Functional and nutritional properties of faba bean (*Vicia faba*) isolates and their application in food

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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ABSTRACT

Replacing protein from animal sources with protein from plant sources is driven by cost, health and environmental factors. *Vicia faba* is a potential source of alternative protein as it contains good amount of protein, carbohydrate, fibre and micronutrients. However, the presence of the anti-nutritional compounds vicine and convicine have limited its utilisation in food. The aim of the project was to prepare protein isolates from *Vicia faba* and apply them to the preparation of beef patties. Four food grade extraction conditions at different pH (7.6 or 10) and temperature (6°C or 20°C) were used to extract *Vicia faba* isolate with a total yield ranging from 26.6 to 30.2 %, and a protein content ranging from 32.5 to 42.7%. Proteins in *Vicia faba* isolates were characterised by SDS-PAGE and confirmed by LC-MS analysis to be mainly legumin, vicilin and favin. The highest protein content (42%) and lowest vicine and convicine (<0.5%) content were found in isolate extracted at pH 7.6 at room temperature. The isolate also contained starch (5%) and fibre (19%). The proximate composition and functional properties were affected by pH and temperature. All *Vicia faba* isolates were shown to have good water holding (1.4 to 1.6 g/g) and fat holding capacity (1.1 to 1.3 g/g). Emulsifying and foaming properties were also observed. Heat-induced gelled was observed in all *Vicia faba* isolates at 12% and 20% w/v. Their functional properties were compared with commercial protein isolates (soy, pea, hemp and faba) and significant differences were found between samples. The best isolate was chosen for the application in beef patties based on the protein composition, quality with low vicine and convicine content. The addition of 20% of *Vicia faba* isolate to beef mince improved product yield significantly compared to 100%

beef patties. The utilisation of faba bean to partially replace meat could contribute towards reducing the cost of nutritious food, and reduce burden on health and the environment.

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Abbreviations

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
FBPC	Faba bean protein concentrate
FHC	Fat holding capacity
G6PD	Glucose-6-phosphate-dehydrogenase
HP	Hemp protein
LC-MS	Liquid chromatography mass spectrometry
PCA	Principle component analysis
PPI	Pea protein isolate
RT	Room temperature
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel
SPI	Soy protein isolate
VF	*Vicia faba*
[VFI 7.6 RT] FD	*Vicia faba* isolate extracted at pH 7.6 room temperature without precipitation
VFI	*Vicia faba* isolate
[VFI 10.5 6C]	*Vicia faba* isolate extracted at pH 10.5 and 6°C
[VFI 10.5 RT]	*Vicia faba* isolate extracted at pH 10.5 and room temperature
[VFI 7.6 6C]	*Vicia faba* isolate extracted at pH 7.6 and 6°C
[VFI 7.6 RT]	*Vicia faba* isolate extracted at pH 7.6 and room temperature
[VFI 7.6 RT] (brown fraction)	*Vicia faba* isolate extracted at pH 7.6 and room temperature brown
[VFI 7.6 RT] (white fraction)	*Vicia faba* isolate extracted at pH 7.6 and room temperature white fraction
[VFI 7.6 RT] (soluble pH 4)	*Vicia faba* isolate pH 4 soluble fraction
WEP	Whole egg powder
WF	Wheat flour
WHC	Water holding capacity

Chapter 1
INTRODUCTION

1.1 Background of study

Legumes represent 27% of total crop production worldwide and have become the second most important crop after cereals (Riascos et al., 2010). Legumes refer to the edible seeds of leguminous plants belonging to the family Leguminosae, which include fresh legumes such as peas and green beans, pulses such as dried beans and lentils and oily nuts such as peanuts, groundnuts and soybeans (Riascos et al., 2010). Legume seeds are important sources of nutrients including protein, low glycaemic index carbohydrates, fibre, minerals, vitamins, carotenoids and polyphenols (Foyer et al., 2016). This makes legumes useful to fight malnutrition in developing countries by making them part of the daily diet. The protein content varies from 20% to 30% of total dry weight, which is higher than most cereals (10-15%). Therefore, legumes have become important in the production of feed and foods (Riascos et al., 2010). Many types of beans have been cultivated for several thousand years for animal or human consumption. Legumes are environmentally beneficial, as they have adapted to grow in most climates with low fertiliser inputs. Commonly consumed species include navy, pinto, lima, kidney, black, white, red, pink beans, lentils, black eye pea, black gram, garden pea, chickpea, horse bean, faba bean, French bean, pigeon pea, moth bean, jack

bean, tepary bean and other oilseeds such as soybeans, peanuts, winged bean and lupin seeds (Sathe, 2002). However, most legumes are still considered as underutilised food crops being traditionally used as an animal feed.

Reasons for their low exploitation include anti-nutritional factors and low level of sulphur-containing amino acids (methionine and cysteine) and tryptophan. Anti-nutritional factors can be removed by processing and the protein quality improved by blending with other dietary proteins. Legume proteins also have interesting functional properties that make them useful in food product development. As an example, soybean (*Glycine max*) protein has been heavily exploited as a food ingredient , used in vegan food as meat analog as it has similar functional and nutritional properties to meat. Soybean protein also shows excellent emulsifying and gelling properties which mimic the functional properties of egg proteins (Ratnayake et al., 2012).

The utilisation of soybeans across the world varies widely (Sánchez-Vioque et al., 1999). According to review by Asgar et al. (2010), the 2015 soybean stats by American Soybean Association (ASA) reported that the world soybean production was estimated to be 107 million metric ton, with production spread amongst countries: United States produced 33%, followed by Brazil 31%, Argentina 18%, China 4%, Paraguay 3%, India 2.5% and other countries contributed 6%. Some parts of the world, especially Asia, uses soybeans to make soymilk, fermented foods such as tofu and in Western countries,

soybeans are mainly used as ingredients such as soy protein. The soy protein has many applications in food products and the applications have increased very rapidly. Important functional properties of soy protein in food systems are gelling capacity, water absorption, fat absorption, emulsification, colour control and elasticity (Singh et al., 2008). Soybean has attracted people's attention as an economic and high-quality plant protein. However, barriers to utilisation include the allergenic properties of soy proteins, their flavour described as grassy, beany, bitter and astringent, and the impact of soybean agriculture on the environment (Asgar et al., 2010). Moreover, in Europe, 70% of soybeans are imported to be used as plant protein required to supplement animal feeds (Pulkkinen et al., 2015). Therefore, exploring new legume protein from the underutilised sources that can grow in various climatic zones and can be utilised throughout the year is a good approach to diversify the sources of plant protein. It is also important to explore alternative legume proteins other than soy protein that exhibit a similar or better functional and nutritional properties. One particular desirable functionality is also the ability to replace egg protein. Egg is commonly used in food products to obtain desirable texture, volume and colour due to foaming, solubility, emulsifying and coagulation properties (Ratnayake et al., 2012). Alternative legume protein that can mimics the functionality of egg, can benefit vegan consumers and people that have allergic towards egg.

The global prevalence of overweight and obesity among adults in 2016 was 39% and 13% respectively which has tripled since 1975 (WHO, 2018). The cause of obesity is mainly due to the imbalance energy intake and energy expenditure exemplified by the intake of high-fat, high sugar food, low in fruits and vegetables and high in red and processed meat and sedentary lifestyle. Health consequences of overweight and obesity are the increased risk of cardiovascular disease, diabetes, musculoskeletal disorders and cancer (WHO, 2018, Rouhani et al., 2014). Meat contains rich amount of saturated fatty acid and cholesterol and meat-based diets are considered as not healthy and sustainable. Research shows that high consumption of meat is associated with significant increased risk of cancers such as colon and gastric cancer (Chan et al., 2011, Song et al., 2014). Besides, the global average per capita of meat consumption is arising due to increase in income per capita in most countries and growth of populations. As the dietary transition continues in many regions of the world, these dietary health risks are expected to get worse. In developing country like Malaysia rapid nutrition transition causes detrimental health impacts. The urbanisation is one of the reason for the rapid nutrition transition that increases the accessibility of food and food purchasing power (Lipoeto et al., 2013). Compared to other rapidly developing Asian countries, Malaysia's per capita consumption of livestock product is relatively high. As an example, meat consumption in 2005 was about 48 kg per capita compared to 24 kg

per capita in Thailand (Lipoeto et al., 2013). Therefore, replacing protein from animal sources with protein from plant sources is one of the way to tackle health issues concerning meat consumption.

The rationale on partial replacement of meat products with legume protein is important for meat eaters to be able to consume meat products with improved nutritional values without changing their food habit. Despite replacing amount of protein from meat, legumes also contain high fibre that can improve fibre content in meat products. The calories of meat products can also be reduced when meat is partially substituted with legume. Legumes have been shown to have beneficial health effect. Higher consumption of legumes is associated with reduced risk of cardiovascular disease, myocardial infarction, cardiovascular and non-cardiovascular mortality and total mortality (Miller et al., 2017). According to Willett et al. (2019), adapting to healthy reference diets from current diets by including 50 g of beans/day could avert 11.1 million death/year, a reduction of premature mortality by 19% in 2030. Springmann et al. (2018) reported that replacing animal-source foods with plant based foods from 25, 50, 75 and 100% led to progressive reductions in premature mortality in 2030 by risk factor. About 18-23% of premature mortality decreased due to increased legume consumption and 8-11% decreased due to reductions in meat consumption across the dietary changes (Springmann et al., 2018). Therefore, a shift in a dietary pattern

focusing on whole grains, fruits, vegetables, nuts and legumes is needed to have beneficial impact upon health.

Despite the health issues, environmental concern of meat consumption has also increased the urgency of exploring more legume or plant protein sources as alternatives to animal protein. According to Scarborough et al. (2014), the production of animal-based foods is associated with higher greenhouse gas emission compared to plant based foods. The greenhouse gas emission for meat-eaters compared to vegetarian is 50% and 54% higher for women and men respectively (Scarborough et al., 2014). Meat has higher environmental effects (greenhouse gases, land use, energy use, nitrogen use and phosphorus use) per serving compared to legumes (Willett et al., 2019).Utilising legume as an alternative protein source would substantially reduce the carbon footprint of protein production, make a valuable contribution to reducing the impact of agriculture and food consumption on climate change. Springmann et al. (2018) reported that replacing 25% to 100% of animal products with plant-based food contributes to reduction in greenhouse gas emission from 20% to 84%. Interest in legume protein is also increasing due to its price being around 80% cheaper than animal protein. According to World Bank report, the total global demand for meat is expected to rise by 56% between 1997 and 2020 (Asgar et al., 2010). This is because the world population has increased and so do the rising income. The growing demand of meat requires more use of water, energy and land

to grow feed for the animals. Legumes have the potential to help meet the increasing worldwide demand for proteins.

Anti-nutritional factors, present naturally in legumes enable them to survive and complete their life cycle under natural conditions. These anti-nutritional factors affect the protein digestibility and the bioavailability of amino acids. Anti-nutritional factors are diverse and include proteinase inhibitors, lectins, polyphenols, phytates. These factors are partially or completely removed by processing (Asgar et al., 2010). In order to improve the commercial exploitation of legumes for food, proteins need to be extracted, isolated and characterised. Extracting protein involves wet processes either in water, acid, salt and alkaline. The effectiveness of the extraction is affected by several factors such as pH, ionic strength, solid to solvent ratio, temperature, particle size of flours, time of extraction (Eromosele et al., 2008, Jyothirmayi et al., 2006) and the type of raw materials. One of the best known methods for extracting proteins is the Osborne protein fractionation process which involves sequential extraction of proteins using water, salt solution, alkaline and alcoholic solution. (Chavan et al., 2001). To ensure that proteins can be applied in food system, proteins need to be extracted using food grade methods. Besides, extracting proteins in harsh environment such as high pH will lead to protein denaturation and affect protein quality. It will lead to the loss of lysine and cysteine with the formation of new amino acids such lysinoalanines ornithoalanine and beta-amino alanine lathionine which

are considered to be nutritionally toxic. Once the extraction has been carried out, proteins need to be isolated to remove the anti-nutritional compounds. The isolated proteins need to be characterised in terms of their qualitative and quantitative analysis and functional properties to select the best extracts for different applications.

Functional properties are influenced by many factors such as the procedure used to make flours, concentrate and isolates, the naturally associated components with the protein such as carbohydrate, lipids, fibres and polyphenols, and the extraction conditions such as pH, temperature and salt which might influence the properties of the protein extracted. All of these factors must be controlled as it is possible to disrupt the protein structure and lead to degradation of certain amino acids which might reduce the functionality to the proteins in the food system**.** All of these factors need to be taken into consideration in order to make the underutilized protein-rich source useful as a functional ingredient in food product (Arogundade et al., 2006). The functional properties that are required from a protein vary with different food systems. However, the most important functional properties that are normally associated with food proteins are solubility, emulsion capacity, foaming capacity and gelling effect. Proteins are often used as food ingredients for their functional properties to impart certain specific characteristics to the final product (Adebiyi and Aluko, 2011).

In this study, bean varieties grown in the UK have been selected because there are easily grown in the UK, but are underutilised in food market. The extracted proteins will be isolated and characterised for their functionality in food products. Despite being very commonly grown in the UK and the world, little is known about the proteins from broad beans (*Vicia faba).* Therefore, these beans are one of the potential legumes that can be exploited on its use for producing high quality of food.

1.2 Literature review

1.2.1 *Vicia faba*

Vicia faba or faba bean, also commonly known as broad bean is considered as an early legume crop. The origin of *Vicia faba* is still unknown but the oldest seeds were found in late 10th millennium B.P. in north-west Syria (Multari et al., 2015). *Vicia faba* is considered as a versatile crop as it has the ability to grow in various climatic zones and it can be utilized throughout the year (Multari et al., 2015). The best development is during the cool temperatures and with that it is normally planted during spring in the northern hemisphere and winter in warm areas. It is able to grow in high altitudes and cultivated in geographical areas where the growing season is shorter than 100 days which makes it suitable to be grown in Boreal zone of Europe, Asia and North America. *Vicia faba* can also fix more nitrogen than other legume species under the same soil conditions and is used as a

break crop in cereal-dominated crop rotation (Multari et al., 2015). *Vicia faba* is a rich source of protein, providing a balanced diet of lysine-rich protein, carbohydrates, fibre and secondary metabolites (Rizzello et al., 2016). It also has the potential to replace soybean and can help meet the demand for protein in European countries who rely on imported soy protein (Rizzello et al., 2016).

Besides the advantages, *Vicia faba* also contains anti-nutritional compounds which makes it still underutilised particularly as human food. In the raw state, *Vicia faba* contains phytic acid, saponins, lectins, alkaloid and others which impairs the digestibility of seeds and lead to some pathological conditions. In particular, *Vicia faba* are rich in pyrimidine glucosides vicine and convicine which are involved in the plant defence against pathogens (Multari et al., 2015). Ten wild-type cultivars of *Vicia faba* grown in the same year and location contained vicine and convicine ranging from 5.16 mg/g to 7.59 g/mg and 2.09 mg/g to 3.63 mg/g dry weight respectively (Pulkkinen et al., 2015). Five low-type cultivars of *Vicia faba* contained vicine and convicine ranged between 0.65 mg/g to 0.65 mg/g dry weight and not detected respectively (Pulkkinen et al., 2015). Upon hydrolysis, vicine and convicine turn into aglycones, divicine and isouramil respectively. These can induce favism, a particular type of anaemia due to genetic mutation that occurs in susceptible individuals with low glucose-6-phosphate-dehydrogenase (G6PD) by oxidising glutathione. Favism was originally noted in Mediterranean and is now widely present not

only in Mediterranean but also in middle east, far east and north Africa where the growth and consumption of faba bean are found (Cappellini and Fiorelli, 2008). The function of G6PD is to regulate the production of NADPH in the red blood cell, and individuals with G6PD deficiency are unable to reproduce reduced glutathione and to control the oxidative stress which lead to acute hemolysis anaemia called favism (Pulkkinen et al., 2015, Rizzello et al., 2016). Upon ingestion of *Vicia faba*, the reaction will take place within 6 to 24 hours with prostration, pallor, jaundice, abdominal pain and dark urine (Luzzatto and Arese, 2018, Cappellini and Fiorelli, 2008). These symptoms result from destruction of red cells, triggered by divicine and isouramil. The global prevalence of G6PD deficiency which leads to favism is 4.9% which is about 400 million worldwide (Nkhoma et al., 2009, Cappellini and Fiorelli, 2008). According to review by Luzzatto and Arese (2018) favism is still associated with several myths that favism can be triggered by inhalation of the pollen from faba plant and other beans can cause attack of favism. A person may be allergic to pollen but that will not lead to haemolytic anaemia and the concentrations of vicine and convicine from other beans are negligible but not faba bean (Luzzatto and Arese, 2018). Most cases on favism are also reported after eating raw beans rather than cooked beans. Therefore, individuals with G6PD deficiency must be aware of their status and prepared to avoid factors that might trigger favism by not eating faba beans. Also, soaking, cooking or roasting should be recommended

before eating faba beans to inactivate the glucosides (Luzzatto and Arese, 2018). However, the level of general awareness of favism in areas with high prevalence of G6PD deficiency is satisfactory because the incidence of favism have greatly reduced with consistent measures of new-born screening and health education (Cappellini and Fiorelli, 2008, Luzzatto and Arese, 2018). Another measure that can be considered is producing products with low or no vicine and convicine. Study reported by Vioque et al. (2012) has successfully reduced vicine and convicine content by more than 99% producing *Vicia faba* protein isolates from flour. Figure 1.1 represents the molecular structures and molecular weight of vicine and convicine with their respective aglycones. Nevertheless, vicine and convicine can be removed by processing the seeds by soaking in water, acid or alkali and roasting or boiling because vicine and convicine are partially thermolabile and soluble in water (Multari et al., 2015).

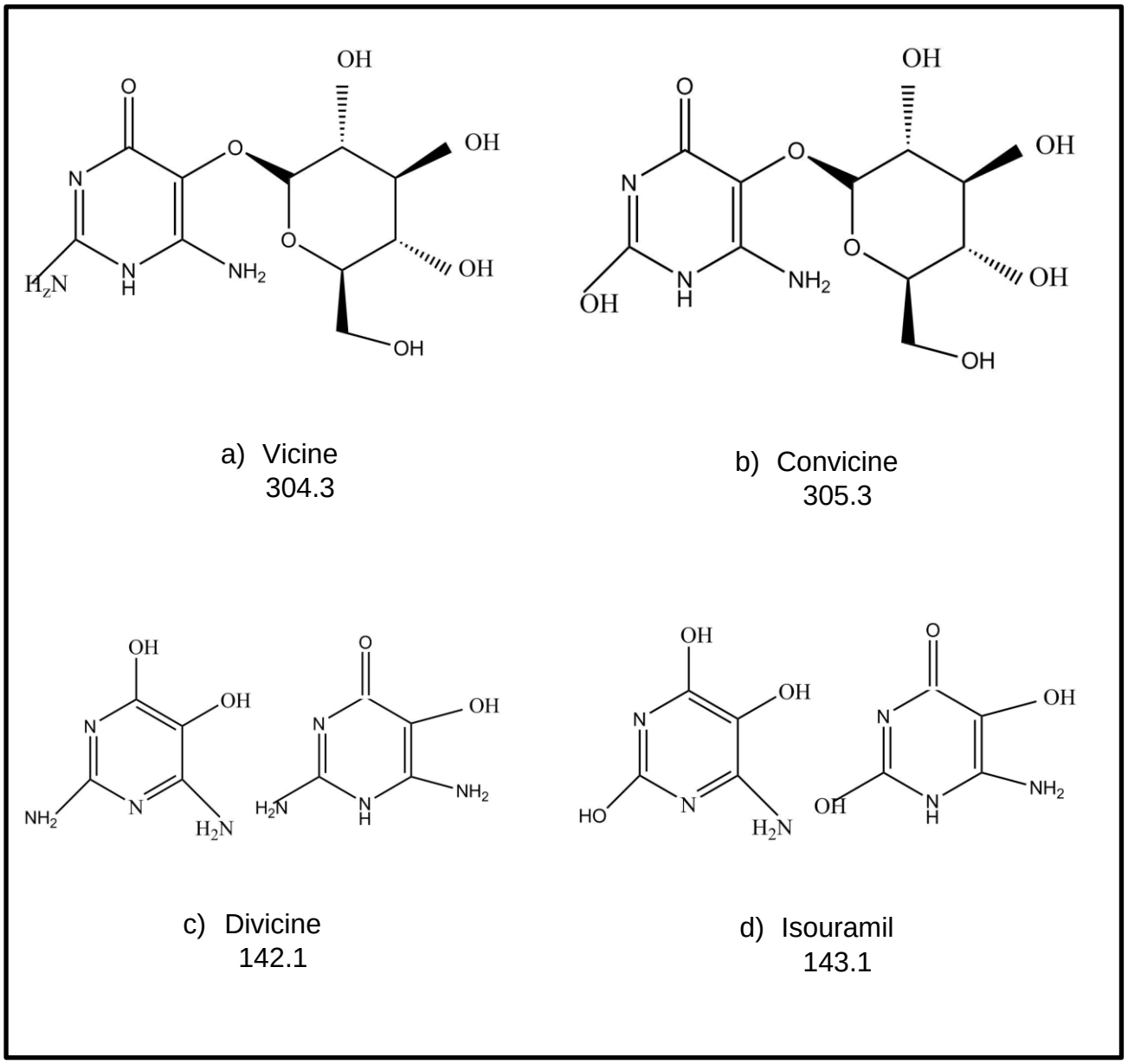

Figure 1.1: Molecular structures and molecular weight of a) vicine and b) convicine and their aglycones c) divicine and d) isouramil

1.2.2 Legume proteins

Legume seeds accumulate large amounts of protein during seed development (Multari et al., 2015). The proteins are termed storage proteins, stored in membrane bound organelles called protein bodies in parenchyma cells of the cotyledon (Duranti and Gius, 1997). Legumes proteins mainly comprise a large number of different proteins, the main ones being albumin and globulins, where globulins are dominant ones. Storage proteins are a source of amino acids and nitrogen for the seed during germination (Asgar et al., 2010). Storage proteins are high molecular-weight oligomeric proteins and are the major components of protein isolates prepared from the seeds (Kaur and Singh, 2007). The albumins are a diverse group of water soluble and comprise enzymatic proteins, protease inhibitors, amylase inhibitors and lectins (Boye et al., 2010a). Their molecular weight ranges between 5 to 80 kDa (Boye et al., 2010a).

Globulins on the other hand are salt-soluble and the major globulins found in legumes are vicilin (7S) and legumin (11S), separated according to their sedimentation coefficient (Boye et al., 2010a). The well-studied 7S and 11S proteins of pea are also named vicilin and legumin (Duranti and Gius, 1997). The proteins from other legumes are normally referred to as vicilin and legumin-like globulins. The 7S and 11S storage proteins are made of polymorphic subunits encoded by multigene families (Schwenke, 2001). The 7S globulins are oligomeric proteins which have a trimeric structure with each

subunit having a molecular weight in the range of 40 to 75 kDa making up a native protein of about 150 to 170 kDa (Boye et al., 2010a, Duranti and Gius, 1997). However, in a review by Sathe (2002), 7S globulins are described as very heterogeneous due to uneven glycosylation of their subunits (Scholz et al., 1983). The 11S globulins are also oligomers with hexameric quaternary structures. The 11S globulins consist of acidic (α) and basic (β) subunits with approximate molecular weights of 40 kDa and 20 kDa respectively (Duranti and Gius, 1997, Boye et al., 2010a). The subunits are bound by disulphide bonds to form a polypeptide of about 60 kDa. Six polypeptides are thought to constitute the native molecule (hexamer) with molecular weight of 320 to 400 kDa (Sathe, 2002). 7S vicilin has no disulphide bonds whereas 11S legumin subunits are linked by disulphide bridges. A third storage protein observed in the seeds of pea is convicilin (Boye et al., 2010a). Convicilin contains very little carbohydrate and has a subunit of 71 kDa and its native form including an N-terminal extension has molecular weight of 290 kDa (Boye et al., 2010a).

Table 1:1shows molecular weight of legume proteins reported from previous studies on faba bean, soy, pea and hemp. Based on Table 1:1, vicilin, legumin and convicilin from various sources of legumes have slightly different molecular weight. However, based on the gel electrophoresis pattern, the order of the protein bands is in agreement, starting with basic and acid subunits of legumin, followed by vicilin, and convicilin. Convicilin is only observed in *Vicia faba* and pea.

According to Chavan et al. (2001) and Adebiyi and Aluko (2011), the expected protein fractions in seed flour extracted sequentially with the four Osbourne solvents are albumin, globulin, glutelin and prolamine respectively. The amino acids of each fraction differ. The albumin/globulin fractions are rich in the essential amino acid lysine, aspargine but contain less leucine, phenyalanine, glutamine, proline and tyrosine compared to the other two fractions. Glutelin on the other hand has high level of glycine and contain higher concentration of methionine and cystine than the globulins which makes it interesting. Finally, prolamin fraction contains high levels of phenyalalnine, isoleucine, proline and glutamine but lower levels of lysine, alanine, threonine, glycine, histidine and aspargine (De Brier et al., 2015). The different compositions of amino acid compositions will influence the functional properties of proteins. All the protein fractions can be characterized by using gel electrophoresis or gel chromatography where subunits of each fraction will be separated. In soy protein, 7S (β-conglycinin) and 11S (glycine) globulins are among the proteins which gives the functional properties to the soy protein (Kimura et al., 2008).

Table 1:1: Molecular weight of globulins proteins from various source of legumes

Legumes	Globulins	Molecular weight (kDa)	References
Vicia faba	7S Vicilin	47 to 50 kDa	(Nivala et al., 2017, Cai et al., 2002)
	11S legumin (acidic)	40 kDa	
	11S legumin (basic)	20 kDa	
	Convicilin	57.5 kDa	(Felix et al., 2018, Liu et al., 2017)
Pea	Vicilin	47 kDa	(Osen et al., 2015)
	11S legumin (acidic)	40 to 45 kDa	(Beck et al., 2017, Cai et al., 2002)
	11S legumin (basic)	21 to 25 kDa	
	Convicilin	~ 70 kDa	(Osen et al., 2015)
Soybean	7S β-conglycinin (α,α' and β subunits)	68, 72, 52 kDa	(Aghanouri et al., 2014)
	Glycinin (acidic subunit)	40 kDa	(Cai et al., 2002)
	Glycinin (basic subunit)	23 kDa	
Hemp	Edestin (acidic subunit)	34 kDa	(Raikos et al., 2015, Raikos et al., 2014)
	Edestin (basic subunit)	18-20 kDa	

1.2.3 Preparation of isolates

The process of preparing isolates depend on the intended end use. Therefore, many studies on the preparation of isolates used different techniques to suit the application of the particular study. Mostly, protocols described in the literature to prepare extracts with high protein content employed alkaline extraction followed by separating the insoluble components, precipitating the protein to isoelectric pH and drying to form powders (Boye et al., 2010b, Zayas, 1997).

1.2.4 Protein extraction and post extraction

Protein extraction involves wet processing in several solvents such as water, salt, acid/alkaline and alcohol. Types of proteins that will be extracted from the raw materials depend largely on the solvent employed. The use of various aqueous extraction solutions would yield different protein fractions that differ in protein functionality. Extraction medium is very important since it will affect the functionality of the isolate. Water is advantageous since it is non-flammable, explosive nor toxic and is economical. In distilled water, the protein extracted tends to be lower compared to aqueous solvent containing salt (NaCl, Na_sSO_4) and NaOH. The use of salt (sodium sulphite) in aqueous solvent is also important to prevent oxidation of polyphenols which may cause the darkening in the protein isolates (Vioque et al., 2012). The method developed by Osborne uses sequential extraction with water, salt, alkaline and alcohol are used to get albumin, globulin, prolamin and glutelin respectively. Many studies have used this

method to fractionate protein before characterising it. The effectiveness of these solvents in extracting protein might also influenced by other factors such as the solvent to flour ratio, pH, temperature, ionic strength, particle size of raw materials. Table 1:2 shows extraction conditions and Table 1:3 post-extractions process on *Vicia faba* protein isolate from previous studies and the outcome of the processing treatments. All of these studies have successfully isolated *Vicia faba* protein and achieved more than 80% protein content, yet, the extraction conditions used were different for each studies. All of these studies also are lacking on the information of the total yield of isolate which makes it difficult to know whether the process is economical.

Table 1:2: Extraction conditions and protein yield of *Vicia faba* protein isolate

Extraction conditions					Total protein content (%)	References
pH	***Temperature***	***Time***	***Solvent***	***Ratio***		
7	20°C	30 mins	water	1:5 (w/v)	92.7	(McCurdy and Knipfel, 1990)
7	21-22°C	30 mins	0.008 M NaOH	1:5 (w/v)	92.5	
10.5	4°C	60 mins	0.25% Na_2SO_4	10% (w/v)	92.4	(Vioque et al., 2012)
9	Not reported	Not reported	alkaline	Not reported	92.2	(Nivala et al., 2017)
9.5	RT	40 mins	1.0 M NaOH	1:10 (w/v)	84.4	(Karaca et al., 2011)
	RT	20 mins	water	1:5 (w/w) pellet:water)		

Table 1:3: Post-extraction conditions of *Vicia faba* protein isolate

Post-extraction conditions					Total yield (%)	References
Centrifugation after extraction	***Isoelectric precipitation pH***	***Centrifugation after precipitation***	***Drying***	***Ratio***		
✓	4.7	✓	water	1:5 (w/v)		(McCurdy and Knipfel, 1990)
✓	4.7	✓	0.008 M NaOH	1:5 (w/v)		
✓	4.0	✓	0.25% Na_2SO_4	10% (w/v)	Not reported	(Vioque et al., 2012)
✓	4.0	✓	alkaline	Not reported		(Nivala et al., 2017)
✓	4.5	✓	1.0 M NaOH	1:10 (w/v)		(Karaca et al., 2011)
			water	1:5 (w/w) pellet:water)		

1.2.5 Effects of processing conditions on the extraction of protein and non-protein content of legume isolate

Extraction process is influenced by many factors which are pH, time and temperature, ionic strength, solid to solvent ratio and particle size.

1.2.5.1 pH

Protein solubility is one of the critical factors to be studied before applying it into a food product. It is clearly stated by Raikos et al. (2014) that protein solubility is dependent on pH and the solubility is increasing from pH 4 to 10. This is because pH 4 is near the isoelectric point, where the net charge is zero for most proteins. The effect of pH can be seen in extracting protein from African yam bean (Eromosele et al., 2008) where the protein extractability was high in both acidic and alkaline medium. However, when the pH reached the isoelectric point (pH 5), the protein extractability decreased to 16% and while it increased to 87% at pH 10. At the isoelectric point, the protein molecules tend to aggregate into an insoluble mass due to a decrease in electrostatic charge repulsion between the particles. As pH increases, the net negative charge increases thus solubility increases. However, extraction at pH more than 10 is not recommended because it causes extreme change in the protein environment. Moreover, extreme pH will induce the formation of a toxic crosslinked amino acids like lysinoalanine by reactions of lysine with dehydroalanine (Peyrano et al., 2016).

1.2.5.2 Time and temperature

High temperature and prolonged extraction time can reduce the nutritional quality as it increases the chances of proteolytic activity. The nutritional quality of food proteins is dependent largely by the composition of essential amino acids and digestibility. At high temperature with an alkaline treatment, there is loss of lysine and cystine, formation of new amino acids such as lysinoalanine, ornithoalanine, beta-amino-alanine, lanthionine which are undesirable nutritionally. Lysinoalanine and lanthionine are said to be formed by heat treatment even at neutral pH (Dev et al., 1986). These effect need to be taken into consideration to compromise between the protein yield and quality while extracting and isolating the protein. However, there is a broad range of optimum temperature for extraction of protein from different sources. The extremely high temperature also contributes to decrease in protein extraction due to coagulation of extracted protein. The effect of time and temperature in protein extraction are very limited and further studies are required.

1.2.5.3 Ionic strength

The addition of salt helps to increase the protein solubility at isoelectric point. The effect of ionic strength is dependent on the medium pH and it is on one of the most important environmental factor in the study of protein functional characteristics. The increase in ionic strength enhances the protein solubility of the particular flour samples thus influence the protein extraction yield. Ionic strength affects protein solubility by its electrostatic, salvation, *salting in* and *salting out* phenomenon. At high pH (10 and 12) the predominant negative charges on the proteins are being neutralized by the Na^+ (from ionic

medium containing NaCl) with increasing ionic strength. And thus, more protein–protein interaction, coupled with the *salting-out* effect of the high ionic strength of the medium which causes continual decrease in its protein solubility as ionic strength increases. While at the other pH (2, 4, and 7), the initial *salting in* effect at low ionic strength may serve to dissociate the protein aggregates, thereby increasing solubility. But further increase in ionic strength of the medium causes a reduction in the predominant positive charges due to the neutralizing effect of Cl^- from the salt (NaCl) this consequently leads to reduction in protein–protein repulsion. Therefore, the concentration of the salt need to be taken into a consideration as the increasing concentration may have a dehydrating effect towards the protein. This combined effect leads to protein aggregation and consequent decrease in its protein solubility (Arogundade et al., 2006).

1.2.5.4 Solid to solvent ratio

The ratio between the solid and the solvent also has some influence during the protein extraction depending on other factors too such as ionic strength and particle size of the solid and samples types. The decrease in protein extraction was observed by Dev et al. (1986) and Eromosele et al. (2008) when higher ratio was used. This is because the higher ratio may lower the ionic strength and this may influence the protein extraction. Higher ratio may cause the protein to be co-extracted with other components of the food (e.g. carbohydrate) which may form insoluble complex aggregates with the soluble protein.

1.2.5.5 Particle size

Different milling treatment gives different particle size to the flours. Smaller particle size of the solid is expected to increase the amount of protein extracted as the surface area between the solid and solvent is increasing. Milling for a short period of time induces fractures in the cell wall, thus decrease the particle size of the flour. However, prolong milling will build up heat between the particles and cause protein breakdown (De Brier et al., 2015). The effect of particle size on the protein extraction and the functionality of protein are very limited and need to be further studied.

1.2.6 Effects of processing on the functional properties of protein isolate.

Functional properties of protein are affected by the molecular and structure of the native protein, processing technique and also environmental factors. Functional properties of proteins are defined as physical and chemical properties including their size, shape, amino acid composition and sequence, net charge, charge distribution, hydrophobicity, hydrophilicity, structures, molecular flexibility in response to external environment or interaction with other food components that influence the functional behaviour of proteins in food systems during processing, storage, cooking and consumption. The functional properties of proteins can be classified into three categories: 1) properties related with hydration (absorption of water/oil, solubility, thickening, wettability), 2) properties related to the protein structure and rheological characteristics (viscosity, elasticity, adhesiveness, aggregation and gelification and 3) properties related to the protein surface (emulsifying and

foaming activities, formation of protein-lipid films, whippability) (Moure et al., 2006). In order to establish the proteins from underutilized legumes into food systems, proteins must possess multiple functionalities. The functional properties that are required from a protein vary with different food applications. The most important functional properties that are associated in food systems are solubility, emulsifying properties, foaming properties and gelling properties.

