maturation'. The outcome of this maturation period is a matured seed which can be defined as a rigid, sectionalized mass of filial structures enclosed within a protective covering of carbohydrates and hemicelluloses. The main aims of seed maturation involves, development of embryo within seeds and form a seed coat around the embryonic leaves to protect the embryo from environmental perils (Agrawal & Rakwal, 2012). Moreover, seed maturation is also responsible for stocking up the embryonic leaves with different storage compounds such as proteins, lipids, starch and carbohydrates to be self-utilized by the seed later (Ruan & Chourey, 2006). These storage compounds are usually synthesized within a seed from the mother plant, when it takes up nitrogen and sucrose from the soil primarily in the form of amino acids (Nowack, Ungru, Bjerkan, Grini, & Schnittger, 2010).

2.2 Protein in seeds: classification

In the process of maturation, seeds synthesize ample amounts of storage proteins (SPs) and stock them in spherical dense organelles, identified as protein bodies or protein storage vacuoles (PSVs). These PSVs are surrounded by a membrane of tonoplast and are generally located within the embryonic leaves/ cotyledons of a seed (fig 2.1) (Herman & Larkins, 1999; Shewry, Napier, & Tatham, 1995). In past, studies on different seeds and proteins have revealed that SPs are edible, can be extracted, and usually constitutes major fraction ($\geq$ 40%) of the overall available protein in a dicot seed (Agrawal & Rakwal, 2012). Thus seed SPs can be defined as the total accumulated edible protein available in a seed, in enough quantity which upon germination acts as a nitrogen source for primary stages of seedling growth and upon maturation is deposited within PSVs (Weber, Sreenivasulu, & Weschke, 2010).

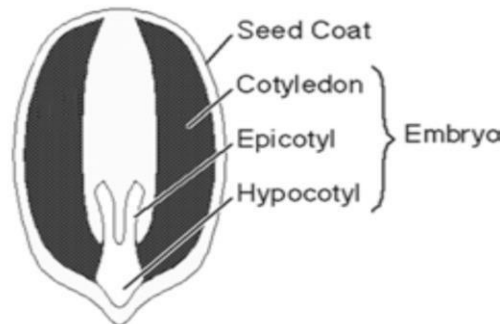

Fig 2.1 Major components of a dicotyledon seed. Sourced from (Agrawal & Rakwal, 2012)

The very first systematic approach in identifying and characterizing SPs from seeds was documented by T.B. Osborne, who summarized his work 'The vegetable proteins' as a monograph in 1924 (Osborne, 1924) and further elucidated the SPs into four groups known as Osborne fractions (Vickery & Osborne, 1928). This system is still widely used for operational reasons and classifies SPs according to their solubility in different solvents; namely water-soluble albumins, saline-soluble globulins, alcohol-soluble prolamins, and alkali-soluble glutelins. Early research suggest that majority of seed SPs are generally globulins (10-45% of the seed dry weight) which is preceded by albumins and then least to no presence of glutelins and prolamins (Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, & van der Goot, 2018). Thus, in the upcoming sections, globulins and albumins of SPs are highlighted and characterized.

The globulin SPs are further differentiated as per their sedimentation coefficients into two major groups: the 11S legumin type and 7S vicilin type. The 7S and 11S seed storage proteins are members of large gene families and the most prominent PSV constituents (Shewry et al., 1995). Both groups depict substantial differences within their structures and properties mainly due to post translational processing of seeds, and have been largely studied on many families of plants such as legumes, soybeans, French beans, peas, tomato seeds etc (M. Chen, Nicolai,Taco, 2016; Gatehouse, Evans, Croy, & Boulter, 1986; Liadakis, Tzia, oreopoulou, & thomopoulos, 1995).

11s globulin protein is structurally hexameric, and typically consists of six pairs of subunits that are attached to each other non-covalently. Each of these pairs contain one basic and one acidic subunit with M_r values 20,000 and 40,000 respectively, linked to

each other via a single disulphide bond. On the other hand a typical molecule of 7-8S proteins of seeds represents a trimer with higher molecular weight of 150,000 to 200,000, consisting of non-identical acidic and basic units bonded by a single disulphide bond (Higgins, 1984).

Globulins are also generally deficient in essential amino acids such as cysteine and methionine levels if compared to 2s albumins. However, the 11s legumin group is reported to have a slightly higher level than the 7s vicilin types. Early research suggest that majority of seed SPs are globulins which are present as oligomers within the seed PSVs and have little or no solubility in water due to their high molecular weight (M_r > 20,000) (Sabelli, 2012).

Albumins is the second major constituent of PSV and are extensively studied in oilseeds and among all major dicot seeds known today. Structurally, all albumins are closely packed protein structures defined in accordance with their respective sedimentation coefficients ($S_{20.w}$) of ~ 2 (Sabelli, 2012). Napin, for example from the family of Cruciferae is a dicot seed which defines a general 2S albumin structure as; two chains of polypeptides with M_r values of 4000 and ~ 9000 connected to each other via interchained disulphide bonds (Ericson et al., 1986). similar heteromeric structure and intertwined disulphide bonds has also been reported by seed researchers from different fields such as cotton seed proteins, pumpkin seed proteins, caster bean proteins and lupin etc. which verifies a similar structure for all dicot seeds (Basra, 2007; Gatehouse et al., 1986).

However, it is to be noted here that, the seeds commonly represent a diverse group of plants with diverse growth conditions and post translational changes. Thus, they consonantly represent a wide range of solubility and structural features. It can also be understood by simply saying that there are always exceptions in a defined set of characteristics for seed proteins which changes as per the physical, chemical, and biological environment of the seed before and after germination (Fukushima, 1991; Shewry, 2000).

2.3 Basic structure of proteins

A continuous succession of 20 amino acids constitutes the primary structure of most of the known proteins today (Chou & Fasman, 1978). However, it is important to note here that, this succession of amino acids within a protein structure is not random and all the proteins (including seed SPs) have a predefined three-dimensional structure (Chothia, 1984). Genes/ information which is transmitted within a seed from the mother plant defines a unique sequence of amino acids for each protein species, which assembles all the molecules within various proteins to exhibit a unique 3D structure (Anfinsen, 1972). These 3D structures are further characterized in accordance with their biological and functional properties as the primary, secondary, tertiary, and quaternary structures of a protein. In order to successfully extract the proteins a sound knowledge of these characteristic properties of proteins is required. Thus, the upcoming sections throws light on different common structures within a protein and their stabilization mechanism.

The primary structure of a protein represents a linear sequence of more than 100 amino acids in a protein which are connected to each other by losing a water molecule (Lehrman, 2017). However, when these chains tend to coil in a characteristic fashion due to vigorous driving force of hydrophobic molecules within a protein gives rise to several localized secondary structures, generally in the shape of α helix or β – pleated sheets. These helix and pleated sheet like structures stabilizes themselves through hydrogen bonding, whereas the folding of protein chains is usually driven by hydrophobic interactions between amino acids and their side chains (Wetlaufer & Ristow, 1973). In the formation of the α helix structures, the chain of amino acids coils upwards, resulting in the formation of a helical backbone with several peptide linkages (3.6 amino acid residues per turn of the helix) where, the side chains of amino acids tend to extend outwards from the structure (Damodaran, 2008). In anhydrous conditions, the α helix structure imparts stoutness simply due to an abundance of hydrogen bonds . Whereas, in aqueous conditions, where the stabilizing effect of hydrogen bonding is minimized, Van der Walls interaction (1-3 Kcal/mol over 3-5 Å) among adjoining residues of the polypeptide chains and hydrophobic links with long apolar side chains are majorly responsible for structural stability of the α helix (Rehman, Farooq, & Botelho, 2021). The regions of β pleated sheets are also predominantly established by hydrogen bonding betwixt adjacent chains of

polypeptides and to a low extent via electrostatic or hydrophobic interactions as it is observed in the α helix structure (Damodaran, 2008).

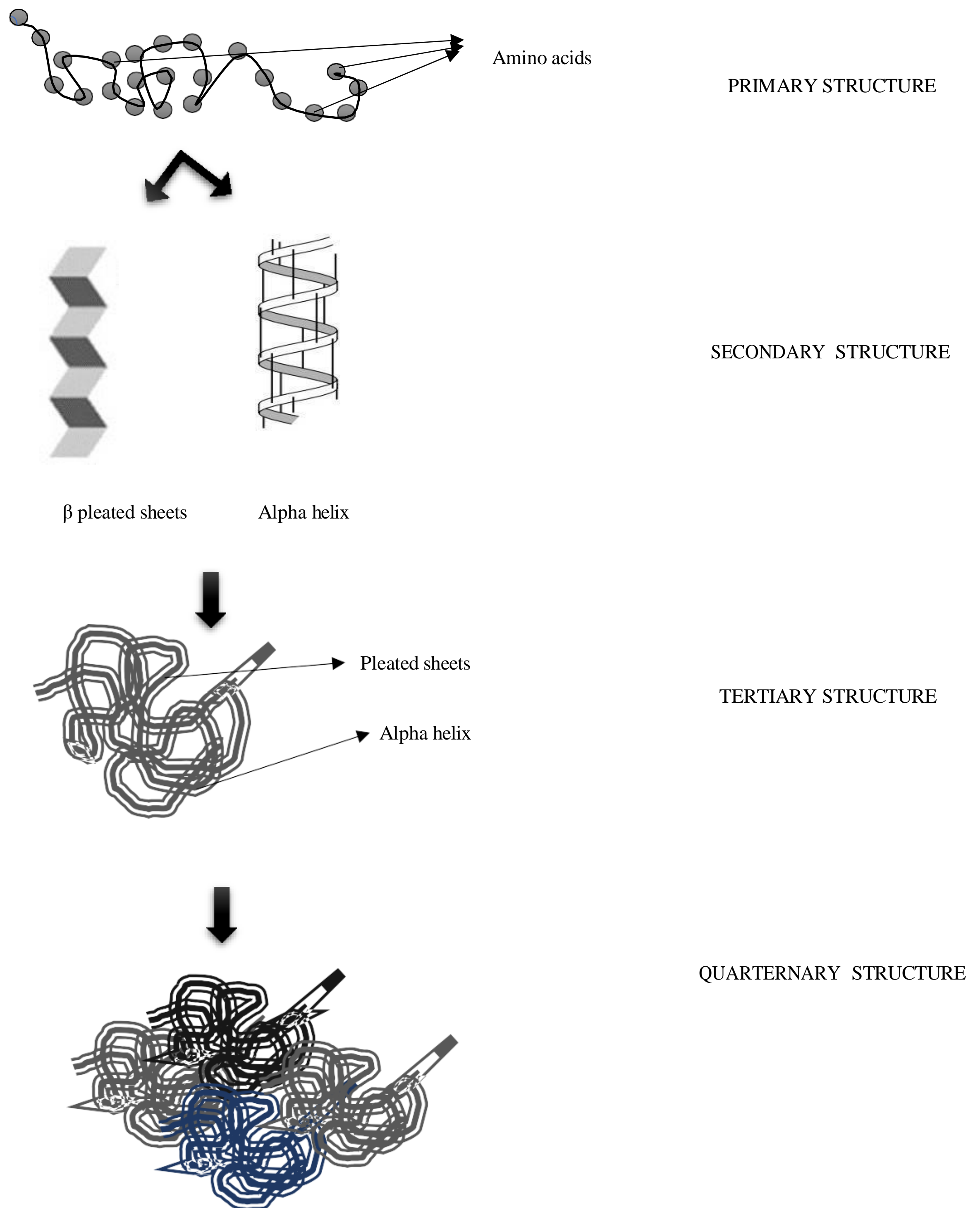

Figure 2.2 levels of protein structures

The tertiary structure is known to be formed when the folded chains of polypeptides (i.e., the helixes and sheets) further coils up to form a tightly bound molecule, where all the polar amino acid residues are positioned on the surface, whereas the apolar residues are dwelled deep within the hydrophobic areas of the protein molecule, from where water is essentially excluded (Bajaj & Blundell, 1984). The folds (Figure 2.2) within the tertiary structure of a protein molecule are crucial in determining functional properties of proteins, as they majorly involve more than 30% of overall charged amino acid residues majorly dominated by molecules like proline and iso-leucine etc. Moreover, the type of bond formation within the protein molecules while folding determines its compactness and degree of folds. For example, insulin is a tertiary structure protein molecule which despite being stabilized by strong (S-S) disulphide bond are loosely bounded, whereas lysozyme (12s amino acid) also stabilized by disulphide bonds with four interpeptide bonds rather than one gives more structural stability to its tertiary structure rather than insulin. As a matter of fact, several forces are known to contribute for a sturdy or a loose tertiary structure of a molecule which are thoroughly discussed in the upcoming sections.

Furthermore, proteins containing more than 28 mole percent of implicit nonpolar amino acids altogether, tends to interact covalently with each other forming a spatial disposition of different ranks of protein structures (primary, secondary, and tertiary) and are known as the quaternary structures of proteins (Klotz, Langebman, & Dahnall, 1970). This self-association of proteins occurs when the portion of hydrophobic residues is exceeded exponentially or are present below a critical point in proteins. In each case, it is impossible for proteins to conceal the hydrophobic residues inside the structure. Thus, a few hydrophobic residues are always unscreened on the surface, which upon interaction with other protein molecules creates hydrophobic patches on the surface of proteins. These patches being hydrophobic in nature forces different ranks of proteins to fold in such a way that, the hydrophobic patches stay isolated from the polar residues resulting in an association of protein subunits collectively known as the quaternary structure e.g., casein, actin, glutenin etc (Bajaj & Blundell, 1984).

2.4 The stabilizing forces within a protein

2.4.1 Hydrogen bonding

In proteins a hydrogen bond, being ionic in nature generally depicts two electronegative

atoms (e.g., Nitrogen, sulphur, carboxyl groups, oxygen, and functional groups etc.) interlinked by sharing one hydrogen molecule, and requires a bond energy of 2-19 Kcal/mol. Usually, the α helix and β sheets (i.e., secondary structures and higher) are the one's which are stabilized via hydrogen bonds. Thermodynamically, the formation of hydrogen bond secures the minimum free energy of the system and as a result the polypeptide chains folds and start interacting with its own molecules (Pace, Scholtz, & Grimsley, 2014)

2.4.2 . Electrostatic interactions

The electrostatic interactions are pH dependent interactions which generally occur between the positively charged (histidine, lysine, arginine etc.) and the negatively charged amino acid residues (glutamic and aspartic acids) within a polypeptide chain. The extent to which these interactions add to the cohesion and structural establishment of proteins is not evidently inferred. However, negligible stabilizing effects have been prophesized. These electrostatic interactions within molecules are widely accepted as vital in determining the functional attributes of proteins. For example, the rheological properties of foams and interfacial film formation are majorly due to net surface charge of protein and electrostatic repulsion among various amino acid residues (Dill, 1990). Furthermore, in recent years protein modification and alteration of functional properties of proteins are also focused upon the net charge and electrostatic interaction of molecules.

2.4.3 Van der walls forces

The Van der walls forces are customarily, non-specific, low range forces which usually determines the packing density of the amino acid residues in a protein requiring a typical bond formation energy of 1-3 Kcal/mol. The intensity of these forces is inversely proportional to the sixth power of the length between the particles. In protein structures, they are usually associated with polar interactions between the induced dipoles and its neighbouring atoms (Dill, 1990).

2.4.4 Hydrophobic interaction

The hydrophobic interactions are basically a set of combined forces initiated within a polypeptide due to the continuous repulsion of apolar groups (alanine, proline, leucine etc.) in an aqueous medium. These forces are one of the principal forces which accounts for protein confirmation, folding and other counterbalancing interactions for structural

integrity of proteins. A protein molecule generally contains several apolar molecules, which unfavourably interact with water and involuntarily enforces water into an organised cage like structure via hydrogen bonding within their immediate vicinities.

Moreover, these hydrophobic interactions with a protein are thermodynamically driven by driving forces of negative entropy which structures water within the protein molecule in such a way that the apolar molecules have a minimized contact with water. Therefore, proteins generally fold into a globular framework where the apolar groups are dwelled in the anhydrous hydrophobic interior, whereas the polar groups are exposed on the surface (Lins & Brasseur, 1995). If a protein is exposed to a temperature driven environment, a downturn in temperature reportedly diminishes the overall hydrophobic interactions whereas increment up to 60° C intensifies it. Basically, as the temperature is dropped, the water becomes largely hydrogen bonded and more structured. Thus, it is usually explained that the entropy of folding the non-polar residues into the hydrophobic interior of proteins is minimized at lower temperatures which further adversely affects the structural stability and hydrophobic interactions. Therefore, on the grounds that hydrophobic interactions are depleted at minimized temperatures, few proteins are known to have a tendency of dissociation at lower temperatures (Chandler, 2005). For instance, soy proteins, casein, and oilseed proteins.

2.5 Protein extraction from seeds and influence of extraction conditions

The extractability of seed proteins can be influenced by many factors. For instance, temperature, pH, time, and ratio of biomass to solvent etc. Protein extraction data from multiple oilseeds and seed meals are tabulated in Table 2.2. All the publications have been reviewed in the next few sections to discuss the significance of each of these important influential process parameters in seed protein extraction. Moreover, the aim of this section is to provide an estimate of upper and lower limits of extraction variables in seed protein extractions which is also briefly discussed in the section.

Table 2.2: A comparison of different common influential parameters of Seed/seed meal protein extraction

Material	Extraction medium	pH	Temperature (^{0} C)	Time (Min)	Solid solvent ratio (g:ml)	Seed protein content	Overall protein yield	References
Rosa rubiginosa seeds	NaCl 0.5 M	9.7	30	90	5	24.6%	90 %	(Moure, Sineiro, & Domínguez, 2001)
Sunflower seed meal	NaCl 1–3 M	6.0	45	60	5	~20%	76–83%	(Pickardt et al., 2009)
Rapeseed meal	NaOH 0.2%	12	-	60	1:10	30-35%	95%	(Pedroche et al., 2004)
Watermelon seeds	NaOH 0.3–1.5%	-	50	5-25	1:70	16.34%	81%	(Wani, Kaur, Ahmed, & Sogi, 2008)
Fenugreek seeds	NaCl 0-1 Mol $^{-1}$ L	9.25	25	30	1-20	~ 10%	89.1%	(Feyzi, Varidi, Zare, & Varidi, 2015)
Tomato seeds	Deionized water & 0.5N NaOH	11.5	50	20	1:30	25.5%	66.1%	(Liadakis et al., 1995) (Mechmeche et al., 2017)

2.5.1 Disruption of selected biomass

The word extraction literally means segregation of the bioorganic substances from its inert components by deploying standardized extraction techniques and solvents. In the case of seeds, the outermost enclosure consists of a convoluted arrangement of polysaccharides along with other major components such as fiber, which are hard to disrupt.(Lerouxel, Cavalier, Liepman, & Keegstra, 2006). Thus, a finely ground powder of the initial biomass as starting materials has become a common standard practice in sample preparation of plant proteomics (Isaacson et al., 2006). The most common methodology to aid the extraction processes is to submerse the seeds in liquid nitrogen followed by crushing with a mortar and pestle to release the plant material, before breaking it down to a fine powder. However, several other economical methods such as pulverizer, mechanical grinders and mills etc. which does not promote use of liquid nitrogen have also been used recently (Deng et al., 2014; Wani et al., 2008).

2.5.2 Removal of lipids

Seeds store ample amount of lipids in the forms of triacylglycerol (TAGs) as food reserves for the early growth stages of seedlings. These spherical shaped oil bodies with diameter 0.5 – 2 µm can be accounted 10 - 40% of the total weight in a matured oilseed (Huang, 1994). These compounds have a shared aromatic, ring shaped structure, with at least one attached hydroxyl substituent. Their interference in the seed extraction process comes down to the fact that they can form irreversible complexes with SPs via hydrophobic interactions. These interactions hinder the protein extraction by showing insolubility in an aqueous solution, majorly due to hydrophobic nature of the complex formed. Thus, a preliminary step of oil removal/ lipid extraction is generally suggested in a seed protein extraction process. Moreover, a suitable choice of solvent for lipid extraction should be made on the basis of factors such as volatility, freedom from toxic or reactive impurities, price etc. (Hara & Radin, 1978).

The use of such volatile solvents in oil removal processes in protein extraction, comes down to the fact that these solvents typically form hydrogen bonds with the available phenolic groups and lipid molecules in a seed meal/flour, which are easier to remove later using simple unit operations such as centrifugation. Moreover, it is also always suggested that removal of phenolics must be repeated twice and should be used in combinations with other technologies like centrifugation for obtaining a high purity

protein (T. Wang, Fuju & Chen, 2008). The most widely used lab scale solvents are n-hexane and chloroform.

2.5.3 Time and Temperature

In Table 2.2, time, and temperature both had significant ($p < 0.05$) effects on the extractability of proteins. Nearly all the literature cited in Table 2.2 settled on an extraction time less than 100 min and an extraction temperature of 30 -60° C. It was equivocally concluded that, higher the protein extraction time and temperature, the higher the yield would be. For example, in the extraction of tomato seed protein with different time and temperature values, the maximum protein yield (66.1%) was observed at 50° C and approximately 20 mins of extraction time. The higher yield of tomato seed proteins at optimized time and temperature combination was linked by the author as an increased driving force for the mass transfer of the protein due to higher time and temperature which can be further related to the molecular weight of the proteins which influences the overall protein yield (Mechmeche et al., 2017). In another approach of extracting protein from watermelon seed meal and understanding the effect of time and temperature through a residual plot analysis, (Wani et al., 2008) states that a temperature of 50°C was optimal for extracting 81% of watermelon seed proteins using NaOH as an extraction solvent with an extraction time of 25 mins. Nevertheless, it has continuously been suggested that, extremely long times for extraction might contribute to microbial growth, thus it is not generally recommended (Shen, Wang, Wang, Wu, & Chen, 2008).

2.5.4 Solvent to biomass ratio

The authors from table 2.2 reported a high ratio of solvent to biomass for a maximized yield of proteins and quicker diffusion of the biomass (protein) into the solvent. On the other hand, lower values of solvent to biomass ratio (e.g. 5:1 – 10:1), were reported to act as a non-conventional driving force upon the protein bodies during extraction, which, rather than extracting the proteins out of their native vacuoles settles them back in, thus negatively affecting the overall protein yield (Shen et al., 2008). For instance, kiwi fruit seeds, tomato seeds and watermelon seeds have maximized (70 -80%) protein extraction at a solvent to biomass ratio of 8:1, 82:1 and 81:1 (ml/gm) respectively which indicates that a general ratio of less than 20ml per gm of seed powder have a negative impact on the extracted yield of proteins (Lestari, Mulder, & Sanders,

2010). Nevertheless, solvent to biomass ratio higher than 50:1 are also not very practical for use at various industrial levels, mainly because of the high cost involved and several environmental issues reported due to excessive use of solvents (Lestari et al., 2010). The research on extraction of tomato seed proteins and kiwifruit seeds reports that increasing the ratio of biomass to solvent after 1:30, had no effects on the extraction yield rather the structure of the proteins were found to be fragmented (Liadakis et al., 1995) (Deng et al., 2014). Thus, it can be inferred that the ratio of solvent to biomass is a significant factor and must be considered while extracting proteins from seeds. Moreover, it was also stated repeatedly by several authors that solvent to biomass ratio have quadratic effects on other influential parameters of seed proteins (Deng et al., 2014; Mechmeche et al., 2017; Pickardt et al., 2009).

