University of Reading

Extraction of Proteins from Soybean Residue (Okara) and Investigation of their Physicochemical Properties and their Application as Emulsifiers

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Department of Food & Nutritional Sciences

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Contents

Abstract

Soybean products and in particular soymilk have received significant attention by the public in recent years. Their increasing popularity is primarily due to their nutritional composition and potential health benefits as well as the fact that they are lactose-free and cholesterol-free which makes them ideal alternatives to cow milk especially by lactose intolerant people as well as people suffering from hypercholesterolemia. Okara is a protein-rich residue produced at significant amounts during soy milk production and thus constitutes an abundant and cheap raw material that can be used for the extraction of proteins with potential applications in the food industry as functional food ingredients. The aim of this research was to evaluate alkaline extraction and ultrasonication for the extraction of okara proteins, build our knowledge on the relationship between the extraction method, the protein structure and its functions, as well as investigate the application of the extracted proteins in a mayonnaise-like emulsion system. The results demonstrated that alkaline extraction in 0.1 M phosphate buffer at pH 12 and at 60 ^{o}C for 60 mins resulted in significantly higher protein extraction from okara (~ 36 % w/w) compared to pH 9,10 and 11 ranging from 4 – 14 % in these cases. The main protein components in the extracts were 11 S (glycinin) and 7 S (β-conglycinin) as observed with SDS-PAGE analysis; further separation and purification resulted in the protein content of ~83-86 % protein compared to the starting okara material. The pH of the extraction had a considerable effect on the structure of the protein as demonstrated by the Fourier transform infrared spectroscopy. It also improved the physicochemical properties of the okara protein isolate (OPI) such as its protein solubility, zeta potential, size distribution and emulsion properties. An ultrasonication method further improved the extraction yield to higher than 100% (compared to the standard solvent method). Ultrasonication also improved the physicochemical properties without affecting the purity of the protein. The rheological data

revealed that ultrasonication derived OPI had the best rheological properties compared to the conventional derived OPI and commercial protein isolate.

Declaration

I confirm the originality of the work presented in this thesis to be mine and materials sourced have been acknowledged.

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List of abbreviations

ANS	1-anilino-**8**-naphthalenesulfonate
DDMP	2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4 moiety
MES	4morpholineethane sulfonic acid
AIL	Acid insoluble lignin
ASL	Acid soluble lignin
AS	Acidic subunit of glycinin
NH_3	Ammonia gas
$(NH_4)_2SO_4$	Ammonium sulphate
AAE	Aqueous alkaline extraction
Atm	atmosphere
BS	Basic subunit of glycinin
H_3BO_3	Boric acid
$CaCO_3$	Calcium carbonate
C=O	Carbonyl
CVD	Cardiovascular disease
CVDs	Cardiovascular diseases
K_{oc}	Casson model intercept
K_c	Casson model slope
η_{ca}	Casson plastic viscosity
τ_{0c}	Casson yield stress
R^2	Coefficient of determination
CSP	Commercial soybean protein isolate
CLSM	Confocal laser scanning microscopy
K	Consistency index
Td	Denaturation temperature
DSC	Differential Scanning Calorimetry
SS	Disulphide bond
db	dry basis
EC	Emulsion capacity
ES	Emulsion stability
ΔH	Enthalpy change
AAE	Enzyme-assisted extraction
EAA	Essential amino acid
n	Flow behaviour index
FI	Fluorescence Intensity
FC	Foaming capacity
FS	Foaming stability
FAO	Food and Agricultural Organisation
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared spectroscopy
FSH	Free sulfhydryl

GN	galacturonans
GC-MS	Gas chromatography-mass spectrometry
11S	Glycinin
GMP	Good Manufacturing Practices
HPLC	High performance liquid chromatography
HCl	Hydrochloric acid
IFT	Institute of Food Technology
pI	isoelectric point
LVR	linear viscoelastic region
LDS	lithium dodecyl sulfate
G"	Loss modulus
LDL	low density lipoprotein
µmpp	micrometer (peak to peak cycle)
MW	Molecular weight
NEAA	Non-essential amino acid
OAC	Oil absorption capacity
o/w	oil-in-water
OPI	Okara protein isolate
OPIs	Okara protein isolates
PEM	Plamil egg-free mayo
PDI	Polydispersity Index
PEG	polyethylene glycol
PDCAAS	Protein digestibility corrected amino acid score
PDI	Protein dispersibility index
PAD	pulsed amperometric detector
RP-HPLC	Reversed phase-high performance liquid chromatography
RG	rhamnogalacturonans
Sat. Fat	Saturated Fat
SEM	Scanning Electron Microscopy
ϒ	Shear rate
τ	Shear stress
NaOAc	Sodium acetate
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
SPC	Soy protein concentrate
STS	Soya Technology Systems process
SPI	Soybean protein isolate
G'	Storage modulus
SRS	Sugar recovery standards
SH	Sulfhydryl
So	Surface hydrophobicity
α, ȧ, β	three subunits of β-conglycinin

TSH	Total sulfhydryl
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
UHT	Ultra-high temperature
US	Ultrasonication
UAE	Ultrasonication-assisted extraction
UV	Ultra-violet
UNU	United Nations University
WAC	Water absorption capacity
WHO	World Health Organisation
XG	Xanthan gum
7S	β-conglycinin

Chapter 1 - General Introduction

1.1 Introduction

Soybean (*Glycine max L.*) is among the major industrial and food crops grown in every continent, with its origin traced to China, and belongs to the pea family of the Leguminosae. Soybean is a good source of nutrients and it is processed into several products for human consumption, among which are soy milk and tofu. Processing of soybean into soymilk or soymilk-based products is increasingly becoming more popular as these products serve as good alternatives to lactose intolerant people and vegetarians, having the most essential amino acids compared to other legumes, with good digestibility. Moreover, they do not contain cholesterol which also makes these products good for people with hypercholesterolemia. In the production of tofu and soy milk, okara (soy bean residue) is generated as a by- product with low commercial value; it is either discarded or used as animal feed, while a small amount is put into food use which is not common to any particular areas of the world (Spring, 2005). It has been estimated that for every kilogram of soybean seed processed into soymilk, about 1.1 kg of wet okara is generated (Khare et al., 1995; Liu, 1997). Despite being underutilised, okara is a good plant source of proteins (26.8 %-37.5 % w/w), fat (8.5 %-23.2 % w/w) and carbohydrates (37.9 %-52.9 % w/w) (Ma et al., 1997; Surel & Couplet, 2005; Vishwanathan et al., 2011). The total carbohydrates in okara are made up of 3.9-6.6% soluble sugars, 0.5-1.8 % starch and 31.8-54.3% total dietary fibre (Surel & Couplet, 2005; Mateos-Aparicio et al., 2010), depending on the processing methods and varieties of soybean seeds used. Pectic polysaccharides (22.47 %) are the major components of the dietary fibre in okara (Yamaguchi et al., 1996) . Other components of the dietary fibre content of okara include (in w/w) 12.1 ± 1.2 % hemicelluloses 5.6 ± 0.9 % cellulose (Guermani et al., 1992). Okara consists mainly of ruptured cotyledon cells and studies have shown that soybean cotyledon polysaccharides consist of two types of pectin, an acidic one (i.e. galacturonic acids, 30 %) and neutral polysaccharides with equivalent amounts of arabinan and arabinogalactan; these have an

arabinose and galactose ratio ranging from 1:1.5-1:2.8 (Aspinall & Cottrell, 1971; Brillouet & Carré, 1983). These ratios are higher than those in other legumes such as peas and broad beans which have arabinose:galactose ratios ranging from 1:0.7-1:0.9. Okara is not only a good source of carbohydrate but of proteins as well, in particular glycinin and β-conglycinin (referred to as storage proteins) (Riblett et al., 2001). Proteins in soybean and okara contain essential amino acids important for human nutrition (Lönnerdal, 1994; Ma et al., 1997). These proteins can be easily released when consumed due to their high digestibility, ranging from 91 % to 96 %, which is similar to that of cow's milk (FAO/WHO/UNU, 1985; Öste 1991). There are three major forms of soy protein ingredients commercially available that can be used as protein supplements or source of proteins in food applications. These are soy flour (about 40-50 % w/w protein content), soy protein concentrate (SPC) (about 60 % w/w protein content) and soy protein isolates (SPI) (about 90 % w/w protein content); the latter two are currently extracted commercially, primarily from soy flour or soybean meal (Endres, 2001). Besides the nutritional quality of soy protein, it also possesses some valuable functional properties ranging from water and oil absorption, foaming and emulsification properties, as well as textural and gelation properties. Among all the soy protein ingredients, SPI is the most functional of all and have found successful applications in food such as cakes, beverages, breakfast cereals, cookie, specialty breads and meat products (Golbitz, 1995; Faller et al., 2000; Lee & Brennand, 2005). In addition to the above mentioned macroelements, okara contains isoflavones, (Jackson et al., 2002), minerals and vitamins (Wang et al., 2010; Mateos-aparicio & Redondo-cuenca, 2010).

The above-mentioned nutritional components in okara render it as a potential raw material for the food industry. The extraction of these components from okara is important in order to valorise it. Some researchers have used either aqueous-alkaline, enzymatic-assisted extraction, or ultrasonication to extract proteins from soybean flakes or soybean meal whereas an aqueous-alkaline method has been exploited for extraction of proteins from okara.

According to Ma et al. (1997), the protein extraction from okara employing an aqueous-alkaline method at pH of 9.0 and 25 ^{o}C for 30 min resulted in low protein recovery (14.1%), where when the process was carried out at pH 9.0 and 80 ^{o}C for 30 min, 53.4% of proteins were recovered. The latter condition may cause protein denaturation as the denaturation temperature of the two major soybean proteins, glycinin and β-conglycinin, is approximately 82 ^{o}C and 68 ^{o}C, respectively (Riblett et al., 2001). Moreover, protein extracted under high temperature conditions would have low solubility and decreased thermal stability (Ma et al., 1997). Alternatively, the enzymatic assisted extraction of proteins from soybean meal has been investigated resulting up to 83 % protein recovery using a combination of proteolytic enzymes (2.5 % Alcalase and 5 % Flavourzyme) for 16 hours (Fischer et al., 2001b). Based on the concentration of enzymes and number of hours required to achieve maximum extraction, enzyme-assisted extraction could be regarded as being cost ineffective and time inefficient. The application of ultra-sonication technology has recently attracted much research interest. Specifically, an ultrasonication-assisted method has been reported to enhance protein extractability from soybean meal and flakes but under high power conditions (Moulton & Wang, 1982; Karki et al., 2010). Protein yields of 78 % were obtained in ultrasonication-assisted extraction under high power of 1280W (Karki et al., 2010). As a result of the high power used, Karki et al. (2009) reported a decrease in the emulsification, foaming stability and rheological properties of the extracted proteins. Therefore, the use of such high level of power is not recommended to produce proteins that are suitable for food applications. The combination of ultrasonication technology with enzymatic extraction has been applied for the extraction of polysaccharides from pumpkin (Wu et al., 2014) and Epimedium leaves (Chen et al., 2012) and resulted in significant increases in their extraction yield. This is attributed to the effect of cavitation on increasing the reaction rate between the enzyme and the substrate as a result of the size reduction of the substrate, creating more surface area for enzyme action.

It is important to follow the concept of cradle-to-cradle, instead of discarding soybean residues. These contain 26.8 % - 40.4 % (w/w) protein content dry basis (Ma et al., 1997; Redondo-Cuenca et al., 2006; Mateos-Aparicio et al., 2010), as these could be reused as raw materials for the production of protein ingredients. Research findings on how this can be effectively achieved would encourage companies to embark on it. At the last IFT conference 2018 (IFTNEXT Food Disruption Challenge), Renewal Mill won the grand prize by pitching an ingredient company that uses okara to create a gluten-free flour ingredient. Following this example, a number of important food ingredients could be recovered from okara using 'green' methodologies that can be developed and scaled up leading to products with high functionality. This research investigates soy protein extraction from using two methods, i.e. a conventional alkaline phosphate buffer extraction method as well as an ultrasonication-assisted alkaline phosphate buffer method, aiming in both cases to optimise the protein yield and obtain protein of high functionality. Moreover, the incorporation of the isolated proteins, functioning primarily as an emulsifier, was also studied in an oil-in-water emulsion system.

1.2 Problems statement and justification of study

Cardiovascular disease (CVD) has been suggested to be one of the major causes of the deaths recorded globally (Mathers et al., 2006; Martinez-gonzalez et al., 2014; ScienceDaily, 2017; WHO, 2018). Also, the alarming increase in the population living with diabetes worldwide has been a significant global issue (Risk & Collaboration, 2016), because of the high rate of morbidity and mortality, as well as the high health-system costs caused by management and treatment of diabetes (Danaei et al., 2014; Seuring et al., 2015). CVD could be prevented by the right diet and lifestyle. As people have become more conscious of what they eat, the demand for functional food products has increased. There is a need to create more sustainable, affordable and functional foods with low fat/sugar/salt content, in order to prevent CVD. This general concept informed the aim of this research.

Okara, which is generated during production of soymilk or soymilk-based products, has high water activity and is rich in nutrients, which makes it readily prone to putrefaction and its disposal difficult. However, okara is a cheap source of protein as this is the most abundant nutrient within soybean and okara. Protein in soybean has high digestibility, comparable to that of cow's milk. In addition to the nutritional quality of soy proteins, studies have shown they could confer health benefits such as reduction in the risk of coronary heart disease, and have also got a Food and Drug Administration (FDA) health claim approval (FDA, 1999; Henkel, 2000); lowering of blood glucose level and insulin sensitivity (Liu et al., 2010). The prevention of cardiovascular diseases and other related diseases such as obesity and renal malfunctions have been linked to soy protein consumption (Anderson et al., 1999; Velasquez & Bhathena, 2007; Anderson, 2008). Therefore, successful extraction of okara protein with environmentally benign technologies would render it a valuable and renewable raw material. Moreover, it is very important to understand the functionality and nutritional value of okara proteins, in order for them to be effectively incorporated into food products and have a beneficial effect. This work would provide more knowledge on protein extraction from okara and more knowledge of okara protein functionality, which would strengthen the concept of utilising okara to produce various functional food products. This approach could be potentially expanded to include other parts of the soymilk industry that produce or utilise okara, thus supporting the circular bioeconomy concept.

1.3 Research hypothesis, aim and objectives

The main research hypothesis of this work was that at a high alkaline pH the extraction of proteins from okara, would give a high extraction yield and most likely result to proteins with good functionality. This is because soy globulins solubilise at pH above their isoelectric point and pH is one of the extrinsic factors that affect the functionality of soybean protein. It was also hypothesised that an ultrasonication-assisted method would increase protein

extraction without adversely affecting its physicochemical properties, as it acts by disrupting the cell wall structure, disaggregating the proteins in okara that might have been aggregated as a result of high temperature process during soymilk production, enabling the cell content to come in contact with the extraction liquid more quickly, and causing the release of proteins that are located in the protein bodies of palisade-like cells in soybean cotyledon. It was also hypothesised that okara protein isolates (OPI) extracted using the ultrasonication-assisted method would produce comparable or more stable emulsions compared to OPI from the conventional alkaline extraction method and that the addition of xanthan gum as a stabiliser would further reduce the droplet size and improve stability.