1.2.6.1 Solubility

Protein solubility is one of the most important functional properties and it is a prerequisite for a protein in order to be useful in food system. It is a physicochemical property that can affect texture, colour and sensory properties of food system. Good protein solubility is required to obtain optimum functionality in gelation, emulsifying and foaming. Factors such as concentration, pH, ionic strength and the presence of other substances influence the solubility of protein. Generally, the solubility of proteins decrease as it comes closer to the isoelectric pH and increase with further increase in pH. Various studies on legume proteins have reported the protein solubility using various methods and buffers.

The effects of environmental pH was studied by Arogundade et al. (2006) which reported protein extractability of broad bean full-fat whole seed flour (BFWSF), broad bean full-fat dehulled seed flour (BFDSF) and broad bean protein concentrate (BPC) at pH 2, 4, 7, 10 and 12. The protein extractability for all three products were in the range of 6 to 33%. The lowest extractability was observed at pH 4. The protein solubility was done only on BPC at various

pH and ionic strength. BPC protein solution prepared at pH 4 have enhanced protein solubility with the increase in ionic strength of 0.05 to 0.4. Study by Nivala et al. (2017) that looked at the soluble protein content of faba bean protein isolates at pH 3 to 10 also reported that the solubility was pH dependent. The highest solubility was observed at pH 10 with about 80% soluble protein and the lowest solubility was observed at pH around 4 to 5. The solubility increased alongside the surface charge (mV) of the protein. According to Adebiyi and Aluko (2011), pea protein fractions (water, salt soluble, alkaline soluble and ethanol soluble fractions) had better solubility at all pH examined compared to the solubility of PPI. Solubility of PPI was between 18 to 20% between pH 3 to 9. The lower solubility was observed at pH around 4. Raikos et al. (2014) also reported that protein solubility of wheat, lupin, green pea, fava bean, hemp and buckwheat flour was pH dependent. The variations in the protein solubility was also observed between flours at all examined pH.

Other studies also looked at the effect of processing such as extraction and post-extraction conditions such as precipitation. Karaca et al. (2011) compared the protein solubility of protein isolates prepared by isoelectric precipitation and salt extraction in phosphate buffer pH 7. Higher solubility for protein isolates prepared by isoelectric precipitation for chickpea, faba bean, lentil, pea and soy protein isolate was observed. The surface charge of (mV) of protein isolate was also lower in salt extraction compared to isoelectric precipitation. Meanwhile, Peyrano et al. (2016) observed the different of the solubility between cowpea protein isolate extracted at pH 8 (91%) and 10

(93%), but found no significant difference. Protein solubility of yellow pea, green lentil, red lentil, desi chickpea and kabuli chickpea protein concentrate prepared by isoelectric precipitation and ultrafiltration was also studied by Boye et al. (2010b) which observed higher solubility in ultrafiltration compared to isoelectric precipitation in all pH of the solution except for yellow pea protein concentrate. The solubility of chickpea isolate prepared with NaOH pH 12 and with sodium sulphite pH 10.5 was also compared. Sánchez-Vioque et al. (1999) reported that chickpea isolate prepared with sodium sulphite exhibited higher solubility.

1.2.6.2 Water holding capacity (WHC) and fat holding capacity (FHC)

Water holding capacity is one of the important functional properties that can determine the potential uses of protein isolates. It is defined as the ability to hold its own and added water during the application of forces, pressing, centrifugation or heating (Zayas, 1997). WHC depends on the type and quantity of the protein alongside with the presence of non-protein components (Sathe, 2002). According to the review by Sathe (2002), most legume proteins hold water less than 5 to 6 times their own weight. This functional property is widely measured using centrifugation technique. Similarly to water holding capacity, fat holding capacity is the ability of protein to hold fat. Most legume proteins hold less than 5 g fat/g of protein (Sathe, 2002).

WHC and FHC are influenced by the amount of hydrophilic and hydrophobic amino acids. Kaushik et al. (2016) reported higher WHC in flaxseed protein isolate compared to FHC could be due to the presence of more hydrophilic sites in its structure. The higher proportion of non-polar amino

acids in proteins results in more lipophilic characteristics Another study by Arogundade et al. (2006) reported that WHC of broad bean protein concentrate to be 1.25g/g showed that it contained less exposed hydrophilic groups. Ionic strength of 0.1 and 0.2 showed an improvement on WHC by 20% and 12% respectively. WHC and FHC of chickpea protein isolate prepared with sodium sulphite pH 10.5 and without sodium sulphite pH 12 have also been reported. Effects of pH (4, 7 and 10) on WHC of various flours was observed by Raikos et al. (2014) and found no significant difference. However, protein content shown to reflect the trend of WHC among all the flours reported (Raikos et al., 2014). Sánchez-Vioque et al. (1999) found that isolate prepared at pH 10.5 with sodium sulphite had lower WHC and FHC compared to isolate prepared at higher pH. Another study by Peyrano et al. (2016) reported that WHC of cowpea protein isolate extracted at pH 8 and 10 were similar. However, when both isolates were heated, increased in WHC was observed due to more unfolding-induced exposure of polar amino acids. WHC and FHC of yellow pea, green lentil, red lentil, desi chickpea and kabuli chickpea protein concentrate prepared with isoelectric precipitation and ultrafiltration were reported. Boye et al. (2010b) observed that WHC of protein prepared by isoelectric precipitation was higher than ultrafiltration, meanwhile, protein prepared by ultrafiltration had higher FHC compared to the ones prepared with isoelectric precipitation. Yet, the reasons are not clearly identified. In addition, differences in WHC and FHC were also observed between different samples.

1.2.6.3 Emulsifying properties

Emulsifying properties can be evaluated by the protein's emulsion stability (ES) and emulsion activity (EA). The ES is a measure of the stability of the emulsion over a certain period of time and EA is a measurement of how much oil a protein can emulsify per unit protein (Boye et al., 2010a). The physicochemical properties of legumes which are molecular size, surface hydrophobicity, net charge, steric hindrance and molecular flexibility have been shown to influence emulsifying properties (Karaca et al., 2011). Surface hydrophobicity and surface charge were proposed to be the most important factors (Schwenke, 2001). Protein is able to simultaneously remain at the aqueous phase and adsorb at the surface of oil droplets due to their amphiphilic nature by stabilising electrostatic forces and steric hindrance (Karaca et al., 2011). Therefore, the net surface charge of protein must be large enough to overcome the attractive forces (van der Waals, hydrophobic, depletion) which can stabilize the electrostatic repulsion between the oil droplets (Karaca et al., 2011). It is also reported that emulsion capacity and emulsion stability increased as the surface charge and solubility increased and emulsion capacity increased as surface hydrophobicity decreased. However, the processing techniques used to prepare isolates (isoelectric precipitation and salt extraction) showed no significant difference on the emulsifying properties (Karaca et al., 2011). Similarly, Boye et al. (2010b) also found no difference in isolates prepared by isoelectric precipitation and ultrafiltration. But both studies found the difference in emulsifying properties for different legume sources. Meanwhile, Sánchez-Vioque et al. (1999) reported that

chickpea isolates prepared with sodium sulphite at pH 10.5 had higher emulsion capacity compared to isolate prepared with NaOH at pH 12. Emulsifying properties of legumes have been reported by many studies but limited studies have been carried out on the effects processing such as extractions and different studies used different techniques of measurement.

Many studies focused on the effects of environmental factors particularly pH and ionic strength. The emulsifying ability and stability of 7S and 11S globulin at 0.5 and 0.08 ionic strength of various legumes (cowpea, French bean, fava bean, pea and soybean) were reported (Kimura et al., 2008). They reported the emulsifying abilities by measuring the sizes of their emulsions. The smaller the size of droplet, the better the emulsifying abilities. French bean emulsion have been found to have the smallest droplet at both ionic strength compared to other legumes. Effects of pH on emulsion stability of PPI and its fractions (water, salt, alkali, ethanol) was also reported by Adebiyi and Aluko (2011). The emulsifying ability and stability of wheat, lupin, green pea, fava bean and buck wheat flour was reported by Raikos et al. (2014) and found that emulsifying properties and stability of all flour increased as the pH increased from pH 4, 7 and 10. In this study, all flour had the highest emulsifying activity and stability at pH 10 and lowest at pH 4. Emulsifying properties of walnut flour, walnut protein concentrate and walnut protein isolate were also shown to be pH dependent (Mao and Hua, 2012).

1.2.6.4 Foaming properties

Foaming capacity and stability are greatly influenced by pH and it is one of the requirements on food manufacturing of ice cream, cakes and meringues (Adebiyi and Aluko, 2011). Adebiyi and Aluko (2011) has reported that PPI and its salt soluble fraction has increased foam stability from pH 4 to 9. This is due to increased charged density that helps to prevent rapid coalescence of the air bubbles and stabilizes the foams by increasing the electrostatic repulsions which reduced the rate of coalescence of foam particles (Adebiyi and Aluko, 2011). Similarly, Arogundade et al. (2006) has also reported that higher pH increased the foam expansion of broad bean protein concentrate. Raikos et al. (2014) reported that foaming capacity of lupin, green pea, fava bean, hemp and buckwheat flour was pH-dependent but the foaming capacity was not the same for all samples. Protein solubility also has been reported to influence foaming capacity (Arogundade et al., 2006, Adebiyi and Aluko, 2011, Toews and Wang, 2013). Besides pH and solubility, source of protein, methods of processing, temperature, ionic strength, protein concentration, mixing time and method of foaming also affect foaming properties (Meng and Ma 2002). Foam stability and the proteins ability to form foams are also important. Another study showed that, the foam stability increased with pigeon pea protein concentration and ionic strength which has reported increased in foaming capacity as protein concentration increased (Akintayo et al., 1999). Foaming stability of broad bean protein concentrate gradually decreased with time regardless of pH and ionic strength, however, foams prepared at pH 4 was the most consistent at all ionic strength compared to foam prepared at pH

2, 10 and 12 (Arogundade et al., 2006). Toews and Wang (2013) reported that poor foam stability of commercial pea and soy protein was due to higher fat content in the sample.

Mostly, these studies have observed the effects of environmental factors on the foaming properties of proteins. Lack of studies on the processing effects (extractions) on the foaming properties have been reported. Study by Jarpa-Parra et al. (2014) on lentil protein isolate reported the effects of extraction pH (8, 9 and 10) on the foaming properties was not significant. However, significant differences occurred when pH of solution changed. Other study by Boye et al. (2010b) has reported that the foaming capacity of pea, chickpea and lentils protein isolates either prepared by isoelectric precipitation or ultrafiltration does not show any impact. However, foaming stability has been reported to be better in isolates obtained by ultrafiltration.

1.2.6.5 Gelling properties

The globular proteins' gelling properties are of big importance in food. It is important to modify the structure and texture of foods such as meat processing to make patties or meatballs. Besides, the gelation properties of egg in foods are one of the important example in the process of making cakes, omelettes and confectionary. Gel formation by globular proteins is a complex process that involves processes which are denaturation, aggregation and network formation. Legume proteins require 10 to 20% w/v and temperature around 70°C to form gel (Sathe, 2002). Globular proteins such as egg white and soybean protein are able to form gels upon heating which is called heat-

induced gel, the most common gelation in plant protein. For a gel to form the functional groups of hydrophobic must be exposed. Least gelation concentration is an indication of better gelling ability of the protein as small amount is required (Adebiyi and Aluko, 2011). Gel formation is complicated, and affected by the concentration of protein, amount of water, ionic strength, time and temperature as well as pH and interaction with other components in the food system (Raikos et al., 2007). The gelling process involves native protein to denature and during that, the denaturation of disulphide bond will form and hydrophobic amino acids are exposed. Further denaturation and heating, the proteins will aggregate and interact with other proteins to form either gel or coagulum.

1.2.7 Non-meat proteins as binder in beef patties

Beef is often consumed by large population worldwide which will have an impact on the greenhouse gas emission. Beef patties are popular kind of meat product eaten around the world. However due to their high meat, fat and cholesterol content, their consumption is associated with several chronic disease. Eliminating meat entirely from daily diet is very difficult. Therefore, finding alternative source of protein from plant sources and incorporate it into meat or meat products like beef patties is one of the way to reduce meat consumption, increase plant consumption, produce lower fat version of beef patty and at the same time reduce the cost.

Beef patties are considered as coarse ground products. Binding of water and fat to the ground meat are important factors to stabilize the meat emulsion (Devadason et al., 2010). Plant or animal proteins are used in meat products

to perform three main functions: 1) emulsification, 2) water retention and 3) structure of meat products (Dzudie et al., 2002). The addition of protein also helps actin and myosin which are proteins in meat by stabilizing the emulsion system by reducing the possibility of breaking, decreasing the fat and moisture loss during cooking (Zayas, 1997). Study reported by Dzudie et al. (2002) observed the difference in WHC, colour, cooking loss and textural properties of sausage formulated with 2.5, 5, 7.5 and 10% of the meat weight with common bean flour (CBF), compared to control sausage. Dzudie et al. (2002) found that sausage batters with common bean flour showed decreased in cooking losses with increased WHC. Cooking losses were the lowest for the sausage that contained 10% common bean flour. However, there no significant difference were found in sausage containing 5, 7.5 and 10%. They also reported that the differences in colour of the cooked sausages is probably due the dilution of myoglobin of meat and to some extent to colour of the flour additives. The hardness of sausage was also the highest for control samples and lowest for the samples containing 5, 7.5 and 10% CBF. This is because substitution of CBF for muscle dilutes the quantity of connective tissue in CBF-extended beef sausages and accounts for lower shear force values. The factors responsible for textural properties in comminuted meat products are mainly the degree of ex-traction of myofibrillar protein, stromal protein content, degree of comminution and type and level of non-meat proteins. Brown and Zayas (1990) also studied on beef patties extended with 10, 20 and 30% of the uncooked weight with hydrated corn germ protein flour. The quality of extended beef patties was evaluated by measuring the amino acid

composition, pH, WHC, cooking loss, cooking yield , textural and sensory properties.

Troy et al. (1999) looked at the effects of beef patties quality when extended with blends containing tapioca starch, oat fibre and pectin and found that the combination of starch, oat fibre and pectin have the potential application of improving the overall palatability of low fat beef burger. Thus, adding binders that contained starch, protein and fibre are useful because each of them has particular role in maintaining the quality of beef patties. Starch aids in the retention and subsequent released of moisture, giving increased succulence, and flavour released. Fibre retains moisture and keep meats from drying out when it is cooled. Protein helps in fat binding which will then improve flavour and texture.

According to review by Petracci (2013), the most common plant proteins that are used in meat products are derived from soybeans or wheat. Pea is becoming more popular in Europe because they are produced from not genetically modified, compared to most soybeans and so it can be used as an alternative to soybeans for meat substitutes. It can be seen that there is no progress in utilising extracted protein from *Vicia faba* into meat products. Petracci (2013) also reviewed that rehydrated flaked textured vegetable proteins from soybean, wheat and pea are widely used in meat products to optimize the cost of formulation by reducing the lean meat content.

1.3 Outline of thesis

1.3.1 Aim

The aim of the study is to utilise *Vicia faba* as an alternative source of plant protein and apply it into food product.

1.3.2 Objectives

1. Extraction, isolation and characterisation of *Vicia faba* isolates.
2. Investigation on the functional properties of *Vicia faba* isolates and commercial protein isolates.
3. Application of *Vicia faba* isolates into beef patties.

1.3.3 Hypotheses

Vicia faba has the potential to be utilised as an alternative source of protein and the isolates prepared will have good level of protein content that will possess good functional properties and it will be able to be used as functional ingredients in beef patties.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Vicia faba (Wizard winter variety) Figure 2.1 were purchased from Senova Limited, Cambridge, UK. The following food samples were purchased: soy protein isolate, whole egg powder (Myprotein, The hut group, Cheshire, UK), hemp protein isolate (Good Hemp Nutrition, UK), pea protein isolate (Pulsin, UK) and faba bean protein concentrate (Theproteinworks, https://www.theproteinworks.com/faba-bean). The plant protein samples were chosen from range of different legumes that were commercially available in the market. Soy, pea and hemp protein were chosen as there were published study on these legumes that can be used to compare the results. The processing method used to obtain the products is not known.

The cost of producing VFI was estimated based on the prices of raw materials used which were *Vicia faba* and chemical. The cost of *Vicia faba* is £1.75/kg and sodium sulphite is £12.03/kg. The cost of equipment and utilities were not calculated.

2.2 Preparation of *Vicia faba* flour

Vicia faba (VF) beans (Figure 2.1) were ground, using Krups Burr grinder GVX 2 and were stored in an airtight plastic bag in a dry place at room temperature prior to use. The *Vicia faba* flour contained a mixture of particle sizes range between 63nm to 710nm.

Figure 2.1: *Vicia faba*

2.3 General reagents

General reagents were of analytical grade and were purchased from Fisher Scientific, UK unless otherwise stated.

2.4 Extraction and isolation of *Vicia faba* protein isolate

There were three different techniques used to produce *Vicia faba* protein isolates.

2.4.1 Vioque's extraction method

The method used to extract and prepare the protein isolate was adapted from Vioque et al. (2012). *Vicia faba* flour was extracted by mixing the flour (10% w/v) in 0.25% (w/v) sodium sulphite (Na_2SO_3) (VWR Chemicals, UK) solution, pH 10.5, by stirring with a magnetic stirrer for 1 hour at 6°C in cold

room. After mixing *Vicia faba* flour with sodium sulphite, the pH of the suspension dropped to 7.6. The pH of the suspension was measured before and after extraction. The resulting extracts were centrifuged at 3857 x *g* for 15 minutes and the supernatant was acidified to pH 4 by using 2 M HCL. The precipitated protein was recovered by centrifugation at 3857*g* for 15 minutes. The pellet was washed with acidified water followed by centrifugation at 3857*g* for 10 minutes and freeze in -20°C overnight. The pellet was then freeze dried for 24 hours to obtain the *Vicia faba* isolate (VFI).

2.4.2 Modified extraction and isolation method

The extraction conditions were modified from Vioque's method 2.4.1 as described in Figure 3.2 *Vicia faba* flour was extracted by mixing the flour (10% w/v) in 0.25% (w/v) sodium sulphite (Na_2SO_3) (VWR Chemicals, UK) solution, pH 10.5, by stirring with a magnetic stirrer for 1 hour. Four extraction conditions were examined in this section to improve the consistency of pH and to increase the yield of *Vicia faba* isolate (VFI). The conditions were at pH 7.6 in 6°C, pH 7.6 in room temperature (RT), pH 10.5 in 6°C and pH 10.5 in room temperature (RT). The pH of the suspension was adjusted to either pH 7.6 or 10.5 using 2 M HCL. The resulting extracts were filtered and pressed through the muslin cloth and the supernatant was acidified to pH 4 by using 2 M HCL. The precipitated protein was recovered by centrifugation at 3857*g* for 15 minutes. The pellet was washed with acidified water followed by centrifugation at 3857*g* for 10 minutes and freeze in -20°C overnight. The pellet was then freeze dried for 24 hours to obtain and isolates prepared from this condition were denoted as [VFI 7.6 6], [VFI 7.6 RT], [VFI 10.5 6] and [VFI 10.5 RT]. Two layers was

obtained after freeze dried, therefore the sample powder for [VFI 7.6 RT] was separated.

2.4.3 Extraction without isoelectric precipitation

This extraction condition was done similarly as described in 2.3.2 without isoelectric precipitation. The extraction was done only at one condition which was at pH 7.6 in room temperature. Briefly, *Vicia faba* flour was extracted by mixing the flour (10% w/v) in 0.25% (w/v) sodium sulphite (Na_2SO_3) (VWR Chemicals, UK) solution, pH 10.5, by stirring with a magnetic stirrer for 1 hour at room temperature. The supernatant was filtered through 2 layers of muslin cloth (Amazon website, UK) for three times to ensure all the starch can be separated from the extracts without pressing through the muslin cloth. Next, the extract was freeze in -20°C for overnight. The frozen extract was freeze dried for 48 hours. The protein prepared from this condition was denoted as soluble [VFI 7.6 RT] FD.

2.5 Proximate analysis

2.5.1 Determination of total lipid content by Soxhlet method

Fat content of *Vicia faba* flour was determined by using the Soxhlet method (Latimer, 2016). Ten grams of *Vicia faba* flour were weighed into a beaker followed by 50 mL of 4 M HCL. The beaker was heated over a Bunsen flame in a fume cupboard. The mixture was boiled for 3 minutes until completely hydrolysed. Whilst still hot, the mixture was filtered through a No.1 fluted filter paper. The contents of the filter paper were washed twice with hot water and was left to air dry in the fume cupboard overnight. A round bottom flask was

placed in an oven at 105°C for 30 min and then cooled in a desiccator before weighing accurately on an analytical balance.

Each filter paper was placed into a Soxhlet extraction thimble after drying for total lipid content of samples. A cotton wool plug was placed into each thimble and the thimble was placed in Soxhlet extractor apparatus. Approximately 150-200 mL petroleum spirit was poured into each of the weighed round bottom flasks. The Soxhlet extraction and the electro thermal heating mantles were switched on and the extraction was carried out for about 10 hours. On the next day, the pressure equalising funnel was used to distill off the petroleum spirit from the round bottom flask until only about 10 mL of solvent remained. The round bottom flask was then placed on a steam bath to remove residual petroleum spirit. When all the solvent has been removed, the outside of the flask was dried before placing in an oven set at 80°C. After drying, the flask was cooled in desiccators for 30 min before weighing accurately. All samples were done in duplicate.

2.5.2 Determination of total protein content using Kjeldahl method

Total protein content of *Vicia faba* and commercial samples were carried out using Kjeldahl method (Latimer, 2016). Sample was weighed and one Kjeldahl catalyst tablet (5g K_2SO_4 + 0.5g $CuSO_4$ x $5H_2O$) (Gerhardt, UK) was added followed by 25 mL concentrated sulphuric acid into the digestion flask. The contents were heated gently on the electro thermal mantle in the fume cupboard until the liquid became clear and the flask was heated strongly for one hour. The digest was allowed to cool and it was then washed into 800mL

distillation flask by using a funnel. The distilled water was then added until the volume in the distillation flask became approximately 400mL. Then, 10 drops of phenolphthalein indicator, 1g of anti-bumping granules (VWR Chemicals, UK) and 1mL of anti-foam agent (Xiameter, UK) were added into the distillation flask. The condenser delivery tube dipping into 500mL conical flask containing 100 mL of 4% boric acid with screened methyl red indicator (Scientific Laboratory Supplus, UK) with the outlet of the delivery tube submerged in the boric acid. Next, 50% sodium hydroxide (VWR Chemicals, UK) was added via the dropping funnel until the test solution became alkaline (pink in colour). The distillation apparatus was sealed plug was replaced and sealed with a few mL of distilled water. The framework was gently rotated to ensure mixing. The distillation flask was heated with Bunsen burner until a minimum of 250 mL distillate has been collected. The delivery tube was washed down into the boric acid solution. Before removing the heat source, the closed system must be released to prevent ‘suck back’. Finally, the ammonia contained in the boric acid solution was titrated with 0.25 M sulphuric acid.

$$Protein\ content = (a \times 0.0070\ g\ nitrogen) * c$$

$$Where\ a\ is\ the\ titration\ volume\ of\ 0.25\ N\ sulphuric\ acid\ and$$

$$c\ is\ protein\ correction\ factor\ of\ 6.25$$

2.5.3 Determination of total dietary fibre using Megazyme kit (AOAC Method 991.43)

2.5.3.1 Preparation of buffer

MES/TRIS buffer was prepared by dissolving 19.52 g of 2(*N*-morpholino) ethanesulfonic acid (MES) and 12.2 g of tris (hydroxymethyl) aminomethane (TRIS) in 1.7 L milli-Q water. The pH was adjusted to pH 8.2 with 6 M NaOH.

2.5.3.2 Procedure

One gram of sample was weighed into 400 mL glass container with lid. Forty mL MES-TRIS blend buffer solution pH 8.2 was added into the glass container. The solution was stirred on magnetic stirrer until sample was completely dissolved in solution in order to prevent lump formation that would make sample inaccessible to enzymes. Fifty µL of heat-stable α-amylase solution was added into the glass container and the container was covered with the lid. The glass container was placed in boiling water bath (100°C) and was incubated for 30 min. Next, the glass container was removed from hot water bath and was let to cool to 60°C. Next, 100 µL of protease solution was added into the sample and the glass container was covered with lid. The sample was incubated in shaking water bath at 60°C for 30 min. After 30 min, the sample was removed from water bath and 5 mL of 0.561 N HCL solution was added into sample and the pH should be between 4.1 to 4.8. Then, 200 µL of amyloglucosidase was added into the glass beaker and samples was incubated at 60°C for 30 min. The sample was transferred into 500 mL conical flask then 225 mL of 95% ethanol (VWR Chemicals, UK) pre-heated to 60°C was added into the samples. The precipitation was allowed to form at room

temperature for 60 min. Next, the weight of sintered glass containing approximately 0.5 g of Celite and 3 layers of Mira cloth that was dried overnight at 80°C was recorded. The bed of Celite in the sintered glass was wet using 15 mL of 78% ethanol. The suction was applied to sintered glass to draw Celite onto sintered glass as an even mat. The precipitated enzyme digested was filtered through sintered glass using vacuum suction. Next the residue from the flask was washed with two 15 mL portions of 78% ethanol (VWR Chemicals, UK) followed by 95% ethanol and finally acetone (VWR Chemicals, UK). The supernatant that was filtered through was used for starch analysis in 2.5.4.2**.** The sintered glass was dried overnight in 80°C incubator. The sintered glass was transferred into desiccator and the weight of sintered glass and fibre was recorded. All samples were repeated three times.

$$Total\ dietary\ fibre\ (\%) = \frac{a-b}{c} X\ 100$$

Where $a\ is\ weight\ of\ sintered\ glass + fibre\ (g)$,

$b\ is\ weight\ of\ sintered\ glass + celite\ (g)\ and\ c\ is\ weight\ of\ sample\ (g)$

2.5.4 Determination of starch content

2.5.4.1 Dinitrosalicylic acid assay (DNS) solution

DNS solution was prepared by dissolving 5 g of 3,5 dinitrosalicyclic acid (Sigma Aldrich) in 250 mL of distilled water preheated to 80°C. The solution was cooled to room temperature and 100 mL of 2 N NaOH was added followed by 150 g of potassium sodium tartrate-4-hydrate (Sigma Aldrich, UK). The solution was mixed well by using magnetic stirrer then volume was completed with de-ionized water to 500 mL.

2.5.4.2 Protocol

A D-glucose (Sigma Aldrich) standard stock solution of 10 mg/mL was prepared by weighing 10 mg of glucose powder and dissolve it in 1 mL of Milli-Q water. Two mL of glucose standards ranges from 0.1 mg/mL to 1.2 mg/mL were prepared from the stock solution in 15 mL centrifuge tubes. Next, 1 mL of DNS was pipetted into each of the glucose standard. Two mL of supernatant from fibre analysis 2.5.3 was pipetted into 15 mL centrifuge tube followed by 1 mL DNS. The glucose standards and sample were incubated in a boiling water bath (Grant SBB 14) for 15 min. All the centrifuge tubes containing sample or glucose standard was cooled in ice. Nine mL of Milli-Q water was added into the centrifuge tubes and the absorbance was measured at 540 nm using Jenway Multi-cell changer spectrophotometer. A standard curve was used to calculate the amount of glucose in sample. The glucose amount was converted to starch content by multiplying by a factor 0.9.

2.5.5 Determination ash content

Ash content was determined using direct method (Latimer, 2016). The sample was prepared by weighing empty crucible and 0.1 gram of sample was weighed into the crucible. The sample was ignited in Phoenix microwave furnace (CEM) at 650°C for 25 mins or until sample light grey ash was produced. The crucible was cooled in a desiccator and weighed after it reached room temperature.

2.6 Microscopy analysis

The microscopy analysis was carried out for *Vicia faba* isolate that was extracted at pH 7.6, room temperature with and without precipitation. The samples were dissolved in water and the mixture was placed on the glass slide by using Pasteur pipette and the structures were analysed under the light microscopy.

2.7 Qualitative protein analysis

2.7.1 Characterisation of protein in isolates using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Protein subunits compositions were characterised using SDS-PAGE. Sample preparation was performed by mixing 40 mg of sample with 1M Tris-HCL pH 6.8 with 10% sodium dodecyl sulphate (SDS). The mixture was vortexed for 30 seconds. Volume of sample that contains 20 µg of protein was mixed with the same volume of 2X SDS-PAGE sample buffer (which contained 62.5mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% *β*-mercaptoethanol, pH 6.8). The sample was heated to 95-100°C for 5 min. SDS-PAGE was carried out by loading 10 µL of samples on to the precast gels (contains 4-15% acrylamide) from Mini-PROTEAN TGX (Bio-Rad, UK). Five µL of protein marker (Precision Plus Protein All Blue Standards, Bio-Rad, UK) was used to estimate molecular weight of protein subunits. The electrophoresis for gel Figure 3.7 and Figure 4.2 were performed at 200V for 30 min. After running for 30 min, the gel was stained overnight with a Coomassie Brilliant Blue R-250 solution (90%) and methanol (10%). The gels

were destained with Milli-Q water and scanned using a Gel Doc™ XR+ System (Bio-Rad Laboratories, Richmond, CA, USA). The electrophoresis for gel Figure 3.8 was performed at 100V for 1 hour and the gel was stained with Gel code blue stain reagent (Thermo Fisher Scientific, UK) for 1 hour and destained with Milli-Q water.

2.7.2 Protein identification

VFI 7.6 RT, VFI 7.6 RT (brown fraction), VFI 7.6 RT (white fraction), VFI 7.6 RT FD, VFI 7.6 RT (soluble pH 4) were sent for protein identification to Mass Spectrometry Facility, The Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, LS2 9JT. The method of protein identification carried out by Mass Spectrometry Facility is in appendix:

2.7.3 Analysis of amino acids composition using Bioinformatic tools

The protein accession numbers obtained from Uniprot databases was used to carry out analysis of primary structure of protein using ProtParam Expasy tool (Liu et al., 2017). Protein secondary structure was analysed using NetSurfP version 1.1.

2.8 Determination of vicine and convicine content

2.8.1 Preparation of vicine standard

Vicine standard (Sigma) was used for quantification of vicine and convicine. Stock solution of vicine was prepared at a concentration of 20 mg/mL in Milli-Q water. The calibration curve was used for quantification of vicine by preparing the standard solution in the range of 0.1 to 0.5 µg/mL.

2.8.2 Extraction of vicine and convicine

Vicine and convicine were extracted from *Vicia faba* flour, all four VFIs prepared from 3.2.2.2 and the commercial faba bean protein concentrate. The extraction was carried out according to Pulkkinen et al. (2015) with some modifications. Briefly, 0.5 g of sample was weighed into 50 mL centrifuge tube and 15 mL Milli-Q water was added. The solution was vortex-mixed for 1 minute and then was centrifuged at 4696*g* for 10 minutes at 4°C. The supernatant was collected and filtered through a 0.45 µm PTFE filter and kept in fridge before analysis by high performance liquid chromatography (HPLC).

2.8.3 Quantification of vicine and convicine using high performance liquid chromatography coupled with UV detection and mass spectrometry (HPLC-MS-UV)

Reversed phase HPLC Shimadzu consisting of a solvent delivery unit, column oven, autosampler, UV-Vis detector and photo-diode array detector. The column used was packed with C18, 4.6mm x 150 mm with 5 µm internal diameter. The column temperature was 30°C with flow rate of 0.8 ml/min and the injection volume was 10 µl. The photo-diode array detection spectra was recorded at 274 nm. The 35 min elution program consisted of isocratic run for 0-8 min with solvent A (0.1% formic acid in Milli-Q water), followed by 9-15 min of 70% of solvent B (0.1% formic acid in acetonitrile). This proportion was maintained at that level until 22 min. The total run time for the next injection was 35 min. (Pulkkinen et al., 2015). MS analysis was performed using electrospray ionization (ESI) interface with a positive ion mode and scanning range of mz 100-400 to detect vicine fraction based on $[M+H]^+$ ion (m/z 305)

for vicine and (m/z 306) for convicine. The MS analysis was later used to confirm the peak corresponding convicine since there is no standard available for convicine.

2.9 Functional properties experiments

2.9.1 Determination of soluble protein by using BCA assay

Soluble protein was measured using Pierce Bicinchoninic acid (BCA) Protein Assay Kit and the protocol was as followed (Thermo Fisher Scientific, UK). Briefly, 0.1 g of sample was mixed with 10 mL of MIlli-Q water and was mixed overnight using vortex genie. The tube was then centrifuged for 20 min at 3500*g*. The supernatant was diluted 5 times Milli-Q water. Twenty-five µL of sample was pipetted into a microtiter plate well and 200µL of the working BCA reagent was added to each well and mixed thoroughly for 30 seconds. The plate was covered and incubated at 37°C for 30 min. The absorbance was measured at 540 nm on a Multiskan FC plate reader. Bovine serum albumin (BSA) was used as standard within the range of 0-200 µg/mL. All of the steps were done similarly for the commercial samples.

2.9.2 Evaluation on the effect of pH on the solubility of protein

Vicia faba flour, [VFI 7.6 RT], FBPC and SPI were extracted in Milli-Q water (10%w/v) for 1 hour by stirring. Then equal volume of suspension was transferred into 15 mL centrifuge tubes and the pH was adjusted using 0.5 M hydrochloric acid or 0.5 M sodium hydroxide to pH 2, 3, 4, 5, 6, 7, 8, 9 and 10. The suspension was vortex mixed for 20 min followed by centrifuged at 2500*g* for 5 minutes. Then the soluble protein from the supernatant was measured

by using BCA assay. The soluble protein of *Vicia faba* flour and [VFI 7.6 RT] at pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 were characterised by sodium dodecyl sulphate polyacrylamide gel electrophoresis as in 4.2.

2.9.3 Zeta potential by Zetasizer

The supernatant obtained for [VFI 7.6 RT] and FBPC from 2.9.2 were used for zeta-potential measurement using Malvern Zetasizer nano ZS (Malvern Instruments, Malvern, UK). The refractive index used was 1.45. Disposable folded capillary cells were used to place sample for measurement. The results were plotted as solubility and zeta potential (mV) versus pH.

2.9.4 Determination of water holding capacity (WHC)

WHC was carried out by weighing 0.5 g of sample followed by 5 mL of Milli-Q water into 15 mL pre-weighed centrifuge tube. The mixture was vortexed overnight using vortex genie. Then, the suspension was allowed to stand for 30 min at ambient temperature and then centrifuged at 4696*g* for 30 min. The supernatant was discarded and the tube with sediment was weighed again. WHC was expressed as the amount of water per gram of sample (Kaushik et al., 2016).