2.5.5 Influence of pH

The pH of the solvent during protein extraction from seeds has two main functions: to modify the cell wall and to modify the functional properties of proteins. Cell wall/ the fibrous coating which forms the outermost layer of the seed provides a significant hurdle for the diffusion of the protein globules into the solvent. Therefore, the easiest pathway for higher protein extraction yields is to modify the cell walls. Such modification can be brought about by the pretreatment of the seeds with a solvent of adequate pH.

However, in case of seed meals, where the cell wall of seeds is torn apart already, the electrostatic charges among the protein molecules is used as a tool to extract the proteins out of their PSVs. Under maximal alkaline or acidic pH, globular proteins become partly unfolded due to a sudden increase in the repulsion amongst charged side chain groups of the protein (Jiang, Wang, & Xiong, 2018). This partial state of unfolding proteins in a solvent is known as ‘molten globule’ which is generally followed by a short incubation period at neutral pH to partially refold the proteins (Goto & Fink, 1989). Such a process, known as pH shift, has been shown to substantially increase the solubility as well as emulsifying and film-forming properties of respective proteins(Jiang & Xiong, 2010). This structure-modifying technology is considered remarkable also because the process is simple, easily adaptable and can be used to convert poorly soluble proteins from an aggregate state into strongly hydrophilic as well as surface active monomers that are suitable for commercial applications.

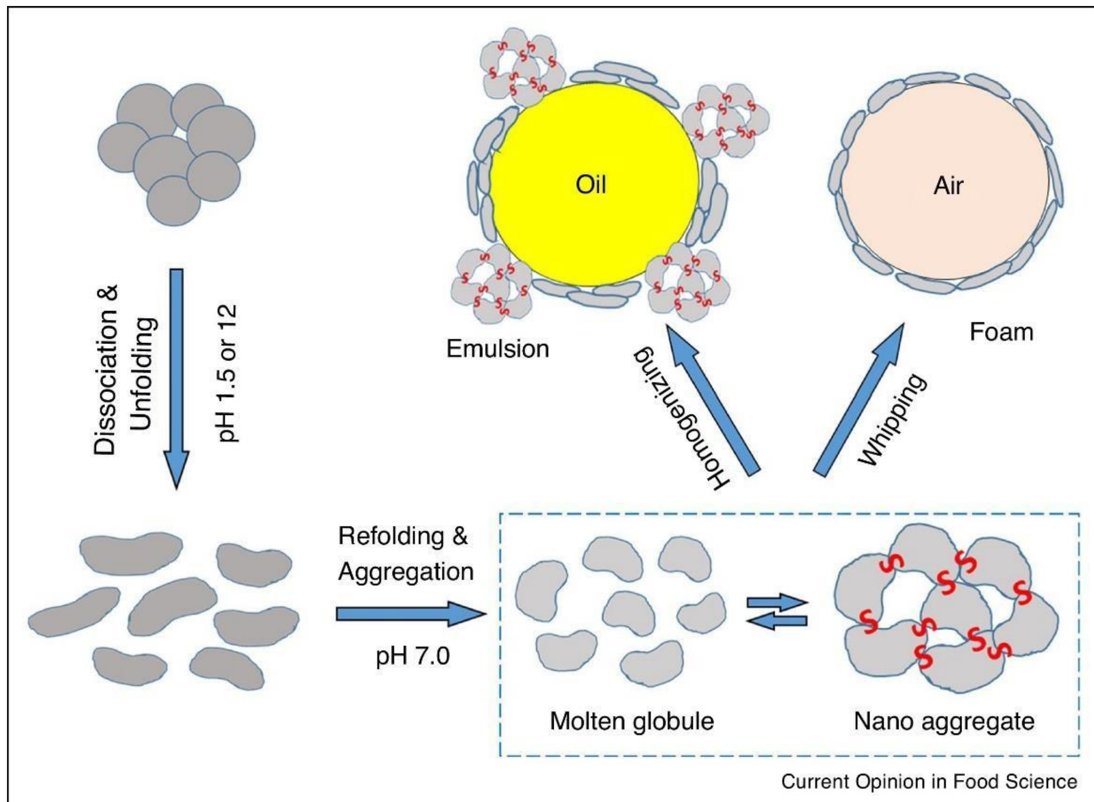

Figure 2.3 The pH shift process and its possible impact on the surface properties of proteins. Reproduced with permission from (J. Jiang, Q. Wang, & Y. L. Xiong, 2018)

Table 2.2 concludes an alkaline pH 8-12 favorable for seed extraction. The seed proteins in general have shown low solubility at acidic pH (2-5) which is also an ideal range of their isoelectric points (3-5). Whereas pH 9-11 in Table 2.2 has reported to disrupt the outermost coverings of the seeds and thus aids in efficient extraction. For example, in Figure 2.3, 83% of soybean protein was recovered using a 9.2 pH and 0.05 M phosphate buffer.

However, it has always been argued that not only pH, but an absolute amount of alkaline is of utmost importance when aiming for maximized protein extraction yields (Zhang, Sanders, & Bruins, 2014). Hence optimization of the processes is generally recommended.

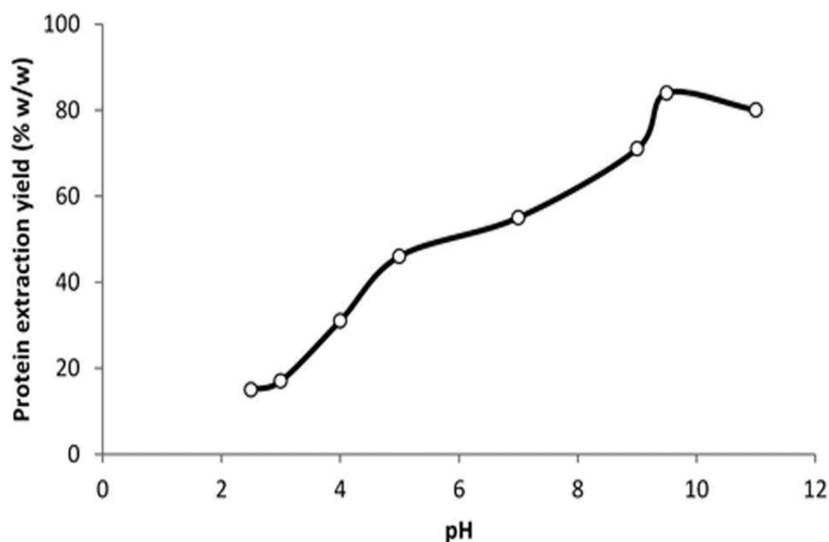

Fig 2.4 Soybean protein extraction yield obtained at various pH showing the dependence of soybean protein extraction on pH and the high recovery at alkaline pH. Reproduced with permission from (Sari, Mulder, Sanders, & Bruins, 2015).

2.6 Conventional protein extraction and rise of green extraction

Several examples are found in the history of Indian, Chinese, Greek, and Egyptian scriptures where extraction of natural products is vastly documented for fulfilling a purpose of food, polymers, or medicines. Later, as science and technology progressed, direct and conventional techniques like Soxhlet extraction, solvent extraction and steam distillation were widely studied and used by the researchers in 90's era as it was commonly believed that the conventional extraction technologies were faster and easier to use than their predecessors. However, in the late 90's it was noticed that conventional techniques have a range of disadvantages when compared to the new and advanced non-conventional techniques for instance, utilization of huge amounts of solvents and lower rates of extraction etc. Thus, an idea "sustainability and waste management in extraction" prospered in that era, divulging the negative footprints of conventional methods for extraction (Anastas, Williamson, Hjeresen, & Breen, 1999; Graedel, 1999; Singh, Szafran, & Pike, 1999) and comparing different extraction procedures.

Paul Anastas, a pioneer in the field of green chemistry published a series of books in 1994 on non-conventional extraction procedures, which is said to initiate a change in the traditional mindset of the scientific community and an impactful read for sustainable extraction in that era (Anastas & Warner, 1998). But, as the conventional techniques were being used worldwide, with standardized procedures and rigid pilot scale

experimental evidence, it was not an easy task in that era whatsoever to propagate the idea of green chemistry. It is said to be the combined efforts by global environmental protection agency who advertised Paul's publications and pushed the idea, are acknowledged in the scientific community for coining a concept of green chemistry in the modern era (Chemat et al., 2017).

The green chemistry thus was based upon finding such processes and designs which on one hand minimize the consumption of energy, promotes the use of organic and alternative solvents; while on the other hand also ensures superior quality extracts and final product (Chemat, Vian, & Cravotto, 2012).Consequently, a term green extract was coined which signifies a non-conventional extracted product whose impact on the environment is least, is recyclable and furthermore can be proved as economically responsible from the farm to extraction, extracted waste, and even further after bioactive removal such as marketing and formulations. In the recent years a competitive environment among the major industries has been developed to thrive sustainably and evolve more innovative and ecological methods for unit operations, like extraction within the industries. Thus, green technologies are highly sought-after techniques in the recent times. To name a few, supercritical fluid extraction technique, subcritical water extraction, microwave assisted extraction and ultrasound assisted extraction are some of the major techniques popularly used in the field.

Six common principles were laid out by (Anastas & Warner, 1998) as recommendations for researchers to demonstrate a green label are mentioned below. These principles, as suggested by the author must reflect entirely on the experimental process.

1. Innovation by selection of a biomass, which is considered as a byproduct from an existing industrial process i.e., a total utilization of food.
2. Deploying bio based or water-based solvents
3. Using green extraction technologies to reduce energy consumption.
4. Inclusion of bio refining industries by developing co products rather than a new waste stream
5. Reduce unit operations, which favors a controlled, safe, and robust overall process.

However, with such diverse principles and methods used for green extraction procedures, it is important to select an adequate technique for further evaluation of the biological activities and characterization of the plant materials (Chemat et al., 2017). Thus, while selection of a procedure, it should be meticulously evaluated that the active constituents are not destroyed, disrupted, or lost while extracting the desired compound.

Table 2.3 puts up a qualitative comparison of some non-conventional and green extraction techniques such as ultrasound, microwave, instant controlled pressure drops (DIC), Supercritical fluid extraction (SFE) and pulsed electric field (PEF) extraction methods (Chemat & Khan, 2011; Da Porto & Decorti, 2009; D. Jadhav, Rekha, Gogate, & Rathod, 2009). It can be said through a comparative research that when conditions of the process were optimized, the ultrasonic extraction showed a better extraction yield than other non-conventional techniques which includes seed meals (Lianfu & Zelong, 2008). Moreover, UAE was also proved to be capable of utilizing minimal solvents and consumption of energy, thus reducing the carbon footprints, and obtaining a green label. However, it has also been convincingly stated that larger scale applications of UAE are not practical and challenging, problems have been encountered in mass diffusion and separation of the biomass in a few acquaintances using UAE and thus proves to be a hindrance for full scale utilization of UAE. It was also reported that frequencies higher than 20kHz sometimes cause unfavorable effects on the plant compounds due to the development of free radicals while using UAE method and thus also possesses a major drawback. However, a recent plant scale trial using UAE for soybean protein extraction is proved to be successful which contrasts with the drawbacks aforementioned (Petigny, Périno-Issartier, Wajsman, & Chemat, 2013).

Table 2.3: A qualitative comparison of modern green extraction technologies

Technique	Overall processing cost	Processing time	Main disadvantage	Main advantage
Ultrasound	Low	Low	Problem for separation	High cell disruption
Microwave	Medium	Low	Hot spots	Cell disruption
SFE	High	Medium	Lack of a defined process and knowledge	Enhance mass transfer
PEF	High	Medium	Difficult operation	Electroporation of wall cells

2.7 Ultrasound assisted extraction technology

2.7.1 Introduction and background

It dates to 1920's when authors Richard and Loomis brought out their first ever paper on the chemical effects of high intensity ultrasound. In their classical work they thoroughly described how the use of power and high intensity ultrasonic waves were able to catalyze the rate of a set of different chemical reactions within a solvent (Richards & Loomis, 1927). Furthermore, the sonic waves were also observed to be able to induce redox processes which can be compared to the induced reactions by ionizing radiations. However, it was still ambiguous until 1935, how and why these effects were occurring at first place. Later, with a thorough understanding of the phenomenon of light, the ultrasonic waves were exposed to water and a spontaneous emission of light was observed, which was later concluded to be the reason of ultrasonic induced cavities. In 1937 (Arnold, 1937) used a high-power sonication for degradation of biological polymers and indicates the beginning of an era of sonochemistry which has continued to flourish since then.

2.7.2 The mechanism actions of high intensity ultrasound

The ultrasonic waves induce several mechanical forces within the solvent which are radiative in nature and result in a combined acoustic streaming force which acts upon the dispersed particles. The occurrence of these forces and their radiative behavior is explained as the movement and penetration of the acoustic waves within the medium (Mason, 1999). However, it was concluded later through a series of sonication experiments that the major energy induced by the acoustic waves was absorbed within the particles, solvent, and dispersed molecules. Thus, not proving strong enough to break the chemical bonds as desired.

Later, an indirect phenomenon was found responsible for the breakage of chemical bonds known as bubble cavitation which arises due to the force of acoustic waves within a liquid medium, and was coined by (Šponer, 1991). Bubble cavitation can be described as a phenomenon which occurs when strong ultrasound waves travel through liquid mediums, which in turn initiates a negative pressure within the solvent and creates bubbles. The bubbles expand and collapse violently which defines the cavitation as stable or transient respectively. A stable cavitation is known to be reached when a bubble occurred due to ultrasonic waves within a liquid medium expands up to a critical point and violently collapses. Whereas the phenomenon of sudden violent collapse of the bubbles gives rise to a transient type of cavitation. These violent collapse and pulsation of the bubbles within a solvent concentrates all the sound energy from the ultrasonic waves and amplifies it by several orders of magnitude. This unparalleled energy, strong enough to cause all the chemical and physical effects within the aspects of ultrasonic extraction thus was considered as a final explanation of why the ultrasound extraction works (Crum, 1995; Price & Clifton, 1999). Moreover, it was also found that cavitation through ultrasonic waves also facilitates hydration and swelling of the matrix to be extracted, specifically with an enlargement of pores that increases the diffusion of solvent into the matrix and increases mass transfer. This hydration and swelling effects are advantageous when a dry matrix is used for UAE for example, seed flour or freeze-dried samples. The cavitation implosion also creates turbulence within the solvent, which promotes unit phenomenon's such as acoustic streaming and fragmentation which aids in the extraction process and are briefly described in the following sections

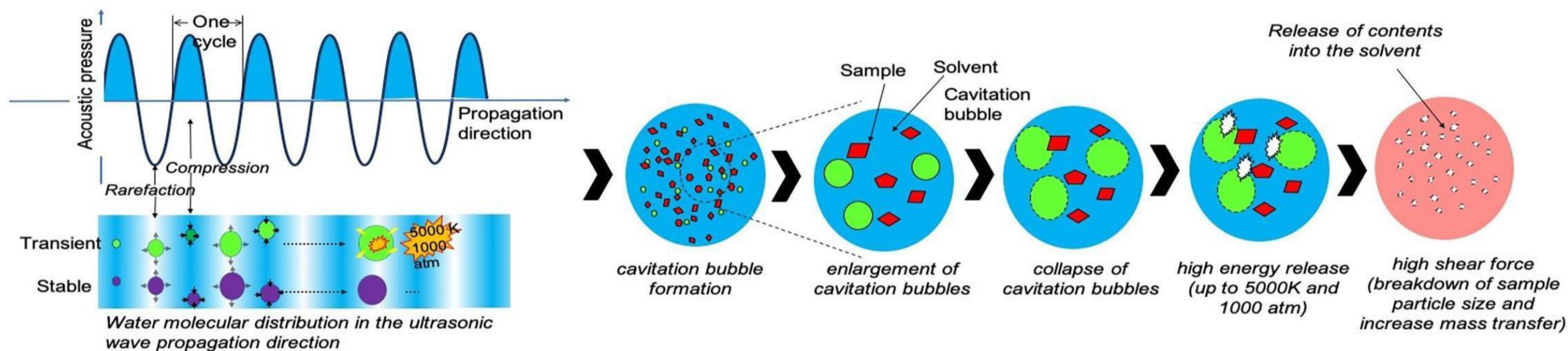

Figure 2.5 Bubble cavitation and sonochemical degradation process. Reproduced with permission from (Fu, Belwal, Cravotto, & Luo, 2020)

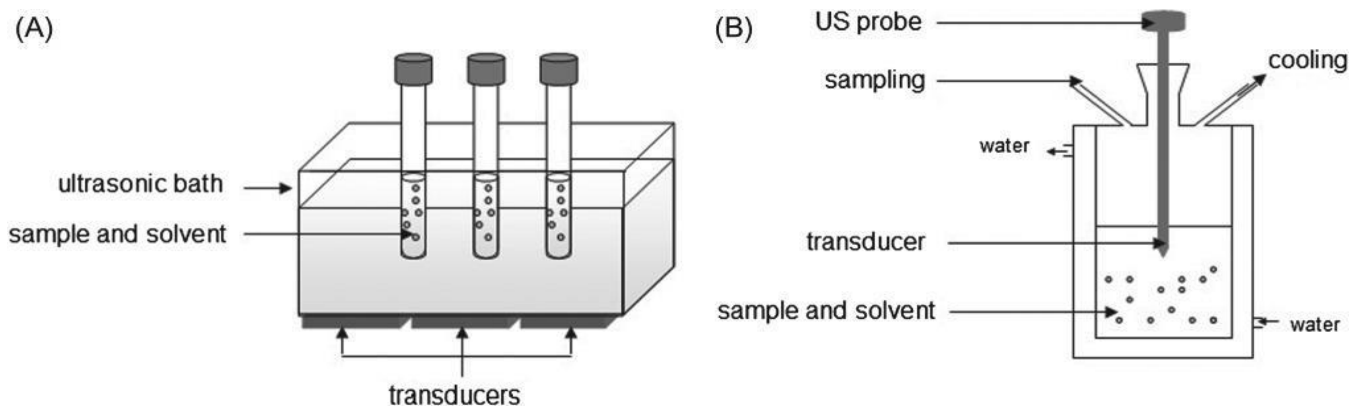

Figure 2.6 Laboratory ultrasound apparatus: (a) ultrasonic bath and (b) ultrasonic probe. Reproduced with permission from (Rombaut, Tixier, Bily, & Chemat, 2014)

2.7.2.1 Fragmentation

Fragmentation phenomenon in terms of ultrasonic extraction can be simply explained as disruption of the plant materials into further smaller particles, thereby enhancing the permeability and reach of the solvent to the target compound. The fragmentation occurs as the sound waves are induced within the solvent, due to collapsing of bubbles with high energies and the inter-particle collisions created from collapsing cavitation bubbles (Kusters, Pratsinis, Thoma, & Smith, 1994). The process can be further explained by studying a comparison report of chlorophyll extraction from spinach leaves using ultrasonic probe. The structure of leaves as compared after one minute of ultrasonication and maceration are described in the Figure 2.7 which shows a linear increase at the beginning of UAE, corresponding to a direct solubilization of chlorophylls. This effect is most probably due to the reduction in particle size occurring during application of ultrasound. Further the spinach residues were analyzed for particle size before and after ultrasonication which also suggested a sharp decrease in the particle size which links to fragmentation (Xiujun et al., 2011). Thus, a direct consequence of the reduction in particle size by ultrasound action is the increase of surface area of the biomasses resulting in higher mass transfer and increased extraction yield.

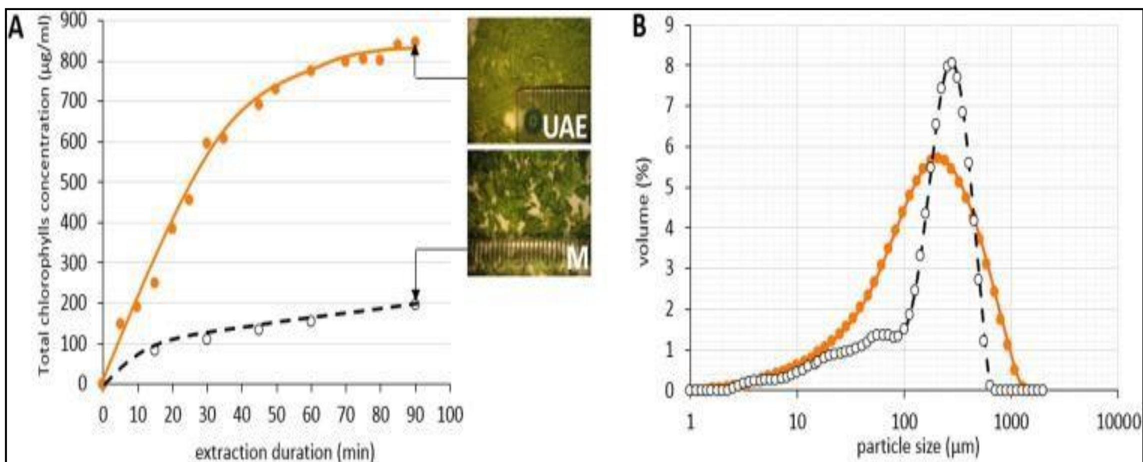

Figure. 2.7 Effect of power ultrasound on spinach leaves. (A: Comparison of chlorophyll extraction kinetics for UAE, US probe 20 kHz (●) and for maceration (M, ○); B: Comparison of particle size repartition (below 1 mm) of spinach residue for UAE (●) and for maceration (○)) Reproduced with permission from (Chemat et al., 2017).

2.7.2.2 Acoustic streaming

Acoustic streaming can be described as a steady fluid motion created under the influence of high-amplitude acoustic waves, when they propagate through a dissipative fluid medium (Frenkel, Gurka, Liberzon, Shavit, & Kimmel, 2001). It occurs due to a vigorous circulatory motion induced by growth and shrinkage of cavitation bubbles during cycles of rarefaction and compression (Margulis, 1991).

Cell-wall disruption due to mechanical effects of ultrasonic cavitation were evident from SEM of seed matrices in several researchers in the past (Both, Chemat, & Strube, 2014; Gogate & Kabadi, 2009). These formations of micro fissures, cell disruption and creating pores on the surfaces thus improve permeability of the matrix. Scanning electron and light microscopic images of the sonicated cellular matrix show the increased accessibility of the solvent to the internal structure, facilitating the release of target compound via the formation of micro fissures and microchannels that improve permeation of solvent into the matrix.

Thus, it can be said that acoustic and cavitational effects are responsible for the extraction of target compounds, resulting in a two-stage mechanism for UAE:

• Entry of solvent into the matrix because of disruption of cellular matrix, reduction in particle size, and mechanical effects, allowing greater penetration of solvent; and,

• Washing out extracts from the matrix due to increase in the contact surface area between the solid and liquid phases.