The overall aim of this research was to develop a "green" method for enhancing protein extraction from okara which would not impact negatively on the physicochemical properties of the protein isolate and its functionality within a food system. The following objectives were targeted:

1. Analyse the nutritional and chemical composition of okara;
2. Evaluate the effect of a phosphate buffer at various pH as an extraction medium for okara protein extraction on the yield, chemical, functional and structural properties of the protein isolate obtained. More specifically, address the following:
    a) Establish the optimum alkaline conditions for okara protein extraction
    b) Evaluate the physicochemical properties of the obtained protein isolates including proximate composition, amino acid composition, surface hydrophobicity and sulfhydryl group content, particle size and zeta potential.
    c) Evaluate the functional properties of the protein isolates including solubility, water and oil absorption capacity, foaming capacity and stability, emulsion capacity and stability.

d) Evaluate the structural properties of the protein isolates using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and differential scanning calorimetry (DSC).

3. Evaluate the potential for optimising okara protein extraction using an ultrasonication-assisted alkaline extraction method and investigating the effects on the physiochemical properties mentioned in objective 2.
4. Evaluate the use of the isolated proteins as an emulsifier in a food emulsion system, i.e. a highly-concentrated oil-in-water emulsion (mayonnaise-like emulsion) and establish the optimum protein concentration to achieve total surface coverage and minimum droplet size reduction.

1.4 Thesis structure

The research presented in this thesis has been divided into 7 chapters. Following the Introduction presented in this Chapter, is Chapter 2 which includes an in-depth literature review covering the key aspects of this research topic, such as soybean production and composition, okara production and detailed chemical composition, the health and functional properties of key okara components, focusing primarily on okara proteins, the extraction methodologies that can be used to extract the proteins and finally, description of protein chemical structure, focusing on the secondary chemical structure and its analysis using Fourier transform infrared (FTIR) spectroscopy. Chapter 3 gives a description of all the methodologies used in this research. Chapter 4 presents the findings from a series of experiments investigating the alkaline extraction of proteins from okara and assessment of the effect of the different extraction conditions on the protein recovery yield and the chemical, physical, structural and functional properties of the extracted proteins. Chapter 5 follows a similar approach to Chapter 4, where the extraction method is based on ultrasonication. Chapter 6 investigates the application of the okara extracted proteins in a model food emulsion system, in this case a highly concentrated

oil in water emulsion system (that resembles mayonnaise in rheology), Finally, Chapter 7 presents a general discussion of the overall findings of this work and proposes future directions for research in this area. **Fig.1.1** presents a schematic diagram describing the research approach taken in this PhD study.

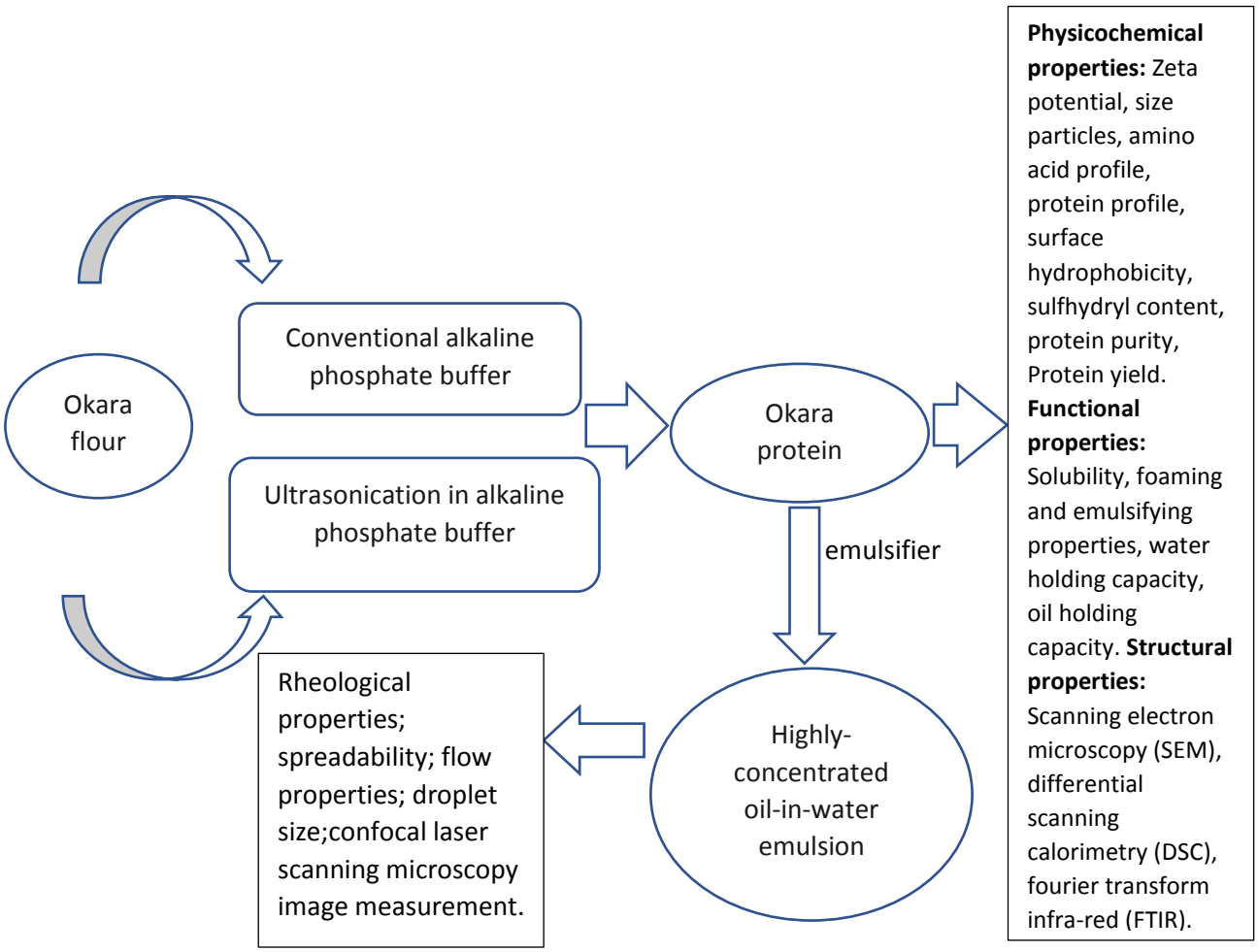


Fig.1. 1: Graphical representation of the research covered in this thesis.

Chapter 2 - Literature review

2.1 Introduction

In recent times, attention is drawing towards the utilisation of industrial food waste and by products for the production of novel or functional ingredients. This is in line with the concept of sustainability and valorisation within the food supply chain. Food waste valorisation is a concept where the generated food processing waste or low value by-products are valorised through recycling and/or recovering, adding-value components that can potentially have applications in a variety of sectors, e.g. food, chemicals, personal care, horticulture, etc. In this respect, okara, which is a by-product of the soymilk industry has significant potential due to the fact that it is an abundant natural material and contains a large amount of nutrients, primarily proteins. This literature review covers the production of soybean-based foods, the composition of okara and its potential health benefits, the potential food applications of okara and its protein components, the functional properties of okara proteins, the possible extraction methods for protein isolates, and finally, the chemical structure of proteins focusing on the use of Fourier transform infrared (FTIR) spectroscopy to analyse the secondary structural components.

2.2 Soybean production

Soybean is a global cash crop that is mainly cultivated for its oil; about 87% of soybean produced globally is converted into soy oil, with soy-derived oil representing more than 50 % of the vegetable oil marketed globally (Theones, 2006). The production of soybean seeds has notably increased from 283 million metric tons in 2013/2014 to 320 million metric tons in December, 2015 (USDA, 2015b). The United States is the largest producer of soybean with 108 million metric tons in December 2015, followed by Brazil with 100 million metric tons. Other countries that contributed to the world estimate of soybean production (in million metric tons) are Argentina (57), Paraguay (8.8), Bolivia (3.10), Uruguay (3.11), China (11.50), South Korea (0.12), North Korea (0.15), Japan (0.22), India (8), Canada (6.24), Russia (2.80),

Ukraine (3.80), European Union (2.05), Indonesia (0.62), Vietnam (0.18), Thailand (0.05), Burma (0.20), Mexico (0.36), Iran (0.20), South Africa (1.14), Nigeria (0.65), Zambia (0.21), Uganda (0.19), Turkey (0.09) and others (0.79) (USDA, 2015b). This global estimate of soybean seed production by country is presented in **Table 2.1.**

Table 2. 1: Countries and quantity of soybean produced in decreasing order.

Countries	**Quantity (million metric tons)**
USA	108
Brazil	100
Argentina	57
China	11.50
Paraguay	8.8
India	8
Canada	6.24
Ukraine	3.80
Uruguay	3.11
Bolivia	3.10
Russia	2.80
European Union	2.05
South Africa	1.14
Others	0.79
Nigeria	0.65
Indonesia	0.62
Mexico	0.36
Japan	0.22
Zambia	0.21
Iran	0.20
Burma	0.20
Uganda	0.19
Vietnam	0.18
North Korea	0.15
South Korea	0.12
Turkey	0.09
Thailand	0.05

Soybean has been grown primarily for its oil, despite that it contains more protein than oil. It is the largest grown oil seed crop in the world for many years. Globally, approximately 87 per cent of all soybean production is crushed into soy meal and soy oil, with the remaining 13 per cent used for direct human consumption. From the soybean crushing process, roughly 80 per cent is extracted as soy meal for use in animal feed, and 20 per cent is extracted as oil for human consumption and as a biofuel feedstock (Product Board, 2011). In recent years,

soybean meal is considered as a relatively cheap source of valuable nutrients and phytochemicals because of the health benefits associated with them, hence, it has started to be used in the formulation of a variety of soy-based foods (Strahm, 2006).

2.3 Soy-based foods

Soybeans have been used to create a variety of traditional foods consumed in many Asian countries such as China, Japan, Indonesia, Korea, India, Thailand, Vietnam and a few African countries (mainly Nigeria and Uganda). The traditional soy foods include miso, tofu (coagulated soy milk), soy milk, tempeh and soya sauce. In recent years, more soy-based foods have emerged which include fresh beans and sprouts, soy bread, soy cheese, soy beverages, soy spreads, soy-containing pasta, meat analogues, and soy cookies. The commercial success of these products was rather low until the Food and Drug Administration (FDA) approved a health claim in 1999 on reduction of risk of coronary heart disease by soy proteins (FDA, 1999; Henkel, 2000). This increased consumer awareness on the potential health benefits associated with the consumption of soybean-based products which are known to contain protein, fat, dietary fibre, carbohydrates, minerals and phytochemicals (e.g. isoflavones, saponins, phytate) and has led to increased demand for soy-based food products, especially soy milk and soy milk-based beverages over the recent years (Golbitz & Jordan, 2006; Granato et al., 2010). Generally, people have the tendency to buy foods associated with physiological health claims more than they would do for foods with psychological health claims (Siegrist, 2008). A report also has it that a rise in the global value sales for functional foods increased approximately by 60 % between 1998 and 2003 and approximately by 40 % in 2008 (Euromonitor, 2009).

2.4 Production of soy milk and its by-product (okara)

Plant derived proteins are more sustainable sources of proteins than animal derived proteins for the human population. Among all the vegetable sources, it is only soy milk that contains almost all the essential amino acids with a digestibility of about 0.90, similar to that

of cow's milk. Soy milk is a hot water extract of wet-milled soybean seeds, it is off-white in colour and contains most of the soluble proteins and carbohydrates as well as the oil present in the soybean seeds (Hajirostamloo, 2009; USDA, 2015a). Soy milk is considered a healthy drink since it is rich in proteins and lower in saturated fat compared to cow's milk as presented in **Table 2.2** (USDA, 2015a; Hajirostamloo, 2009).

Table 2. 2: Chemical composition of soy milk and cow's milk (100 g)

Chemical composition	**Soy milk**	**Cow milk**
Protein (g/100g)	2.8-4.8	3.3
Cholesterol (mg)	0.0	10.4-14.0
Lactose (g)	0.0	1.7
Total fat (g)	1.9-3.0	3.7
Saturated fat (g)	0.2	2.3
Fibre (g)	1.3	0.0
Folate (µg)	33.6	5.0
Energy (Kcal)	32.2-41.0	60.6-64.0
References	(USDA,2015a);(Hajirostamloo,2009); (Shakeel et al., 2015)	(USDA,2015a);(Hajirostamloo, 2009)

Soy milk does not contain lactose or cholesterol (see **Table 2.2** for chemical composition of soy milk), thus it is suitable for lactose intolerant people and for those at risk of cardiovascular diseases (Kris-Etherton et al., 2002; Mozaffarian & Ludwig, 2010; Anand et al., 2015). In addition, soy milk contains isoflavones (genistein, daidzein and glycetein), which have been suggested to potentially impact health benefits to humans, such as decreasing the risk of cancer, preventing the risk of cardiovascular diseases, lowering of cholesterol, minimising the risk of osteoporosis, and alleviating menopausal symptoms (Shu et al., 2001; Zhou et al., 2004; Messina et al., 2004; Messina, 2006; Bolla, 2015). Commercially, soy milk is available in various forms such as plain soy milk, flavoured soy milk, dairy-type soy milk, soy beverages, cultured products, and blends of soy milk with other vegetable or dairy milks. As soy milk production is increasing continuously due to the increased demand, this results in high accumulation of the main soybean processing residue, i.e. okara. It is estimated that for every kilogram of soybean seed processed into soymilk, approximately 1.1 kg of wet okara is

generated (Khare et al., 1995; Liu, 1997). Below, the two general processing methodologies for soy milk production are presented and discussed.

2.4.1 Traditional process

This method involves washing the soybean seeds, soaking overnight, grinding the seeds, and subsequently forming a slurry by adding cold water; this is then followed by sieving or pressing through a cheese cloth to obtain the milk. The remaining residue is called okara. The obtained soymilk is heated and strained again before filling into containers. This method is old and known to be used in China at household scale. The produced soy milk is normally characterised by a strong beany flavour and a chalky mouthfeel (Berk, 1992).

2.4.2 Soya Technology Systems (STS) Process

This method is carried out in modern food production plants under Good Manufacturing Practices (GMP) and ensures that the produced soymilk is safe as well as nutritionally and organoleptically acceptable for human consumption. The processing steps are shown in **Fig.2.1** (STS, 1986). The modern production of soymilk starts with the selection of the soybean variety and the storage of the seeds in a system with moisture and temperature control. The seeds are then cleaned, and the damaged ones are sorted out to avoid fatty acid oxidation due to the activity of the lipoxidase enzymes. At this point a dehulling step takes place in order to produce a white and appetising soymilk. The remaining seeds are blanched in order to inactivate endogenous enzymes (lipoxidases) and are grinded in a hot water solution of sodium bicarbonate to further eliminate undesirable enzyme activity through formation of thick slurry and to enhance soy protein extractability. The soybean residue is separated using a decanter centrifuge. The extracted milk is deodorised by using vacuum to deaerate the soymilk and further heating in the deodorizer and standardized to obtain the desired protein composition and then, flavoured, fortified and homogenised to obtain uniform product. The milk product obtained is subjected to ultra-high temperature (UHT) treatment and aseptically packaged.