2.9.5 Determination of fat holding capacity (FHC)

FHC was carried out by weighing 0.5 g of sample followed by 5 mL of sunflower oil (local shop Tesco, UK) into 15 mL pre-weighed centrifuge tube. The mixture was vortexed overnight using vortex genie. Then, the suspension was allowed to stand for 30 min at ambient temperature and then centrifuged at 4696*g* for 30 min. The supernatant was discarded and the tube with

sediment was weighed again. FHC was expressed as the amount of water per gram of sample (Kaushik et al., 2016).

2.9.6 Determination of foaming properties

Foaming capacity (FC) and foam stability (FS) were determined using the method of Toews and Wang (2013) with some modifications. Three grams of sample were homogenised in 50 mL MIlli-Q water using POLYTRON PT2500E homogenizer at a speed of 10,000 rpm for 1 min. The dispersion was rinsed into a bowl with 100 mL Milli-Q water then whipped with caged wire blades at setting 6 using Kitchen aid food mixer for 6 min. The solution was transferred into a 1 L or 500 mL measuring cylinder. The readings were taken at 1, 10, 30, 60, 90 and 120 minutes. FC was expressed as the foam volume at 1 min per liquid volume before whipping (%). The stability of the foam volume over time was expressed as a percentage of the initial foam volume.

$$FC\ (\%) = \frac{a - b}{b} \times 100$$

$Where\ a\ is\ volume\ after\ whipping\ (mL)\ and\ b\ is\ volume\ before\ whipping\ (mL)$

$$FS\ (\%) = \frac{a}{b} \times 100$$

$Where\ a\ is\ foam\ volume\ (mL) after\ time\ and\ b\ is\ initial\ volume\ (mL)$

2.9.7 Determination of emulsifying properties

Emulsifying activity (EA) and emulsifying stability (ES) were determined according to Mao and Hua (2012) with some modifications. One g of sample was homogenised using POLYTRON PT2500E homogeniser at a speed of 10,000 rpm in 25 mL Milli-Q water. The protein solution was mixed with 25 mL

of sunflower oil (local shop Tesco, UK) followed by homogenization at a speed of 10,000 rpm for 1 min. The height of the emulsified layer was measured. After overnight and a week, the height of the emulsified layer was measured again.

$$EA\ (\%) = \frac{a}{b} \times 100$$

$$Where\ a\ is\ height\ of\ emulsified\ layer\ (cm), b\ is\ height\ of\ content\ (cm)$$

$$ES\ (\%)\ after\ overnight = \frac{a}{b} \times 100$$

$$Where\ a\ is\ height\ of\ remaining\ emulsion\ (cm), b\ is\ EA$$

2.9.8 Determination of gelling capacity

2.9.8.1 Preparation of heat induced gel

The heat induced gel was prepared according to method by (Zhao et al., 2015). 12% and 20% w/v of gel was prepared by dissolving sample in water containing 100 mM of NaCl. The mixture was stirred on a hot plate magnetic stirrer (Gallenhamb) at setting number 1 until the sample was completely dissolved and thickened for about 30 min. Then the pH of dispersion was measured and poured into a 5 mL syringe. The syringe containing dispersion was heated for 40 min in 80°C GLS Aqua 12 Plus Grant water bath, followed by cooling to room temperature in an ice bath for 30 min. The dispersion was refrigerated overnight at 4°C. The gel was carefully poured out from the syringe and used to measure the firmness using Texture analyser (TA.XT plus).

2.9.8.2 Determination of gel hardness

The method of measuring gel hardness was according to Chang et al. (2014) with some modifications. All gel samples with the height and diameter of 10 mm were analysed using compression test mode using Texture analyser (TA.XT plus) at room temperature. The probe used was SMSP/75 with pre-test speed 5.0 mm/s, test speed 1.0 mm/s, post-test speed 1.0 mm/s, maximum load 2 kg, distance 2.5 mm, force 5 gHardness (N) value was measured as the maximum force of the peak.

2.10 Beef patties experiments

2.10.1 Preparation of beef patties

Beef patties were made using Irish Beef Steak Mince (local shop Tesco, UK) which contained 15% fat. Eight formulation of beef patties were prepared according to Table 2:1. The amount of binder added was 20% from the amount of uncooked ground beef mince. The ingredients were then mixed in Kenwood mini processor for 5 minutes. The batter was moulded into round shape with diameter of 5 cm and 1 cm thickness. The beef patties were weighed before frying. The beef patties were then cooked on Tefal fryer for 2.5 min on each side. Then, the weight of cooked beef patties was recorded.

Table 2:1: Beef patties formulation

Formulation	Beef mince (%)	Binder (%)
Control	100	-
[VFI 7.6 RT]	80	20
SPI	80	20
PPI	80	20
HP	80	20
FBPC	80	20
WF	80	20
WEP	80	20

2.10.2 Determination of cooking loss, product yield and drip loss of beef patties

The quality of beef patties was measured according to Modi et al. (2004) and Shariati-Ievari et al. (2016) with some modifications. Three beef patties from each formulation were weighed before and after cooking to determine percentage of cooking loss and product yield as follows:

$$Cooking\ loss\ (\%) = \frac{(a-b)}{a} \times 100$$

$$Product\ yield\ (\%) = 100 - cooking\ loss$$

The drip loss was determined by weighing filter paper before and after placing cooked beef patties using the following equation:

$$Drip\ loss\ (\%) = \frac{c-d}{a} \times 100$$

$$Where\ a\ is\ weight\ before\ cooking\ (g), b\ is\ weight\ after\ cooking\ (g),$$

$$c\ is\ weight\ of\ filter\ paper\ with\ drip\ (g)\ and\ d\ is\ weight\ of\ filter\ paper\ (g)$$

2.10.3 Determination of moisture content

The measurement of moisture content of beef mince and cooked beef patties were carried out using Halogen moisture analyser (Mettler Toledo, UK). Briefly, the method was as followed. The weight of sample was determined according to the food samples. As for beef, 5 g of samples were distributed evenly on an aluminium sample pan (Mettler Toledo, UK).

2.10.4 Determination of hardness of beef patties using Texture analyser

The measurement of hardness was carried out using Compression test mode using Texture analyser TA.XT plus. Hardness measurement was conducted in triplicate on each sample at room temperature. The conditions of texture analysis were adapted from Park et al. (2016) with some modifications using SMSP/75 probe: pre-test speed 1.0 mm/s, test speed 2.0 mm/s, post-test speed 10.0 mm/s, maximum load 2 kg, distance 5.0 mm, force 5 g. The hardness (N) value of beef patties was measured as the maximum force of the peak.

2.11 Statistical analysis

All experiments were performed in triplicate and reported as the mean ± standard error. A one-way and two-way analysis of variance (ANOVA) were used to measure statistical differences in experiments. One-way anova was used to see the differences between samples and two way anova was used to see the effects of pH and temperature on the compositions and functional properties at 5% confidence level. Model assumptions were used to

check the normality of residuals, consistency of error variance, outliers and influencing case. Multiple comparison was analyzed using Tukey HSD. Principle component analysis was used to analyze the relationship between samples compositions and functional properties. PCA was performed based on single value decomposition using built in R-function prcomp. All statistical analyses were analyzed using R software.

Chapter 3
Extraction, Preparation and Characterisation of *Vicia faba* isolates

3.1 Introduction

Vicia faba also known as faba bean or broad bean has traditionally been used as animal feed. Recent interest in the sustainability of diets has increased demand for plant proteins and has brought attention to *Vicia faba* as a potential source of plant protein for human nutrition. However, legumes have a number of issue that limit their utilisation in food. Firstly, functional properties of legumes protein is poor in their native state. This is because legume proteins are stored in membrane bound organelles in parenchyma cells of the cotyledon and are tightly packed in protein bodies which make them poorly soluble. The proteins are termed storage proteins and are globular in shape consists of high molecular-weight oligomeric storage proteins which require certain amount of ionic strength to help them solubilise in aqueous media (Kaur and Singh, 2007, Schwenke, 2001). Proteins in legumes can be fractionated based on their solubility in water, salt solution, alkaline solution and alcohol. This fractionation method is called the 'Osborne' method (Osborne, 1924). According to Chavan et al. (2001) and Adebiyi and Aluko (2011), the expected protein fractions sequentially extracted from seed flour are albumin, globulin, glutelin and prolamine respectively. *Vicia faba* proteins mainly comprise the storage proteins albumins and globulins, where globulins are the dominant ones including 7S vicilin and 11S legumin (Multari et al.,

2015). Secondly, raw or unprocessed legumes contain anti-nutritional factors and toxins which reduced the digestibility and limits the utilisation as food products (Khattab and Arntfield, 2009). The low digestibility of legume can be improved by reducing the anti-nutritional compounds by processing legume. *Vicia faba* also contain the pyrimidine glucosides, vicine and convicine. These glucosides are hydrolysed into highly reactive aglycones called divicine and isouramil. These aglycones cause oxidative stress in humans with deficient glucose-6-phosphate dehydrogenase (G6PD) which can lead to acute haemolytic anaemia. This condition is referred to as favism. High performance liquid chromatography (HPLC) is widely used for the quantification of vicine and convicine. Vicine and convicine has 1 different amino group which makes the difference between them is 1Da. Study reported by Pulkkinen et al. (2015) used mobile phase containing formic acid and detection wavelength of 273 nm. Vicine standard was used in the quantification of vicine and convicine standard are not available, however HPLC-MS can be used to confirm peak using the mz. Other anti-nutritional factors which are protein inhibitors, lectins, tannins and phytates are also present in *Vicia faba*. Thirdly, legume protein have lower nutritional value compared to animal protein due to low essential amino acids particularly cysteine and methionine. However, this is not a serious problem unless legume is the only source of protein. This is because legume protein can be combined with other dietary protein source to help meet the daily amino acid requirement. The preparation of protein isolates, involving extraction and precipitation of protein, is an important process to obtain ingredients with high protein content, reduced levels of anti-nutritional factors

and a neutral flavour, which can be used in food (Multari et al., 2015). Functional properties can be tuned by controlling the extraction and post-extraction processing conditions including pH, temperature and solvent used. The ability of protein to interact with and bind to water or lipid and to form gels, will influence the type of products that the protein isolates are added to. These functional properties of protein isolates are dependent on the structural and physicochemical properties of the protein constituents, which are heavily influenced by the extraction method (Mune and Sogi, 2015). An efficient food-grade extraction method is required to maximise extraction-yield and optimised composition to achieve high protein content with low or no anti-nutritional compounds. Most studies on the extraction of proteins from legumes only focused on obtaining isolates with high protein contents, without reporting the yield obtained. Table 3.1 shows previous studies on VFI which reported their extraction conditions and protein content. All studies produced VFI that contained more than 80% of protein. However, neither study reported yield, which is important to establish if extraction is efficient. Besides, the reported aims and methods were different which makes it difficult to compare with the present study. Even though the studies exploring the functional properties of various leguminous protein isolates is quite extensive, it is difficult to compare their functional properties because the processing techniques used to extract and isolate them are different.

Table 3:1: Previous studies on the extraction of *Vicia faba* isolates

Extraction conditions						Total yield (%)	Protein content (%)	Scale	References
pH	***Temperature***	***Time***	***Solvent***	***Ratio***	***Precipitation pH***				
7	20°C	30 mins	water	1:5 (w/v)	4.7	Not reported	92.7	Lab scale	(McCurdy and Knipfel, 1990)
7	21-22°C	30 mins	0.008 M NaOH	1:5 (w/v)	4.7		92.5	Pilot scale	
10.5	4°C	1 hour	0.25% Na_2SO_4	10% (w/v)	4.0	Not reported	92.4	Not reported	(Vioque et al., 2012)
9	Not reported	Not reported	alkaline	Not reported	4.0	Not reported	92.2	Not reported	(Nivala et al., 2017)
9.5	RT	40 mins	1.0 M NaOH	1:10 (w/v)	4.5		84.4	Not reported	(Karaca et al., 2011)
	RT	20 mins	water	1:5 (w/w) pellet:water)		Not reported			

3.2 Aim of the chapter

The aim of the work described in this chapter was to produce *Vicia faba* isolates (VFIs) using food grade methods at medium scale and to select the method that achieved high overall yield, high protein content, low vicine and convicine content and optimal protein profile. Summary of analysis used to analyse protein and non-protein compounds in VFIs. Table 3.2 shows a summary of the methods used to analyse protein, non-protein and anti-nutritional factors, used as criteria for selection. The composition and solubility of VFIs were compared to commercial legume protein isolates.

Table 3:2: Summary of analysis used to analyse protein and non-protein compounds in VFIs

	Methods
Protein analysis	
Quantification analysis	Total protein content by Kjeldahl method
Qualitative analysis	Molecular weight of protein subunits by SDS-PAGE
	Protein identification by LC-MS
	Bioinformatic tools: ProtParam ExpaSy to analyse primary structure of protein
	Protein charge by zeta potential
Non-protein analysis	Starch content by DNS method
	Total dietary fibre by enzymatic-gravimetric method
	Ash by dry ashing method
	Anti-nutritional compounds (vicine and convicine) by LC-MS

3.3 Results and Discussion

3.3.1 Extraction, preparation and microscopy analysis of *Vicia faba* isolates.

Whole *Vicia faba* beans were ground into flour and the composition is presented in Table 3:3. It was decided not to defat *Vicia faba* flour prior to extraction as the fat content was less than 1%. The protein content of *Vicia faba* flour was comparable to the values reported by Karatas et al. (2017), Karaca et al. (2011) and Vioque et al. (2012). The difference in the value of protein content can be due to different varieties of *Vicia faba*. Extraction of *Vicia faba* flour was initially performed according to Vioque et al. (2012) refer to method 2.4.1 and the flowchart of the extraction is shown in Figure 3.1. The extraction was carried out for 1 hour in the cold room (6°C) by mixing the flour and 0.25% sodium sulphite pH 10.5. However, the pH was not consistent throughout the extraction period as the pH decreased to 7.6 after extraction of *Vicia faba* flour. VFI produced in this study contained 80% protein which was comparable to protein content of VFI by Vioque et al. (2012) and Karaca et al. (2011). The yield of VFI was 9.6%, however it could not be compared to any of the previous studies as yield was not reported. For this study, all prepared samples were labelled as isolate, even though the level of protein content was lower than 80%. There is no universal classification separating a protein concentrate from an isolate for all legumes (Karaca et al., 2011).

Table 3:3: Composition of *Vicia faba* flour

Composition	VF flour (%)
Protein	26.7
Fibre *	25
Carbohydrate *	58
Fat	0.1

***Values were provided by supplier, Senova Ltd.**

Due to the inconsistency of pH, four extractions conditions were established from Vioque's method: pH 7.6 6°C, 7.6 RT, 10.5 6°C or 10.5 RT to achieve medium-scale extraction (see Figure 3.2). The centrifugation step after the extraction was replaced by filtration through muslin cloth (green coloured box) tom improve the scale of process. Finally, the soluble protein was also collected. Modifications made as presented in Figure 3.2 were coloured with green. The fixed extraction condition produced stable pH and filtration process increased the yield of VFI.

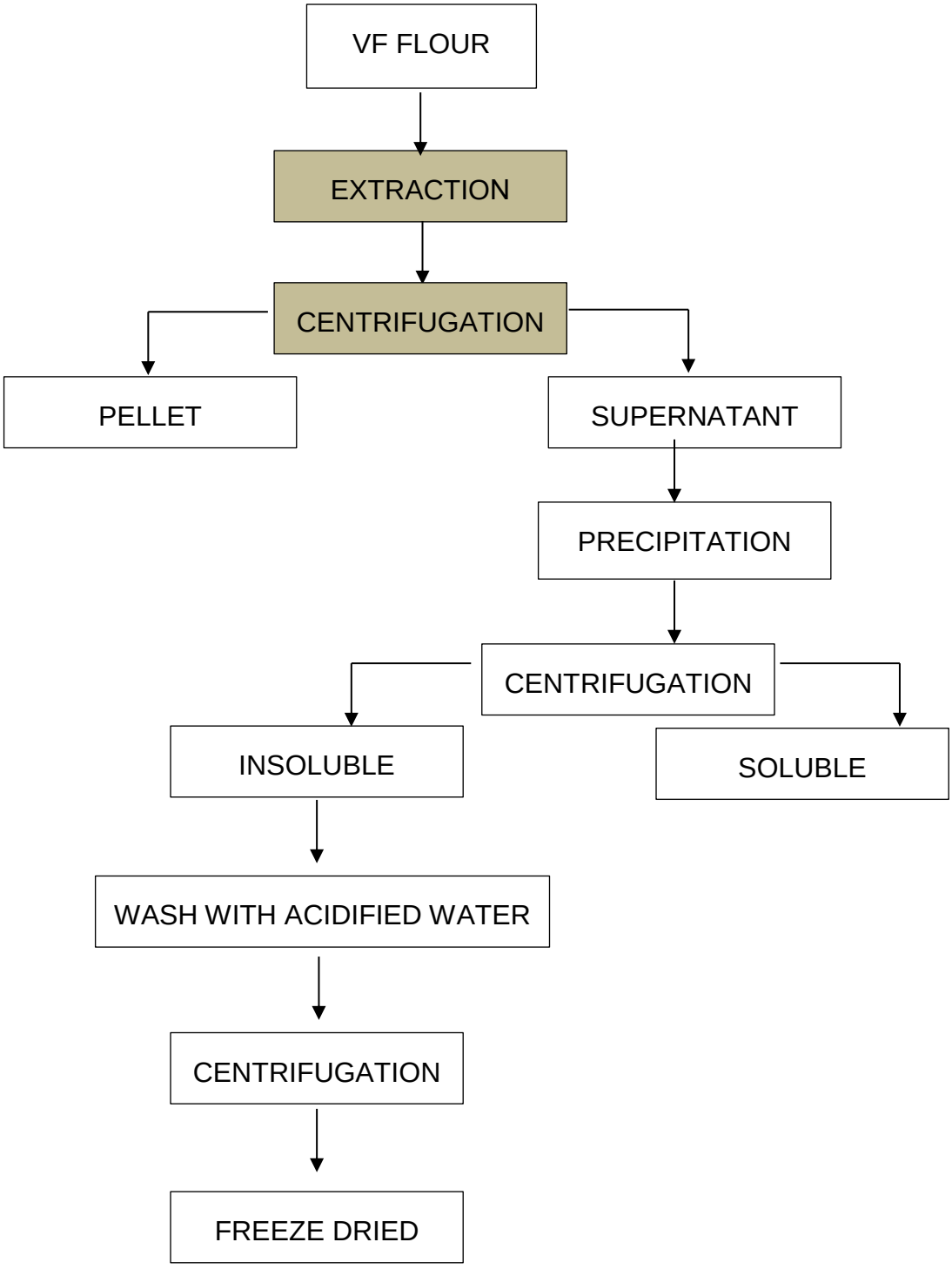

Figure 3.1: Extractions of VFI according to Vioque's method

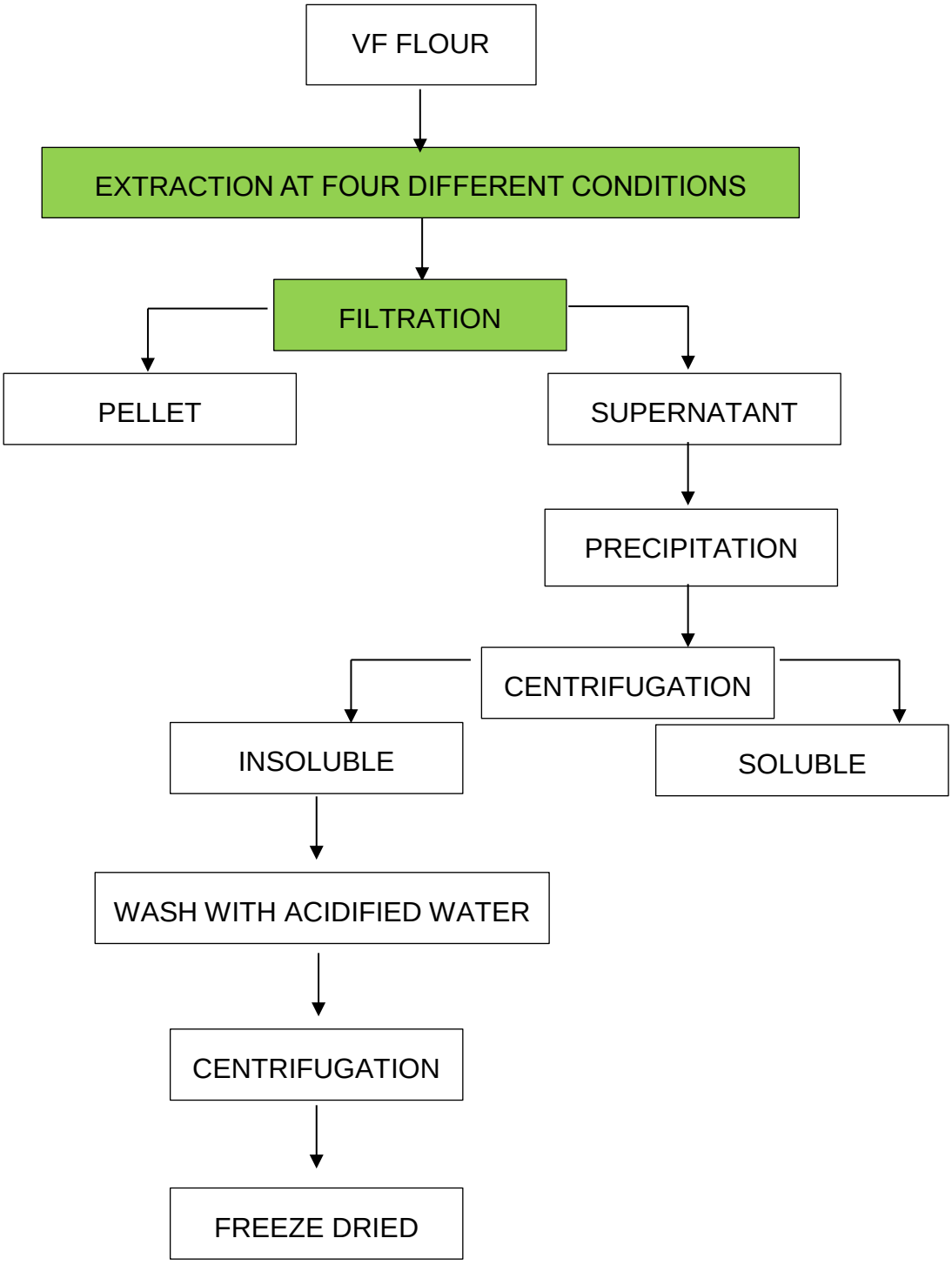

Figure 3.2: Modified extractions of VFIs from Vioque's method

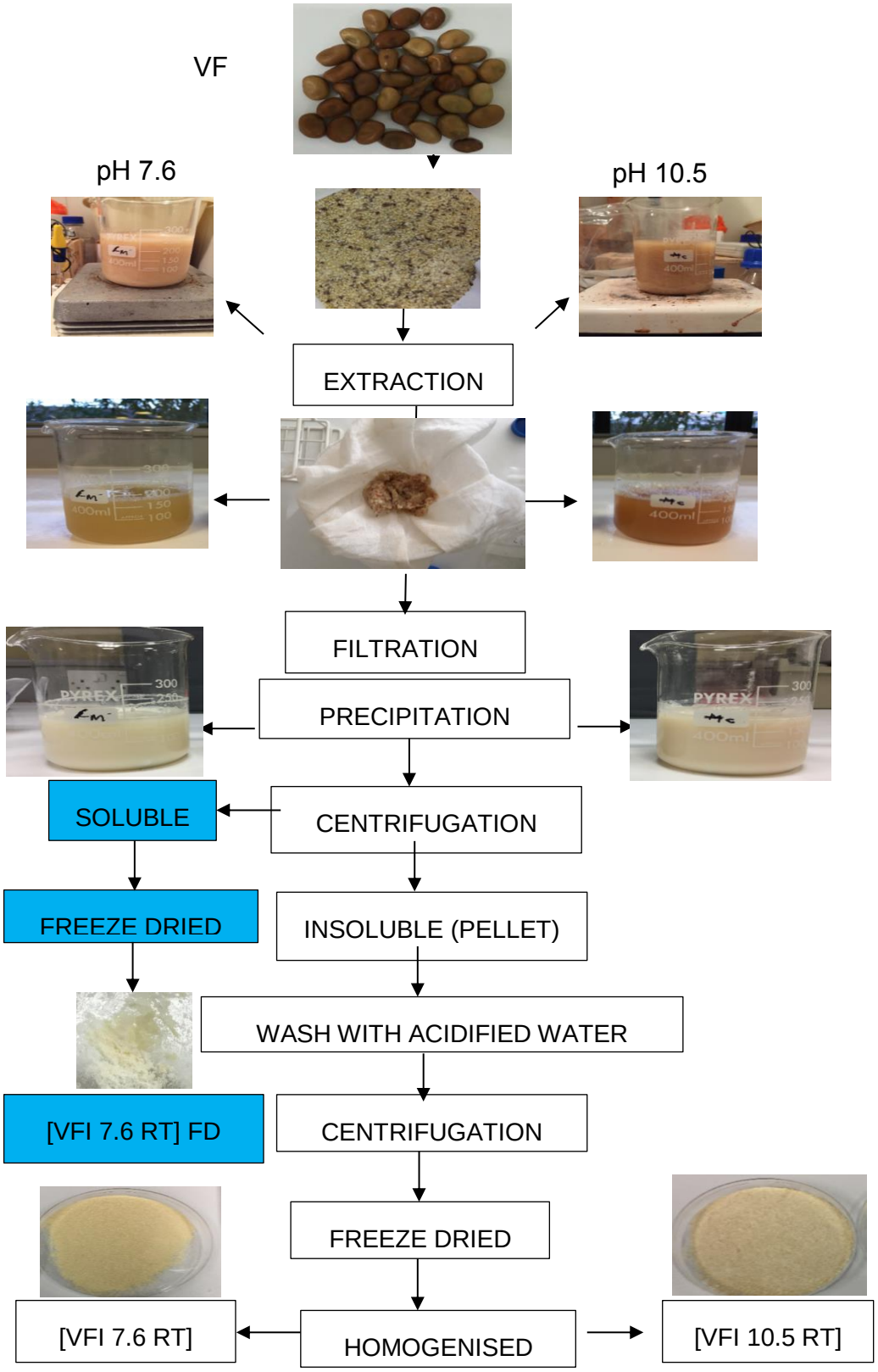

Figure 3.3: The processing steps of preparing [VFI 7.6 RT] (left) and [VFI 10.5 RT] (right).

Figure 3.3 shows the outline of the preparation of VFI at room temperature. The brown colour of the extracts may be due to the polyphenols that present in *Vicia faba* flour. Polyphenols are said to have health benefits and can be considered as antioxidants. The most abundant polyphenols in *Vicia faba* are tannins (Kosińska et al., 2011). Darker supernatant was observed in extract at pH 10.5 compared to extract at pH 7.6 indicating oxidation of polyphenols which are known to oxidise at high pH. The brown colour due to polyphenols in the supernatant decreased as the pH of supernatant decreased to isoelectric point. Changes in pH affect the stability of pigment. The extraction of protein carried out by Kosińska et al. (2011) at pH 7.5 involves ammonium sulphate precipitation instead of extraction at high pH and isoelectric precipitation to avoid phenolic compound-protein complex formation during isolation process. Polyphenols are attracted to protein mainly because some of the amino acids have aromatic groups which form hydrophobic interaction between them (Kosińska et al., 2011). However in this study, the amount of total polyphenols in the protein isolate was not measured. But it is good indication that this protein isolate might have antioxidant or antibacterial properties that can be useful as a food ingredient. In the study done by Vioque et al. (2012) polyphenols were extracted using 75% acetone prior to protein extraction. However, acetone is not food grade and was not used in this study.

Table 3:4 shows yield of VFIs at different extraction conditions. Total yield was defined as the mass of extract obtained from 100 g of *Vicia faba* flour. From the

observation, extraction pH and temperature have significant effect ($p<0.05$) on the total yield of VFIs. The highest yield of VFI was obtained when the extraction was done at higher pH. The results are in agreement with Jarpa-Parra et al. (2014) which reported pH and solid to solvent ratio have significant impact on the total yield of extraction on lentil protein. They observed that the yield increased with increasing pH at solid to solvent ratio between 1:20 and 1:10. This is because high alkaline concentration improves solubility by breaking down the hydrogen bonds to dissociate hydrogen from carbonyl and sulphate groups. The increased surface charge on protein molecule then leads to enhance solubility in the solvent system (Jarpa-Parra et al., 2014).

Table 3:4: Total yield of VFIs produced at different extraction conditions.

Processing conditions		Total yield of VFI (mean ±SE) %
pH	*Temperature*	
7.6	6	27.59±0.28a
7.6	RT	26.61± 0.01a
10.5	6	30.20± 0.19b
10.5	RT	30.03± 0.02b

Each value is expressed as mean ± standard error (n=3 extractions). Data followed by different letters in a column are significantly different by multiple comparison test using two-way ANOVA ($p<0.05$).

Effects of isoelectric precipitation on the total yield and protein content was observed by doing another extraction of *Vicia faba* at pH 7.6 in room temperature without isoelectric precipitation. Flowchart on the extraction of [VFI 7.6 RT] FD was shown in Figure 3.3. [VFI 7.6 RT] FD obtained was more homogenous with whitish colour than the one prepared with precipitation as there was no separate fraction. The total yield obtained was lower than VFI produced by precipitation which was 3.74% because the powder produced was much lighter compared to VFI produced with precipitation. Summary of the extraction and preparation of all VFIs produced in this study is presented in Figure 3.4.

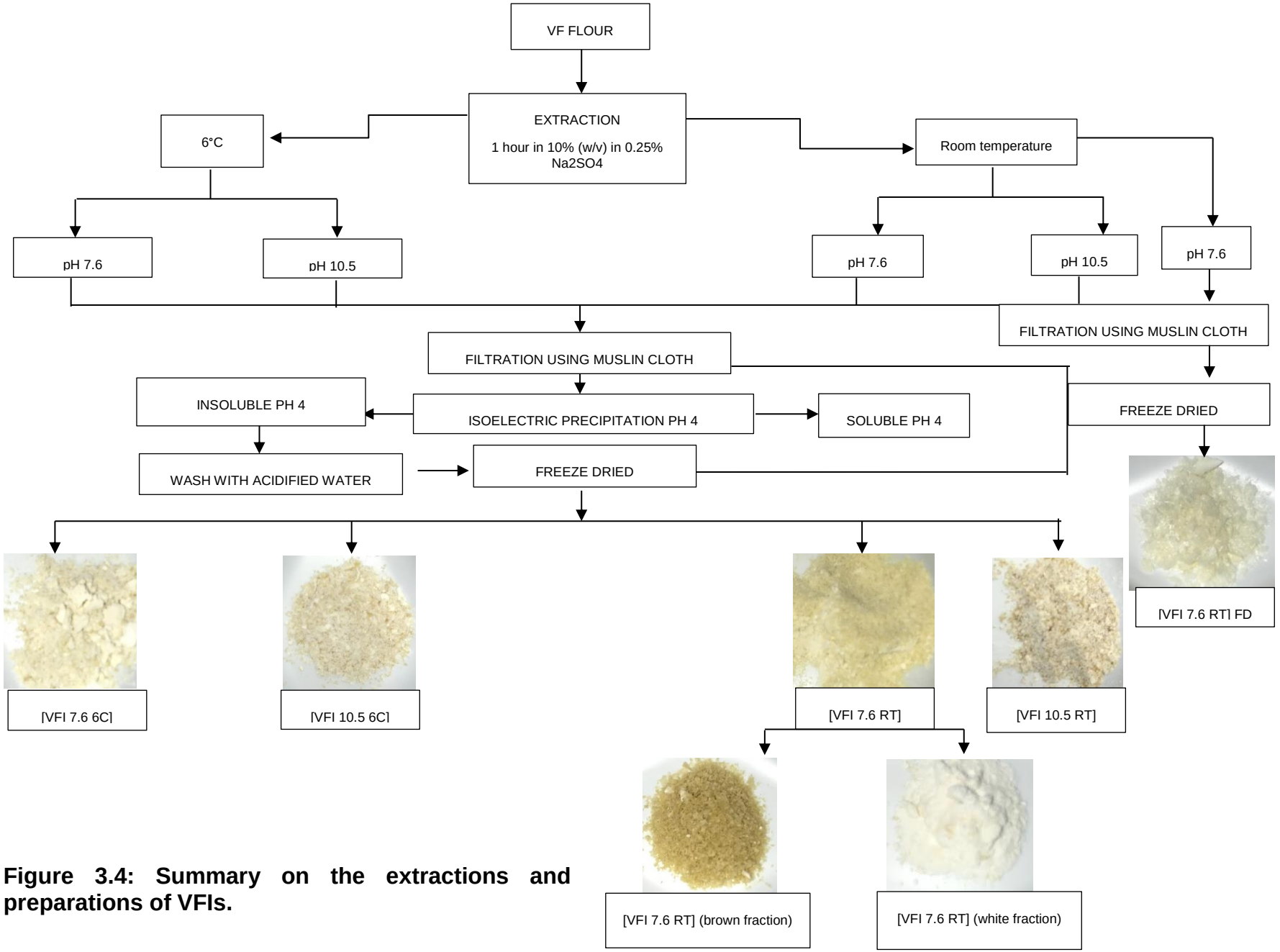

Figure 3.4: Summary on the extractions and preparations of VFIs.

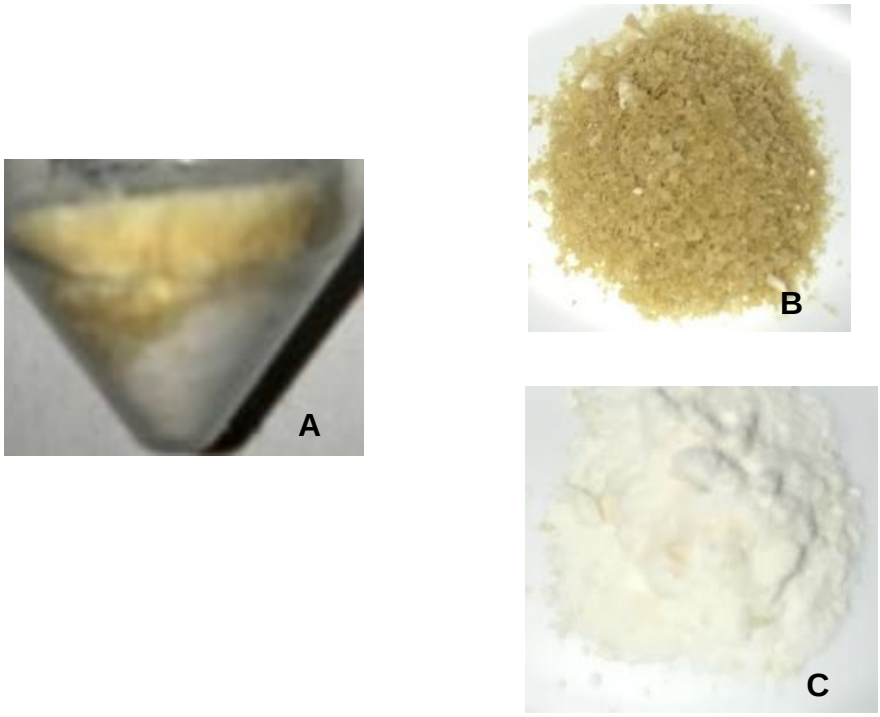

Figure 3.5: A: [VFI 7.6 RT] B: brown and C: white fractions

The freeze dried sample collected in a tube contained two separate fractions before homogenising (refer Figure 3.5), were brown fraction at the top and white fraction at the bottom. The brown fraction could probably composed of protein and fibre, meanwhile white fraction could probably be starch. Microscopy analysis of brown and white fractions were carried out to see the structures. In this study only brown and white fractions from [VFI 7.6 RT] were chosen and separated for protein qualitative analysis.