2.8 The experimental system design for ultrasonic extraction

As outlined above, there is considerable scientific evidence demonstrating the suitability of ultrasonic system to enhance extraction yields in short extraction times using clean or green solvents. This implies that, while the technology could exhibit great promise, it will have to be carefully developed and scaled up for large-scale commercial applications. In general, the terms ultrasound power, ultrasonic intensity or acoustic energy density are commonly used as the primary design parameter, as they account for energy entering the extraction system. Further, there are two main types of transducers commonly used for

ultrasonic applications: piezoelectric and magnetostrictive. Various types of transducers has been discussed by other authors but are not considered here in detail (Frost, 1979; Ramadan, Sameoto, & Evoy, 2014). The placement of these transducer is important for extraction efficiency, process intensification and energy losses. The transducers can be placed on either side of the extraction vessel, so ultrasonic waves will be transmitted through the outer wall of the extraction vessel (Figure 2.5). When transducers are in direct contact with the sample in the presence of a suitable solvent, extraction efficiency is enhanced, whilst minimizing acoustic energy losses.

Two kinds of designs are further utilized for delivery of high-power ultrasound to the system: an ultrasonic probe system and a bath design (Figure 2.6). Both bath and probe-type ultrasonic equipment are commercially available for extraction purposes operating at various frequencies. There are several types of probe shape, including uniform cylinder, exponential, taper, linear taper, or cone and stepped. Volume of the sample and type are two critical factors which govern the selection of probe. High-power ultrasonic probes are generally preferred for their extraction efficiency and can enhance yields in short extraction time (Bajerová, Adam, Bajer, & Ventura, 2014). The probe system (Figure 2.5) basically concentrates the propagation of ultrasound waves into a solvent by a providing a decreased surface area for the outlet of the sound waves. It consists of a tip, which delivers the intensified shear forces and other effects of sonication on the biomass. A common procedure for using a probe system includes immersion of the probe into the reactor which ensures a direct delivery of sound energy, reduction in the loss of energy. However, the probe system is also reported to be limited up to only 20 kHz intensity as temperature sharply rises due to concentrated sound waves, thereby further altering with the native biological state of the biomass (Adam, Abert-Vian, Peltier, & Chemat, 2012). Whereas the bath system is reported to uniformly distribute the ultrasonic waves throughout the system which helps it to evenly distribute the sound intensity at higher ultrasonic frequencies with minimal rise in the temperature.

2.8.1 The influential parameters of ultrasonic extraction

Several factors which have major roles in defining the targeted molecule extraction yield can be subdivided into two major groups namely the physical parameters and

medium/solvent parameters. The following section details these parameters as their profound knowledge is of utmost importance to understand the effects of parameters on the extraction yield. It helps in enhancing the overall extraction efficiency, if adequate parameters have been deployed by giving an idea of minimizing the resources and solvents to obtain a green label.

2.8.1.1 Ultrasonic power and frequency

Ultrasonic power or intensity is proportional to the ultrasonic amplitude. Higher amplitude does not necessarily improve process efficiency and cavitation. The level of cavitation and erosion of transducers depend on the amplitude. Higher amplitude enhances the agitation and reduce the level of cavitation. However, higher amplitude can be selected based on sample properties; for higher viscosity solvents, it is advisable to optimize the amplitude/power level to achieve the desired agitation and cavitation for various sonochemical reactions and extraction efficiencies. Selection of optimum conditions of amplitude/intensity is necessary to avoid undesirable degradation of the extracted compound. Moreover, to achieve the desired cavitation, ultrasonic intensity should be increased with increase in frequency to overcome the cohesive forces of the solvent and the sample (Niazi, Hashemabadi, & Noroozi, 2014). For example, 10 times more power is required to induce cavitation in water at 400 kHz than at 10 kHz (Lickiss, 1989).

2.8.1.2 The size and shape of ultrasonic probe

Several literatures have suggested an effect of the diameter and shape of an ultrasonic probe on the extraction dynamics. There have been documented various shapes of probe which are used in the modern sonochemistry however, a stepped-up probe and an exponential probe are more commonly used. The probes are usually made of a titanium alloy to provide it a thermal resistant and anti-corrosive properties. Stepped up probe is designed particularly for achieving higher magnification of amplitudes, thus is more common in large scale applications of ultrasounds, whereas an exponential probe is more of a micro based design used for lab scale and micro applications respectively. In a study, (Bajerová et al., 2014) carried out ultrasonic extraction of antioxidants from various plants by using an ultrasonic bath and an ultrasonic probe. In most of the cases, they observed significantly higher antioxidant capacity and levels of antioxidant extraction while the probe system was

used as compared to the ultrasonic bath system. However, (Adam et al., 2012) observed higher values for antioxidant compounds using an ultrasonic bath and no significant differences in the antioxidant capacity of antioxidants extracted from various plant samples under comparable extraction conditions. Both studies employed similar ultrasonic frequencies and power levels, the obvious differences are mainly attributed to the matrix and other extrinsic and intrinsic factors. Hence, generalizing that the probe-based systems are superior to ultrasonic baths may not be appropriate. However, in terms of process efficiency and energy losses, the ultrasonic probe-based system can be preferred for extraction purposes.

2.8.1.3 The solvent

A choice of solvent for ultrasonic extraction is mainly determined by the solubilizing ability of the target molecules. However, literature also suggests that several physical parameters of the solvent such as viscosity, vapor pressure and surface tension etc. might affect the desired output (Mason, 1999). These physical parameters influence the cavitation threshold and negative pressure generation in the solution which determine the bubble formation, expansion and amount of energy produced by cavitation. During the process of bubble cavitation, the bubbles formed are filled with vapor induced from the solvent surroundings. Thus, solvents with extremely lower vapor pressure are reported to indurate the process of cavitation due to unavailability of the vapors (Santos, Lodeiro, & Capelo-Martínez, 2009). On the other hand, extremely volatile substances are also not generally used as they promote cushioning of the bubbles due to enormous vapor pressure within the solvent. The cushioning phenomenon stops the bubble from expanding which hinders acoustic streaming of the system and further diffusion of solvent into the target molecules (Flannigan & Suslick, 2010). Several suggestions of specific surfactants and buffers are common in the case of seed protein extraction. For instance, the use of tris, phosphate and lysine buffers have particularly been promoted as they have proved optimally efficient for several ultrasonic assisted seed protein extraction (Deng et al., 2014; Quanhong & Caili, 2005).

2.8.1.4 Temperature

Temperature change during the ultrasonic extraction process is inevitable, mainly due to immense sound energy produced in the system. Thus, temperature is a factor that must be controlled externally by circulating cold/hot water during extraction. High solvent temperature during extraction enhances the diffusion rate and assists in breaking solute-matrix interaction (Santos et al., 2009).However, the phenomenon of cavitation is also reported to be reduced at higher extraction temperatures because the voids within the solvent matrix and biomass are filled with solvent vapors, leading to less violent collapse. (Barroso & Rostagno, 2013) investigated into the effects of temperature on reduction in particle size using UAE. It was observed that while the temperature was raised from 70 to 90^0C, the delivery of ultrasonic intensity to the solution was decreased, whereas a rise in the temperature from 20 to 70^0C was proved to be beneficial for extraction as a fair amount of reduction in particle size was observed. The reduction in particle size was linked to increase in the solid – solvent contact area resulting in a high nuclei cavitation of the bubbles. Moreover, a drop in particle size as temperature reached boiling point of the solvent (100^0 C) was recorded and was explained as generation of vapor pressure within the solvent due to temperature rise, which delayed the bubble collapse process and thus negatively affect the final extraction efficiency of the target compounds. Hence, a controlled temperature is strongly suggested for UAE to control degradation of the target compounds and to provide a controlled vapor pressure to the system.

2.9 The modern experimental design and process optimization

The term optimization is generally referred to enhance a process or a products performance so that maximum benefit can be obtained from it. In analytical chemistry, optimization is commonly used as a way of analyzing or discovering specific parameters, which if applied to a procedure, produces a maximized response (Araujo & Brereton, 1996). The experimental designs in their initial days traditionally used method which could only analyze the influence of one factor at a time on the response variable. examples of which can be found in several literatures (Tzia & Liadakis, 2003) (Lundstedt et al., 1998). However, measuring each factor, and its interaction with response was found to be time consuming. Moreover, it also increased the overall cost of experiment and utilization of

materials and reagents. Thus, to overcome this gap in optimizing analytical procedures, a multivariate statistical approach was developed, known as response surface methodology (RSM). Most of the optimization studies of seed protein extraction have used RSM for the successful optimization of a procedure, which makes this statistical tool extremely reliable, admissible and easy to use for extraction optimization process (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008).

2.9.1 Response surface methodology (RSM)

RSM was developed by Box and collaborators in the 50s when graphical representation of a mathematical model was popularly used only in chemometrics (Bruns, Scarminio, & de Barros Neto, 2006). RSM consists of a group of mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to the experimental design. Toward this objective, linear or square polynomial functions are employed to describe the system studied and, consequently, to explore (modeling and displacing) experimental conditions until its optimization (Teófilo & Ferreira, 2006). RSM overcomes the problem of interaction analysis of variables by analyzing a set of responses for a set of variables, rather than doing one variable at a time (Araujo & Brereton, 1996). Before application of RSM it is important to choose a suitable experimental design, which aids in defining experimental region and levels for experimental accuracy. Generally, linear functions are used as a basic experimental design, whereas response approximation uses a quadratic response (Hanrahan & Lu, 2006). A few commonly known quadratic response experimental designs are Box Behnken design, central composite design and Doehkert designs. The following section primarily reviews the basic staging principles to use response surface methodology in analytical chemistry. Later, it focuses on the basics of Box Behnken experimental design, its advantages, and disadvantage. The following section also broaches the process of optimization in analytical chemistry, and analysis of the response data.

2.9.1.1 Screening of variables

Many factors are present in an experimental setup, that can either be controlled or not, and have impacts on the response of the experimental systems. Thus, the very initial stage of optimization is to select the factors which have major impacts on the desired response. In

order to analyze these variables, screening designs must be produced to determine significant factors and interactions. Generally, a full or fractional level design is used for this purpose as they have a proven record specifically in the field of food technology and sciences (Lundstedt et al., 1998).

2.9.1.2 Selection of a regression model

Least square multiple regression methodology is generally deployed to investigate a correlation amongst independent and dependent variables. A multiple regression equation is presented here that can be utilized to fit a second order polynomial equation.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 \sum_{1 \leq i \leq j}^{k} \beta_{ij} X_i X_j + \varepsilon$$

In the equation presented above, y signifies a response which is predicted, β_0 is the constant term, β_i, β_j and β_{ij} resembles the linear, quadratic, and interactive effects, respectively. Whereas X_i and X_j represents factors and k is the number of factors chosen for the experiment (Baş & Boyacı, 2007).

2.9.1.3 Coding the factor levels

Succeeding the process of variable screening, factors are defined and fenced around their maximum and minimum limits. The step is extremely important to consider as success of an optimized process largely depends on it. The codification further converts each value which is being studied in the experiment to a range with no dimension (Baş & Boyacı, 2007).

2.9.1.4 Verification of the fitted model

It is known that an estimated response and regression coefficients can be generated using the statistical technique RSM. However, to be confident about accuracy of the optimized processes for practical applications, numerous techniques have been made available which verify the fitted empirical model, such as lack of fit tests, prediction of error, sum of squares, residuals, and analysis of the residuals etc. However, the most used technique in the recent times is coefficient of determination or R^2 value, to analyze the predictive potential of the fitted equation or model. The technique measures a total number of

changeability in a response by applying the repressor variables in a model (Vieira & Hoffmann, 1989). However, it has always been noticed that by simply increasing the number of variables within a model, a higher value is always expected. Thus, the R^2 value alone is not enough to measure the of accuracy of a model (Baş & Boyacı, 2007). Average absolute deviation, which literally means an average of all the deviations from the central point of the fitted model, has been used in combination with the R^2 values for enhanced accuracy (Cornell, 1990). Furthermore, a quick analysis of residuals is also one of the valuable tools commonly used for verification of the fitted models along with other methods. In the analysis of residuals, observed error if consistent with the stochastic errors in the experimental data is represented using graphical representation. It is generally recommended that the residuals must be centered on zero all-round the range of fitted values. Moreover, the residuals must not be either consistently high or low, they should be spread constantly throughout the range.

2.9.1.5 A graphical representation of the model and analysis

The predicted model and equation can be visually represented and analyzed at various levels and points using potential response surface plots as depicted in Figure 2.8. These graphical representations are basically a three-dimensional, theoretical approach of the response surface methodology to elucidate the link between independent and dependent variables. Whereas the contour plots are two-dimensional representation of the three-dimensional response surface plots in which, the dependent variables are constantly ranged upon a plane of independent variables. If the target point is maximized within a model system, the contour plots represent a circle or ellipses most possibly at the center of the plots. However, parabolic, and hyperbolic curves are generated, if neither a maximum nor a minimum point is reached. Thus, these representations enhance the understanding of complex interaction effects of the variables on the desired response. However, it is ought to be noted that the graphical representation is just a depiction of the estimated responses, not the actual responses. Thus, a combination of verification and graphical methods are thus recommended to finalize the fitted model in an experimental analysis.

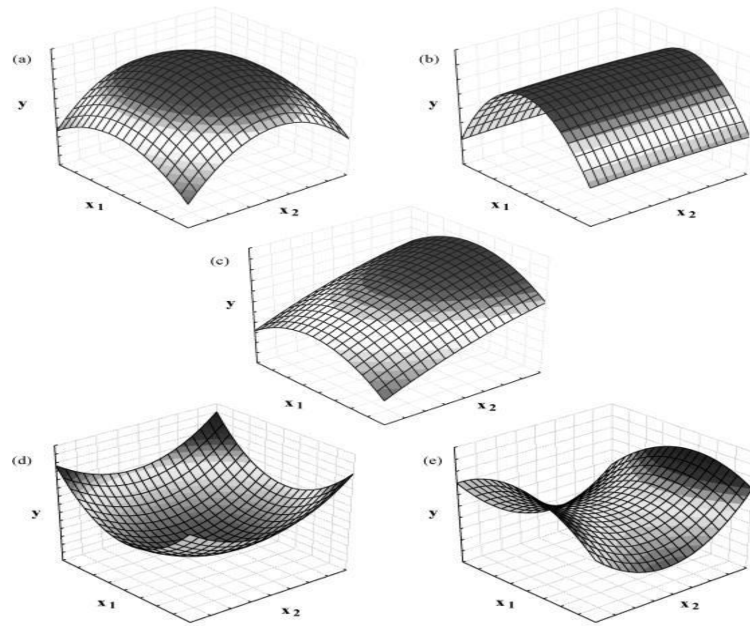

Fig 2.8 surface response generated from a quadratic model in the optimization of two variables. (a) maximum, (b) plateau, (c) maximum outside the experimental region, (d) minimum, and (e) saddle surfaces. Reproduced with permission from (Bezerra et al., 2008)

2.9.1.6 The Box-Behnken design

Significance of successfully applied RSM is denoted by deploying an appropriate experimental design of experiment. This design of experiments (DOE) helps to choose the points where the response must be studied with an experimental approach. BBD (Box Behnken design) encompasses a subset of a combination of factorial from 3^k designs, where k represents the number of variables used in an experiment. The design is made in such a way that the chosen points of the experiment are located on a hypersphere which are at the equal distance from the center (Fig 2.9). The Box-Behnken (BB) is another approach in response surface methodology where the outcome is to find the optimal variables to produce optimum response/ output. According to Maran et al. (2013), Box- Behnken design is considered as a design without the embedded factorial or fractional factorial point that could be identified as the variable condition located at the midpoint edges of the variables space as well as at the center (Ghorbani, Kaffashi, Shokrollahi, Seyedjafari, & Ardeshirylajimi, 2015). The box Behnken design is generally sought after

and admired in food processing field due to its economic design and flexibility of levels and variables. Moreover, the BBD also eliminates impractical and extreme points which are helpful in determining the success of the experiment. In addition, it is also viewed generally that no more than 3 levels of factors can be chosen while using BBD, which is considered as one of its major drawbacks.

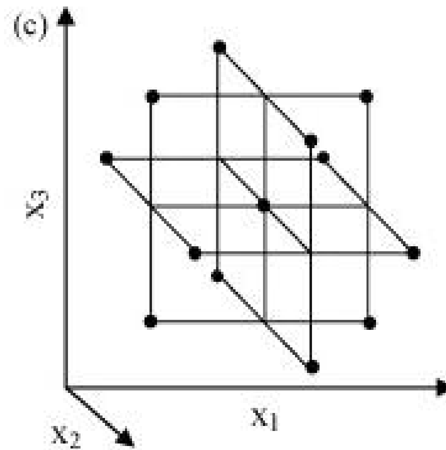

Fig. 2.9 Experimental designs based on Box–Behnken design for the optimization of three variables. Reproduced with permission from (Bezerra et al., 2008).

2.10 Amino acids in proteins

2.10.1 Amino acids

Proteins are also popularly known as polymers of amino acids because several single units of amino acids combine to form a basic structure of protein. Moreover, different groups and types of amino acids as per their chemical characteristics and the order in which they are arranged within the protein structure also affects the final three-dimensional design of a protein molecule (Akram et al., 2011). In the modern era it is becoming more apparent that the overall quality of a novel plant protein source is majorly determined by its amino acid levels. Therefore, it seems worthwhile to review different amino acids in the following sections. However, the literature on amino acids is voluminous and it is not possible to embrace every aspect of amino acids in this review. Therefore, the following section

focuses on the chemical characteristics, nutritional importance, and structural chemistry of amino acids.

2.10.1.1Structural and chemical characteristics

Nearly all amino acids (with exceptions of proline and hydroxyproline) can be defined as a chemical compound which must possess at least one amino and one acidic group attached to a chiral carbon atom, where the amino group should be in the α position to the carboxyl group while the rest of the molecule representing a unique functional group (R) covalently attached to the same carbon atom (Wu, 2009). In the formation of the primary structure of a protein molecule, the basic linkage in the backbone of the polymer is a peptide bond formed by reaction between the amine group of one amino acid and the carboxyl group of the another. This reaction is represented in the Figure 2.10 below which indicates removal of a water molecule during the protein formation process. The functional group (R) as depicted in the figure represents a crucial part of these chemical compounds, as they primarily determine the unique properties of each amino acid. The uniqueness of different proteins is then determined by which amino acids it contains, how these amino acids are arranged in a chain, and further complex interactions the chain makes with itself and the environmental matter.

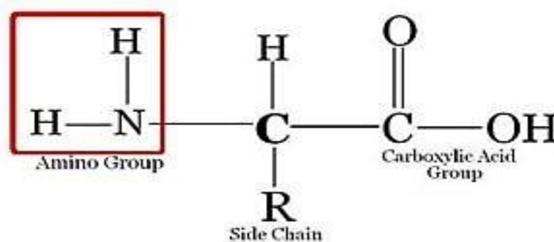

Fig. 2.10 A generic structure of amino acids

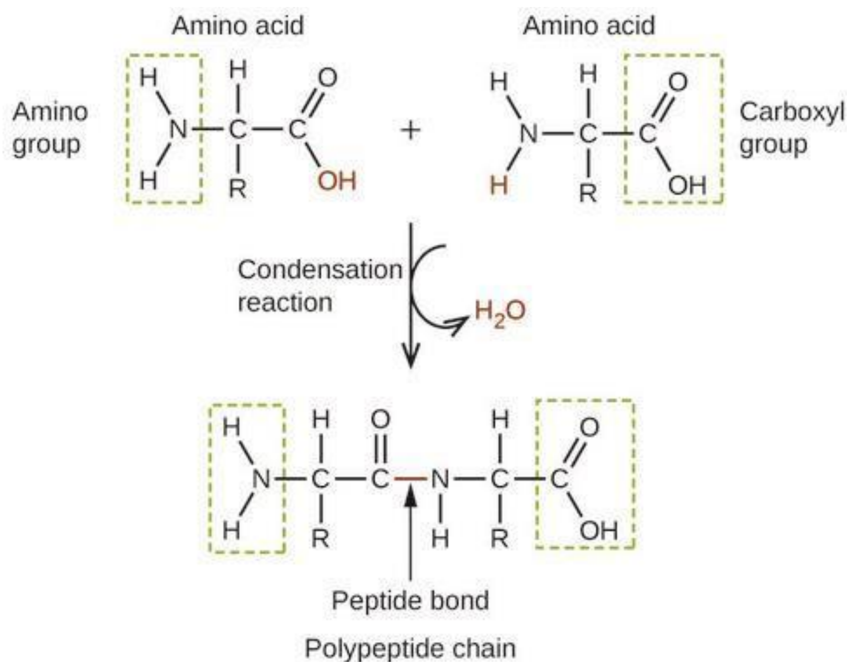

Fig 2.11 Peptide bond formation

Moreover, amino acids are amphoteric in nature as they typically exhibit dual characteristic as acid as well as base. This amphoteric nature of amino acids is due to the presence of a carboxylic acid group which ionizes as an acid within the molecule, and an amino group which ionizes as a base (Jalkanen, Elstner, & Suhai, 2004). It can also be understood in the terms of zwitterionic forms (Figure 2.12) where amino acid abides as dipolar (zwitterions) with both amine group as well as the carboxyl group ionized at the same time. This degree of ionization of molecules is universally indicated by a range of pH value/values at which the group is partially associated and partially dissociated at the same time thus representing the pK_a value of that group. The α carboxyl group being acidic in a generic structure of amino acids have a pK_a value in the range of pH 2 -3, whereas that of the amine group is approximately pH 10. Thus, in the pH range of 4 to 9 (i.e., the range between acidic and basic groups) nearly all the amino acids exist as a zwitterion with no overall charge on it.

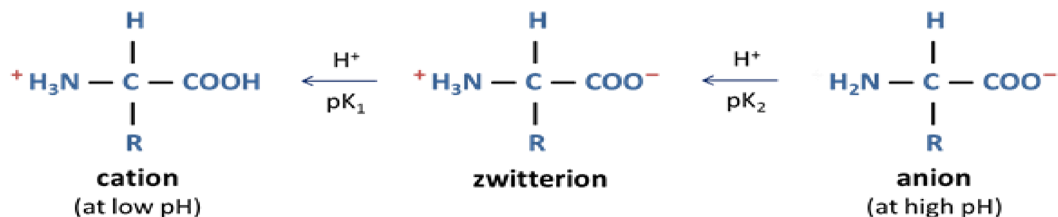

Fig 2.12 Zwitterionic form of amino acids

2.10.1.2 Nutritional importance

In the past two centuries, hundreds of amino acids were discovered by the scientific communities worldwide, who categorized them into three major classifications. Namely, non-essential amino acids, essential amino acids, and conditional amino acids. However, only about 20 -22 amino acids are needed to make all the proteins found in the human body and generally account for making up the bulk of monomer units in major proteins we know today. Out of these twenty amino acids, eleven are commonly found as constituents of human peptides itself, which implies that these chemical compounds are produced by our body naturally, thus were addressed as the non-essential amino acids. Whereas the other nine amino acids which a human body is unable to synthesize through their metabolic pathways and must be ingested through various external mediums such as foods, diets and drinks are collectively labelled as essential amino acids. Moreover, the conditional amino acids are usually expressed as non-essential amino acids itself, except when conditions like illness and stress occur. In these conditions our body stops producing these non-essential amino acids and thus must be supplied through an exogenous diet.

