Characterization And Solubility Properties of Pumpkin Seed Flour for Functional Beverage Development

A thesis submitted in partial fulfilment of the requirements for the
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Dedication

I thank Almighty God for all the grace and blessing throughout my life.

To my father Mathewkutty V.G., my mother Mariyamma Mathew, my brother Jibal Mathew and my sister Meekhal Mathew, thank you for your support, prayers and motivation throughout this journey.

I also thank everyone who has supported and guided me to become a good human being.

Abstract

The rise in demand for plant-based protein beverages has given opportunities to explore various alternatives protein rich sources like pumpkin seed flour (PSF). This research mainly focuses on the functional and structural properties of PSF which originated from hull less pumpkin seeds. The aim of the study was to check the suitability of pumpkin seed flour in beverage applications. The initial proximate on the pumpkin seed flour, determined high crude protein (53%) and crude fat (15.9). In addition to this, PSF also contains notable ash and carbohydrate levels which making PSF a strong potential nutritional source.

By eye it was evident that the commercial pumpkin seed flour studied was comprised of heterogenous particles – some dark green and others light green. The functional impact of this visual variation was evaluated via a sieving experiment performed across standard mesh sizes. The darker green particles concentrated in the larger mesh size while the lighter green particles tended to be smaller. The ash and the protein content in the various particle sizes however showed minimal variation, which justifies the usage of whole flour for solubility studies. Water solubility was conducted at different pHs (3, 6.6 and 8) and temperatures (30ºC, 60ºC and 80ºC). The result showed that the solubility of PSF improved with rising temperatures and with alkaline conditions. The proximate analysis of the solubility sediments, resulted in lower amounts protein and fats, when comparing with the supernatants.

The colour analysis of the sediments showed significant variations in L*, a* and b* values which mostly depend on the application of heat and pH. It also shows potential colour degradation and Maillard reaction during varying conditions. SDS – PAGE analysis confirms the solubilization of certain protein bands mostly on higher temperatures and under alkaline conditions. The notable soluble bands are Cucurbitin, 11S basic subunits and 7S globulins. Confocal laser studies gave evidence of protein and fat interactions in varying conditions. The fat and proteins were more dispersed and mixed structures in supernatant during high solubility conditions meanwhile, dense protein aggregates were visible in the sediment fractions.

Generally, this study indicate that alkaline conditions are necessary to manufacture a beverage. However, the properties are highly depended on varying processing conditions, mainly certain temperature and pH. These findings help to give an idea for developing a high protein content plant-based drink with the use of pumpkin seed flour as a sustainable and functional ingredient.

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List of Abbreviations

PSF	Pumkin Seed Flour
PS	Pumkin Seed
PSO	Pumkin Seed Oil
OS	Oxidative stress
PI	Protein Isolates
SME	Small and Medium level Enterprise
PUFAs	Poly Unsaturated Fatty Acids
BPH	Benign Prostatic Hyperplasia
WHO	World Health Organisation
FAO	Food and Agriculture Organisation
MUFAs	Mono Unsaturated Fatty Acids
LDL	Low-Density Lipoprotein
HDL	High-Density Lipoprotein
BME	β – mercaptoethanol
IgE	Immunoglobulin E

1 Introduction

The one of the oldest cultivated crops, Pumpkin (*Cucurbita spp.*) is largely grown around many regions of the world including Asia, Africa, Europe and the United States. Pumpkin belongs to the family Cucurbitaceae and famous for its versatility due to edible parts of the plant- flesh, seeds, flower and even leaves are used for making different varieties of cuisines and medicinal purposes. *Cucurbita pepo, Cucurbita maxima* and *Cucurbita moschata* are the ones mainly used for food applications among the other different species present. Even though pumpkins are mainly cultivated for its flesh, pumpkin seeds are gaining popularity because of its functional and nutritional benefits. Unfortunately, in most countries these seeds are considered as waste or byproducts after the processing of pumpkin. Present day studies suggest that these seeds can be converted into seed flours which can be a valuable food ingredient in formulating bakery, confectionery and beverages applications (Ningthoujam et al., 2018).

Pumpkin seeds are well endowed with macronutrients and bioactive components. They have abundant amounts of protein, essential fatty acids mainly linoleic and oleic acids, fibre and minerals like iron, magnesium, zinc and phosphorus (Akintade Adeyanmola et al., 2019) (Ningthoujam et al., 2018). These nutritional components enhance the Pumpkin seed Flour (PSF) as an excellent foundation for functional foods and beverages. (Amin et al., 2019) reported that Bangladeshi native *Cucurbita maxima* seeds' have 21.2% proteins and 23.5% fat content along with rich amounts of minerals. Moreover, PSF shows excellent antioxidant capacity as the presence of phytosterols, polyphenols and tocopherols, which can result in preventing chronic medical conditions like cardiovascular disease and cancer (Ceclu et al., 2020; Dhiman et al., 2009).

Numerous researchers have investigated the therapeutical values of pumpkin seeds. They reported various benefits like anti-inflammatory, antimicrobial, hypotensive, hypoglycaemic and anthelmintic properties, which all attributes to the bioactive components of Pumpkin Seed (PS) (Dhiman et al., 2009). For instance, (Ali et al., 2022) stated that Pumpkin Seed Oil (PSO) contains fair quantities of tocopherols and sterols, which helps in improving lipid profiles and minimizing Oxidative Stress (OS) (Ningthoujam et al., 2018). These results are evident in stating that PS are not only a source of nutrients, moreover, helps in promoting health and preventing diseases.

PSF exhibits beneficial properties like water and oil absorption capacities, foaming capacity, emulsifying ability and gelation potential, which are the crucial steps in food product development (Akintade Adeyanmola et al., 2019). These functional capabilities create a variety potential in various food systems including bakery products, snacks and beverages. Such as, (Das et al., 2021) successfully utilised PSF to enhance the protein and mineral content of biscuit, like improving their nutritional values for the targeted audiences: children and elderly populations.

In the last few years, the worldwide food industries gained a specific demand for developing plant-based beverages in order to satisfy the demands of health-conscious customers. These functional drinks are made to increase nutritional and physiological benefits and not only for just hydrating the body (Dhiman et al., 2009). In beverage sector, plant-based milk, smoothies, protein drinks and fortified fruit juices are one of the fast-growing products in the market. Generally, these products depend on soy, almond and oat as the key protein ingredient, and these may not be readily available or expensive in many parts of the world. However, PSF can be easily available locally with affordable rates and rich in nutrients, and therefore could be an excellent alternative for beverage formulation, particularly in different parts of the world where the cultivation of pumpkin is mostly done, and seeds are thrown as waste or byproducts (Fedha, 2014).

Few researchers have investigated the ways to incorporate pumpkin seed extract and flour in formulation various food items. (Öztürk & Turhan, 2020) conducted a study on adding powdered pumpkin seed to grounded beef as a fat replacer and reported the enhancement in fatty acid profile, cooking loss and the overall composition like ash, protein content and the carbohydrates have been increased in the meat Their study also supports the use of PS as an ingredient in food formulation to improve functional and nutritional benefits without negotiating the palatability and visual appearances. In another research conducted by (Elanany et al., 2023), they used a mixture of PSF and broken rice to formulate a infant formula and reported enhanced nutritional benefits and protein digestibility.

The possible advantage of using PSF in production of beverages are not limited to nutrition content. In a point of economic and environmental aspects, the utilization of PSF helps in maintaining sustainability and decrease in food wastage. The process of converting the ignored by products to some valuable ingredients, this method is well matched with sustainable development goals and circular economy (Dhiman et al., 2009). In addition to that, small and medium food industries of developing countries can take advantage by benefits economically as well as selling value added products made from pumpkin seeds.

Furthermore, PSF incorporated beverages can play a crucial role in reducing protein malnutrition, especially in disadvantaged communities. In majority of developing countries, access to good quality of protein source like milk and meat products are limited due to their cost and availability (Ceclu et al., 2020). In these scenarios, plant-based protein sources like hemp, and pumpkin seed can be an alternative source for accessing hight quality protein. According to (Chatain et al., 2017), PS have storage proteins like albumins and globulins which have beneficial amino acid profiles. When these proteins are incorporated into the beverage, it can make an overall improvement in protein intake of the population.

Although, the incorporation of PSF in beverages are still under researched. There are many flaws in information about formulating methods, shelf life, sensory acceptances and consumer demands. More investigations are to be done to point out the best processing methods to make a nutritional and functional beverage without any loss during production. In addition to that, there is no standardized guidelines for the using PSF in different types of beverages.

In short, PSF is a very assuring ingredient in formulating functional drinks due to its high and quality protein content, bioactive components, rich mineral profile and beneficial functional properties. The incorporation of PSF in a beverage not only adds nutritional value but also improves sustainability by using productional by products. This thesis aims to study proximate content, particle size, solubility behaviour and micronutrients interaction of the pumpkin seed flour in various treatments. The sample PSF is the byproduct, or the press cake after the extraction of pumpkin seed oil. The end results are expected to provide a foundation in upcoming growing research on plant based functional beverages and recommends the utilization of pumpkin seeds and flour in various novel food applications.

2 Literature Review

2.1 Pumpkin Plant and its agricultural importances

2.1.1 Taxonomy and Classification of *Cucurbita* Species

The pumpkin belongs to a genus *Cucurbita*, within the family Cucurbitaceae. In this family there are around 800 species of dendritic trailing plants like melons, squashes and gourds. Within these, *Cucurbita pepo, Cucurbita maxima* and *Cucurbita moschata* are majorly produced and consumed throughout the world in both culinary and medicinal purposes (Fedha, 2014). The morphology, adaptability and nutritional value vary species by species. For instance, *Cucurbita pepo* are the common pumpkins and summer squashes; meanwhile *Cucurbita moschata* is mainly grown in tropical and subtropical parts due to it heat tolerance and in case of *Cucurbita maxima,* their seeds are nutritional richness and economic source of protein, minerals, vitamins and other components which are essential for human health (Habib et al., 2015).

Phytologically, pumpkin plants are annual climber or trailing plant which consists of large, lobe structured leaves and yellow monoecious flowers. The fruits can be in various shape and sizes ranging from small and round to large and oblong (Kaur et al., 2020). These fruits also contain larger numbers of green, flat and edible seeds. The seed normally has a hard outer shell or hull, meanwhile some variety like Styrian oil Pumpkins has hull less seeds, they mostly suitable for oil extraction and flour production (Fruhwirth & Hermetter, 2007).

2.1.2 Major Species: *C. pepo, C. maxima* and *C. moschata*

Individually all the main *Cucurbita* species show different traits that influence their suitability for food application. The most genetically diverse variety is *C. pepo* and are grown on tropical as well as temperate climates. The varieties come under are zucchini, acorn squash and oilseed pumpkins(Dhiman et al., 2009). *C. moschata* (**Figure 2**) is also known as butternut squash, these are widely grown in different parts of Asia and Africa. It gained its demand for its adaptability to heat and resistances to pests (Ceclu et al., 2020).

C. maxima (**Figure 1**) is a crucial variety for food processing sector, due to its large sized seeds, soft hull and high nutrient density. Researchers reported that *C. maxima* seeds have higher lipid and protein content while comparing *C. pepo* and *C. moschata* (Chatepa, 2022).

Figure 1: Image of Pumpkin species *C. maxima* (Fedha, 2014)

Figure 2: Image of Pumpkin species *C. moschata* (Fedha, 2014)

2.1.3 Cultivation, Yield and Post Harvest Utilization

The largest producers of pumpkin worldwide are China, India and USA. As a crop, pumpkin can withstand warm climates with well-drained soil and requires very less agricultural input, which makes it a favourable crop in both commercial and subsistence farming (Ahmad & Khan, 2019; Kaur et al., 2020). In parts of Sub-Saharan Africa and Southeast Asia, pumpkins are cultivated as a backyard or intercropped vegetables and harvested for their flesh, seeds are often thrown as waste (Fedha, 2014). **Table 1** shows the major producers of pumpkin in regarding with their area and yield.

Table 1: Global Production of Pumpkin (Dhiman et al., 2009)

Country	Production (Tons)	Area (ha)	Yield (H/ha)
India	3500000	360000	97222
China	6315000	328000	192530
USA	864180	39500	218780
Germany	83100	2200	377727
Japan	237000	17000	139412
Spain	315000	7500	420000
South Africa	95000	12000	79166
Pakistan	255000	26000	98076
Mexico	17000	5500	212727
Columbia	60000	3650	164384

The recent expansion in agriculture gave prominence to PS in food innovation and sustainability. **Figure 3** shows the inclining of pumpkin production from 2020 to 2027. The amount of oil produced by PS and their protein content make them valuable for agro processing industries. For example, in Malawi, even though pumpkins are cultivated in large number, they only utilized their flesh and pulp while the seeds are discarded due to lack of awareness as well as processing technology (Chatepa, 2022). The Post harvest handling mainly consist of manual separation of seeds, sun drying and stored in sealed containers. The novel processing techniques like roasting, defatting and milling gave ways for more useability of PS in food applications. Products like PSF, Protein Isolates (PI) and PSO are being studied as functionals ingredient for various sectors like bakery, snacks and beverages (Sharma & Lakhawat, 2017).

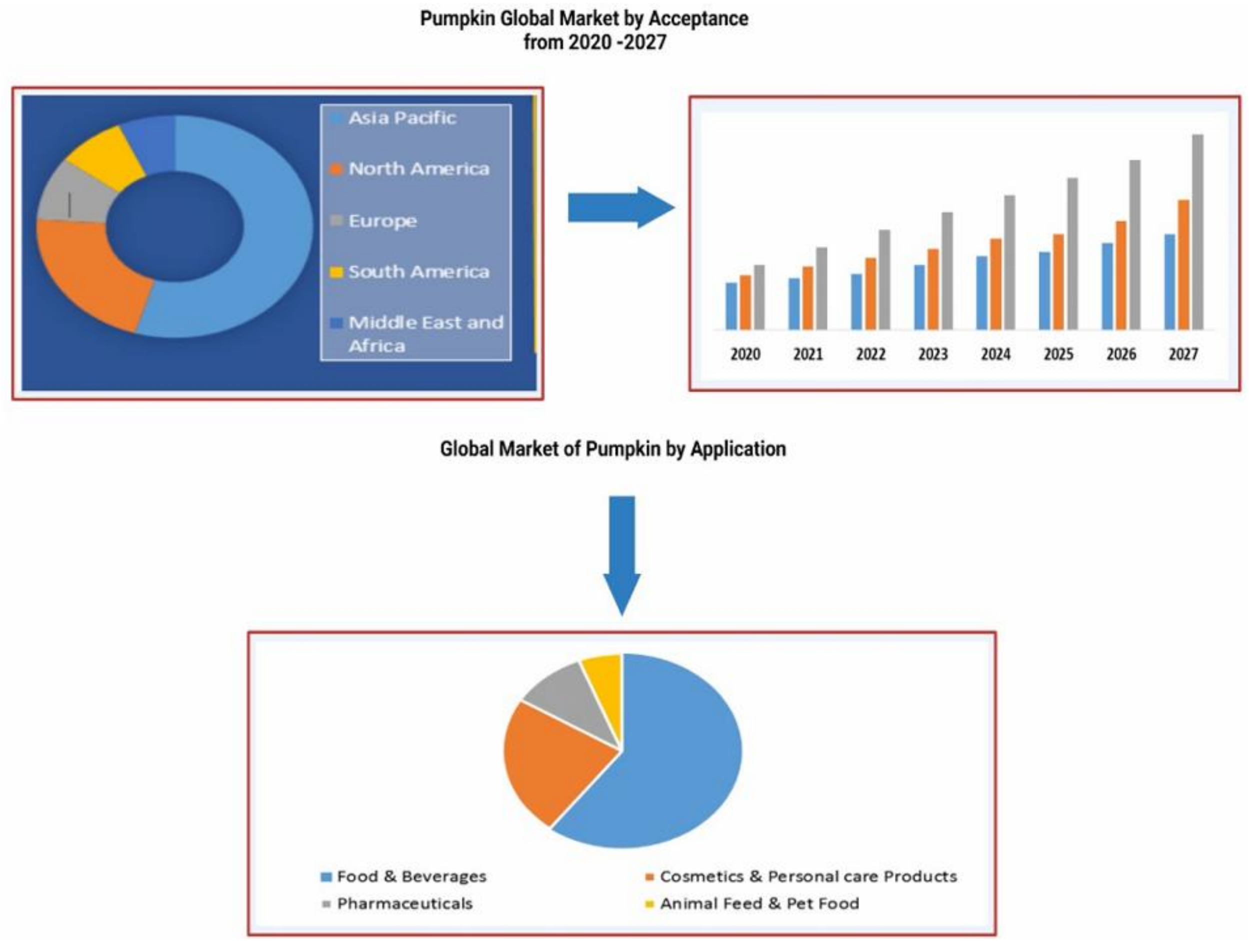


Figure 3: Inclining Market of Pumpkin (Aziz et al., 2023)

2.1.4 Pumpkin as a Traditional and Functional Crop in Developing Countries

Pumpkin plays an important role in food security in countries like India, Bangladesh, Kenya and Nigeria. These countries, pumpkin is consumed during monsoon or lean season because of its high yield and long shelf life. The flesh of pumpkin has high beta carotene and dietary fibre and seeds are rich source of protein and fat which can be a crucial in minimizing protein energy malnutrition (Maria et al., 2022). Even though with all the benefits, cultural biases and lack of awareness is always a con in regards with utilisation of PS. Studies shown that incorporating PSF into traditional and modern food systems can enhance nutritional intake of disadvantage populations (Das et al., 2021).

Additionally, encouraging PS processing at small and medium level enterprise (SME) can help in improving rural economy by creating job opportunities and wages from a wasted or ignored resource (Villamil et al., 2023). These efforts help in attaining global sustainable development goals associating with food waste reduction and nutritional resilience.

2.2 Pumpkin Seed and Pumpkin Seed Oil

2.2.1 Physical Characteristics and Morphology of Pumpkin Seeds

Pepitas is another name for Pumpkin seeds, they are usually flat and oval shaped seeds which are situated at the hollow interior of fruit. Pumpkin's exterior appearance and internal compositions can change according to the species and cultivar. Mostly, PS are white or cream in colour when its raw. Pumkin seeds have a thick outer shell in many species. However, specific species like Styrian oil pumpkin (*Cucurbita pepo subsp. pepo var)* are hull less species, which are more suitable for processing oil and flour (Fruhwirth & Hermetter, 2007). **Figure 4** represents the industrial treatment flowchart of pumpkin to extract seeds and other potential materials.

The usual size and weight of PS can change according to species. For example, seeds of *Cucurbita maxima* are normally bigger and softer in comparison with *Cucurbita moschata* or *Cucurbita pepo*. These variation in physical appearances can directly affect the processing steps like drying, dehulling and milling. According to (Fedha, 2014), the shelf life and processing capability can significantly change according with seed coat thickness, surface smoothness and moisture content. Moreover, hull less seed variety shows more oil extraction efficiency and are mostly selected for functional applications in food as well as oil extracting industries (Gohari Ardabili et al., 2011).

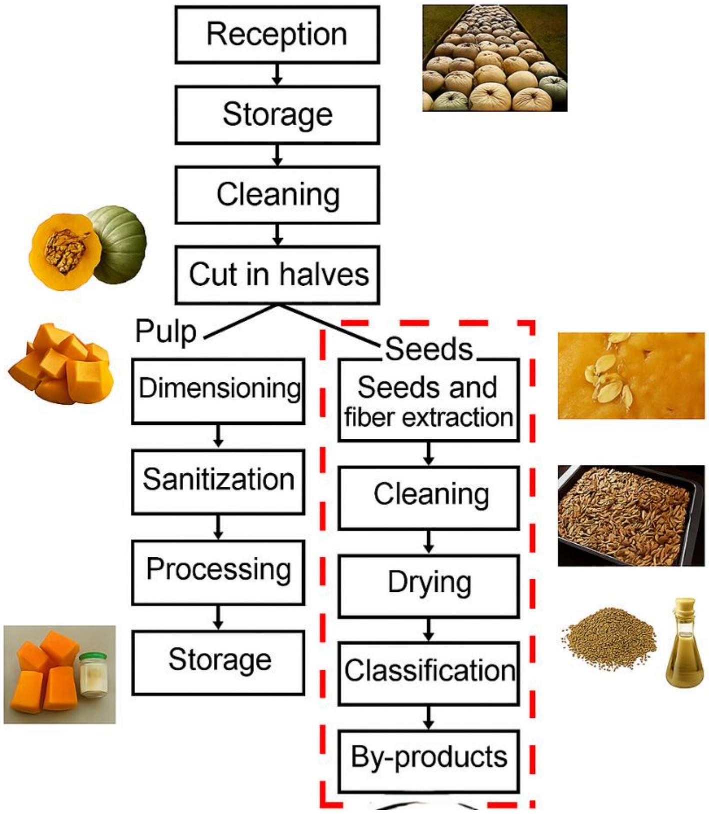


Figure 4: Process flow chart of pumpkin processing (Lemus-Mondaca et al., 2019)

2.2.2 Oil Yield, Extraction Methods, and Composition

Pumpkin Seed Oil is valued as a high demand and value product due to its high content of essential fatty acids and bioactive compounds. The oil yield and quality level are directly linked to the variety of pumpkin, seed pre- treatment and extraction methods. Normal extraction methods are cold pressing, hot pressing and solvent extraction, in these cold pressing is often used as it preserves natural antioxidants and reduce thermal degradation of sensitive components (Hien & Minh, 2021). In pumpkin seeds, cold pressing methods helps in retaining its dark green colour, nutty flavour and high content of tocopherols and phytosterols. Meanwhile, hexane is used in solvent extraction method which helps in higher oil yields but also need refining techniques to remove residual solvents. In **Figure 5** shows a brief flowchart of different extraction methods of PS. The research conducted by (Amin et al., 2019) found that oil content in *Cucurbita maxima* seeds can be in between 35% to 50% depending on whether they are native species or hybrid.