3.3.2 Microscopy analysis of *Vicia faba* isolates

Preliminary microscopy analysis (Figure 3.6) of [VFI 7.6 RT] (brown fraction), [VFI 7.6 RT] (white fraction) and [VFI 7.6 RT] FD were carried out to confirm that the white fraction mainly consist of starch. Clear starch granules were observed in [VFI 7.6 RT] (white fraction) compared to [VFI 7.6 RT] (brown fraction. As for [VFI 7.6 RT] FD, more compact structure was observed. From this microscopy analysis it provided some information that [VFI 7.6 RT] contained starch. However, particles in [VFI 7.6 RT] and [VFI 7.6 RT] FD need further investigation to confirm existing polymers.

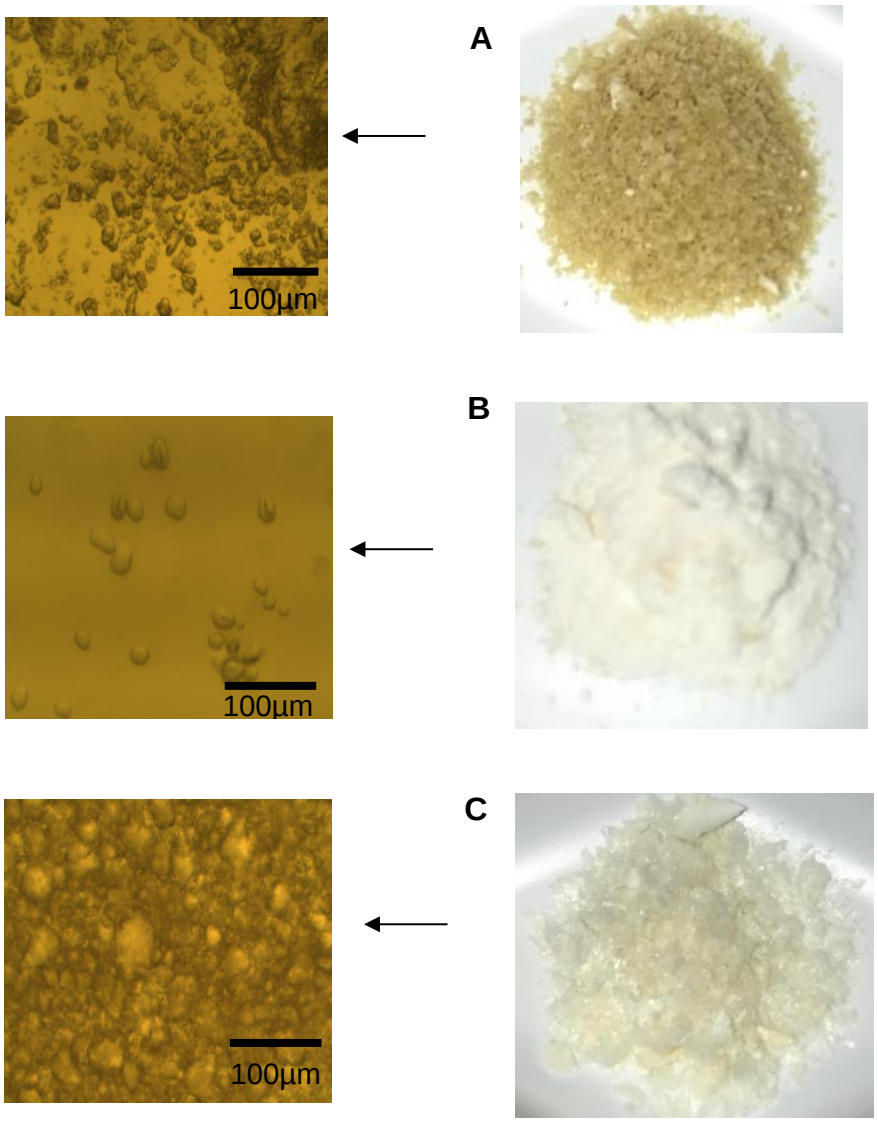

Figure 3.6: Light microscopy of 1) [VFI 7.6 RT] (brown fraction), 2) [VFI 7.6 RT] (white fraction) and 3) [VFI 7.6 RT] FD.

3.3.3 Qualitative protein analysis of *Vicia faba* isolates

SDS-PAGE and protein identification by LC-MS were used to characterise proteins in VFIs to see the effect of extraction and precipitation on their protein profiles. These two analyses were used to decide which VFIs will be used to analyse quantitatively.

3.3.3.1 Characterisation of proteins in *Vicia faba* isolates and commercial products using SDS-PAGE

Proteins in VFIs and commercial products were characterised by using SDS-PAGE as shown in Figure 3.7 and Figure 3.8. In Figure 3.7, the second lane was loaded with 20 µg of BSA which showed as a very intense single band with molecular weight of 62 kDa. BSA was used as positive control for the SDS-PAGE experiment. Regardless of pH and temperature of extractions, protein profile and main bands of VFIs appeared to be similar. Bands for all four samples of VFI and FBPC showed existence of several protein of various molecular weights, ranging from 90 kDa to 15 kDa with the existence of about 11 bands. This suggests that pH and temperature modified the amount of extracted protein but not its polypeptide composition. The protein bands with MW of 15 to 20 kDa were proposed to be β-legumin, while the protein with molecular weight of 37 kDa was proposed to be α-legumin and vicilin with the molecular weight of about 47 kDa. Study by Liu et al. (2017) reported convicilin to be at 57.5 kDa, but there was no clear band observed in SDS-PAGE in this study that represents convicilin. Another study by, Felix et al. (2018) also proposed bands at 16 to 21, 40, 49 to 75 kDa to be β-legumin, α-legumin and vicilin respectively. Previous study on faba bean protein curd by Cai et al.

(2002) also reported bands at 22 kDa and 41 kDa corresponded to β and α-legumin respectively but did not characterise any bands to be convicilin. Meanwhile, study carried out by Nivala et al. (2017) on faba bean protein isolate reported bands at 20 kDa to be β-legumin, 40 kDa to be α-legumin and bands at 50 and 70 kDa to be vicilin and did not characterise bands above vicilin to be convicilin. Most studies on SDS-PAGE of faba bean protein only characterise protein bands as β-legumin, α-legumin and vicilin. There is no study on gel electrophoresis of faba bean has confirmed which band is convicilin, apart from study by Liu et al. (2017) that identified convicilin based on LC-MS and the molecular weight from amino acid sequence to be 57.5 kDa. Study by Felix et al. (2018) proposed one of protein bands to be convicilin but the molecular weight is not mentioned clearly in the study. All of these studies on SDS-PAGE gels of faba bean protein were in agreement on the electrophoretic pattern of the proteins bands which were first β-legumin followed by α-legumin, vicilin and convicilin. The SDS-PAGE also suggests that the 11S legumin and 7S vicilin-like proteins are the major protein components in VFIs. Based on the previous study reported by Meng and Ma (2002), on the study of red bean globulin, the 11S legumin was detected at about 40 kDa and 22 kDa for acidic and basic subunits respectively. The 11S subunit is dissociated into their acidic and basic subunits because the proteins have been treated with mercaptoethanol that cleaves the disulphide bond that bind the subunits. According to Chakraborty et al. (1979) and Carbonaro et al. (2005), they suggested that legumin comprises 45% of the total protein fraction whereas vicilin comprises 33% of faba bean proteins.

Figure 3.6 also showed proteins from commercial samples SPI, PPI and HP. SPI showed slightly different protein subunits with three major bands compared to VFI. Separation of SPI proteins showed three major components with molecular weight of 37 kDa, 20 kDa and 70 kDa which were acidic subunit of legumin, basic subunit of legumin and vicilin respectively which is also in agreement with (Cai et al., 2002). Interestingly, PPI exhibited more protein bands than VFI ranged between 15 kDa to 90 kDa. This includes low molecular weight protein subunits ranged between 15 to 25 kDa and 40 kDa which could represent albumin, β-legumin and α-legumin subunit respectively. Vicilin and convicilin subunit in PPI were represented by the bands at about 47 kDa and 70 kDa respectively. These findings were supported by studies done by Beck et al. (2017) and Osen et al. (2015) where the pea proteins composed of legumin, vicilin and convicilin at molecular weight of 20 kDa for β-legumin, followed by α-legumin with a molecular weight of 40 kDa, vicilin (47 kDa) and convicilin (70 kDa). HP on the other hand showed three major bands of protein subunits at about 19-20 kDa for basic subunit and 35 kDa for acidic subunit. The protein subunits of HP is called edestin which is like legumin-like globulin which consists of acidic and basic subunit which is in agreement with study by (Raikos et al., 2015, Raikos et al., 2014). Protein subunits of SPI are glycinin (11S), a hexamer protein which comprises of acidic and basic subunit linked by disulphide bond and beta conglycinin (7S) is a trimer glycoprotein which composed of three subunits which are α, α’ and β associated by hydrophobic interaction (Aghanouri et al., 2014).

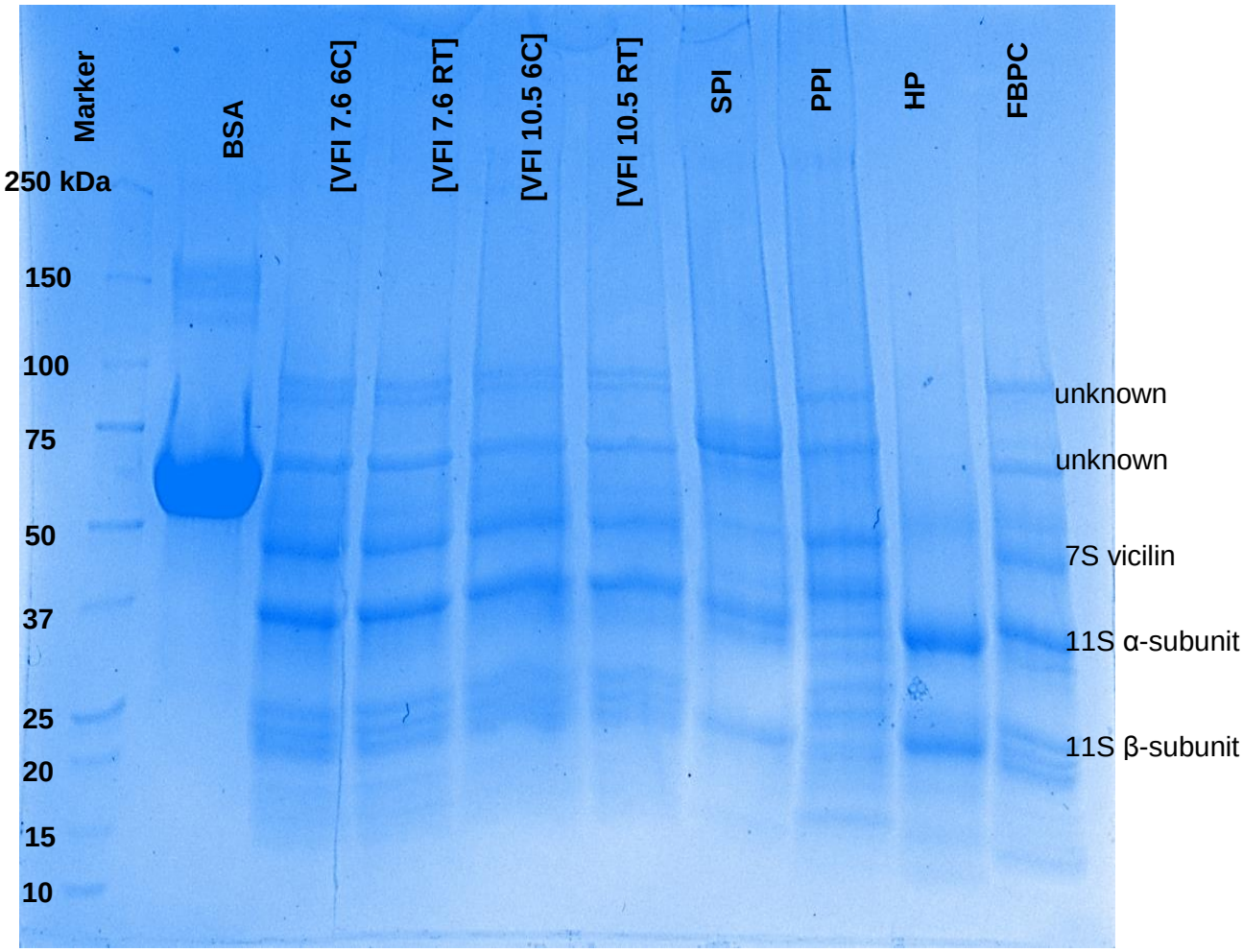

Figure 3.7: SDS-PAGE of VFIs and commercial samples. Lane 1 was loaded with 5 µl of protein marker. Lane 2 was loaded with 20 µl bovine serum albumin (BSA). Lane 3, 4, 5, 6, 7, 8, 9, and 10 were loaded with 20 µg of protein.

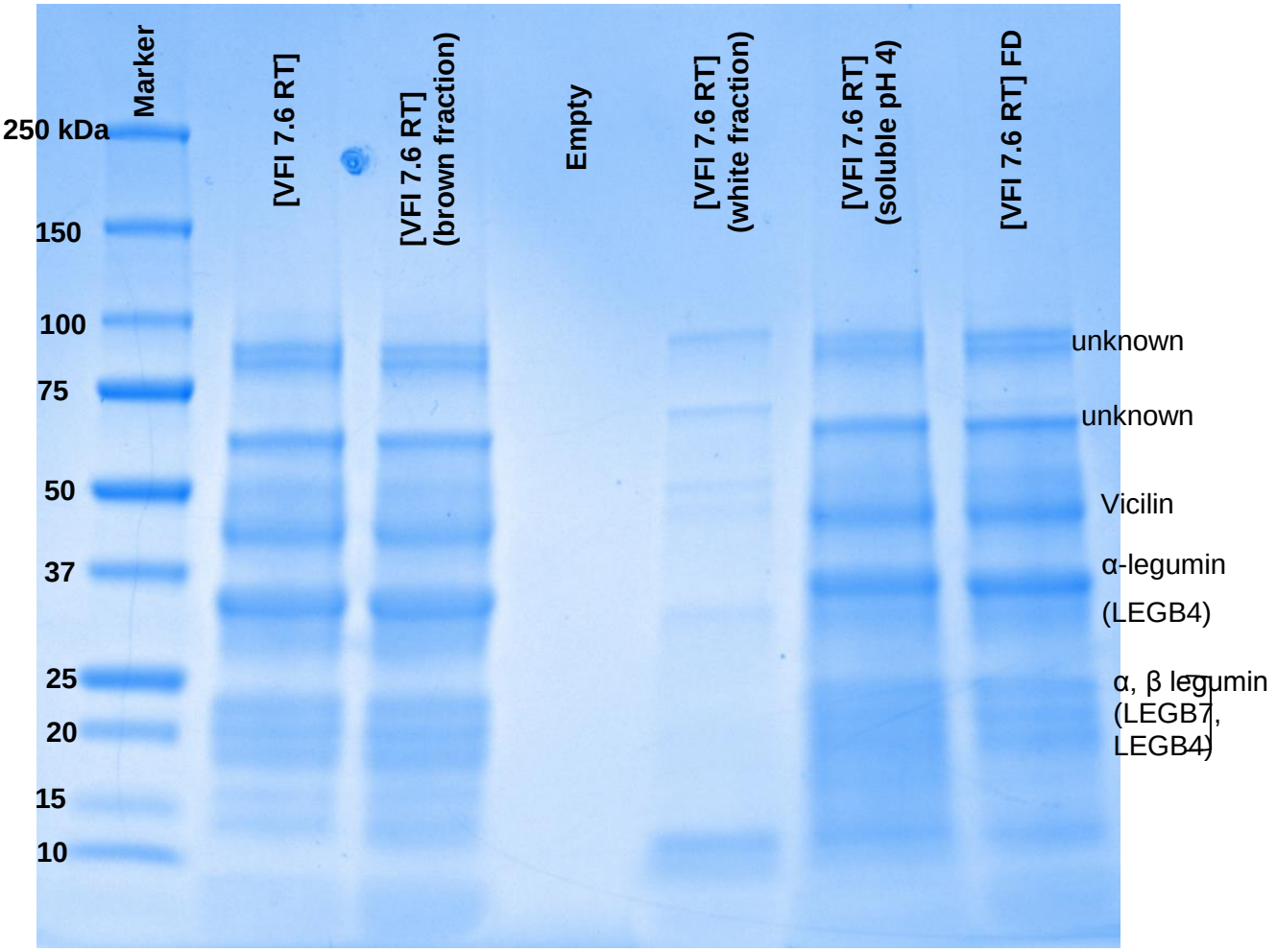

Figure 3.8: SDS PAGE of VFI 7.6 RT proteins and its fractions. Lane 1 was loaded with 7 µl of protein marker. Lane 2, 3, 5, 6 and 7 were loaded with 20 µg of protein.

Table 3:5: Molecular weight of proteins subunits observed in SDS-PAGE Figure 3.7 and Figure 3.8

Samples	Molecular weight (kDa)	Proteins as confirmed by literature
[VFI 7.6 6C]	14, 15,18, 20, 22 kDa	β-legumin
[VFI 7.6 RT]	37 kDa	α-legumin
[VFI 10.5 6C]	54 kDa	11S legumin
[VFI 10.5 RT]		
FBPC	47, 70 kDa	Vicilin
	57.5 kDa	Convicilin
SPI	20 kDa	Basic subunit of legumin
	37 kDa	Acidic subunit of legumin
	70 kDa	Beta conglycinin (7S)
PPI	15 kDa	Albumin
	25 kDa	β-legumin
	40 kDa	α-legumin
	47 kDa	Vicilin
	70 kDa	Convicilin
HP	19-20 kDa	Basic subunit of legumin
	35 kDa	Acidic subunit of legumin

Effect of precipitation on protein profile was investigated using SDS-PAGE (Figure 3.8) and LC-MS (3.2.3.2). [VFI 7.6 RT] (brown fraction) and [VFI 7.6 RT] FD showed similar protein profile as to [VFI 7.6 RT] and [VFI 7.6 RT] (soluble pH 4) but with presence of more intense low molecular weight protein bands (approximately 10 kDa), However, [VFI 7.6 RT] (white fraction) showed weak protein bands as it is thought to contain mostly starch as shown in light micrographs (Figure 3.6).

Table 3:6: Molecular weight of proteins subunits observed in SDS-PAGE Figure 3.6 and 3.7 for VFI 7.6 RT

Samples	Protein identified from SDS-PAGE			Protein identified from MS	
	Protein	*Molecular weight (kDa)*	Reference	*Protein*	*Molecular weight (kDa)*
[VFI 7.6 RT]	β-legumin	14, 15, 18, 20, 22 kDa	(Nivala et al., 2017, Felix et al., 2018, Cai et al., 2002)	LEGB4	54.4
[VFI 7.6 RT] (brown fraction)	α-legumin	37 kDa		LEGB7	37.8
	Vicilin	47kDa		Vicilin	52.7
	Convicilin	57.5 kDa		Favin	25.5
[VFI 7.6 RT] (white fraction)	Protein inhibitors	~10 kDa		LEGB4	54.4
	α-legumin	37 kDa		LEGB2	37.8
	Vicilin	47, 70 kDa		Vicilin	52.7
	Convicilin	57.5 kDa		Favin	25.5

[VFI 7.6 RT] (soluble pH 4)	Protein inhibitors	~10 kDa	, Bowman Birk type-proteinase	6.9
	β-legumin	20, 22, 24 kDa	Subtilisin inhibitor	7.1
	α-legumin	37 kDa	LEGB4, LEGB6, LEGB2, LEGB7	54.4,37.8,37.8
	Vicilin	47 kDa	Vicilin	52.7
	Convicilin	57.5 kDa	Favin	25.5
[VFI 7.6 RT] (FD)	Protein inhibitors	10 kDa	Vicilin	52.7
	β-legumin	20, 22, 24 kDa	LEGB4	54.4
	α-legumin	37 kDa	Favin	25.5
	Vicilin	47kDa		
	Convicilin	57.5kDa		

3.3.3.2 Analysis of amino acids composition in [VFI 7.6 RT] using Bioinformatic tools.

The sequence of amino acids for legumin LEGB4, legumin LEGB7, vicilin and favin were analysed by using ExPASy ProtParam tool to predict the amino acid composition of proteins in [VFI 7.6 RT] identified from LC-MS. The amino acid compositions of these four proteins were also compared with BSA and β-lactoglobulin from cattle and β-conglycinin, α-conglycinin and glycinin from soy protein primary structure of proteins.

The amino acid composition of proteins in VFI, BSA, beta lactoglobulin and soy protein is presented in Table 3:7. There was not much difference in amino acid compositions among LEGB4, LEGB7, vicilin and favin from VFI except for cysteine and methionine. Cysteine composition was the highest in LEGB4. In VFI, low amount of sulphur containing amino acids cysteine and methionine and tryptophan limit the nutritional quality of VFI. However, this can be overcome by making sure other protein sources rich in sulphur amino acids are also present in the diet for example egg and meat which have good protein quality. The most abundant amino acids found in LEGB4, LEGB7, vicilin and favin was leucine (9.9%), glutamic acid (11%), leucine (11.4%) and threonine (12.4%) respectively. Amino acids composition of LEGB7 are in agreement with study by (Liu et al., 2017). The sulphur containing amino acid (cysteine and methionine) in the four proteins was 1.2%, 0.3%, 0.4% and 0% in LEGB4, LEGB7, vicilin and favin respectively. The essential amino acid for all four proteins accounted for 34.7%, 33.3%, 40.2% and 48.9%. Previous studies on *Vicia faba* protein

isolate by Vioque et al. (2012) reported 0.6% on the composition of cysteine and methionine. Leucine was found to be the highest for BSA, beta lactoglobulin, beta conglycinin and alpha conglycinin which accounted for 10.7%, 15.2%, 10.1% and 8.3% respectively. Glutamine (8.7%) has the highest amount in glycinin. Essential amino acid composition in BSA, beta lactoglobulin, glycinin, beta conglycinin and alpha conglycinin was 43.7%, 47.2%, 33.8%, 37.8% and 32.6% respectively.

Table 3:7: Amino acids compositions in VFI, BSA, beta-lactoglobulin and soy protein. VFI proteins were highlighted in green and soy proteins were highlighted in orange. Essential AA were highlighted in blue.

Amino acid (%)	Legumin LEGB4	Legumin LEGB7	Vicilin	Favin	BSA	Beta-lactoglobulin	Glycinin	Beta-conglycinin	Alpha-conglycinin
Ala (A)	5.4	6.3	4.3	6.9	7.7	10.7	3.9	5.3	4.2
Arg ®	8.1	8.4	5.6	1.7	4.3	1.7	6.4	7.0	7.9
Asn (N)	6.4	7.5	7.8	6.4	2.3	2.8	6.4	7.9	6.6
Asp(D)	3.3	3.0	4.5	5.6	6.6	5.6	4.7	5.0	5.0
Cys ©	1.0	0.3	0.2	0.0	5.8	3.9	1.6	NA	0.2
Gln (Q)	7.9	8.4	6.0	2.1	3.3	5.6	8.7	7.9	8.5
Glu (E)	9.3	11.0	9.7	4.7	9.7	9.0	8.1	8.9	14.0
Gly (G)	6.8	7.5	5.8	8.2	2.8	2.8	7.9	4.6	4.4
His (H)	2.3	2.1	1.9	1.7	2.8	1.1	2.9	1.9	1.1
Ile (I)	5.4	4.5	6.0	6.0	2.5	5.6	3.3	6.2	5.5
Leu (L)	9.9	8.7	11.4	6.9	10.7	15.2	7.9	10.1	8.3
Lys (K)	2.9	3.0	6.9	5.2	9.9	9.0	3.7	5.0	5.9

Met (M)	0.2	NA	0.2	NA	0.8	2.8	1.0	NA	0.2
Phe (F)	2.9	3.0	5.2	6.4	4.9	2.2	3.5	6.7	5.0
Pro (P)	6.0	4.8	4.5	5.2	4.6	4.5	7.4	5.0	7.0
Ser (S)	8.5	6.9	8.2	6.4	5.3	3.9	8.3	7.5	7.2
Thr (T)	4.5	3.9	3.0	12.4	5.3	5.1	4.1	2.4	2.0
Trp (W)	0.6	0.6	NA	2.1	0.5	1.1	0.8	NA	0.2
Tyr (Y)	2.7	3.0	2.8	3.9	3.5	2.2	2.9	2.9	2.4
Val (V)	6.0	7.5	5.6	8.2	6.3	5.1	6.6	5.5	4.4

NA: Not available

Table 3:8: Essential Amino acids requirements for children age 0 to 5 years old and amount of amino acids in proteins required to satisfy the daily need.

Amino acid (AA)	Requirements for children age 0 to 5 years old (mg/kg per day) *	#Amount of AA (g/100g of proteins)/legumin LEGB4 (g) needed to meet the AA requirement	#Amount of AA (g/100 g of proteins)/ legumin LEGB7 (g) needed to meet the AA requirement	#Amount of AA (g/100 g of proteins)/vicilin (g) needed to meet the AA requirement	#Amount of AA (g/100 g of proteins)/favin (g) needed to meet the AA requirement
His (H)	22	2.29/0.96	2.1/1.05	1.90/1.16	1.70/1.30
Ile (I)	36	5.39/0.67	4.49/0.80	5.99/0.60	5.99/0.60
Leu (L)	73	9.89/0.74	8.69/0.84	11.39/0.64	11.39/0.64
Lys (K)	64	2.90/2.21	3.0/2.14	6.89/0.93	6.89/0.93
Met (M) + Cys ©	31	1.20/2.59	NA	0.40/7.76	NA
Phe (F) + Tyr (Y)	59	5.59/1.05	5.99/0.98	7.99/0.74	10.29/0.57
Thr (T)	34	4.49/0.77	3.9/0.87	3.00/1.13	12.38/0.27
Trp (W)	9.5	0.60/1.56	0.6/1.59	NA	2.10/4.5
Val (V)	49	5.99/0.82	7.49/0.65	5.59/0.88	8.19/0.60

Amino acid	#Amount of AA (g/100 g of proteins)/ BSA (g) needed to meet the AA requirement	#Amount of AA (g/100 g of proteins)/ beta lactoglobulin (g) needed to meet the AA requirement	#Amount of AA (g/100 g of proteins)/glycinin (g) needed to meet the AA requirement	#Amount of AA (g/100 g of proteins)/ beta conglycinin (g) needed to meet the AA requirement	#Amount of AA (g/100 g of proteins)/alpha conglycinin (g) needed to meet the AA requirement
His (H)	2.80/0.79	1.10/2.00	2.90/0.76	1.90/1.16	1.10/2.00
Ile (I)	2.50/1.44	5.59/0.64	3.30/1.09	6.19/0.58	5.49/0.66
Leu (L)	10.69/0.68	15.18/0.48	7.89/0.93	10.09/0.72	8.29/0.88
Lys (K)	9.89/0.65	8.99/0.71	3.70/1.73	4.99/1.28	5.89/1.09
Met (M) + Cys ©	6.59/0.47	5.49/0.56	2.60/1.19	NA	0.40/7.76
Phe (F) + Tyr (Y)	8.39/1.21	4.39/2.69	3.50/1.69	6.69/0.88	7.39/0.80
Thr (T)	5.29/0.64	5.09/0.67	4.09/0.83	2.40/1.42	2.00/1.70
Trp (W)	0.50/1.90	1.10/0.86	0.80/1.19	NA	0.20/4.76
Val (V)	6.29/0.78	5.09/0.96	6.59/0.74	5.49/0.89	4.39/1.12

*Data from (Pillai and Kurpad, 2012). NA-Not available. #Values were calculated based on average molecular mass of amino acid=112g/mol.

Data from Table 3:7 on the amino acid compositions of proteins in VFI was used to estimate the amount of essential amino acids in VFI's proteins. Table 3:8 presented the daily requirement of amino acids for 0 to 5 years old children and amount of amino acids in each proteins and also amount needed to satisfy the requirement of amino acid. The highest amount of amino acid found in all proteins was leucine except for threonine for favin. The lowest amount of amino acid found in all proteins was cysteine and methionine except for beta lactoglobulin. Beta lactoglobulin is major whey protein in milk. All proteins meet the daily requirement for all essential amino acids except for cysteine and methionine. Only beta lactoglobulin meet the daily requirement of cysteine and methionine. Intake amount of legumin (LEGB4), legumin (LEGB7), vicilin and favin required to meet the requirement needed ranged between 0.6 g to 7.7 g. Similar range was observed in SPI's proteins alpha conglycinin, beta conglycinin and glycinin which required intakes from 0.7 to 7.7 g. The highest amount required to meet requirements were from legumin (LEGB4) and vicilin which required intakes of 2.59 and 7.76 g to satisfy the requirement for daily cysteine and methionine due to the low content of both amino acids. Meanwhile, only 0.56 g of beta lactoglobulin was needed to meet the daily requirement of cysteine and methionine. This shows that legumes are deficient in sulphur containing amino acids, cysteine and methionine Sathe (2002) compared to protein from milk. However, deficiency of cysteine and methionine in VFI can be overcome by consuming other sources of dietary proteins such as meat or complementing it with cereal proteins.

3.3.3.3 Secondary protein structure using NetSurfP

NetSurfP was used to predict the secondary protein structure and the results are presented in Table 3:9. The secondary structures of protein will be used to discuss the functional properties.

Table 3:9: Secondary protein structures from NetSurfP

Proteins	*exposed*	*buried*	*polar exposed*	*polar buried*	*non-polar exposed*	*non-polar buried*	*alpha helix exposed*	*beta strand expose*	*coil exposed*	*alpha helix buried*	*beta strand buried*	*coil buried*
LEGB4	264	220	199	78	65	267	0.103818	0.091374	0.350242	0.072911	0.208968	0.173831
LEGB7	193	142	145	48	48	93	0.111651	0.10306	0.361382	0.060491	0.20137	0.163774
Vicilin	250	213	201	61	49	152	0.10079	0.086076	0.353127	0.081909	0.209253	0.168931
Glycinin	277	239	217	95	60	144	0.10487	0.096079	0.3358	0.085351	0.197256	0.180605
Beta-conglycinin	214	201	173	64	41	137	0.08216	0.08823	0.348867	0.0470747	0.24247	0.194804
Alpha-conglycinin	341	201	270	62	71	139	0.175862	0.08278	0.37185	0.04795	0.17703	0.14518

From these results, it can be seen that the number of exposed polar and non-polar amino acid of glycinin, beta and alpha conglycinin from soy are higher than LEGB4, LEGB7 and vicilin. Polar and non-polar amino acids compositions from Table 3:9 will be useful to see the different in the functional properties of VFIs and SPI proteins for example in WHC and FHC properties.

3.3.4 Quantification analysis of *Vicia faba* isolates

Based on the qualitative protein analysis of VFIs, precipitation was efficient to remove anti-nutritional compounds and therefore only [VFI 7.6 6C], [VFI 7.6 RT], [VFI 10.5 6C] and [VFI 10.5 RT] were being used in the quantitative analysis in order to decide which VFIs will be used in the next chapter.

3.3.4.1 Total protein, starch, total dietary fibre and ash composition of *Vicia faba* isolates and commercial protein powders

The proximate compositions of VFIs obtained at four different conditions are presented in Table 3:10. The interaction effects of pH and temperature on the total protein content, starch and fibre were observed and significant differences were found ($p<0.05$) based on two-way ANOVA analysis. Protein content of VFIs ranged between 32.5 to 42.7%. [VFI 7.6 RT] had significantly higher protein content followed by [VFI 10.5 RT], [VFI 10.5 6] and [VFI 7.6 6]. Starch content of VFIs which ranged between 5.16 to 8.27%. The highest starch content was found in [VFI 10.5 6] (8.27%) followed by [VFI 7.6 6] (7.54%), [VFI 10.5 RT] (7.25%) and the lowest starch content was found in [VFI 7.6 RT] (5.16%). Fibre content of VFIs ranged between 12.4 to 19.8%. [VFI 7.6 RT] was significantly higher (17%) compared to the other three VFIs. [VFI 10.5 6C] had the lowest fibre content (12%). A lower protein content was

observed in [VFI 10.5 6] and [VFI 10.5 RT] could be due to protein denaturation at alkaline pH which induced protein aggregation. This finding is in agreement with Jarpa-Parra et al. (2014) on the extraction of lentil protein isolate which observed lower protein content when samples were extracted at higher pH. Other reason could be due to high amount of starch in the isolate. Milling process could damage starch granules which made it more susceptible during alkaline extraction as the solubility increases at high pH (Jarpa-Parra et al., 2014). Also during filtration, some of the solubilised starch pass through the muslin cloth and precipitated alongside with the protein (McCurdy and Knipfel, 1990). This can also happens for products containing small starch particles such as cow peas where lower protein purity is obtained (Cloutt et al., 1987). Previous studies on *Vicia faba* isolates as tabulated in Table 3:1 did not look on the effects of pH and temperature on the protein extraction.

Table 3:10: Proximate composition of *Vicia faba* isolates (VFI) extracted at four different conditions.

Processing conditions		**Protein (%)**	**Starch (%)**	**[1]Fibre (%)**	**Ash (%)**
pH	*Temperature*				
7.6	6	*32.5±0.1d	7.54±0.24ab	13.3±0.5c	ND
7.6	RT	42.7±0.6a	*5.19±0.30c	*19.8±0.7a	3.27±1.84
10.5	6	36.8±0.6c	8.27±0.02a	12.4±0.1c	0.97±1.67
10.5	RT	39.6±0.2b	7.25±0.17b	16.5±0.3b	0.32±0.56

Mean values ± standard error for 3 technical replicates except * (two values). Data followed by different letters in a column are significantly different by multiple comparison test using two-way ANOVA ($p<0.05$) except for ash content.

Ash content in [VFI 7.6 RT] was 3%, followed by 1% in [VFI 10.5 6C] and 0.3% in [VFI 10.5 RT]. The starch content of VFI produced in this study was higher compared to study by Karaca et al. (2011) and Vioque et al. (2012) where carbohydrate content of faba bean protein isolate for both studies were 4%. Meanwhile, the value for ash content reported by Karaca et al. (2011) and Vioque et al. (2012) was comparable with ash content in [VFI 7.6 RT]. Fibre content reported by Nivala et al. (2017) was lower (5%) than fibre content reported in this study for all VFIs. The ash level also indicated that strong alkali or acid used in isoelectric precipitation methods may result in salt formation and a subsequent higher ash level in the protein isolate relative to the flour. Similarly, salts remaining after dialysis would contribute to higher ash contents in the isolates compared to starting materials (Karaca et al., 2011).