Table 2.3 essential and non-essential amino acids

The 20 major amino acids	**Essential amino acids**
Alanine	Phenylalanine
Arginine	Valine
Asparagine	Tryptophan
Aspartic acid	Threonine
Cysteine	Isoleucine
Glutamic acid	Methionine
Glutamine	Histidine
Glycine	Leucine
Histidine	Lysine
Isoleucine	
Leucine	
Lysine	
Methionine	
Phenylalanine	
Proline	
Serine	
Threonine	
Tryptophan	
Tyrosine	
Valine	
Selenocysteine	
Pyrrolysine (not used in human protein synthesis)	

2.11 Functionality of proteins

Besides having several nutritional benefits, physicochemical properties of proteins also play a role in the sensory characteristics of major processed foods; this includes altering organoleptic features such as taste, flavour, and texture. Due to the amphiphilic nature of proteins (as mentioned in previous sections) and their versatile structural abilities they can interact with several other food constituents such as fats, water, minerals, carbohydrates etc. which plays a significant role in determining the end quality of a processed food product. For example, the textural characteristics of products from bakery industries are a manifestation of the dough forming and viscoelastic properties of wheat gluten; the textural and sensory properties of cakes and desserts can be attributed to the functional properties of egg proteins and curd formation in dairy products are a result of casein micelle colloidal structure etc. Thus, in food processing industries proteins are considered as one of the most versatile ingredients which enhances and alters the sensory properties of foods as per requirement.

Functional properties of proteins are defined by several authors and have a diverse range of definitions. However, (Zayas, 2012) precisely defines protein functional properties as "*the physico-chemical properties of proteins from any sources that influences functional behaviour of proteins in food which include their size, shape, amino acid composition and sequence, net charge, charge distribution, hydrophobicity, hydrophilicity, structures* (*secondary, tertiary and quaternary*), *molecular flexibility/rigidity in response to external environment* (*pH, temperature, salt concentration*), *or interaction with other food constituent".* Due to a heterogeneous structure and its ability to interact with a broad range of food components, protein functional properties are also hugely diversified, which has resulted in a lack of commonly accepted scheme of classification for protein functionality. However, attempts to classify proteins as per their source types like plant, milk, meat etc. have been extensively published since the last decade (Moure, Sineiro, Domínguez, & Parajó, 2006). Characteristics of a protein such as its molecular weight, molecular shape, structural diversity, and conformation are popularly known to affect its functionality. Moreover, sources of protein, environmental factors and processing parameters also possess effects on its functionality (Kinsella, 1982). Thus, the following

section reviews some of the major functional properties of proteins and highlights the crucial factors affecting them.

2.11.1 Solubility

The solubility of proteins can be defined as the amount of soluble nitrogen which can be detected in the soluble state under predefined conditions. The solubility of proteins is primarily known to be affected by composition of amino acid, molecular weights, and conformation of non-polar and polar groups within amino acids. This can also be understood in terms of hydrophobicity that higher the hydrophobicity of a protein molecule and higher the charge frequency on the interaction surface, higher would be the solubility of the protein (McGowen & Mellors, 1979). This empirical relationship however is scrutinized in several cases due to a prolonged diversity in the type and sources of proteins (Kinsella, 1982). As one of the first functional property to be tested and applied in protein chemistry, solubility stands out from other functional properties as it can be related to many other functional properties, specially foamability, gel formation and emulsification. Solubility determination of proteins is particularly crucial for several beverages and liquid food manufacturers as a highly soluble protein displays a finely dispersed colloidal system and thus possess good dispersibility benefiting beverage and liquid food industries.

Factors affecting solubility of proteins

pH of the medium

pH can be called as a determining factor for protein solubility. The quantifiable solubility of a protein in any aqueous medium is usually linked with hydrophobic and electrostatic interactions within the protein molecules. The solubility tends to increase if a higher electrostatic repulsion is recorded than the interactions of hydrophobic molecules. To obtain a maximum solubility, interactions of the proteins with the solvent's is crucial (Kinsella & Melachouris, 1976). The proteins have net zero charge at the isoelectric point (pI) which creates a strong attractive force within the molecules thus hindering their interactions with solvents, resulting in insolubility. This can be also explained by an increasing protein solubility above or below the pI values of different proteins due to a net positive or negative charge within them. However, minimal interactions occur if the values

of pH are not very far away from the pI values. Hence, solubility and extraction are greater at higher pH values where the net electrical charge within the solvent molecules is high enough to interact with the charged surface of proteins. For example, Soy protein in an alkaline medium were shown to increase their extraction yield and solubility, especially the 7S and 2S globulins (Kinsella, 1979).

Temperature

The solubility of proteins is inversely proportional to the time of heating and high temperatures as several irreversible instances of precipitation at higher temperatures have been reported. The solubility of a protein is generally reported to increase within a range of 0 – 50^0C and decline further. However, the type of heat i.e., moist heat or dry heat have different and complex effects on the solubility. The nitrogen solubility index of soy protein flour concentrates and isolates for example, showed an increase of 20% as the temperature of the system was raised above 50^0C (Kinsella, 1979). Moreover, heating also evidently alters the conformational and structural changes in proteins thus can also be accounted for identification of degree of denaturation within proteins (Zayas, 2012).

2.11.2 Water holding capacity

The water holding capacity of proteins can be defined as the ability to retain water against gravity physiochemically and physically. The character of protein – water and protein-protein interactions chiefly determine if the function of proteins in the food would turn out to be a colloidal dispersion, gel, or insoluble precipitate. Thus, the water binding or water holding capacity relies largely on the conformation and composition of the protein molecules. The mechanism of water interacting with protein can be explained simply by the phenomenon of hydrogen bonding. As the molecules of water and hydrophilic protein ends interact by hydrogen bonding, significant proportion of water is retained by the polar polypeptide groups of the proteins. Generally, polar hydrophilic groups such as carboxyl, amino, hydroxy, carbonyl etc. are responsible for the bonding and retention of moisture within the protein molecule. The number and type of these polar groups in a protein polypeptide chain thus affects the moisture retention properties which can also be deciphered by analysing the amino acid composition of a protein molecule. It is commonly reported that, higher the amount of charged amino acids in the protein, larger would be its

water retention capacity. The amino acids as per their water binding abilities have been further classified into polar amino acids (highest water retention capacity); non-ionized amino acids (Intermediate water binding) and hydrophobic amino acids (Minimal to negligible binding capacities). The polar amino acid groups are majorly active sites for water protein interactions and thus are classified to have a high-water binding affinity. The absorbed water encloses the molecules of protein in several layers of tightly bound water at sites within the molecules, which includes the adsorbed layer and surface of the proteins (Zayas, 2012).

2.11.3 Emulsifying properties

The emulsions in foods can be described as macroemulsions with a droplet size of 0.2 - 50µm, which represents a heterogeneous mixture of fat globules in an aqueous solution or vice versa. The emulsions are generally classified as water in oil (W/O) with greasy textural properties or oil in water (O/W) with a creamy texture. The common attributes to analyze the aspects of protein emulsification properties are namely, emulsifying activity index, emulsifying capacity of the proteins (EC), and emulsifying stability (ES). ES can be further defined as the ability of proteins to form and stabilize newly created emulsions, whereas EC can be quantified by measuring the amount of oil (ml) emulsified by 1g of protein under specified conditions. Several interconnected physical and chemical parameters influence the stability, textural properties, and formation of fat – protein – water emulsions such as molecular flexibility of proteins, protein solubility and surface hydrophobicity etc (Kinsella & Morr, 1984). In food chemistry, proteins with higher number of apolar amino acids are generally considered as “surface active agents” due to their capacity of minimizing interfacial tension between hydrophilic and hydrophobic parts of different foods. When a protein participates in an emulsification process, the surface-active properties of proteins result in surrounding the fat globules with a protective barrier of adsorbed amphiphilic protein molecules (having high surface hydrophobicity) on the oil/water interface. This prevents the fat/water droplet from collapsing by reducing interfacial tension between the lipid and aqueous phases, thereby assisting the formation and stabilization of the emulsion system (Kato & Nakai, 1980).

2.11.4 Foamability of proteins

Foam in food production and manufacturing can be defined as an extremely complex system of liquids, gases, solids, and surfactants. This phase of air cells separated by a thin layer of liquids and stabilized by surfactants also known as lamellar phase, results in uniform distribution of small stable bubbles within the system which provides body, lightness, and smoothness to the foods. Commonly used proteins as foaming agents among food industries are gelatins, casein, egg white and soy protein etc. The mechanism of formation of foams principally involves three basic stages as described by (Kinsella, 1981). Primarily, the globular storage proteins diffuse into the interface of air/water, and minimize the surface tension, which in turn increases the elastic and viscous properties of the liquid phase. The proteins unfold at the interface, orienting the hydrophobic and hydrophilic ends towards fat and aqueous phases respectively. Increase in the viscous and elastic properties of liquid phase forms continuous strong films around the gas bubbles due to protein- protein polypeptide interaction and associations through hydrogen bonding, hydrophobic, electrostatic interactions.

2.11.5 Gelation properties

Protein gels from a macroscopic point of view can be defined as an aggregated network of protein molecules in an aqueous solution that restrains deformation when tractive forces are applied (Kutsepalov & Matveev, 2015). This irreversible conversion of fluids into solid plays an important role in production of a range of foods with unique textural properties since antiquity. Several methods have been adopted in the past to induce gelation of proteins which includes, addition of salts in aqueous mediums, high pressure application, acidification, and enzymatic reactions etc. However, the most popular method used is heating and cooling of the protein-aqueous solution simultaneously. To efficiently form gels, a protein must depict a dense and well defined secondary and tertiary structures such as that of plant globulins, egg white proteins and whey proteins (de Jongh, 2003). The process of gelation or aggregation initiates with heating of globular proteins in an aqueous solution, which in turn allows the polypeptide chains to lose their rigid ternary structure and become more mobile in the solution. The amino acids within the native protein structures thus becomes available to form disulphide covalent bonds with other proteins

molecules such as two cysteines, hydrogen bonds with water molecules and other hydrophobic interactions. The rate of aggregation of protein molecules in an aqueous solution is often analysed by the rate at which the proteins within the solution denature and become accessible for bonding. Therefore, the rate of aggregation and gelation induced by heating increases with increasing temperature and can often be characterized by an activation energy (E_a). Moreover, the protein aggregates can be further quantified with a power law relationship between radius (R) and molar mass (M) of the protein molecules: $M = (R/R_o)^{df}$, where d_f is the so-called fractal dimension of the protein network formed by gelation. The R_o denotes size of the fractal aggregates and is generally reported to be significantly higher when compares to the individual protein structures. A schematic representation of irreversible aggregation and gelation is shown in Figure 2.13 and 2.14.

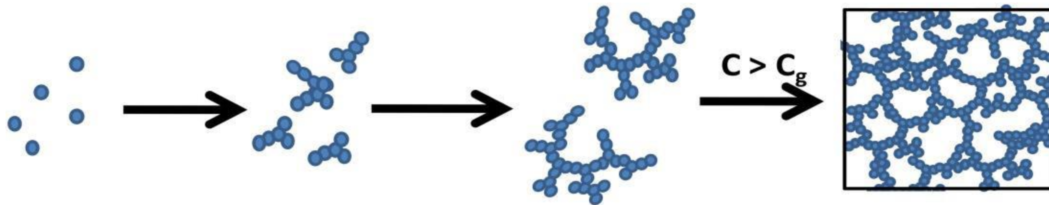

Fig 2.13 Schematic representation of random aggregation and gelation of globular proteins (Nicolai, 2019).

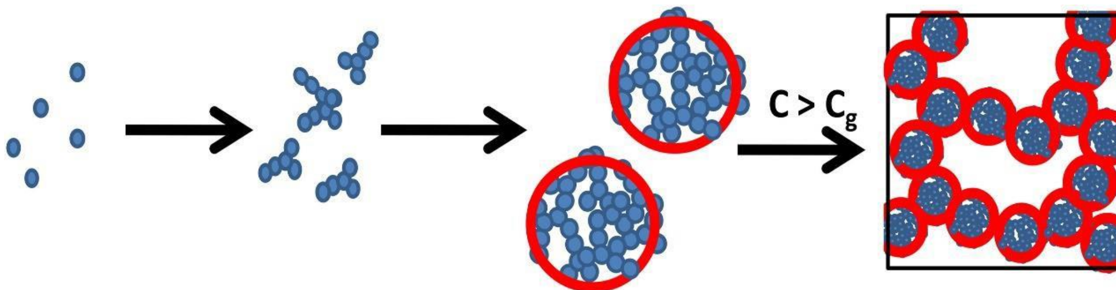

Fig 2.14 Schematic representation of microphase separation of globular proteins followed by aggregation and gelation of microgels (Nicolai, 2019)

Additional attractive or repulsive interactions between water and protein molecules also influences the irreversible aggregation process. Repulsive interaction leads to a network of connected strands as this favors chain formation, whereas additional attractive interaction causes coarser networks to form. The extent to which the system coarsens depends on the strength of the attractive interactions, which controls the rate of phase separation, and the

probability to form permanent bonds, which controls the rate at which restructuring is arrested. This process may thus be considered as spinodal phase separation arrested by the formation of permanent bonds Fig 2.5. General features of the interaction between food proteins in aqueous solutions leading to phase separation, aggregation and/or gelation have been thoroughly reviewed by (van der Linden & Foegeding, 2009).

2.12 Conclusion

It can be concluded from the review of the literature that kiwi fruit seeds are indeed a significant source of protein that can be extracted and are available as a potential waste from food and cosmetic industries. It can also be concluded that, copious amounts of studies were conducted in the past on a wide variety of seeds, highlighting various extraction parameters, optimizing procedures and techniques used which was helpful to design the optimizing procedure of kiwifruit seed in this study. A potential gap found was lack of substantial literature on the morphology of a kiwifruit seed and recent data of kiwifruit seed waste in New Zealand, which is also a limitation of this research. Moreover, greener technologies and their benefits were profoundly explored in this review which offers the possibility of using greener process such as ultrasonication than traditional extraction processes. Further studies are needed to understand kiwifruit seed as a waste protein source, reliable data is needed on how much kiwifruit seed waste is happening in the country. This would help to optimise protein extraction processes for kiwifruit seeds in the future and to confirm and identify the benefits of ultrasonic extraction on protein yields and the properties of the extracted protein.

Chapter 3: Materials and Methods

3.1 Introduction

This chapter describes the materials and scientific methodologies used in this study to achieve a specific set of objectives as previously mentioned in Chapter 1. This research was basically branched into three parts:

- The first part was aimed to maximize protein extraction from kiwifruit seed meal using ultrasonication, an environment friendly extraction technology in combination with statistical tools such as response surface methodology, box Behnken design and response optimizer.
- Secondarily, the research aims a thorough functional, and physicochemical analysis of KSP in order to have a deeper understanding of the novel protein and highlights its possible utilization by the food industries in future.
- The research finally attempts to investigate the effects of ultrasonic extraction on the functionality of KSPC and compares it with KSPC obtained via conventionally stirred extraction procedure.

Optimized results with maximized protein extraction were obtained using ultrasound extraction technology and analyzing data through response surface methodology in Minitab 19. Bradford method was subjected to quantify the amount of protein present in the extracted samples whereas the protein purity was analysed by standard Kjeldahl method of protein estimation with conversion value of 6.25.

The protein concentrates of KSP were obtained via freeze drying process and were subjected to various functionality tests and amino acid analysis for analysing the potential of KSP as a functional ingredient.

3.2 KSP Extraction and optimization

This section discusses materials and methods used for the extraction and optimization of KSP from kiwifruit seeds. Completely processed dried kiwifruit (*Actinidia deliciosa*) seeds (with no chemicals added while processing) were purchased from Pure ingredients ltd. New Zealand. A Hielscher UIP 1000hd ultrasonic processor and a standard 50 mm sonotrode

BS2d50 were used for ultrasonic extraction in this study. The Bradford reagent and standard bovine solution (0.125 – 1.0 mg/ml) for Bradford protein estimation was purchased from Bio Rad New Zealand. All other chemicals and reagents; Hexane (analytical grade, Sigma-Aldrich, Switzerland) Na2HPO4 (analytical grade, Sigma-Aldrich, Switzerland), NaH2PO4 (analytical grade, Fisher chemical UK) and Na3HPO4 (analytical grade, Sigma-Aldrich, Switzerland) used in this study were of analytical grade and were provided by Massey university.

3.2.1 Preparation of defatted kiwi seed flour

Kiwifruit seeds were crumbled using a plate mill (Type S 100m). The seed cake obtained was defatted using n-hexane (analytical grade, Sigma-Aldrich, New Zealand) mixed in a ratio 1:3 (w/v) and stirred continuously using a magnetic stirrer for 90 mins. After stirring, the solution was transferred to 250 ml centrifuge bottles and the mixture was centrifuged at 4700 RPM for 17 min at 4^0C. The supernatant was removed, and the entire process was repeated twice to increase the extraction efficiency. The defatted meal was left overnight for drying at room temperature under a fume hood, and finally sifted through 500 µm sieve to obtain the defatted kiwifruit seed meal flour which was stored at 4^0C for further extraction purposes.

3.2.2 Preparation of phosphate buffer saline (PBS)

A 0.05M phosphate buffer solution was prepared for protein extraction by calculating the amounts of acid and conjugate base for different pH using Henderson Hasselbalch equation. Disodium phosphate (Na_2PO4) and its conjugate bases (Na_2HPO4, NaHPO4 etc.) were used for reaching the pH of 3.5, 7.5 and 11.5. Approximately 800 mL distilled water was taken in a glass beaker and the calculated amounts of acid, and its conjugate base was mixed and stirred using a magnetic stirrer until the solution was clear. A pH meter was then used to adjust the pH up to desired values using 1M HCl or 1M NaOH. Finally, the solution was made up to 1 liter and stored at room temperature for experiments.

3.2.3 Box Behnken design (BBD) for response optimization

In Minitab 19 the low/high values for each factor were defined (Table 3.1) and then BBD was used to outline different extraction parameters (Appendix: Table 8.1) for optimized protein extraction from kiwifruit seeds.

Table 3.1: Low and high values for experimental factors

Factor	**Name**	**Low**	**High**
A	pH	3.5	11.5
B	Power	20	100
C	Time (min)	20	100
D	Ratio (g:ml)	4:100	20:100
E	Temperature (^{o}C)	10	100

3.2.4 Ultrasonication and protein extraction

A flat bottom steel container was placed on the top of a stand scissor rack with a regular in and outflow of water from a water bath to control and stabilize the temperature. Different ratios of defatted meal and 0.05 M PBS was prepared in a beaker and was placed in the steel container (aforementioned). An ultrasonic processor was then used to produce the sound waves and a standard 50 mm sonotrode was dipped more than half into the beaker to evenly disburse the waves into the mix. Power and time were also controlled through the ultrasonic processor which helped to run the samples for precise time and power as required. A velcro strap was intently used to keep the beaker in place during the experiments as high intensity sound waves tends to displace the beaker while extraction. After ultrasonication, the samples were kept at 4°C for an hour in a regularly calibrated laboratory refrigerator and were later transferred into 250 ml centrifuge tubes. The samples were then centrifuged at 3000 RPM for 15 mins at 4^{0}C; and the procedure was repeated twice for enhanced extraction. The supernatant was finally collected and stored at -20°C for further analysis. All the experiments were done in triplicates. A Standard 300 ml glass

beaker was used for all the experiments conducted. Figure 3.1 outlines the setup ultrasonic protein extraction.

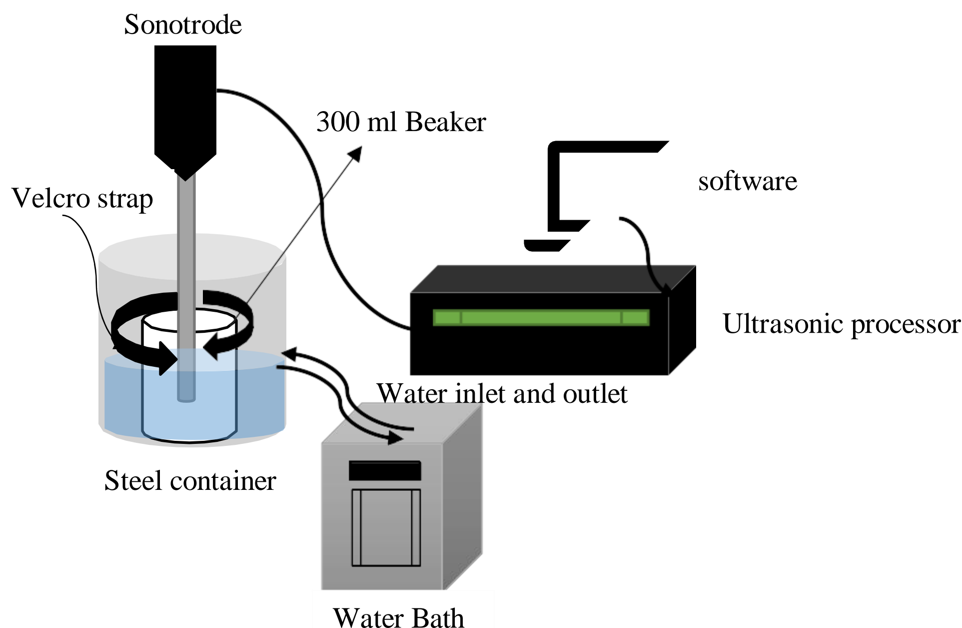

Figure 3.1 The ultrasonic extraction setup

3.2.5 Bradford method and protein estimation

Standard 1ml protocol of Bradford method was used as per (Bradford, 1976) for estimating protein in unknown kiwi seed protein samples. A standard solution of bovine serum albumin (BSA) was prepared by mixing the phosphate buffer (pH 3.5,7.5 and 11.5) and 96% BSA (2mg/ml) using a standard volume flask and stored in different aliquots at -20^0 C for further use. The aliquots were taken out from the freezer and brought to room temperature before the standard solution was serially diluted. As per table 3.1 different amounts of standard solution were plunged into labelled trimax tubes in triplicates along with 1ml of the Bradford reagent and vortexed for 2 mins each. The Bradford reagent concentrate was purchased from Bio Rad New Zealand and was diluted using deionized water in a ratio 1:4 and was stored at room temperature for analysis. The serial dilution was transferred to disposable 1ml quartz cuvettes and were incubated at room temperature for

10 mins each before analysis. In order to obtain a standard curve, a spectrophotometer was set at 595 nm, and was zeroed using a blank sample (Table 3.2). Each cuvette was placed into the spectrophotometer and the absorbance was noted. For the unknown samples, 20µl of each sample was taken in the triplicates in different test tubes and 1ml Bradford reagent was poured into it. The tubes were vortexed and incubated for 10 mins and transferred into 1ml quartz cuvettes before analyzing the absorbance at 595 nm using a spectrophotometer.