The main constituents of PSO are unsaturated fatty acids, in which linoleic acid (omega-6) and oleic acid (omega-9) are mostly outstanding. Also it contains a few amounts of carotenoids, squalene and phenolic compounds these compounds helps in contributing to antioxidant properties (Fruhwirth & Hermetter, 2007).

Figure 5: Various Oil extraction methods of Pumpkin seeds (Šamec et al., 2022)

2.2.3 Comparison of Native vs Hybrid Seed Varieties

The selection between native and hybrid pumpkin varieties is a very important factor in determining oil quality, nutritional composition and functional benefits. Hybrid pumpkin varieties are usually grown for higher yields, disease resistance and oil content. However, native varieties sometimes give better nutritional content, improved fatty acid ratios and higher mineral content (Amin et al., 2019). For instance, it is reported that in local *Cucurbita maxima* seeds they have higher amount of magnesium, potassium and dietary fibre which make them more suitable for flour production and oil pressing. On the other hand, hybrid cultivars help in more oil yields with better oxidative stability which is useful in a commercial perspective for food formulations and shelf stable products.

Native seeds mostly show greater genetic variability, which is an advantage for local survival and sustainability in a small-scale farm setting system (Dhatt et al., 2024). This makes them a favourable resource not only for edible purposes but also for breeding programs which helps in improving both nutritional and agronomic characteristics.

2.2.4 Nutritional and Medicinal Properties of Pumpkin Seed Oil

Pumpkin seed oil is a high nutritional oil which has been using long before as traditional medicine. PSO has a desirable amount of PUFAs (Poly Unsaturated fatty acids) like linoleic acids, which helps in heart health, oleic acid, helps in minimizing inflammation and supporting brain functions. PSO is also a good source of vitamin E (specifically γ-tocopherols), phytosterols and squalene, these compounds are connected to antioxidant and cholesterol lowering effects (Šamec et al., 2022). Several researchers have reported that PSO helps in prostate and urinary tract health. They also reported its anti-inflammatory and diuretic properties in management of BPH (Benign Prostatic Hyperplasia) and bladder irritation. The study conducted by (Ali et al., 2022) supports these claims and pointed out that effectiveness in minimizing symptoms related to prostate enlargement.

In addition to this, the high antioxidant content in PSO helps in fight oxidative stress and protect from chronic diseases like atherosclerosis, diabetes and some types of cancer. In the process of PSO consumption, it shows lowering blood pressure and enhancing lipid profiles. This makes PSO a valuable ingredient in adding to functional foods and nutraceutical products (Sharma et al., 2020).

2.2.5 Functional Food Potential of Pumpkin Seed Oil

The increased usage of Pumpkin seed oil in functional food products are due to the interesting sensory profile, nutritional benefits and health promoting properties. PSO has be used as salad dressing, spreads, supplement and as plant based dairy alternative. Moreover, PSO has also been used as an ingredient for gourmet cuisines due to its special flavour and notable deep green hue (Aziz et al., 2023). Not only in cooking applications, but Pumpkin Seed oil is also an interesting ingredient for developing a functional food or beverage due to its stable bioactive compounds. The natural stabilizer present in the oil helps in stabilizing oil-in-water emulsions, which is crucial factor in formulating nutrients rich, shelf stable beverages. Several ongoing research are investigating the formulation methods to maintain PSO's health benefits in beverage products (Pham et al., 2017).

2.3 Nutritional Composition of Pumpkin Seeds

Pumpkin seeds are notable for their rich nutritional value, which includes a considerable amount of macronutrients and micronutrients. PS also has high levels of oil and protein content. In addition to this, dietary fibre, vitamins, minerals and many other bioactive compounds are present in PS. This section reviews the nutritional benefits of PS with comparison with various species (*C. maxima, C. pepo and C. moschata*). A more expansive review of purported health benefits of pumpkin seeds is given in the (**Appendix A**).

2.3.1 Macronutrients: Protein, Fat, Fibre, and Carbohydrates

Pumpkin seeds are a rich source of plant-based protein and oil. In a comparative study conducted on three common species of pumpkin and its parts like flesh, peel and seed are briefly listed on **Table 2**. Normally, unprocessed PS contains roughly 30 – 40% protein and 30 – 40% fats by dry weight, the accurate value will be differ with species and cultivar. In a study conducted on PS (*C. maxima Linn.*), it contains about 34.6% protein and 36.7% fat on dry basis (Habib et al., 2015). Likewise, research on hull less variety of PS (*C. pepo* subsp. *pepo var.* Styriaca) reported 25.4% protein and 41.6% oil found in their seeds (Gohari Ardabili et al., 2011). *C. moschata* seeds contain around 35 – 36% protein and 34 – 35% fats, a little above to *C. maxima* (Fedha, 2014). These results shows that all three species yield protein and oil rich seed, but some cultivars can show variations. For example, a study based in India stated that seeds of *C. pepo* are an excellent source of protein that is 39.35% (Das et al., 2021).

The high levels of oil content in PS always make them comparable to other oilseeds. Some pumpkin cultivars like the hull less variety or the naked seed are grown for oil extraction process, the oil percentage comes in the range of 40 – 50%. A study conducted by (Gohari Ardabili et al., 2011) found certain type of *C. pepo* found in Iran contains about 43 – 56% oil, meanwhile 41 – 54% of oil was found in PS species found in West African region (Fedha, 2014). These oil levels resemble that of high oil nuts or seeds like sunflower. The presences of seed coat affect the oil percentage as the fibrous material on the hull dilutes the protein and fat. In naked or the hull less varieties, show a higher oil and protein content as per the weight of seed. The naked variety is due to a spontaneous mutation occurred during breeding, which is useful in efficient use in cooking applications as well as oil extractions (Dhatt et al., 2024). The seed coat removed or dehulled PS has more oil than the whole PS with hulls, even though hull contain little bit of fat in it. Similarly, protein content is also slightly higher in dehulled seeds than the seeds with hulls or the whole seeds. In comparison with other protein rich seeds and nuts, PS has a better protein profile, much higher than of cashew and sesame (Fedha, 2014).

Dietary fibre is one of the important macronutrients, present in the seed coat or the hull of pumpkin seeds. The PS has a notable amount of fibre, which is mostly insoluble fibre from the seed coat. In various studies, it is stated that PS has about 11 – 13% crude fibre in that only 4% is from the seed without seed coat (Fedha, 2014). This also indicates that the outer coating of PS is highly rich in fibre (Arora et al., 2023). An investigation conducted by (Fedha, 2014) stated that whole unhulled seeds provide to the dietary fibre intake almost half of the fibre content of sesame seeds as per weight basis. This fibre is crucial for digestion purposes and adds quantity to the diet, even though it dilutes the caloric and nutrient density of the seed. Hulled PS are low in fibre but has a high amount of protein and fat. In a nutritional point of view, both forms have their own importances – consuming the unhulled seed gives more roughage and fibre whereas consuming hulled seeds helps to get more nutrients with a softer texture.

In pumpkin seeds, carbohydrates are mainly found as structural carbohydrates and residual starches or sugars. However, the digestible fraction of carbohydrates is very less while comparing with other macronutrients present in PS. In a study, *C. maxima* seeds tend to contain 1.1% free sugars and 2.2% starch (Habib et al., 2015). The total non- fibre carbohydrates present in PS comes in a range of 5 – 15% by weight. These carbohydrates act as the minor storage components in the seed, and all the seed's energy are stored in the form of oils. The low glycaemic index of PS indicates its low sugar content, while the presence of starch and fibre contributes to some prebiotic and stool – bulking effects. In short, PS have high levels of protein and fats as the primary macronutrients, medium to low levels of carbohydrates and some levels of dietary fibre, if the hull is also consumed. In the most common species, the exact macronutrients can be different, that is *C. pepo* an oil seed variety of pumpkin has more percentage of oil, meanwhile *C. moschata* and *C. maxima* varieties are mostly used for flour or food applications, tend to have balance number of protein and fat (Fedha, 2014). Even though with all these variations, all pumpkin seeds are highly rich in nutrients and helps to achieve daily fat and protein intake requirements.

Table 2: Comparative proximate study of common pumpkin variety (g/kg raw weight) (Kim et al., 2012).

	Part		**Species**	
		C. pepo	***C. moschata***	***C. maxima***
Carbohydrate	Flesh	26.23 ± 0.20^{a}	43.39 ± 0.84^{b}	133.53 ± 1.44^{c}
	Peel	43.76 ± 0.74^{a}	96.29 ± 1.11^{b}	206.78 ± 3.25^{c}
	Seed	122.20 ± 7.47^{a}	140.19 ± 7.60^{b}	129.08 ± 8.25^{ab}
Protein	Flesh	2.08 ± 0.11^{a}	3.05 ± 0.65^{a}	11.31 ± 0.95^{b}
	Peel	9.25 ± 0.12^{a}	11.30 ± 0.99^{a}	16.54 ± 2.69^{b}
	Seed	308.83 ± 12.06^{b}	298.11 ± 14.75^{ab}	274.85 ± 10.04^{a}
Fat	Flesh	0.55 ± 0.14^{a}	0.89 ± 0.11^{b}	4.20 ± 0.23^{c}
	Peel	4.71 ± 0.69^{a}	6.59 ± 0.41^{b}	8.69 ± 0.99^{c}
	Seed	439.88 ± 2.88^{a}	456.76 ± 11.66^{b}	524.34 ± 1.32^{c}
Fibre	Flesh	3.72 ± 0.02^{a}	7.41 ± 0.07^{b}	10.88 ± 0.35^{c}
	Peel	12.28 ± 0.15^{a}	34.28 ± 1.37^{c}	22.35 ± 0.01^{b}
	Seed	148.42 ± 0.55^{b}	108.51 ± 8.36^{a}	161.54 ± 6.79^{c}
Ash	Flesh	3.44 ± 0.04^{a}	10.36 ± 0.01^{b}	10.53 ± 0.11^{c}
	Peel	6.30 ± 0.06^{a}	13.96 ± 0.16^{c}	11.20 ± 0.64^{b}
	Seed	55.02 ± 1.00^{c}	53.15 ± 0.20^{b}	44.22 ± 0.36^{a}
Moisture	Flesh	967.70 ± 0.15^{c}	942.31 ± 0.08^{b}	840.43 ± 0.17^{a}
	Peel	935.98 ± 0.27^{c}	871.86 ± 0.09^{b}	756.79 ± 0.44^{a}
	Seed	74.06 ± 0.91^{c}	51.79 ± 6.04^{b}	27.51 ± 0.21^{a}

Values are mean ±SD. Different superscript letters within a row indicate significant differences by Duncan's multiple range test ($P < 0.05$).

2.3.2 Micronutrients: Mineral Content of Pumpkin Seeds

Pumpkin seeds are a good source of minerals, consisting of a wide range of both macro – minerals and trace minerals. The specific notable minerals are phosphorus (P), magnesium (Mg) and potassium (K) also with a significant amount of zinc (Zn) and iron (Fe). Due to the rich mineral profile, PS also knows as the powerhouse of nutrients by many researchers (Arora et al., 2023).

The three common varieties of pumpkin seeds have identical mineral composition with a slight variation in the quantity across species. The amplest mineral found in PS is the Phosphorus, due to the presences storage phosphate compounds that are used for the germination of plants. The phosphorus level comes between 700 – 1000 mg per 100 g. *C. moschata* seeds has about 981 mg phosphorus and approximately 538 mg magnesium per 100 g (Fedha, 2014). Magnesium is also one of the abundant minerals found in PS. Pumpkin seeds are also famous for the top natural sources of magnesium in the diets. The dehulled pumpkin seeds offer 500 g per 100 g magnesium, which covers an adult's daily magnesium requirement. Potassium content is also rich PS but while comparing with phosphorus and magnesium, it's not that high in level. This is because of the lower concentration in storage tissues. The normal range comes between 300 – 800 mg per 100 g. However, the sodium content is very low, that is approximately 20 – 40 mg per 100 g (Fedha, 2014). Due to this high potassium and low sodium level, it is considered to be advantageous for cardiovascular health and also helps in managing blood pressure (Arora et al., 2023).

In the trace mineral category, zinc is mineral which stands out the most. A number of studies found that pumpkin seeds are one of the best plant-based sources of zinc. It also improves the immune system and maintain prostate health in males (Arora et al., 2023). The whole PS has around 7 – 10% mg of zinc per 100 g and dehulled PS has bit higher level than whole PS that is 10 – 15% mg per 100 g. In a study on *C. moschata*, dehulled seeds has 11.1 mg zinc while the whole seeds contain only 8.1 mg per 100 g. The zinc level in normal PS can contribute to 70 – 100% of daily zinc intake requirement for an adult male so, PS is considered as a zinc supplement in vegetarian or vegan diets.

The moderate presences of iron in PS have some advantages (Aziz et al., 2023). The iron levels are roughly 5 – 15% mg per 100g. In *C. moschata* seeds, the iron levels for whole seeds are 7.4 mg and 13.6 mg for hull removed seeds. This indicates that better bioavailability and concentration of iron is when the seed coat or the hull is removed from the seeds. However, the iron present in the PS is non – heme iron. The absorption for non - heme iron is lower than that of iron getting from animal sources, regardless PS helps in contributing to iron intake like every other iron rich food. The other trace minerals found in PS are manganese, copper and selenium but in minor amounts (Gohari Ardabili et al., 2011). In research on *C. maxima* seeds, found that approximately 1.9 mg copper and 3.1 mg of magnesium in dehulled seeds and the levels are slightly lower in whole seeds. These trace minerals are required only in microgram to milligram amount as daily intake requirement, these help in increasing the overall nutritional benefits of pumpkin seeds.

The level of mineral content is higher in dehulled pumpkin seeds while comparing with whole seeds is due to the dilution of hull. The seed coat of PS is mainly consists of fibre with very negligible amount of minerals (Fedha, 2014). In the case of magnesium and zinc, the removal of seed coat helps in concentration of these minerals. Meanwhile, in the case of calcium, whole PS sometimes shows higher levels than dehulled seeds. Even so, the presences of calcium in PS are relatively very low that's makes PS a bad example of calcium rich food (Arora et al., 2023). All the mineral strength of pumpkin seeds lies in phosphorus, magnesium, zinc and iron also with moderate levels of potassium and trace amounts of copper and manganese. These mineral rich seeds will be an advantage in reducing deficiency related to micronutrients. A daily consumption of PS can help to contribute the daily intake of zinc and magnesium (Fedha, 2014). All common pumpkin varieties contain broad range of essential minerals. Even though there are slight differences in the level, all specie's seeds are very beneficial mineral source for human diet.

2.3.3 Amino Acid Profile of Pumpkin Seeds

The protein present in PS is not only in high in number but also high in quality for a plant-based protein, which containing all essential amino acids. The protein in PS is massively consist of globulins and albumins which acts as storage protein for the growing embryo. In several studies, characterisation of amino acids profile shows certain amino acid like glutamic acid, arginine and very lower levels of sulfur – containing amino acids. In study conducted by (Akintade Adeyanmola et al., 2019) shows the detailed amino acid breakdown for whole pumpkin seeds. In that, ample amount of amino acid was glutamic acid, around 13g per 100g of seed flour, followed by arginine with 10.2g, aspartic acid with 8.1g and alanine with 5.5g for 100 g of seed flour.

In the essential amino acids group, *C. maxima* have 5.8 g of lysine and 4.5 g of valine for 100 g of seed flour (Akintade Adeyanmola et al., 2019). These numbers indicate the balance of amnio acid pattern when scaled to protein content. For instance, in 100 g of protein, PS protein contributes to 17 – 18% glutamic acid and 14 – 15% arginine, these rich contents of amino acids are beneficial for metabolism as well as cardiovascular health of an individual. Many articles stated the high amount of arginine as a unique feature of PS. Arginine helps in wound healing, proper immune functioning and also contribute to improve cardiovascular health (Arora et al., 2023).

Pumpkin seed protein consists of all essential amino acids, due to the limiting amount of lysine and sulfur containing amino acids like methionine and cysteine, it is not always meeting as an ideal reference pattern (Fedha, 2014). In very earlier studies, they have reported the deficient in lysine and sulfur amino acids. The level of lysine comes around 5 – 6% of protein, which is slightly less than of soybean protein (6.5% lysine) but mostly higher than other available cereal proteins (Fedha, 2014). In *C. maxima* seed protein, methionine content is very low as 2.3g that is roughly 0.7% of protein and cysteine about 0.8g per 100 g seed flour. While combining both sulfur containing amino acids, it reaches only up to 1.5 – 2% of the protein, which is lower than Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) advised level that is approximately 2.5 – 3% for an adult. However, to rectify this limitation, PS can always pair with grains or seeds that are low in lysine and high in methionine. Despite the limitation, the overall amino acid range present in PS is beneficial. All the other essential amino acids like leucine, phenylalanine and valine are present moderate to high levels and meets the daily intake requirements for adults (Akintade Adeyanmola et al., 2019).

The dominant amino acid, glutamic acid comes around 15 – 20% of total amino acid is significant for glutamate and its amide form glutamine which is very important for cellular metabolism and contributes to the umami flavour of PS. The 10% of total amino acid of PS is arginine which has potential metabolic benefits. Due to the presences of arginine, PS are often recommended for athletes for cardiovascular improvement. Tryptophan is present in PS as very less quantity but has a crucial nutraceutical property. PS is suggested as a natural source of tryptophan which help in improving sleep and mood. In a health benefit point of view, consuming PS, which consists of tryptophan and magnesium helps in better sleep quality (Arora et al., 2023). Cucurbitin is a recognized amino acid compound found in PS. It has been investigated for its anti-parasitic properties in ancient or traditional medicines.

While comparing with various species, the amino acid profiles of *C. maxima, C. pepo* and *C. moschata* seeds kind of look alike. The accurate concentration can be different with various seed protein content and genotype. However, no unique amino acids tend to be found. All species shows a complete protein profile characteristics of cucurbit seeds. A study conducted in Kenya found that *C. maxima* and *C. moschata* seeds has 35 – 37% protein with similar quality (Fedha, 2014). Meanwhile another study on *C. pepo* hull – less variety has a slightly lower levels of total protein percentage, because of the presences of very high amount of oil, but the distribution of amino acids was similar with same major components. Hence, the pumpkin seed protein from different species can be used interchangeably for the nutritional benefits such as all are rich in glutamic acid, arginine and leucine, also consist of notable amounts of lysine and a very few amounts of methionine and cysteine. (Gohari Ardabili et al., 2011).

This protein quality cannot compare with animal proteins in every aspect, but it is sufficiently high that PSF is used to enrich the nutritional values of different cuisines. For example, PS protein has been incorporated into bakery products as well as in meats products to achieve desired nutritional benefits (Öztürk & Turhan, 2020; Shevchenko et al., 2022). In short, proteins from pumpkin seeds are nutritionally valuable protein, which is beneficial in human diets and functional food formulations.

2.3.4 Fatty acid profile of pumpkin seeds

The pumpkin seeds contain high concentrations of unsaturated fatty acids, mainly linolic acid (omega – 6) and oleic acid (omega – 9). The combination percent of both fatty acids come around 70 – 80% in most of the species such as *C. maxima* and *C. pepo* (Gohari Ardabili et al., 2011). During research, Indian grown pumpkin seeds shown 38% of linoleic acid and 28% of oleic acid (Ningthoujam et al., 2018). However, in Styrian *C. pepo* this ratio has some variations like linoleic percent is similar but oleic acid came up to 39% (Gohari Ardabili et al., 2011). The presences of these fatty acids result in similar quality when compared with sunflower and peanut oil, which supports in cardiovascular and metabolic health advantages. Variation in species and cultivars shows differences in fatty acid profiles. *C. maxima* have a high polyunsaturated fats, meanwhile *C. pepo* and *C. moschata* has a more balanced amount of mono and poly unsaturated fats (Kim et al., 2012). In a comparative study reported that, 56.8% of PUFA is present in *C. maxima* seed oil and in C. pepo it shows more of an oleic rich profile. These variations can be occurring due to the genotype, climate and difference in post-harvest methods (Ningthoujam et al., 2018).

In the case of saturated fatty acids, the main acids are palmitic and stearic acids which make up 10 – 25% of total lipids. Palmitic acid comes in the range of 10 – 17% and stearic can be different between 5 – 15% due to the variety of PS. Other components like palmitoleic and arachidic present in very minute amounts. In the process of extraction of oil, cold pressing and solvent extraction might affect the concentration of fatty acids however, some studies state that the overall profile will be identical with different extraction methods (Gohari Ardabili et al., 2011). Some factors like seed drying temperature and storage conditions can alter the accurate values but cannot make differences in the dominance of unsaturated fatty acids which indicates the seed's nutritional and functional benefits (Montesano et al., 2018).

2.4 Food Applications of Pumpkin Seed Flour

Pumpkin Seed Flour gained an interest among the people due to its functional and nutritional values. The increased desire of health-conscious individuals to consume protein rich, plant-based foods, PSF shows a favourable solution because of rich amounts of protein amino acids, dietary fibre, healthy fats and important minerals like zinc, magnesium and potassium. Meanwhile, the low glycaemic index level and the lack of gluten makes PSF a potentially useful ingredient in diabetic and gluten-free foods.

2.4.1 Bakery products

Pumpkin seed flour is mostly used as a natural ingredient in bakery products for improving nutritional benefits. PS contain notable amounts of proteins, fats, minerals and dietary fibres (Ningthoujam et al., 2018). The presences of these compounds can make PSF an excellent supplement which can be combined with wheat flour to make breads, biscuit and other bakery products to improve in their protein and micronutrient content. In an investigation on wheat bread, 10% of wheat flour is replaced with PSF result in an increase in mineral content of the bread mainly iron, zinc, magnesium and phosphorus. The iron content in the investigated bread was 6.7 times greater than the normal bread (Zlateva et al., 2022). This shows that with small changes greater nutritional benefits can be achieve by incorporating PSF.