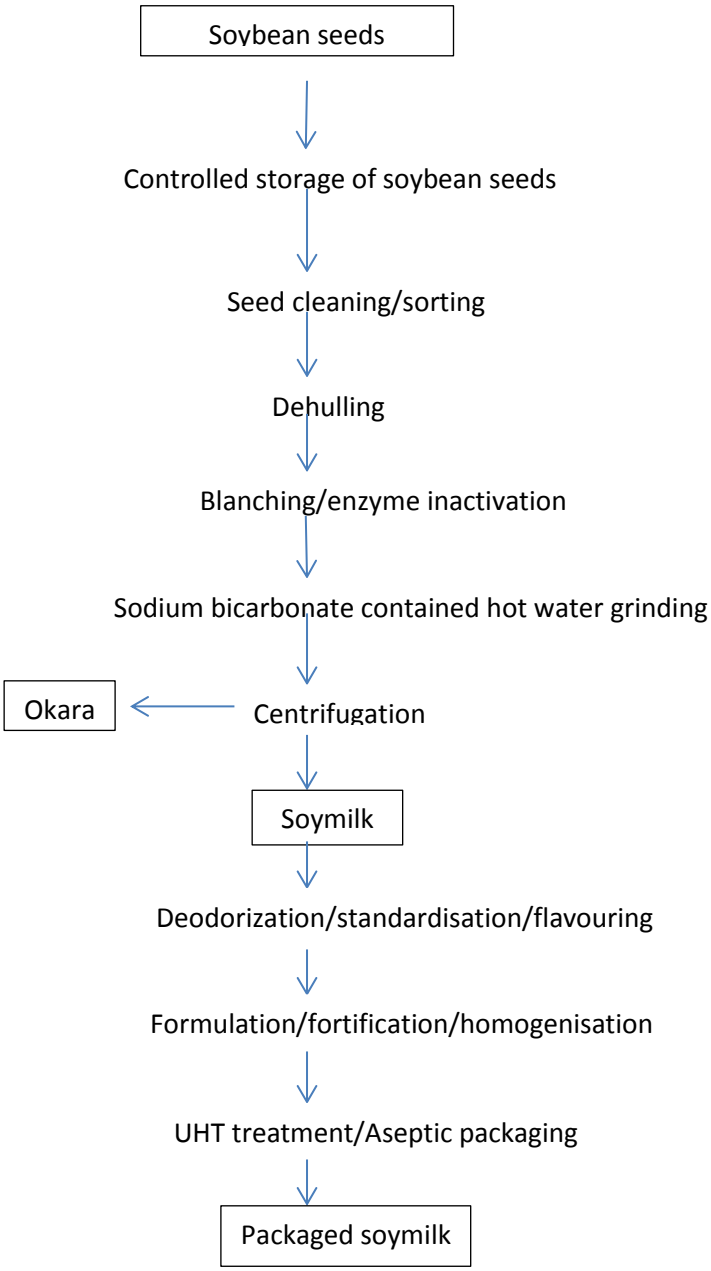


Fig.2. 1: Flow chart of STS soymilk/okara processing steps (Adapted from (STS, 1986).

In addition to the STS process, other similar processes have been developed, such as the Illinois process (Anon., 1987).

2.5 Composition of okara and their associated health benefits

Okara is the residue generated during soymilk production and has a low commercial value. It was estimated that for every 1000 litre of soymilk produced via commercial process

or traditional process 250 kg or 398 kg of okara is generated respectively (Gavin and Wettstein, 1990). Taking into account the significant rise in the sales of soymilk, which is expected to reach 16.3 billion litres in 2018 (Statista, 2018), approximately 6.5 billion Kg of okara are expected to be produced. The driving factor for the rise in the soymilk market is the increasing number of people that are allergic to cow milk or are lactose-intolerant and people that have hypercholesterolemia. On the other hand however, okara is a good plant source of proteins (26.8 %-37.5 % w/w), fat (8.5 %-23.2 % w/w) and carbohydrates, which include primarily dietary fibre (37.9 %-52.9 % w/w) (**Table 2.3**) (Ma et al., 1997; Surel & Couplet, 2005; Vishwanathan et al., 2011). Other soy components that remain in okara include isoflavones, minerals, vitamins, saponins and phytates. Some of these components, in particular isoflavones and soy proteins have attracted the most attention, as they have been associated with positive health-promoting properties such as reduction of the risk of cardiovascular diseases and cancer, as well as antioxidant activities (Shu et al., 2001; Zhou et al., 2004; Xu & Chang, 2012). These will be discussed in more detail in the sections below, for each class of okara component.

Table 2. 3: Proximate composition of okara (g/100 g dry basis)

Protein	Fat	Ash	Carbohydrate	Dietary fibre	References
25.4-28.4	9.3-10.9	3.0-3.7	3.8-5.3[a]	52.8-58.1	(Van der Riet et al., 1989)
15.2	8.3	3.9	4.1[b]	42.4	(Li et al., 2008)
28.5	9.8	4.5	5.1	55.5	(Redondo-Cuenca, 2008)
33.4	8.5	3.7	3.9[c]	54.3	(Mateos-aparicio and Redondo-cuenca, 2010)
37.5	20.6	3.9	6.6	31.8	(Surel and Couplet, 2005)

[a]Monosaccharides, oligosaccharides, and starch; [b]reducing sugar; [c]low-molecular weight carbohydrate

2.5.1 Proteins

There are different types of proteins that make up the soy proteins but the two major ones consisting about 70-90 % of total protein in soy and its products are the 7S (β-conglycinin) and 11S (glycinin) (Liu, 1997). They are storage globulins, and have been found to possess various functional properties attributed to their distinct structures, which influence the overall

functional properties of soy protein ingredients (Garcia et al., 1997; Kwon et al., 2002; Kwon et al., 2003). Glycinin (known as 11S) is a hexamer with a molecular weight of about 360 kDa and consists of 12 polypeptides split into 6 acidic (34-44 kDa) and 6 basic (20 kDa) ones (Adachi et al., 2003). The polypeptides exist as acidic-basic pairs linked by a single disulphide bond (A-SS-B). The disulphide linked acid-basic polypeptide pairs associate with each other by hydrophobic and hydrogen bonds to form two trimers of 6 polypeptides each giving it a more compact structure (Marcone et al., 1998). *β*-Conglycinin (7S) is the other major storage protein of soy and it is a trimer with a molecular weight ranging between 150-200 kDa (Kannan et al., 2012). It has three major subunits, ά, α, β with molecular weight of 58 kDa, 57 kDa and 42 kDa, respectively (Thanh and Shibasaki, 1978), without disulphide linkages (Utsumi & Kinsella, 1985; Mujoo et al., 2003; Tezuka et al., 2004). These three subunits come together by strong hydrophobic and hydrogen bonds without disulfide bond to form a trimer. *β*-Conglycinin is a cysteine-rich glycoprotein. It is N-glycosylated as the carbohydrate unit is linked to the aspartic acid residue at the N-terminal end of the molecule (Marcone et al., 1998).

The three major soy protein ingredients in the market include soy flour (full fat and defatted), soy protein concentrate (SPC) and soy protein isolate (SPI) and are classified according to their protein composition as well as the production methods. Full fat soy flours are produced by milling soybeans after the hulls have been removed and contain about 35-40 % (w/w) of protein, while defatted flours are derived from soybeans after the hulls and oils have been removed and contain about 44-54 % (w/w) of proteins (Golbitz and Jordan, 2006). Soy protein concentrates are derived from defatted flour or flakes after the sugar, water and alcohol have been removed by either alcohol or water wash method and have protein content between 65-70 % (w/w) (Endres, 2001; Golbitz & Jordan, 2006). Soy protein isolates are derived from defatted flour or flakes after removal of sugars and other water-soluble materials including fibres by conventional aqueous alkaline extraction method and have a protein content

in excess of 90 % (w/w) (Endres, 2001). The proximal composition of these soy protein fractions is given in **Table 2.4**. More so, the active form of isoflavones, mainly genistein and daidzein occur abundantly in protein isolates probably because of alkaline hydrolysis that might have occurred during alkaline extraction (see **Table 2.7** for the isoflavone content of the soy protein ingredients).

Table 2. 4: Chemical composition of soy protein ingredients

Soy protein fractions	Crude Protein (%)	Ash (%)	Crude fat (%)	Total carbohydrates[a] (%)	Moisture (%)
DSF	52.0-54.0	5.0-6.0	0.5-1.0	30.0-34.0	6.0-8.0
SPC	62.0-69.0	3.8-6.2	0.5-1.0	19.0-24.0	4.0-6.0
SPI	86.0-87.0	3.8-4.8	0.5-1.0	3.0-4.0	4.0-6.0
OPI	77.0	2.0	4.1	7.5	4.5

[a]Total carbohydrate is calculated by mass difference. Sources: (Endres, 2001; Kim & Kim, 2015). DSF: Defatted soy flour; SPC: Soy protein concentrate; SPI: Soy protein isolates; OPI: Okara protein isolates

Soy protein ingredients, in particular soy protein isolate (SPI) and soy protein concentrate (SPC) are widely available plant proteins that can provide quality proteins based on the amount of essential amino acids (EAA) they contain, which compared favourably or even higher than the FAO/WHO recommended level. Soybean flour as a source of protein also contained appreciable amount of EAA as can be seen in **Table 2.5**. Moreover, the protein digestibility corrected amino acid score (PDCAAS) data showed that SPI and SPC are highly digestible by having PDCAAS scores ranging from 0.92 to 1.00 and 0.99 for SPI and SPC, respectively (FAO/WHO, 1991; Sarwar, 1997). These scores compare well with the PDCAAS values (0.92-1.0) reported for animal proteins but are higher than the values ranging from 0.23-0.73 reported for other vegetable proteins (FAO/WHO, 1991; Ahrens et al., 2005). Okara has been shown to provide protein ingredients (protein isolates) that contain EAA at the level comparable or higher than the FAO/WHO recommended level (Ma et al., 1997; Kumar et al., 2016). The results presented in **Table 2.5** and **2.6** also revealed that the OPI amino acid contents are similar to those of SPI, suggesting that okara proteins could be used instead of commercial soy

proteins. Proline is the only non-essential amino acid (NEAA) in OPI found to be below the range reported for SPI and SPC. This is not a big concern though as proline can be synthesised by the human body. From **Table 2.5**, among the essential amino acids, methionine/cysteine was found to be slightly below the FAO/WHO recommended level and tryptophan was not reported.

Table 2. 5: Essential amino acid composition of soy protein (mg/g of protein)

Essential amino acid	**FAO/WHO[a]**			**Defatted[b] Soy flour**	**Defatted[c] Okara**			
	2-5	**10-12**	**Adult**			**Soy protein Concentrate[d]**	**Soy protein Isolate[d]**	**Okara isolate[e]**
Histidine	19	19	16	26	27	23-26	23-27	28
Isoleucine	28	28	13	46	69.8	43-47	45-49	51
Leucine	66	44	19	78	108.5	74-77	78-82	81
Lysine	58	44	16	64	54	61-63	61-65	65
Methionine + Cystine	25	22	17	26	16.5	27-29	24-26	26
Phenylalanine +tyrosine	63	22	19	88	97.5	84-88	86-92	95
Threonine	34	28	9	39	37	36-37	36-38	41
Tryptophan	11	9	5	14		12-13	12-14	ND
Valine	35	25	13	46	46.6	47-48	49-51	51

Sources: [a](FAO/WHO/UNU, 1999); [b](Cavins et al., 1972); [c](Kumar et al., 2016); [d](Hughes et al., 2011); [g](Chan & Ma, 1999). Soy protein isolate and concentrate reported are commercial products.

Table 2. 6: Non-essential amino acid composition of soy/okara protein ingredients (mg/g protein)

Amino acids	**SPI[a]**	**SPC[a]**	**OPI[b]**
Proline	52-53	52-53	36
Alanine	42-43	39-40	46
Arginine	73-86	75-86	75
Glycine	40-42	40-42	46
Glutamic acid	189-196	198-202	195
Serine	49-51	49-51	50
Aspartic acid	112-125	118-129	117

Sources: [a](Hughes et al., 2011); [b](Chan & Ma, 1999); SPI: Soy protein isolates; SPC: Soy protein concentrates; OPI: Okara protein isolates.

2.5.2 Dietary fibre

Dietary fibre is a key component of plant cell wall material that consists normally of the soluble fibre (types of hemicellulose) and insoluble fibre (e.g. resistant starch, cellulose, types of hemicellulose). Dietary fibre resists digestion in the small intestine and is fermented in the colon by the resident gut microbiota. Dietary fibre has been associated with a number of physiological effects which include lowering of the risk of coronary heart disease, lowering of serum cholesterol concentration, lowering levels of cardiovascular disease risk factors, improving gastrointestinal function and glycaemic control and enhanced weight loss (Anderson et al., 1994; Pereira et al., 2004; Anderson et al., 2009; Mann & Cummings, 2009). Okara contains both soluble dietary fibre (4.2-14.6 g/100 g dry matter) and insoluble dietary fibre (40.2-50.8 g/100g dry matter) (Van der Riet et al., 1989; Redondo-Cuenca et al., 2008; Mateos-aparicio & Redondo-cuenca, 2010). It is suggested that the dietary fibre in soybean could exert glycaemic control in diabetic patient (Holt et al., 1996). In addition to the above, it has been suggested that consumption of soybean dietary fibre could increase faecal excretion of bile acids with a concomitant reduction in fat absorption (Jenkins et al., 2003). Moreover, there is possibility of soybean dietary fibre causing a reduction in the calorific density of some foods (Liu, 1999), which suggests that soybean fibre could be potentially used as a functional ingredient for formulation of specialty foods targeting low sugar/high fibres for mostly individuals looking to lose weight to prevent or control obesity.