Table 3.2: The serial dilution of BSA for standard curve (Bio rad standard protocol)

Tube #	**Standard volume (µl)**	**Source of standard**	**Diluent volume (PBS)**	**Final protein (µg/ml)**
1	70	2mg/ml stock BSA	0	2000
2	75	2mg/ml stock BSA	25	1500
3	70	2mg/ml stock BSA	70	1000
4	35	Tube 2	35	750
5	70	Tube 3	70	500
6	70	Tube 5	70	250
7	70	Tube 6	70	125
8	-	-	70	0

3.3 KSP analysis and protein yield

This section outlines the methods used for proximate analysis of KSP and protein yield of KSP after freeze drying. The materials and chemicals used for protein estimation and crude fat analysis were of analytical grade provided by Massey University. A list of materials used in the analysis is mentioned in the Appendix section for reference.

3.3.1 Determination of Total Moisture content (dry basis)

Air oven method was applied as described by (AOAC, 1925) in order to determine the total moisture content of Kiwifruit seed flour, ultrasonicated and non-sonicated KSP samples. Empty aluminium moisture dishes with fitting covers were initially dried in a hot air oven at 105^0C for at least 10 hrs and kept in a desiccator at room temperature prior to use. The weight of empty moisture dishes along with their lids was measured up to four decimal

places. Approximately one gram of freeze dried KSP samples was weighed, and the moisture dishes were again kept in the hot air oven at 105 ±0.5^{0}C for 12 hours with lid opened. While weighing empty dishes and samples, gloves were worn all the time to avoid extra moisture in the dishes and samples. After drying, dishes were also covered before removing them from the oven. The dried sample was weighed again, and the moisture percentage was calculated as per the following equation.

$$\text{Total moisture content (\%)} = \frac{W2 - W3}{W2 - W1} \times 100 \quad \textbf{(Eq.3.1)}$$

Where W1 = weight (g) of moisture dish + lid; W2 = weight (g) of moisture dish + lid + KSP sample; and W3 = weight (g) of moisture dish + lid + dried KSP sample.

3.3.2 Determination of protein purity

The protein analysis of freeze dried KSP samples was conducted using Kjeldahl method in conformity with AOAC official method 2001.11 (Chemist, 2005). 1 gram of each KSP sample was accurately weighed in empty digestion tubes followed by addition of two Kjeltabs (each containing 3.2 g K_2SO_4 and 0.00035g Selenium, Foss analytical, United Kingdom) and 15-16ml of concentrated sulphuric acid. The digestion was carried out using K_{424} digestion unit (Buchi labortechnik, Switzerland) at 420^{0}C for at least an hour or until a clear solution was obtained. After digestion, the samples were cooled at room temperature until the digestion tubes were okay to touch with bare hands. Steam distillation was carries out using a Kjeltec 8100 distillation unit (FOSS Analytical AB, Höganäs, Sweden). 80 ml of 40% NaOH was automatically added into the digestion tubes, the distillate was received in a conical flask containing 25ml of 4% (w/v) boric acid solution [With added 0.1% (w/v) methyl red and 0.1% (w/v) bromocresol green made up with alcohol]. Distillation was caried out for 3 mins and titration was subsequently performed against 0.1 M HCl solution until a grey, mauve end point was reached. The crude protein content was finally calculated using Equations 3.2 and 3.3, using a conversion factor of 6.25 (AOAC, 1925).

$$Nitrogen\ (\%) = \frac{(A \times B) \times 14 \times 100}{1000 \text{ X } C} \quad \textbf{(Eq. 3.2)}$$

$$Crude\ protein\ (\%) = \%\ nitrogen \times F \quad \textbf{(Eq 3.3)}$$

Where A = volume (ml) of HCl used; B = exact molarity of HCl; C = weight (g) of KSP sample used; and F = conversion factor.

3.3.3 Crude fat content of Kiwi Seeds, Kiwi Seed Flour and KSP

A standard Soxhlet analysis was carried out as per (AOAC, 1925) in order to determine the overall fat percent in the kiwifruit seeds before and after the process of defatting. In addition, the freeze-dried sample of KSP was also analysed for crude fat content in order to have insights for lower or higher extraction yields. All the glassware's were initially washed with petroleum ether and dried in a hot air over at 105^0C for 12 hours before analysis. A completely dried 5 gm of sample was weighed and placed in a cellulose thimble (Whatman™ High Purity Glass Microfiber). The thimble with sample was placed in the Soxhlet extractor along with a 150 ml round bottom flask (Previously dried and weighed accurately up to four decimal places) with 90mls petroleum ether. The flask was dipped in a boiling water bath at 80^0C for at least 6 hours to boil the petroleum ether. The round bottom flask with dissolved fat contents was collected afterwards and placed in a hot air oven (overnight) at 104^0C. Finally, the crude fat percentage was calculated by subtracting the total weight of the flask before and after fat extraction.

3.3.4 Determination of extracted protein yield

The yield of sonicated and unsonicated KSP extracts obtained was calculated according to the method of (Kaushik et al., 2016) as follows:

$$\text{Yield (\%)} = (P/S) \times 100 \qquad \textbf{(Eq. 3.4)}$$

where 'P' is the weight (g) of protein concentrate powder obtained after extraction and freeze drying from kiwifruit seed meal and 'S' is the weight (g) of actual seed meal taken for protein extraction.

3.3.5 Ash content (Dry basis)

The ash content of the samples was measured as per (Chemist, 2005) using a high temperature muffle furnace and porcelain crucibles**.** Prior to analysis, the crucibles were kept in the muffle furnace for one hour at 520^0C and cooled at room temperature in a silica gel desiccator. Approximately one gram of powdered KSP were weighed accurately up to four decimal places in a crucible and the sample was heated using a hot plate under a fume hood for several minutes until there was no visible smoke. The crucibles were kept in a

muffle furnace at 520^{0}C for at least 12 hours and were cooled before getting the final weight.

3.3.6 Color analysis of KSP

Color characterization of KSP concentrates was measured using a Hunter colorimeter, Model D25, fitted with an optical Sensor (Hunter Associates Laboratory Inc., Reston, VA., USA). A glass cell containing freeze dried KSP was placed above a light source which was covered with a white plate, the brightness, indicated by L^* (0 = black, 100 = white) was recorded. Three measurements from three different samples were performed.

3.4 Characterization of KSP

The following section describes the methods used for determining various physicochemical properties of KSP.

3.4.1 Particle size analysis

A mastersizer 3000 [General Purpose (Emulated MS2000 / MS2000E)] was used to determine the mean diameter size of KSP particles with water (pH 7) being the dispersing medium. A 1 % KSP dispersion (w/v) was prepared and poured drop by drop in the mastersizer beaker to determine the Sauter-average diameter d (3, 2) and volume-mean diameter d (4, 3) until the laser obscuration reached between 4 and 20 %. The refractive index of general proteins and water used in the protocol for measurement were 1.46 and 1.33, respectively. The average of ten measurements was calculated as mean particle size diameter of KSP using the mastersizer software.

3.4.2 Scanning Electron Microscopy (SEM) Analysis

To investigate the influence of extracted technique on the structure of the materials and to understand the extraction mechanism, the KSP samples (Sonicated and unsonicated) were visually studied through SEM analysis. The KSP samples were dried in air prior to the sample particles were fixed on the silicon wafer and sputtered with gold to a thickness of about 100 nm. The shape and the surface characters of the samples were observed and recorded on the scanning electron microscope (Quanta-200, FEI Ltd., Holland).

3.4.3 Amino acid analysis

Amino acid analysis was conducted to determine the amino acid compositions of KSP samples. The KSP samples were sent to an external laboratory (Nutrition Laboratory, Massey Institute of Food Science and Technology, Massey University, Palmerston North, New Zealand) for acid stable amino acid profile test (Reference number: TNT21 - 20). All samples were delivered in powdered form.

3.5 Functional properties of KSP

3.5.1 Determination of solubility and pI (isoelectric point) of KSP

The method of (Lawal, 2004) was used for checking the percentage solubility of KSP at different pH conditions with minor modifications. 24 hours before the analysis, KSP fraction [1% (w/v)] was dispersed with distilled water in 200ml glass beakers and the pH was adjusted from 2 -11 using 0.1M HCl or 0.1M NaOH. The dispersion was further stirred at a room temperature for 60 mins using a magnetic stirrer and centrifuged immediately at 4400 × g for 20 mins at an ambient temperature. The supernatant obtained was filtered through Whatman filter paper in order to obtain a clear solution and were analysed for total protein content using the Kjeldahl method. A 5ml sample (supernatant) was used for the Kjeldahl method along with a conversion value of 6.25. The solubility of KSP was determined as the percentage of proteins remaining in the supernatant against the overall protein content in the sample. Eq. 3.5 was used to calculate the % solubility. Furthermore, the pI of KSP was defined as the value of pH with least solubility.

$$\text{Solubility (\%)} = (\text{protein in the supernatant/total protein}) \times 100 \ldots\ldots \quad \textbf{(Eq. 3.5)}$$

3.5.2 Water Absorption capacity (WAC)

Water absorption capacity was determined using the method described by (Gong et al., 2016). 1 g of KSP samples were weighed into 15 mL pre-weighed centrifuge tube. Then 10 mL of distilled water was added in small increments to the tube under continuous stirring with a glass rod. The solution was then allowed to stand at ambient temperature for 30 mins before the tube was centrifuged at 2000 g for 20 min. The tubes were finally carefully decanted, and the weight of tube with sample was accurately recorded. All

analysis was performed in triplicate. WAC expressed as grams of water per gram of sample, was calculated by:

$$\text{WAC \%} = \frac{W2 - W1}{W_0} \times 100 \qquad \textbf{(Eq. 3.10)}$$

Where W_0 is the weight of dry KSP in grams, W_1 is the weight of the tube plus the KSP sample and W_2 is the weight of decanted tube after centrifugation.

3.5.3 Fat absorption capacity (FAC)

The FAC was determined as described by (Gong et al., 2016). 1 g of KSPC was mixed with 10 mL of soybean oil in a pre-weighed 15 ml plastic centrifuge tube. The mixture was vortexed thoroughly with a vortex mixer and allowed to stand at room temperature for 30 mins. The centrifuge tubes were then centrifuged for 6000×g for 15 min, the oil was carefully decanted, and the bottom sediment of the mixture was weighed. The FAC was calculated by formulae:

$$\text{FAC \%} = \frac{F2 - F1}{F0} \times 100 \qquad \textbf{(Eq. 3.11)}$$

Where, F0 is the weight of KSP (g), F1 is the weight of the tube plus KSP (g), and F2 is the weight of the tube plus the sediment (g).

3.5.4 Least gelation concentration (LGC)

The least gelation concentration (LGC) was evaluated using a method of (Wang & Damodaran, 1991). The KSP was dispersed [2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 % (w/v)] in different test tubes with 5 mL of distilled water and was heated at 90 °C for 1 h in a water bath. The contents of a were then cooled under tap water and kept for 5 h at $4 \pm 2\ ^0$C. The least gelation concentration was determined as that concentration when the sample from inverted tube did not slip. Thus, the sample was tapped twice and observed if the sample falls.

3.8 Statistical analysis

All experiments were done in triplicate from 3 independent experiments using a randomized experimental design and values were expressed as mean values ± SD. Data were subjected to simple classification analysis of variance and in pertinent cases to

Tukey's mean comparison analysis. Significance was established at $p \leq 0.05$. Data analysis was performed with the statistical software Minitab 19.

Chapter 4: Structural morphology and chemical composition of KS and KSF

4.1 Introduction

The grinding process is often been applied to seeds and other plant materials because of its nutritional and zootechnical impact. This effect is explained by an increase of the ratio surface/volume of the substrate and by the breakdown of cell walls structures, leading to an increased accessibility to cellular contents such as proteins. As this study is also focused on extraction of one of such cellular component's (edible protein fraction) from kiwifruit seeds, it is crucial to define structural morphology and compositional analysis of kiwifruit seeds, kiwifruit seed meal and defatted kiwifruit seed flour prior to protein extraction. Thus, this chapter tries to present a proximate and SEM assisted optical analysis of various stages of processing KS to KSM and finally KSF. The discussion of results in this chapter helps to understand the compositional aspects of KS which are recommended as crucial for determining seed protein extraction parameters.

4.2 Chemical Composition and SEM analysis of KS, and KSF

Table 4.1 Proximate analysis comparison of kiwifruit seeds (KS) and defatted kiwi seed flour (KSF)

	Moisture%	**Fat %**	**Ash %**	**Protein % (N = 6.25)**	**Carbohydrate (%)**
KS	6.23 ± 0.19%	35.78 ± 0.328 %	3.13 ± 0.004%	13.39 ± 0.24%	41.47%
KSF	6.8 ± 0.14%	2.1 ± 0.004%	1.84 ± 0.008%	11.33 ± 0.045%	77.43%

Proximate results (Table 4.1) indicate that KS are predominantly rich in oil (35.78%) which was in agreement with the previous results from (Cravotto et al., 2011) who reported approximately 40% of crude fat in *Actinidia Chinensis* seeds. Subsequently, the protein content in KS was found to be 13.39 % on dry matter basis which was lower than what

was reported by (Deng et al., 2014) (15.67%). A possible reason for this difference could be due to differences in seed maturation conditions, environmental conditions such as location, temperature, soil conditions, humidity etc. These parameters have been reported in the past to have significant effect on seed components including protein (Amen, 1968; Burstin et al., 2007). In addition, the ash content of KS was 3.13% on dry basis. The results were similar with other oilseeds found in literature, such as flaxseed (3.5%), walnut (2.1%) and peanuts (2.2%) (Barreto et al., 2016).Finally, the carbohydrate content of the seed and flour samples were also recorded by subtracting the overall sum of nutrients from 100%, which indicated a higher presence (41.47%) in KS and (77.83%) KSF before and after defatting.

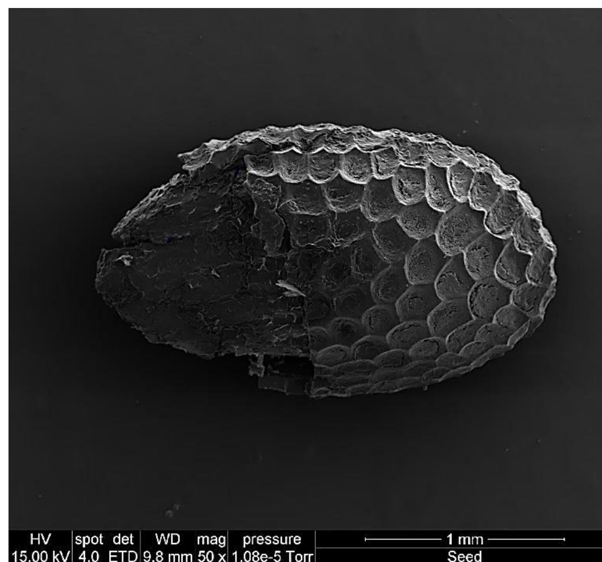

Figure 4.1 SEM image of a Kiwifruit seed. (Magnification x50)

This higher percentages of carbohydrate can be associated with the kiwifruit seed structure (Figure 4.1) which seems to have an ellipsoidal shape with rough and rigid seed coat having sharp angular surface. Thus, it can be assumed that the outer shell is composed of a higher proportion of fiber than other parts of KS which is also common in structurally similar seeds such as red pepper seeds (Firatligil-Durmus & Evranuz, 2010) and watermelon seeds (Wani et al., 2008). Generally, seed coats are difficult to grind and result in larger particles of carbohydrates. Thus, it can be assumed that a higher percentage of carbohydrates has been incorporated in the KSF while milling. Previous studies have also explained that plant

parts containing a high proportion of fibres and sugars (both belonging to carbohydrates) are harder to grind and thus results in larger particles of carbohydrate content in the seed meals and flours (Waiss et al., 2020).

However, as the kiwifruit seeds were milled and defatted, more than 95% drop in oil percentage was recorded via crude fat analysis of the defatted flour (Table 4.1). In addition, a minor decrease in crude protein content (11.33%) and ash content (1.84%) of kiwifruit seeds was also observed indicating a mild reducing impact of milling on the overall nutritional profile of KSF. A possible explanation for the differences in the protein % before and after milling and defatting could also be heat generation while grinding KS in a plate mill, which in turn is likely to lead to aggregation of fiber fragments with oil and protein bodies within KS. This decreasing effect of milling on crude KSP further explains the need to avoid the use of heat induced mills for crushing KS before extracting KSP in future research. As heat was an unavoidable factor involved in using a plate mill, it implies that a small number of proteins in KS had been exposed to heat and have been lost or washed away while milling and defatting. The statement can be backed by the SEM analysis of KSM (Figure 4.2) where large, aggregated particles can be seen which majorly represent a sum of protein, fiber, and kiwifruit seed oil.

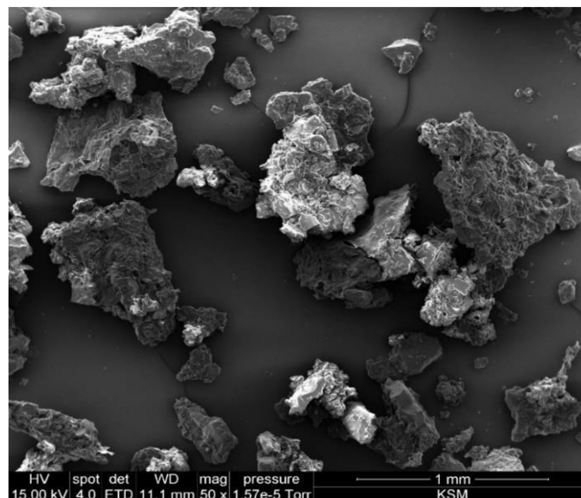

Figure 4.2 SEM image of KSM (Kiwifruit seed meal) showing aggregated starch, fat, and protein bodies. (Magnification x50)

Furthermore, the proximate analysis of flour of kiwifruit seed obtained after defatting consisted of protein fractions (11%) along with ample quantities of carbohydrates. This can also be confirmed from morphological images of KSF where a mixture of fibre and KSP with rough surfaces and small pores was observed at 50x magnification (Figure 4.3). Thus, from the proximate results and visual analysis it can be concluded that the fibers and proteins are grouped to form aggregates in defatted KSF whereas some individual granules of fibers and proteins have been randomly dispersed because of the milling process. In addition, from Figure 4.3 it can also be concluded that most of the oil from the flour was lost as the aggregated molecules were significantly reduced in size.

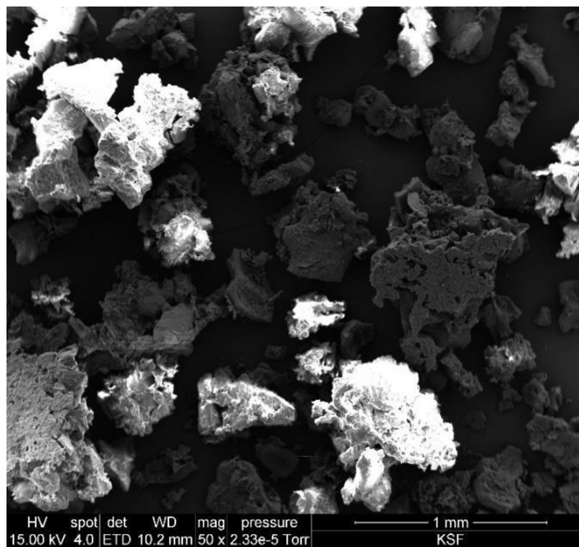

Figure 4.3 SEM images of KSF (kiwifruit seed flour) showing fragmented units of protein and fiber aggregation. (Magnification x50)

4.3 Conclusion

Overall, it can be said that the values are well correlated with those reported in the literature and the initial findings show that kiwifruit seeds have a potential to be used as a plant protein source. The overall protein content in KS can be closely compared with pomegranate seed proteins where similar amount of protein (13.7%) was reported by (Talekar, Patti, Singh, Vijayraghavan, & Arora, 2018).

Chapter 5: Ultrasonic KSP extraction, optimization, and yield

5.1 Introduction

From Chapter 4 it was surmised that adequate amount of edible protein percentage was present in KSF which can be extracted. Thus, in order to achieve the secondary objective of this research and extract as much protein as possible from kiwifruit seeds, this Chapter presents results from an optimized ultrasonic KSP extraction procedure, unsheathed upon five variables: pH, temperature, extraction time, ultrasonic power, and ratio of solvent to KSF. The extraction and statistical analysis were carried out using a Sonicator probe, Minitab 18, and a Box Behnken design of experiment respectively whereas a response optimizer was further used to optimize the results. This chapter aims to highlight effects of significant variables on the extraction yield of KSP and discuss in detail the significance of each variable in the ultrasonic extraction procedure of KSP.

5.2 Optimization of process parameters

Defatted KSF was extracted following 46 combinations of five variables (pH, temperature, flour/solvent ratio, extraction time and ultrasonic power) as indicated by the experimental BBD (Chapter 3). The results indicate effects of five extraction parameters: ratio of seed flour to buffer, pH, extraction temperature, ultrasonication time and ultrasonic power on KSP extraction. The amount of protein acquired in each run are presented in Table 5.1 and the data suggests that percent protein extracted from kiwi seed flour increased from 4.32% to 50.74% under various set of conditions provided. The data of total protein content extracted from each experimental combination was analysed using multiple regression and was fitted to a second order polynomial equation as follows:

$$Y = -0.717 + 0.4018X_1 - 0.00784 X_2 + 0.00053 X_3 - 2.49 X_4 + 0.02156 X_5 - 0.01663 X_1 * X_1 + 0.000014 X_2 * X_2 + 0.000009 X_3 * X_3 - 5.19 X_4 * X_4 - 0.000231 X_5 * X_5 + 0.000175 X_1 * X_2 - 0.000119 X_1 * X_3 + 0.101 X_1 * X_4 + 0.001079 X_1 * X_5 + 0.000020 X_2 * X_3 + 0.0374 X_2 * X_4 - 0.000003 X_2 * X_5 - 0.0328 X_3 * X_4 + 0.000028 X_3 * X_5 - 0.0096 X_4 * X_5 \quad \ldots\textbf{(Eq. 5.1)}$$

Where Y = Total extracted KSP (mg/ml), X_1 = pH, X_2 = power, X_3 = time, X_4 = ratio and X_5 = temperature.

Using the second-order polynomial Equation 5.1, predicted values of Y were obtained and compared with the experimental values . The R^2 (adj) value which represents the total sum of variation in the response factor (i.e., KSP content) due to extraction conditions provided came out to be 97.25% and was not much different from the R^2 (pred) value of 94.21% which indicates adequacy of the applied model (Mizubuti, Júnior, de Oliveira Souza, & Ida, 2000; Sogi, 2003). Moreover, the coefficient of variable (R^2) which is used to determine the overall adequacy of the model came out to be 0.984 for this study. This implies that 98.4% of the variations within the experiment could be explained by the fitted model which were in accordance with previous studies reporting R^2 ranging from 71.00% to 99.20% as an accepted range for model adequacy in the field of DOE. Furthermore, the lack of fit which measures failure of the model to represent data in experimental domain at points which are not included in the regression was also recorded as non-significant (0.127) for the assessed variables. This concludes that this model fitted the data well and therefore is sufficiently reliable for predicting the relevant response.