In the case of bread, one study found that adding 15% PSF while making bread, improved its total nutritional properties (Shevchenko et al., 2022). The incorporated bread gained higher protein and fibre levels while comparing with the normal ones. In another similar study, PSF has been added to biscuit and wafers to enhance their bioactive compounds which resulted with greater functional benefits (Das et al., 2021; Karaś et al., 2024). In short, several investigations prove that incorporating PSF in bakery products like cookies, biscuit, cakes and other bakery products, largely enhances their nutritive profile without compromising their taste and texture.

2.4.2 Snack products

Pumpkin seeds have been used as a snack in very long time. The method of consumption was either roasted or used as a topping for salad in many communities (Beni & Edy, 2018). From the traditional use, PS is finding its path in modern day snack products. The addition of PSF in snack products can improve protein, fibre and micronutrients mainly in starch-based products to make them more nutritious choice. Defatted PSF, which is rich in protein content is added in recipes of crackers and chips to increases their protein availability (Ceclu et al., 2020). These fortification are very beneficial for vegan and gluten free consumers to attain daily protein intake and also creating a healthier snack version among common consumers. Extrusion is a crucial method in making ready to eat snack products. In an investigation, PSF has be added with corn masa to achieve protein rich puffed snacks. The result stated that, while PSF is added moderately the end product shows good expansion and, the protein content also drastically increased while comparing with common corn chips (Navarro-Cortez et al., 2016). The presences of several antioxidant compounds in PSF helps in longer shelf life and health advantages. The PSF can be used in many other products such as breakfast cereal and nutrient bar as it is convenient form of snacks. These convenient form of snacks can cater consumer demands like protein rich options, vegan options and health considerations.

2.4.3 Meat Alternatives

The presences of high protein and functional compounds in Pumpkin seed flour can be beneficial for utilizing as a natural ingredient for meat alternatives. When the fats are removed from the seeds, PSF contain more than 50% protein which also has well balanced amino acid profiles (Montesano et al., 2018). The presences of glutamate, arginine and other branched amino acids are responsible for nutrition and flavour. Cucurmoschin is a bioactive peptide found in PS, which has notable arginine and glutamic acids in them. The protein present in PS might mimic umami taste and has a good water and fat binding capacity which gives desirable texture which is crucial for meat analogues.

Many studies have taken place to check whether PS can be used as meat alternative. In an investigation, PS protein isolate was incorporated into a sausage formulation. By adding minor percent of isolate, the nutritional levels of the sausage were largely improved without compromising flavour and texture (Dan et al., 2022). In another study, gels made by heat treatment of PS protein isolate are made into meat like chunks which resembles the texture of chicken (Baig et al., 2023). These results can help in proving PS protein can be used as an ingredient in meat alternatives like soy and pea proteins.

2.4.4 Dairy and Dairy free Alternatives

Pumpkin seeds and flour can be used as both dairy and dairy free alternatives. One of the main PS dairy application is its pumpkin seed milk which is the blend of PSF in water that's gives a creamy and white – green emulsion. Naturally, it does not contain any lactose and cholesterol which is favourable for allergic and health-conscious consumers. PS milk contains all the nutrients that present in the seed such as proteins, healthy fats, minerals and antioxidant compounds. However, like every other seed milks, PS milk can also undergo phase separation when it is not stabilized. Many studies have taken place to improve this stability issue in PS milk and in one of the study with roasted PS shows no phase separation for 30 days shelf-life study where else in PS without treatment showed very faster separation (Yu et al., 2023). This convenient heat treatment improved the overall stability of PS milk which attracts more consumers by its visual satisfaction. PSF also has capabilities in making non-dairy cheese and yogurt alternatives. As PS contains high protein and oil content, it can provide creamy textures. PS milk can be also used to make soft cheese spreads and yogurt like products by fermentation process and adding stabilizers. In short, PS can be used to make dairy alternatives and vegan cheese and yoghurts. These ability can be advantageous for consumers seeking dairy alternatives with higher health benefits.

2.5 Allergenicity and Allergens in Pumpkin Seed

Usually, pumpkin seeds are considered as an ingredient of food allergy, however few cases have been reported (Rodríguez-Jiménez et al., 2009). PS contains allergenic proteins, during a study it has been identified that 2S albumin and 11S globulin are mainly responsible for triggering IgE – mediated reactions (Bueno-Díaz et al., 2021). These storage protein allergen compounds are found widely in many nuts and seed which can be related to pumpkin like melon and which are not related to pumpkin like cashew nut (Fritsch et al., 1997). Even though with least cases, these allergic compounds can bring serious allergic reactions.

The medical reports have both children and adults getting serious health issues with PS allergy. In a report, a 2-year-old child had symptoms of anaphylactic reactions with PS but tolerated pumpkin pulp. With an allergic skin prick testing it was confirmed a strong IgE sensitivity to the seed but no visual reaction to the pulp which directs us that allergic compounds are found in the PS not in the flesh (Gawryjołek et al., 2021). Likewise, in another report an adult person was hospitalized with IgE medicated anaphylactic shock after eating PS showing that these allergic reaction can come in any stage of life (Doll et al., 2017). PS consumption in allergic person can led to rapid swelling in the face and difficulty in breathing which need emergency medical attention (Rodríguez-Jiménez et al., 2009). These reports highlight that, even though these allergy reaction are very rare to occur in humans but can cause severe and systemic reactions to the susceptible peoples. In a food safety point of view, the presences of allergens can lead to a strict risk management in novel products. The usage of PS in many food items for its functional and nutritive benefits makes the PS allergy concern more prevalent. For example, an extreme allergic reaction was reported in a child after eating multigrain bread which contain pumpkin seeds (Chatain et al., 2017). To overcome these accidents, strict allergen control in formulations should be done. Novel products should contain clear labelling and warning to aware the consumers (Bueno-Díaz et al., 2021). By acquiring these steps can act as a preventive measure for the allergenicity of PSF and cater the potential consumers without compromising safety of consumers.

2.6 Beverage Fundaments and Classifications

A beverage is termed as any liquid food or drink that is for human consumption (Sharma et al., 2021). There are a wide range of beverages like normal water, milk, fruit juices and formulated drinks. Functional beverages are made up with health benefit compounds to impart positive benefits when consumed (Sharma et al., 2021). Nowadays, functional beverages are fortified with bioactive compounds to improves greater values. These beverages not only satisfy thirst but also imparts good health.

The main properties of a beverage are palatability, nutritional content and physiochemical stability. Palatability indicates the good flavour, aroma and the mouthfeel of the drink which is very important for consumer acceptances. Nutritional availability can be of important vitamins, minerals, antioxidants and other macro nutrients present in the beverage (Ferruzzi et al., 2020). Physicochemical stability is related to the behaviour of the beverages like phase separation or nutritional degradation while in storage conditions. It a challenging task for beverage makers to balance all these properties in their product (Neha & Neelam, 2023). The acceptances of functional beverage in market mainly depend on the satisfying consumer expectations. The recent consumers are drawn to the health conscious and functional advantages of beverage; however, these consumers also expect to get all the nutritional values with the same taste as in traditional drinks. So, the developer face challenges in incorporating bioactive components without compromising flavours.

2.6.1 Classification by pH

Beverages can be grouped into 3 categories by their pH levels. pH is one of the factor that affects the taste, shelf life and processing of beverages.

Acidic beverages: The drink that comes below 7 pH are normally classified as acidic. Many commercial acidic beverages range from 3 – 4 pH. Fruit juices and carbonated soft drink comes under this category, where its pH measures around 3 or less (Vogley & McClintock). These acidic beverages have a sour or tart taste due to the presences of organic acid content. These high levels of acid also help in eliminating microorganisms and extend shelf life of the product.

Neutral beverages: The drinks that comes around 6 – 7 pH are classified as neutral beverages. One of the common examples are milk, which is around 6.7 – 6.9 pH and pure water, which is 7 pH (Seychelle, 2023). Neutral drinks are slightly bland or not so acidic in taste. Microorganisms can easily survive and multiply in these beverages so treatment like pasteurization and sterilization are done to secure its safety and shelf life.

Alkaline beverages: The drinks contain pH level greater than 7 are consider Alkaline beverages. Alkaline drinks are not common in markets, however plant based or fortified drink can come in this category. They have a smooth or mild taste. Similarly, like neutral beverages, these beverages also cannot prevent microbial degradation so, they are undergone some microbe irradicating treatment and stored properly. Almond, soy milks and some specific vegetable juices comes under alkaline beverages.

3 Research Hypothesis

3.1 Research Aim

The aim of this project is to explore opportunities to increase PSF use in both powdered drink applications and liquid drink applications. The scope of the research will include reconstituted beverages using low shear and high shear to reflect spoon dispersible and smoothie-type applications respectively. Heat stability studies will include pasteurisation and UHT temperatures to reflect the refrigerated and ambient stable liquid beverage markets.

3.2 Research Objectives

1. To determine the homogeneity of pumpkin seed flour as a function of particle size distribution
2. To evaluate the solubility of pumpkin seed flour in as a function of temperature and pH ranges that are typical in beverage preparation.
3. To determine the protein – fat interaction and their distribution in the sediment collected after solubility characteristics by confocal microscopy.
4. To assess the different proteins that become soluble as a function of beverage preparation and processing using SDS – page.

3.3 Research Significance

This experiment will contribute to the crucial growing demand and interest in plant-based beverages by natural and functional ingredient that is Pumpkin seed flour. Even though with its rich nutritional benefits and sustainable method, it is not widely used as commercial beverage around the world.

By checking the different solubility patterns across different pH and temperature, this study result in some key data, which will help to design high protein plant-based drinks. This way it also helps to attain sustainability goals by utilizing and promoting discarded materials like pumpkin seeds. This study also outlines the challenges or barriers faced during different solubility studies with pumpkin seed flour.

4 Materials and Methods

4.1 Materials

4.1.1 Raw Material Collection

The raw materials were procured from Summer Hill Seeds (Whanganui, New Zealand). Husk less or naked variety of seeds were used to make the pumpkin seed flour. Visual inspection and proper grading are done to eliminate foreign material, damaged seeds and other debris prior to making the flour. These pumpkin seeds are cold pressed to extract the oil before grinding the press cake into flour. The collected pumpkin seed flour was stored under freezing conditions (-20°C) in airtight containers to prevent excess moisture absorption and preserve its initial quality.

4.1.2 Chemical Reagents

The chemical reagents used throughout the experiments are Milli – Q and reverse osmosis (RO) water, methanol, glacial acetic acid, concentrated sulfuric acid (H_2SO_4), potassium sulphate (K_2SO_4), selenium catalyst, boric acid, bromocresol green (0.1%), methyl red (0.1%), hydrochloric acid (HCl) of 0.1 M and 3 M, sodium hydroxide (NaOH) of 1M, Coomassie Brilliant Blue G – 250 stain, sample buffer with and without β – mercaptoethanol (BME), 10× Tris – Tricine SDS running buffer, Fast Green FCF and Nile Red. A protein standard of Precision Plus Protein™ All Blue was also used for SDS – PAGE standardization. All the mentioned used reagents were of analytical grade.

4.2 Methods

4.2.1 Proximate Analysis of Pumpkin Seed flour

Proximate analysis is conducted to understand the nutritional content of the PSF, which includes moisture, ash, protein, fats and carbohydrates. This nutritional content can give us idea on main composition in the flour which can help in functional food applications. The experiments are done based on standard AOAC procedures with few modifications which are detailed below.

- Moisture content: The moisture content was calculated using standard hot air oven method. Firstly, clean and dry aluminium dishes including their lids where preheated in the hot air oven for 60 minutes at 105°C to remove the residual impurities. Using tongs and gloves, the preheated moisture dishes were transferred to a desiccator for another 30 to 45 minutes and recorded the weight. 2 g of PSF samples were placed into the dish and noted the weight of dish and lid with the sample. And the dish is transferred to the hot air oven set at 105°C with the lid underneath. The sample was kept in hot air oven for overnight drying to eliminate all the moisture. After completely dried, the dishes are transferred to desiccator using tongs and gloves and allowed to cool for 30 to 45 minutes and noted the weight. Each of the samples was done triplicate and the average reading was reported. Moisture content was calculated using the following formula.

$$\% \text{ Total Moisture} = \left(\frac{W2-W3}{W2-W1}\right) \times 100$$

Where, W1= Weight of moisture dish and lid (g)

W2 = Weight of moisture dish + lid + sample before drying (g)

W3 = Weight of moisture dish + lid + sample after drying (g)

- Ash content: The Ash content is evaluated by burning a specific amount of PSF in the muffle furnace. Firstly, a clean and dry metal crucible was preheated for 1 hour in muffle furnace in 550°C to remove any residual impurities. With the help of heat-resistant gloves and tongs, the crucibles are transferred to the desiccator for about 30 minutes for cooling and the weight of empty crucible is noted. About 2.5 g PSF sample is added to the crucible and noted the weight of both sample and crucible. This is then transferred to hotplate under a fume hood and slowly heated so that the sample can be charred. Charring is done to avoid excessive smoke production and when smoke production is stopped it indicates that the charring process is completed. This charred sample is carefully transferred to the muffle furnace for 5 hours at 550°C. After this ashing process, the crucible is transferred to a desiccator for 60 minutes to cool down and weight is noted. Each of the sample was done triplicate and the average reading was reported. Ash content can be calculated with the help of the following formula.

$$\% \text{ Ash Content} = \left(\frac{W3-W2}{W1-W2}\right) \times 100$$

Where, W1 = Weight of crucible +Sample before incinerating (g)

W2 = Weight of crucible + Sample after incinerating (g)

W3 = Weight of empty crucible (g)

- Crude Protein: The crude protein content was determined using Kjeldahl method which includes digestion, distillation and titration to calculate the total nitrogen content. A conversion factor of 6.25 was used to calculate the crude protein from nitrogen.

  Digestion

  0.35 g of sample flour was weighted and transferred to a digestion tube. Two Kjeltabs (each containing 3.5 g of potassium sulphate and 0.0035 g of selenium catalyst) were added to the tube and 15 mL of conc. Sulphuric acid (H_2SO_4) were also added to it and left the sample overnight. On the next day, digestion was carried using block digestor. The temperature was slowly increased to 420°C and maintained for 45 to 60 minutes until the solution turns clears, shows the digestion is completed. Then the tubes are cooled under the fume hood for about 10 minutes and 40 – 50 mL of RO water is added slowly into the tubes.

  Distillation and Titration

  For distillation process, 25 mL of 4% boric acid solution (is made by dissolving 400 g boric acid in 6 litres of hot distilled water with 100 mL of 0.1% bromocresol green and 70 mL of 0.1% methyl red in alcohol and then diluted to 10 litres) was added to 250 mL conical flask. Then the digestion tube is attached to the distillation unit, and the receiver conical flask was kept in the specific position. Distillation process undergoes until all the ammonia has been transferred to the boric acid solution. After the distillation process, the conical flask was removed, and the solution was titrated with 0.1 M hydrochloric acid (HCl) to an end point of grey mauve. The crude protein is calculated using the below equation.

$$\%\text{Nitrogen} = \frac{(A\times B)\times 14\times 100}{1000\times C}$$

Where, A = mL of HCl used

B = molarity of HCl

C = accurate sample weight

With the nitrogen value multiplied by the conversion factor that is 6.25 to get crude protein content.

- Fat content: The fat content of PSF was analysed by giving to a certified nutrition analysing laboratory. The method used was Soxtec extraction method (AOAC 991.36). The test results were directly sent towards us which provides the total fat content of PSF.
- Carbohydrates: The carbohydrate content of PSF was calculated using a different standard approach that is the subtraction method. This involves subtracting the sum of moisture, ash, crude protein and fat content from 100. The equation follows

$$\text{Carbohydrates\%} = 100 - (\text{Moisture} + \text{Ash} + \text{Crude Protein} + \text{Fat})$$

This gives us the total carbohydrates that is the soluble and insoluble carbohydrates, present in the system.

4.2.2 Sieving and Particle size Distribution

To evaluate the different particle size of PSF, method of separation used was sieving. A set of stainless-steel sieves with various mesh sizes of 850 µm, 710 µm, 600 µm, 500 µm, 424 µm, 355 µm, 300 µm, and 212 µm were descending stacked on the mechanical sieve shaker (Electromagnetic Sieve Shaker EMS-8). Small stainless-steel balls of were introduced to overcome the sieve clumping during sieving process. After placing 100 g of PSF on the top sieve and the process carried out to run at 20 power for 30 minutes.

The total amount of flour settled on each sieve was carefully collected and recorded the weight with the weight of empty sieves. The percentage of flour remaining on each sieve was calculated and the readings was used to classify the particles into fine (≤ 300µm), moderate (>300 - < 600 µm) and coarse (≥600 µm) size categories.

4.2.3 Ash Content and Crude Protein Analysis on Different Particle Size Distribution

After the sieving, the powder in each of the three size categories was analysed for ash content, to check the mineral deposition in various particles. The ash content was evaluated with same procedure mentioned in Section 4.2.1

To examine the crude protein content of different particle sizes, the sieved powder was grouped into different categories based on its texture and fineness, coarse particles (≥600 µm), moderate particles (>300 - < 600 µm) and fine particles (≤ 300 µm), this grouping was done manually by observing the texture, colour and particle consistency. The crude protein evaluation for the three groups was done using Kjeldahl method, as explained in section 4.2.1. This experiment helps us to understand the distribution of ash and crude protein in different particles size in PSF.

4.2.4 Solubility Determination Using Moisture Dish Analysis

Sample preparation

The solubility determination for the PSF in water was determined under different temperature (30°C, 60°C and 80°C) and pH (3.0, 6.6 and 8.0) ranges. The pH of the solution was adjusted to the target pH after the addition of PSF. This study was adapted from (Natarajan, 2021) to understand the effect of both pH and temperature on protein solubility and the solubility of the PSF in aqueous solution.

For all the solubility testing, 200 mL of reverse osmosis (RO) water is taken into a 250 mL stainless steel beaker. Then the beaker is placed in a heat-controlled water bath to reach required temperature (30°C, 60°C and 80°C) and the temperature was monitored using a thermometer throughout the experiment. A flat blade propeller stirrer was used for the uniform mixing. When the desired temperature is attained, 13.2 g of PSF sample is slowly added to the stainless-steel beaker to avoiding clumping, while the propeller was stirring at 350 – 400 rpm. After adding all the sample into the beaker, the stirring speed is increased to 700 – 750 rpm.

For all the temperature range, pH was adjusted according to with the help of chemical reagents. To make the solution acidic (3.0 pH) 3 M HCl is used. In 6.60 pH, no adjustment was done as it is the natural pH of the PSF. In making of alkaline (8.0 pH), 1M NaOH is used. The amount of the reagent added to the solution during different temperature varies, as the following.

3 M HCl for 3.0 pH

- 30°C – 4.20 mL
- 60°C – 3.75 mL
- 80°C – 3.10 mL

1 M NaOH for 8.0 pH

- 30°C – 1.72 mL
- 60°C – 2.73 mL
- 80°C – 4.53 mL

Beakers were covered with aluminium foil during the solubility test to minimize the evaporation. The stirring was for 30 minutes for every conditions.

Moisture dish analysis

When determining the solubility, the bulk solution as well as the centrifuged supernatant is evaluated to find their solid content by the moisture dish method. About 2 g of bulk solution is pipetted into a pre heated moisture dish using Pasteur pipette. The total weight of the moisture dish, lid and sample is noted. Then the dish is heated in a steam bath of 100°C for 10 – 15 minutes and transferred to the hot air oven of 108°C overnight. The next day, these dishes are transferred to a desiccator for cooling down before the final weight is noted.

The leftover sample is poured into 50 mL centrifuge tube and centrifuged (ThermoFisher Scientific Multifuge X1R, Germany) at 700×g for 10 minutes to separate the supernatant. From the supernatant 2 g aliquot is pipetted to the pre heated moisture dishes and followed the same drying in hot air oven and weighting process.

The formulas to calculate the moisture and the total solids are,

$$\% \text{ Total moisture} = \% \text{ Total Moisture} = \left(\frac{W2-W3}{W2-W1}\right) \times 100$$

Where, W1= Weight of moisture dish and lid (g)

W2 = Weight of moisture dish + lid + sample before drying (g)

W3 = Weight of moisture dish + lid + sample after drying (g)

% Total Solids = 100 - % Total Moisture

And solubility of PSF was determined using the following formula,

$$\text{Solubility \%} = \frac{Total\ Solids\ of\ Supernatant}{Total\ Solids\ of\ Bulk\ Solution} \times 100$$

All the solubility tests are done in duplicates to ensure consistency and accuracy.

4.2.5 Proximate Analysis in the Sediment

After the solubility tests, the sediments are carefully taken out to estimate proximate analysis to check the residual nutritional profile. Moisture, ash content and carbohydrates were calculated using the same procedure outlined in section 4.2.1, however for the crude protein and fat content, the sediments are given to an accredited nutrition laboratory for evaluation. Crude protein was evaluated using Dumas method (AOAC 968.06) with a conversion factor of 6.25, while Soxtec extraction method (AOAC 991.36) was used to determine fat content.

4.2.6 Colour Analysis

The colour analysis was done on the centrifuged sediments that have been collected from the solubility studies to evaluate the effects of temperature and pH on the visual appearances of PSF residues. The sediments were carefully separated from the supernatant after the centrifugation and stored in freezing conditions.

The colour values are measured using Minolta Chroma Meter CR – 400 (Konica Minolta, Japan). It is a very accurate colourimeter which is designed to measure the colour of food powders and granules. The instrument was calibrated using white calibration tile with a refence value of Y = 86.6, x = 0.3162 and y = 0.3232 before every measurement to ensure the accuracy. The colour parameters are estimated according to the CIELAB colour space. In L* value represent the lightness of the sample, which ranges from 0 (black) to 100 (white). In a* value shows the redness and greenness of the sample; positive value represents red and negative value represent green. In b* value shows the yellowness and blueness of the sample; positive value represents yellow and negative value represent blue. The measurements are done in duplicates and average value of L*, a* and b* will be estimated for every sample.