2.5.3 Isoflavones

Phytoestrogens are non-nutritional components with estrogenic activity, which are found in plants. Isoflavones are a class of phytoestrogens with both estrogen-agonist and estrogen-antagonist activities (Lampe, 2003); they are polyphenolic compounds with biological activities resembling that of human hormone estradiol (Hutabarat et al., 2001). Among all the identified edible legumes, soybean seeds contain the largest amount of

isoflavones, present in a bound state as glycosides (Fletcher, 2003; Munro et al., 2003). This suggests that soybean and its products are the main food sources of isoflavones. Isoflavones in bound forms include genistin, daidzin, and glycitin. Upon processing (such as fermentation and heating), the free forms are released, which are known as aglycones and they include genistein, daidzein and glycitein. Genistein and daidzein have received considerable attention due to their bioactive properties (Messina, 1999; Choi & Kim, 2013; Kaur & Badhan, 2015; He et al., 2015). Genistein and daidzein content in okara are present at around 58 mg/100 g and 33mg/100 g dry matter, respectively (Kumar et al., 2016). The current commercial soy protein ingredients also contain isoflavones at different levels and the data are presented in **Table 2.7**. This means their method of extraction leaves appreciable amount of isoflavone in them. Foods containing phytoestrogens are regarded as functional foods because of the physiological effects they have on human health conditions after consumption. Consumption of soy-foods has been suggested to potentially offer protection against the risk of cancer development in humans (Messina & Barnes, 1991; Shu et al., 2001; Zhou et al., 2004). Since the 1998 report (Potter et al., 1998) that isoflavones improve bone health, subsequent trials have not significantly supported this claim (Alexandersen et al., 2001). To this end, the action of soy isoflavones on reducing the rate of bone loss and osteoporosis is based on Asian epidemiologic data that revealed the association of higher intake of soy isoflavone with higher bone mineral density (Messina et al., 2004). Although genistein may potentially serve as an alternative to hormone therapy to address post-menopausal symptoms, its action is more effective if the consumption has started earlier in life than during menopause (Piekarz & Ward, 2007; Mardon et al., 2008). Research has also shown that isoflavones are vascular reactivity modulators and possess anti-inflammatory properties through the stimulation of estrogen receptors (ER) and by exerting intracellular kinase signalling cascades (Li et al., 2006). Interestingly, during the production of

soymilk, the majority of isoflavones in soybeans are left in the residue (okara) (Jackson et al., 2002) because isoflavones are present in the plumular axis of soybean seed.

Table 2. 7: Isoflavone composition of soy protein ingredients (%)

Products	Daidzein	Genistein	Glycitein
Whole soy flour	38	49	13
Defatted soy flour	36	59	6
Soy protein isolates	30	66	5
Soy protein concentrate	32	62	5

Source: (Genovese et al., 2007).

2.5.4 Other components

Soybean and its products contain phytate in the range of 0.1-2.22 g/100g dry matter (Al-Wahsh et al., 2005; Lestienne et al., 2005). A small amount of phytate (0.5-1.2 g/100 g dry matter) remains in okara after soymilk production (Van der Riet et al., 1989). Phytate is considered an anti-nutrient because it interferes with the bioavailability of some essential micronutrients such as calcium, iron, zinc, magnesium and copper, which may subsequently lead these micronutrient deficiencies (Moser et al., 1988; Harland et al., 1988; Greger, 1999). On the other hand, in the last 2 decades, research has shown that phytate could be used to lower blood glucose and cholesterol level (Jariwalla et al., 1990; Lee et al., 2007) by reducing the glycaemic index of foods (Thompson et al., 1987; Lee et al., 2006). In addition, the effect of phytate on the prevention of renal stone formation (Grases and Costa-Bauza, 1999) has been reported. Phytate may also exhibit antioxidant and anticancer activities (Shamsuddin, 1995; Xu & Chang, 2012). In order to minimise the negative effect phytate of on micronutrient bioavailability, an approach has been proposed where phytate-containing foods are consumed with protein rich foods; this may decrease the micronutrient blocking effect of phytate as suggested by research carried out by Sandström et al. (1989). It was suggested that practicing a well-balanced diet would render the supposed side effect of phytate on micronutrients insignificant while achieving a huge success in prevention of cancer, kidney stone, high blood

and cholesterol issues (Schlemmer et al., 2009). In this regard, the consumption of okara, or okara derived products might find commercial applications.

Another important class of component present in okara is the micronutrients. These include some minerals, B vitamins and fat-soluble vitamins (**Table 2.8**). These are important compounds with physiological and therapeutic properties which include antioxidant property, enhancement of the immune system, prevention of cardiovascular diseases (Quitain et al., 2006).

Table 2. 8: Micronutrients in okara

Nutrients	**Contents (mg/100g)**
Potassium, k	936-1233
Sodium, Na	16-96
Calcium, Ca	260-428
Magnesium, Mg	130-257
Iron, Fe	0.6-11
Copper, Cu	0.1-1.2
Manganese, Mn	0.2-3.1
Zinc, Zn	0.3-6.4
Chromium, Cr**	0.2
Phosphorus, P*	396-444
Thiamin, B1*	0.5-0.6
Riboflavin, B2*	0.03-0.04
Nicotinic acid, B3*	0.8-1.0

Sources: (Van der Riet et al., 1989)*; (Wang et al., 2010)**; (Mateos-aparicio and Redondo-cuenca, 2010).

Saponin is also present in okara, although at a lower concentration (0.1 g/100 g dry matter) compared to that found in soymilk (0.26-0.39 g/100 g dry matter) (Kitagawa et al., 1984; Ireland et al., 1986; Anderson & Wolf, 1995). The soyasaponins belong to the group of triterpene saponins and are divided into 3 major groups, A, B and E; they have sugar chains attached to carbon atom position C-3 and C-22 for group A and at only C-3 position for group B and E. Groups B and E also contain a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4 (one DDMP) moiety at C-22 position. Soybean and its products are good dietary sources of soyasaponins and reports have shown that these have antiviral, immune-stimulatory and anti-

angiogenetic properties (Lásztity et al., 1998; Bae et al., 2002; Gurfinkel & Rao, 2003). Okara, being a product of soybean, contains high amount of polyunsaturated fatty acids. The composition of the fatty acid content is given as ~15 % palmitic acid (C16:0), ~3 % stearic acid (C18:0), ~27 % oleic acid (C18:1), ~50 % linoleic acid (C18:2), ~5 % α-linolenic acid (C18:3) (Kumar et al., 2016). The presence of omega-3 fatty acid in okara (Kumar et al., 2016) could potentially predispose okara as a non-fish source of this compound which confers several health benefits (Sena and Seiça, 2007) such as reduction in the risk of cardiovascular disease, cystic fibrosis, rheumatoid arthritis and prevention of cognitive impairment (Terano et al., 1999; Darlington & Stone, 2001; Hu et al., 2002; Cleland et al., 2003; Beckles- Willson et al., 2002).

2.6 Health benefits of soy/okara proteins

The proteins present in soybeans and to large extent in okara as well, have been associated with a number of beneficial health effects, based on a number of in vitro and in vivo studies. The majority of these studies have been conducted with soy protein concentrates (SPC) or soy protein isolates (SPI) extracted from either soy flour or soybean meal. Certain key areas of research and findings are discussed in the sections below although comprehensive discussion on these topics can be found in a number of reviews (Chao, 2008; Chen et al., 2012; Messina, 2006).

2.6.1 Reduction of risk of cardiovascular diseases

Cardiovascular diseases (CVDs) are the leading cause of death globally, representing one-third of the total deaths occurring throughout the world (ScienceDaily, 2017; WHO, 2018). In 2016, the deaths caused by CVDs were estimated to be 17.9 million, which accounts for 31 % of all deaths that occurred globally and about 85 % of the deaths caused by CVDs were due to heart attack and stroke (WHO, 2018). CVDs are a class of disorders of the heart and blood vessels and include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis and pulmonary

embolism (WHO, 2018). Consumption of soybean proteins has increased in recent times, because they are linked with a reduction in the risk of cardiovascular diseases (Scheiber et al., 2001). Soy protein has a health claim confirmation by Food and Drug Administration (FDA) in 1999, which states that 25 grams of soy protein consumed a day could reduce the risk of coronary heart disease (FDA, 1999) by lowering blood cholesterol levels. Anderson et al. (1995) demonstrated that consumption of soy protein could reduce the levels of both blood cholesterol and lipids. Soy proteins have demonstrated atheroprotective activity in animal models (Adams et al., 2002; Adams et al., 2004). Moreover, several clinical trials demonstrated that consumption of 25-50 g of soy protein daily could cause a reduction on serum cholesterol level, low density lipoprotein (LDL) cholesterol level and triglycerides levels (Anderson et al., 1995; Reynolds et al., 2006; Weggemans & Trautwein, 2003; Zhan & Ho, 2005).

2.6.2 Diabetes

Soy proteins have been suggested to control hyperglycemia, hyperlipidemia and hyperinsulinemia, (Lavigne et al., 2000; Velasquez & Bhathena, 2007; Kwon et al., 2010). In addition, consumption of soybean protein may help reduce the risk of renal disease in type 2 diabetes (Jenkins et al., 2003). A soybean diet could also be used for the management of type 2 diabetes, having demonstrated some beneficial effects on hypertension, hypercholesterolemia, atherosclerosis and obesity which are commonly seen in type 2 diabetes (Holt, 1996). However, the mechanisms through which soy proteins exert these beneficial effects still remain unclear and are under investigation (Lavigne et al., 2000;Velasquez & Bhathena, 2007;Villegas et al., 2008).

2.7 Methodologies for protein extraction from okara

Soy protein isolate is one of the soy protein ingredients, with a protein content in excess of 90 % on a dry basis (Johnson and Kikuchi, 1989). The protein extraction of soybean and okara has been investigated by various researchers using different methods. More specifically,

the extraction of proteins has been carried out using acid and alkaline conditions, enzyme assisted extractions and more recently with the aid of ultrasonication technology. The ultrasonication process is a new technology which has not been fully exploited or studied comprehensively with regards to the extraction of protein from okara.

2.7.1 Aqueous alkaline extraction (AAE)

This procedure is a common approach for the extraction of the major soy bean proteins (globulins). It involves the dissolution of defatted soy flour or okara flour in water (w/v), followed by pH adjustment to alkalinity (pH value of 8.0, 8.5 or 9.0) using 2N NaOH and extraction either at room temperature or heated temperature (between 40-80 °C) for 30 min to 2 hour under stirring (Ma et al., 1997; Karki et al., 2010; Vishwanathan et al., 2011); . This protein extract could be further purified via precipitation at the isoelectric point of pH of 4.5 using 2N HCl, after which the precipitated protein is washed with distilled water (Karki et al., 2009). The precipitated protein could subsequently be re-dispersed in water and the pH of the solution is adjusted to 7 for complete solubilisation. The solubilised protein is then either spray dried or freeze dried to obtain soy protein isolate or okara protein isolate (Wu et al., 2000; Karki et al., 2009; Karki et al., 2010). Ma et al. (1997) reported a protein recovery of ~53 % (w/w) and ~14 % (w/w) of the total protein in the original material (okara flour) for okara protein isolate extracted at 80 ^{o}C and 25 ^{o}C respectively. The SDS-PAGE analysis of these proteins showed no major differences in the intensity of the bands. Another group of researchers (Liu et al., 2007), have reported a protein extraction yield of 25.1 %, which they expressed as a percentage of the isolated dried fraction weight to defatted soy flour weight from soy flour under optimised conditions. The condition of the extraction was 0.03M Tris-HCl buffer (pH 8.5) containing 0.01M sodium bisulphite as extraction medium, extraction twice at 45 ^{o}C for 1 hour and 1: 15 ratio (w/v) of flour: Tris-HCl. In another study reported by Karki et al. (2010), the protein yield was about 27 % from soybean flakes extracted at pH 8.5 at 60 ^{o}C

for 30 min, with flakes to water ratio of 1:10 (w/w basis). To increase protein recovery from okara that has undergone some severe heat treatment during soymilk production, Vishwanathan et al. (2011), tried a three stage protein extraction process using 2N NaOH to adjust the pH of the slurry to 9.0 and the extraction carried out at 80 ^{o}C for 30 min; the results demonstrated that approximately 87 % and 80 % of protein could be recovered from okara flour and okara flakes respectively. The three-stage process could be labour intensive and may not be economical and environmentally friendly due to the large amount of liquid extractant that may be required to complete the three extraction steps. Moreover, the extraction temperature was above and close to the denaturation temperatures of the two major soybean proteins 68 ^{o}C and 82 ^{o}C (Riblett et al., 2001). Overall, the alkali extraction method is a conventional method that is chemical and energy intensive and results in the recovery of up to half of the proteins present in soybean meal or okara (Ma et al., 1997; Vishwanathan et al., 2011), and therefore, it is energy intensive.

2.7.2 Enzyme-assisted extraction (EAE)

This method involves the use of enzymes for the treatment of okara or soybean flakes to enhance protein release and extraction yield. Enzyme-assisted extraction is considered to be a milder extraction method than alkaline extraction and has lower impact on the environment (Shen et al., 2008), but it is time consuming and expensive as enzyme concentrations of more than 1 % are usually required for better results (Fischer et al., 2001). The enzyme-assisted extraction of the protein from soybean meal has been investigated with up to 83 % protein recovery obtained, using a combination of proteolytic enzymes (2.5 % Alcalase and 5 % Flavourzyme) for 16 hours (Fischer et al., 2001a). A high yield of protein of approximately 90 % was obtained from soybean meal when the extraction was assisted with enzymes Protex 40XL (used at pH 11), Protex P (used at pH 10) and Protex 5L (used at pH 9.5) (Sari et al., 2013). The alkaline conditions of the enzyme treatment adopted by Sari et al. (2013) increased

the protein extractability from soybean meal compared to carrying out the enzymatic treatment at neutral pH, as carried out by Fischer et al. (2001). Okara is reported to be difficult to digest by enzymes because it contains high amount of indigestible components (insoluble fiber) (Kasai et al., 2004) and that it would involve series of digestion which include autoclaving for 20 mins at 121 ^{o}C which was effective to loosen the glues holding the cells together (Cassab, 1998); followed by cellulase digestion (1 %, 40^{o}C, 15 h) to digest the primary cell wall and pectinase pectinex digestion (1 %, 40 ^{o}C, 15 h) to obtain approximately 83 – 85 % w/w protein yield.