Meanwhile, fibre content from Karaca et al. (2011), McCurdy and Knipfel (1990) and Vioque et al. (2012) were not determined. Protein content in [VFI 7.6 RT] (brown fraction) and (white fraction) were 70.6% and 6.6% respectively. Higher protein content in brown fraction compared to white fraction confirmed that particles in brown fraction as shown in light microscopy (Figure 3.6) were mainly protein while the white fraction were mainly starch.

Table 3:11: Compositions of commercial samples soy protein isolate (SPI), pea protein isolate (PPI), hemp protein (HP) and faba bean protein concentrate (FBPC).

Samples	*Total protein content using Kjeldahl (%)[a]	Composition from label (%)			
		Protein	**Starch**	**Fibre**	**Ash**
SPI	70.6±5.2	90	0.1	1.0	NA
PPI	69.2±3.3	80	0.1	1.4	NA
HP	44.4±6.3	47	5.4	21.0	NA
FBPC	47.3±0.5	56	2.8	11.0	NA

*Mean values ± standard error for 2 technical replicates. NA-not available from label.

The total protein content varied significantly ($p<0.05$) among protein powders from different plant sources Table 3:11. SPI had the highest protein content (62%), followed by PPI (63%), FBPC (43%), [VFI 7.6 RT] (42%), [VFI 10.5 RT] (39%), HPI (38%), [VFI 10.5 6C] (36%) and finally [VFI 7.6 6C] (32%). As observed in this study, centrifugation step was important to isolate protein from other components like starch and fibre. Protein content of *Vicia faba* isolates reported by McCurdy and Knipfel (1990) Karaca et al. (2011), Vioque et al. (2012) and Nivala et al. (2017) was higher compared to VFIs produced in this study because they used centrifuge to isolate protein from other non-protein materials. However, the total yield would be low and therefore the process would be uneconomic. A review made by Schutyser et al. (2015) is in agreement with the findings in this study where high purity (high protein content) of isolate is accompanied by a decreased yield. Nevertheless, the use of less refined form of isolates can be advantageous because it requires fewer raw materials to produce the final product. Moreover, some applications into

food systems do not require 100% protein purity because other components help to improve the functional properties when it is added into food. The non-protein compound like fibre and other micronutrients like antioxidants in VFI could provide health benefit when it is applied into food product such as beef patties. Variability in the composition of ingredients and nutritional value can develop new food products with improved quality.

To the best of our knowledge, there were no similar study to compare the total yield and proximate composition of VFI as reported studies shown in Table 3:1 by McCurdy and Knipfel (1990) Karaca et al. (2011), Vioque et al. (2012) and Nivala et al. (2017) did not report the total yield and centrifugation was used to separate the supernatant from the solid instead of filtration in this study. All of these studies also did not observe the effects of pH and temperature on the yield and protein content. A study on other legume sources, lentil protein isolate by Jarpa-Parra et al. (2014) reported 14.5% total yield with protein content of 82% was achieved at pH 9 with ratio of 1:10. The total yield is useful indication to decide if the extraction is efficient.

3.3.4.2 Quantification of vicine and convicine using high performance liquid chromatography coupled with UV detection and mass spectrometry (HPLC-MS-UV)

Vicine and convicine content of *Vicia faba* flour, VFI and FBPC were measured by using LC-MS after being extracted by using water and the results are presented in Table 3:12. Vicine and convicine content were calculated based on the vicine calibration curve Figure 3.9. Good separation of vicine and convicine was achieved with 0.1% formic acid in water used as an eluent. The UV chromatogram of 0.1 µg/µl vicine standard Figure 3.10 showed one peak at about 3 minutes and the peak was further confirmed by MS which showed that the dominant ion was vicine with m/z [M+H] of 305. Different retention times for vicine (~5.4 mins) and convcine (~6.1 mins) were obtained in study by Pulkkinen et al. (2015) and Pulkkinen et al. (2016), meanwhile comparable retention times were obtained based on study by Vioque et al. (2012) for vicine (~2.5 mins) and convicine (3.8 mins). Difference in the retention time by Pulkkinen et al. (2015) and Pulkkinen et al. (2016) as compared to this study could be due to the hydrolysis of vicine and convicine to their agylcone divicine and isouramil which might happen during the isoelectric precipitation at pH 4. Aglycones of vicine and convicine, divicine and isouramil reported by Pulkkinen et al. (2016) were eluted at the beginning of the chromatogram with retention times for divicine and isouramil were found to be stable at 2.16 ± 0.04 and 2.50 ± 0.01 min. Although different retention time was obtained in this study, similar trend was observed as the retention times for vicine was earlier followed by convicine. Besides, in this study retention time of vicine standard

was also achieved at 3 minutes. Vicine and convicine are polar compounds which make them soluble in water. Vicine was less retained compared to convicine because it has extra amino group. Therefore, when formic acid was used as an eluent, vicine as a weak base was eluted first followed by convicine as a weak acid. Although commercial convicine standard was not available, vicine standard can be used for the quantification of convicine due to the similarity response of vicine and convicine (Pulkkinen et al., 2015). The identity of convicine was also confirmed by MS spectra of m/z $[M+H]^+$ of 306.

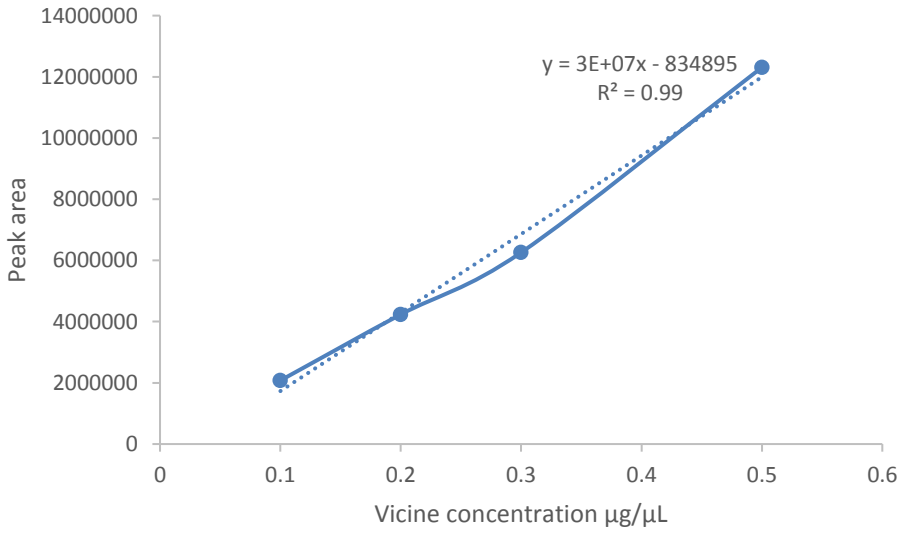

Figure 3.9: The vicine calibration curve for peak area against concentration of vicine. Each point represents average value of three replicates.

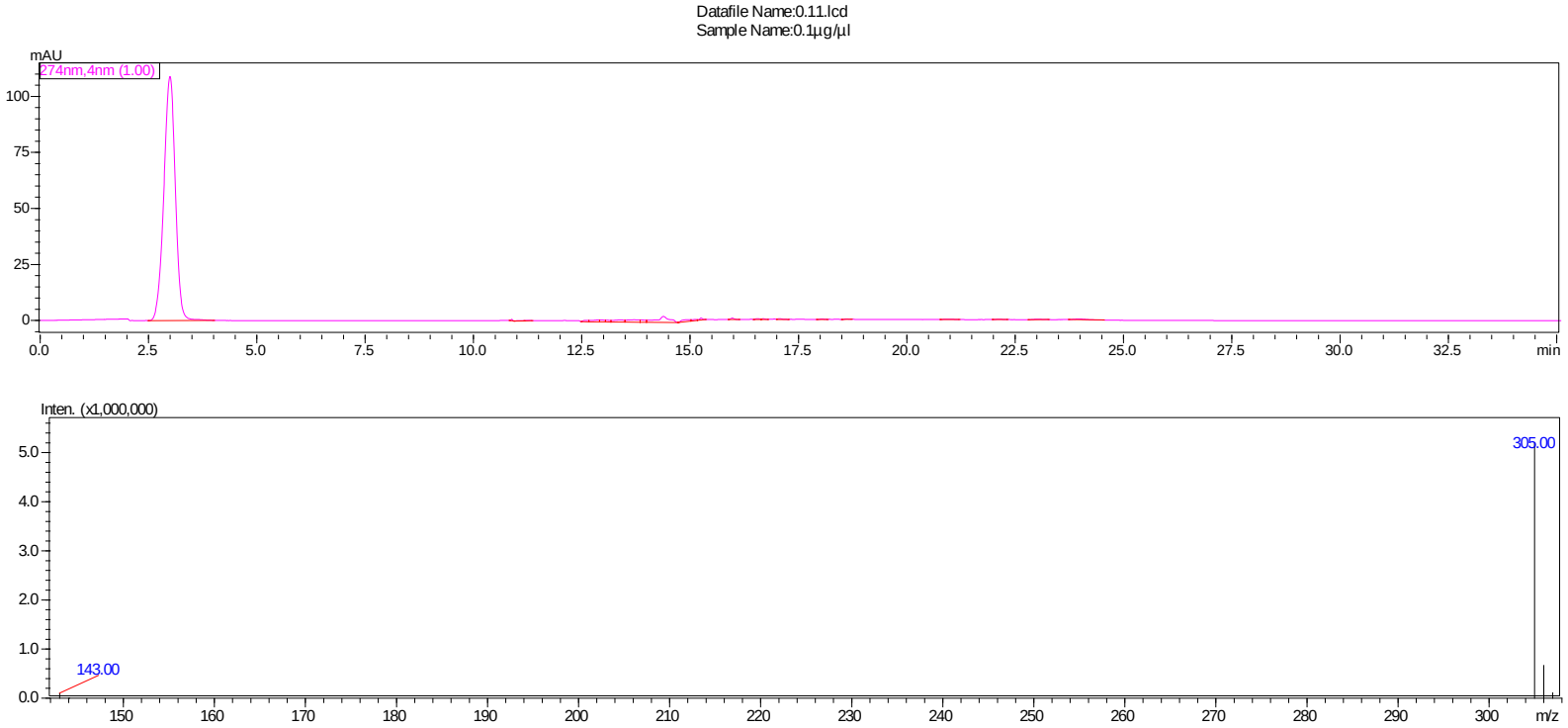

Figure 3.10: A HPLC-UV chromatogram (A) and MS spectra (B) of an m/z $[M+H]^+$ of 305 of 0.1 µg/µl vicine standard.

The highest vicine and convicine content which accounted to 1.7 % was found in FBPC which was higher than *Vicia faba* flour. Vicine and convicine in [VFI 7.6 6C], [VFI 7.6 RT], [VFI 10.5 6C] and [VFI 10.5 RT] were all less than 0.5% and reduced significantly ($p<0.05$) by 70% for vicine and 60% for convicine after extraction and isolation process. The extraction of VF flour was successful in reducing the vicine and convicine content to less than 1%. The lowest vicine and convicine content were found in [VFI 7.6 RT]. The vicine and convicine content of all VFIs in this study is in agreement with vicine and convicine content of protein isolate produced by Vioque et al. (2012) from *Vicia faba* flour which is less than 1%.

Table 3:12: Vicine and convicine content of *Vicia faba* flour, VFI and FBPC using HPLC-UV-MS.

Samples	(g/100 g of samples)		
	Vicine	Convicine	Total
Vicia faba flour	0.881±0.003b	0.368±0.002b	1.249
[VFI 7.6 6C]	0.219±0.002d	0.135±0.000c	0.354
[VFI 7.6 RT]	0.198±0.003e	0.121±0.003c	0.319
[VFI 10.5 6C]	0.267±0.001c	0.146±0.000c	0.413
[VFI 10.5 RT]	0.270±0.001c	0.148±0.001c	0.418
FBPC	1.056±0.009a	0.642±0.017a	1.698

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$).

3.4 Conclusion

Extracting proteins from *Vicia faba* are complex and difficult process because the total yield, total protein content and anti-nutritional compounds were influenced by the processing conditions. Extraction temperature and pH had significant effect on the total yield and pH 10.5 was found to be optimal for the total yield of VFIs. Interaction effects of pH and temperature on total protein and starch content were found to be significant. The optimal condition for extracting protein with lowest amount of vicine and convicine content was pH 7.6 at room temperature. VFI prepared by isoelectric precipitation has better protein profile as shown in SDS-PAGE and LC-MS. Therefore, [VFI 7.6 6C], [VFI 7.6 RT], [VFI 10.5 6C] and [VFI 10.5 RT] and commercial protein isolates will be characterised for their functional properties in Chapter 4 and will be used in beef patties in Chapter 5.

Chapter 4
Functional properties of *Vicia faba* isolates and commercial protein isolates

4.1 Introduction

Functional properties of proteins can be defined as physicochemical properties of food components which influence the behaviour of food during preparation, processing, storage and consumption and contribute to the quality and sensory attributes of food systems (Zayas, 1997). The functionality of proteins is related to their molecular structure and ability to interact with other food ingredients such as carbohydrate, fibre, lipid, ion and water. Functional properties of protein are affected by the effects of the processing isolate and environmental factors, namely pH, temperature and ionic strength.

The presence of protein can contribute to desirable functional properties which can be broadly classified into three different groups (Sathe, 2002). The first of these groups are, hydration-related properties which include solubility, wettability, swelling, water absorption/adsorption. Secondly, surface properties which influence the formation of emulsion, foams and films through protein interaction with components such as fat, water and air. Thirdly, the rheological properties which are related to thickening and gelling, elasticity, grittiness, cohesiveness, chewiness, aggregation, gelation, stickiness, viscosity, texturization, fibre formation, dough-forming ability, extrudability and adhesion. Functional properties are important determinants of the application of proteins in food formulations. As an example in minced meat products,

gelling, emulsifying, and water and fat binding properties during mixing and cooking are important functional properties which influence the quality of the final product and acceptability by the customer.

As mentioned in previous chapters, legumes are underutilised, sustainable and cheap source of proteins and other important nutrients including carbohydrates, fibres, vitamins and minerals. In the previous chapter, a process of isolating proteins from *Vicia faba* was investigated. The results showed that the process was successful at reducing the amount of anti-nutrients (vicine and convicine), however, the isolate was a mixture of protein, starch and fibre. All these components are likely to impact the functional properties.

Previous studies have investigated functional properties of pea, faba bean, hemp, buckwheat flour (Raikos et al., 2014), pea protein isolate fractions (Adebiyi and Aluko, 2011), broad bean protein concentrate (Arogundade et al., 2006), 7S and 11S globulin from pea, faba bean, cowpea, French bean and soybean (Kimura et al., 2008) , *Vicia faba* protein isolates (Vioque et al., 2012), and chickpea flour and protein isolates (Sánchez-Vioque et al., 1999). However, most of these studies focused on functional properties without considering it into the final food products and were focusing on the environmental factors such as pH, ionic strength and ratio on the functional properties. Table 4:1 summarises previous studies of functional properties of various protein sources. Limited studies have been carried out to establish the impact of processing conditions particularly the extraction and preparation of VF on the functionality of protein flours, concentrate and isolates.

In this chapter, the functional properties of *Vicia faba* isolates prepared in different ways are evaluated. Five functional properties were chosen due to their relevance to the replacement of egg in the production of beef patties. These functional properties are: protein solubility, water holding capacity (WHC), fat holding capacity (FHC), and foaming, emulsifying properties and gelling properties under natural pH conditions.

Table 4:1: Studies on functional properties of proteins

Samples	Functional properties	Factors that affect the functional properties	References
Cowpea protein isolate	Protein solubility, water holding capacity, least gelation concentration, viscosity	pH of protein extraction (8 and 10) and treated with thermal and pressure	(Peyrano et al., 2016)
***Vicia faba* protein isolate**	Water and fat absorption	_	(Vioque et al., 2012)
Lupin, green pea, fava bean, hemp, buckwheat flour	Protein solubility, Water holding capacity, foaming emulsion capacity, gelation	pH (4, 7 and 10)	(Raikos et al., 2014)
Chickpea, faba bean, lentil, pea, soy flour and isolates	Emulsion capacity	Isoelectric precipitation and salt extraction	(Karaca et al., 2011)
Pea protein isolate and pea protein isolate fractions: **1) Water soluble** **2) Salt soluble** **3) Alkaline soluble** **4) Ethanol soluble**	Solubility	pH (3 to 8)	(Adebiyi and Aluko, 2011)
	Emulsion and foam properties	pH 4, 7 and 9	
	Least gelling concentration	Ratio (2% to 20% w/v) pH (4, 7 and 10)	

Yellow peas, green lentils, red lentils, desi chickpea, kabuli chickpea	Solubility, water holding and fat absorption capacity, emulsifying, foaming and gelling properties	Isoelectric precipitation and ultrafiltration	(Boye et al., 2010b)
Faba bean protein concentrate	Protein solubility	pH (2, 4, 7, 10 and 12) Ionic strength (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2 M)	(Arogundade et al., 2006)
	Foam properties	pH (2, 4, 10 and 12) Ionic strength (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2 M)	
	Water absorption capacity	pH 6 Ionic strength (0.1, 0.2, 0.4, 0.6, 1, 2 M)	
7S and 11S pea, faba bean, cowpea, French bean and soybean	Protein solubility, Emulsion properties	pH (3 to 9) Ionic strength (0.5 and 0.08 M)	(Kimura et al., 2008)
Chickpea flour and protein isolate	Solubility, Water and fat absorption capacity, emulsion capacity	Effects of preparing isolates (with or without sodium sulphite)	(Sánchez-Vioque et al., 1999)
Pigeon pea protein concentrates	Foaming and gelling properties	Effects of pH, ionic strength, salt and protein concentration	(Akintayo et al., 1999)

4.2 Aim of the chapter

The aim of this chapter was to investigate the effects of processing on selected functional properties of the VFIs preparations which were produced in chapter 3. The functional properties of VFIs were also compared to SPI, PPI, HP and FBPC.

4.3 Results and Discussion

4.3.1 Solubility of *Vicia faba* isolates and other commercial samples by using BCA assay

The solubility of protein in water regardless of environmental factors was investigated by measuring the concentration of protein in the supernatant using BCA assay. The solubility of VFIs were measured without adjusting the pH of the solution. The results presented in Table 4:2 showed that the interaction effects of pH and temperature of extraction were significant ($p<0.05$) on VFIs solubility in water. VFIs were partially soluble in water where only 13%, 10.6%, 8.3% and 10.3% was soluble for [VFI 7.6 6C], [VFI 7.6 RT], [VFI 10.5 6C] and [VFI 10.5 RT] respectively. VFI extracted at pH 7.6, 6°C had significantly higher solubility compared to other VFIs. This can be explained by the milder extraction at pH 7.6 compared to higher pH of 10.5. All of the studies tabulated in Table 4:1 did not look at the effects of extraction pH and temperature on the solubility except Peyrano et al. (2016) which reported cowpea protein isolate extracted at pH 10 had slightly higher solubility (93%) compared to isolate extracted at pH (8). However, the difference was found insignificant which is in contrast to the present study. Meanwhile, study by Sánchez-Vioque et al. (1999) is in agreement with

this study which reported that chickpea isolate prepared with sodium sulphite at lower pH (10.5) exhibited higher solubility than isolate prepared with NaOH at higher pH (12). This is because extraction at lower pH may maintain the native conformation of the proteins compared to high pH (Sánchez-Vioque et al., 1999). During extraction, strong acid or alkali may denature *Vicia faba* proteins which exposed the hydrophobic sites which explain the lower solubility in VFIs extracted at pH 10.5. The low solubility of VFIs is also reflected to the processing technique used which partially insolubilize the protein during the precipitation of the extracts to pH 4. This finding is in agreement with study by Adebiyi and Aluko (2011), where pea protein fractions (water, salt soluble, alkaline soluble and ethanol soluble fractions) had better solubility at all pH examined compared to the solubility of PPI. Solubility of PPI was between 18 to 20% between pH 3 to 9 and the lowest solubility was found at pH 4.

The insolubilisation is caused by polymerization of the protein by the formation of disulphide bonds and by the irreversible denaturation of protein (Zayas, 1997). Besides, plant proteins have poor solubility due to the structure of globulin protein. Large globular proteins like legumin, have significant amount of surface area being buried into subunit-subunit interface. Therefore, the accessible of the surface area is reduced by the subunit contact area. Inter subunit volumes are not accessible by the solvent which made legume protein less soluble (Schwenke, 2001). Presence of other substances also influences the solubility of protein. In the present study, VFIs composed other non-protein compounds which were starch and fibre which could limit their solubility in water.

Table 4:2: Effects of extraction conditions of *Vicia faba* isolates (VFIs) on protein solubility in water.

Extraction conditions		Protein solubility g/100g of total protein)
pH	*Temperature*	
7.6	6	12.99±0.30c
7.6	RT	10.63±0.51b
10.5	6	8.24±0.51a
10.5	RT	10.26±0.11b

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using two-way ANOVA ($p<0.05$).

Freezing and drying can change the properties of proteins causing the reduction in protein solubility. During freezing the protein pellet was highly concentrated and the formation of ice crystals involves the removal of water which then caused all protein molecules to be brought closer together or aggregated. In the native protein molecules, sulfhydryl groups are not exposed to interaction, however during freezing and drying, free sulfhydryl and disulphide bonds are exposed at the surface of proteins which results in insolubulisation (Zayas, 1997). Vicilin-like and legumin-like globulins which are the major proteins in VFI can form polymers through the formation of disulphide bonds.

Solubility of VFIs in water was also compared with commercial samples as presented in Table 4:3. FBPC had the highest solubility at 63% followed by PPI 59%, SPI 40% and finally HP 10%. Study reported by Boye et al. (2010b) observed that green lentil, red lentil, desi chickpea and kabuli chickpea protein concentrates prepared by isoelectric precipitation had lower solubility compared to those prepared by ultrafiltration regardless of pH. Another study by Karaca et

al. (2011) found the solubility of legume protein isolates prepared by isoelectric precipitation was higher compared to salt extraction. In this study, the surface charge of (mV) of protein isolate was also lower in salt extraction compared to isoelectric precipitation which explained the low solubility in salt extraction. Another study reported by Karaca et al. (2011) found that the protein solubility of chickpea (91%), faba bean (89%), lentil (90%), pea (61%) and soy protein isolate (96%) in sodium phosphate buffer pH 7 was much more higher than in the present study. Another study was performed by Johnston et al. (2015) also reported high protein solubility for faba bean protein at 85%. The variations in the protein solubility are due to pH, buffer and technique used to measure the protein solubility and also method in preparing the isolates. Protein solubility obtained from all four VFIs was significantly lower than FBPC, although both proteins were from the same type of beans. This could be because FBPC had higher protein content than VFIs and it is also an indication that the FBPC was prepared differently from VFIs. Solubility in VFIs were also lower compared to PPI and SPI which could also be due to the lower protein content of VFIs compared to PPI and SPI and their methods of preparation. It should be noted that the methods of preparation of commercial samples are not known. PCA in

Figure 4.7 later also shown that solubility and protein content were in the same component which suggests that solubility is affected by protein content. Variations in solubility have also been reported for different legume proteins (Boye et al., 2010b, Raikos et al., 2014).

However, protein could change conformations under different pH values, ionic strength, temperature and solvent which could improves the solubility. Study

reported by Arogundade et al. (2006) on broad bean protein concentrate (BPC) observed increased solubility of BPC protein solution at pH 4 as the ionic strength increased from 0.05 to 0.4. Therefore, it may suggests that the isolated protein at pH 4 from *Vicia faba* will have enhanced solubility in the presence of salt which makes them a potential ingredients in food formulation. Effects of pH on the solubility of VFI was studied later. In this present study, it is clear that protein solubility are not only influenced by the environmental factors, solvent/buffer types but also by processing either during extraction or post-extraction treatments.

Table 4:3: Protein solubility of *Vicia faba* isolates (VFIs) and commercial samples in water.

Samples	Protein solubility (g/100g of total protein)
[VFI 7.6 6C]	12.99±0.30c
[VFI 7.6 RT]	10.63±0.51b
[VFI 10.5 6C]	8.24±0.51a
[VFI 10.5 RT]	10.26±0.11b
SPI	40.79±0.95c
PPI	59.87±0.55d
HP	10.07±0.35b
FBPC	63.68±0.26e

Mean values ± error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$).

4.3.2 Effects of pH on the solubility of protein from *Vicia faba* flour, [VFI 7.6 RT], SPI and FBPC.

Effects of pH on the solubility of protein from *Vicia faba* flour, [VFI 7.6 RT], SPI and FBPC were measured at pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 was shown in Figure 4.1. In this section, only [VFI 7.6 RT] was studied because it had the highest total protein content compared to the other three VFIs. The initial pH of FBPC, SPI, *Vicia faba* flour and VFI when added to Milli-Q water were 6.66, 7.95, 6.61 and 2.92 respectively. The increasing trends of all four samples were observed as the pH increased. The minimum solubility occurred at pH 3 for FBPC (11%), pH 4 for SPI (1%), and *Vicia faba* flour (10%) and VFI (3%) had minimum solubility at pH 4.

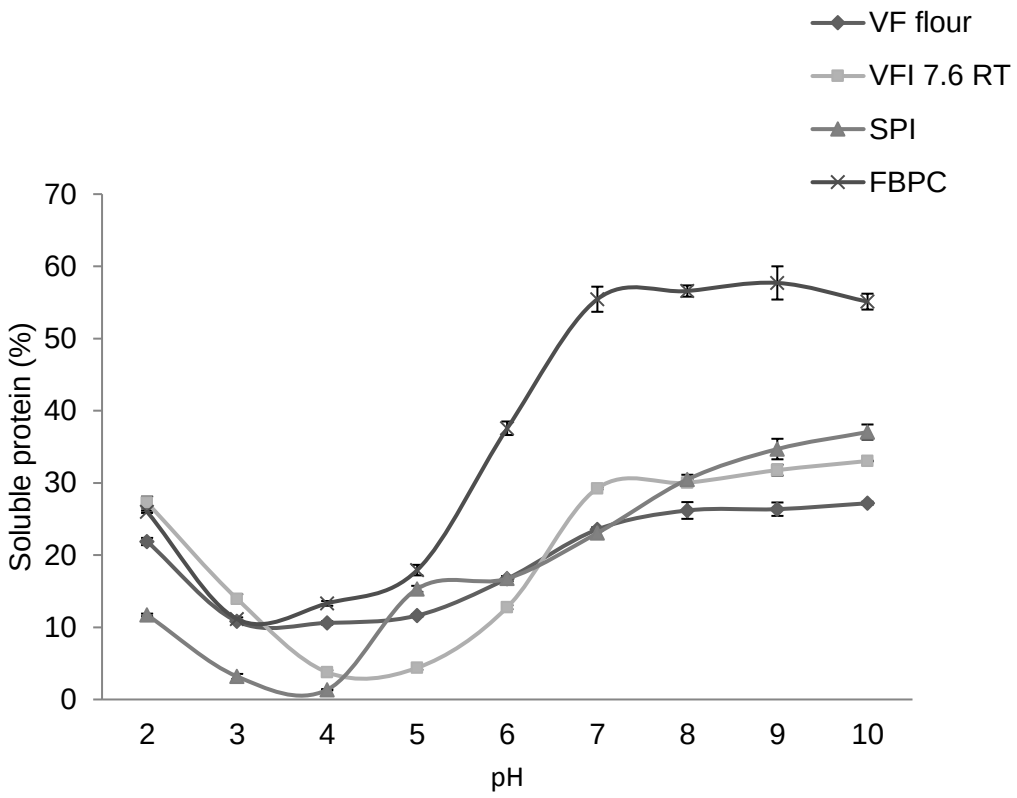

Figure 4.1: Effects of pH on the solubility of Faba bean protein concentrate (FBPC), Soy protein isolate (SPI), *Vicia faba* flour (VF flour) and [VFI 7.6 RT] in water at pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 as measured by BCA assay.

The minimum solubility for all four samples at around pH 4 indicated that this pH is close to the isoelectric point of legume proteins. This finding is in agreement with Nivala et al. (2017) which observed the minimum solubility of faba bean protein isolate at pH 4. Study reported by Raikos et al. (2014) on the solubility of wheat, lupin, green pea, fava bean and buckwheat flour also observed minimum solubility at pH 4. Previous studies by Kimura et al. (2008), Sánchez-Vioque et al. (1999), Adebiyi and Aluko (2011) and Boye et al. (2010b) also observed minimum solubility for all legume protein at the isoelectric point. Precipitation of protein made the solubility of VFI at pH 4 was 7% lower than *Vicia faba* flour. As the pH increased, the solubility for all samples were increased as seen in Figure 4.1. At pH 7 the protein solubility of FBPC, SPI, *Vicia faba* flour and [VFI 7.6 RT]

were 55%, 23%, 23% and 29% respectively and at pH 9 57%, 34%, 26% and 31% of protein from FBPC, SPI, *Vicia faba* flour and [VFI 7.6 RT] were soluble. Two main interactions are important in protein solubility which are protein-protein and protein-solvent interactions. The protein-protein interaction is facilitated through hydrophobic interaction to form precipitation and protein-solvent interaction promotes protein hydration and solubility. However, most of the protein will still remain in globular forms due to very strong internal hydrophobic interactions of aromatic and nonpolar amino acid. The cores of globular subunits are buried with polar and hydrophilic amino acids on the surface to prevent unfavorable interactions between a polar solvent and hydrophobic amino acid side chains; therefore, there is no interaction between the parts of protein chains inside the cores with other protein subunits (Aghanouri et al., 2014). The exposed amino acids on the surface of proteins will be able to interact either with protein or solvent.

Soluble protein of [VFI 7.6 RT] at pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 were characterised by using SDS PAGE to observe the protein profile at different pH. From Figure 4.2, bands at about 50 kDa were observed in all pH except pH 4 and 5.. At pH 2 and 10, the band became more visible compared to other pH.

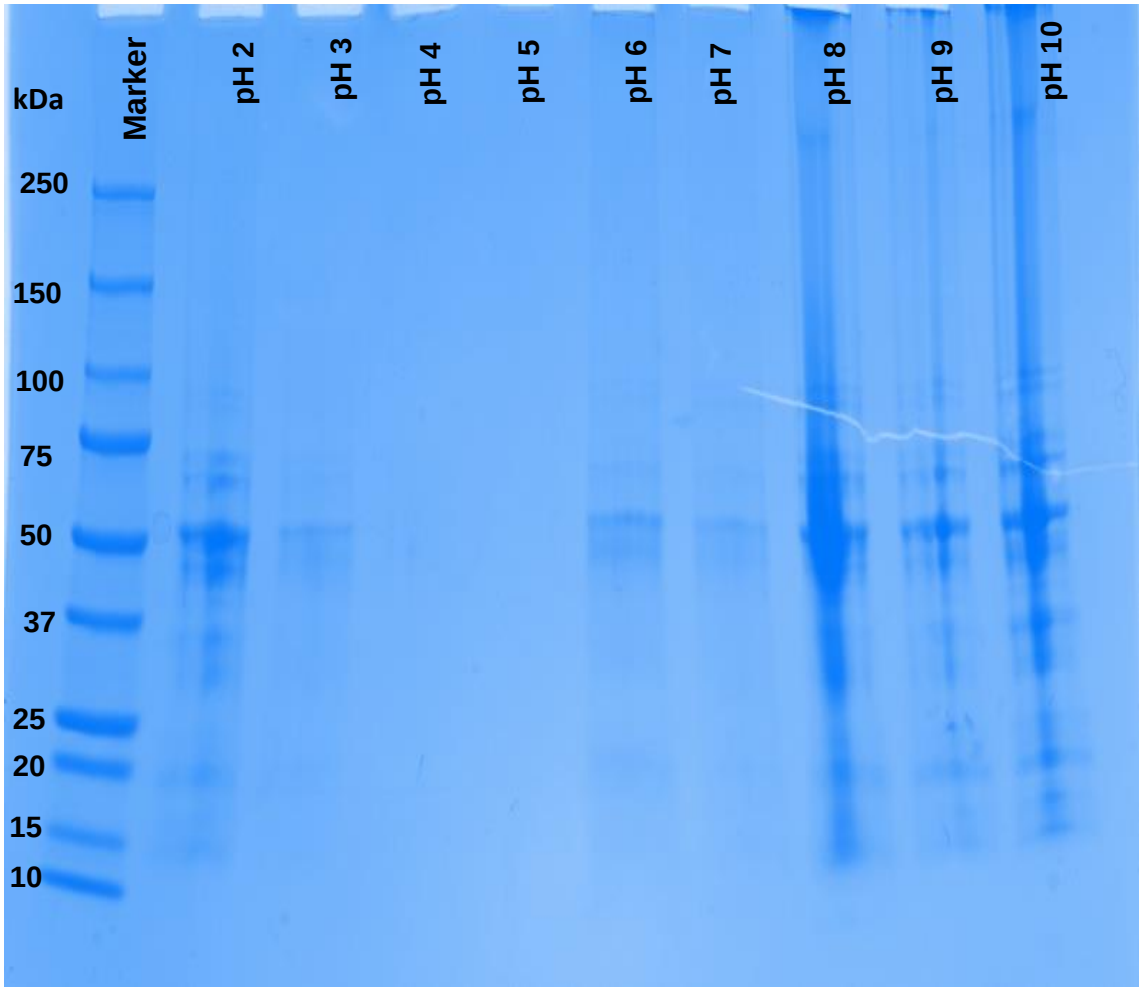

Figure 4.2: SDS PAGE gel of [VFI 7.6 RT] proteins Lane 1: Protein marker, lane 2, 3, 4, 5, 6, 7, 8, 9, 10 was soluble protein at pH 2 to 10.

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4.3.3 Zeta potential

Surface charge of protein is important as measured by the zeta potential. The surface charge (zeta potential) of [VFI 7.6 RT] and FBPC were determined by measuring the electrophoretic mobility of protein solutions at pH 2 to 10. Zeta potential measurement as a function of pH is shown in Figure 4.3. At pH 2 and 3 for [VFI 7.6 RT], zeta potential values were positive and as the pH increased from 4 to 10, the values were negative. Similar results were observed for FBPC as the pH increased from 3 to 10, the values were negative. At pH 4, the charge of [VFI 7.6 RT] was near to zero (-3.0) and for FBPC the charge was near to zero (-1.2) at pH 3. This indicates that pH 4 and 3 were the isoelectric point for [VFI 7.6 RT] and FBPC respectively.