Further statistical analysis of the coefficients of model revealed that pH, ratio, and temperature were the most relevant factor ($p < 0.005$), both in linear and squared forms which exhibited a significant effect on KSP extraction in this study. The coefficient values of the temperature variable showed a positive value in linear terms and negative in squared terms as shown in Table 5.1, which indicates a quadratic effect of the variable on the response and a parabolic trend of temperature on the KSP extraction. This implies that an increase in temperature positively affected the KSP extraction up to a limit and then seizes the extraction significantly. The ratio variable on the other hand, showed an inverse correlation with KSP extraction, as negative coefficient value was recorded for the ratio variable in both linear and squared terms. This depicts that higher the ratio of KSF to solvent, lower was the protein extraction which can also be visualized from Figure 5.1 where individual effects of variables are demonstrated on KSP extraction.

Table 5.1: KSP extraction results

S.No.	pH	Power (%)	Time (min)	Ratio (g/ 100ml)	Temperature (º C)	KSP extracted* (%)
1	7.5	100	100	12	55	38.82
2	7.5	100	20	12	55	36.20
3	7.5	20	100	12	55	33.09
4	7.5	60	100	4	55	43.13
5	3.5	20	60	12	55	13.48
6	7.5	60	20	12	100	32.17
7	7.5	100	60	20	55	34.59
8	7.5	60	60	4	100	35.07
9	3.5	100	60	20	55	11.52
10	7.5	100	60	4	55	39.44
11	7.5	60	100	20	100	34.09
12	7.5	20	60	20	10	24.30
13	3.5	60	60	12	10	4.76
14	7.5	20	20	12	55	33.01
15	3.5	60	20	12	55	13.50
16	7.5	100	60	12	10	24.84
17	7.5	60	100	12	10	19.43
18	7.5	60	60	20	10	16.53
19	11.5	60	60	4	55	50.74
20	7.5	60	60	20	100	22.70
21	7.5	60	20	4	55	42.41
22	7.5	60	60	12	55	34.87
23	7.5	100	60	12	100	30.03
24	7.5	60	20	12	10	21.60
25	7.5	60	60	12	55	35.81
26	7.5	60	20	20	55	35.20
27	11.5	60	60	20	55	42.69

28	7.5	20	60	12	100	29.94
29	11.5	60	100	12	55	48.34
30	3.5	60	60	20	55	5.074
31	7.5	60	60	12	55	36.61
32	3.5	60	100	12	55	13.50
33	11.5	60	20	12	55	49.86
34	7.5	60	60	12	55	35.24
35	7.5	20	60	4	55	43.97
36	11.5	60	60	12	100	47.59
37	7.5	60	60	12	55	35.30
38	7.5	20	60	20	55	29.54
39	11.5	100	60	12	55	50.63
40	3.5	60	60	4	55	15.717
41	7.5	60	60	4	10	26.12
42	7.5	60	100	20	55	27.52
43	7.5	60	60	12	55	38.51
44	11.5	20	60	12	55	50.35
45	3.5	60	60	12	100	4.32
46	11.5	60	60	12	10	32.49

**Extracted protein % represents the overall protein in the supernatant obtained (mg/ml) divided by the overall protein in the flour sample taken for extraction. All experiments were done in triplicates.*

Table 5.2: Analysis of variance (ANOVA) of the second-order polynomial model for optimization of protein extraction.

Source	Coefficient value	F-Value	P-Value
Linear	1.8031	269.52	0.000*
pH	0.9089	1188.89	0.000*
power	0.0262	0.99	0.329
time	-0.0189	0.52	0.479
ratio	-0.2586	96.27	0.000*
temperature	0.2057	60.92	0.000*
Square	-0.2660	48.00	0.000*
pH*pH	0.0223	55.57	0.000*
power*power	0.0144	0.39	0.538
time*time	-0.0332	0.16	0.690
ratio*ratio	-0.4685	0.87	0.361
temperature*temperature	0.0280	172.29	0.000*
2-Way Interaction	-0.0191	2.53	0.029
pH*power	0.0324	0.28	0.601
pH*time	0.1943	0.13	0.720
pH*ratio	0.0318	0.38	0.545
pH*temperature	0.1197	13.58	0.001*
power*time	-0.0055	0.36	0.552
power*ratio	-0.1051	5.16	0.032
power*temperature	0.0512	0.01	0.918
time*ratio	-0.0347	3.97	0.057
time*temperature	1.8031	0.94	0.341
ratio*temperature	0.9089	0.43	0.517

(*) = Terms with significant effect ($p < 0.005$)

The results from ANOVA also revealed that the interaction between pH and temperature exhibited a significant effect on protein extraction which highlights two basic things in this experiment. Primarily it can be implied that these factors modulate the response oppositely, that is, when pH and temperature are increased simultaneously, the individual effects of these parameters on protein extraction are canceled. Secondarily it also underlines pH as one of the most influential variables in KSP extraction as the significance and the high coefficient values for the linear and squared terms of pH, demonstrates its positive and relevant correlation in this study. A similar effect between pH and temperature on protein extraction from watermelon and *Prosopis cineraria* (L.) druce seeds was also observed by (Wani et al., 2008) and (Garg, Chakraborty, & Gokhale, 2020), respectively where a canceling effect on proteins was observed when pH and temperature of the experiment were raised simultaneously.

5.3 Effect of pH on ultrasonic extraction of KSPC

In Table 5.2 the extracted protein percentage was notably increased as pH of proteins gets towards extreme alkaline conditions (>10). However, at acidic pH extreme inverse effects on KSP extraction were observed as the concentration of protein gets minimized critically in acidic conditions. This can be explained by solubility of a protein in a solvent, which is a function of its pH and is generally least at its isoelectric point (pI) where the net charge on proteins is zero. Therefore, the values of KSP showing least extraction at lower pH demonstrates an increase in hydrophobic interaction within KSP, which would have possibly resulted in aggregation of KSP molecules. Whereas the alkaline pH would have increased the net negative charge of the carbonyl groups, amino acids, and side chains of acidic amino acids in KSP making them highly dissociable in the solvent with a net negative charge on the surface. Therefore, the dissociation of acidic groups within the extracting buffer would have increased, positively affecting the extraction of KSP at higher pH. However, along with the pH, ultrasonic treatment also contributes to enhance the mass transfer and leads to increase in the rate of extraction which should also be considered here.

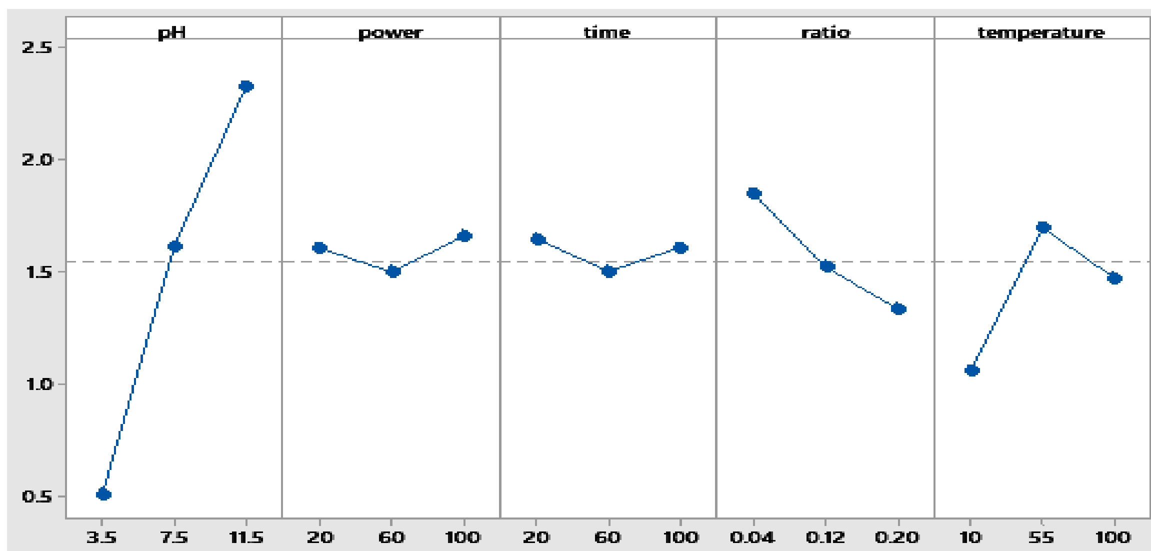

Figure 5.1 Main effects plot for KSP conc. (mg/ml) against experimental variables

5.4 Influence of temperature on ultrasonic extraction of KSPC

In Figure 5.1, a drop in extracted protein concentration was recorded when the temperature goes beyond 55^{0}C. This confirms the parabolic trend of temperature on KSP extraction as discussed in the previous section of this chapter and explains the interactive effects of pH and temperature. It could be since higher temperature initiates denaturation of proteins, which contributes to lowering in extraction yield. The reason behind the changes is unfolding protein or the breaking of hydrophobic bonds at lower cavitational effect followed by breaking strong bonds at the higher cavitational effect that occur by low-power sonication for a long time. However, along with denaturation, another variable which contributes to lowering extraction is effect of temperature on sonication. This can be represented as, at lower temperatures limited bubbles are formed, but they collapse with comparatively high intensity which enhances the cell disruption. Thus, at a temperature around 50 °C the KSP extraction was relatively higher since low temperature offers a better sonication effect and bubble collapse with higher intensity. Moreover, it can also be stated that vapor pressure of the solvent increases with an increase in the temperature and more solvent vapor fills the cavitation bubbles which collapse with less intensity resulting in a reduction in cavitation. Thus, at higher temperature (>50^0), surface tension was decreased, which might have affected the bubble formation and collapse which generated bubbles

collapse with low intensities eventually resulting in overall decrease in the mass transfer rate.

5.5 Effect of ratio on KSPC extraction

Figure 5.1 showed that as the ratio of the solvent was increased, the concentration of KSP was increased. However, increasing the ratio of solute degraded the overall protein concentration because of limited solvent available for the extraction. The higher amount of solvent offers a greater driving force for the mass diffusion during the extraction process. It has been found that an increase in solvent ratio, extraction yield has enhanced linearly due to greater availability of the solvent which gives a larger protein concentration difference between the protein present in the solid and in the solvent phase. However, as the amount of solvent was increased (4:100 gm/ml), the accessibility of the solvent for the extraction was increased thus the extraction was higher (Bird, 2002). Furthermore, sonication could also have incited the formation of cavitation bubbles in the sample and resulted in cell breakage which in return would have increased the porosity of KSF. Thus, it can be visualized that intracellular material of KSF was easily interacted with the phosphate buffer, facilitating the extraction of KSP in this experiment.

5.6 Response surface methodology

The fitted polynomial equation 5.1 was plotted as three-dimensional response surfaces (Figure 5.2 a, b, and c) to visualize the relationship between experimental levels of evaluated factors and response in this study. Effects of two independent variables on protein content are shown, while the others are maintained at their respective central levels (center value of the testing ranges). The highest extraction percentages in each figure is represented as the white area in the given 3D figures.

It is evident from Figure 5.2a that pH significantly enhanced the amount of extracted protein. The maximum protein extraction was observed when pH of the sample was above 10 and temperature was below 60^0C. It can also be noted here that neither temperature, nor pH exerted a linear effect on the response. Thus, it can be said that a combined effect of pH and temperature modulated the response in a positive way. However, it was interesting

to note from Figure 5.2 that despite lower temperatures, the protein extraction at alkaline pH values was more than 50%.

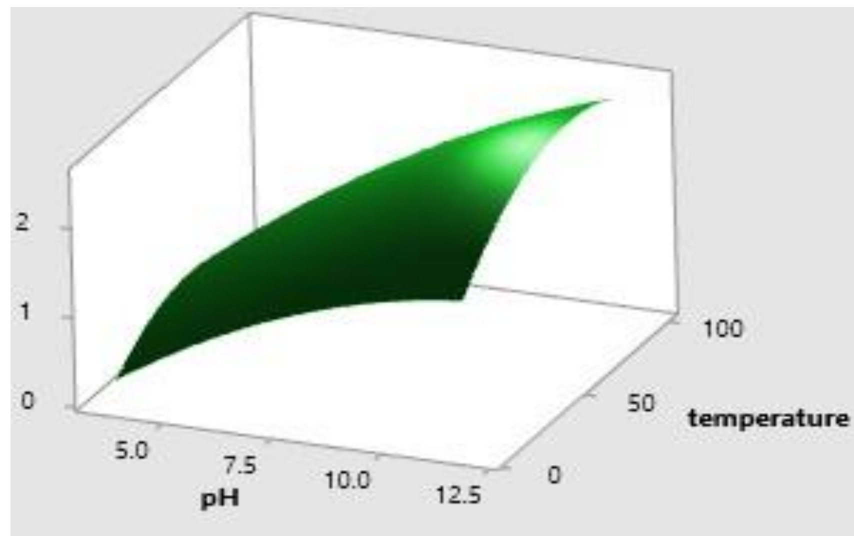

Figure 5.2a Response surface plot for the effects of temperature and pH on protein extraction from KSF.

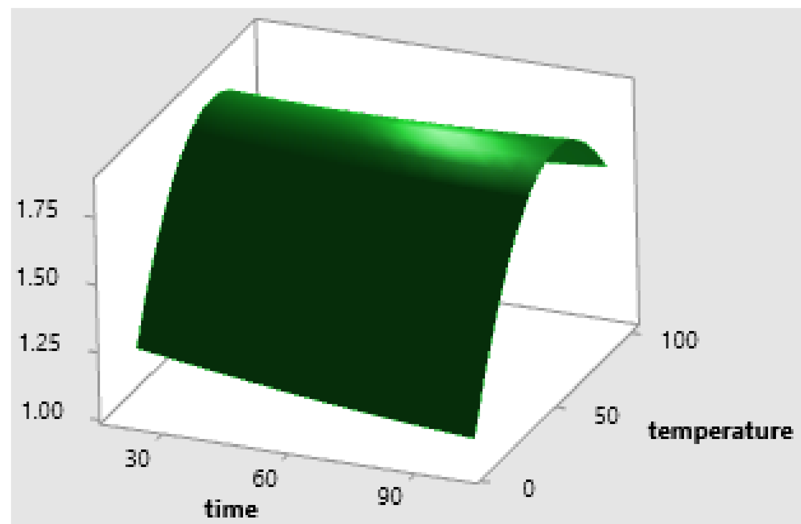

Figure 5.2b Response surface plot for the effects of temperature and time on protein extraction from KSF.

The effects of extraction time and temperature on KSP extraction at pH 7.5, power 60 and ratio (w/v) 12:100 is shown in Figure 5.2b. The results indicated that extraction time displayed a linear effect on KSP extraction, and the amount of extracted protein slightly decreased with an increase in extraction time. However, temperature demonstrated a

quadratic effect on the response; hence extracted protein increased up to about 55^0C, followed by a decline with its further increase. As time was previously noted as a non-significant variable, it was obvious that there was no significance between the percentage protein drop at higher temperature and an increase in extraction time. This can also be stated in a way that despite what the extraction time for the experiment was, at a temperature range above 50^0C a significant increase in the extraction percentage of KSP was observed.

In the following Figure 5.2c interactive relations between ratio and temperature are displayed on KSP extraction. The pH, temperature and power were maintained at values 7.5, 60^0C and 60 respectively. The ratio variable as explained in the previous sections as well, displays a negative trendline for protein extraction when is considered as a factor alone. However, as the temperature was increased in the experiment along with lower ratio values, an increase in the protein extraction was observed. Maximum protein extraction was noted when temperature was ranged from 50 to 80^0C and the ratio was below 6gm of KSF per 100ml of the solvent (Fig. 5.2a).

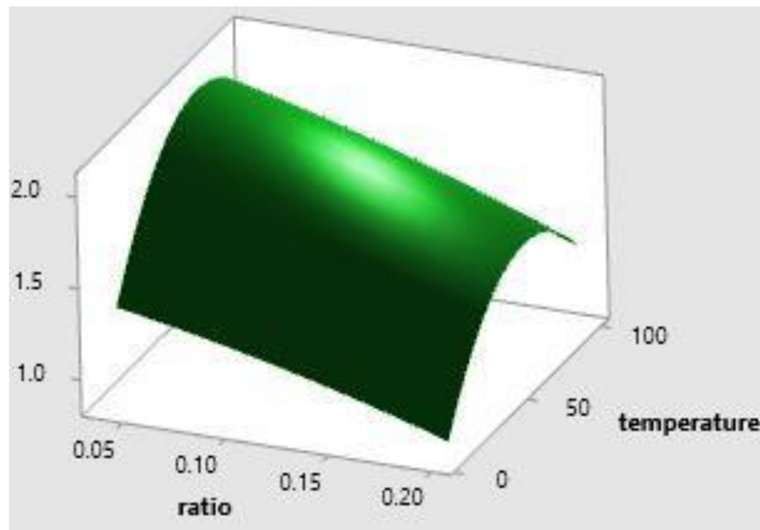

Figure 5.2c: Response surface plot for the effects of temperature and ratio on protein extraction from KSF.

5.7 Optimal extraction conditions

Optimum conditions for KSP extraction from defatted KSF following 46 trials of protein extraction were obtained and are presented in Table 5.3. Response optimizer (Minitab 18) was used as a tool to analyze the maximum response of each variable and conditions. The response optimizer results predicted that if the ratio of KSF to solvent is (4:100), pH of the solvent is 11.5, Ultrasonic power is maintained at 20%, extraction time is 100 mins and temperature 55°C, 0.9mg protein concentrate per ml of the supernatant can be extracted of the KSF. The suitability of the predicted optimum response was further tested by additional independent experiments using the recommended optimum conditions and results have been tabulated in Table 5.3 as extracted protein concentration. The results indicated that the experimental protein value (0.90 mg/ml) was not significantly different from the predicted protein value (0.92 mg/ml) which further validates adequacy of the experimental model presented in this study.

Table 5.3 Optimized parameters for ultrasonic extraction of KSP

Ratio (g/ml)	pH	Temperature (°C)	Ultrasonic power	Time (min)	Extracted protein concentration (mg/ml)	Predicted protein concentration (mg/ml)
4/100	11.5	55	20	100	0.90	0.92

5.8 Extraction yield

KSP was also extracted as per (Deng et al., 2014) using conventional stirred extraction method in order to compare the extraction yield with ultrasonically extracted KSP. It was observed that the ultrasound-assisted extraction gives 32.5% extraction yield of KSPC as against 21.5% using conventional stirred extraction process. This can be attributed to thermal and mechanical influences induced by sonication, which caused cell disruption and opening of pores in the kiwifruit seed meal used in this study (Figure 5.3A). It can be imagined that sonication subsequently resulted in the increase in the rate of mass transfer and improvement in contact frequency in the release of protein. Therefore, the results

proved that ultrasonic extraction could improve the protein yield from kiwifruit seed meal if compared with the conventional stirred extraction technology.

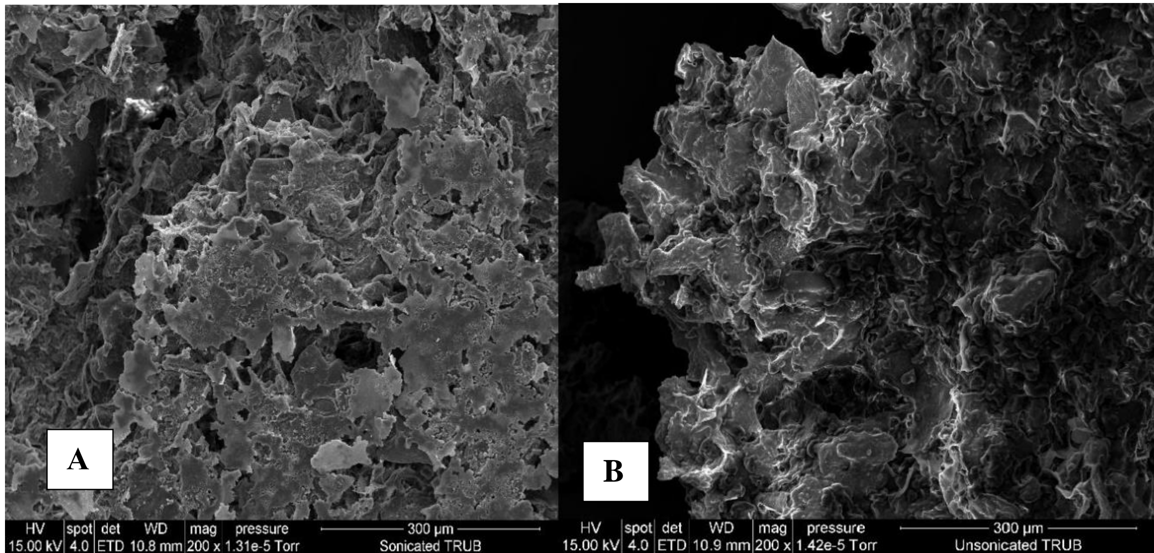

Figure 5.3: SEM images of KSF residue after protein extraction. (A) Using ultrasound (B) Conventional stirred extraction

5.9 Conclusion

In this chapter, the response surface methodology was successfully deployed to analyze critical extraction parameters of kiwifruit seed. The results show that ultrasound extraction significantly increased the overall protein yield from the defatted seed flour by 12% if compared to the conventional method. The optimized extraction parameters for the ultrasonic extraction of defatted *Actinidia Chinensis* seed meal were pH 11.5, temperature 55^0C, ratio of seed flour to solvent (4:100) and 100 mins of ultrasonic extraction. pH was identified as the most significant factor involved in successful extraction of proteins from kiwifruit seeds whereas temperature and ratio were respectively significant factors after pH. Moreover, the results also demonstrate that the extraction time displayed a linear effect on KSP extraction, and the amount of extracted protein slightly decreased with an increase in extraction time whereas temperature demonstrated a quadratic effect on the response; hence extracted protein increased up to about 55^0C, followed by a decline with its further increase.

Chapter 6: Physicochemical and functional properties of KSPC

6.1 Introduction

This chapter includes results of characterizable properties of the novel KSPC. The main aim of presenting this chapter is to understand the functional properties of KSP and discuss how it could possibly be utilized in the food industries by comparison with other major oilseeds in market today. The functional properties explored in this chapter are effect of pH on solubility, water holding and oil binding capacities, and the least gelation capacity of KSPC. Moreover, this chapter also compares the KSPC obtained via ultrasonic extracted KSPC with conventional stirred extraction method which help to understand the effects of sonicated extraction on different functional properties of the novel protein.

6.2 Characteristic properties of KSPC

6.2.1 Compared chemical composition and colour characterization of KSP extracted using two different methods.

Table 6.1 Proximate analysis comparison of KSP extracted with two different methods

	Moisture	Crude Fat	Ash	Crude protein	Carbohydrate (subtracted from 100)	Color		
						L^*	a^*	b^*
KSPC (ultrasound)	4.23 ± 0.09%	1.13 ± 0.002 %	0.02%	62.1 ± 0.14%	32.5%	82.5	2.5	17.52
KSPC (conventional extraction)	4.12 ± 0.034%	1.18 ± 0.007%	0.07%	55.8 ± 0.24%	38.83%	81.1	1.2	18.26

The given chemical composition of KSPC in Table 6.1 was obtained via two different extracting procedures as described in Chapter 3. The results indicate that the protein concentrate obtained via ultrasonic extraction of KSF displayed a higher percentage of crude protein (62.1%) if compared with the solvent extracted KSPC (55.8%). Moreover, a

significant presence of carbohydrates (>30%) and approximately 1g of fat in both protein concentrates was seen apart from 4% average moisture and negligible ash traces. When ultrasound is utilized to assist with the extraction of protein, the process results in denaturation of protein based on sonication power and treatment times employed. The time and power deployed to extract protein from kiwifruit seed meal in this experiment as indicated by optimized parameters were high time low power. This could be a possible explanation for an increased protein content in sonicated KSPC as it has also been widely published that a high time, low power ultrasonic extraction has increased protein extraction from plant materials due to an enhanced unfolding of protein or the breaking of hydrophobic bonds at lower cavitational effect followed by breaking strong bonds at the higher cavitational effect that occur by low-power sonication for a long time (Mir, Riar, & Singh, 2019).