4.2.7 Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to identify the structural arrangement and interaction of fat and protein in the centrifuged sediment.

A small amount of sediment was uniformly spread into microscopic slide. Two fluorescent dyes are used to identify the major components. Nile Red dye was used to stain for lipids and Fast Green FCF for proteins. Around 10 µL of both the dyes are added to the sample on microscopic slide and cover slip is used for covering the sample. The microscopic evaluation was conducted using a Zeiss LSM 710 confocal laser scanning microscope which has a 40× oil immersion objective lens. The captured images are analysed using ZEN 3.11 (ZEN lite) software, which allows us to see the distribution and arrangements of fats and proteins.

4.2.8 SDS- PAGE Analysis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS – PAGE) was done to determine the protein profile of raw PSF and the supernatant which collected from the solubility. A readymade gel of 16.5% Tris – Tricine Criterion™ (Bio – Rad Laboratories) was used for the test to resolve the proteins. This study will help us to compare the molecular distribution and the protein solubility under different conditions.

Approximately about 0.1g of test samples are being taken in a microtube and add 1 mL of Milli – Q water. This sample is vortexed to get full dispersion and a 5 µL aliquot is transferred to another microtube and made up with 95 µL of Milli – Q water to get the concentration of 1mg/mL. Two types of sample were prepared, first in reducing condition, in which 100 µL of sample is mixed with 100 µL of sample buffer with 2% β – mercaptoethanol (BME) and second type, non – reducing condition, in which 100 µL of sample is mixed with 100 µL of sample buffer without 2% β – mercaptoethanol (BME). These prepared samples are then heated in a water bath at 95°C for 5 minutes for the protein to denature.

The electrophoresis unit was assembled and filled with running buffer, which is made up by diluting 100 mL of 10×Tris – Tricine buffer with 900 mL of Milli – Q water. Then about 20 µL of prepared sample and 10 µL of protein standard (Precision Plus Protein™ All Blue prestained Protein standard) were loaded into their respective wells. The gel was run at a fixed voltage of 125 V and current ranging from 79 – 60 mA, till the dyes reach at the very end of the gel.

After the electrophoresis, the gel is transferred to a container and rinsed it for three times using Reverse Osmosis (RO) water to eliminate the residual salts. Then the container is filled with Bio – Rad Coomassie G – 250 stain and stained the gel for 60 – 90 minutes in a slower container shaker. After the staining, the gel is washed again with RO water and immersed in RO water for 30 minutes to reduce background staining. This gel is then stored in RO water at 5°C condition. The scanning of gels to see the protein bands was done using Molecular Imager Gel Doc XR system and images were analysed using Image Lab™ software version 6.1.0 build 7. This software helps in clear identification of protein profiles between raw and solubilized samples.

4.3 Statistical Data analysis

Microsoft Excel was used to present the data by calculating the mean ± SD (Standard Deviation) and Standard Error. Origin, 2025 and Microsoft® Excel® for Microsoft 365 MSO (Version 2507 Build 16.0.19029.20136) 64-bit were used to represent the result graphically. The mean values were determined in significant differences between the various treatments by one – way analysis of variance (ANOVA) Tukey HSD Test at $P<0.05$ significance level using Astatsa software.

5 Results and Discussion

5.1 Proximate Analysis of Pumpkin Seed Flour

The proximate analysis of PSF, used in this work, is shown in the **Table 3**. These values indicate that PSF is a low moisture, protein rich flour which contains moderate residual fat and notable mineral content. The nutritional content of the flour can depend upon the variety of PS and the grinding technique.

Table 3. Proximate values of Pumpkin Seed Flour

PROXIMATE	COMPOSITION (%)
MOISTURE CONTENT	10.8 ± 0.10
ASH CONTENT	8.9 ± 0.25
CRUDE PROTEIN	53.0 ± 0.23
CRUDE FAT	15.9*
CARBOHYDRATES	11.2**

Each value was calculated by the average of two duplicates ± standard deviation. The superscript * shows the value given by Nutritional Laboratory, Massey University. ** value was calculated by indirect subtraction method.

The PSF has a moisture content of 10.8%, this low moisture content is very beneficial for storage, it improves the shelf stability and microbial attacks (Öztürk & Turhan, 2020). The ash content was around 8.9 to 9%, which shows that there is notable amount of minerals present in the PSF. The most known minerals in PSF are phosphorus, magnesium, zinc and iron (Habib et al., 2015). The presences of the components is why the ash value is high.

Crude protein was around 53% of the total weight. This level of protein was very high in regards of plant-based flour. This also brings out the potential application of PSF as a protein source. When comparing with raw PS the protein levels come around 30 – 40% and in the hull less varieties it comes around 35% (Chatepa, 2022; Fruhwirth & Hermetter, 2007; Habib et al., 2015). The high protein levels found in the examined samples are due to the oil extraction process, which concentrated the proteins in the meal. Similarly, high protein concentrations are reported in defatted pumpkin seed flours. (Pham et al., 2017) reported around 56.8% protein in a hexane defatted *C. pepo* seed meal. The protein content of PSF is comparable with defatted soy flour in regard to the protein content and increase the chance of utilising the PSF to enhance or enrich the human diets. In the case of amino acids profile, the proteins of PSF have wide variety of essential amino acids, but there are limitations for some specific amino acids. Several research finalized that PS proteins can meet many amino acid requirements however, they are low in lysine and in some sulphur containing amino acids (Fruhwirth & Hermetter, 2007; Pham et al., 2017). This can be overcome by combining PSF with other protein sources which are high in lysine, so a balanced amnio acid profile can be achieved. Overall, the good quality protein present in the PSF can be help in making nutritionally valued ingredients.

The crude fat content was found around 15.9% in the sample, which indicates that the rest of fats were removed as oil by extraction methods. The leftover fats consist of essential fatty acids and tocopherols. The PS oil has a notable amount of unsaturated fatty acids like linoleic and oleic acids (Öztürk & Turhan, 2020). The presence of fat in the defatted PSF can improve the palatability and provide energy however, it also can affect the product's shelf life and product formulation. The presences of high amount of polyunsaturated fatty acids, PSF can undergo oxidative rancidity during improper storage (Öztürk & Turhan, 2020).

In point of making a beverage out of PSF, the oil present can help to bring a smooth mouthfeel and nutritional profile, but it can also, if there is not sufficient emulsification result in undesirable free oil and phase separation in a beverage.

By subtraction of fat, protein, ash and moisture contents, the carbohydrate present in the sample was calculated to be 11.2% of whole PSF. This low level of carbohydrate is normal for an oilseed derived flour and is similar to values reported as containing very low levels of starch and free sugars in the literature for PSF (Pham et al., 2017). The largest carbohydrate fraction is likely to be the non – starch polysaccharides and dietary fibre, only minor fraction is contributed by simple sugars. In an experiment conducted on defatted PSF, the crude fibre was around 3 – 4% with few amounts of soluble sugars and starch (Pham et al., 2017). In the case of hull less PS, the fibre present in the flour comes from seed kernel itself due to lack of the seed coat and resulting in moderate fibre amount. The presence of fibre can be advantage as it enhance the texture and water holding capacity of the flour during various food applications.

The whole pumpkin seeds from different Cucurbita species shows 25 – 39% of protein, 30 – 50% of fats and around 3 – 5% ash and the rest will be fibre and moisture (Syed et al., 2019). (Habib et al., 2015) reported 34.6% protein and 36.7% fats in PSF of *C. maxima* variety and of 3.8% ash. In another study in Malawi shows, 32.5% protein and 46.4% fat in *C. maxima* seeds which are not defatted (Chatepa, 2022). The flour made from a hull less variety of PS has a greater amount of protein and low amount of fat due to the oil extraction. This is similar to other defatted PS products, in which the protein levels can go higher than 50% as the residual fat is decreases (Pham et al., 2017). The ash content of 8.9% is significantly greater than that of raw and un defatted PS. PSF is a great source of minerals, for example, 100 g of PSF can satisfy roughly 148% of daily intake requirements for magnesium and a notable amount of zinc and phosphorus (Syed et al., 2019). In short, hull less variety PS with partial defatting can make up the flour with high amounts of proteins and minerals when compared to the normal PS which has the seed coat.

5.2 Sieving and Particle Size Distribution

The sieving of 100 g PSF gave us a wide variety of particle distribution within 850 – 212 µm range (**Figure 6**). As a result, a major part of the flour is of intermediate size, around one – third (~34 g) of the sample was collected on 300 µm sieve. The amount collected on 500 µm and 710 µm sieves are 16.6 g and 14.9 g respectively, which indicates that flour contains relatively coarse granules. The least collected sieves are 800 µm, 600 µm, 425 µm and 355 µm with 8.7 g, 9.1 g, 8.8 g, and 7 g respectively meanwhile, on 212 µm sieve only 2.2 g of particle were collected. These finding show that the PSF needs further milling, due to the large number of moderate to coarse particles and very little amount of fine particles.

The further milling is necessary as it helps to enhance the application of the beverage as well as other products which can be incorporated into. The coarse size particles often bring gritty mouthfeel and irregular mixing in the product formulation. In addition to this, large particles can disturb the water hydration, binding and textural properties which are very crucial in any type of food products.

In a visual observation, there was clear distinction between coarse and fine particle in the flour. The coarser particle fraction collected on the larger mesh sizes ≥ 500 µm, were a dark greenish – brown colour however, the finer fraction collected on the smaller mesh sizes were a lighter green colour. The coarser particles were postulated to contain more of outer seed pigments while, the finer particles are consisting of interior components. If this were true, then it would be likely that the composition of the particles would differ. Therefore, a proximate analysis of the fractions was undertaken.

During the sieving process, PSF particles tends to stick to the sieve meshes and formed clumping. To overcome this challenge, 10 stainless steel balls (Diameter = 12.61 mm and Weight = 8.35 g individually) were introduced to each mesh during the sieving process. The formation of lumps was believed to be the high content of lipids and protein present in the sample. The husk less variety of PS is naturally rich in oil content and prone to stickiness. In the sample, the presences of oil content would be the reason for particles to adhere to the sieve mesh. The protein in the PSF can also be adhesive when moist, these can be the reason for the clumping behaviour of PSF. The addition of stainless-steel balls helped in breaking the adhesion. After the sieving, each of the particles where collected and weighted separately to determine the particle size distribution.

There are not many studies done on the particles size distribution of PSF, most of the studies are conducted on the unsieved flour or the protein isolate from PS. However Tarek-Tilistyak & Stamusz, 2022, in a fortification experiment of biscuit using PSF used finely milled flour that had 72% of the particles smaller than 212 µm (Tarek-Tilistyak & Stamusz, 2022). The difference in the particle size can depend on the milling method as well as the fat content. The fat content of the flour taken for fortifying the biscuit was 5% as it was greatly defatted before milling to get fine, powdery consistency. The fat content was high in the sample PSF of this thesis and also it was milled in moderate conditions, the high oil content not only caused clumping issues, but it also potentially limited the particles to grind properly, as oil can lubricate and prevent fragments from further shattering.

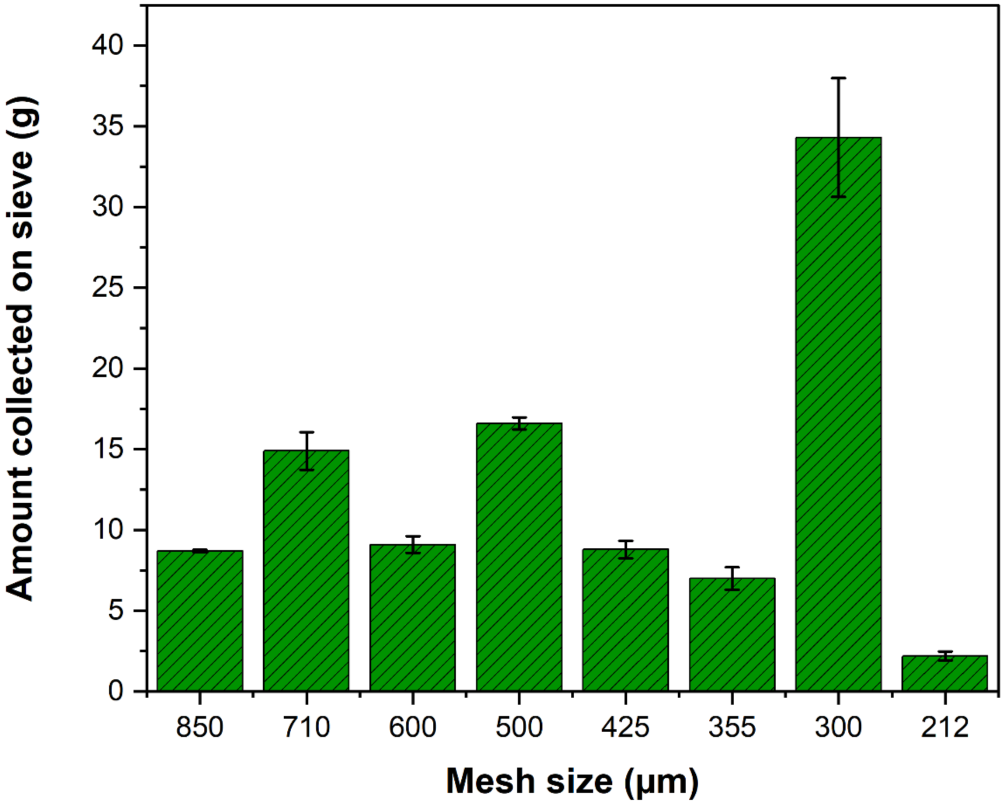


Figure 6: Different particle size after the sieving process. Data were obtained in duplicates and are calculated to standard error.

5.3 Ash and Crude Protein Composition as a Function of Particle Size

After the particle size distribution, the ash content was measured for each fractions. The ash content was between 8.1% to 9.9% for different sizes (**Figure 7**). The coarsest particle size fraction, those greater than 600 µm, showed the least level of ash content, which was around 8.2%. The medium sized particles, those retained on the 500 µm sieve, had the highest level of ash which was about 9.9%. The particles retained on the 425 µm and 355 µm sieves had an ash content levels of 9.1% and 8.9% respectively. Meanwhile, the 300 µm fine particle decline in ash level to 8.3% and in the finest particles of 212 µm, the ash content increased to about 9.1%. This non uniform pattern shows that the ash content levels not just increase and decrease with the size of particles.

The distribution of minerals in the flour can be linked to how the various seed components are getting partitioned during milling. The coarse particles consist of bigger granules and flakes of seed show the least levels of ash content. These particles are mostly from the inner cotyledon material which are usually rich in oil and proteins. The mid-size particles that are around 500 µm, may consist of seed coat or other structural components that are high in inorganic components. The husk less variety of pumpkin seed does not have a seed coat over its seeds, however they still have a thin seed coat called testa which is dark green in colour and fibrous material (Peričin et al., 2009). So, while the milling process, the fragile testa can break into small particles and fell into medium sieve range and increase the ash content of those particles. The increase in ash content at 500 µm can be of mineral rich particles while the finer 300 µm particles might consist of mostly the fine powdered cotyledon with very less ash content particles. The rise of ash content again in particles less than 300 µm can be the presence of fine powdered testa, making the ash levels to increase.

The raw pumpkin seed normally contains about 3 – 5% of ash content. (Uduwerella et al., 2021) reported that there is approximately 4.5 – 5.5% ash content in raw pumpkin seed which has seed coat. In another studies, PSF showed 3.8 – 4.4% ash content levels and 5.9% ash content levels which has the natural oil. The sample flour exhibits a higher level of ash content that is 8 – 10%. This can be due to the partial oil extraction and also due to the husk less variety. When 40 – 50% oil is extracted from the PS, this results in a relative increase in the concentration of minerals in the defatted meal. According to (Apostol et al., 2020), the oil pressed byproduct from dehulled pumpkin seed has a notable amount of minerals. Normally in PS with husk, the husk contributes to the ash content and fibre. However, the sample PS are husk less which leads to a uniform distribution of minerals across the different particle sizes.

The crude protein content was examined by Kjeldahl method, by grouping the various particles sizes into three groups based on their visual differences. The three groups are: coarse sized particles (≥600 µm), medium sized particles (>300 - < 600 µm) and fine sized particles (≤300 µm). Even though there were differences in the ash content, the protein content remained nearly uniform across the different groups. The coarse group consist of an average of 53.2% crude protein content, the medium sized group with 53.0% and the fine sized group with 52.9% crude protein content (**Figure 8**). In all the three groups contain around 52 – 53% of crude protein. The slight variation can be an experimental error. The reading indicates that different particle size does not affect the protein concentration in the sample PSF. Each of the grouped particle sizes were analysed in triplicates and showed high protein content with minimal deviation.

The readings can be justified by the nature of hull less PS. The protein present in the seed is stored in the cotyledons, which are milled into various particle sizes. Due to the lack of seed coat, there is not another components to disproportionately end up in the various particle sizes. All the particle sizes can mainly consist of the same material, which is the powdered cotyledon, in different sizes. So, the crude protein is similar across the particles. These crude protein values are impressively high for a plant-based flour. This can be of two main reasons, the intrinsic richness of PS in protein content and the elimination of the oil from the PS. The normal PS contains around 25 – 37% protein in them, meanwhile the seed also contain oil, which is nearly half of seed's weight that is to be extracted out, so the left meal will be concentrated with protein. The crude protein in the sample PSF, which is only partially defatted is nearly double of the normal PS. A fully defatted PS crude protein can be around 80% and to convert it into an isolate will involves additional steps to eliminate non – protein components.

In short, the particle distribution of hull less variety of PS had shown some variation in the ash content, meanwhile there was no notable difference in the crude protein levels within coarse, medium and fine sized particles. The coarser particles were darker in colour and has lower levels of ash content, which can be mostly composed of components that present on the surface of the seed, while the medium and fine sized particles mainly composed of the interior materials of the seed and well powdered testa particles, which makes the ash content to incline. The crude protein content was around 53% across the different particle sizes.

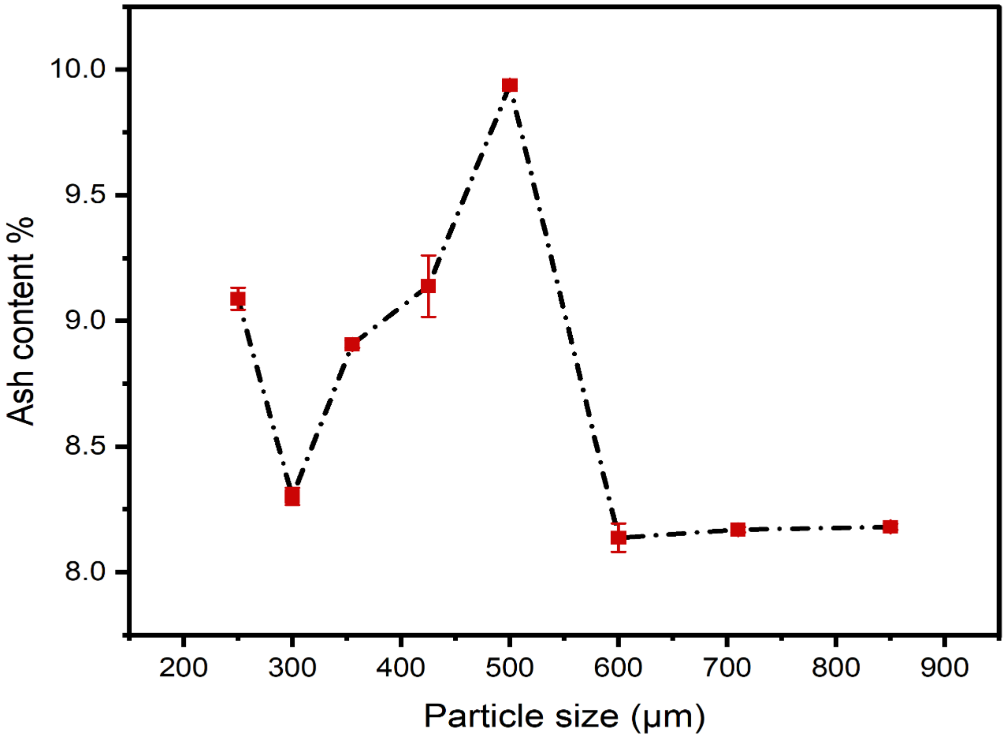


Figure 7: Ash content across various particle sizes of pumpkin seed flour. Data were obtained in duplicates and are calculated to standard error. Data were obtained in duplicates and are calculated to standard error.

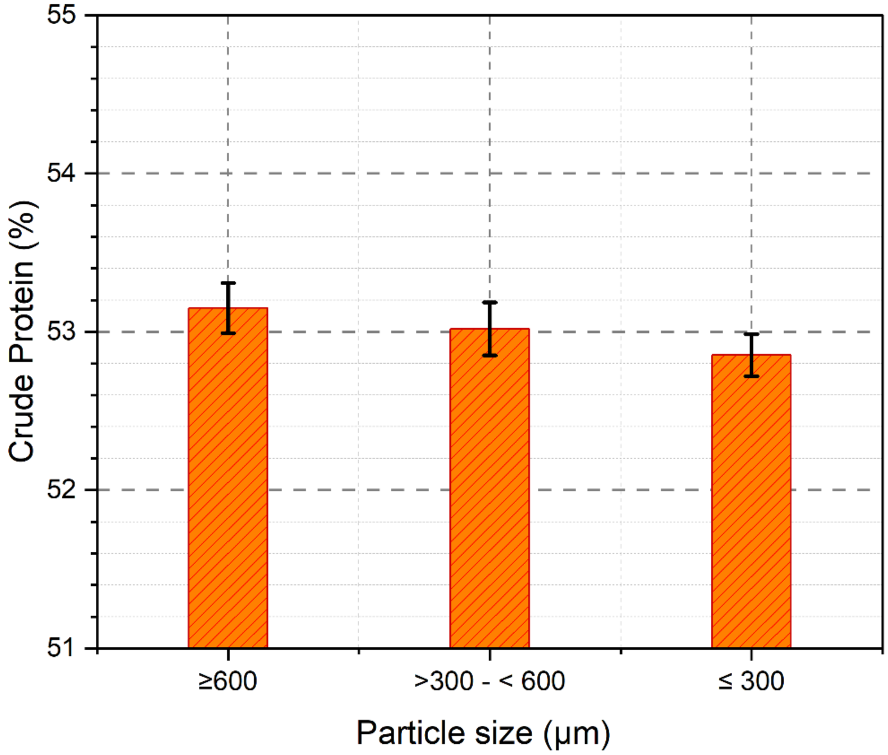


Figure 8: Crude protein content of pumpkin seed flour across the various particle size groups. Data were obtained in duplicates and are calculated to standard error.