The higher solubility at both sides of isoelectric point as shown in Figure 4.3 was also related to the increased surface charge of protein at different pH. As the pH was far from the isoelectric point, the charge increased significantly and so did the surface charge due to the predominant charge of the amino acids. The pH was increased by adding base which then deprotonated the amines leaving the net negative charge and decreasing the pH by adding acid which then protonated the carboxyl ion. Both protonation and deprotonation enhance protein–solvent interaction because the same charged proteins keep them apart due to the electrostatic repulsion which encourages protein interaction with solvent as a consequences increases protein solubility. But at the isoelectric point, (pH 4), the dipolar species of *Vicia faba* protein predominates, resulting in minimum repulsion and high protein–protein interaction, forming insoluble

aggregates, thus decreased protein solubility (Arogundade et al., 2006, Raikos et al., 2014).

At pH 7 to 10, the surface charge of [VFI 7.6 RT] and FBPC were -34.3 to -29.7 and -36.4 to -29.1 respectively. Protein with charge 30 and above are said to be stable. Results are in agreement with Nivala et al. (2017) for oat protein isolate and faba bean protein isolate which reported the zeta potential value for faba bean protein isolate was -30 to -35 from pH 7 to 10. Karaca et al. (2011) also reported similar surface charge at pH 7 for faba bean protein isolate to be -23 mV which produced by isoelectric precipitation and lower surface charge for isolate that was prepared by salt extraction (-18.3 mV). At this pH region, proteins carry a net negative charge as all are above their isoelectric point (where zeta potential is 0 mV). The net negative charge arises primarily from the negatively charged R groups found on the aspartate ($pK_R = 3.65$) and glutamate ($pK_R = 4.25$) amino acids spatially located on the protein surface (Johnston et al., 2015). The high solubility of VFI on either sides of IEP could be due to prevalent charge of the constituent amino acid of the proteins. It is obvious that pH being a major factor that affects the protein charge as at high pH all the amines are deprotonated, leaving a net negative charge while the reverse is the case at low pH .

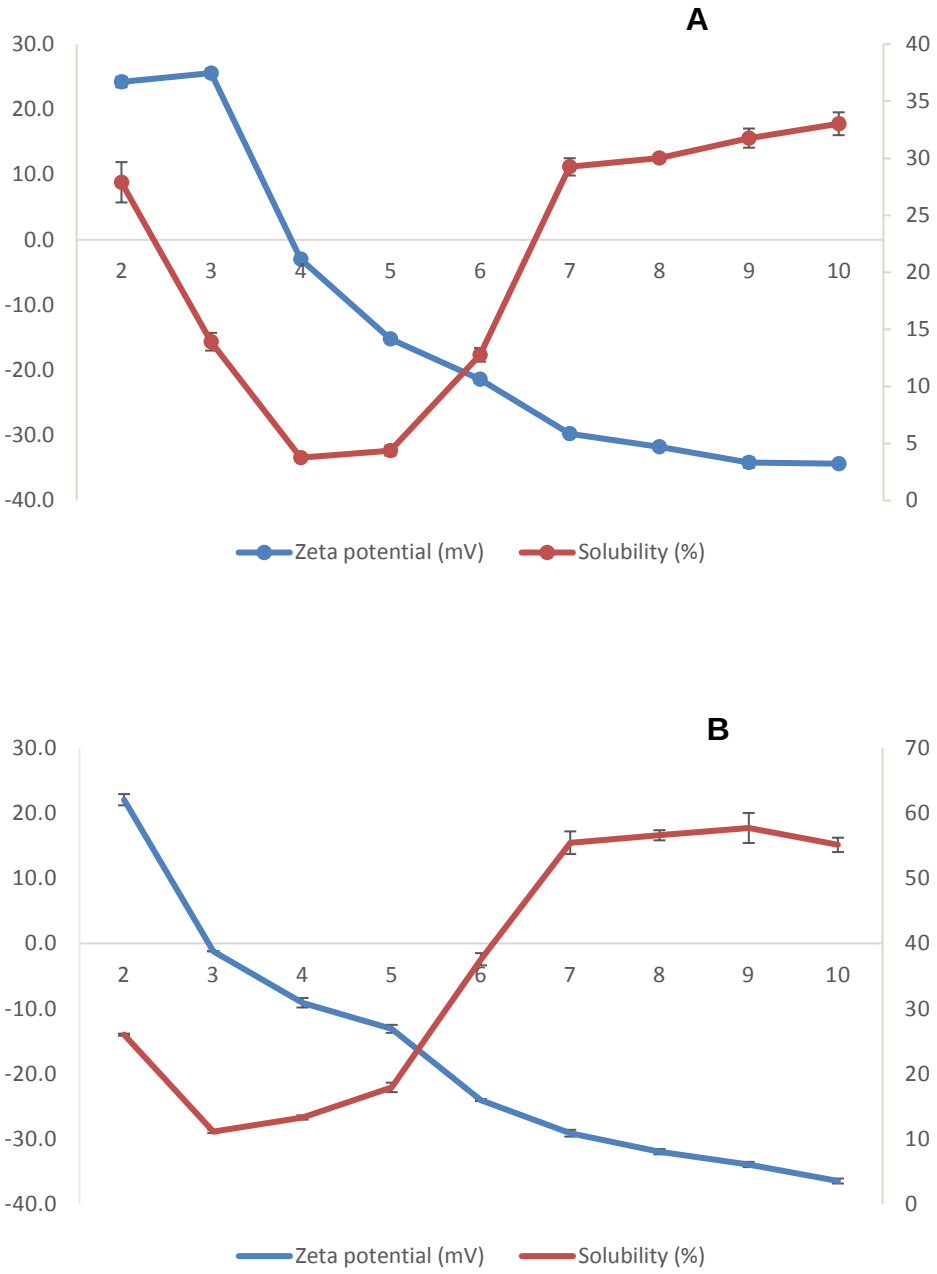

Figure 4.3: Effects of pH on the solubility as well as zeta potential of A) [VFI 7.6 RT] and B) FBPC from pH 2 to 10 for protein aqueous solutions. Error bars represent standard deviations of 3 replicates.

4.3.4 Water holding capacity (WHC)

WHC of VFIs produced at different pH and temperature showed that only temperature of extraction was significant on WHC of VFIs ($p<0.05$) and pH was not significant ($p>0.05$) (Table 4:4). Higher temperature during extraction could unfold the polypeptide chains due to denaturation and will undergo transition of conformation from globular to random coil. This may result in a reduction in the availability of polar amino acid groups for binding water (Zayas, 1997). Previous study by Peyrano et al. (2016) on WHC of cowpea protein isolates did not show any significant different at different extraction pH (8 and 10) at 20°C. However, when both isolates were heated, increased in WHC was observed due to more unfolding-induced exposure of polar amino acids. In contrast to study by Sánchez-Vioque et al. (1999), WHC of chickpea isolate produced at pH 12 (3.43g/g) showed higher WHC compared to chickpea isolate produced at pH 10.5 (1.99g/g). This is because lower losses of soluble proteins in chickpea isolates produced at pH 12 using NaOH (Sánchez-Vioque et al., 1999).

Table 4:4: Effect of temperature on Water holding capacity of *Vicia faba* isolates.

Temperature	*WHC (g water/g samples)
6°C	1.56±0.03b
20°C	1.45±0.01a

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using two-way ANOVA ($p<0.05$).

Significant difference ($p<0.05$) was also observed in WHC of [VFI 7.6 RT] brown fraction compared to white fraction and other VFIs samples. This can be related to higher protein content of [VFI 7.6 RT] (brown fraction) compared to [VFI 7.6 RT] and [VFI 7.6 RT] (white fraction). WHC of samples were positively correlated to the total protein content of the samples ($r=0.7017$, $p=0.0024$) and negatively correlated to starch ($r=-0.4896$, $p=0.0543$) and fibre ($r=-0.5745$, $p=0.0199$). WHC of VFIs were also compared to commercial protein samples (Table 4:5). The water holding capacity of VFIs and commercial protein samples ranged between 1.4 to 4.1 g/g. The highest WHC was found in SPI followed by PPI, [VFI 7.6 RT] (brown fraction), [VFI 7.6 RT] (white fraction), [VFI 10.5 6], [VFI 7.6 6], [VFI 7.6 RT], [VFI 10.5 RT], FBPC and HP.

Table 4:5: Water holding capacity of *Vicia faba* isolates and commercial samples.

Samples	WHC (g of water/g of samples)
[VFI 7.6 6]	1.52±0.04a
[VFI 7.6 RT]	1.45±0.03a
[VFI 10.5 6]	1.60±0.03a
[VFI 10.5 RT]	1.44±0.02a
[VFI 7.6 RT] (brown fraction)	2.31±0.02c
[VFI 7.6 RT] (white fraction)	1.66±0.10ab
SPI	4.17±0.18d
PPI	2.18±0.23bc
HP	1.26±0.07a
FBPC	1.30±0.09a

Mean values ± standard error for 3 technical replicates.

Data was also compared with WHC of faba bean protein concentrate, *Vicia faba* protein isolate, green pea, hemp, buckwheat, wheat and fava bean flour, chickpea flour and isolates from previous studies (Table 4:6). Raikos et al. (2014) reported that pH has no effect on WHC and the data showed lower WHC of faba bean flour (1.5g/g) compared to lupin flour (2.4 g/g) which composed of 30% and 40% protein content respectively. Effect of ionic strength on WHC was studied by Arogundade et al. (2006) and low ionic strength of 0.1 improved WHC of of faba bean protein concentrate (1.5g/g) from the control (1.25 g/g) but reduced at ionic strength of 0.6 to 1.0. But the value was comparable to WHC of VFIs in this study. WHC of VFIs in the present study was slightly lower compared to previous study by Vioque et al. (2012) on *Vicia faba* isolate (3g/g) because it composed of higher protein content (92%). In agreement with both studies, protein content appeared to be one of the factor contributing to WHC. Another study by Sánchez-Vioque et al. (1999) found lower WHC in chickpea isolate that contained higher protein content compared to chickpea isolate produced by alkaline extraction without sodium sulphite. WHC of chickpea isolate produced using sodium sulphite (1.9g/g) was comparable with WHC value for VFIs in this study. This suggests that additional factors other than protein content contribute to WHC. Factors like the availability of polar amino acids exposed for protein-water interaction (Toews and Wang, 2013, Arogundade et al., 2006) and different protein conformation among different legumes are also important in WHC (Withana-Gamage et al., 2011). According to (Kaur and Singh, 2005) the presence of hydrophilic constituent such as polysaccharides also attributed to high WHC. However, in

this study it is unlikely that starch contributed to WHC because starch is insoluble in water and certain temperature condition was needed for starch to contribute to WHC. According to Zayas (1997), carbohydrate plays an important role during incubation time because starch granules swell extensively in the presence of water during heating which caused gelatinization. Therefore, higher temperature and longer incubation time during WHC could enhance starch swelling and increase water binding. However, in this study, highest WHC in SPI could be related to the swelling ability of the hydrated protein in SPI and to the change in protein conformation which increased the binding sites for water (Zayas, 1997). Swelling of protein particles have been used as an indicator of water holding without protein being dissolved. In Figure 4.4, SPI swell up to 3 or 4 times its weight compared to other samples which enhance its water holding capacity. For VFI, FBPC and HP high amount of fibre (Chapter 3) may also contributed to lower WHC compared to more refined protein isolate in SPI and PPI with the absent of fibre content.

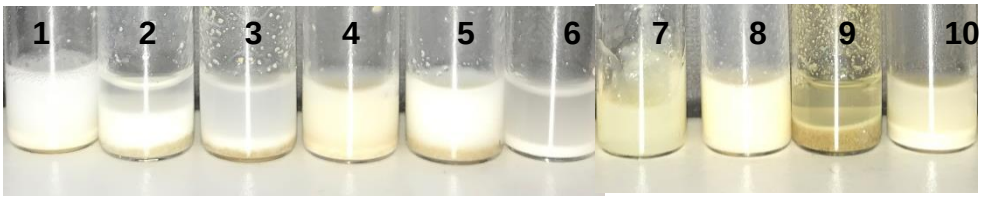

Figure 4.4: Vials containing samples with water being held for 1 hour after overnight mixing. Vial 1: [VFI 7.6 6], 2: [VFI 7.6 RT], 3: [VFI 10.5 6], 4: [VFI 10.5 RT], 5: [VFI 7.6 RT] (brown fraction), 6: [VFI 7.6 RT] (white fraction), 7: SPI, 8: PPI, 9: HP, 10: FBPC.

Table 4:6: WHC of previous studies on legume samples.

Samples	Test Condition	WHC (g of water/g of samples)	Conclusion	References
Chickpea protein isolate	*Extraction pH 8*	1.05	Extraction pH did not affect WHC	(Peyrano et al., 2016)
	Extraction pH 10	0.95		
Wheat flour	*pH 4, 7 and 10*	0.8	pH of water did not affect WHC	(Raikos et al., 2014)
Green pea flour	*pH 4*	1		
	pH 7	1.3		
	pH 10	1		
Lupin flour	*pH 4*	2.3		
	pH 7	2.4		
	pH 10	2.5		
Fava bean flour	*pH 4*	1.6		
	pH 7	1.5		
	pH 10	1.4		
Hemp flour	*pH 4*	1.7		
	pH 7	1.6		
	pH 10	1.7		
Buckwheat flour	*pH 4*	1.4		

	pH 7	1.3		
	ph 10	1.2		
***Vicia faba* protein isolate**	-	3.04		(Vioque et al., 2012)
Faba bean protein concentrate	*Control*	1.25	Ionic strength of 0.1 improved WHC then reduced at 0.2 ionic strength and above	(Arogundade et al., 2006)
	Ionic strength (0.1)	1.5		
	Ionic strength (0.2)	1.3		
	Ionic strength (0.4)	1.25		
	Ionic strength (0.6)	0.9		
	Ionic strength (1)	0.9		
	Ionic strength (2)	1.1		
Yellow pea	*Isoelectric precipitation*		Protein extracts produced by isoelectric precipitation had higher WHC compared to ultrafiltration	(Boye et al., 2010b)
Green lentil **Red lentil** **Desi chickpea** **Kabuli chickpea**	*Ultrafiltration*			
Chickpea flour	-	1.79	Extraction affects WHC	(Sánchez-Vioque et al., 1999)
Chickpea protein isolate (A)	*Alkaline extraction without sodium sulphite*	3.44		

Chickpea protein isolate (B)	*Alkaline extraction with sodium sulphite*	1.99

4.3.5 Fat holding capacity (FHC)

Effects of pH and extraction conditions were not significant on fat holding capacity of VFIs based on two-way ANOVA. Therefore, fat holding capacity of VFI and commercial protein samples were analysed using one-way ANOVA to see the different between samples. The FHC ranged between 1.3 to 8.8 g/g (Table 4:7). SPI and PPI had significantly higher FHC which were 8.78 g/g of samples and 8.46 g/g of samples respectively compared to the rest of samples ($p<0.05$). [VFI 7.6 RT] brown fraction (2.6 g of oil/ g of sample) showed significantly higher WHC ($p<0.05$) compared to the rest of VFI samples. However, effects of pH and temperature of extraction of VFIs on FHC were not significant ($p>0.05$). FHC of samples were positively correlated to the total protein content of the samples ($r=0.8721$, $p=0.00001$) and negatively correlated to starch ($r=-0.5322$, $p=0.0338$) and fibre ($r=-0.7336$, $p=0.0012$). FHC of [VFI 7.6 RT] (brown fraction) was comparable to study observed by Vioque et al. (2012) on *Vicia faba* protein isolate (2.3 g/g) but not in agreement with other VFIs samples because both have higher protein content compared to the rest of VFIs. FHC of flax seed protein isolate had 2.8 g/g (Kaushik et al., 2016). FHC of VFIs were comparable with study by Boye et al. (2010b) on yellow pea, green lentil, red lentil and desi chickpea and kabuli chickpea protein concentrate which ranged between 1.2 to 1.3 g/g.

Based on the data observed for FHC in this study, higher protein content exhibited higher FHC as demonstrated by SPI, PPI and VFI brown fraction. According to Sathe (2002), most bean proteins held less than 5 g oil/g. In this

study SPI and PPI had more than 5g/g FHC. This higher value could be due to oil trapped in between two layers of protein after mixing for overnight as observed in Figure 4.5. Besides concentration of protein in samples, FHC was enhanced by denaturation of proteins by exposing the hydrophobic groups of proteins which favours fat binding during processing (Zayas, 1997). The secondary structure of proteins also influenced FHC of proteins (Mune Mune et al., 2018). This is because FHC involved the physical entrapment of oil as well as noncovalent bonds involved in lipid-protein interaction. According to study on oil holding capacity of cowpea and Bambara protein concentrate, FHC increased with increasing β-sheet content which favoured the exposure of hydrophobic amino acids (Mune Mune et al., 2018). Besides, higher proportion of the non-polar amino acids in proteins results in more lipophilic characteristics which contributed to the increased in FHC. The dissociation of 11S protein into their subunits would involve exposure of hydrophobic β chains that are hidden in the native legume. The dissociation of 11S legumin in VFIs were observed in SDS-PAGE Figure 3.7 and Figure 3.8 at 14-22 kDa which were β-subunit and 37 kDa which were α-subunits. The accessibility of β chains would also facilitate the interaction with non-polar compounds like lipids and increased fat absorption (Sánchez-Vioque et al., 1999).

Table 4:7: FHC of *Vicia faba* isolates (VFIs) and commercial proteins samples.

Samples	FHC (g of oil/g of samples)
[VFI 7.6 6]	1.30±0.06a
[VFI 7.6 RT]	1.31±0.04a
[VFI 10.5 6]	1.34±0.08a
[VFI 10.5 RT]	1.11±0.11a
[VFI 7.6 RT] (brown fraction)	2.58±0.07c
[VFI 7.6 RT] (white fraction)	2.15±0.33bc
SPI	8.78±0.17d
PPI	8.46±0.18d
HP	1.30±0.07a
FBPC	1.47±0.08ab

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$)

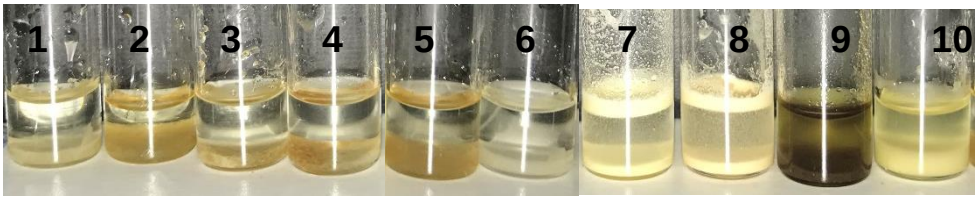

Figure 4.5: Vials containing samples with sunflower oil being held for 1 hour after overnight mixing. Vial 1: [VFI 7.6 6], 2: [VFI 7.6 RT], 3: [VFI 10.5 6], 4: [VFI 10.5 RT], 5: [VFI 7.6 RT] (brown fraction), 6: [VFI 7.6 RT] (white fraction), 7: SPI, 8: PPI, 9: HP, 10: FBPC.

4.3.6 Emulsifying properties

Emulsifying properties of the sample was described as emulsion capacity (EC) and emulsion stability (ES). EC measured the capacity of protein to aid in the formation and stabilisation of the emulsion made. ES measured the ability of protein to provide strength to the emulsion over period of time. The emulsion was made by using VFIs and commercial samples. The pH of the emulsion was not measured. As seen visually from Figure 4.6, VFIs and commercial samples were able to produce emulsions. The emulsifying properties of the samples were observed at three different times. The first observation was made after the emulsion was prepare, the second observation after 1 hour and the third observation after 1 week. After 1 hour the samples had separated into two layers, water and cream. The emulsion made from HP and FBPC started to flocculate after 1 week. From the emulsion prepared, all samples could produce emulsion except HP. Emulsion made with HP looked unstable at the very beginning where the emulsion had tiny bubbles on the top layer. Effects of pH and the temperature of the emulsion activity and emulsion stability of VFIs after 1 hour and 1 week are shown on Table 4:8. The interaction effect of pH and the temperature of VFI extraction was significant ($p<0.05$) on the emulsion capacity. VFI extracted at pH 7.6, room temperature (20°C) had the lowest emulsion capacity compared to the other three VFIs. Very few studies compare the emulsifying properties of legume protein processed at different extraction conditions and processing techniques. In the studies that were reported in Table 4.1, only Boye et al. (2010b) and Karaca et al. (2011) looked at the effect of processing (precipitation) on emulsifying

properties, yet the method used to measure the emulsifying properties is different which makes comparison of a results difficult. Boye et al. (2010b) reported that even though the method of preparation of the pulse proteins was different, being prepared by either isoelectric precipitation or by ultrafiltration, this had little impact on the emulsifying properties of pulse protein and no significant difference was observed. But differences were found for different types of pulse namely pea, chickpea and lentil. In contrast to study by Karaca et al. (2011), isolates prepared by isoelectric precipitation had higher emulsion capacity and emulsion activity . However, there is no similar study that investigated the effects of extraction pH and temperature on emulsifying properties.

Table 4:8: Effects of extraction pH and temperature on emulsifying activity of emulsion made from [VFI 7.6 6], [VFI 7.6 RT], [VFI 10.5 6], [VFI 10.5 RT]

Extraction conditions		Emulsion capacity (%)	Emulsifying stability after 1 hour (%)	Emulsifying stability (%) after a week
pH	*Temperature*			
7.6	6°C	94.3±0.3b	65.9±0.5a	65.9±0.5a
7.6	Room temperature	91.3±0.9a	75.9±0.9ab	70.7±0.7b
10.5	6°C	95.4±0.4b	71.8±0.9ab	65.5±0.3a
10.5	Room temperature	95.6±0.6b	82.±1.2b	81.0±0.6c

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using two-way ANOVA ($p<0.05$).

Emulsion capacity of VFIs were also compared with commercial proteins and soy lecithin (Table 4:9). Emulsion capacity ranged between 80 to 95% by the addition of 2% VFI or commercial samples. The emulsion capacity of VFIs were comparable with SPI, soy lecithin and HP, and the emulsion capacity FBPC and PPI were the lowest. Soy lecithin was used as a control and 95.9% of oil was emulsified by 2% of soy lecithin. Pea had the lowest emulsion capacity. After 1 hour, the stability of the emulsion capacity was reduced for all samples. Emulsion made from SPI was the most stable after 1 week followed by [VFI 10.5 RT], [VFI 7.6 RT], HP, [VFI 10.5 6], PPI, FBPC, [VFI 7.6 6] and soy lecithin. Previous studies used different units and indices to calculate the emulsion properties which makes comparison of results quite difficult.

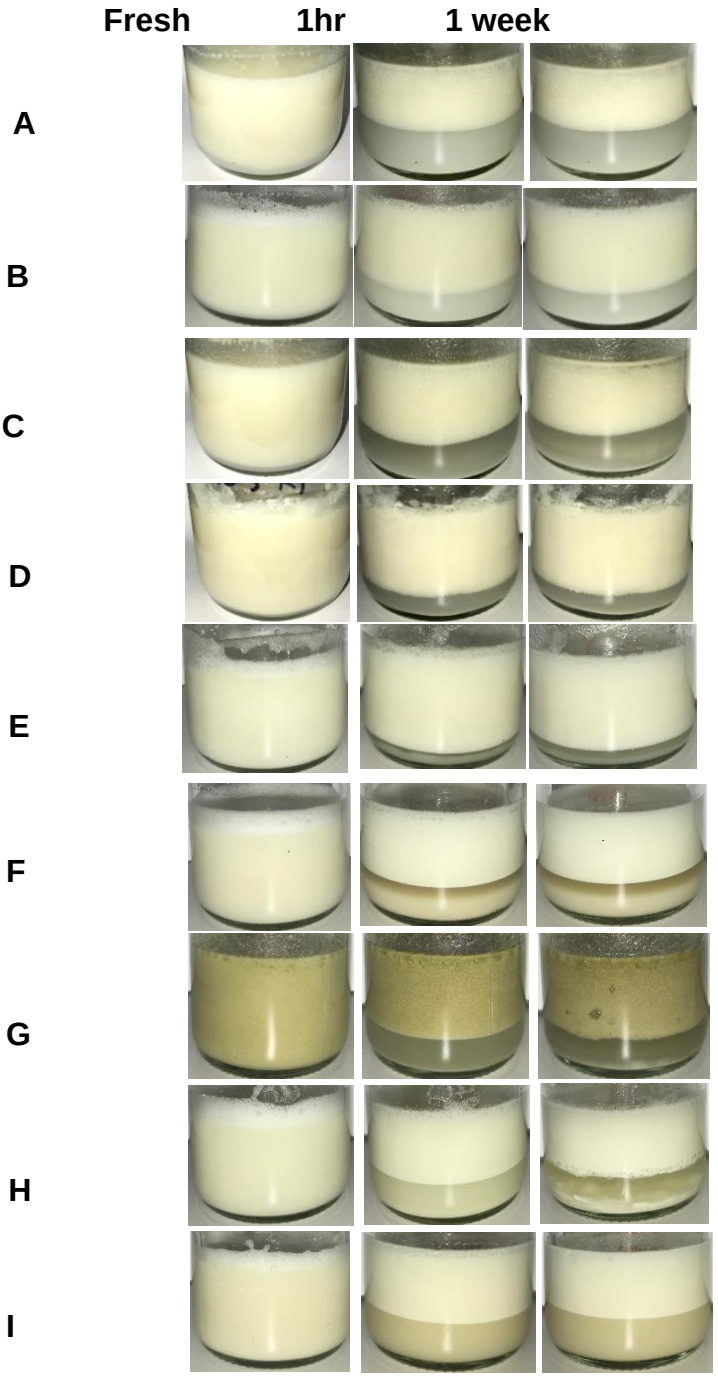

Figure 4.6: Emulsion made using A) [VFI 7.6 6], B) [VFI 7.6 RT], C) [VFI 10.5 6], D) [VFI 10.5 RT], E) SPI, F) PPI, G) HP H) FBPC, I) Soy lecithin (control) at (50/50 oil in water emulsion) fresh, after 1 hour and a week at room temperature.

Table 4:9: Emulsifying activity of emulsion made from [VFI 7.6 6], [VFI 7.6 RT], [VFI 10.5 6], [VFI 10.5 RT], SPI, PPI, HP and soy lecithin powder (positive control) and their stability after 1 hour and a week.

Samples	Emulsion capacity (%)	Emulsifying stability after 1 hour (%)	Emulsifying stability (%) after a week
[VFI 7.6 6]	94.3±0.3bc	65.9±0.5a	65.9±0.5ab
[VFI 7.6 RT]	91.3±0.9b	75.9±0.9c	70.7±0.7b
[VFI 10.5 6]	95.4±0.4bc	71.8±0.9bc	65.5±0.3ab
[VFI 10.5 RT]	95.6±0.6bc	82.0±1.2d	81.0±0.6c
SPI	95.1±1.1bc	91.8±0.9e	85.0±2.1c
PPI	80.9±0.9a	71.1±0.6abc	67.7±1.4ab
FBPC	84.5±1.1a	75.1±2.1c	70.0±2.3b
HP	96.7±1.7c	69.1±1.2ab	65.6±0.8ab
Soy lecithin	95.9±0.4c	67.7±0.4ab	64.0±0.2a

Mean values ± standard error for 3 technical replicates. *Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$).

Although protein concentration is important for emulsion formation, the type of protein is also important for reducing the interfacial tension and the formation of a protective layer around the oil droplet. EC and ES of the 7S protein fraction were higher than the 11S protein fraction due to a higher rate of diffusion to the interface and possibly due to disulphide bonding in the 11S legumin which inhibit unfolding and decreases the interactions at the oil/water interface (Zayas, 1997). The emulsion capacity of VFIs and FBPC as compared to SPI, PPI and HP were different could be due to the composition of 11S and 7S proteins which was observed qualitatively from SDS-PAGE (Chapter 3 Figure 3.7). Kimura et al. (2008) reported that French bean 7S

globulin exhibited excellent emulsion stability compared to 11S pea. Enhanced emulsifying properties at alkaline pH were observed in wheat, lupin, green pea, fava bean, hemp and buckwheat flour (Raikos et al., 2014). At higher pH levels, zeta potential (mV) increased with increasing solubility (Figure 4.3) due to higher surface charge. The more soluble the protein is, the better the ability of protein molecules to diffuse fast and adsorb at interface. Further, improved solubility at alkaline pH, dissociation and partial unfolding of globular proteins at high pH also improve emulsifying properties. This results in exposure of hydrophobic amino acid residues which then increase the surface activity and adsorption at the oil and water interface (Sánchez-Vioque et al., 1999). In the present study, the effects of pH on the emulsion were not studied. However, results from zeta potential demonstrated that by changing the pH of a solution, the EC can be improved. Based on the literature, the physicochemical properties of legumes, that is are molecular size, surface hydrophobicity, net charge, steric hindrance and molecular flexibility have been shown to influence emulsifying properties (Karaca et al., 2011). Karaca et al. (2011) observed that the emulsion capacity increased as the surface charge and solubility increased and surface hydrophobicity decreased. At alkaline extraction pH, the dissociation of globulins into their subunits, resulted in increased surface hydrophobicity due to the exposure of the originally buried hydrophobic side chain groups.

4.3.7 Foaming properties

Effects of extraction pH and temperature on the foaming properties of VFIs are presented in Table 4:10. Foaming capacity is the ability of proteins to adsorb at the air/water interface during whipping and foaming stability was the ability of the multilayer, cohesive film which surrounds air bubbles to resist liquid drainage and droplet coalescence (Raikos et al., 2014). Extraction pH and temperature of VFIs significantly affect foaming capacity ($p<0.05$). [VFI 10.5 RT] had a significantly higher foaming capacity followed by [VFI 7.6 6] and [VFI 7.6 RT] but [VFI 7.6 RT] had the highest foaming stability followed by [VFI 10.5 6] and [VFI 7.6 6] and [VFI 10.5 RT]. Previous studies by Raikos et al. (2014) on the foaming capacity of faba bean flour at pH 4 (40%), 7 (55%) and 10 (70%) and by Arogundade et al. (2006) on foaming capacity of broad bean protein concentrate were different compared to the value of foaming capacity of VFIs in this study. This could be due to different pH used during hydration and whipping, protein concentration and the method of measurement. However, the foaming stability value reported by Raikos et al. (2014) for all pH (below 10%) was comparable with VFI foaming stability in this study. Based on the literature, there were no similar studies to compare the effects of pH and temperature on the foaming properties of VFIs. However, Jarpa-Parra et al. (2014) observing the effect of extraction pH on the foaming properties of lentil protein isolate found no significant difference.

In comparison to commercial proteins in this study as presented on Table 4:11, PPI had the highest foaming capacity and stability followed by FBPC and SPI. HP and commercial whole egg powder did not form any foam due to the

presence of lipid. This finding is supported by Toews and Wang (2013) who reported that chickpea protein had the lowest foaming capacity and was improved when chickpea was defatted. Lipid is one example of foam inhibitors as it could rupture off protein film and lead to the collapse of bubbles (Zayas, 1997). In the case of whole egg powder, yolk phospholipid lecithin exhibits strong anti-foaming capacities. Positively charged lecithin can interact with negatively charged protein and disrupt the association of protein at the air/water interface. Foaming properties were influenced by protein solubility, pH of the medium, protein concentration, surface flexibility of proteins and surface hydrophobicity of proteins (Zayas, 1997). In this study, effects of the pH medium were not studied, however a significant correlation was established between protein solubility and foaming capacity of samples ($r= 0.8461$, p value=0.000004). PCA in Figure 4.7 also showed strong correlation between protein content, solubility and foaming capacity of samples. The highest protein solubility was observed in PPI followed by FBPC, SPI and VFIs and HP samples. Previous study on pea protein isolates also observed higher foaming capacity of its water soluble fraction due to its higher protein solubility, and this improvement of foam capacity was observed with increasing pH (Adebiyi and Aluko, 2011). Increased foaming capacity was observed in pigeon pea samples with highest protein content and with ionic strength of 0.5 compared to control (Akintayo et al., 1999). According to Kaur and Singh (2007), the higher the amount of protein in the sample, then more protein will be available to form foam. Another study by Arogundade et al. (2006) on the effect of ionic strength and pH on broad bean protein concentrate observed

high pH (pH 12) had high foaming capacity and low foaming capacity at pH near the isoelectric point due to the protein solubility factor. This may also indicates that foam capacity of VFIs can be improved by altering the pH of a medium. Solubility is important in foam formation as it enhances protein flexibility which facilitates unfolding and rapid diffusion of protein to form interfacial protein membranes at the air-water interface. This enhances the encapsulation of air bubbles, which are readily adsorbed at the air-water interface with hydrophilic groups oriented to the aqueous phase and hydrophobic groups oriented towards the air (Adebiyi and Aluko, 2011, Meng and Ma, 2002, Jarpa-Parra et al., 2014).

Generally, foam stability of all samples decreased with time. PPI and FBPC had the highest foaming stability at 120 mins followed by SPI, [VFI 7.6 RT] and [VFI 10.5 6] with an absence of foams in other samples. This finding is in agreement with Arogundade et al. (2006) who observed decreased foam stability of broad bean protein concentrate with time. Arogundade (2006) also noted however that foams prepared at pH 4 were the most consistent at all ionic strengths when compared to foam prepared at pH 2, 10 and 12. This suggests that foam stability of VFIs can be improved by increasing the ionic strength of the medium. The lower foam stability in all VFIs samples could be due to reduced or inadequate intermolecular interactions of electrostatic repulsions which leads to higher protein-protein interactions and foam collapse (Toews and Wang, 2013). Foam stability could also be influenced by extraction procedures. Additionally, foam stability is also controlled by a protein's structure which is influenced by pH and ionic strength of the medium

which then directs the charge density and electrostatic repulsion of protein (Adebiyi and Aluko, 2011, Chavan et al., 2001, Toews and Wang, 2013). Therefore, protein needs to have enough flexibility and sufficient charge density to maintain the foam.

Table 4:10: Effects of extraction pH and temperature on foaming capacity (FC) and foaming stability (FS) over time for *Vicia faba* isolates (VFIs).

Extraction conditions		Foaming capacity (%)	Foaming stability (%) over time				
			10min	***30min***	***60min***	***90min***	***120min***
pH	*Temperature*						
7.6	6°C	51.4±0.7b	8.3±0.1a	2.3±0.1a	-	-	-
7.6	Room temperature	22.8±1.4a	18.8±0.6b	13.3±0.7b	10.5±0.3a	10.3±0.3b	10.2±0.2b
10.5	6°C	60.0±1.2c	22.5±0.3c	17.5±0.2c	10.0±0.2a	5.0±0.2a	5.0±0.2a
10.5	Room temperature	67.9±0.7d	25.1±0.6d	2.1±0.1a	-	-	-

Mean values ± standard error for 3 technical replicates. *Data followed by different letters in a column are significantly different by multiple comparison test using two-way ANOVA ($p<0.05$)

Table 4:11: Foaming capacity (FC) and foaming stability (FS) of *Vicia faba* isolates (VFIs) and commercial samples.