The colour parameters of KSPC are also shown in Table 6.1 comparing two protein concentrates obtained via different extraction procedures. From results it can be deduced that ultrasonic KSPC was whiter having larger values for L^*(82.5%) than the solvent extracted sample (81.1%). Whereas the yellowness (b^* values) and redness of the sample (a^* values) were found to be higher in the conventionally extracted sample (18.26). The values justify the fact that ultrasonic KSP was lighter in colour than the conventionally extracted KSP thus a significant effect of ultrasonication on whiteness index of KSPC was confirmed. In food industries higher values of whiteness index of a protein makes it preferable for use as a food component as it can be added to foods without significant alteration of the colour of the foods. The results were in correlation with (Chittapalo & Noomhorm, 2009) where ultrasound-assisted extraction of protein from defatted rice bran showed that unsonicated protein concentrate was darker and more reddish-yellow than sonicated one.

6.2.2 Amino acid analysis of KSPC

Table 6.2 compares the essential amino acid composition of KSP with standard protein requirement guidelines of World Health Organization (WHO) for adults and a few other major oilseeds. Among the 10 essential amino acids involved, Leucine (0.72), lysine (0.71) and valine (0.70) appeared to be the most abundant essential AAs found within KSP.

Whereas tryptophan (0.14) was regarded as the least available essential AA. Moreover, tyrosine (0.51) and isoleucine (0.46) were the second and third limiting essential AA followed by Sulphur amino acids such as Cystine and Methionine which were only 0.25 and 0.34 g/100gm of KSP concentrate. Furthermore, KSPC was also found to be rich in Glutamic acid (2.25 g/100 g) followed by arginine (1.33 g/100 g), aspartic acid (1.05g/100g), glycine (0.97g/100g) and proline (0.79g/100g) respectively which represents a typical amino acid profile of plant seed proteins. Similar glutamic and aspartic contents were also observed by (Yuan et al., 2009) for soy glycinin polypeptides.

The overall sum of essential AAs in KSP was 4.64g/100g and it can be interpreted that amino acid profile of KSP is balanced which contains all the essential AA. However, if compared with other oilseeds it can be clearly stated that essential amino acid composition of KSP was the least. All the values of amino acid composition of KSP were found to be good agreement with other oil seeds reported. If compared with other oilseeds KSP had most similar AA profile as flaxseed protein whereas the sunflower protein had second most similar essential AA profile to KSP. It was also interesting to note here that the sulfur amino acid levels of Kiwifruit seeds, flaxseed and Sunflower seed proteins looks similar despite the difference in their overall protein content. Thus, it can be said that KSP has competitively equal or greater ratio of sulfur amino acid as found in other oilseeds.

Essential amino acids in oil seeds contribute to good health and wellbeing. For example, a deficiency of lysine could possibly lead to physical and mental handicap (Papes, Surpili, Langone, Trigo, & Arruda, 2001) whereas arginine is proven to be beneficial for prevention of cardiovascular disease (Balasubramanian, Seetharamulu, & Raghunathan, 1980). Similarly, Valine promotes mental vigour, muscle coordination and calm emotions and Lysine safeguards appropriate absorption of calcium in a human body which not only help in the formation of collagen, but also aids the production of antibodies, hormones, and enzymes. On the other hand, Lysine deficiency may result in tiredness, inability to concentrate, irritability, bloodshot eyes, retarded growth, hair loss, anaemia, and reproductive problems. The results obtained in this study proves that amino acid profile of KSP contained all the essential as well as non-essential amino acids hence, KSP can be considered as a cheaper source of nutritional proteins for infants as the available AAs are

low as compared with WHO profile for adult requirement of EAAs. Moreover, as KSP also contains equivalent amount of lysine which many legumes and beans lack. Therefore, it can also be stated that KSP can be combined with various legumes or beans to formulate fortified foods for children or infants. Improvement in nutritional quality of KSP can also alternatively be achieved by mixing them with other plant proteins that are rich in sulphur-containing amino acids.

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Table 6.2 Essential amino acid comparison of KSP and major oilseeds [g/100g]

Essential AA	Standard (WHO)	Kiwifruit seeds (12% protein)	Flaxseed (brown) (18% protein)	Sunflower seeds (25% protein)	Hemp seeds (24.2% protein)	Rapeseed meal (19% protein)
Ile	3.01	0.46	0.87	0.92	1.29	1.25
Leu	5.30	0.72	1.18	1.40	2.16	2.51
Lys	4.50	0.71	0.75	0.86	1.28	2.04
Met	2.21	0.34	0.32	0.53	0.93	0.47
Cys		0.25	0.32	0.38	0.67	0.59
Phe	3.81	0.45	0.95	1.05	1.45	1.44
Tyr		0.51	0.53	0.57	1.26	0.99
Thr	2.30	0.36	0.72	0.81	1.27	1.59
Trp	0.61	0.14	0.30	0.35	0.37	0.43
Val	3.90	0.70	1.07	1.11	1.78	1.55
AA sum	25.64	4.64	7.01	7.98	12.46	12.86
References	(WHO, 2007)		(Klimek-Kopyra, Zajac, Micek, & Borowiec, 2013)	(Danish Food Composition databank, 2019)	(Map, 2012)	(Mejicanos & Nyachoti, 2018)

6.2.3 Surface morphology of KSPC (SEM analysis)

The surface morphology of extracted protein concentrates was examined using SEM as shown in Fig. 6.3 (A) and Fig. 6.3(B). The surface of KSPC extracted using ultrasound had a flaky texture and a rough surface at first glance. The protein concentrate was fragmented into small evenly sized fragments along with irregular aggregates. Since the chemical composition of KSPC defines protein and carbohydrate as the most abundant macromolecules within KSPC, it could be assumed that the observed aggregated particles by SEM are aggregates of KSP and carbohydrates of KSF. However, if compared with the conventional stirred extracted KSPC (Figure 6.3b), the aggregates were absent in the later; despite a smooth surfaced protein network was confirmed with minor fractures and pores.

The aggregates shown in fig 6.3A and absence of aggregates in Fig. 6.3B could be broadly attributed to low intensity ultrasonic sound waves used for higher time periods (100min) in this study. Similar aggregates were also reported by (Hu et al., 2013) who visualized the morphology of soy protein isolates treated with ultrasound waves and found that longer exposure time even with lower ultrasonic power resulted in aggregates due to exposing more free SH groups that reacted with themselves or underwent oxidization. Moreover, it has also been widely stated that with increasing power in ultrasonication, the protein structure becomes relatively smaller, and particle size distribution broadens due to microstreaming and higher turbulent forces of ultrasonication (Resendiz-Vazquez et al., 2017). This could explain the shear effect of ultrasonication which may have created smaller particle and more free SH groups within available KSPC that react with themselves or are oxidized to make aggregates. Thus, could be a possible explanation to difference in the SEM structures of the two KSPC powders shown in the figure below.

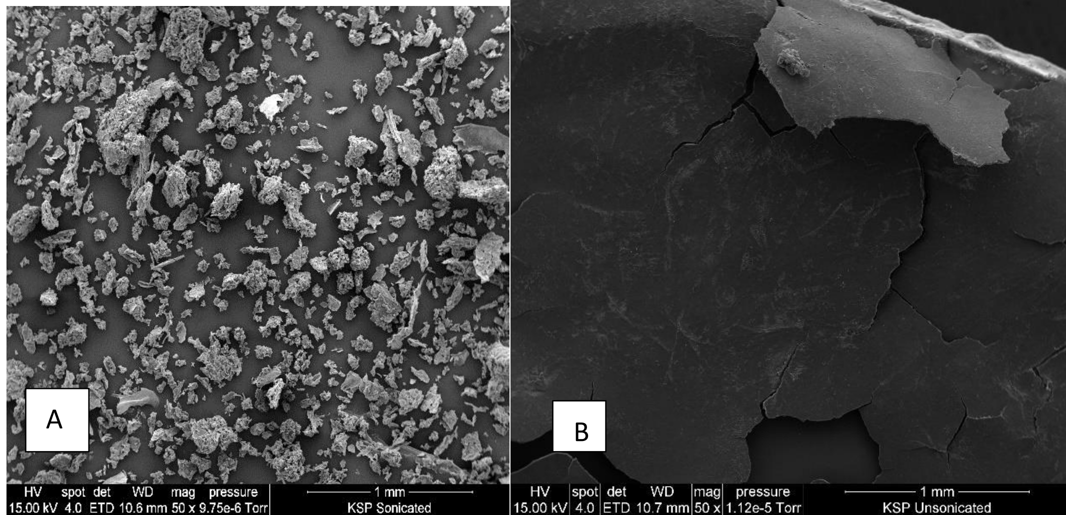

Figure 6.3 A. 50x mag SEM image of KSPC powder extracted and freeze dried using ultrasonic extraction process; B. 50x magnified SEM image of KSPC extracted using conventional stirring method.

6.3 Functional Properties of KSPC

6.3.1 Solubility profile

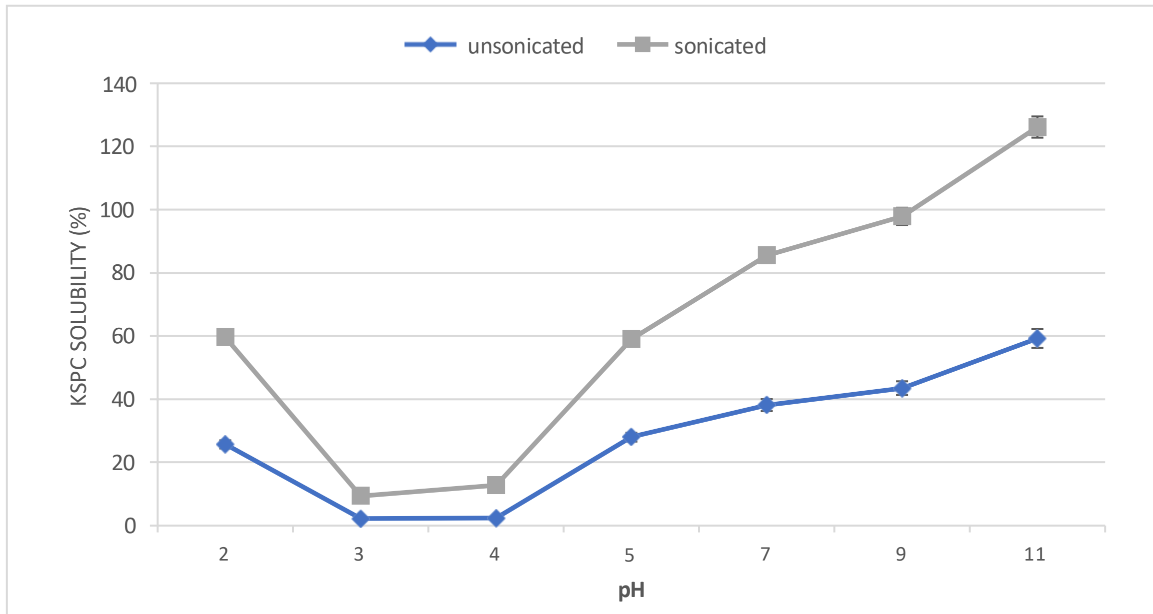

Figure 6.4 Protein solubility profile of Actinidia deliciosa seed protein concentrate extracted by ultrasonication and conventional stirred extraction method as affected by pH. Error bars represent the standard deviation of three replications.

Effects of pH on the solubility profile of two kiwifruit seed protein concentrates (sonicated and non-sonicated) are presented in figure 6.3. The values of solubility showed a typical oilseed profile for KSPC which was high at alkaline values (10-11) and least at acidic pH (3). Several other researchers have shown that most plant seed proteins generally have the least solubility at acidic pH values which is also an ideal range of their isoelectric points (3-5) (Bera & Mukherjee, 1989; S. Jadhav, Lutz, Ghorpade, & Salunkhe, 1998; Otegui et al., 1997). Thus, it can be predicted that isoelectric point of KSP is around pH value 3 -4 which was also in agreement with the isoelectric point of KSP reported by (Deng et al., 2014).

Moreover, solubility of KSPC extracted via ultrasonic extraction was found to be significantly more soluble ($p<0.05$) than the KSPC extracted via solvent extraction at higher pH. This increase in solubility can be attributed to an increase in protein-water interaction formed by the partial unfolding of KSP molecules while high intensity ultrasound was applied for protein extraction. The effects of ultrasound treatment are related to high temperature and pressure formed by acoustic cavitation, fragmentation, and shock waves which induce unfolding of proteins, non-covalent bond damage, secondary and tertiary structure changes in protein molecules (Kadam, Tiwari, Álvarez, & O'Donnell, 2015), (Shirsath, Sonawane, & Gogate, 2012). These changes in the physical and chemical structure would have unfolded the KSP orienting the charged (NH_4^+, COO^-) and hydrophilic groups inside of protein towards water which would have contributed to the protein-water interactions resulting in better solubility (Moulton and Wang 1982). Moreover, high pressure and fragmentation phenomenon associated with ultrasonic extraction are known for reduction of particle size and intermolecular bonds cleavage which promotes the protein-water interactions with a larger protein surface area (Arzeni et al., 2012; L. Jiang et al., 2014), thus an exposure of charged (NH_4^+, COO^-) and hydrophilic groups inside of protein can contribute to protein-water interactions resulting in better solubility. In agreement with this finding, (Malik, Sharma, & Saini, 2017) reported that the solubility of sunflower protein isolates increased linearly with increasing treatment time of high intensity ultrasound. Moreover, (Nazari, Mohammadifar, Shojaee-Aliabadi, Feizollahi, & Mirmoghtadaie, 2018) reported a similar effect of ultrasound treatments on the solubility of millet protein concentrate. Similar results were also found in ultrasound

applied for chickpea protein isolates (Wang et al., 2020), perilla seed protein isolates (Zhao et al., 2022), fava bean protein isolates (Martínez-Velasco et al., 2018) and black bean protein isolates (L. Jiang et al., 2014).

6.3.2 Water holding capacity (WHC)

WHC values of KSPC at a neutral pH 7 (sonicated samples and solvent extracted samples) are compared in the Figure 6.5. The values suggest that the protein samples extracted using ultrasonication exhibited significantly larger ($p<0.05$) hydration capacity i.e., 0.93mL of H_2O retained per gram of KSPC than the hydration capacity of unsonicated samples (0.74 mL/g) following application of mild centrifugal force. The values if compared to some major oil seeds such as rapeseed protein isolate (0.99 mL/g), sunflower seed protein isolates (1.38 mL/g) and Soybean commercial concentrate (*Promosoy*) (1.96 mL/g) reveal that KSPC has notably equal amount of water absorption capacity at neutral pH (Moure et al., 2006). However, the lower values of WHC in KSPC can be further related to the "protein concentrate" used in this study which generally exhibit poor water-binding capacity compared to that of the isolates likely because protein isolates have greater ability to swell, dissociate and unfold than the protein concentrates (Kinsella, 1982). Thus, it can be visualized that when KSPC was dissolved in water, the carbohydrate and other components of the protein concentrate impaired the overall exposure of enough binding sites for hydrogen bonding with water molecules. However, it can also be noted here that the increase in WHC for KSPC treated with ultrasound might have attributed to unfolding of the polypeptide chain within KSP, which might have exposed more hydrophilic groups to the water molecules than KSPC extracted with stirred extraction.

In food industries today water retention properties are of high importance in formulated food products where a rate of water retention by a protein determines whether it should be added as powder or rehydrated before the addition to the mixture. The information related to protein powder-moisture interaction is also deployed in developing kinds of packaging materials necessary to maintain the required moisture content in the product.

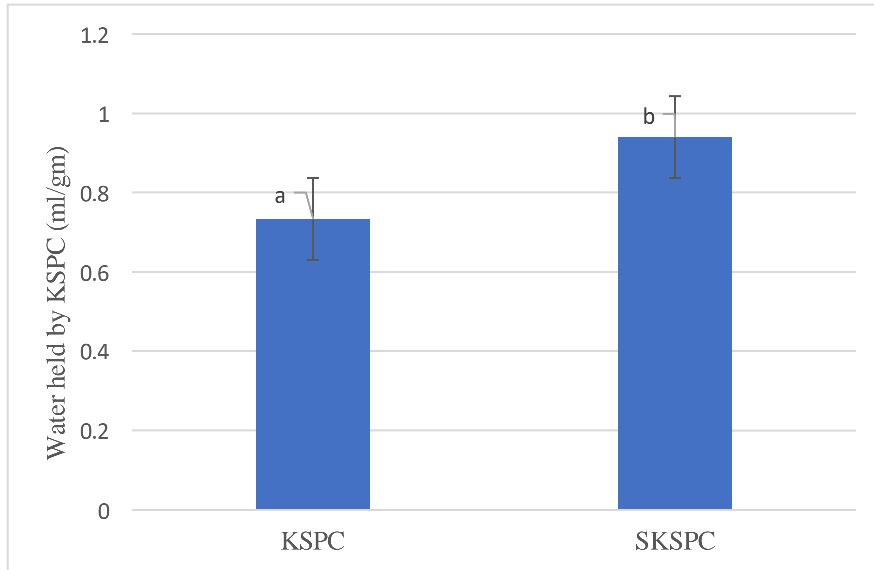

Figure 6.5 Water holding capacity of sonicated (SKSPC) and conventionally extracted kiwifruit seed protein concentrates (KSPC) at pH 7. Different letters indicate a significant difference (p<0.05). Error bars represent the standard deviation of three replications.

6.3.3 Oil binding capacity (OBC)

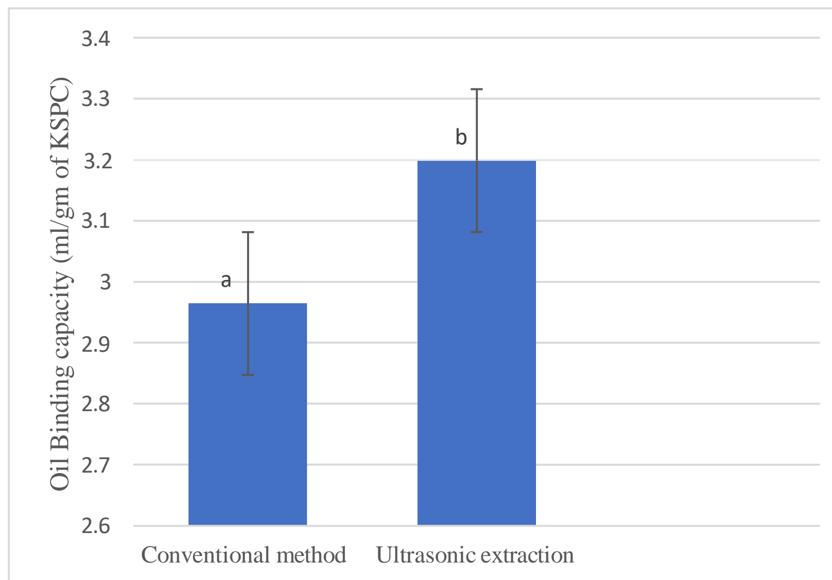

Figure 6.6 Oil binding capacity of conventionally extracted and ultrasonically extracted kiwifruit seed protein concentrates at pH 7. Different letters indicate a significant difference (p<0.05). Error bars represent the standard deviation of three replications.

The results obtained for the oil holding capacities in the present study shows (Figure 6.6) that the OHC of the ultrasonically extracted protein concentrates were significantly higher as compared to that of conventionally extracted KSPC. Generally, the major chemical components in proteins are composed of both hydrophilic and hydrophobic components which are known to significantly affect the oil holding capacity (Malik, Sharma, & Saini, 2017). In this study, the reason for increase in OBC of the protein concentrates might be attributed due to unfolding of the polypeptide chains as well as conformational changes in the protein structure which are caused by the collapse of the cavitation bubbles near the protein molecules due to ultrasonication (Biswas & Sit, 2020). It could be thus deduced from results that these conformational changes might have exposed the non-polar hydrophobic side chains of amino acids present in KSP, which in turn would have increased binding of the oil molecules to the protein molecules (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). Moreover, the variation in the OBC of ultrasonically extracted samples could also be due the different degrees of unfolding of the polypeptide chains, which is a function of both intensity of ultrasonication as well as time of treatment.

Since oil holding capacity is of significant importance in improving the mouth feel of foods and acts as flavor retainer, it is considered very important property of proteins for incorporation in various food formulations. Knowledge of oil holding capacity (OHC) of proteins is important as it is directly related to the emulsifying properties of the proteins. Stability of the emulsion as well as the oxidative stability of the fats or oils present in a food system is dependent on the binding of the fats or oils to other components of the food system particularly protein.

6.3.4 Least gelation concentration (LGC) of KSPC

As shown in Table 6.3, the ultrasonic extraction improved the gelling capacity of KSPC with a lower LGC from 18% to 14%. (Resendiz-Vazquez et al., 2017) reports that ultrasound treatment (20 kHz; 15 min) reduced the LGC from 10% to 4% in jack fruit seed protein by increasing solubility which is known to contribute to the 3-dimensional network structure of protein gels. Moreover, the exposure of the buried hydrophobic groups and the sulfhydryl groups by cavitation effect also results in the thermal aggregation of the protein by forming disulfide, hydrogen, hydrophobic, and Van der Walls bonds which results in a

robust and lower concentration gels (Arzeni et al., 2012). In our study, a significantly higher solubility profile of UKSPC is potential evidence of increased hydrophobic bonds within KSP to form a gel with less protein concentration. Thus, it can be stated that, partial denaturation and reduced particle size due to ultrasonic extraction increased the flexibility of KSP could be required for the successful formation of 3- dimensional gels in UKSPSC which eventually resulted in a lower LGC value.

Table 6.3 Least gelation capacity of KSPC in H_2O. The (+) signs indicate a strong gel formed whereas (-) sign indicates fluid state. UKSPC stands for ultrasonic extracted kiwifruit seed protein whereas, CEKSPC stands for the conventionally extracted kiwifruit seed protein concentrate.