5.4 Solubility of Pumpkin Seed Flour

The solubility of PSF is done on two main parameters that is pH (3.0, 6.6 and 8.0) and temperature (30, 60 and 80°C). Whole flour was used for this testing due to the difficulty in separating sufficient quantities of coarse, medium, and fine fractions and additionally that the difference in the crude protein and ash content across the different particle sizes was minor.

Effect of pH on PSF solubility: The PSF shows a very low solubility in water at pH 3 and 6.6, however, when the pH is adjusted to 8, the solubility increased significantly. At 30°C, the flour suspension in the water was only about 16 – 19% soluble under pH 3 and 6.6 conditions. In contrast the solubility was around 46% when dissolved at 8 pH (**Table 4**). This behaviour indicates that neutral and slight acidic conditions inhibit the suspension of the flour while in moderate alkaline conditions promote much better suspension. Similar findings regarding showing pH dependent solubility have been found in various studies. According to (Lazos, 1992), defatted PSF shows less nitrogen solubility between 3 and 7 pH but in alkaline conditions the nitrogen solubility sharply inclined. In addition to this, a study conducted by (Innocent-Ukachi & T, 2010) reported that the solubility was minimum in pH 6 – 8 range in case of *C. pepo* seeds and maximum solubility was observed at 10 pH.

In many seed flours, 6 – 7 pH ranges are the point of lowest dispersibility, which is due to the pH values resulting in the net charge of the flour components to be very low which in turn inhibits water – particle interactions. In the case of alkaline pH like 8 and above, the proteins and the polysaccharide in the flour have a tendency to hold more negative charge, which causes an electrostatic repulsion among the particles and enhance their ability to stay in the suspension (Rehab Abd-El Salam, 2013). In some cases, highly acidic media can also increase the solubility, such as in the study on PSF, from fluted pumpkin (*Telfairia occidentalis*) which shows better dispersion rate at pH 2 (Rehab Abd-El Salam, 2013). The improved solubility at these very low pHs is probably due to a high net positive charge as the pH moves away from the lowest isoelectric point of pH 3 for 11s proteins (Lazos, 1992). The solubility data reported here reflects the findings of Lazos (1992) who reported that nitrogen solubility for both *C. pepo* and *C. maxima* derived flours were minimal between pH 3 and pH 7 which they attributed to the range of isoelectric points for pumpkin seed proteins. At or near the isoelectric point proteins have minimal charge and tend to be insoluble. The high solubility found here at pH 8, which on the alkali side of the protein isoelectric points, is therefore likely due to increased negative charge (Lazos, 1992).

Effect of temperature on PSF solubility: Temperature has a great influence on the suspension and hydration of PSF. In all the pH ranges, the increase of temperature from 30°C to 60°C and to 80°C promoted a better solubility levels. At 8 pH levels, the solubility increased from around 46% at 30°C to around 64% at 60°C and again to around 65 – 70% at 80°C (**Table 4**). Similarly, in low pH levels this trend was also observed, although to a lesser degree. This can be due to the improved hydration of flour and particle dispersion in rising temperature. Heating can usually improve the water and food solids interactions: as water molecules attain a greater kinetic energy resulting in a decrease in viscosity and an increase in Brownian motion which collectively increase water ingress and diffusion through particles. The increase in water content in turn can then disrupt the intermolecular forces which hold the particles of flour together.

In the case of cereal and legume flours, heat treatments can disorient the physical structure of cell wall and promotes the conversion of the insoluble components into soluble form (Caprita et al., 2011). Heating can cause solubilisation of some components like dietary fibre and other polysaccharides present in the flour which is indicated by the increase of water extractable solids during heating (Caprita et al., 2011). The temperature solubility profile for the sample PSF data reported here follows this pattern - and is therefore likely to be due to temperature induced solubility of soluble fibres and other macronutrients from the PSF particles and increase the solid content in the supernatant.

The maximum solubility outcome was obtained under the highest heat treatment in alkaline condition that is 8 pH at 80°C, nearly two thirds of the flour mass remained dispersed in the water following centrifugation. This indicates that the alkaline condition and heat application can enhance flour hydration and dispersion. The alkaline condition helps increase electrostatic repulsion between the flour components, and the application of heat helps in soften the particles and increases the molecular mobility. There are several studies which supports the finding which are also conducted on plant-based protein and flours. Studies reported that PSF and protein isolates are very less soluble in neutral and slight acidic pH however, solubility levels are very much enhanced when it comes to alkaline pH (Innocent-Ukachi & T, 2010; Lazos, 1992). The solubility enhancement using heat treatments are also reported with other seed flours. Wheat and barley flours which are heated to 100°C had an increase level of soluble fibre fraction by 20 – 30%, which shows a conversion of insoluble components to soluble components (Caprita et al., 2011). In addition to this, there were no signs of coagulation of flour compounds during the heating process. Some high protein flours tend to coagulate with high temperature. However, the heating process done on sample PSF was helpful for better flour dispersion and hydration.

Table 4: The pH and temperature influencing the solubility in pumpkin seed flour.

PH	TEMPERATURE	SOLUBILITY
3	30°C	16.7 ± 0.3^{a}
	60°C	20.0 ± 0.1^{a}
	80°C	22.0 ± 0.5^{a}
6.6	30°C	17.8 ± 0.8^{a}
	60°C	21.3 ± 0.8^{a}
	80°C	22.3 ± 3.0^{a}
8	30°C	46.2 ± 0.3^{b}
	60°C	64.5 ± 0.3^{c}
	80°C	66.5 ± 3.8^{d}

Data were obtained in duplicates and are calculated to standard deviation. Different superscript letters within a row indicate significant differences by Tukey HSD test ($P < 0.05$).

5.5 Proximate Analysis in the Sediment

The sediment was in a wet condition while collecting it after the centrifugation process. The sediment proximate analysis is shown on **Table 5** and **Figure 9**. The moisture levels where around 76.8% - 87.9% across the varying pH and temperature parameters (i.e. total solids of sediments varied from 12 – 23%). The moisture content is indicative of the degree of water absorption by the flour and the water holding capacity of the insoluble (sedimentable) fraction. At pH 3 there is a slight increase in moisture content from 30°C to 60°C but no further increase in moisture content with heating at 80°C.

In order for the moisture content of the sediment to increase there must be increased non-compressible voids (under centrifugation conditions) and/or increased water association with sediment components. Non-compressible voids could be produced by the solubilisation of fat, protein, or carbohydrate bodies which would leave a void. If this were the dominant mechanism, for increased sediment moisture content then one would expect to see a correlation between their sediment content and moisture content.

To investigate this theory sediment moisture content was plotted as a function of the composition of each sediment. These plots are shown in **Figure 10** to **Figure 13** together with a trendline and correlation coefficient. The correlation coefficient for these plots shows that there is no correlation of moisture content with either ash (R^2=0.22) or carbohydrate content (R^2=0.18). The plot of fat against moisture content (**Figure *11***) shows that there is a somewhat weak correlation (R^2=0.40) with the moisture content increasing with increasing fat solubilisation. In contrast to the other components the plot of protein against moisture content (**Figure 10**) shows that there is a strong correlation (R^2=0.91) with the moisture content increasing with increasing protein solubilisation.

Turning to the coefficients for both the fat and protein equations we see that for every percent of protein that becomes soluble there is a 1 % increase in sediment moisture content. Solubilisation of fat however has twice the impact on moisture content as protein solubilisation.

The analysis above suggests that the main factor resulting in the increase in sediment moisture content is through water entrapped in non-compressible voids resulting from migration of protein and fat bodies from the flour to the soluble phase.

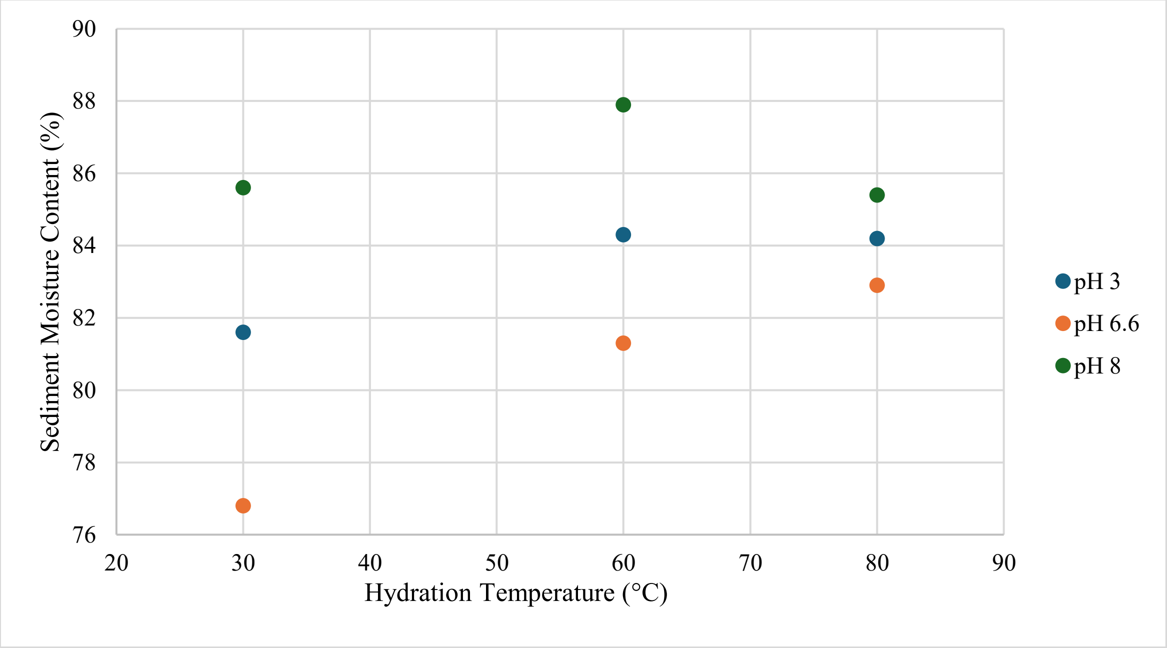


Figure 9: Plot indicating hydration behaviour of pumpkin seed flour in various temperature and pH treatments.

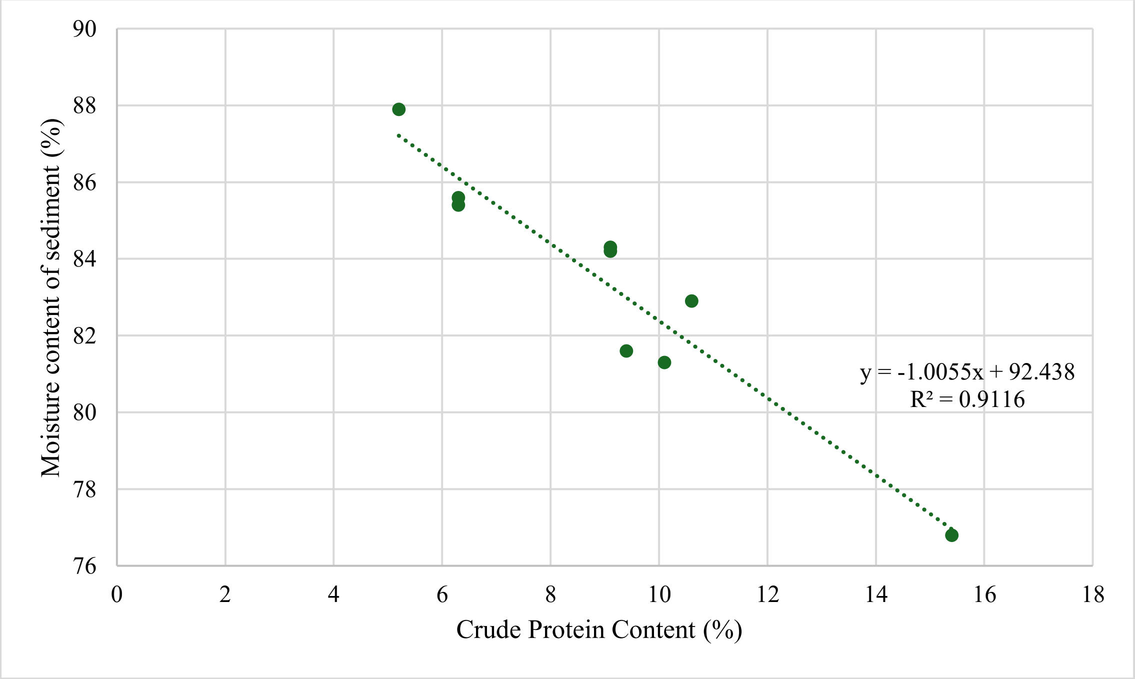


Figure 10: Plot indicating moisture content of the sediments in related to the crude protein content.

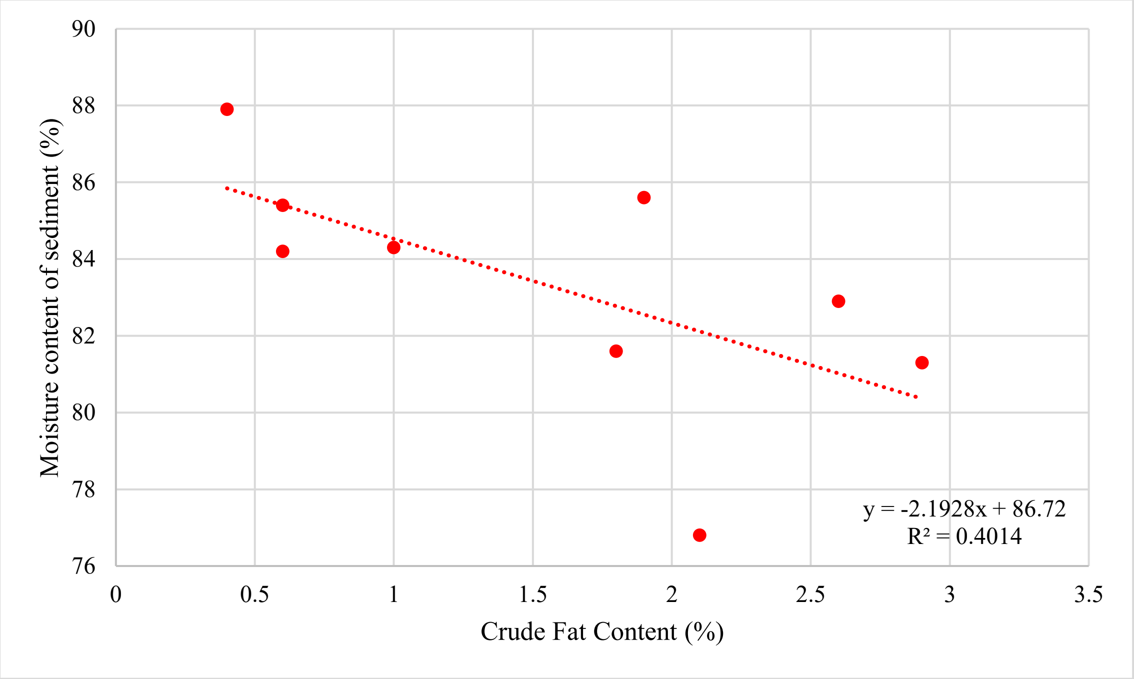


Figure 11: Plot indicating moisture content of the sediments in related to the crude fat content.

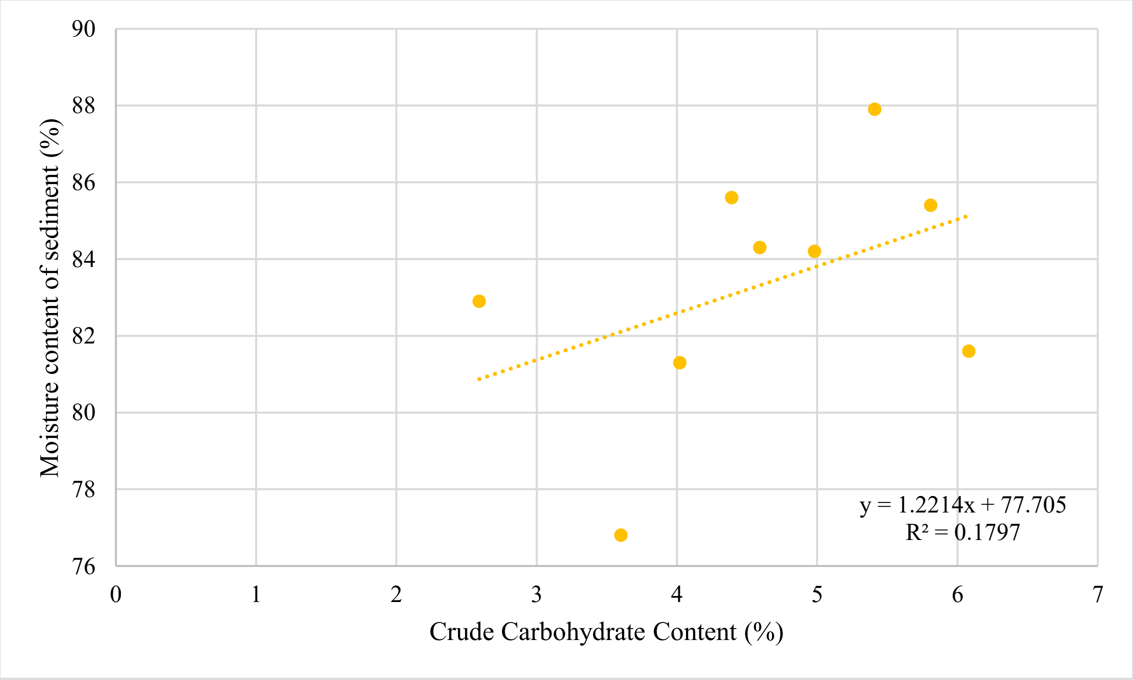


Figure 12: Plot indicating moisture content of the sediments in related to the crude carbohydrate content.

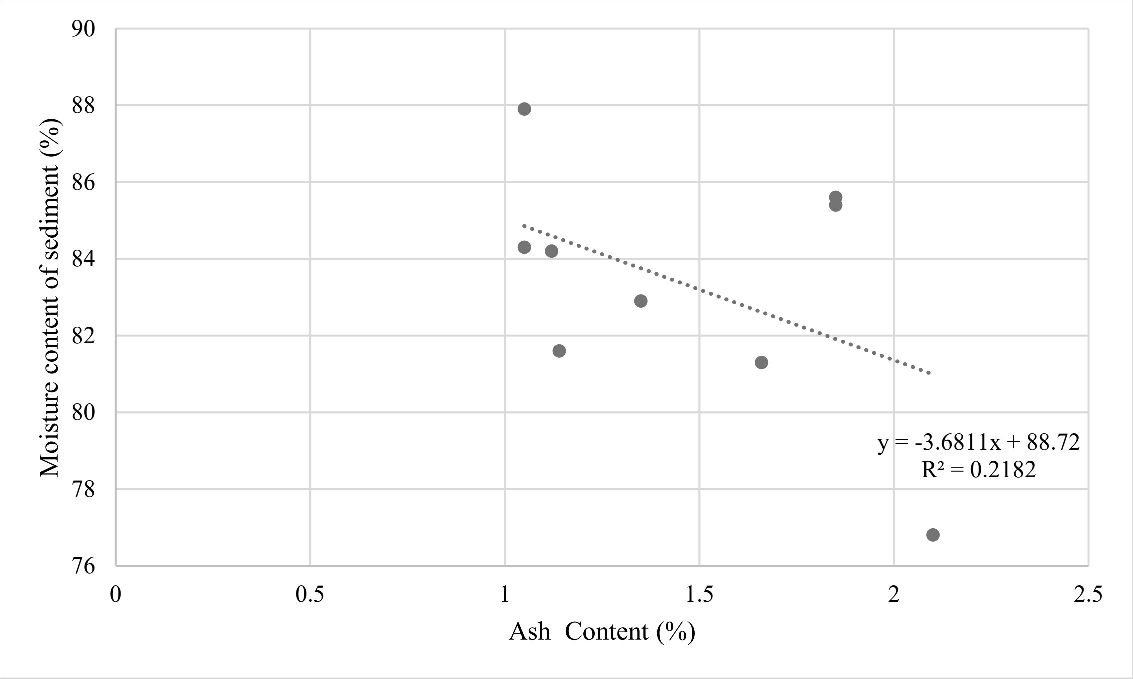


Figure 13: Plot indicating moisture content of the sediments in related to the ash content

The main components present in the dry matter are protein, minerals, fat and carbohydrates. At neutral conditions, the protein content in the sediments were high specifically at low temperature, about 15.4% of protein was present at 30ºC. However, at alkaline conditions, the protein in the sediments were much lower levels, about 6.3% at 30ºC, which indicates that most of the proteins got solubilized in the water. Under acidic conditions, protein comprised around 9.4% of the sediment weight at 30ºC and a similar value was found at 6.6 pH, however, increasing temperature from 30ºC to 60ºC reduced the protein content in the sediments from around 15% to 10%, indicating that protein solubility increases with increases in temperature levels. Similar observations have been made by other researchers investigating protein solubility and temperature (Gao et al., 2021; Sert et al., 2022).