Samples	Foaming capacity (%)	Foaming stability (%)				
		10min	***30min***	***60min***	***90min***	***120min***
[VFI 7.6 6]	51.4±0.7b	8.3±0.1a	2.3±0.1a	-	-	-
[VFI 7.6 RT]	22.8±1.4a	18.8±0.6b	13.3±0.7a	10.5±0.3a	10.3±0.3b	10.2±0.2b
[VFI 10.5 6]	60.0±1.2c	22.5±0.3bc	17.5±0.2ab	10±0.2a	5±0.2a	5±0.2a
[VFI 10.5 RT]	67.9±0.7d	25.1±0.6c	2.1±0.1a	-	-	-
SPI	107.0±0.7e	90.7±0.7d	86.7±0.7bc	56.1±0.7b	28.5±0.2c	24.2±0.3c
PPI	296.5±0.9g	284.3±2.1f	273.3±1.7d	262.4±1.2d	253.9±2.0e	253.3±2.0e
HP	-	-	-	-	-	-
FBPC	152.7±1.5f	142.2±1.2e	141.3±0.7c	142±1.0c	135.0±1.7d	133.3±1.7d
Whole egg powder	-	-	-	-	-	-

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$).

4.3.8 Gelling properties

A gel is a network between denatured molecules cross-linking to form aggregates containing large amounts of trapped water (Raikos et al., 2014). Heat induced gel was made using 12 or 20% of VFIs and commercial samples in the presence of 100mM NaCl regardless of pH. Table 4:12 visually shows gel made from all of the samples. All samples gelled at 12% and 20 % (w/v) except for PPI, HP and FBPC which produced paste rather than cohesive gels at 12% (w/v) but PPI gelled at 20% (w/v). Gel made at 12% and 20% (w/v) of [VFI 7.6 6] and [VFI 10.5 6] were white compared to [VFI 7.6 RT] and [VFI 10.5 RT] which were slightly brown but all had a smooth appearance. Gel made with 12% SPI was translucent compared to 20% (w/v). Gel made with 12% PPI was also comparable to [VFI 7.6 6], [VFI 10.5 6] and SPI gels. HP and FBPC did not form gel but rather formed a thick paste and as a result, there were no hardness values for HP and FBPC. The pH value and the temperature of extraction have a significant effect on heat-induced gel hardness which was measured using texture analyser. This information is presented on Table 4:12. [VFI 7.6 6] produced gel with the highest hardness for both 12% and 20% (w/v) followed by [VFI 10.5 6], [VFI 10.5 RT] and [VFI 7.6 RT]. The effect of pH and temperature on the hardness of gel was more pronounced with 20% (w/v). [VFI 7.6 RT] had the lowest gel strength for both 12% and 20% (w/v). [VFI 7.6 RT] had the highest protein content and lowest starch content compared to [VFI 7.6 6], [VFI 10.5 6] and [VFI 10.5 RT].

Table 4:12: Gel made using either 12% or 20% (w/v) of *Vicia faba* isolates (VFIs).

Extraction conditions		Percentage (%)	
pH	*Temperature*	*12*	*20*
7.6	6°C		
7.6	Room temperature		
10.5	6°C		

10.5	Room temperature		
	Commercial samples		
SPI	-		
PPI	-	Not gelled	
HP	-	(Produced paste) Not gelled	(Produce paste) Not gelled
FBPC	-	(Produced paste) Not gelled	(Produce paste) Not gelled

(n=3 biological replicates)

The effects of pH and the temperature of extraction on gel hardness are presented in Table 4:13 and the interaction effects were significant ($p<0.05$) for both 12 % and 20%. [VFI 7.6 6] had the highest gel hardness at both 12% and 20% followed by [VFI 10.5 6], [VFI 10.5 RT] and [VFI 7.6 RT]. Gel hardness increased as the concentration of the sample increased from 12 to 20%. There are no similar studies that look at the effects of extraction pH and temperature on the hardness of gel and the previously reported studies by Peyrano et al. (2016), Boye et al. (2010b) and Akintayo et al. (1999) measured gelling properties using least gelling concentration (LGC). Peyrano et al. (2016) studied LGC of cowpea isolate and observed no significant difference between isolates prepared at pH 8 and 10. This study did not report on gel hardness. However, the gel was characterised as a very firm gel based on LGC for both isolates as the sample concentration increased from 6 to 16% w/v which is in agreement with the present study. Boye et al. (2010b) also reported gelling properties based on LGC and found that isolates prepared by ultrafiltration has lower LGC (better gelling) compared to the ones produced using isoelectric precipitation. Similarly, samples with a higher protein concentration resulted in firmer gel, however results vary between different pulses. Another study by Akintayo et al. (1999) observed the effects of pH, ionic strength and salt concentration on gelation of pigeon pea protein concentrate using LGC. The study observed that gelling ability was improved at the presence of 0.25% NaCl indicated by a lower LGC. This could be due to higher protein solubility linked to the presence of salt. However, the gelling ability decreased at higher salt concentrations. This suggests that higher

concentration of salt could neutralise the charges stabilising the gel and therefore reduced the viscosity of gel (Akintayo et al., 1999). Similarly, higher ionic strength was found to decrease the gelling ability as it neutralised the charges stabilising the gel. The best LGC was found at 0.5 ionic strength at a concentration of 4% w/v and 0.1 ionic strength at a concentration of 8% w/v. As for pH, the study found that pH 2 and 6 enhanced the gelling ability while alkaline pH decreased the gelling ability. This previously reported study also concluded that NaCl, at a concentration above 0.5% w/v is able to neutralise the charge.

The gel hardness of VFIs were compared with commercial samples using one-way ANOVA presented in Table 4:14. The hardness of SPI gels, was lower than VFIs gels for both 12% and 20%. PPI gel produced at 20% (w/v) had the lowest hardness compared to all other samples. All VFIs had more starch content than protein compared to SPI and PPI suggesting that gelation is also due to other non-protein components such as polysaccharides and lipids. Previous study on wheat and buckwheat flour which contained the highest levels of carbohydrate also reported the lowest LGC which suggests better gelling capacity (Raikos et al., 2014). Gel made from fava bean flour at 14% (w/v) pH 4, 12% (w/v) pH 7 and 14% (w/v) pH 10 which composed of 30% protein and 63% carbohydrate were reported to have the lowest LGC (Raikos et al., 2014). Similarly, Adebiyi and Aluko (2011) reported that water soluble and salt soluble fractions of PPI were unable to produce a firm gel between 1% to 20% (w/v) which were composed of 86 and 80% protein content respectively. This indicates that, protein content is not the only determinant of

gel strength and the type of protein and non-protein components are also relevant. Also, processing techniques and environmental factors contribute to gelling properties. The gel produced by VFIs can be considered as two-component gel due to the mixture of starch and protein (Makri et al., 2006).

Table 4:13: Hardness (N) of gel containing 12% or 20% (w/v) *Vicia faba* isolates (VFIs) by compression test using texture analyser.

Extraction conditions		Hardness (N)	
pH	*Temperature*	12%	20%
7.6	6°C	*2.7152±0.0279c	5.3270±0.3835c
7.6	Room temperature	0.1546±0.0308a	0.7518±0.0732a
10.5	6°C	0.4438±0.0410b	2.400±0.2174b
10.5	Room temperature	0.2907±0.0281ab	2.8227±0.3826b

Mean values ± standard error for 3 technical replicates except * with 2 readings. Data followed by different letters in a column are significantly different by multiple comparison test using two-way ANOVA ($p<0.05$).

Table 4:14: Hardness (N) of gel containing 12% or 20% *Vicia faba* isolates (VFIs) and commercial samples.

Samples	Hardness (N)	
	12%	*20%*
[VFI 7.6 6]	2.5798±0.1363d	5.3270±0.3835c
[VFI 7.6 RT]	0.1546±0.0308ab	0.7518±0.0732a
[VFI 10.5 6]	0.4438±0.0410c	2.400±0.2174b
[VFI 10.5 RT]	0.2907±0.0281bc	2.8227±0.3826b
SPI	0.1623±0.0263ab	0.5099±0.0480a
PPI	-	0.0876±0.0105a
HP	-	-
FBPC	-	-

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$).

4.3.9 Principle component analysis (PCA)

Since VFIs were mixtures of protein, starch and fibre, and all of these compositions may influence the functional properties of VFIs, PCA of composition and functional properties was conducted (Figure 4.7).

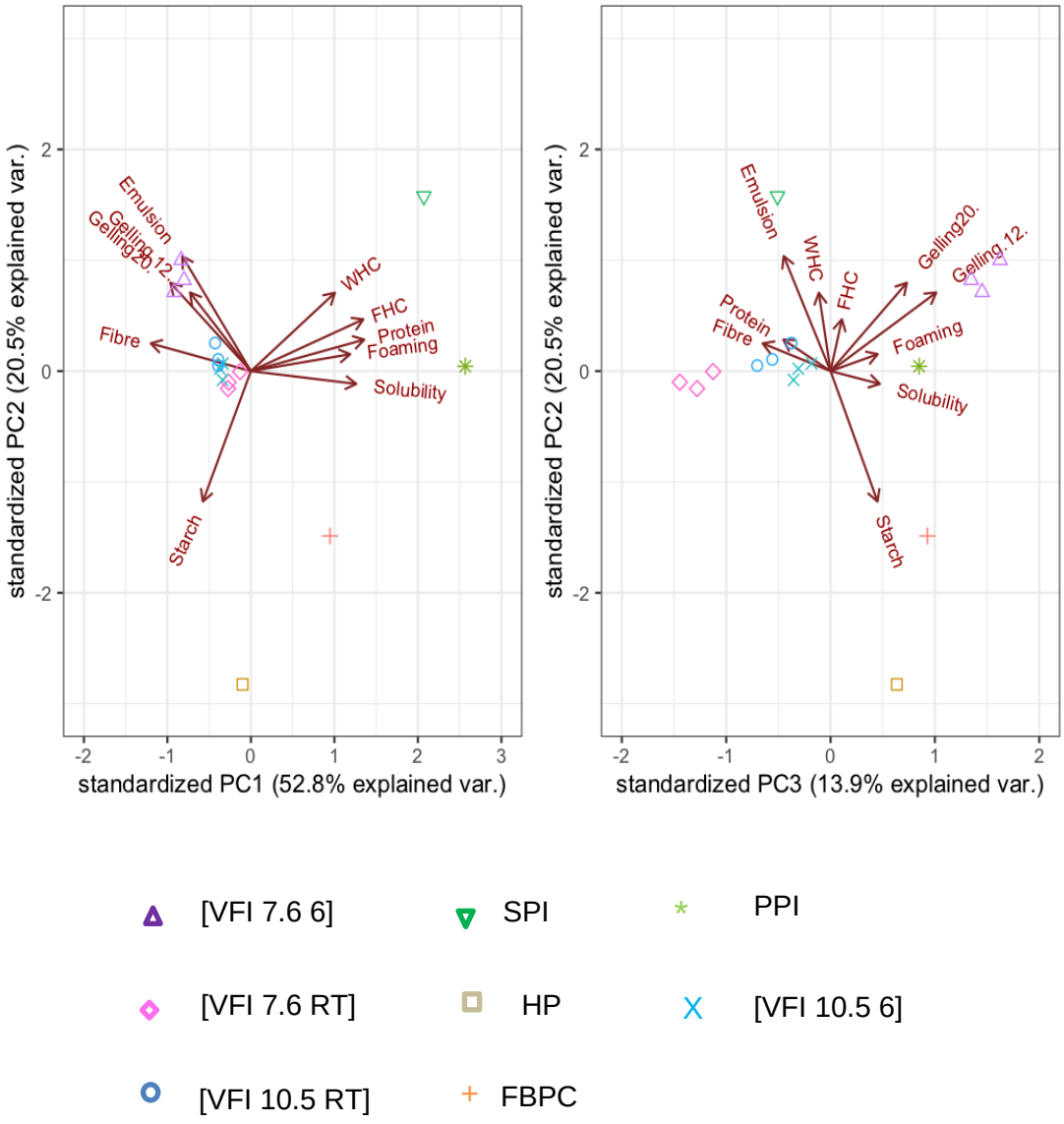

Figure 4.7: Principle component analysis (PCA) shows loadings of protein, fibre, starch, solubility, emulsion, foaming and gelling and scores for VFIs, SPI, PPI, HP, FBPC.

The selection of appropriate principle components was based on the rule of eigenvalues greater than 1.0, the scree plot and proportion of explained variance. The first three PCs which accounted for 87.1% of the explained variance could be used to show correlation between compositions of VFIs and commercial samples with solubility, water holding, fat holding, foaming, emulsion and gelling properties. Functional properties and compositions that were positioned beside each other on loading plots were highly correlated to each other. PC1 and PC2 showed higher correlation of compositions and functional properties compared to PC2 and PC3. According to PC1 and PC2, WHC, FHC and foaming were strongly correlated to protein content but not correlated to starch and fibre content. Gelling and emulsion properties were also strongly correlated which indicates that emulsifying and gelling properties plays major role in stabilising one another. PC2 and PC3 showed that protein, starch and all of the functional properties were in the same component and correlated with each other.

According to PC1 and PC2, VFIs were separated into three groups. However, PC2 and PC3 were able to separate VFIs into four groups. These were consistent with the results obtained for compositions and functional properties of VFIs where all these four isolates were different in terms of compositions and functional properties.

4.4 Conclusions

Experimental procedures, including how VFIs were prepared, the test conditions for functional properties (e.g. pH and concentration), compositions of VFIs and the presence of non-protein compounds were responsible for variations in the functional properties of VFIs. Environmental pH affected protein solubility and surface charge of VFIs and subsequently could improve emulsifying, foaming and gelling properties. [VFI 7.6 RT] (brown fraction) could be suitable for the preparation of food products that requires high water and fat absorption. Meanwhile, [VFI 7.6 6], [VFI 7.6 RT], [VFI 10.5 6] and [VFI 10.5 RT] have potential applications as emulsifying, foaming and gelling agents. VFIs possessed similar WHC and FHC except for [VFI 7.6 RT] (brown fraction). However different emulsion, foaming and gelling properties were observed in VFI samples. VFIs have potential as functional ingredients in replacing commonly used binders in meat products due to their WHC, FHC, emulsion and gelling properties.

Chapter 5
Application of *Vicia faba* isolates in beef patties

5.1 Introduction

The increased demand for the food industry to substitute meat with alternative sources of protein such as legumes is driven by cost, consumer health and environmental concerns. High levels of meat consumption have been shown to be detrimental to human health, high intakes being associated with raised cholesterol and a higher prevalence of obesity, cardiovascular disease and various forms of cancer. The negative health effects are attributed to meat's high saturated fat and cholesterol content (Rouhani et al., 2014). Meat production also contributes to higher greenhouse gas emissions compared to plant based foods (Alexander et al., 2017, Scarborough et al., 2014, Asgar et al., 2010). Livestock contributed to a quarter of all the protein and 15% of energy consumed during food production (Alexander et al., 2017). It also dominates agricultural land and use by area and is a major source of greenhouse gases. Beef contributed to 26.6 kg CO_2-eq/kg produce compared to legumes (Clune et al., 2017). However, meat is a good source of protein and other nutrients and, as such, its consumption has been rising in all countries, especially in developing countries such as Malaysia. Meat consumption is associated with nutrition transitions, especially in urban populations.

There has been an increasing interest in the partial replacement of meat with extenders/binders/fillers that not only minimizes the product cost but also

improve or at least maintain nutritional and sensory product quality (Modi et al., 2004). Red meat is also a suitable vehicle for further fortification with nutrients targeted at elderly people, such as protein. Addition of dietary proteins from sources such as pulses or legumes in fortified meat products could fulfil requirements to partially reduce meat consumption, in compliance with clean label requirements and could reduce calorific intake and reduce greenhouse gas emission effectively (Asgar et al., 2010, Baugreet et al., 2016).

According to reviews by Petracci et al. (2013) and Brewer (2012) extenders/binders/fillers can be sourced from plant hydrocolloids, or from animal by products (collagen derivatives, blood protein) milk proteins (whey proteins) and egg albumen. The functions of extenders/binders/fillers in meat products are water retention, fat emulsification, gelling, to form structure of meat products, increasing product yield and reducing formulation costs (Dzudie et al., 2002, Zayas, 1997, Devadason et al., 2010). The most common plant proteins that are used in meat products are derived from soybeans, wheat and pea. However, soybeans and pea are common source of allergens. Therefore, finding alternative extenders/binders/fillers from underutilised legumes could be useful. In the present study, we used the word binder instead of extender or filler because binder is defined as a substance with high-protein content that is able to bind fat and water.

Different sources of binders have different functionalities but in order to have a good criteria as a meat binder, the protein must be able to produce beef patties with good stability, palatability, textural properties, nutritive value

and a good yield. Previous studies have used extenders/binders from various plant sources in meat products such as patties (Park et al., 2016, Modi et al., 2004, Shariati-Ievari et al., 2016, Serdaroglu, 2006, Kilic et al., 2010, Elgasim and Al-Wesali, 2000, Sheridan and Shilton, 2002, Alakali et al., 2010, Holliday et al., 2011), meatballs (Serdaroglu et al., 2005, Kilic et al., 2010, Anderson and Berry, 2000), sausage (Dzudie et al., 2002, Holliday et al., 2011) and nugget (Devadason et al., 2010). These studies reported increase in yield and decrease in cooking loss, reduction in formulation costs and enhancement of the texture of meat products. As mentioned in previous chapter, VFIs displayed good protein profile as well as containing protein, starch and fibre (Table 3:10) and promising functional properties on WHC, FHC and heat induced gelling which can provide a particular role in maintaining the quality of beef patties. In this chapter [VFI 7.6 RT] was chosen to be added into beef patties because it has the highest protein content with lowest vicine and convicine as compared to the other three VFIs. To the best of our knowledge, there is no study on the application of *Vicia faba* isolates as extender/binders and meat replacers in beef patties. The percentage of 20% was chosen in the study based on the previous research which reported higher cooking loss for the addition of more than 20% soy protein. Besides, 20% was also a considerable amount that can be used to reduce the cost of the formulation of beef patties (Sulaiman et al., 2018).

5.2 Aim of the chapter

The aim of the work described in this chapter was to produce beef patties enriched with 20% of [VFI 7.6 RT]. Addition of 20% VFIs were also aimed to bind beef mince, replace commonly used binders such as egg or wheat flour to improve the quality of beef patties and reduce the cost of production. Quality characteristics of VFIs-enriched beef patties were compared with beef patties made with 100% beef mince and 20% enriched with SPI, PPI, HP, FBPC, wheat flour and whole egg powder. The objectives of the study were to evaluate the effect of substitution of VFIs and commercial protein products on the cooking losses, product yield, drip loss and textural properties of beef patties.

5.3 Results and Discussion

5.3.1 Effects of adding 20% of *Vicia faba* isolates on the processing of beef patties and effects on visual quality

Eight beef patties were made using beef mince (15% fat) where one of them is a control which made with 100% beef mince. The other seven were replaced with 20% [VFI 7.6 RT], SPI, PPI, HP, FBPC, wheat flour and whole egg powder which were used as binders presented in Figure 5.1. Beef patties were blended with 20% binders using a food processor for 5 minutes. When blending with beef patties, some binders were difficult to handle as it became wet and stuck to the blades and walls of the food processor. This was observed for beef patties enriched with FBPC and whole egg powder as the shapes were not round compared to other beef patties. Colour of beef patties were

also different based on the binders used. The colour of beef patties enriched with VFIs were comparable to the control, and to beef patties enriched with SPI, PPI and wheat flour. Beef patties enriched with HP had the darkest colour due to the colour of the protein powder which is green. The colour of beef patties enriched with FBPC and whole egg powder were also comparable. All beef patties retained their round shape after frying except for beef patties made with FBPC and whole egg powder.

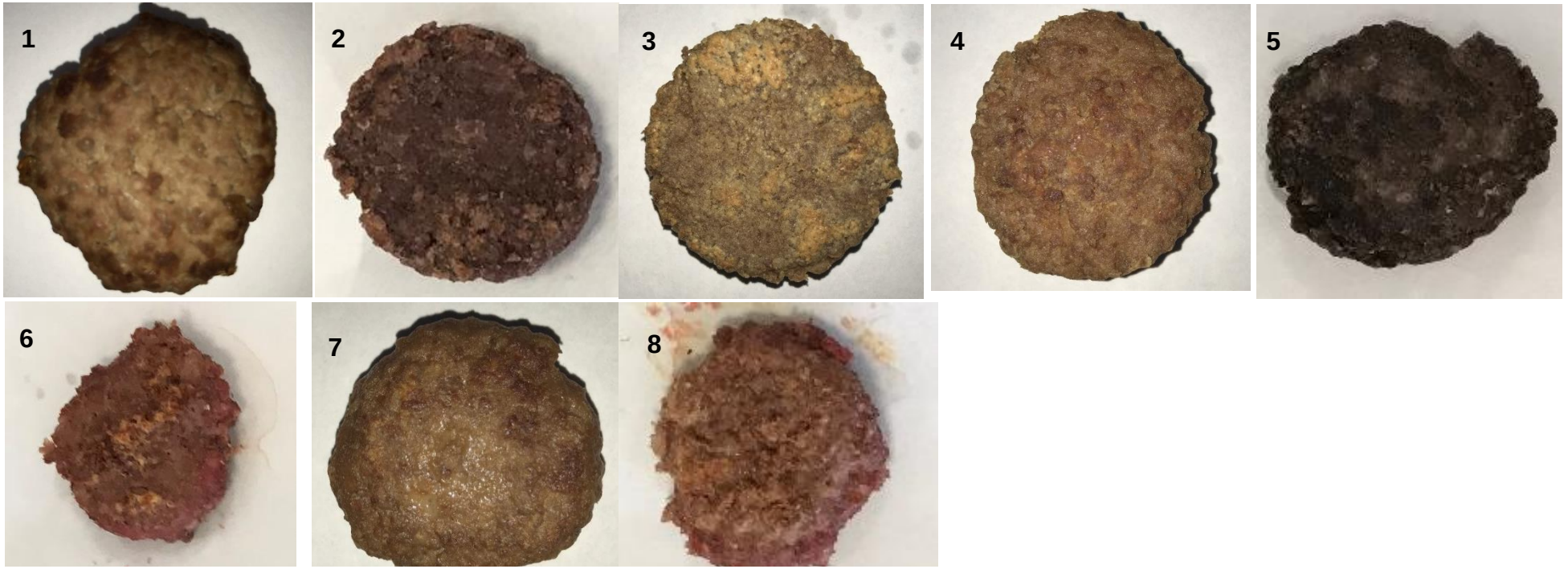

Figure 5.1: 1) Control beef patties (No binder), beef patties made with 20% 2) [VFI 7.6 RT], 3) SPI, 4) PPI, 5) HP, 6) FBPC, 7) Wheat flour and 8) Whole egg powder after frying.

The effects of adding different binder on the cooking quality of beef patties were studied. Table 5.1 shows cooking loss on beef patties enriched with binders were improved except for FBPC and whole egg powder. Beef patties made with VFI, SPI, PPI, HP and wheat flour had significantly lower cooking loss ($p<0.05$) compared to 100% beef patties. Sheridan and Shilton (2002) reported that cooking loss is due to the loss of moisture and fat. The improvement in cooking loss is due to the addition of binders that is related with fat and water holding capacity (Alakali et al., 2010). In VFI, protein, starch and fibre present could increase water retention in beef patties and prevent moisture migrating from the patties (Alakali et al., 2010, Petracci et al., 2013).

Table 5:1: Beef patties cooking quality made with different binders.

Binders	Cooking loss (%)	Product yield (%)	Drip loss (%)	Moisture content (%)
No binder	17.66±1.36b	82.34±1.36b	0.29±0.04a	57.98±0.65d
VFI 7.6 RT	8.97±0.80a	91.03±0.80c	0.14±0.01a	54.63±0.48bc
SPI	9.56±0.21a	90.44±0.21c	0.37±0.09a	53.89±0.32ab
PPI	10.83±0.45a	90.93±0.45c	0.27±0.07a	55.18±0.36bc
HP	10.83±0.53a	89.17±0.53c	0.33±0.03a	55.37±0.38bc
FBPC	30.71±1.76c	69.29±1.76a	0.96±0.09b	56.79±0.56cd
Wheat flour	6.25±0.41a	93.75±0.41c	0.13±0.03a	55.93±0.19bcd
Whole egg powder	26.74±0.90c	73.26±0.90a	0.97±0.12b	52.02±0.44a

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$).

The results are in agreement with (Kilic et al., 2010) which reported significantly lower cooking loss of beef meatballs with the addition of 10 and 20% of soy protein. According to the review by Brewer (2012) and a study by Dignam et al. (1979) and Kilic et al. (2010) up to 20% textured soy protein can be incorporated into ground beef to decrease cooking loss and evaporative loss but the addition may have an effect on sensory characteristics. The amount of 20% was chosen in this study was based on the previous research which reported higher cooking loss for the addition of more than 20% soy protein. Besides, 20% was also a considerable amount that could be used to reduce the cost of beef patties. The cost of a beef patty with added VFI in this study was reduced by 17% compared to 100% beef patties and partially replacing meat in making beef patties while still maintaining other sensory characteristics.

The yield of beef patties after frying were the lowest in beef patties made with FBPC, followed by whole egg powder and 100% beef patties. Beef patties made with VFI, SPI, PPI, HP and wheat flour had significantly higher product yield ($p<0.05$) compared to 100% beef patties which suggests that binders improved the emulsion of the beef patties. Results are in agreement with Modi et al. (2004) which looked at the quality of buffalo beef burger when legume flour was added where all burger had 90% yield with the addition of 8% soya, bengal gram, green gram and black gram flour. This shows that VFI with mixtures of protein, starch and fibre produced stable meat emulsions and improved emulsification, water and fat binding and gelation of beef mince compared to beef patties made without binder. Protein and starch in VFI

incorporated into cooked beef patties can be considered as heat-induced gels (Zayas, 1997). Interaction between faba bean protein and myofibrillar meat proteins occurred during cooking and this interaction encourages the formation of a gel matrix which improves the quality characteristics of meat products (Kilic et al., 2010). Meanwhile, starch in VFI will gelatinise during cooking, forming mixed protein/starch gels in the presence of bean and meat proteins. The lowest product yield found in beef patties enriched with FBPC and whole egg powder can be related to wet batter produced when binders were mixed with beef mince. In Chapter 4, Table 4:3, it was observed that FBPC had the highest solubility which could also explain the stickiness and wetness of the batter when FBPC was mixed with beef patties. Drip loss was the highest observed in beef patties made from whole egg powder followed by FBPC, PPI, no binder, HP, SPI, [VFI 7.6 RT], and wheat flour. Drip loss is the loss of moisture after beef patties were fried and with the addition of a binder, drip loss was significantly reduced except when FBPC and whole egg powder were used. In this study, moisture and fat content of raw beef mince was 65% and 15% respectively. After frying, 100% beef mince was found to have the highest moisture content ($p<0.05$) compared to beef patties enriched with binders. VFI added beef patties has significantly lower moisture content compared to 100% beef patties. This is probably due to the increased in the total solid content of beef patties enriched with binders. This finding supports Elgaism and Al-Wesali (200)is who reported decreased in the moisture content of cooked beef patties with the addition of soy protein and Samh flour. Meanwhile, the study by Alakali et al. (2010) reported an increase in the

moisture content of beef patties with the addition of Bambara groundnut seed flour. Serdaroglu (2006) also reported increased moisture content with the addition oat flour in cooked beef patties. The differences observed compared to the present study could be due to the composition of binders. During cooking, the loss of moisture from beef patties was through drip and evaporation (Sheridan and Shilton, 2002).

5.3.2 Textural properties of beef patties

Texture is the most important sensory property in meat especially in minced meat products such as burgers which refer to the evenness, consistency and uniformity of raw and cooked product. Hardness is one of the common textural properties of beef patties and the texture is evaluated based on the binding ability of meat particles and elasticity and hardness of the final product (Shariati-Ievari et al., 2016). The hardness of cooked beef patties presented in Table 5.2 is the instrumental hardness. Different binders have significant effects on the hardness of cooked beef patties ($p<0.05$). VFI, FBPC, HP, wheat flour and whole egg powder enriched beef patties had hardness lower than no binder beef patties. These results suggest that the addition of a binder meant that more fat and moisture were retained in beef patties. This could be due to higher fibre content in VFI and FBPC compared to SPI and PPI which contributed to the tenderness of cooked beef patties. In the study by Shariati-Ievari et al. (2016), the shear force of a burger containing chickpea and green lentil flour decreased significantly compared to the control. Beef patties incorporated with pea fibre have been reported to improve tenderness and cooking loss compared to the 100% beef patty control (Anderson and

Berry, 2000). Similar findings from Holliday et al. (2011) reported that incorporation of green lentil flour at the 42.5 and 50% levels in formulations of beef burgers containing 20% fat resulted in more tender cooked burgers compared to 100% meat. This suggests that [VFI 7.6 RT] can potentially replace egg and other commonly used binders in beef patties. Reducing the amount of meat is also successful as the quality of cooking and texture were comparable to the addition of [VFI 7.6 RT]. Hardness of beef patties made with [VFI 7.6 RT] was lower than with no binder. This finding is in agreement with study by Park et al. (2016) which observed reduced value of hardness when buckwheat and fermented buckwheat were added into pork patties as compared to the control.

Table 5:2: Textural property (hardness) of cooked beef patties enriched with 20% binders and without binder

Binders	Hardness
No binder	41.05±1.62c
VFI 7.6 RT	35.68±3.92bc
SPI	69.59±4.50d
PPI	69.27±4.84d
HP	21.71±0.37ab
FBPC	19.29±3.87a
Wheat flour	19.38±1.77ab
Whole egg powder	23.36±3.01ab

Mean values ± standard error for 3 technical replicates. Data followed by different letters in a column are significantly different by multiple comparison test using one-way ANOVA ($p<0.05$).

SPI and PPI had significantly higher hardness compared to the control (no binder). These two proteins had higher protein and lower fibre content than the other legume isolate, as observed in Chapter 3 Table 3:11. The toughness or hardness of ground beef has shown to be related to the degree of extraction in myofibrillar protein, stromal protein content, degree of comminution and type and level of non-meat proteins (Dzudie et al., 2002). This finding is also supported by (Brown and Zayas, 1990) who reported the addition of corn germ protein flour (CGPF) give the diluting effects on these proteins. In this study, it has to be noted that the addition of 20% binders from different samples contained various levels of protein, starch and fibre. Thus, in order to compare which composition contributes to the stable emulsion of beef patties, the comparison needs to be made between the protein, starch and fibre basis.

5.4 Conclusion

All cooking quality characteristics of beef patties were improved when 20% VFI was added when compared to 100% beef patty control, with the exception of mositure content. The cooking quality of beef patties made with VFI was also improved compared to whole egg powder. The instrumental textural property of VFI and whole egg powder beef patties were comparable. In conclusion, beef patties enriched with [VFI 7.6 RT] can partially replace meat and reduced the formulation cost as well as having binding properties.

Chapter 6
General Discussion, Future Work and Conclusion

6.1 General discussion

There are three main drivers for utilising legumes in a way that reduces meat consumption:1) cost, 2) health, 3) sustainability.

1) Cost

Adding legumes to meat products could reduce the cost of protein rich food. In this study, the cost of patty was reduced by 17% when 20% of VFI was added to beef mince as compared to 100% beef mince when only the cost of beef mince was evaluated However, when the cost was estimated based on the price of VFI and beef mince, beef patty with 20% added VFI is 11% cheaper than 100% beef mince. The cost of beef mince used in this study was £5/kg and VFI was £1.78/kg. The cost of VFI was evaluated based on raw materials which were *Vicia faba* and sodium sulphite. Beef patties in this study were made with either 100% beef mince or with 20% added VFI. For commercial purposes, other ingredients costs, such as salt, pepper, spices and preservatives, need to be taken into considerations in the formulation of beef patties.

Legumes are cheap to produce in a range of climatic and agricultural conditions, and are available most of the year around. Therefore, they are reliable sources of ingredients that could provide a suitable protein alternative to other more utilised protein sources, including cereals and animal produce.

2) Health

The health benefits associated with utilising legumes as a protein ingredient in meat products is two-fold. Firstly, VFI contains plant protein and some additional nutrients such as starch and fibre. The consumption of legumes is associated with health beneficial outcomes on diseases such as cardiovascular disease, diabetes and the management of obesity (Rebello et al., 2014). Legume consumption was shown to be inversely associated with cardiovascular mortality, non-cardiovascular mortality and total mortality (Miller et al., 2017). While *Vicia faba* is not an intact legume, it contains some of the components associated with health such as protein and fibre. The removal of anti-nutritional factors will make legumes suitable to be used as food ingredients, since there is a 5% prevalence of favism in the population. However, bioinformatics amino acid analysis of VFI indicated low levels of the indispensable amino acids methionine and tryptophan compared to animal proteins such as beta lactoglobulin and bovine serum albumin as presented in Table 3:7. Thus, complementing *Vicia faba* with other sources of dietary protein is needed to ensure amino acid sufficiency. Mixing meat proteins with legume proteins would provide a complete protein source.

The second benefit is associated with the reduction of meat intake. Replacing 20% of meat with legume protein could have an impact on health, as epidemiological studies have shown that high red meat intake is associated with increased risk of colorectal, colon and rectal cancer (Chan et al., 2011). It is suggested that an increased intake of 50 g/day for processed meat and 100 g/day of unprocessed meat increased the relative risk of colorectal cancer

by 18% and 17% respectively (Rohrmann and Linseisen, 2016). The positive association between processed meat consumption and risk of cardiovascular disease particularly myocardial infarction is also reported. It is estimated that the reduction of meat intake to less than 20g/day could reduce the total mortality by 3.3% (Rohrmann and Linseisen, 2016). Due to the high saturated fat and cholesterol content in meat, meat consumption is also related to an increased risk of obesity (Rouhani et al., 2014). In developing countries like Malaysia, a decreasing trend in cereal consumption has been observed between 1961 to 1997 where the calories obtained from cereals have reduced from 61% to 41%, meanwhile, calories intake from meat, eggs and fish have increased from 6.2% to 14.3% (Noor, 2002). According to Mohamad Hasnan A (2015) about 75% of Malaysian adults failed to meet the recommended intake of legumes. This study also reported that legumes and nuts are the food group least likely to be consumed at or above the level of the daily recommended intake followed by vegetables and milk and dairy products. On the other hand, three food groups that are consumed over the recommended intake are: 1) fish, 2) fruits and 3) meat, poultry and egg. Miller et al. (2017) also reported that the mean intake of fruits, vegetables and legumes in Malaysia were about 3.8 servings per day which were below the dietary recommendations. This trend in food consumption may be due to the nutrition transition that Malaysia and other developing countries are experiencing as a result of rapid economic development and urbanisation. According to Mohamed et al. (2017), the current food consumption trend among Malaysians is associated with high cholesterol and calories and accordingly the population

has turned away from plant based food. This unbalanced diet has been associated with overweight and obesity which are the risk factors for diabetes and cardiovascular diseases. In 2013, the prevalence of being overweight or obese are 43.8% and 11.8% for men, 18.6% and 16.7% for women, 22.5 % and 8.8 % for boys and 19.1% and 7.2% for girls respectively (Ng et al., 2014). In Malaysia, meat substitute products have been on the market for a while, yet due to low sensorial characteristics associated with it, consumers avoid these foods (Mohamed et al., 2017). Thus, one of potential way to increase legume intake is by having a mixture of plant and animal proteins in processed foods, in the sense that the animal ingredients can provide specific sensory characteristics and the plant proteins can reduce the content of the undesirable components such as fat as shown in the present study. Blending beef with legume protein is one alternative to reduce the calorie intake and improved the total protein and dietary fibre. Table 6:1 shows the calculated protein and fibre content of beef patties and product yield (Sulaiman et al., 2018). This shows that replacing meat with legume proteins is estimated to have an impact on health. According to the healthy reference diet, the recommended intake of legumes is 50 g/day, as an alternative protein source to reduce red meat and beef or lamb intake to 7g/day (Willett et al., 2019). VFI can be used as one of the legume sources that can potentially meet the legume intake requirement. Approximately, 117 grams of VFI per day is required to meet the requirement intake. For example, Tesco finest beef burger UK contains 426 grams of beef. Therefore, if 20% of the original

formulation is replaced by VFI, the recommended legume intake has been met up to 72%.