2.7.3 Ultrasonication-assisted extraction (UAE)

Ultrasonication technology has been applied in various processes as a pre-treatment step to enhance or improve the entire process of extraction of several components from plant materials, such as phenolic compounds from coconut shell powder (Rodrigues and Pinto, 2007) and hesperidin from penggan (*Citrus reticulate*) peel (Ma et al., 2008). Ultrasound is a green technology that allows a sustainable extraction process by increasing extraction efficiency, reducing solvent utilisation, number of unit operation, extraction time, energy utilisation and environmental impact (Yagoub et al., 2017; Chemat et al., 2017). Ultrasound refers to sound waves or mechanical vibrations that travel through solids, liquids or gases with frequencies greater than the upper limit of human hearing (Mason, 1998). The movement of sound waves in a medium involves the application of expansion and compression cycles. During the expansion cycle, bubbles are created in the liquid system that generate negative pressure as they form, grow and collapse systematically. When cavity collapse occurs close to a solid boundary, high-speed jets of liquid are produced which are known to exert strong impact on the solid surface (Luque-Garcıa and De Castro, 2003). The phenomenon upon which ultrasonic extraction is based is the production of localised cavitation which causes particle or cell disintegration (Khanal et al., 2007). The disintegration of cell walls by cavitation exposes the

hidden compounds in the cells to the extracting medium, hence promoting higher extraction at shorter time (Mason et al., 1996). Utilisation of ultrasound can be categorised based on the frequency and power of the machine into: low frequency/high power (<16-100 kHz and power from 10 to 1000 W/cm^2) and high frequency/low power (100 kHz to 10 MHz and power ranging from <1 W/cm^2) ultrasound (McClements, 1995; Demirdöven & Baysal, 2008; Soria & Villamiel, 2010). Low power ultrasound has found application in medical diagnostics and analytical techniques while high power ultrasound has found its application mostly in the industry to cause physical and chemical changes in biological matrices by exerting mechanical, cavitational and thermal effects (McClements, 1995; Demirdöven & Baysal, 2008; Soria & Villamiel, 2010). Application of ultrasound often leads to generation of localised high temperature of above 5000 K and high pressure of about 500 atm, together with resultant formation of free radicals by sonolysis of water (Rokhina et al., 2009). However, this effect can alter the native conformational structure of proteins with resultant change in the functional properties of the proteins (McClements, 1995). Based on this fact, ultrasound has been employed not only for extraction processes but also for enhancement of the functional properties of the ingredients to be extracted. Karki et al. (2010) reported an increase of 46 % and 50 % for protein and total sugar extraction from soybean flakes respectively, after applying 120 s of sonication at 84 μmpp amplitude, compared to the aqueous-alkaline method. The conditions employed in those experiments were energy intensive as high energy of about 1280 W was used and there could be structural destruction of the proteins which led to the decrease in emulsification, foaming stability and rheological properties, as observed previously by Karki et al. (2009). An ultrasound-assisted enzymatic extraction process has been applied for the extraction of polysaccharides from pumpkin (Wu et al., 2014) and Epimedium leaves (Chen et al., 2012) and they reported significant increase in the extraction yield when ultrasound was used simultaneously with enzymatic extraction than when these were used separately (Chen et

al., 2012; Wu et al., 2014). Overall, ultrasonication is a promising technology which can be potentially used for extraction of proteins from okara, since proteins are usually stored in protein bodies confined inside the cell walls, which need to be disrupted or disengage to allow the protein molecules to solubilise or hydrolyse in the extractant. The different extraction methods of proteins from okara and soybean flakes are presented in **Table 2.9**.

Table 2. 9: Extraction methods of proteins from okara and soybean

Materials	Extraction conditions	Isolation	Yield	References
Okara	25°C and 80°C at pH 9.0 adjusted with 2N NaOH	Acid precipitation at pH 4.5	14 % and 53 % (w/w) (for 25 °C and 80 °C respectively	(Ma et al., 1997)
Okara	3-step extractions: 1:30; 1:20; 1:10 (w/w) solid to liquid ratio adjusted to pH 9.0 with 2N NaOH at 80 °C	Not given	93.4 % (w/w)	(Vishwanathan et al., 2011)
Okara	Series of digestion of 2g of okara in 13.4 mL of water was carried out as autoclaving (121 °C; 20 mins); cellulase digestion (1 %, 40 °C, 15 h); Pectinase pectinex digestion (1 % v/v, 40 °C, 15 h)	Not given	83-85 %	(Kasai et al., 2004)
Defatted soy flake	Conventional method (1:10 w/w flakes to water ratio, pH 8.5, 60°C with continuous stirring; Ultrasonication pretreatment (1:20 w/w flake to water ratio, 84 µmpp, 120 s sonication time, 65°C)	Acid precipitation at pH 4.5 using 2N HCl	~54 %db for conventional method and ~73 % db for ultrasonication.	(Karki et al., 2009)
White flakes	pH 8.5 adjusted with 2N NaOH, 1:10 w/v soyflour :water ratio, 60 °C for 30 mins	Acid precipitation at pH 4.5 using 2N HCl	~46 % w/w	(Wang et al., 2004)
Extruded flakes (PDI 60)	pH 8.5 adjusted with 2N NaOH, 1:10 w/v soyflour :water ratio, 60 °C for 30 mins	Acid precipitation at pH 4.5 using 2N HCl	~25 %	(Wang et al., 2004)
Extruded flakes (PDI 35)	pH 8.5 adjusted with 2N NaOH, 1:10 w/v soyflour :water ratio, 43 °C for 60 mins		~34 %	(Wang et al., 2004)
Defatted low temperature desolventised soybean flakes	Ultrasonication method (pH 8 adjusted with 0.1 N NaOH, 36 °C, 1:10 db soy flake/water ratio, 60 mins, 13.4 Watts per gram of protein) performed in a sonic cleaning bath (Model 90C 1) Ultrasonic Industries Inc.	Acid precipitation at pH 4.5 with 1N HCl	53 % (w/w)	(Moulton and Wang, 1982)
Defatted soy flakes	Multifect pectinase at 5 % (wt/g of protein), 50 °C, pH 4.0, 1:6 flakes: water ratio for 3 h, while stirring at 13 rpm. Additional 30 mins extraction at 60°C, and pH8.5.	Acid precipitation at pH 4.5 with 2N HCl	~47 %	(Jung et al., 2006)

PDI: Protein dispersibility index

2.8 Functional properties of soy/okara proteins

The functional properties of soy proteins are important to be determined as they influence their potential application in food products. Differences in the structure of soy proteins affect the functionalities they exhibit when incorporated into food product systems. Protein functionality is influenced by a combination of physicochemical and structural properties of the proteins (intrinsic factors) and the interaction of these intrinsic factors with their environment (extrinsic factors) during processing (Damodaran, 1994). Intrinsic factors include the protein size, shape, its hydrophilic/hydrophobic characteristics and amino acid composition, as well as the conformational structure of the protein. The extrinsic factors that influence functionality of proteins in food systems include temperature, pH, ionic strength, and interaction between protein molecules and other food components such as water, lipids and carbohydrates. The major soybean proteins, glycinin, 11S (52 %) and β-conglycinin, 7S (35 %) (Hettiarachchy & Kalapathy, 1998; Riblett et al., 2001), differ in their physicochemical properties as well as in their adaptability to changes in the processing conditions like pH, temperature and ionic strength due to their structural differences. For instance, 7S (β-conglycinin) proteins possess only 2-3 cysteine groups per mole with no distinct disulphide bond and their average molecular weight (size) is 180 kDa while 11S (Glycinin) proteins possess 18-20 disulphide groups with average molecular weight of 340 kDa. As a result, the higher molecular weight and disulphide group of glycinin contributes to better gel formation with increased turbidity (Utsumi et al., 1997). On the other hand, *β*-conglycinin which has no disulphide linkages and has lower molecular weight, forms weak and transparent gels (Maruyama et al., 1999; Maruyama et al., 2002). These properties of *β*-conglycinin contribute to its enhanced emulsifying and foaming properties (Utsumi et al., 1997). Garcia et al. (1997) stated that solubility, water-holding capacity, viscosity, gelation, foaming and emulsification are important soy protein functionalities relevant to food applications.

2.8.1 Solubility

Protein solubility is affected by the concentration of protein in the liquid or solvent, and is an important factor to be determined in food application because it could influence the other functional properties of proteins (Hailing & Walstra, 1981; Vojdani, 1996). This is because proteins must be solubilised when incorporated in a food system in order to exert their foaming, emulsification, and water and oil binding capacities, as well as thickening stability, leading to the formation of food systems with desirable qualities. Soy globulins, mainly 7S (β-conglycinin) and 11S (glycinin) are the most abundant storage proteins in soybeans. Soy globulins are soluble in dilute solutions of neutral salts but insoluble in water. This is because ionic strength is one of the factors that influences the solubility of soybean proteins. Soy globulins are more soluble at pH away from their isoelectric point which is around pH 4.5-4.8, where they show the highest insolubility. This implies that the solubility of soy globulin is pH dependent. The solubility of soybean proteins can be compromised by extensive denaturation (Wagner & Anon, 1990; Ma et al., 1997) through heating. Okara hydrolysate showed lower solubility than soy hydrolysate, suggesting that okara proteins might have undergone denaturation as a result of severe heat treatment during soymilk manufacture (Chan & Ma, 1999a). Heating is a treatment utilised mostly in the food industries and it affects the solubility of globulins as it denatures proteins. This suggest the need to determine of the solubility of novel protein sources to ensure their successful utilisation in the food industry. Generally, determination of protein solubility is carried out by measuring the fraction of the protein that has been solubilised after subjecting the whole solution to a centrifugal force. Partial enzymatic hydrolysis could be used to improve the solubility of soybean proteins (Jung et al., 2005; Tsumura et al., 2005; Kempka et al., 2014). Protein solubility of okara was improved by partial enzymatic hydrolysis using Flavouzyme by up to 46 % when compared with non-hydrolysed protein (Kempka et al., 2014). An increase in the solubility of okara protein isolate (OPI) could

be achieved by increasing the level of deamidation through acid modification (Chan & Ma, 1999b). This approach could enhance the potential applications of okara as an ingredient within the food industry.

2.8.2 Emulsifying properties

The ability of plant proteins to stabilise emulsions is very important in a wide range of food products such as emulsified meat, cake batters, mayonnaise, salad dressings, frozen desserts, margarine, butter and frankfurter (Singh et al., 2008). The major aim of the addition of an emulsifier is to improve the emulsion formation and its stability (Hasenhuettl and Hartel, 2008). Studies on the emulsifying properties of glycinin and β-conglycinin showed that β-conglycinin can form more stable emulsion than glycinin, probably because of its lower molecular size and higher hydrophobicity than glycinin (Hayakawa and Nakai, 1985). Also Aoki et al. (2006) demonstrated that the emulsifying capacity and emulsion stability of a β-conglycinin-rich fraction were higher than those of a glycinin-rich fraction. Since the rate of adsorption at the interface contributes towards the emulsion formation, glycinin adsorbs slowly because of the presence of both inter- and intra-subunits of disulphide bonds which slow down the unfolding of the amino acid residue of glycinin at the interface (McClements, 2016). The presence of higher net charge in glycinin could cause higher repulsion between charged molecules around the interface which may subsequently retard adsorption. This suggests that the proportion of the two proteins would influence the emulsifying properties of the soy protein ingredient. Acid modification has been shown to improve the emulsifying activity but did not affect the emulsifying stability index (Chan & Ma, 1999b). However, Fierens et al. (2016) suggested that the emulsion stability of OPI could be improved through enzyme hydrolysis and enzymes that could yield hydrolysates with higher degree of hydrolysis included the commercial enzymes Alcalase and Esperase. This result is in contrast to the findings of Chan & Ma (1999a), where trypsin was used to hydrolyse OPI.

2.8.3 Foaming properties

Foaming properties are very important in some food applications in order to impart a desirable textural quality. Some of the aerated foods and beverages that require foaming agents to attain the desired aesthetic quality acceptable to consumers include ice cream, whipped cream, bread, cakes, extruded and expanded cereal based products, meringues, chocolate bars, marshmallow, champagne and beer (Campbell & Mougeot, 2000; Green et al., 2013). Foamability, which is the effectiveness of gas encapsulation, and foam stability, which is the life time of the foam, are two separate foaming properties that require consideration in food application (Wilde and Clark, 1996). OPI at extraction temperatures of 80 ^{o}C and 25 ^{o}C showed higher foaming stability than commercial soy protein isolate (Supro 610) (Ma et al., 1997), most likely because OPI might have more appropriate conformation that favours foaming properties which suggested that the protein molecules of OPI might have acquired high net charge density. The net charge density could influence the foaming stability by allowing the formation of highly viscous films and subsequently, formation of most stable foams (Nakai, 1983; Maeda et al., 1991; Phillips et al., 1994). Enzymatic hydrolysis of OPI did not lead to an improvement in foaming properties (Fierens, 2016). Foaming properties (both foamability and foam stability) were decreased after hydrolysing the OPI with trypsin (Chan & Ma, 1999a).

2.8.4 Water and fat binding capacities

Water holding capacity refers to the ability of proteins to strongly retain water and fat holding capacity is the ability of proteins to entrap fat in their matrices. Both properties are attributed to physical entrapment of either water or fat. These are important parameters in foods that require textural improvements such as comminuted meat, baked doughs and extruded doughs. The water holding capacity of OPI (4.3 mL/g – 5.1 mL/g) obtained using an alkaline extraction method compared favourably with that of commercial soybean protein isolate, Supro 610 (5.2 ml/g) (Ma et al., 1997). However, enzymatic hydrolysis could improve water holding

capacity but not fat binding capacity (Chan & Ma, 1999a). Vishwanathan et al. (2011) reported an improvement in both the water and fat binding capacity of OPI by subjecting OPI to membrane filtration to remove non-protein substances. Ma et al. (1997) reported higher fat binding capacity than Supro 610. Chan & Ma (1999b) also reported that OPI showed higher fat binding capacity than Supro 610, and that deamidation did not change the fat binding capacity of OPI, because it did not cause any effect on the bulk density of OPI.

2.9 Food applications of okara flour and okara proteins

Due to the high content of okara in a number of macro and micro-nutrients, research on okara has intensified over the last few years. Okara can either be used wholly or its ingredients in food product formulation. As manufacturers and researchers became aware through research of the inherent nutrients in okara and their physicochemical properties that would enable their application in food formulation, several products have been tested which include cookies with good quality and nutritional value (Park, 2015), molded sweet biscuits with 30 % (w/w) addition of okara and the level was considered adequate for confectioneries (Grizotto et al., 2010). Okara flour was successfully used to substitute wheat in bread making at 10 % level (Wickramarathna & Arampath, 2003). Okara can also be used as a fermentation stock for the production of seasonings, spices and tempeh (Wang and Cavins, 1989). There is existing market on incorporation of soy protein ingredients food products. Functional soy protein ingredients have found huge application in processed meat with about 55 % of approximately 1 million metric tons of functional soy protein ingredients produced annually being applied in processing of meat and seafoods (Hoogenkamp, 2007). Imitation meat products made with soy products found in the market include a range of meat-free products (eg. Tofurky and Gardein product). Soy protein isolates have found application in dairy-type products distributed mainly in China and Japan due to their fine particle size, dispersibility, emulsification properties, colour, and flavour. They have been used in different types of toppings to replace sodium

caseinate such as dry and liquid coffee whitener, liquid whipped toppings, prewhipped toppings (Singh et al., 2008). Imitation cheese such as tyne chease at Yumbles and toppings such as parmesan grated topping containing soy product are found in the market currently. There are different brands of egg-free mayo in the market containing soy protein. These include plamil egg-free mayo, primal kitchen mayo, and granovita Mayola. Soy protein isolates has also found applications in breakfast cereals due to people looking for convenient and highly nutritious meals. Soy proteins have been used to develop edible films to reduce fat absorption in deep-fried foods (Rayner et al., 2000). Alpro is a company that manufactures a range of plant-based dairy-free beverages including soy-milk beverage in the Europe. Overall, the market for soy-based products will continue to expand as the demand for healthy and nutritious food is increasing.