In alkaline conditions (pH 8), there was a very little protein present (6%) in the sediments at 30ºC and when the PSF was hydrated at 60ºCthe protein content in the sediment reduced to 5.2%. There was no further decrease in sediment protein contents when the hydration temperature was increased to 80°C.

Meanwhile, in pH 3 conditions the protein levels were around 9% and did not vary with increasing hydration temperature. The alkaline conditions showed that most of the protein have been solubilised in the water and least amount have been retained in the sediments whereas, around the neutral pH had most of the protein remained in the sediments with little solubilized in the water. Similar findings have been reported in different studies conducted on pumpkin seed proteins. The higher pH greatly improves the solubility of proteins and when the pH is near to neutral, the solubility will be reduced to minimal levels (Sert et al., 2022). The sample PSF proteins also show minimal solubility at neutral pH ranges and a comparatively high protein solubility in both high acid and alkaline pH. During the pH 8 conditions, protein contain a negative charge which help them to dissolve in the water than settle in the sediments. Meanwhile, at pH 3, below the isoelectric point, protein acquires a positive charge and can be solubilised to the maximum (Vinayashree & Vasu, 2021). From the results, alkaline conditions are much more effective in maximum protein solubility than acidic conditions.

In 6.6 pH at 30ºC, the sediment has around 23% dry matter content with around 77 % moisture, 2.1% ash, 15.4% protein, 2.1% fat and 3.6% of carbohydrates. Meanwhile, in pH 8 at 60ºC, the sediments have around 12% dry matter content with around 88% moisture, 1.05% ash, 5.2% protein, 0.4% fat and 5.4% carbohydrates. The rest of the conditions are within these extremes. For instance, pH 3 at 30ºC sediments consist of 82% moisture, 9.4% protein, 1.8% fat, 1.1% ash and 6.1% carbs meanwhile, 6.6 pH at 80ºC sediments has around 83% moisture, 10.6% protein, 2.6% fat, 1.35% ash and 2.6% carbohydrates.

The moisture content is inversely related to the protein levels that is when more amount of proteins is present in the sediments, the moisture levels of the sediments will be little bit lower. This illustrated in **Figure *10***. For example, the high protein sediment was in pH 6.6 at 30ºC, which had the lowest moisture content that is 76.8%, likewise low protein sediment was in pH 8 at 60ºC had a high amount of moisture that is 87.6%. This is because the sediment containing more dry solids will contain less water proportionally. Additionally, protein and fibre present in the sediment promotes water holding capacity, so the differences in protein and fibre levels in sediment can influence the presences of moisture retained in sediments (Rehab Abd-El Salam, 2013).

Even though there was variation in the protein levels, the ash content present in the sediment remained in less levels and did not change notable in various parameters. The ash content was in a range between 1 – 2.1% across the all the samples. When this value is converted to dry solids, it comes around 5 – 10% of minerals are present in the sediments. In the results, the highest ash content is noted on neutral pH and low temperatures meanwhile, lower ash content was noted in some heated and extreme pH parameters. The small decline in ash content in pH 8 at 60ºC can be the leaching of minerals to the water under the influence of heat or pH. Moreover, the ash content levels reduced from 2.1% to 1.35% when the temperature is increased from 30ºC to 80ºC, which indicates that in higher temperature some of the minerals can leach or solubilize into the water, but these differences are very minute (Syam, 2023). Most of the minerals present in PSF are in the sediments, it also indicates that influence of external parameters may not affect mineral solubility.

The presences of crude fat in the sediments were very low as the PS was defatted before grinding into flour. The crude fat levels are between 0.4% - 2.9% across all the samples. In dry solids point, the crude fat contributes to 5 – 13% in the sediments. During the neutral pH, the fats in sediment was 2.1% at 30ºC and 2.6 – 2.9% at 60ºC & 80ºC meanwhile, in extreme acidic and alkaline conditions fats tended to decrease with the temperature increasing. In pH 3 the fat level declined progressively from 1.8% at 30ºC to 0.6% at 80ºC. Similarly, in pH 8 the fat level declined from 1.9% at 30ºC to 0.4% at 60ºC. These finding suggest that increasing hydration temperature can be advantageous in eliminating fats from sediments by dissolving the residual fats and promoting its migration into the water phase.

The carbohydrates were calculated using indirect difference method, it contributes to around 3 – 6% of the total sediments. On a dry solids basis this equates to around 10 – 16%. The main components present in the sediment are the fibre and other non-protein, and non-fat organic materials. There was not a significant trend in carbohydrate composition across different parameters. The carbohydrate fraction is likely to be fibre in the PSF which mainly comes from the seed coat part and other insoluble polysaccharides. These component always remains in the sediment under all conditions by providing a carbohydrate level in proximate analysis.

Table 5: Proximate analysis of PSF sediments undergone various pH and temperature variations.

pH	Temperature (ºC)	Moisture content (%)	Ash content (%)	Crude protein* (%)	Crude fat** (%)	Carbohydrates***(%)
3	30	81.6 ± 0.8^{b}	1.14 ± 0.02^{a}	9.4	1.8	6.08
	60	84.3 ± 0.7^{c}	1.05 ± 0.04^{a}	9.1	1	4.59
	80	84.2 ± 0.4^{c}	1.12 ± 0.04^{a}	9.1	0.6	4.98
6.6	30	76.8 ± 0.3^{a}	2.1 ± 0.10^{c}	15.4	2.1	3.6
	60	81.3 ± 0.4^{b}	1.66 ± 0.002^{b}	10.1	2.9	4.02
	80	82.9 ± 0.1^{bc}	1.35 ± 0.04^{ab}	10.6	2.6	2.59
8	30	85.6 ± 0.1^{c}	1.85 ± 0.09^{bc}	6.3	1.9	4.39
	60	87.9 ± 0.1^{d}	1.05 ± 0.04^{a}	5.2	0.4	5.41
	80	85.4 ± 0.2^{c}	1.85 ± 0.07^{bc}	6.3	0.6	5.81

Data were obtained in duplicates and are calculated to standard deviation. Different superscript letters within a row indicate significant differences by Tukey HSD test ($P < 0.05$). * and ** values are tested in nutritional laboratory, Massey University. *** value is calculated through indirect difference method.

5.6 Colour Analysis of sediments

The colour analysis was done on centrifuged sediments samples collected from the solubility studies with variation in pH and temperature (**Table 6**). This analysis helped to obtain visual quality metric and consumer insight on PSF sediments after it has been to different pH and temperature hydration processes.

There was a uniform trend in all different pH levels. The L* values increased with temperature rise at 3 pH, meanwhile the value decreased at pH 6.6 and pH 8. At pH 3, the L* value increased from 70.55 at 30°C to 73.61 when reaching 80°C, which shows a gradual brightening of the sediment samples. This can be due to the reduced pigment stability and leaching process happening in acidic and high temperature conditions. At 6.6 pH, L* value declined from 66.53 at 30°C to 63.99 when reaching 80°C and at 8 pH, it declined from 64.69 at 30°C to 56.48 when reaching 80°C, this can be due to the browning reaction like Maillard reaction, which can happen under neutral to alkaline conditions as well as increasing temperature.

The a* values were negative in all conditions, showing that sediments have mainly green hues. The highest negative values were in 8 pH at 30°C around -5.16, this can be the retention of chlorophyll under low temperature in alkali conditions. When the temperature increased, the a* values showed less negative, indicating that degradation of pigments and browning effects mask the green hues. This gradual dimmish of green hue in rising temperature in neutral and acidic conditions, leads the breakdown of pigment and formation of brown coloured compounds that covers the initial hues. In the case of b*, at 3 pH the value declined from 14.87 at 30°C to 11.45 when temperature was 80°C, mostly because of the heat degradation of yellow colour pigments such as xanthophylls. However, at 8 pH the b* value increased from 15.41 at 30°C to 16.42 at 60°C and again decreased to 15.94 at 80°C. These differences can be the interaction between heat related colour degradation and the development of Maillard reaction.

In short, the results indicate that both pH and temperature have a strong influences on the visual quality of sediments. Alkaline conditions with high temperature showed the most browning effect and acidic conditions with low temperature maintained brighter colour.

Table 6: Colour analysis of PSF sediments undergone various pH and temperature parameters.

pH	***Temperature (ºC)***	***L****	***a****	***b****
3	30	70.6 ± 1.3^{c}	-3.5 ± 0.03^{bc}	14.9 ± 1.4^{ab}
	60	71.4 ± 0.5^{c}	-2.8 ± 0.04^{cd}	12.7 ± 0.4^{ab}
	80	73.6 ± 0.1^{c}	-2.6 ± 0.1^{d}	11.4 ± 0.1^{a}
6.6	30	66.5 ± 1.1^{bc}	-4.2 ± 0.2^{b}	14.6 ± 0.6^{ab}
	60	65.6 ± 0.7^{b}	-3.5 ± 0.2^{bc}	15.0 ± 0.5^{b}
	80	64.0 ± 1.1^{b}	-3.3 ± 0.02^{c}	13.9 ± 0.2^{ab}
8	30	64.7 ± 0.9^{b}	-5.2 ± 0.1^{a}	15.4 ± 0.9^{b}
	60	58.5 ± 0.5^{a}	-3.9 ± 0.1^{bc}	16.4 ± 0.2^{b}
	80	56.5 ± 0.9^{a}	-3.6 ± 0.2^{bc}	15.9 ± 0.4^{b}

Data were obtained in duplicates and are calculated to standard deviation. Different superscript letters within a row indicate significant differences by Tukey HSD test ($P < 0.05$).

5.7 Confocal Laser Scanning Microscopy (CLSM) Analysis on Sediments and Supernatants

The confocal laser scanning microscopy was done to see the various distribution and interaction between fats and protein in the sediments as well as the supernatants which collected from the solubility experiments. Different images on varying pH and temperature, helps us to get an idea of how these parameters can influence the distribution and structural changes of fat and protein.

In pH 3 at 30ºC, the protein is dense with irregular aggregates, which shows large protein settlement in the sediments (**Figure 14**). The fat can be visible as small droplets and few larger droplets. Some of the fat droplets are embedded within and some are adjacent to the proteins. A noticeable large fat droplet is partly attached to the protein aggregates and numerous small fat droplets are scattered across the protein aggregates. This indicates that the protein had aggregated and trapped fat droplets with them. The protein aggregates prevent the movement of fat droplets by entrapping them. While some larger fat droplets have undergone partial mixing with the protein, and the smaller droplets remain separate from mixing with the aggregate. The sediment reflects low protein solubility at pH 3 with large amounts of aggregation with also entraps fat droplets.

In pH 3 at 80ºC, the protein is still in aggregated state, but the cluster seems finer and fragmented while comparing with 30ºC which shows that the protein networks break upon rising in temperature. A spherical larger fat droplet with very little protein aggregation around it, no partial or fully mixing of fats and protein can be noticed (**Figure 14**). The large fat droplets indicates that on increasing the heat, the merging of smaller fat droplets take place and proteins denatured with minimal stability. Some smaller fat droplets can be seen however, they are still attached with the proteins, which shows the preserving of some emulsified fat. The association of fat and protein is continued but in lesser extent. The emulsification has been weakened by the application of heat. The smaller fat droplets combined into one bigger droplet instead of uniform dispersion. This sediment shows presences of protein in pH 3, and fragmented protein aggregates became smaller. Meanwhile, the oil droplets combined into one big oil droplet.

In pH 8 at 30ºC, proteins show aggregates indication some fraction of protein bodies are still collocated however the clusters seem to be less dense with loose network when comparing with the pH 3. The big spherical fat globule can be seen at the bottom centre and smaller fat droplets across the protein aggregates (**Figure 14**). This shows that the soluble proteins are coating the oil droplets. The presences of big and smaller oil droplets indicate partial mixing of components. The occurrences of partial emulsification when the large oil droplets show insufficient interfacial stabilization that inhibit mixing and indicating emulsified fat by the presences of small oil droplets in the protein aggregates. This sediment shows a mixed state with moderate protein aggregates; the lipids are in bigger as well as smaller droplets. pH 8 helps in improving protein solubility however, there are some protein in the sediment fraction which interact with the oil.

In pH 8 at 60ºC, the conversion from the solid dispersion to aggregates, the protein gets partial denatured. Several oil droplets can be seen across the protein aggregates. The proteins form different clumps and networks in which some are similar to spherical, and some are filamentous (**Figure 14**). The large oil droplets can be seen however the presence of fine sized droplets have been increased. This can be due to application of moderate heat which turned bigger droplets into fine sized droplets. The majority of oil droplets are surrounded by the protein fragments indicating stronger emulsification effect. The occurrences of small droplets state that 60ºC can help in better emulsification than 30ºC, this is likely due to the partial unfolding of protein to increase interfacial adsorption before getting aggregation. In the sediments, the protein and fats are partially distributed in which oil is stabilized by aggregated protein.

In pH 8 at 80ºC, the proteins have been fully denatured and highly aggregated with removing oil droplets. In the image, there are several large spherical components which has small internal voids like patterns (**Figure 14**).

These void-like patterns support the theory discussed in section 5.5 which hypothesised that the observed increase in moisture content as a function of protein and fat solubility might be due to non-compressible voids. The presence of these void-like structures was more prevalent in sediments that possessed higher moisture contents.

In the supernatant of pH 3 at 30ºC, a very few oil droplets can be seen as dispersed. From the image it is visible that the green and red droplets are scattered evenly (**Figure 15**). However, large fat droplets with surrounding smaller fat droplets rings can be seen and inside the big fat sphere, a bright green ring is visible. These indicates an oil in water droplet; the green ring is formed by the protein fraction solubilized at pH 3 and coated the oil surface. Most of the protein particles have not solubilized and settled in the sediments leaving the supernatant with very few particles. The emulsification network is weak with limited protein availability and only some droplets remained to exhibit incomplete coating.

In pH 8 at 30ºC, most of the soluble proteins appear as uniform sized protein bodies in the range of about 1 µm in size. This size is significantly smaller than the oval shaped protein bodies (7 x 10 µm) that were observed in cotyledon cells extracted from germinating pumpkin seeds by (Hara & Matsubara, 1980). In the image, a noticeable big oil droplet which has green stains inside as well as on the edges can be seen with few small droplets of protein at the surroundings (**Figure 15**). This indicates that the big oil droplet has been partially coated with the proteins. The green stain presents inside as well as the edges can be the proteins coated within the droplet by forming interfacial complexes. The absences of smaller fat droplets states that the oil droplets have merged to single droplet, due to low shear and emulsification.

In pH 8 at 60ºC, the supernatant contains a large spherical oil droplet with small oil droplets surrounding around it (**Figure 15**). There are visible green stains at the edge of the big oil droplet, which indicates the solubilized protein have been stabilized the droplet. The rise in temperature causes partial destabilization, making the oil droplet bigger and many small oil droplets remain in the surroundings due to not fully mixing or distributed. The supernatant contains mixed sized emulsion such as the presences of big as well as several small oil droplets. When comparing with 30ºC, there was only one droplet present however at 60ºC there are heterogeneous distribution of droplets can be seen with partial denaturation. The supernatant shows oil in water dispersion but the application of increasing temperature results in structural changes.

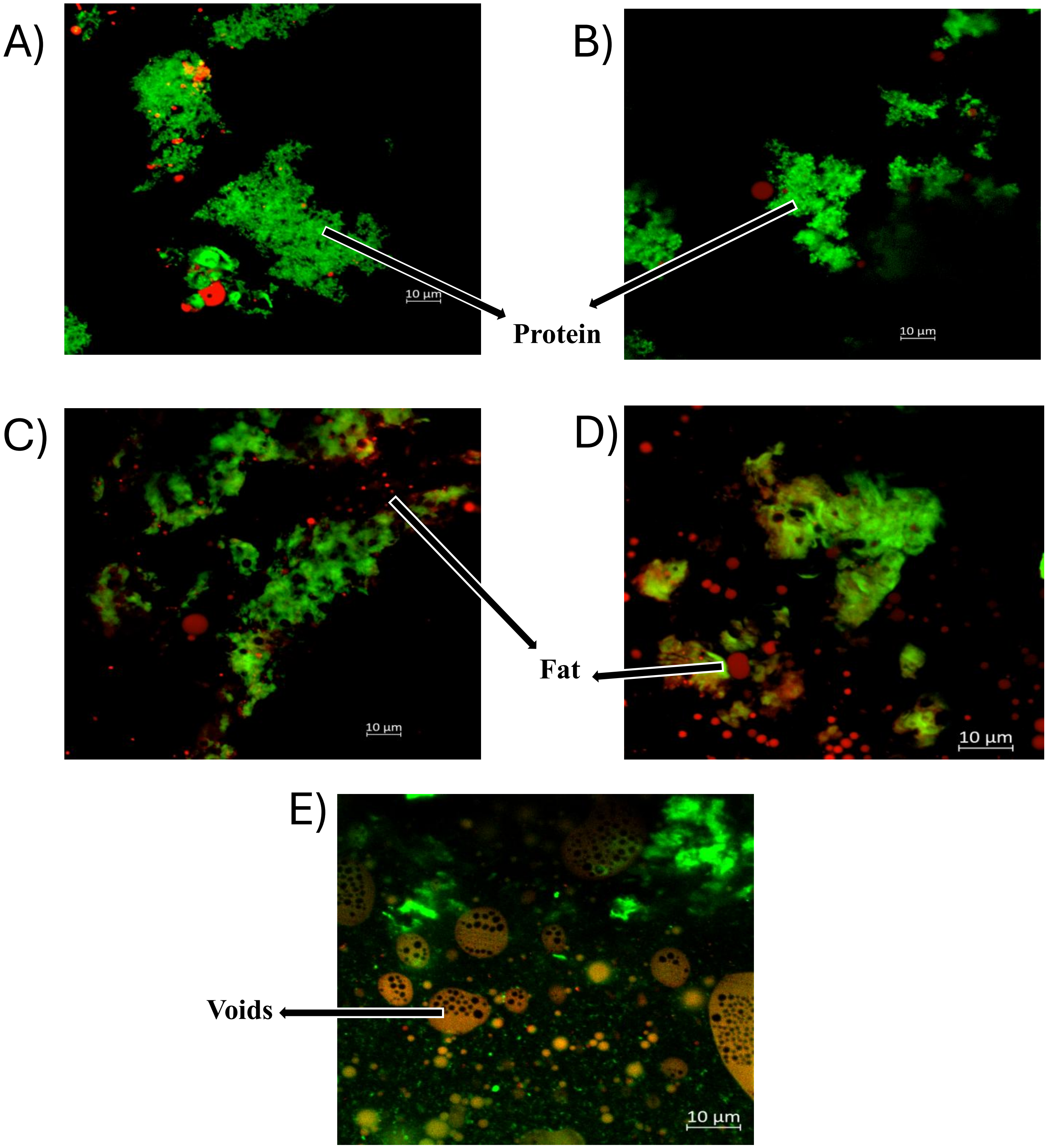


Figure 14: Confocal images of pumpkin seed flour solubility study sediments. **A)** pH 3 30ºC, **B)** pH 3 80ºC, **C)** pH 8 30ºC, **D)** pH 8 60ºC, **E)** pH 8 80ºC

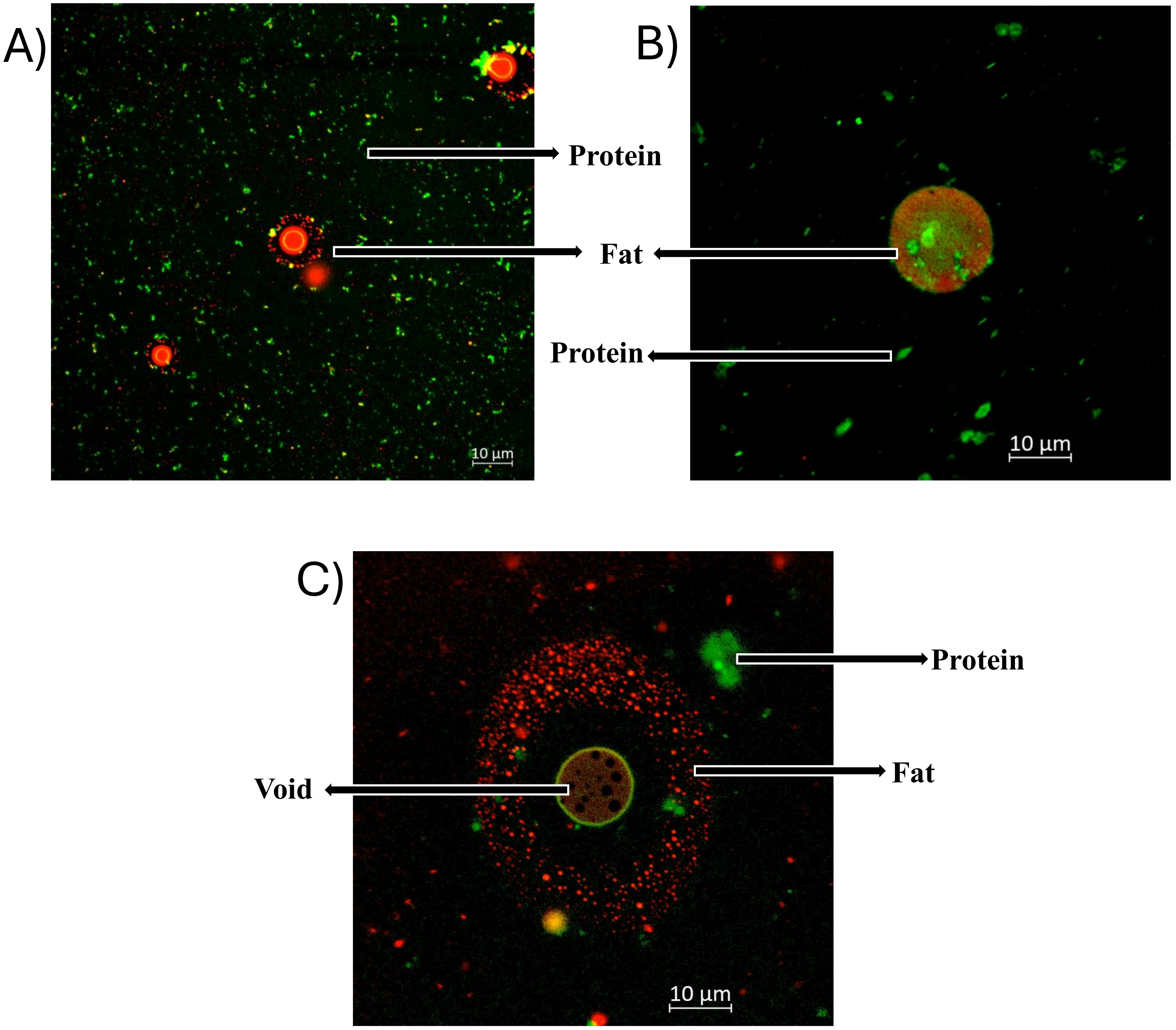


Figure 15: Confocal images of pumpkin seed flour solubility study supernatant. **A)** pH 3 30ºC, **B)** pH 8 30ºC, **C)** pH 8 60ºC