Table 6:1: Theoretical value of protein and fibre content in beef patties and product yield

Samples	Protein (%)	Fibre (%)	Product yield
Beef patty	19.7	0.0	82.3
Beef +VFI patty	24.3	3.5	91.0

Most plant-based burgers available in the market currently contain texturized soy protein, rehydrated egg and wheat flour. These ingredients restrict consumers that are allergic to soy, egg and wheat, as well as vegan consumers from consuming this type of plant-based burger. Thus, VFI could be a potential source of ingredients for producing new plant-only-based foods that cater for people with allergen restrictions and/or vegans. However, the nutritional and allergenic properties of *Vicia faba* proteins should be investigated further.

3) Environment

Again, the benefit of utilising legumes is two-fold. Legumes grow well in most climates and are useful rotation crops that add nitrogen to soils. The environmental impact of food production cannot be reduced to zero, indeed soy production has been linked to deforestation and increased water use. However, legume production has less impact than meat production, in terms of energy utilisation and greenhouse gas emissions. The energy utilisation of meat is about 1500 kJ/28 g serving, meanwhile legume is about 125 kJ/ 28 g serving (Willett et al., 2019). Beef produces higher greenhouse gas emissions,

contributing 16.6 kg CO_2/kg of produce compared to legumes and pulses at 0.51 kg CO_2/kg (Clune et al., 2017).

As meat contributes to the highest energy consumption and greenhouse gas emissions, meat eaters are recommended to reduce the amount of beef and lamb they consume to just 7 g/day and increased legume intake to 50 g/day (Willett et al., 2019). VFI could play a role in helping the environment by adding it into meat products. Theoretically, reducing beef mince in beef patties by adding 20% VFI would reduce greenhouse gas emissions from 26.6 kg CO_2/kg to 22.3 kg CO_2/kg of produce(Clune et al., 2017, Sulaiman et al., 2018). This shows that replacing meat with legume proteins is estimated to have an impact on the environment.

6.1.1 Outcome of the extraction of *Vicia faba* isolates

Extraction is one of the economical process to concentrate the protein and reduce the anti-nutritional compounds in *Vicia faba*. Several studies that evaluate the extraction of protein extracted from legumes have focused on the protein content and functional properties of the protein extracted. Yet, studies focusing on the yield, cost, sustainability and application of utilising the end product are scarce. As mentioned in Chapter 3, the yield of extraction seems to be neglected by most studies when extracting protein. Studies by (Karaca et al., 2011, McCurdy and Knipfel, 1990, Vioque et al., 2012, Nivala et al., 2017) successfully produced isolates with up to 80% protein content, but the yield was not reported. In this study, the total yield of isolates obtained ranged between 26 to 30% leaving 70% solid residue after extraction. Though the isolates produced were lower than the waste, it could be considered as a

reasonable amount in this study as only 20% of VFI was used in the preparation of beef patties and the aim was not to achieve a pure form of protein isolate. But, in other approaches such as extraction on the industrial scale where higher amounts and pure forms of product are needed for application purposes, sequential extraction of the residues can be done to increase the total yield of isolates because there was still about 60% protein remaining in the solid residue. Besides, the residues can be regenerated into different products that contains high fibre like Okara, a regenerated waste produced from the production of the soy milk manufacturing industry. A study by Vioque et al. (2012) reported that *Vicia faba* residues had a high content of fibre (57%) but there is no further study on the application. Therefore, the residue may be utilised as an ingredient in a variety of foods as fibre has binding and gelling functionalities, and also to increase the fibre content of the food. To date, there is still no study on the application of the *Vicia faba* protein isolate in any food products including beef patties. Extensive studies on the functional properties of proteins from other legumes especially soy and pea provided information on how *Vicia faba* proteins were expected to behave, but, these studies were not comparable because soy has no starch and pea has green pigments that makes all of them different. Overall, literature data on the functional properties of legume proteins are contradictory due to the diversity of methods and pre-treatments employed, as well as the large variety of products investigated.

Based on this study, the extraction condition that is recommended for upscaling is 7.6 at room temperature because it produces isolates with the

highest protein and fibre content and lowest vicine and convicine content and with considerable amount of total yield. In addition, this condition can be considered the cheapest compared to the other three combinations due to not needing cooling or heating. Moreover, less NaOH is needed as compared to higher extraction pH. In this case, this extraction condition would be useful for the scaling up process.

Based on all the results, extraction conditions had different effects on different functional properties. Thus, there is no clear conclusion as to which extraction conditions had the best properties for all functional properties. Most studies have also reported that the effects of environmental factors especially pH can improve the functional properties of proteins. pH influences the solubility, surface charge and other properties of proteins at a molecular level. Thus, it is decided that the best extraction conditions will be based on the proximate composition (highest protein content), protein quality and level of vicine and convicine.

The functional properties of solubility, WHC, FHC, emulsifying, foaming and gelling were observed in [VFI 7.6 RT]. Although, some functional properties like solubility, WHC, FHC and foaming properties were lower compared to SPI. Solubility is highly dependent on the pH of the medium. SPI had higher solubility compared to [VFI 7.6 RT] which could be due to the pH of the medium. In this study the pH of the solution was not controlled. However, [VFI 7.6 RT] had a lower pH compared to SPI when it was dissolved in water. Besides pH, processing of [VFI 7.6 RT] could denature the proteins which exposes the hydrophobic amino acids and thus reducing the solubility of [VFI

7.6 RT] in water. Precipitation to isoelectric point also contributed to the lower solubility. Protein solubility was also influenced by the secondary structure of protein. The increase in unordered structure with decrease in β-strand has been reported to increase protein solubility (Mune Mune et al., 2018). The results from the secondary structure in this study indicates very little difference in exposed and buried coil and beta strand structure between [VFI 7.6 RT] and SPI. Thus, the predicted secondary structure is not very useful in explaining the solubility and a further instrumental study might give more insight in the secondary structure of the proteins. Moreover, both of these isolates are mixtures of proteins and purification could be useful in explaining which proteins contributes to solubility with the knowledge of secondary protein structure. Gel hardness and emulsifying properties and hardness of gel for [VFI 7.6 RT] and SPI were comparable. The difference in the functional properties of [VFI 7.6 RT] and SPI is due to several factors. Firstly, [VFI 7.6 RT] is composed of protein and non-protein materials which contribute to the functional properties. The results indicated that WHC and FHC of [VFI 7.6 RT] and SPI were influenced by the amount of protein content (Chapter 4). Besides protein content, the availability of polar and non-polar amino acids in isolates also contributed to WHC. The results obtained from the secondary structure predicted using bioinformatics tools and Table 3:9 indicated that SPI had higher polar amino acids exposed compared to [VFI 7.6 RT]. The higher polar amino acids exposed for protein-water interaction might be the reason for the superior WHC obtained in WHC. Presence of starch in [VFI 7.6 RT] could also be the reason for the lower WHC compared to SPI because starch

is insoluble in water and sufficient heating is required for starch to contribute to WHC in [VFI 7.6 RT]. The fractionation of [VFI 7.6 RT] into brown and white fractions indicated that WHC improved in the brown fraction. Therefore, purification could be advantageous in increasing WHC. FHC also were influenced by protein content. Higher amounts of non-polar amino acids in SPI were observed from the secondary structure shown on Table 3:9 when compared to [VFI 7.6 RT] which reflects the high FHC in SPI. Similarly, fractionation into brown fraction increased FHC in [VFI 7.6 RT]. FHC is also said to increase with increasing beta sheet content and decrease with an increasing alpha helix or loop (Mune Mune et al., 2018). The results from the secondary structure indicates that [VFI 7.6 RT] had slightly more beta strands either exposed or buried, lower coil structure and higher alpha helix compared to SPI which is contradictory to the literature. Thus, to provide a better estimation secondary structure analysis need to be performed.

The emulsion capacity of [VFI 7.6 RT] and SPI were slightly lower but comparable, but the emulsifying stability of SPI was higher than [VFI 7.6 RT]. Emulsifying properties were influenced by solubility, surface charge and surface hydrophobicity of protein (Schwenke, 2001). Surface charge was measured using zeta-potential. The linear relationship of surface charge with solubility were observed as presented in Figure 4.3. A stable emulsion needs to have a surface charge above 30. Thus, the higher stability of emulsion made with SPI could be due to a higher surface charge compared to [VFI 7.6 RT]. Beta turn secondary structure also influenced emulsion stability together with solubility and surface hydrophobicity. Emulsion stability increases with

increasing solubility at high proportions of beta turn secondary structure and decreases with increasing solubility at low beta turn structure (Mune Mune et al., 2018). The limitation of the data from predicted secondary structure could not explain the relationship between the structure and emulsion stability. SPI also had higher foaming properties compared to [VFI 7.6 RT]. The higher solubility and protein concentration of SPI could be responsible for higher foaming properties compared to [VFI 7.6 RT] which were observed in this study. In addition to this, foaming capacity is also influenced by the unordered structure proportion. The increase in foaming is observed as the solubility increased at high unordered structure proportion and decreased with increasing solubility at low unordered structure proportion (Mune Mune et al., 2018). Surface hydrophobicity is not favourable to foaming and a similarly increased beta turn is favourable to foaming ability (Mune Mune et al., 2018). Therefore, higher foaming properties of SPI compared to [VFI 7.6 RT] could be due to several other factors besides being more soluble. In gelling properties, the hardness of gel was comparable with [VFI 7.6 RT] and SPI gel even though the compositions of both isolates were different. This indicates that the presence of several other components in [VFI 7.6 RT], fibre and starch contributed to the gel strength. Since [VFI 7.6 RT] gel consisted of starch, retrogradation is expected to happen during storage. The water excluded from the gel during freeze and thaw is normally used to see the extent of the retrogradation. The amount of water loss is due to the increased intermolecular and intramolecular hydrogen bonding between starch chains during freezing (Hoover et al., 1997). The differences in functional properties between [VFI 7.6

RT] and SPI could also be due to their different protein profiles and the amount of 11S and 7S proteins which observed qualitatively from SDS-PAGE as presented in Figure 3.7.

The findings on the functional properties of [VFI 7.6 RT] have shown that it has potential for application in food products. This also indicates that, the end product does not necessarily require pure proteins to have functional properties (Sathe, 2002). However, once again, it all comes back to the purpose of the end used. The importance of processing legumes using food grade extraction is important to produce VFI so that it can be used in food products. With the extensive studies on the extraction of legumes, one can decide to choose the best method of processing by considering the application purpose.

6.1.2 Partially replacing meat in beef patties

All the properties observed in [VFI 7.6 RT] particularly WHC, FHC, emulsion and gelling showed this isolate is useful in reducing meat content in the process of making beef patties. The ability to bind water and fat, emulsified and produced gel when heated are useful properties to stabilise meat emulsion. The results indicates that by adding 20% [VFI 7.6 RT] into the final weight of beef mince, cooking loss, product yield and drip loss were improved as compared to 100% beef patties. The properties of [VFI 7.6 RT] in beef patties were also comparable with SPI. Hardness of beef patties were also comparable with 100% beef patties which suggests the textural properties of beef patties was not affected by reducing the percentage of meat. This is because apart from protein, starch and fibre helps this isolate bind water and

fat in beef patties during cooking contributing to the texture of beef patties (Anderson and Berry, 2000).

6.1.3 Sensory and consumer evaluation of beef patties

The sensory study is useful to evaluate consumer acceptability of beef patties formulated in this study. The acceptability test can be used to score the appearance, flavour, firmness, chewiness, taste and colour and odour of beef patties made with VFI. The acceptability tests use a 9-point hedonic scale to assess the degree of liking for each of the product's attributes. The number of participants for the acceptability test ranged between 25 up to a maximum of 75 depending on the number of samples tested to complete balanced block design (Stone et al., 2012). Participants were normally aged 18 years old and above and generally in good health. Exclusion criteria needed to be applied to those who have an allergy to any type of food, pregnant or lactating mothers, and also in this study, those who are vegetarians or vegans and suffer from favism. Sensory study is also useful because the sensory properties can be closely related to instrumental texture analysis of beef patties (Park et al., 2016). For example, the hardness of beef patties tested using texture analyser in this study can be compared with a firmness score if a sensory study is carried out.

6.1.4 Limitations of the research

In the present study, due to time limitations, commercially available plant protein samples were used to compare with VFIs. The comparison of functional properties is limited because the method of processing and the varieties of the samples are not known. Besides, the full cost evaluation on the production of VFIs and beef patties including equipment, utilities and environmental impact were not calculated. Also, sensory study was not tested because of time limitations and there is no access to larger scale equipment to produce isolates for at least 60 participants as was needed here.

Nutritional quality of raw and cooked 100% beef patties and beef patties added with 20% binders (VFI and commercial protein isolates) were also not tested in this study. Macronutrient values which are protein, fibre, fat, carbohydrate, ash and energy content are useful determinant to see the improvement of nutritional quality of beef patties blended with protein isolates. These values can provide the information to consumers on the healthier choices of beef patties products. Also, environmental assessment on the production of VFI and beef patties were not carried out in this study. The greenhouse gas emissions value of 100% beef patties and 20% added VFI beef patties were only calculated based on the legumes and beef patties greenhouse gas emissions values from previous literature. Other environmental assessments which are cropland use, freshwater use, nitrogen and phosphorus application would give better indications on the effectiveness of beef patties added with plant proteins on reducing the environmental footprints.

6.2 Recommendations for further work

Based on the findings of this study, further works could be carried out in the future as follows:

1) Protein structure analysis for secondary structures using Fourier Transform Infrared (FTIR) spectra to compare with bioinformatics tools that predicts the secondary structure of VFIs. This information would be useful to further correlate the secondary structures of proteins with functional properties of VFIs. Peak from the spectra could be used to characterise the presence of secondary structures. The spectra are characterized by the presence of antiparallel *β*-sheets (1632 and 1684 cm^{-1}), overlapped *α*-helices and random coil signals (1651 cm^{-1}), and β-turns (1670 cm^{-1}) (Mune Mune et al., 2018) (Jarpa-Parra et al., 2014). The intensity of bands observed from the spectra can be an indicator of secondary structures of proteins.
2) Nutritional analysis could include amino acids analysis and protein digestibility using Protein digestibility corrected amino acid score (PDCAAS) to determine the protein quality of VFIs. The amino acid analysis can be done by hydrolysing samples in 6N HCL at 110 C for 24 hours followed by determination by HPLC (Vioque et al., 2012).
3) Protein digestibility of VFI to have better understanding of protein quality. This nutritional parameters can be calculated using amino acid composition. Particularly biological value (BV) estimates the amount of ingested protein would be incorporated in organism. Amino acid scores

(AAS) predict dietary protein quality and protein efficiency ratio (PER) estimates protein quality. Allerginicity could also be explored.

4) Perform sensory study on beef patties made with 20% VFI and commercial proteins to see the acceptability for consumers of the taste, appearance and texture so that the products can be commercialised. An acceptability test using the 9-point hedonic scale can be used to assess degree of liking of the attributes of beef patties. Participants who normally consume beef patties and were generally healthy with no food allergies are the best candidates for the study.
5) Extraction conditions at the pilot scale to see the feasibility of extraction. This could be explored with VF and other legume species (such as black eyed peas and mung beans).
6) Assessing nutritional value of raw and cooked beef patties. Macronutrients values which are protein, fibre, fat, ash and carbohydrate and energy content can be evaluated using AOAC standard method.
7) Total evaluation on the environmental footprints on the production of VFI and beef patties added with VFI by looking at the greenhouse gas emissions, cropland and freshwater use and nitrogen and phosphorus application. This is useful to demonstrate the effects of reducing meat consumption by substituting it with plant-based food for reducing the environmental effects of animal food.

6.3 Conclusion

Functional and nutritional properties of *Vicia faba* isolates can be improved by extracting interest compounds to achieve isolates with a good amount of protein and total yield and reduced amounts of anti-nutritional compounds. Presence of non-protein compounds which are starch and fibre are also useful in some functional properties and the application in beef patties. Thus, VFI is a potential source of food ingredients that can provide an alternative to animal protein, exhibit functional properties that will be useful in other food products, reduce the cost of food formulation and help with sustainability, particularly to reduce greenhouse gas emissions associated with the production of meat. Extracting and isolating compounds from legumes is a useful strategy to increase the utilisation of legumes for food production.

Chapter 7 References

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Appendices

Protein identification method:

The samples were reconstituted in 8M guanidine, reduced with 10mM DTT at 56°C for 60 min, alkylated with 30mM iodoacetamide for 30 min at RT in the dark. The samples were then diluted with 50mM ammonium bicarbonate such that the guanidine concentration was 1M. 2 µg trypsin was then added to each sample and incubated for 18 h at 37°C. Samples were quench by the addition of 5µL formic acid and concentrated by SPE using Sep-Pak tC18 cartridges. The samples were then dried under vacuum and reconstituted in 50µL 0.1 % TFA ready for injection. LC-MS was performed.

LC separation of the peptide mixtures was performed on an ACQUITY M-Class UPLC (Waters UK, Manchester). 1 µL of each sample was loaded onto a Symmetry C18 trap column (180 µM i.d. * 20 mm) and washed with 1% acetonitrile/0.1% formic acid for 5 min at 5 µL min^{-1}. After valve switching, the peptides were then separated on a HSS T3 C18, 75 µm i.d. x 150 mm analytical column (Waters UK, Manchester) by gradient elution of 1-60% solvent B in A over 30 min. at 0.3 µL min^{-1}. Solvent A was 0.1% formic acid in water, solvent B was 0.1% formic acid in acetonitrile.

The column eluent was directly interfaced to a quadrupole-ion mobility - orthogonal time of flight mass spectrometer (Synapt G2Si, Waters UK, Manchester) via a Z-spray nanoflow electrospray source. The MS was operated in positive TOF mode using a capillary voltage of 3.0 kV, cone

voltage of 40 V, source offset of 80 V, backing pressure of 3.58 mbar and a trap bias of 2 V. The source temperature was 80°C. Argon was used as the buffer gas at a pressure of 8.6×10^{-3} mbar in the trap and transfer regions. Mass calibration was performed using [Glu]-fibrinopeptide (GFP) at a concentration of 250 fmol μL^{-1}. GFP was also used as a lock mass calibrant with a one second lock spray scan taken every 30 s during acquisition. Ten scans were averaged to determine the lock mass correction factor. Data acquisition was using data dependent analysis with a 0.2 s scan MS over *m/z* 350-2000 being followed by five 0.5 s MS/MS taken of the five most intense ions in the MS spectrum. CE applied was dependent upon charge state and mass of the ion selected. Dynamic exclusion of 60 s was used. Data processing was performed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Peptide MS/MS data were processed with PEAKS Studio (Bioinformatic Solutions Inc, Waterloo, Ontario, Canada) and searched against the Uniprot databases (release 2017_12). Carbamiodomethylation was selected as a fixed modification, variable modifications were set for oxidation of methionine and deamidation of glutamine and asparagine. MS mass tolerance was 20 ppm, and fragment ion mass tolerance was 0.05 Da. The false discovery rate was set to 1%.

1) LEB4 amino acids sequence

MSKPFLSLLSLSLLLFTSTCLATSSEFDRLNQCRLDNINALEPDHRVESEAGLTETWNPN
HPELRCAGVSLIRRTIDPNGLHLPSYSPSPQLIYIIQGKGVIGLTLPGCPQTYQEPRSSQ
SRQGSRQQQPDSHQKIRRFRKGDIIAIPSGIPYWTYNNGDEPLVAISLLDTSNIANQLDS
TPRVFYLVGNPEVEFPETQEEQQERHQQKHSLPVGRRGGQHQQEEESEEQKDGNSVLSGF
SSEFLAHTFNTEEDTAKRLRSPRDKRNQIVRVEGGLRIINPEGQQEEEEEEEEKQRSEQ
GRNGLEETICSLKIRENIAQPARADLYNPRAGSISTANSLTLPILRYLRLSAEYVRLYRN
GIYAPHWNINANSLLYVIRGEGRVRIVNSQGNAVFDNKVTKGQLVVVPQNFVVAEQAGEE
EGLEYLVFKTNDRAAVSHVQQVFRATPADVLANAFGLRQRQVTELKLSGNRGPLVHPQSQ
SQSN

2) LEB7 amino acids sequence

GIPYWTYNNGDEPLVAISLLDTSNIANQLDSTPRVFYLGGNPEVEFPETQEEQQERHQQK

HSLPVGRRGGQHQQEEESEEQKDGNSVLSGFSSEFLAQTFNTEEDTAKRLRSPRDKRNQI

VRVEGGLRIINPEGQQEEEEQEEEEKQRSEQGRNGLEETICSLKIRENIAQPARADLYNP

RAGSISTANSLTLPILRYLRLSAEYVRLYRNGIYAPHWNINANSLLYVIRGEGRVRIVNS

QGNAVFDNKVRKGQLVVVPQNFVVAEQAGEEEGLEYLVFKTNDRAAVSHVQQVFRATPAD

VLANAFGLRQRQVTELKLSGNRGPLVHPHSQSQSN

3) Vicilin amino acids sequence

MAATTLKDSFPLLTLLGIAFLASVCLSSRSDQDNPFVFESNRFQTLFENENGHIRLLQKF

DQHSKLLENLQNYRLLEYKSKPHTIFLPQQTDADFILVVLSGKAILTVLLPNDRNSFSLE

RGDTIKLPAGTIGYLVNRDDEEDLRVLDLVIPVNRPGEPQSFLLSGNQNQPSILSGFSKN

ILEASFNTDYKEIEKVLLEEHGKEKYHRRGLKDRRQRGQEENVIVKISRKQIEELNKNAK

SSSKKSTSSESEPFNLRSREPIYSNKFGKFFEITPKRNPQLQDLNIFVNYVEINEGSLLL

PHYNSRAIVIVTVNEGKGDFELVGQRNENQQGLREEYDEEKEQGEEEIRKQVQNYKAKLS

PGDVLVIPAGYPVAIKASSNLNLVGFGINAENNQRYFLAGEEDNVISQIHKPVKELAFPG

SAQEVDTLLENQKQSHFANAQPRERERGSQEIKDHLYSILGSF

4) Favin amino acids sequence

TDEITSFSIPKFRPDQPNLIFQGGGYTTKEKLTLTKAVKNTVGRALYSLPIHIWDSETGNVADFTTTF
IFVIDAPNGYNVADGFTFFIAPVDTKPQTGGGYLGVFNGKDYDKTAQTVAVEFDTFYNAAWDPSNGKR
HIGIDVNTIKSISTKSWNLQNGEEAHVAISFNATTNVLSVTLLYPNLTGYTLSEVVPLKDVVPEWVRI
GFSATTGAEYATHEVLSWTFLSELTGPSN

5) Glycinin amino acids sequence

MGKPFFTLSLSSLCLLLLSSACFAITSSKFNECQLNNLNALEPDHRVESEGGLIETWNSQHPELQCAG
VTVSKRTLNRNGSHLPSYLPYPQMIIVVQGKGAIGFAFPGCPETFEKPQQQSSRRGSRSQQQLQDSHQ
KIRHFNEGDVLVIPLGVPYWTYNTGDEPVVAISPLDTSNFNNQLDQNPRVFYLAGNPDIEHPETMQQQ
QQQKSHGGRKQGQHRQQEEEGGSVLSGFSKHFLAQSFNTNEDTAEKLRSPDDERKQIVTVEGGLSVIS
PKWQEQEDEDEDEDEEYGRTPSYPPRRPSHGKHEDDEDEDEEEDQPRPDHPPQRPSRPEQQEPRGRGC
QTRNGVEENICTMKLHENIARPSRADFYNPKAGRISTLNSLTLPALRQFGLSAQYVVLYRNGIYSPDW
NLNANSVTMTRGKGRVRVVNCQGNAVFDGELRRGQLLVVPQNPAVAEQGGEQGLEYVVFKTHHNAVSS
YIKDVFRVIPSEVLSNSYNLGQSQVRQLKYQGNSGPLVNP

6) Beta conglycinin amino acids sequence

LKVREDENNPFYFRSSNSFQTLFENQNGRIRLLQRFNKRSPQLENLRDYRIVQFQSKPNTILLPHHAD
ADFLLFVLSGRAILTLVNNDDRDSYNLHPGDAQRIPAGTTYYLVNPHDHQNLKIIKLAIPVNKPGRYD
DFFLSSTQAQQSYLQGFSHNILETSFHSEFEEINRVLFGEEEEQRQQEGVIVELSKEQIRQLSRRAKS
SSRKTISSEDEPFNLRSRNPIYSNNFGKFFEITPEKNPQLRDLDIFLSSVDINEGALLLPHFNSKAIV
ILVINEGDANIELVGIKEQQQKQKQEEEPLEVQRYRAELSEDDVFVIPAAYPFVVNATSNLNFLAFGI
NAENNQRNFLAGEKDNVVRQIERQVQELAFPGSAQDVERLLKKQRESYFVDAQPQQKEEGSKGRKGPF
PSILGALY

7) Alpha conglycinin amino acids sequence

VEKEECEEGEIPRPRPRPQHPEREPQQPGEKEEDEDEQPRPIPFPRPQPRQEEEHEQREEQEWPRKEE
KRGEKGSEEEDEDEDEEQDERQFPFPRPPHQKEERKQEEDEDEEQQRESEESEDSELRRHKNKNPFLF
GSNRFETLFKNQYGRIRVLQRFNQRSPQLQNLRDYRILEFNSKPNTLLLPNHADADYLIVILNGTAIL
SLVNNDDRDSYRLQSGDALRVPSGTTYYVVNPDNNENLRLITLAIPVNKPGRFESFFLSSTEAQQSYL

QGFSRNILEASYDTKFEEINKVLFSREEGQQQGEQRLQESVIVEISKEQIRALSKRAKSSSRKTISSE
DKPFNLRSRDPIYSNKLGKFFEITPEKNPQLRDLDIFLSIVDMNEGALLLPHFNSKAIVILVINEGDA
NIELVGLKEQQQEQQQEEQPLEVRKYRAELSEQDIFVIPAGYPVVVNATSNLNFFAIGINAENNQRNF
LAGSQDNVISQIPSQVQELAFPGSAQAVEKLLKNQRESYFVDAQPKKKEEGNKGRKGPLSSILRAFY

8) BSA amino acids sequence

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVN
ELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPERNECFLSHKDDSPDL
PKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPK
IETMREKVLTSSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGD
LLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCK
NYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQ
NLIKQNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYL
SLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEK
QIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA

9) Beta lactoglobulin amino acids sequence

MKCLLLALALTCGAQALIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEG
DLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQ
CLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI

Vicia faba: a cheap and sustainable source of protein and its application in beef products

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Introduction

- The negative impacts of meat consumption on the environment and health are increasingly of importance to consumers [1,2]. According to National Diet and Nutrition Survey 2008/10, adults aged 19-64 accounts consumed on average 110g/day, which is considered as high meat consumption. Beef products also contribute significantly to energy and saturated fat intake.
- Therefore, finding alternative sources of protein from non-meat sources could be of benefit to human health and the environment [2,3].
- Vicia faba is a widely grown legume that is rich in protein and fibre and has the potential to improve the nutritional characteristics of meat products. However its application in food is limited due to presence of vicine and convicine which causes favism in susceptible individuals.

Aim of the study

- Optimise the preparation of *Vicia faba* protein isolate (VFPI).
- Characterise the nutritional and functional properties of VFPI when combined with beef mince to make patties

Methods

Extraction and preparation of VFPI from Vicia faba

- Characterisation of protein by SDS-PAGE and LC-MS
- Quantification of total protein content using Kjeldahl
- Quantification of total dietary fibre using Megazyme kit
- Quantification of vicine and convicine content using LC-MS

Application of 20% VFPI into beef mince

Results

Samples	Protein (%)	SD	Fibre (%)	SD
VFPI	42.7	1.0	17.6	3.8
Soya PI	70.5	7.3	1.0*	ND
Pea PI	69.2	4.7	2.5*	ND
Beef	19.7*	ND	0.0*	ND

Table 1. Total protein and dietary fibre content of VFPI (values show mean of 3 independent extractions), commercial soya and pea protein isolates (PI). *values from the product label, ND = not determined.

Samples	Protein (%)	Fibre (%)	Product yield (%)
Beef patty	19.7	0.0	82.3^{a}
Beef + VFPI patty	24.3	3.5	91.0^{b}
Beef + Soya PI patty	29.9	0.2	91.1^{b}
Beef + Pea PI patty	29.6	0.5	90.9^{b}

Table 2. Calculated protein, fibre content and experimental product yield (values show mean of 3 independent preparations). The values with different letter superscripts are significantly different ($p<0.05$).

- VFPI is rich in both protein and fibre.
- The most abundant proteins identified in VFPI by SDS-PAGE followed by LC-MS were legumins, vicilin and favin.
- VFPI contained significantly less ($p<0.05$) of vicine (0.2%) and convicine (0.13%) compared to VF flour (0.7% vicine and 0.3% convicine).
- Adding 20% of VFPI with beef mince improved the product yield by improving moisture retention.
- The carbon footprint of beef patty enriched with VFPI could be reduced by 16% from 26.6 kg CO_2/kg to 22.3 kg CO_2/kg of produce [4].
- The energy content of the beef patty was theoretically reduced by 12% from 209 kcal/100 g to 185 kcal/100 g of beef patty.

Conclusion

Vicia faba could be used as an alternative source of protein to replace 20% of meat in this popular food with potential positive implications for health and the environment.

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FUNCTIONAL PROPERTIES OF FABA BEAN (*VICIA FABA*) PROTEIN ISOLATES AND THEIR APPLICATION TO BEEF PRODUCTS

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BACKGROUND OF STUDY

- Plant proteins can be utilised to improve the nutritional quality, texture and other functional properties of food.
- Faba bean (*Vicia faba)* is a relatively underutilised legume crop.
- Consumer demand for sustainable plant-derived proteins, driven by environmental and health concern and sustainable dietary choice increase the potential use of *Vicia faba* particularly as a protein source for human consumption (Multari,2015).
- A good solution to increase consumption of plant-protein and reduce consumption of red meat is by adding plant-protein to meat products.

AIM OF THE STUDY

- Optimise the preparation of *Vicia faba* protein isolate (VFPI).
- Explore the functional properties of VFPI.
- Explore the potential application to the formulation of beef products.

1. EXTRACTION, PREPARATION AND CHARACTERISATION OF VFPI

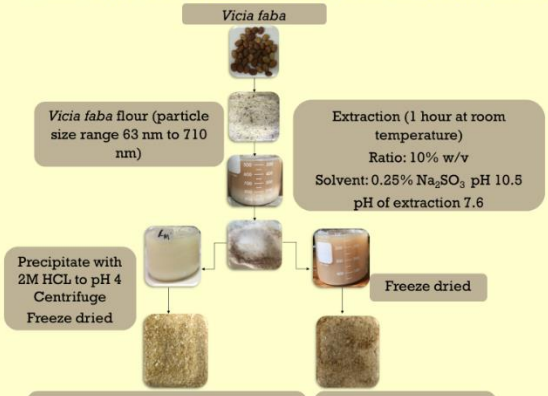
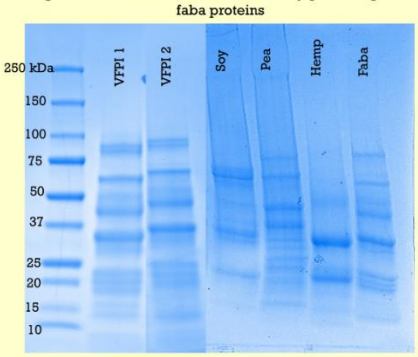


Figure 1: The preparation of VFPI

Figure 2: SDS-PAGE of VFPI 1, VFPI 2, soy, pea, hemp and faba proteins



The identity of the proteins of VFPI 1 and VFPI 2 were confirmed by LC-MS against the Uniprot databases to be:
1) Legumin type B (Accession number: sp|P05190|LEGB4_VICFA)
2) Vicilin (Accession number: sp|P08438|VCL_VICFA)

2. FUNCTIONAL PROPERTIES OF PLANT PROTEINS

Functional properties	VFPI 1	VFPI 2	Soy protein	Pea protein	Faba protein	Hemp protein
Protein (%)	42.7	37.3	75.7	50.3	46.8	38.1
Fibre (%)	17.6	13.3	1.0*	2.4*	11.0*	21.0*
Solubility (%)	4.5	37.0	28.8	41.4	30.1	4.5
Water holding capacity (g of protein per g of water)	2.8	15.0	8.2	4.2	2.6	2.5
Fat holding capacity (g of protein per g of fat)	2.7	37.0	16.9	16.2	3.0	2.8
Emulsifying properties (%)	93.0	93.0	93.0	72.4	82.8	100.0
Emulsion stability after a week (%)	77.7	59.3	82.8	62.0	72.4	60.0
Foam volume (%)	16.0	57.0	70.6	196.6	103.3	4.0

Table 1: Functional properties of plant proteins. Values with * are provided by the food manufacturer

3. APPLICATION OF PLANT PROTEINS IN BEEF PATTIES

Beef patties quality	No binder	VFPI 1	Soy protein	Pea protein	Faba protein	Hemp protein
						
Cooking loss(%)	17.66	8.97	8.92	9.07	30.57	10.83
Product yield (%)	82.34	91.03	91.08	90.93	69.43	89.17
Hardness (N)	38.45	28.34	61.25	60.15	12.55	21.26

Table 2: Quality characteristics of beef patties made from 20% plant proteins

4. DISCUSSIONS

- VFPI 1 and VFPI 2 contain legumin and vicilin-like protein
- Solubility, water holding capacity, fat holding capacity and foaming capacity of VFPI 2 are better than VFPI 1, in agreement with Wong et al., (2013) and Mune and Sogi, (2015) who suggest preparation of protein isolates by freeze drying without precipitation of protein improves functionality.
- Beef patties made from 20% VFPI 1 show better moisture retention compared to 100% beef patties, similar to soy and pea commercial protein isolates.

5. CONCLUSIONS

- A functional protein isolate was prepared from *Vicia faba* presenting good functional properties for food applications.

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