2.10 Protein structural chemistry

Protein structure is a combination of different amino acids by a peptide bond to form a three-dimensional network of amino acid chain. This amino acid chain can be arranged in different conformations leading to proteins with different structures and different functions. Reversible conformational changes could occur to give rise to different conformational isomers with different functional properties from the original protein structure. On the other hand, the protein structure can be viewed as ordered arrangement of secondary structural elements or components, forming a three-dimensional structural network. The native structure of proteins exhibits different sizes ranging from 1 nm to 100 nm involving tens to thousands of amino acids. Hence, based on physical size, proteins are classified as nanoparticles (Brocchieri & Karlin, 2005). Although, subunits of proteins can interact to form a very large protein aggregate.

2.10.1 Levels of Protein Structure

There are four distinct levels of protein structural conformation. These are:

a) Primary Structure: Linear sequence of amino acids linked by peptide bonds to form amino acid chain referred to as unfolded proteins.

b) Secondary Structure: The main or regular secondary structural components of proteins are α-helices and β-sheets which are formed by hydrogen bonds between the C═O and NH groups of the amino acid make up. There could be existence of turns, which links the regular secondary structural elements (α-helices and β-sheets), possesses three dimensional configurations and occurs on the surface of the protein structure, most importantly where the polypeptide chain changes its overall direction and lastly is the coil, which is any other component of the protein structure that is neither α-helices, β-sheets, nor turns.

c) Tertiary Structure: This is the actual three-dimensional conformation of the polypeptide chain, stabilized by interactions between the side chains ('R' groups). Examples of these interactions are ionic interactions, hydrogen bonds, van der waals dispersion forces and sulphur bridges.

d) Quaternary Structure: This occurs as a result of the aggregation of two or more three-dimensional polypeptide chains or protein subunits to give rise to a globular structure with a specific functional property (multimer). The stability of the multimer is achieved by non-covalent interactions and disulfide bonds, similar to the interactions that stabilizes the tertiary structure.

2.10.2 Determination of protein structure

There are quite several techniques available for determination of the structural conformation of proteins. The x-ray crystallography is the main technique that has been used for determination of protein structures. It operates by measuring the three-dimensional density distribution of electrons in the proteins, in crystallised state. The nuclear magnetic resonance (NMR) has been employed for protein structure determination but not as often as the x-ray

crystallography. When very large protein complexes are being determined, the cryo-electron microscopy is used, although, its resolution is lower than that of x-ray crystallography and NMR. Specifically, for secondary structure composition determination, circular dichroism is mainly used. While infrared spectroscopy is used to characterise the secondary structural conformation of peptides, polypeptides and proteins, the two-dimensional infrared spectroscopy is a preferred technique to study the structure of flexible proteins that cannot be characterised by other methods.

2.10.3 Infrared Spectroscopy

Infrared spectroscopy (IR) is an important technique used for analysis of the secondary structural composition of proteins and polypeptides (Smith, 2011). FTIR measures the wavelength and intensity of absorption of infrared radiation by the sample. It has been shown that polypeptides and proteins produce nine distinct IR absorption bands, which are amide A, B, and I-VII as given in the **Table 2.10** below when analysed with FTIR. Amongst all the characteristic bands, amide I and II are the most important bands of the protein residue (Krimm & Bandekar, 1986; Surewicz & Mantsch, 1988; Smith, 2011). Amide I band (1700-1600 cm-1) is the most sensitive spectral region and it is caused by C═O stretch vibrations of the peptide linkages (approximately 80 %), while amide II is caused by in-plane NH bending which is about 40-60 % of the potential energy) and from the CN stretching vibration (approximately 18-40 %) which rendered it lesser sensitive to conformational changes when compared with amide I band (Smith, 2011). Hence, Amide I is further analysed and used to determine the secondary structural composition of proteins and polypeptides because it is highly sensitive to small alteration in its geometrical and hydrogen bonding orientation.

Table 2. 10: Description of the infrared bands of the peptide backbone

Band names	Frequency range (cm^{-1})	Interpretation
Amide A	3300	NH stretching
Amide B	3100	NH stretching
Amide I	1600-1700	C═O stretching
Amide II	1480-1575	CN stretching, NH bending
Amide III	1229-1301	CN stretching, NH bending
Amide IV	625-767	OCN bending
Amide V	640-800	Out-of-plane NH bending
Amide VI	537-606	Out-of-plane C═O bending
Amide VII	200	Skeletal torsion

Source: (Krimm & Bandekar, 1986; Bandekar, 1992).

Based on the fact that proteins are made up of different secondary structural elements, proteins can be quantitatively analysed. There are different methods that have been used to quantitatively elucidate the different types of secondary structural components in proteins from their original IR amide I spectra. These methods include Fourier self-deconvolution (FSD) curve fitting (Kauppinen, Moffatt, Mantsch, & Cameron, 1981; Byler & Susi, 1986) second derivative analysis (Byler & Susi, 1986; Dong, Huang, & Caughey, 1990); partial least-squares analysis (D. C. Lee, Haris, Chapman, & Mitchell, 1990), and data basis analysis (Sarver & Krueger, 1991). The most commonly used methods are the FSD-curve fitting and second derivative analysis. However, amide I band second derivative spectra could be assigned to each type of the secondary structural elements based on their characteristic impact to C═O stretching frequency which is enabled because of their unique molecular geometry and hydrogen bonding pattern. Established second derivative amide I band assignment to each type of secondary structural elements for proteins in both deuterium (D_2O) and water (H_2O) is given in **Table 2.11** below. Measurement in H_2O is preferable than D_2O, because D_2O tend to change the properties of the sample slightly when compared with the same sample in native state.

Table 2. 11: Assignment of deconvoluted amide I band frequencies to secondary structural components of proteins

Frequency (H_2O)	Assignment	Frequency (D_2O)	Assignment
1624±1.0	β-sheet	1624±4.0	β-sheet
1627±2.0	β-sheet	1631±3.0	β-sheet
1633±2.0	β-sheet	1637±3.0	β-sheet
1638±2.0	β-sheet	1645±4.0	Random
1642±1.0	β-sheet	1653±4.0	α-helix
1648±2.0	Random	1663±4.0	β-turn
1656±2.0	α-helix	1671±3.0	β-turn
1667±1.0	β-turn	1675±5.0	β-sheet
1675±1.0	β-turn	1683±2.0	β-turn
1680±2.0	β-turn	1689±2.0	β-turn
1685±2.0	β-turn	1694±2.0	β-turn
1691±2.0	β-sheet		
1696±2.0	β-sheet		

Source: (Byler & Susi, 1986; Dong, Huang, & Caughey, 1992; Dong, Caughey, Caughey, Bhat, & Coe, 1992)

Chapter 3- Materials and methods

3.1 Preparation of defatted okara flour

Yellow soybean seeds (*Glycine max*) were soaked in water (1:5 w/v) for 8 hours and hulls were removed manually by washing. Dehulled, washed beans were ground with a hammer mill. Milk was separated from ground soybean slurry using a clean linen cloth and the obtained wet okara was dried in an oven dryer (60±2 ^{o}C) for 4 hours. The dried okara flakes were ground into a fine flour of particle size less than 1.0 mm. The okara flour was defatted using hexane in the ratio of 1:10 (w/v) for 30 min while continuously stirring in a beaker prior to use. The summary of the okara flour processing procedure is presented in **Fig.3.1**.

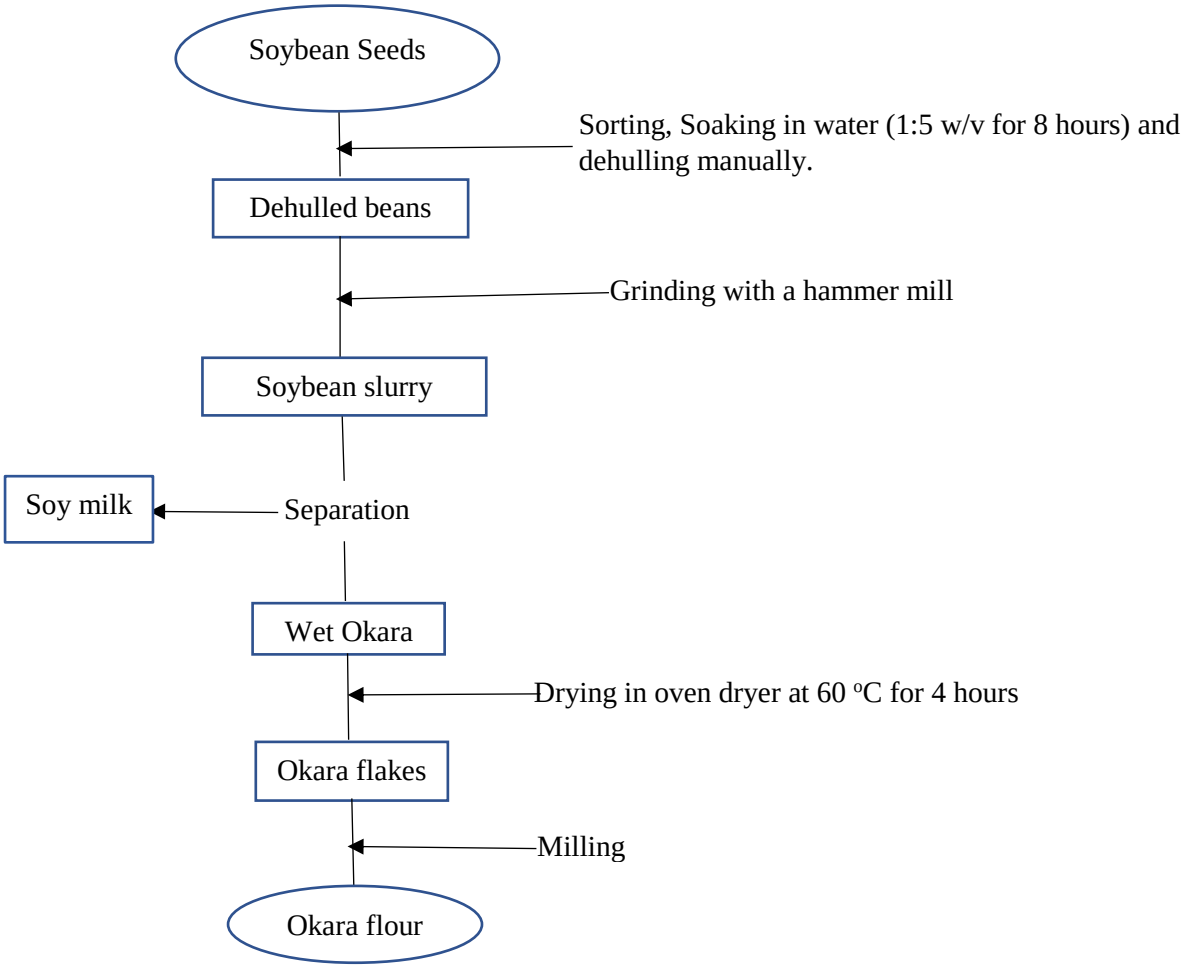


Fig.3. 1: Okara flour processing flow chart

3.2 Conventional method of protein extraction process

Defatted okara flour was mixed with 0.1 M phosphate buffer (pH 8-12) or carbonate buffer (pH 9-11). In preliminary trials, various solid : liquid ratios were tested (1:20, 1:10 and 1:5), and the ratio of 1:20 was selected due because it gave the highest protein recovery results. Preliminary studies also showed that most of the proteins were extracted between 30 min to 40 min, and thus, 1 hour was chosen as the duration of the extraction. The sample slurry was placed in a 60 ^{o}C water bath with stirring for 1 h. The protein extract/supernatant was recovered by vacuum filtration using Buchner funnel and the protein extract was precipitated at pH 4.5-4.8 with 2 N HCl and cooled to 4 ^{o}C overnight to enhance the precipitation. The precipitate was recovered after centrifugation at 10,000 *x* g for 10 min. The obtained protein pellet was washed twice and re-dissolved in distilled water, the pH was adjusted to 7.0 using 2N NaOH and then freeze dried to obtain the protein isolate using Virtis sp scientific freeze dryer (2KBTES, Warminster, Pennsylvania). The process scheme is presented in **Fig.3.2**.

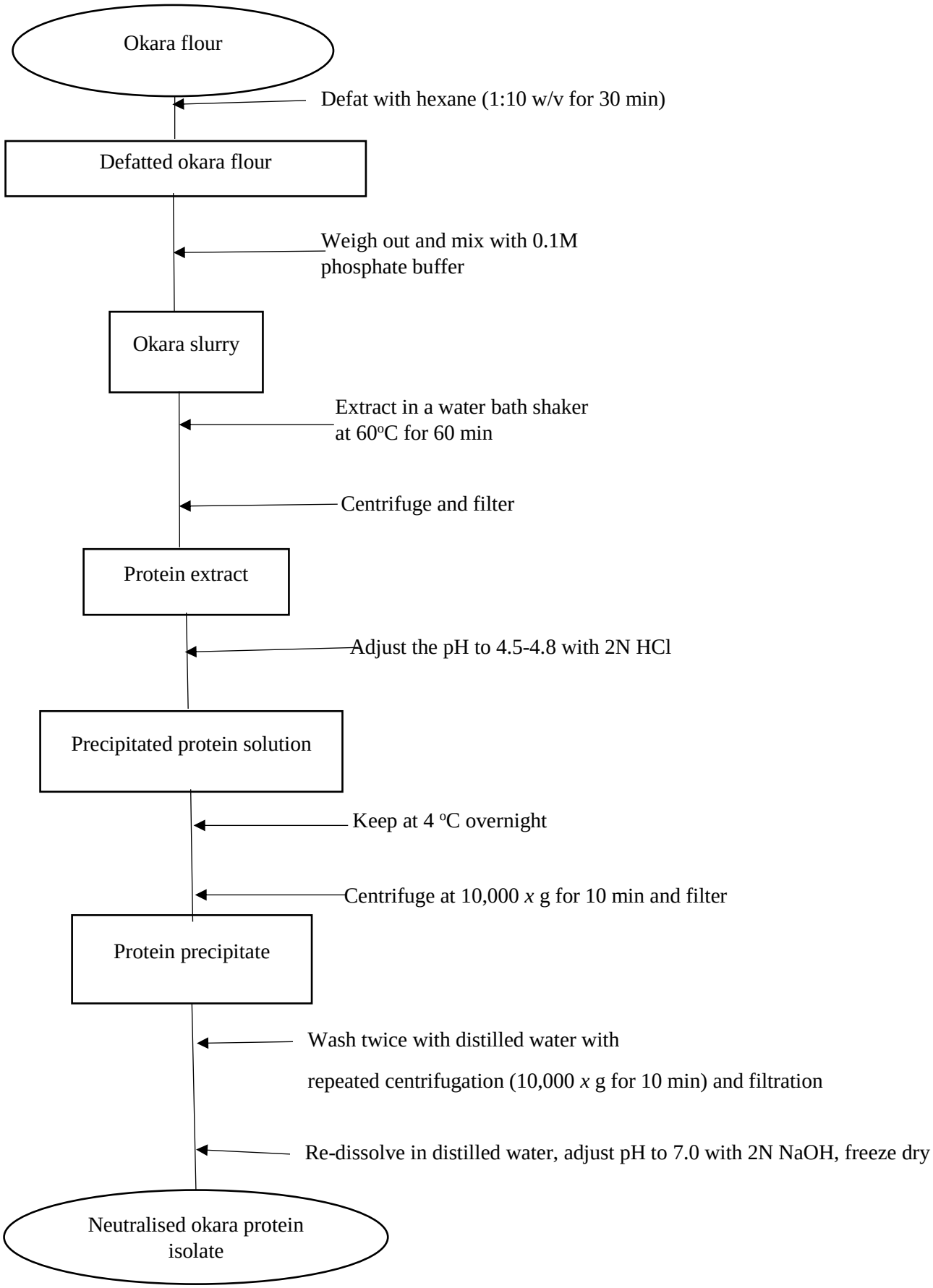


Fig.3. 2: Aqueous alkaline protein extraction process scheme.