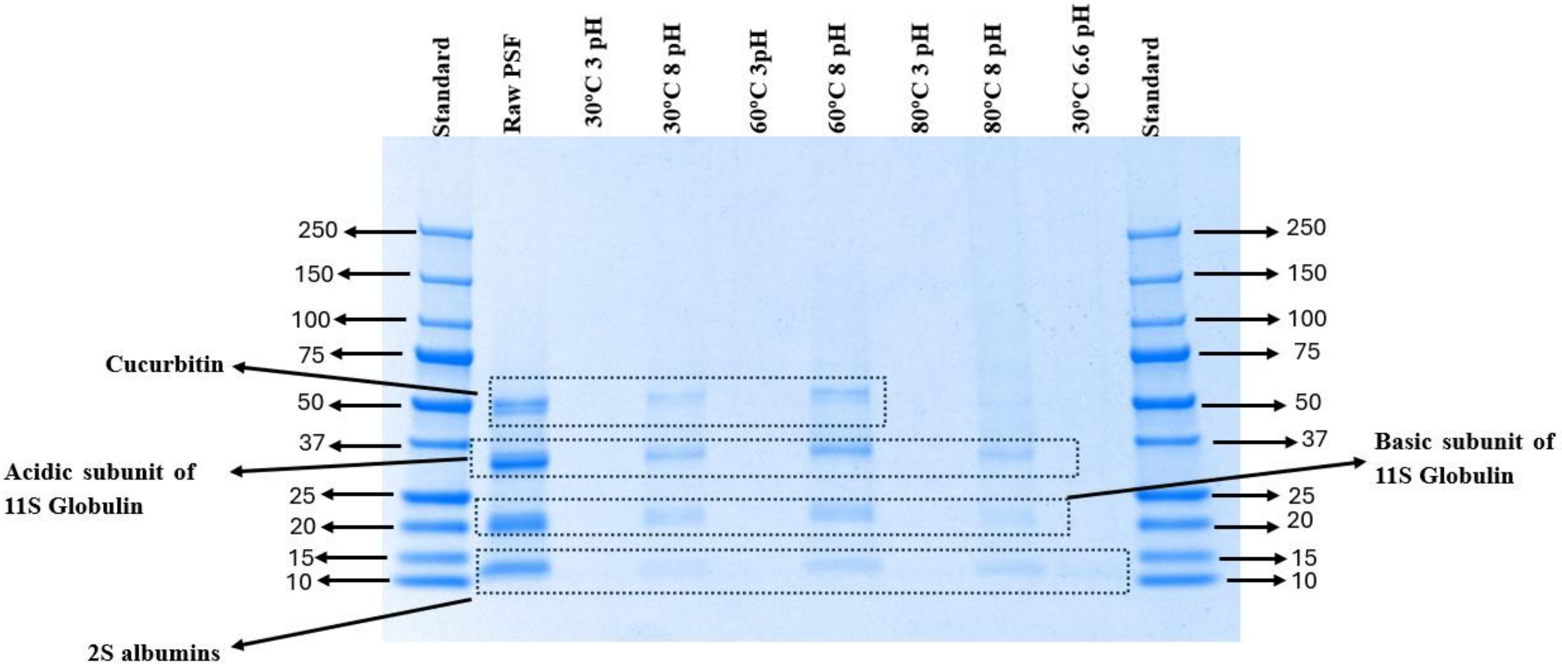
5.8 SDS – PAGE Analysis of Pumpkin Seed Flour Proteins Under Various Conditions.

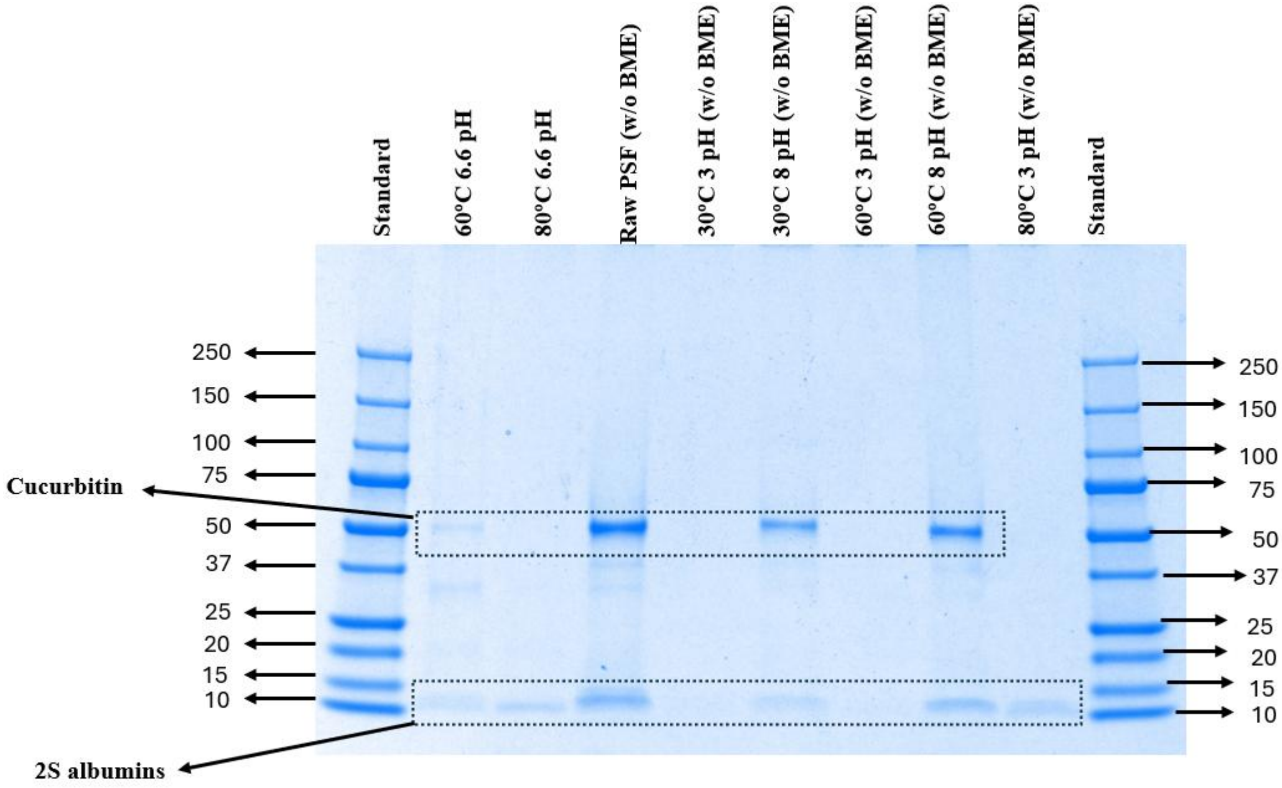
The SDS – PAGE analysis provides a tool to identify the proteins that are rendered soluble as a function of hydration conditions i.e. across different pH and temperature treatments (**Figure 16**).

The main types of protein present in the pumpkin seeds are11S globulins (50 – 60 kDa), 7S globulins (40 – 42 kDa) and 2S albumins (12.5 kDa). The 11S or Cucurbitin has 2 subunits: an acidic subunit (7S globulins) and a basic subunit (20 – 22 kDa)(Chelliah et al., 2018; Fang et al., 2010). Under reducing conditions (with β – mercaptoethanol), with raw PSF bands were visible at 52.7 kDa, 31.2, 18.4 kDa and 11.8 kDa. In the sample treatment with 30ºC, 60ºC and 80ºC in pH 3 (with BME), the presences of less molecular weight proteins (11 – 13.3 kDa) are more. This indicates that there was poor solubility of high molecular weight proteins in the acidic conditions. Studies reported that PSF shows a significant decline in protein solubility below pH 5(Gao et al., 2021). During alkaline conditions that is 8 pH, there are several visible bands which having higher molecular weight, mainly at increasing temperatures. The bands at 70.7 kDa, 68.1 kDa, 58.6 kDa, 52.8 kDa, 35 kDa, 33.8 kDa and 21 kDa. During 6.6 pH, there was moderate levels of solubility. The visible bands are 52.5 kDa, 32.8 kDa and 10.6 – 13.3 kDa at 60ºC and 80ºC. (Vinayashree & Vasu, 2021) stated that PS proteins show moderate solubility levels around neutral pH with clear visible bands of globulins and albumins.

In non-reducing conditions, that is without β – mercaptoethanol, there was variation in the molecular weight of protein bands, due to the presences of disulfide bond interactions. The raw flour had bands at 49 kDa, 38.3 kDa and 32.9 kDa. The native disulfide aggregates have a high molecular weight but breakdown to form their lower molecular weight constituent parts when a reducing agent is present (Gao et al., 2021). In alkaline conditions without BME, there were clear bands on 58.5 kDa and 52.1 kDa at 30ºC and 60ºC also smaller peptide bands on 13.3 kDa and 11.9 kDa.

Overall, in alkaline conditions, the higher molecular weight proteins maintained a good level of solubility but were less soluble in acidic conditions. Lower molecular weight proteins were more soluble under acidic conditions.


Standard
Raw PSF
30ºC 3 pH
30ºC 8 pH
60ºC 3pH
60ºC 8 pH
80ºC 3 pH
80ºC 8 pH
30ºC 6.6 pH
Standard
250
150
100
75
50
37
25
20
15
10
Cucurbitin
Acidic subunit of 11S Globulin
Basic subunit of 11S Globulin
2S albumins


Standard
60ºC 6.6 pH
80ºC 6.6 pH
Raw PSF (w/o BME)
30ºC 3 pH (w/o BME)
30ºC 8 pH (w/o BME)
60ºC 3 pH (w/o BME)
60ºC 8 pH (w/o BME)
80ºC 3 pH (w/o BME)
Standard
250
150
100
75
50
37
25
20
15
10
Cucurbitin
2S albumins

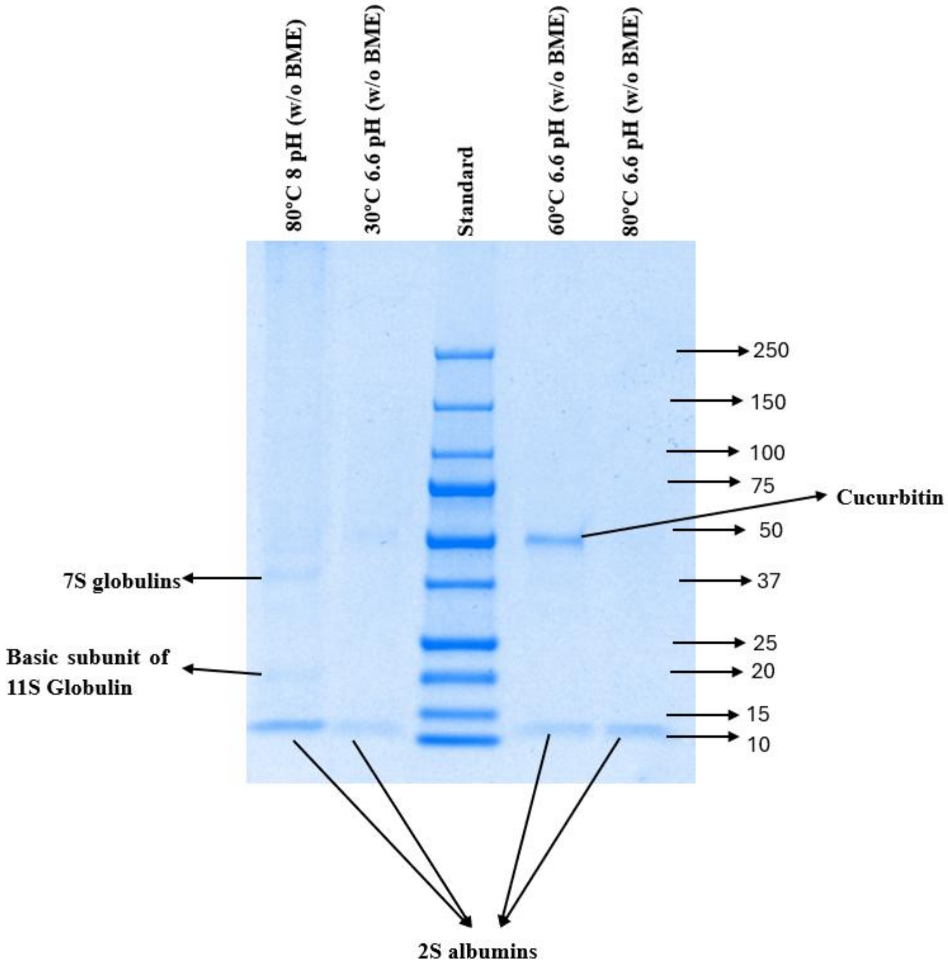


Figure 16: SDS - PAGE images of raw and various treatment undergone pumpkin seed flour samples.

6 Conclusion and Future Recommendations

The study was conducted to examine the potential of hull – less variety of pumpkin seed flour as a protein rich beverage application. With a various experiments like proximate analysis, particle size distribution, solubility in different treatments and structural interactions, the functional characteristics of PSF was evaluated.

The initial proximate analysis resulted in that the raw PSF sample has a rich protein content (53%) and notable levels of fats (15.9%) which makes PSF a nutrient rich ingredient for functional and beverages application.

The sieving experiments showed that from a composition viewpoint, with only slight variations in protein and ash content across the different particle sizes, the PSF flour consists of homogeneous particles despite there being visible differences in the colour of the different size fractions.,

This conclusion led to whole PSF to be used for the solubility studies. The solubility analysis in different temperature and pH parameters reflected the range of beverage types that the ingredient could be used in: acid, neutral, and slightly alkaline beverages; powdered beverages reconstituted at 30°C representing a cold beverage, 60°C representing a hot beverage and 80°C representing a pasteurisation temperature that might be used for a ready to drink beverage.

The solubility was greater in higher temperatures like 80ºC and alkaline conditions while the solubility was poor when reconstituted in acidic conditions These outcomes are consistent with other experiments done on plant-based flours.

Analysis of the sediment gave an understanding of the mechanisms underpinning hydration and solubility. The sediment moisture content was increased as a function of protein and fat solubility which led to the development of a theory that the higher moisture content may be due to non-compressible voids left behind by the solubilisation of fat and protein bodies. Confocal microscopy confirmed the presence of these voids which are then able to entrap water.

The confocal microscopy study also showed that at higher pH and temperature, there were better emulsification and protein distribution. The sediments samples of very low solubility levels have thick protein aggregates and poor dispersion causing minimal fat – protein interaction.

SDS – PAGE was used to identify the protein bands in the supernatant; the bands of higher molecular weight proteins were solubilized at alkaline pH and high temperatures, while lower molecular weight proteins tended to be more soluble across all conditions. This indicates that reconstitution conditions will impact the type of proteins that are solubilised and thus the ability of the soluble phase to participate in beverage functional properties such as foaming and emulsification. The functional properties of the soluble phase were outside the scope of the current work, and it is recommended that this be investigated in future studies.

The study confirmed that PSF has various functional and structural properties which are suitable for beverage applications. This study provides a foundational understanding of PSF solubility effects in water system and laying groundworks for product formulation and optimization in future works. In increasing consumer demand for plant based and protein rich beverages, pumpkin seed flour can be a significant ingredient that has both nutritional and functional properties.

This study gives important information about the solubility properties, proximate composition and interaction between fats and protein under different pH and temperature parameters, further research is needed to understand the potential applications of PSF in food systems. The main future recommendation is to progress a UHT beverage using PSF. For this formulation should be done on various concentrations, use of different stabilizers and emulsifiers. Sensory evaluation and consumer acceptance is also a crucial part in which the consumer will access the flavour, texture, colour and market viability of the PSF beverage.

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8 Appendix A

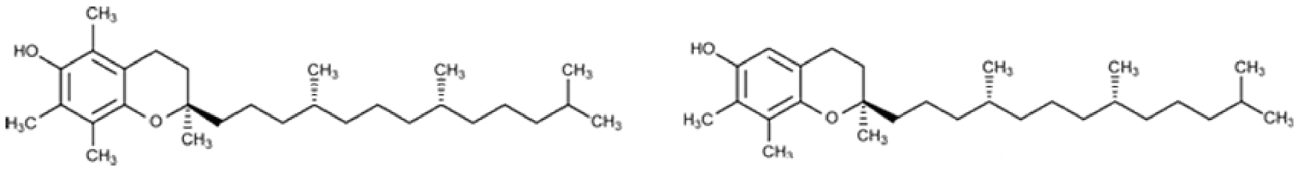
8.1 Review of Bioactive compounds and potential health benefits of pumpkin seeds

8.1.1 Bioactive Compounds: Tocopherols, Sterols, Phenolics, and Other Phytochemicals

Not only their macro and micronutrients, but pumpkin seeds are also loaded with different types of phytochemicals which helps in functional and medicinal values. Tocopherols, phytosterols, phenolic compounds and other compounds like squalene and phytoestrogenic lignans are mostly observed. These compounds may be very lesser in quantity compared to the other bulk nutrients, but they have significant antioxidant and health enhancing properties.

Tocopherols (Vitamin E): PS and PSO consists of large amount of vitamin E, mostly in the form of α-tocopherol and γ -tocopherol (**Figure 17**). Research on Styrian pumpkin seeds, the extracted oil has total tocopherol about 882mg per 1 kg of oil (Gohari Ardabili et al., 2011). This can be converted to 88mg of vitamin E per 100g of oil. This amount of vitamin E is significantly high when comparing with the other plant-based oils like soyabean or olive, which comes in a range between 10 – 50 mg per 100g. The dominance of γ -tocopherol is often considered as a unique characteristic of PSO. γ -tocopherol is an antioxidant and has a special anti-inflammatory property in the body.

The study conducted by (Beni & Edy, 2018) stated that both α-tocopherol and γ -tocopherol helps in its oxidative stability and health benefits. These tocopherols defend the fatty acids present in the seed from oxidation. For example, the study on *C. maxima* seed oil from Indonesia found high amounts of γ -tocopherol and α-tocopherol, which directs the total vitamin E content into a high levels making PSO a dietary source of vitamin E (Beni & Edy, 2018). During cold press extraction of PSO, vitamin E helps as a resistant from rancidity, due to the presences of natural antioxidants.



(i) α-tocopherol (ii) γ-tocopherol

Figure 17: Various Tocopherol Structures found in Pumpkin Seed (Beni & Edy, 2018).

Phytosterols: Pumpkin seeds are packed with high amount of phytosterols. These plant sterols may look structurally similar to cholesterol but can help with cholesterol lowering properties. In PSO, about 1 – 2% of total sterol content can be found in the oil (Gohari Ardabili et al., 2011). In the *C. pepo* seed oil from Iran, the total sterol was 1.86% which is higher than most of the other oils. β – sitosterol and Δ7 – sterols such as spinasterol, Δ7 – stigmasterol and campesterol are major sterols found in pumpkin seeds (Gohari Ardabili et al., 2011). The Δ7 – stigmasterol is a relatively an unusual sterol variant, which has a double bond in the 7th position of sterol molecule, that has been investigated for improving prostate health values. In traditional medicine, PS and PSO are used to minimize BPH symptoms.

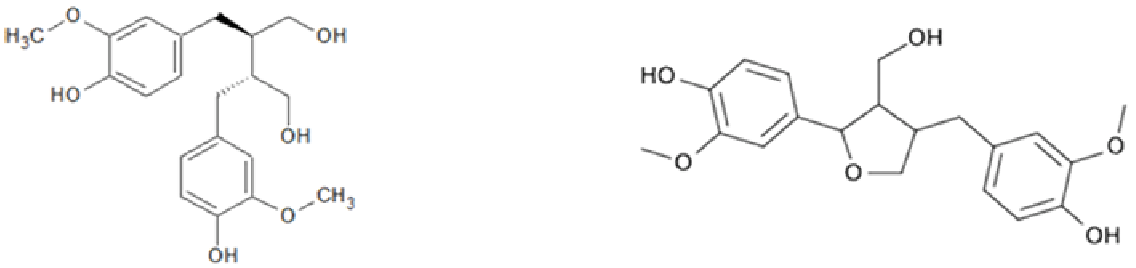
A study published that, *C. maxima* var. Berrettina seeds have high sterol quantity of unsaponifiable matter, that is even higher than tocopherol content in the same variety (Beni & Edy, 2018). A different analysis stated that supplements can be made using PS due its rich content in minerals and sterols (Gohari Ardabili et al., 2011). The phytosterols present in the pumpkin seeds have shown health benefits like lowering LDL (low-density lipoprotein) cholesterol by reducing absorption of cholesterol in the intestines, when it is taken as part of a diet. There can be up to 1500 – 2000 mg of phytosterols in 100 g of PSO, which greater than in equal amount of olive or canola oil (Hien & Minh, 2021). Therefore, pumpkin seeds are one of the rich sources of phytosterols among other seeds and has ability to improve heart health.