% KSP IN H_2O	UKSP	CEKSP
2	-	-
4	-	-
6	-	-
8	-	-
10	-	-
12	-	-
14	+	-
16	+	-
18	+	+
20	+	+

The results also indicate that UKSPC had least gelation concentration at around 14% which was comparable with other major oilseeds such as soy protein (14%) and mung bean protein (12%) (Ismail-Fitry et al., 2017). (Ruiz, Xiao, van Boekel, Minor, & Stieger, 2016) reports that extraction in extreme alkaline pH (>pH 10) had significant negative effects on the heat-induced gelation ability of quinoa proteins due to the formation of insoluble large aggregates resulting in a weaker tendency to form a network (Ruiz et al., 2016). A possible reason for this could again be the partial unfolding of the protein structure and active groups exposure at higher alkaline pH. At higher pH protein-protein interactions are facilitated during the formation of gel, particularly at the beginning stage of heating (Jarpa-Parra et al., 2014). A strong alkaline (>pH 10) might cause hydrolyzing native proteins into fractions with lower molecular weights, where the low molecular fractions associate

together through hydrophobic interactions and intermolecular disulfide bonds and form insoluble aggregates (Ruiz et al., 2016; Valenzuela, Abugoch, Tapia, & Gamboa, 2013)

Protein gelation is essential in food products such as pudding, jelly, desserts, and meat formulations. LGC can determine the gelling concentration in a 3-dimensional network structure formed by covalent and non-covalent bonds to form a self-supporting gel. Several factors influence gel formation, such as protein size, structure, flexibility, intermolecular interactions, hydrophobic and structural distribution of other amino acids (Wang & Damodaran, 1991).

6.4 Conclusion

The main ideas and results from this chapter can be summarized as following.

- The protein concentrate obtained via ultrasonic extraction of KSF displayed a significantly higher percentage of crude protein (62.1%) if compared with the solvent extracted KSPC (55.8%).
- An acidic profile for *Actinidia Chinensis* seed protein amino acids was revealed in this study, where glutamic and aspartic acid were the dominating amino acids. The most abundant essential amino acids were Leucine, Lysine and Valine respectively and the least available EAAs were the sulphur AAs such as Cysteine and Methionine.
- The basic functional properties of KSPC obtained in this study indicates that water hydration capacity (0.93g/ml), oil binding capacity (3.8g/ml) and least gelation concentration (14%) of the seed protein concentrate used in this study was comparable with rapeseed protein, flaxseed, and soy protein concentrates. Specifically, the oil/fat binding capacity of *Actinidia Chinensis* seed protein concentrate was found superior to most of the oilseed proteins compared followed by the least gelation capacity. This defines that Actinidia Chinensis seed proteins have credible potential to be utilized for food industries to change the organoleptic properties of foods. Moreover, aforementioned functional properties of KSPC were also compared based on their extraction procedure i.e., ultrasonic, and solvent extraction procedures. The results confirm significant increase in functional properties when ultrasonic extraction procedure was used.

Chapter 7: A general discussion and avenues for future work

7.1 Introduction

Novel plant proteins are one of the hot topics of the decade and scientific community are finding new sources of proteins every day. Protein sources from plants not only reduce the footprints of carbon emission from our planet, but also promotes sustainability and food security for generations ahead (Aiking, 2011). Kiwifruit, a perennial woody vine fruit is of considerable importance for New Zealand's economy as the country is one of the leading exporters of the fruit in the world today. The seeds from kiwifruit are considered a waste from various industries such as beverages and oil extraction and remains considerably underutilized. As per our review of literature, reliable data for kiwifruit seeds, the seed meals, flour, and protein were scarce which outlines a major gap for further research in the area. Thus, this study tried to fill that gap by presenting a reliable optimized procedure of extracting proteins from kiwifruit seeds using a green extraction method; ultrasonication. Moreover, characterizable properties of the novel protein along with functional properties were also explored.

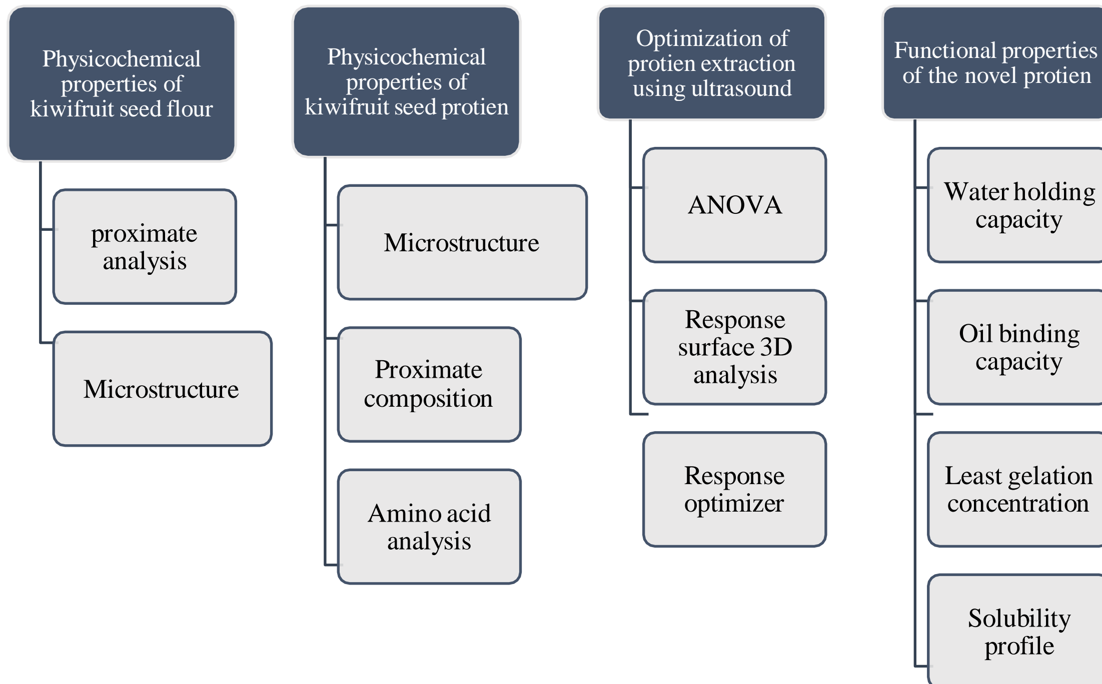

Figure 7.1: Overview of the investigated experiments in this study

7.2 Kiwifruit seed protein as a valuable plant protein source.

The structural, morphological, and compositional aspects of an *Actinidia Chinensis* seed had never been explored to the best of our knowledge. Thus, chapter 4 primarily analysed the proximate composition of *Actinidia Chinensis* seeds. The values showed a protein percentage of 13% in the seeds which was found to be adequate for recovery and extraction. The seeds structural morphology was also explored for the first time to the best of our knowledge using SEM analysis which defined a rough, sharp, angular surface for kiwifruit seeds. The seeds were then milled in a plate mill and then defatted using n Hexane. Proximate analysis was carried out again to compare the fat percentage in *Actinidia Chinensis* flour and meal. A significant drop of more than 95% was recorded in the fat percentage with a minimal drop in overall protein percentage of the seeds. Plate mill used in this study for pulverizing the seeds could be a possible reason for the decreased protein values as uncontrollable heat was generated due to friction of plates smashing into each other. Thus, it is not recommended to use plate mills for seed protein extraction and must be avoided in the future works. As this study also aimed at green extraction i.e., without use of solvents thus chemicals were avoided to pulverize the seeds.

7.3 Ultrasonic extraction can be used as a clean and green method to extract proteins from *Actinidia Chinensis* seeds

Chapter 5 investigated the effect of extraction conditions pH (pH 3 to 11), temperatures (10 to 100^{0}C), ratio of seed flour to solvent (4:100 to 20:100), Time of ultrasonication (10 to 100 min) and power (20 to 100) on the overall extraction percentage of proteins. The results showed a significant increment when ultrasonication was used as a mode for protein extraction. This confirms that ultrasonication holds a definite potential to be used as a green technology to extract protein from kiwifruit seeds for future research which is in agreement with authors who previously worked on oilseeds and protein extraction from seed meals (Dabbour, He, Mintah, & Ma, 2019; Dong et al., 2011; Malik et al., 2017). A highly alkaline pH (11.5) with lowest ratio of flour to solvent along with controlled heating (55^{0}C) were found to be the most favourable conditions for *Actinidia Chinensis* seed protein extraction. Moreover, a low power high time combination of ultrasonic extraction was observed for maximum protein extraction from the given seed profile. Thus, it can be

interpreted that controlled heating and high pH extraction along with low power high time ultrasonic extraction disintegrated the kiwifruit seed matrix well and contribute to the releasing of more proteins out in the aqueous phase. This was more clearly understood by SEM analysis of the leftover substrate of KSM which was found to be disrupted while using ultrasonication. The role of pH, temperature and ratio was found to be most significant in extraction of KSP. Moreover, it was notably understood that increasing temperatures beyond 60^0C did not increase protein extraction from kiwifruit seed meals. This could be explained by the denaturation of proteins at high temperatures and might have caused aggregation or precipitation, thus reducing the yield of extraction. Overall, it can be said from the results that ultrasonication had consequential effects in increased protein extraction from the kiwifruit seed meal.

7.4 Amino acid analysis of *Actinidia Chinensis* seed proteins

To the best of our knowledge this study was the first to present amino acid analysis of *Actinidia Chinensis* seed proteins. The profile for amino acid in this study represents a typical amino acid profile of oilseeds where glutamic and aspartic acid were dominating amino acids which defines an acidic profile for *Actinidia Chinensis* seed proteins. Moreover, the most abundant essential AAs were Leucine, Lysine and Valine respectively and the least available AAs were the sulphur AAs such as Cysteine and Methionine. Overall, the seed protein showed a balanced profile with all essential and non-essential amino acids present in *Actinidia Chinensis* seed protein. However, if compared with other major oilseeds, the overall AA content in *Actinidia Chinensis* seed proteins was the lowest. The lower values for amino acids can be related to the low protein percentage in the *Actinidia Chinensis* seeds.

7.5 Functionality of *Actinidia Chinensis* seed proteins.

The functional properties of *Actinidia Chinensis* seed proteins had a comparable profile to some of the major oilseeds commercially being used in the food industry today. As the results indicate, the water hydration capacity (0.93g/ml), oil binding capacity (3.8g/ml) and least gelation concentration (14%) of the seed protein concentrate used in this study was comparable with rapeseed protein, flaxseed, and soy protein concentrates. Of all the functional properties explored, oil binding capacity of the *Actinidia Chinensis* seed protein

concentrate was found superior to most of the oilseed proteins compared in this study. Moreover, the least gelation capacity of KSPC was also comparably equal to the soy protein concentrates which defines that *Actinidia Chinensis* seed proteins have credible potential to be utilized for food industries to change the organoleptic properties of foods.

7.6 Avenues for Future Work

Developing suitable methods for extracting plant proteins from plant-based materials is critically important for the food industry and scientific community. This study provided a systematically optimized method of ultrasonic extraction of *Actinidia Chinensis* seed proteins and details which conditions/processes can lead to greater amounts of soluble proteins. There are some limitations in the current study that have opened opportunities for future research and have been summarised below.

- The seed of kiwifruit has never been investigated in detail, thus cryogenic SEM images of *Actinidia Chinensis* seeds could be done in the future to better understand the overall composition and exact placement of protein storage vacuoles in the seeds
- The KSP analysed in this study contained not only protein but other solubilised components as well such as carbohydrates, therefore a study can be conducted on different methods of obtaining seed meals from seeds and its effect on overall protein concentration.
- The Osborne fractionation of proteins could not be carried out in this research due to time constraints which could be a possible work to better understand the novel protein.
- Different extraction procedures have different influences on the protein extraction, thus more green technologies such as pulsed electric field extraction, microwave extraction etc, can be deployed to compare and optimize the best procedure for protein extraction from *Actinidia Chinensis* seeds.
- Significant portions of fiber and carbohydrate were analysed in kiwifruit seeds which could be utilized after protein extraction from seeds.
- Flour obtained from kiwifruit seeds as analysed in this research can be looked further into for different product formations.

- As only the ultrasonic probe was used to assist extraction proteins from kiwifruit seeds in this study, other mode of sonication such as ultrasonic baths can also be looked to compare the extraction results.

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Number of figures/tables	1
Will you be translating?	No
Title	Ultrasound assisted extraction and optimization of kiwi seed proteins, physicochemical and functional properties
Institution name	Massey University
Expected presentation date	Feb 2022
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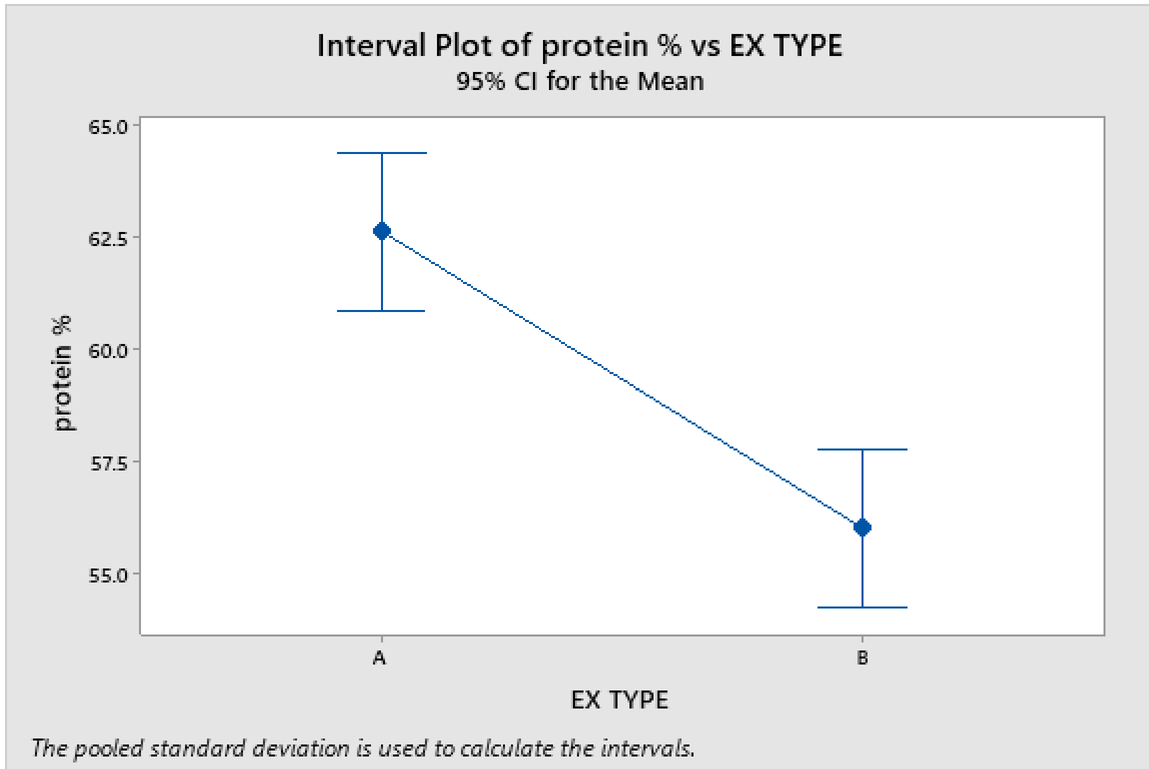
Materials and chemicals used for proximate analysis in this study

Test	Materials	Chemicals	Supplier name
Protein estimation	Digestion unit, Kjeltec Distillation unit, digestion tubes, glassware	Kjeltec tabs, Conc. H_2SO_4, 0.1 N HCl, 4% Boric acid solution.	Sigma Aldrich, New Zealand. Massey University
Crude fat analysis	Weighing balance, Soxhlet apparatus, Drying oven, Thimbles, water bath (for heating), Glassware	Petroleum ether	Sigma Aldrich, New Zealand
Ash analysis	Muffle furnace, Crucibles,	-	Massey University

THE BOX BEHNKEN DESIGN OF EXPERIMENT

RUNORDER	PTTYPE	BLOCKS	PH	POWER	TIME	RATIO	TEMPERATURE
1	2	1	7.5	100	100	0.12	55
2	2	1	7.5	100	20	0.12	55
3	2	1	7.5	20	100	0.12	55
4	2	1	7.5	60	100	0.04	55
5	2	1	3.5	20	60	0.12	55
6	2	1	7.5	60	20	0.12	100
7	2	1	7.5	100	60	0.2	55
8	2	1	7.5	60	60	0.04	100
9	2	1	3.5	100	60	0.12	55
10	2	1	7.5	100	60	0.04	55
11	2	1	7.5	60	100	0.12	100
12	2	1	7.5	20	60	0.12	10
13	2	1	3.5	60	60	0.12	10
14	2	1	7.5	20	20	0.12	55
15	2	1	3.5	60	20	0.12	55
16	2	1	7.5	100	60	0.12	10
17	2	1	7.5	60	100	0.12	10
18	2	1	7.5	60	60	0.2	10
19	2	1	11.5	60	60	0.04	55
20	2	1	7.5	60	60	0.2	100
21	2	1	7.5	60	20	0.04	55
22	0	1	7.5	60	60	0.12	55
23	2	1	7.5	100	60	0.12	100
24	2	1	7.5	60	20	0.12	10
25	0	1	7.5	60	60	0.12	55
26	2	1	7.5	60	20	0.2	55
27	2	1	11.5	60	60	0.2	55
28	2	1	7.5	20	60	0.12	100
29	2	1	11.5	60	100	0.12	55
30	2	1	3.5	60	60	0.2	55
31	0	1	7.5	60	60	0.12	55
32	2	1	3.5	60	100	0.12	55
33	2	1	11.5	60	20	0.12	55
34	0	1	7.5	60	60	0.12	55
35	2	1	7.5	20	60	0.04	55
36	2	1	11.5	60	60	0.12	100
37	0	1	7.5	60	60	0.12	55
38	2	1	7.5	20	60	0.2	55
39	2	1	11.5	100	60	0.12	55
40	2	1	3.5	60	60	0.04	55
41	2	1	7.5	60	60	0.04	10
42	2	1	7.5	60	100	0.2	55
43	0	1	7.5	60	60	0.12	55
44	2	1	11.5	20	60	0.12	55
45	2	1	3.5	60	60	0.12	100
46	2	1	11.5	60	60	0.12	10



Here A represents Ultrasonic extraction whereas B represents conventional stirred extraction.

WORKSHEET 1

One-way ANOVA: protein % versus EX TYPE

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
EX TYPE	2	A, B

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
EX TYPE	1	65.737	65.737	54.41	0.002
Error	4	4.833	1.208		
Total	5	70.570			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.09920	93.15%	91.44%	84.59%

Means

EX TYPE	N	Mean	StDev	95% CI
A	3	62.633	0.932	(60.871, 64.395)

B	3	56.013	1.244	(54.251, 57.775)

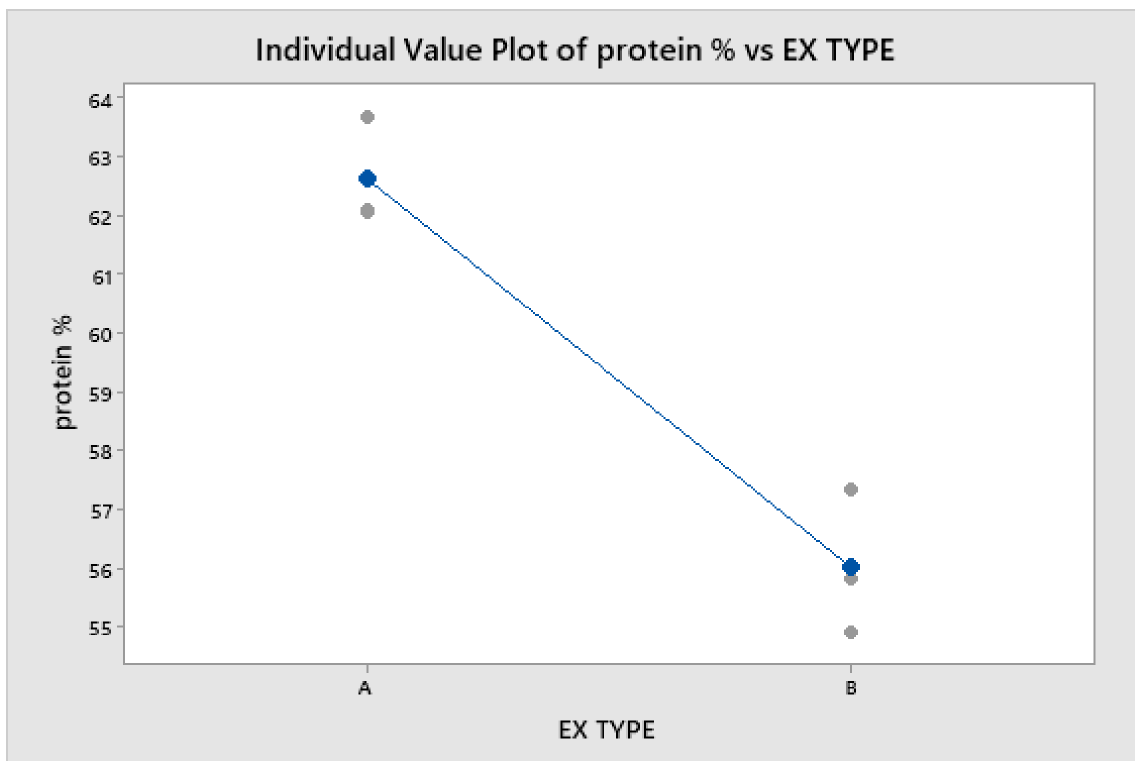
Pooled StDev = 1.09920

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

EX TYPE	N	Mean	Grouping	
A	3	62.633	A	
B	3	56.013		B

Means that do not share a letter are significantly different.



Amico acid results

1/2

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TO:	Ashutosh Bisht	AT:	SF&AT
SUBJECT:	Final Report	DATE:	23/02/21
TRIAL:	TN21-20	SAMPLES RECEIVED:	18/01/21

Number of pages in this report: 2 Client Reference:
Testing initiated: *18/01/21* Testing completed: *23/02/21*

TN21-20 Results are on an as received basis

AMINO ACIDS	Kiwifruit Seed Protein
Aspartic Acid	1.05
Threonine	0.36
Serine	0.50
Glutamic Acid	2.29
Proline	0.79
Glycine	0.97
Alanine	0.51
Valine	0.70
Isoleucine	0.46
Leucine	0.72
Tyrosine	0.51
Phenylalanine	0.45
Histidine	0.30
Lysine	0.71
Arginine	1.33
Cysteine	0.25
Methionine	0.34
Tryptophan	0.14
Units	mg/100mg

Methodology

Amino Acid Profile (Acid Stable) : HCl hydrolysis followed by RP HPLC separation using AccQ Tag derivatization. AOAC 994.12

CYS/MET : Performic acid oxidation, AOAC 994.12

* *Tryptophan : Alkaline Hydrolysis, sub-contracted (accredited)*

*Tests marked with an asterix are currently outside the scope of the Nutrition Laboratory's accreditation

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FINAL REPORT-v2

Biswas, B., & Sit, N. (2020). Effect of ultrasonication on functional properties of tamarind seed protein isolates. *Journal of food science and technology, 57*(6), 2070-2078.

Malik, M. A., Sharma, H. K., & Saini, C. S. (2017). High intensity ultrasound treatment of protein isolate extracted from dephenolized sunflower meal: Effect on physicochemical and functional properties. *Ultrasonics sonochemistry, 39*, 511-519.

Wouters, A. G., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety, 15*(4), 786-800.