3.3 Ultrasonication-assisted alkaline extraction

The system included an ultrasonication transducer that operated at a nominal frequency of 20 KHz and was submerged to a depth of 1-2 cm in the sample as shown in **Fig.3.3**. The flow scheme of the ultrasonication protein extraction process is shown in **Fig.3.4**. The ultrasonicator maximum amplitude and power are 16 μmpp and 100 W. The samples were contained in a double wall flow cell through which water was circulated to maintain the temperature of extraction constant (60^{o}C). Two different sonication amplitudes were monitored, and these are referred to as low (5 μmpp), and medium (10 μmpp) amplitudes at two different times (50 min and 25 min). Defatted milled okara flour was mixed with 0.1 M phosphate buffer pH 12 in the ratio of 1:20 (w/v) in a beaker and the pH adjusted back to 12 before the slurry was transferred into a double walled flow cell for ultrasonication extraction. The extraction was carried out for 50 min. The obtained slurry was centrifuged at 10,000 *x* g for 10 min at 4 ^{o}C. The pH of the supernatant was reduced to 4.5 using 2 N HCl to isolate the proteins, refrigerated at 4 ^{o}C overnight, and centrifuged at 10,000 *x* g for 10 min at 4 ^{o}C to recover the isolated protein precipitate. The precipitate was washed twice with distilled water, centrifuged after each washing at the same conditions and was dispersed in distilled water with its pH adjusted to 7.0 with 2 N NaOH. The neutralized protein was frozen and then freeze-dried, using Virtis sp scientific freeze dryer (2KBTES, Warminster, Pennsylvania), ground and packaged in an air-tight container and stored in a desiccator until further use.

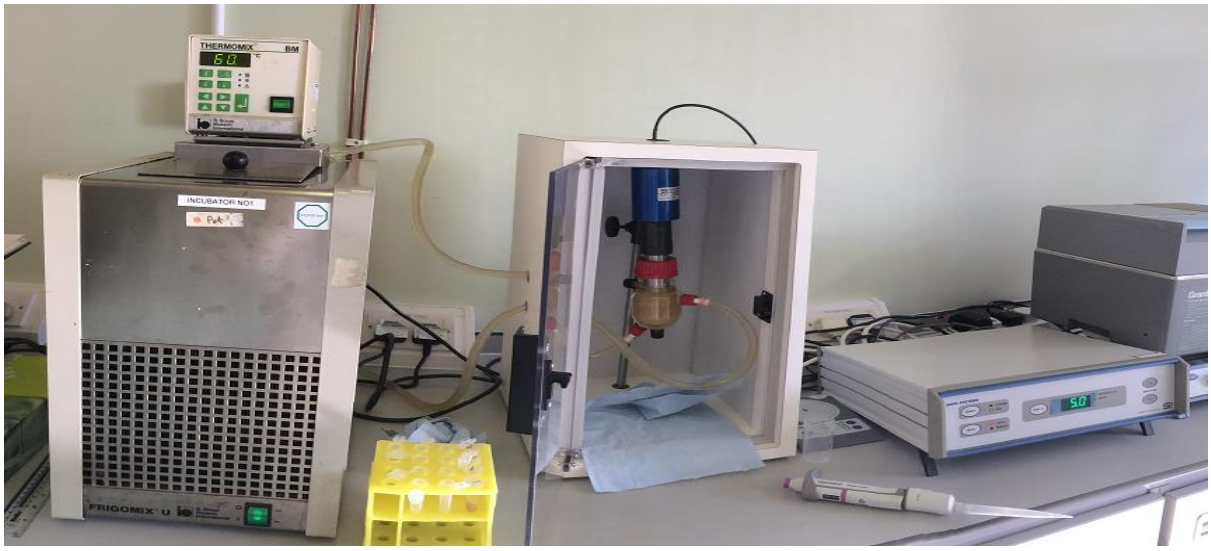


Fig.3. 3: Ultrasonication set-up with recirculating water bath for protein recovery from okara

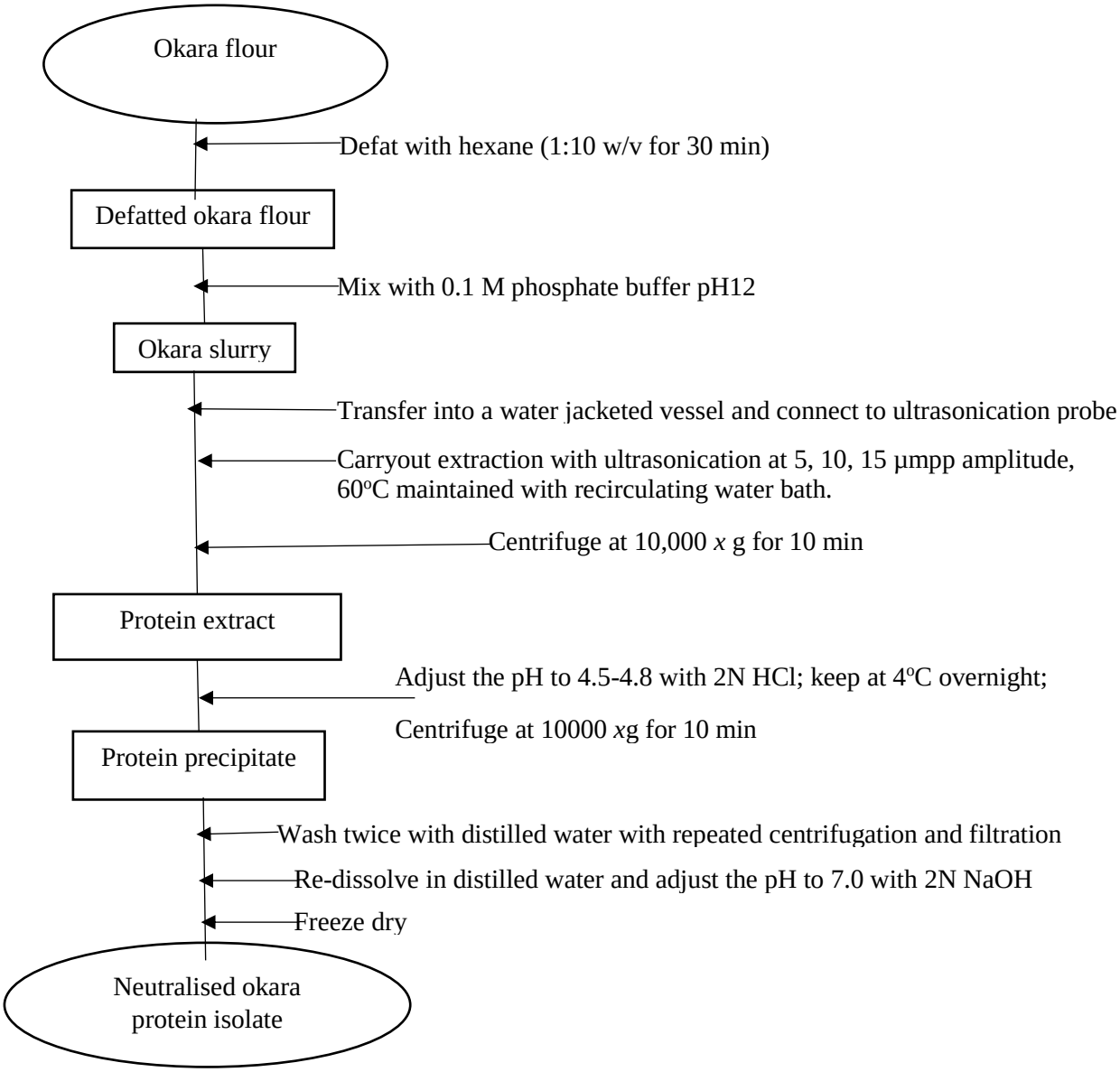


Fig.3. 4: Flowchart of ultrasonication method of protein extraction from okara flour

3.4 Chemical composition determination

Moisture, fat, ash and protein contents were determined according to the methods of AOAC (2000).

Fat content was determined by soxhlet method using soxhlet extractor with a reflux condenser and a 200 mL round bottom flask was set up. Two grams (2 g) of the flour samples were weighed into a labelled thimble and plugged with cotton wool. The round bottom flask was filled with 50 mL of petroleum ether. The Soxhlet apparatus after assembling was allowed to reflux for about 5 hours. The thimble was removed with care and the petroleum ether was removed with rotary evaporator set at 55 ^{o}C. The flask was then dried at 60 ^{o}C overnight in an oven (to remove any residual solvent) and then cooled in a desiccator before weighing.

Calculation

$$\%\ Fat\ \ content\ =\ \frac{weight\ of\ \ fat}{weight\ of\ \ sample}\ \ x\ \ 100\,(\text{w/w}) \qquad (3.1)$$

Protein was determined by kjeldahl method, as digestion converts any nitrogen in the food sample into ammonia which binds to the sulphate ion and remains in the solution as ammonium sulphate, protein in okara was determined based on the total nitrogen content. Okara samples (2 g) were weighed in ashless paper and put in Kjeldahl digestion tubes. Two tablets of Kjeldahl catalyst (each tablet contains 5 g potassium sulphate and 5 mg of selenium) were added followed by the addition of 25 mL of concentrated H_2SO_4 (97%, v/v). The flask with the content was heated in a heating block placed under a fume cupboard until the solution became clear for about 60-90 min. The solution was allowed to cool down before placed in the distillation unit.

$$N(food) \longrightarrow (NH_4)_2SO_4 \qquad (3.2)$$

The digestion tube containing the digest was transferred to distillation apparatus and a 100-ml conical flask, (receiving flask) containing 50 mL of 2 % (w/v) boric acid (H_3BO_3) and two drops of methyl red indicator was placed under the condenser of the distillation apparatus. The solution in the digestion tube was then made alkaline by the addition of sodium hydroxide, which converted the ammonium sulphate into ammonia gas:

$$NH_4SO_2 + 2NaOH \rightarrow 2NH_3{\uparrow} + 2H_2O + Na_2SO_4 \qquad (3.3)$$

The ammonia gas liberated was distilled into the receiving flask containing boric acid and gets converted into ammonium ion and boric acid into borate ion:

$$NH_3 + H_3BO_3 \text{ (boric acid)} \longrightarrow NH_4^+ + H_2BO_3^- \text{ (borate ion)} \quad (3.4)$$

The solution in the receiving flask (ammonium borate) was titrated with 0.1 N H_2SO_4 and was indicated by a pink end point enabled by the presence of methyl red indicator. The same procedure was carried out on the blank (sucrose) and standard (glycine).

$$H_2BO_3^- + H^+ \longrightarrow H_3BO_3 \quad (3.5)$$

Calculation:

$$\%\ Nitrogen\ of\ the flour\ sample(\%N) = \frac{(V_S - V_B)}{W}\ x\ N\ acid\ x\ 0.01401\ x\ 100$$

(w/w) (3.6)

Where V_S = Volume (ml) of acid required to titrate the sample.

V_B = Volume (ml) of acid required to titrate the blank (sucrose).

N acid = Normality of acid (0.1N)

W = Weight of sample in gram

% Crude protein = %N x 6.25 (conversion factor).

The ash content was analysed by dry method. The flour samples (2 g) were weighed into preheated, cooled and weighed crucibles. The samples were first charred on a Bunsen flame inside a fume cupboard and then transferred into a preheated muffle furnace at 550 ^{o}C for 4 hours until a white or light grey ash was observed. Then, the samples were cooled down in a desiccator and their weight was recorded. The ash content was calculated as follows:

$$\%\ Ash\ content = \left(\frac{W_3 - W_1}{W_2 - W_1}\right)\ x\ 100\ (w/w) \quad (3.7)$$

Where W_1 = Weight of empty crucible

W_2 = Weight of crucible + weight of sample before ashing

W_3 = Weight of crucible + weight of sample after ashing.

Moisture content was analysed by dry basis. Briefly, the crucibles were washed thoroughly, dried in the oven at 100 ^{o}C for 1 hour, cooled in a desiccator and their weight was

recorded. Two grams of the flour sample were weighed into the crucible and placed in an oven at 100 ^{o} C. The weight of the samples was recoded periodically until a fairly constant weight was obtained.

$$\%\ Moisture\ content\ = \left(\frac{W_2 - W_3}{W_2 - W_1}\right)\ x\ 100\ (w/w) \qquad (3.8)$$

Where W_1 = Initial weight of empty crucible.

W_2 = Weight of crucible + weight of sample before drying.

W_3 = Weight of dish + weight of sample after drying.

3.5 Estimation of protein in the extract

The protein content of okara extracts was measured according to Bradford (Bradford, 1976). The procedure is based on the formation of a complex between the dye (Brilliant blue G) in the Bradford reagent (Sigma-Aldrich, UK) and the protein present in the sample. An aliquot of 1.5 mL of the Bradford reagent was mixed with 50 µL of appropriately diluted protein solution (based on the concentration of the protein solution) and the absorbance of the mixture was read at 595 nm with a spectrophotometer (BioMate 3, Madison, WI 53711 USA). Bovine serum albumin (BSA), (Sigma-Aldrich, UK) was used as standard for the calibration curve. The yield of the protein based on the extract was estimated as the ratio of weight of the protein in the protein extract to the weight of the protein in the defatted okara.

$$\%\ \text{Protein yield} = \frac{Weight\ of\ protein\ in\ the\ extract}{Weight\ of\ protein\ in\ the\ defatted\ okara}\ x\ 100\ (w/w) \qquad (3.9)$$

3.6 SDS PAGE analysis

SDS-PAGE was performed using a XCell SureLock Mini-Cell device and NuPAGE Bis-Tris Mini Precast Gels (4 - 12 %, 1.0 mm, 12 wells) with MES SDS Running Buffer. The concentration of all protein samples was standardised to 1 mg/mL prior to use and 50 µL of the diluted protein sample were mixed with 25 µL of NuPAGE LDS sample buffer. 10 µL of NuPAGE reducing agent and 15 µL of deionised water were also added in the mixture. Samples were heated at 70 ^{o}C for 10 min and 10 µL of each of the sample mixtures was loaded into a

Tris Mini Precast Gels well along with 7.5 µL of a prestained Molecular Weight Marker (MWM). NuPAGE antioxidant was mixed with the running buffer in the set up to maintain the proteins in their reduced state during electrophoresis. On completion of the electrophoresis, the gels were washed for 5 min three times with deionised water and were stained with the SimplyBlue SafeStain for 60 min. The staining solution was discarded, the gel was rinsed once with deionised water and was gently shaken in deionised water overnight in an orbital shaker (Stuart Scientific mini orbital shaker-SO5, UK) to allow for proper resolution of the bands which were captured using a SYNgene G:Box (Chemi XR5, UK) image capturing device .