Phenolic Compounds: The phenolic compounds are mainly present in the hull or husk of the PS and lesser fraction in the seed. This category includes polyphenols like phenolic acid (vanillic, syringic, p – hydroxybenzoic acids) and flavonoids. The exact amount of total phenolic content of a PS can be measurable, an analysis reported that around 66 mg of gallic acid present per kg of oil, which shows the presence of antioxidants in the unsaponifiable fraction (Gohari Ardabili et al., 2011). Even with these measures, PS is not a source of polyphenols as compared to olive oil but still manages to contribute to overall antioxidant capacity. The *C. pepo* Styrian seeds have dark green seed coat which contain phenolic pigments that gives its oil a dark green colour. It also has derivatives of protocatechuic and caffeine acids, which serves as antioxidants.

In some studies, PS has been connected to anti – inflammatory and anti – cancer benefits (Gohari Ardabili et al., 2011). For instance, a methanolic extract of pumpkin seed cake that is defatted cake consists of phenolic antioxidants that can scavenge free radicals. In a review published by (Gohari Ardabili et al., 2011) stated the antioxidative phenolic compounds and also marked PS as a rich source of antioxidative phenolic compounds along with proteins and unsaturated fats.

Other Functional Phytochemicals: The other bioactive compound present in PS is squalene, coumarins, lignans and triterpene, which is mostly found in seed oils. There is trace amount of squalene found in PSO, which is a parental compound for sterols that imparts healthy skin benefits. In a nutraceutical review paper, PSO's squalene presences has the ability to mimic skin's natural oil and also acts as an antioxidant (Arora et al., 2023). Lignans are another category of functional phytochemicals. The common lignans found in PS are secoisolariciresinol and lariciresinol (**Figure 18**).

In a review paper published by (Beni & Edy, 2018) has reported that secoisolariciresinol and lariciresinol are phytoestrogens which are extracted from PS. The compounds found in pumpkin seeds contribute to hormonal and anticancer effects (Dhiman et al., 2009). In addition to these compounds, there are *cucurbitacin* triterpenoids in less amounts, mostly found on leaves and flesh and might present on seed coat. Cucurbitacin are bitter components which imparts anti – tumour and anti – parasitic properties (Ceclu et al., 2020; Dhiman et al., 2009). However, the PS doesn't contain these bitter components due the breeding process which help to minimise the cucurbitacin content in the new developed seeds.



(i) Secoisolariciresinol (ii) Lariciresinol

Figure 18: Various Phytoestrogens Structure (Beni & Edy, 2018).

8.1.2 Anti-Nutritional Factors of Pumpkin Seeds

Pumpkin seed also contain minority of antinutritional compounds, despite their rich nutritional value. Phytic acid, oxalates and trypsin inhibitors are such antinutritional factors present in PS. These compounds can inhibit the absorption capability of other nutrient compounds like zinc, iron and magnesium. Study conducted by (Ningthoujam et al., 2018) state that the phytic acids are a bit higher in whole seeds than dehulled seeds. Their amount is much lower in PS than other legumes and grains. The levels of antinutrients can be reduced by simple food processing techniques like roasting, fermentation and germination. It is also found that these techniques not only reduce their levels but also enhance protein bioavailability and improves palatability and digestibility (Akintade Adeyanmola et al., 2019).

8.2 Functional and Health-Promoting Properties of Pumpkin Seed Flour

Pumpkin seed flour is obtained by grounding pumpkin seeds, which has a lot of nutrients as well as a number of bioactive compounds that imparts functional and health promoting properties. The *Cucurbita* seed have been using from very long time as in traditional medicines and in modern diets for their antioxidant, anti-inflammatory, antidiabetic, cardioprotective, antimicrobial and other therapeutic properties. These properties are contributed by various phytochemicals and nutritional compounds like unsaturated fatty acids, amino acids, vitamins, minerals and phenolic compounds (Arora et al., 2023). In the present days, PS and PSF are analysed as a nutraceutical ingredient, which has a capability to manage and minimize chronic and health conditions.

8.2.1 Antioxidant Properties

Pumpkin seed flour has a strong antioxidant capacity due to the nutrient component and various phytochemicals present in it. The PS also contain natural antioxidant vitamins, tocopherols and carotenoids (Arora et al., 2023). These antioxidant compounds scavenge free radicals and protect the cells from oxidative damage. In 100g of PS there are around 35 mg of γ -tocopherol and also with other antioxidant micronutrients like selenium, zinc and manganese which act as a cofactor for antioxidant enzymes (Arora et al., 2023). The other components present in PS are polyphenolic compounds which contributes to the overall antioxidant activity. In the process of grinding the seed into flour, the antioxidant components remain bioactive making the flour naturally antioxidant rich.

In several studies shows the ability of pumpkin seeds and flour to scavenge free radicals. A study reported that PS oil extracts have significant scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and also constrain the lipoxygenase enzyme, which is a contributor to inflammatory oxidative reactions (Xanthopoulou et al., 2009). Both results of radical scavenging and lipoxygenase inhibition shows that PS compounds have the ability to interrupt lipid peroxidation chain reactions and minimize oxidative stress which leads to inflammation. In research on different Cucurbitaceae seeds, it shows that their extracts not only inhibit free radical but also protect the DNA from oxidative damage (Beni & Edy, 2018). In that study PS extract to show genoprotective effects and minimize genotoxicity by harmful chemicals. These findings can help PSF to be a functional ingredient that improves antioxidant activity of our diets.

The antioxidant ability of PSF is used in different food applications. In one research study, PSF was incorporated with wheat/oat flour to make high protein content biscuit, this addition increased the total phenolic count and DPPH radical scavenging activity of the biscuit while comparing with control sample (Das et al., 2021). The blend with high percentage of PSF (30% PSF) showed the maximum antioxidant activity, that proves that the natural antioxidant present in PSF imparts in final product. The addition of PSF to cereal-based food to enhance their oxidative stability and health value is due to the presences of phenolic compounds, carotenoids and vitamin E. The antioxidant properties of PSF are not only crucial for protecting cells from oxidative stress but it also helps to minimize the diseases occurred due to free radical damage like cancer and degenerative disorders (Arora et al., 2023).

8.2.2 Anti-inflammatory Effects

Pumpkin seeds and flour have an ability of anti-inflammatory effects. Chronic inflammation such as common underpinning of arthritis, metabolic syndrome and other chronic diseases can be treated with the bioactive components present in the PS. The extracts of PS can inhibit lipoxygenase which is an enzyme that creates pro – inflammatory leukotrienes from fatty acids. So, when this pathway is obstructed, PSF compounds minimize the inflammation at molecular level. Other than enzyme inhibition, PSF also consist of monosaturated and omega – 6 fatty acids like oleic and linoleic acids, which affects eicosanoids production and cell signalling that reduce inflammatory responses (Beni & Edy, 2018). The presence of phytosterols and other compounds are also showing anti – inflammatory properties.

In traditional medicines, PS have been used long before for anti – inflammatory benefits for specific conditions like arthritis. PS is also used as folk medicine to reduce joint pains and present-day studies shows support for this practice. According to (Dhiman et al., 2009) review paper, PS has anti – inflammatory properties that can be potentially useful for arthritis. In several research conducted on animals, PSF extracts shows minimizing inflammatory markers and oxidative damage in joints tissues. For example, in a study of induced arthritis on rats, PS extracts resulted in reducing swelling and inflammatory cytokine levels in joint tissues, which indicate a remedy effect against arthritis related inflammation (Arora et al., 2023). Some anti – inflammatory components like peptides and polyphenols, alleviate the release of inflammatory mediators. The presence of these bioactive compounds in PSF should be incorporated into daily diets to helps in lowering chronic inflammation with time.

8.2.3 Antidiabetic and Hypoglycaemic Effects

Pumpkin seed flour gained a large attention for its glycaemic control and diabetes management. During ancient times, various parts of pumpkin plant were used to treat diabetes. In some communities and cultures, consuming pumpkin believed to be helps in hypoglycaemic properties (Dhiman et al., 2009). PS have low quantities of sugar and starch naturally; however, protein and healthy fats are high which shows low glycaemic affects when consumed. In addition to this, they also provide magnesium and other minerals responsible for blood glucose regulation. Magnesium is a critical factor in many enzymes that take part in carbohydrate metabolism and insulin signalling. Consumption of foods rich in magnesium significantly reduce the risk of type 2 diabetes. In a study reported that, there is 30% reduction of happening type 2 diabetes in people taking magnesium rich food daily (Arora et al., 2023). Therefore, the magnesium present in the PSF can enhance insulin sensitivity, glucose absorption by the cell and improved blood sugar levels (Arora et al., 2023).

In an investigation conducted on rats, PS extract and defatted PSF shows reduction in blood glucose level and enhance glucose tolerances in comparison with untreated diabetic controls. This effect is believed due to the combination of factors like, the high fibre content likely to reduce absorption. The protein and healthy fats in PS improve satiety and reduce glucose spike and the mineral like magnesium, zinc and other antioxidant compounds helps in pancreatic function and insulin action (Arora et al., 2023). The presences of amnio acids and peptides in PS influence incretin hormones and modulate enzymes involved in glucose metabolism. A published review paper also supports anti diabetic effects in PS and magnesium contribution for remedying glycaemic control (Caili et al., 2006).

8.2.4 Cardiovascular and Lipid-Lowering Potential

The consumption of PS on regular basis has significant health benefits like improving blood lipid profiles and maintaining healthy blood pressure. Naturally pumpkin seeds are high in unsaturated fatty acids, mainly monosaturated fatty acids (MUFA) like oleic acid and polyunsaturated fatty acids (PUFA) like linoleic acid (Beni & Edy, 2018). During the processing of defatting PSF, oil is extracted along with these fats. However, whole PSF has these heart heathy fats. Oleic acid is mainly known for reducing bad or LDL (low-density lipoprotein) cholesterol and elevate good or HDL (High-density lipoprotein) cholesterol in the blood, these effects are also shown by olive oil. Due to this, PS helps in preventing coronary artery disease (Das et al., 2021). The phytosterols like plant sterols present in PS are structurally similar to cholesterol so, it minimizes the cholesterol absorption in the intestines.

In a nutritional evaluation paper among the common edible nuts and seeds, pumpkin seeds have the highest phytosterol content (Beni & Edy, 2018). These phytosterols reduce the LDL cholesterol levels by blocking the uptake of dietary cholesterol and also help in minimize hepatic cholesterol synthesis. The presence of sterols in PS provides reductions in blood cholesterols and lower the chance of atherosclerosis (Arora et al., 2023). The sterols and unsaturated fats combined, can help in heart protective properties, reduce the chance of heart attacks and improves lipid profiles (Arora et al., 2023).

PSF is a beneficial compound for blood pressure regulation and cardiovascular functions. The flour consists of very less sodium and high amounts of potassium. This combination of minerals is favourable for maintaining healthy blood pressure levels (Arora et al., 2023). The foods with low sodium and high potassium ratio are well known for maintaining blood pressure and minimizing hypertension risks. The amino acid, L – arginine is a vasodilatory molecule which is very important for blood vessel relaxation. Some studies suggest that consumption of PS helps in improves production of nitric oxide in the body. Nitric oxide helps in dilation of blood vessels, improves blood circulation, lowering blood pressure levels and preventing arterial plaque(Arora et al., 2023). The high magnesium content present in the PS also has an important role in cardiovascular health. Magnesium is useful to relax blood vessels, acts as a natural calcium channel blocker, maintaining blood pressure and reduce the chances of arrhythmias (Arora et al., 2023). In an animal model of study, PS extract supplements reduced the cholesterol levels and protected against diet induced hyperlipidaemia (Beni & Edy, 2018). This feature is due to the presences of phytoestrogens, which creates estrogen like effects on digestion and the healthy fats that improves plasma lipid profile. There is historical evidence on PS benefits like in Eastern Europe traditional medicine suggest that PS consumption is beneficial for individuals with high cholesterol or atherosclerosis and in Soviet times, its reported that regular consumption of pumpkin helps in reducing blood cholesterol level in obese people (Dhiman et al., 2009). Thus, lowering cholesterol levels, enhances lipid ratios and improves blood pressure controls, PSF can be used as a functional ingredient that supports cardiovascular health and minimizing the risk of heart diseases.

8.2.5 Anticancer and Chemo preventive Effects

As a functional food ingredient, PS are also researched for their ability in anticancer and chemo preventing properties. In epidemiological observations, foods rich in pumpkin seeds shows in reduced risk of certain cancer such as stomach, breast, lungs, prostate and colon cancers (Arora et al., 2023). In women, during postmenopausal stage consuming more pumpkin seed have shown lowering risk of breast cancer, indicating protective estrogen maintaining effect. In review paper reported that pumpkin seeds are rich in phenolic acids and lignans like pinoresinol and lariciresinol (Arora et al., 2023). These phytochemical has a strong antioxidant property, scavenging free radicals and preventing DNA damage, which leads to prevention of cancer (Beni & Edy, 2018). By minimizing oxidative stress, compounds present in pumpkin seeds prevent the occurrence of carcinogenesis and act as a chemo preventive function.

In the review paper published by ram reported that PS extracts obstruct the growth of cancerous and hyperplastic cells during in vitro conditions while sparing normal cells (Beni & Edy, 2018). In addition, these effects were not interfered by sex hormone receptors and PS compounds can work through other pathways. In the same paper its mentioned that ethanolic and aqueous PS extracts influences the apoptosis in prostate cancer cells by causing oxidative stress and mitochondrial dysfunction. In both cell and animal-based studies, PS extracts stunts prostate tumour growth. In an animal-based study, giving PS as supplement to rats showed a drastic inhibition of testosterone induced prostate growth (Beni & Edy, 2018). In humans-based studies on a group of men with early symptoms of prostate problems experienced in lowering urinary symptoms after three months of PS extract medication (Beni & Edy, 2018). These results agree with the traditional uses of pumpkin seeds in managing prostate problems and benefits in reducing hormone related cancer progression. Cucurbitacin and a ribosome inactive protein known as moschatin, which acts as an antiproliferative agents. When cucurbitacin extracted from PS shows reducing in apoptosis in cancer causing cells by signalling pathways (Beni & Edy, 2018). Moschatin, also reported in minimizing melanoma cell growths. In addition, these compounds with other antioxidants present in PS helps to suppress tumour development (Beni & Edy, 2018). In short, several investigations support anticancer abilities in PSF. Pumpkin seed compounds are considered in inhibiting chemo preventive agents that help in minimizing cancer risk.

8.2.6 Antimicrobial and Antifungal Activities

Pumpkin seed flour has shown notable antimicrobial as well as antifungal effects. This property can be related to the usage of PS in traditional medicine, as remedy to prevent infections (Ceclu et al., 2020). Present day studies point out that PS have bioactive components and phytochemicals that helps in inhibiting the growth of different pathogens. For example, (EI-Aziz, 2011) conducted an experiment by isolating protein extracts from PS and exhibiting on a broad spectrum of antimicrobial effects against common bacterial strains. Incredibly, these protein extracts show antimicrobial properties even with the exposure to high dose of gamma irradiation (EI-Aziz, 2011).

These findings help to underline the ability of PSF as a natural preservative ingredient that hinders the microbial growth.

Several studies have done to evaluate the antifungal property of PS. In a published review paper stated that, pumpkins seeds were consumed by individuals for their antifungal and antimicrobial uses in earlier times (Ceclu et al., 2020). Researcher have found that certain peptides have this ability of antifungal property. Cucurmoschin is a peptide extracted from PS, which shows a great inhibition power on yeast and molds during a lab analysis (Wang & Ng, 2003). Meanwhile, it is also reported the growth obstruction of Candida albicans, which is a common opportunistic fungus (Beni & Edy, 2018). The antimicrobial action mode is when the peptides bind with the microbial cell membranes and disrupting microbial metabolic enzymes. PS not only has the antimicrobial effect, PS also has immune supporting nutrients like zinc and other bioflavonoids, which helps to increase the body's natural defence mechanism against from microbes and infections (Arora et al., 2023). In brief, PSF has natural antimicrobial compounds that helps PS to be a functional ingredient that has the ability to enhance food safety.

8.2.7 Gastrointestinal and Urinary Health Benefits

One of the various benefits from consumption of PS is gastrointestinal and urinary health. PSF is a rich source of dietary fibre, which helps in regulating bowel movements and enhance the gut health (Fatima et al., 2025). PSF enriched foods have shown improvement in their nutritional and functional qualities, such as biscuits enriched with PSF resulted in increased fibre and antioxidant content (Das et al., 2021). This presences of fibre with other anti – inflammatory compounds help in increase gastrointestinal health. The normal nutritional profile of PSF contains fibre, healthy fats and antioxidants indicates a protective effects on gastrointestinal tract. The easy digestibility feature of PS was used in traditional medicine as laxative to relieve constipation. Magnesium content present in PS regulate gut movements and the anti – inflammatory effects soothe the gut lining. Incorporating PSF in cuisines can helps in improving digestive health and minimize the inflammation of gut lining.

PS have beneficial values for urinary health, mostly focused on prostate and bladder functions. PS have been using as a natural medicine for urinary problems long back. For instances, in North

American and European traditional medicines suggests that consumption of PS helps in reducing symptoms of overactive bladder and BPH (El-Aziz, 2011). A large study conducted on 2,245 men with BPH resulted that improvement in their urinary symptoms without any side effects on regular intake on PS extract supplements (Beni & Edy, 2018). In a similar clinical trial, PS extracts help in improved urinary flow and minimize the prostate enlargement within a three months of treatment. In the nutraceutical review paper, giving PS to rats helps them to reduce testosterone induced hyperplasia and managing BPH. The mode of mechanism is when phytosterols like β - sitosterol obstruct 5 – α – reductase and anti – inflammatory actions on prostate cells (Beni & Edy, 2018). PS beneficial values can be useful for women suffering from overactive bladder or post – menopausal bladder discomfort, shown relief when PS extracts are consumed. It also shown improvement in bladder control and minimize the urgency (Arora et al., 2023). In short, PSF can support urinary health by maintaining normal bladder functions and slowing prostate related problems, which justifies its usage in traditional medicines.

8.2.8 Immune Modulation and Wound Healing

Pumpkin seed flour consists of many nutrients and bioactive compounds that maintain the immune system and also helps in repairing the tissues. The zinc content of PS is a critical mineral that support immune cell development and function (Arora et al., 2023). 100g of PS has enough zinc content that satisfies the daily zinc requirements, which aids in stronger immune defences and also minimize the occurrences of infections. PS also has antioxidants like vitamin E, carotenoids and selenium, which shields immune cells from oxidative stress (Dhiman et al., 2009). These antioxidant compounds also help in regulate chronical inflammation and gives aid to a balance immune response. In some of the investigation, it is noted the presences of immunomodulatory peptides in PS. For instances, in a clinical study, some protein parts from the PS stimulate the activity of natural killer cells and macrophages (Beni & Edy, 2018).

PS have also resulted in wound healing and tissue regeneration. This is due to the large combination of protein, vitamins and polyunsaturated fatty acids. The rich content of manganese and vitamin K plays an important role in collagen production and blood clotting which initiates the wound repair (Arora et al., 2023). In folk medicines, practice of applying PS paste or oil to wounds to heal faster. In a laboratory study on rats, application of cold pressed PS extract on wound healing properties shows a faster healing and wound closure when compared to the untreated wound (Beni & Edy, 2018). The PS treatment supports skin cells regeneration, which is due to the action of tocopherols and essential fatty acids that minimize the inflammation at the area of wound. The anti – inflammatory ability of PS compounds is beneficial in healing and reduce excessive inflammation and scarring. The incorporation of PSF in diets has advantageous value like faster healing and closure of wounds and injuries. Overall supports to the body's natural defences mechanism.

8.2.9 Anti – parasitic and Anthelmintic Properties

The oldest documented medicinal use of pumpkin seed is its natural anti – parasitic feature. Different cultures and communities usually consume PS to eradicate intestinal parasites like tapeworm (Kaur et al., 2020). In review paper, stated that eating raw or roasted PS can acts as that is to get rid of worms from the digestive tract (Ceclu et al., 2020). In North America, PS is used as a traditional remedy for anthelmintic and it is also used as supportive therapy for disorderly functions of bladder. These findings can prove the bioactive factor present in PS that eradicate parasites (EI-Aziz, 2011).

Pumpkin seeds consist of a compound known as cucurbitacin, which is a tetracyclic triterpenoid, which might be partly responsible for the vermifuge action. The cucurbitacin and its related compounds can paralyze these parasites by interfering with their neuromuscular system and getting rid from our body. In addition to this, an amino acid known as Cucurbitin which can be extracted from PS can also contribute to anthelmintic effects by similar stunning of parasites (Grzybek et al., 2016). It is also important to keep in mind that, in all the cases PS may not achieve complete elimination of parasites in the body. However, the anti – parasitic effect of PSF is a good example of nutraceutical effects from natural source ingredients, which can be incorporated into our daily diets for functional and medicinal benefits.

8.2.10 Additional Therapeutic Effects

Pumpkin seeds are not only limited to some main therapeutic values, but some of other therapeutic areas are sleep regulation and anxiety management. PS are a natural source of tryptophan, which is an important amino acid that helps to make serotonin and melatonin. These neurotransmitter enhance in quality sleep and regulation of mood; thus, PS can be a natural remedy for sleep disorder and mild depressions (Yadav et al., 2010).

The neurological stability and metal wellbeing effects by PS is due to the high content of magnesium and zinc. Zinc is an important mineral for neurogenesis and synaptic plasticity and magnesium helps in calming nervous system (Fatima et al., 2025). When the minerals combined with bioactive peptides, it greatly decreases stress related symptoms and anxiety.

9 Appendix B

I have used the help of AI tools while writing this thesis, to improve my language quality and the sentence flow. The tools are Grammarly and Quillbot, which were used to correct the grammatical errors. These tools were used language rectifying purposes only; all the research ideas and analysis are my own work.