3.7 Reversed phase-high performance liquid chromatographic (RP-HPLC) analysis

The profile of the proteins in okara protein isolates and commercial soybean proteins (CSP) was determined with RP-HPLC according to the method described by Garcia, et al. (1997) with some modifications. The protein isolate samples were dispersed in distilled water and the concentration confirmed using Bradford method. Afterwards, the samples were standardized to 6 mg/mL. The column used was ACE 5 C18, 250 × 4.6 mm, 5 mm, 100 Å; on a 1100 Agilent HPLC. The volume of each sample injected into the system at a flow rate of 1 mL/min was 50 µL and the column temperature was maintained at 50 ^{o}C. The separation of the proteins in the samples according to their increasing hydrophobicity was detected by UV absorption at 254 nm wavelength. A mixture of two solvents was used to carry out the gradient elution. Eluent A which was the weak mobile phase was 0.1 % TFA in water and Eluent B which was the strong mobile phase was 0.1 % TFA in acetonitrile. A 5step linear gradient was used and the first 3 steps which included; 5-20 % B; 20-25 % B; 25-35 % B; were performed for 10 mins each; followed by a 35-45 % B allowed for 30 seconds. Then, a linear reversed gradient of 45-5 % B was performed for 5 mins in order to re-equilibrate the column for the next sample.

3.8 Total and free sulfhydryl contents

Total and free sulfhydryl (SH) content of okara protein isolates and the commercial soybean protein isolate was determined using the modified method of Tang et al. (2009). In brief, free sulfhydryl (FSH) content was determined by dissolving 50 mg of protein isolate samples in 10 mL of Tris-glycine buffer (86 mM Tris, 90 mM glycine, 4 mM ethylenediaminetetraacetic acid, pH 8.5) containing 8 M urea and then kept overnight at room temperature with gentle mixing using an orbital shaker. The protein solution was centrifuged in 50 mL centrifuge tube at 10 000 *x* g for 10 min at room temperature and the supernatant was collected. Protein concentration in the supernatant was determined by Bradford method and then diluted to 0.1 mg/mL with Tris-glycine buffer. A 1 mL aliquot of the sample reacted with 10 µL of Ellman's reagent (0.4% 5,5'-dithiobis-[2-nitrobenzoic acid] in 10 mM Tris-glycine buffer, pH 8.5] for 10 min at room temperature and another 1 mL aliquot of the same sample was taken without Ellman's reagent (used as blank), and absorbance was read at 412 nm using ultraviolet-visible (UV) spectrophotometer (BioMate 3, Madison, WI 53711, USA) and plastic cuvettes (1 cm path length). For total SH content determination, 50 mg of protein isolate was dissolved in Tris-glycine buffer (pH 8.5) containing 8 M urea and kept overnight at room temperature with continuous gentle mixing. A 1 mL aliquot of the protein solution was diluted with 4 mL of Tris-glycine buffer (pH 8.5), then 50 µL of 2-mercaptoethanol was added and the mixture was left to stand for 1 h at room temperature. Then, 10 mL of 12 % (w/v) trichloroacetic acid (TCA) were added to the mixture, left for 1 h at room temperature, and centrifuged at 10,000 *x* g for 10 min at 4 ^{o}C to collect the precipitate. The precipitate was re-suspended in 5 mL of 12 % (w/v) TCA with subsequent centrifugation at 10,000 *x* g for 10 min to remove residual 2-mercaptoethanol. The washed pellet was re-dissolved in 2 mL of Tris-glycine buffer (pH 8.0) containing 8 M Urea and protein concentration determined using the method of Bradford with BSA as a standard and afterwards, diluted to final concentration of 0.1 mg/mL.

A 1 mL aliquot of the sample was reacted with 10 µL of Ellman's reagent (0.4% 5,5'-dithiobis-[2-nitrobenzoic acid] in 10 mM Tris-glycine buffer, pH 8.5) for 10 min at room temperature and another 1 mL aliquot of the same sample was taken without Ellman's reagent (used as the blank to zero the instrument). The absorbance was read at 412 nm using ultraviolet-visible (UV) spectrophotometer and plastic cuvettes (1 cm path length).

Total and free SH (µmol/g) contents were determined with the equation below:

$$\text{SH} = \frac{75.35 \times A \times D}{C}, \quad (3.10)$$

where A is the absorbance, D is the dilution factor, and C is the protein concentration (0.1 mg/mL) while 75.35 is a constant derived from a conversion factor (10^6) to change molar basis of the protein to a µmol/mL basis and from milligrams of solids to grams of solids, divided by molar absorptivity ($1.36 \times 10^4 M^{-1}cm^{-1}$).

Disulphide bonds content (SS) for each sample was determined based on the equation below:

$$\text{SS} = \frac{(SHtotal - SH\ free)}{2} \quad (3.11)$$

3.9 Amino acid determination

Okara protein extracts (0.1 g each) were mixed with 6 M HCl in a sealed container and nitrogen was flushed into it to prevent oxidation reactions and then the suspension was hydrolysed for 24 hours at 110 ^{o}C. The hydrolysate was neutralised with $CaCO_3$ powder prior to derivatization. The neutralised hydrolysate was analysed for amino acid content using the EZ-Faast amino acid derivatization kit for GC-MS (Phenomenex, Torrance, CA) (Husek 2000). The derivatization was carried out on 100 µL of each sample using the EZ-Faast amino acid analysis kit for free amino acid analysis by GC-MS. The same procedure was carried out on the standards. The derivatised amino acids were analysed in electron impact mode using

Agilent 5975 system (Agilent, Palo Alto, CA). An aliquot (1 µL) of the derivatised amino acid solution was injected in split mode (40:1) at 280 ^{o}C onto a zebron ZB-AAA capillary column (10 m × 0.25 mm; 0.25 µm film thickness), with the flow rate of the carrier gas held at 1.5 mL per min, throughout the run. The oven temperature was kept at 110 ^{o}C for 1 min and then increased at 30 ^{o}C per min to 310 ^{o}C, while the transfer line and ion source were kept constant at 320 ^{o}C and 230 ^{o}C respectively. Samples and standards were analysed in duplicate and the retention time of the standards were used to identify the respective amino acids peaks and the concentrations extrapolated and calculated as follows:

$$\text{Amino acid (mg/mL)} = \frac{MWx\ C}{0.1g} xVxDF, \quad (3.12)$$

where,

V= Volume of the hydrolysate

DF= dilution factor

MW= molecular weight of the amino acid

C= Concentration of the amino acid in µM/L

3.10 Surface hydrophobicity content determination

Surface hydrophobicity of the okara protein isolates was determined following the method of Hayakawa & Nakai (1985) with some modifications. Protein solutions were prepared by suspending 200 mg of the isolates in 10 mL of 0.01 M sodium phosphate buffer pH 7.0 and incubating at room temperature for 3 hours with continuous gentle mixing, after which the suspensions were centrifuged at 10,000 *x* g for 10 min at 4 ^{o}C. Protein concentration of the supernatant was determined with Bradford method (Bradford, 1976). Surface hydrophobicity was determined by adding 40 µL of 8 mM ANS (prepared in 0.1 M phosphate buffer pH 7.0) to the final protein concentration and without the addition of 8 mM ANS (served as control) and then incubated in the dark for 30 min. Fluorescence intensity (FI) was measured at wavelengths of 390 nm (excitation) and 470 nm (emission) at 20 ^{o}C±0.5 ^{o}C with a constant

excitation and emission slit of 5 nm with Varian Cary Eclipse Fluorescence spectrophotometer. The net FI (sample with ANS minus sample without) was plotted against protein concentrations and surface hydrophobicity was taken as the slope. All measurements were performed in duplicate.

3.11 Analysis of sugar composition

The determination of the carbohydrate content in okara was carried out according to the method by Sluiter et al. (2011). Briefly, samples (300 mg) were put into a test tube with 3 mL of 72 % (v/v) sulphuric acid and mixed thoroughly. The tubes were incubated in a water bath set at 30 ^{o}C for 60 min with continuous shaking at 200 rpm. At the end of the first hydrolysis, the content of the tubes were added in Duran bottles and were diluted with 84 mL of distilled water to 4 % (v/v) of sulphuric acid concentration, thoroughly mixed and incubated at 121 ^{o}C for 30 min. Sugar recovery standards (SRS) were also used for the determination of sugar losses during hydrolysis and were mixed with 4% (v/v) of 72 % sulphuric acid and incubated at 121 ^{o}C for 30 min. The hydrolysed sample solutions were vacuum filtered. The solid residues were used to analyse acid insoluble lignin, by drying first at 105 ^{o}C for 4 hours until constant weight. Then, the crucible and dried residue were transferred to muffle furnace to be ashed at 575 ^{o}C for 6 hours. For the determination of acid soluble lignin, part of the filtrate was used, and its absorbance was measured with a spectrophotometer (BioMate 3, Madison, WI 53711 USA) at 320 nm. The remaining part of the filtrates were neutralised with $CaCO_3$ powder to pH 5-6. The neutralised samples were allowed to settle, and the clear supernatant was recovered and filtered with 0.2 μm filter and placed into an autosampler vial for the analysis of monomeric sugars. The latter was performed in a Dionex ion chromatography system (Sunnyvale, CA) consisting of a pulsed amperometric detector (PAD), an autosampler and a gradient pump. The system was also equipped with a CarboPac PA1 (4 × 250 mm) analytical column and a CarboPac PA1 (4 × 50 mm) guard column (Dionex Corp., Sunnyvale, CA). The injection

volume was 20 μL and the flow rate was maintained at 1.0 mL/min at room temperature. The mobile phase used were 0.1 M NaOH and 0.1 M NaOAc and the gradient scheme used to run the sample was as follows: 90:10 % A:B for 20 min; 80:20 % A:B for 10 min; 50:50 % A:B for 30 min. Peaks were identified using the respective monomeric sugar standards (rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose) in solutions of known concentrations. Data analysis was performed using a Chromeleon V6.8 (Thermo, UK).

3.12 Functional properties analysis

3.12.1 Protein solubility

Determination of protein solubility was carried out by dispersing 20 mg of protein isolate samples in 20 mL distilled water, adjusted to pH 1.5- 10 with either 0.1 N HCL or 0.1 N NaOH. Samples were magnetically stirred at room temperature for 20 min and centrifuged at 10,000 *x* g for 10 min. Protein content of the supernatant was determined by Bradford (Bradford, 1976).

3.12.2 Water absorption capacity (WAC)

Water absorption capacity of the OPI and CSP was determined following the method described by Wang et al. (2006) with some modifications. Protein samples were dispersed in distilled water 2 % (w/v) and the pH was adjusted to 7. The mixture was mixed vigorously using a vortex for 2 min and incubated at room temperature for 20 min before it was centrifuged at 5,000 *x* g for 10 min at 20 ^{o}C. The supernatant was removed, and its volume was recorded in order to calculate the WAC as follows:

$$\text{WAC (in mL/g)} = \frac{mL\ of\ liquid\ added - mL\ of\ liquid\ removed}{mass\ of\ protein\ sample} \quad (3.13)$$

3.12.3 Oil absorption capacity (OAC)

The OAC was measured as the volume of oil absorbed per mass of protein sample (Wasswa et al., 2007) with some modifications. Protein sample was mixed with olive oil 10 % (w/v) and vortexed vigorously for 30 seconds before incubation at room temperature for 30

min. The oil dispersion was centrifuged at 13,600 *x* g for 10 min at 25 °C and the volume of oil was recorded for calculation of the OAC as follows:

$$\text{OAC (in mL/g)} = \frac{mL\ of\ oil\ added - mL\ of\ oil\ removed}{mass\ of\ protein\ samples} \quad (3.14)$$

3.12.4 Emulsion capacity and stability (EC & ES)

The emulsion capacity of okara protein samples was determined according to Naczk et al. (1985) with some modifications. Protein samples (0.05 g) were dispersed in 10 mL of distilled water and the pH of the solution was adjusted to 7 before 10 mL of olive oil were added and then the whole mixture was homogenized using Ultra Turrax T25 S7 (IKA-Labortechnik, Germany) homogenizer for 1 min at room temperature at 13,500 rpm. The formed emulsion was centrifuged at 1,300 *x* g for 5 min and the EC was calculated as follows:

$$\text{EC (\%)} = \frac{Volume\ of\ emulsified\ layer\ after\ centrifugation}{Total\ volume\ of\ emulsion\ formed\ before\ centrifugation}\ x\ 100 \quad (3.15)$$

The ES was determined by subjecting the formed emulsion to heat for 30 mins at 80 °C in a water bath before centrifugation at 1,300 *x* g for 5 min. The remained emulsified layer was recorded, and the ES was calculated as follows:

$$\text{ES (\%)} = \frac{Volume\ of\ remaining\ emulsified\ layer\ after\ heating\ \&\ centrifugation}{Volume\ of\ emulsion\ formed\ immediately\ after\ homogenization}\ x\ 100 \quad (3.16)$$

3.12.5 Foaming capacity and stability (FC & FS)

The FC and FS of the OPI and CSP were determined following the method of Liu et al. (2010). Protein solutions (0.5 % w/v) were prepared and the pH adjusted to pH 7. The protein solution was homogenised at 13,500 rpm using Ultra Turrax T25 S7 (IKA-Labortechnik, Germany) homogenizer for 2 min. The total volume of the whipped sample was recorded, and the FC was calculated as follows:

$$\text{FC (\%)} = \frac{Total\ volume\ after\ whipping}{Total\ volume\ before\ whipping}\ x\ 100 \quad (3.17)$$

To calculate for the FS, the whipped sample was allowed to stand for 5 min and the total volume was recorded.

$$\text{FS (\%)} = \frac{Total\ volume\ after\ standing\ for\ 5\ \text{min} - Total\ volume\ before\ whipping}{Total\ volume\ before\ whipping}\ x\ 100 \quad (3.18)$$

3.13 Measurement of zeta potential

The zeta potentials of all the protein isolates were determined by a laser doppler electrophoresis and phase analysis light scattering (PALS) technique using a Malvern Zetasizer Nano-ZS (model: ZEN3600) instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The protein samples were dispersed in distilled water (0.5 % w/v), allowed to stand for few minutes and 1 mL was collected without shaking the protein dispersion and transferred into an electrophoresis cell (model: DTS 1060C, Malvern Instruments Ltd., Malvern Worcestershire, UK). The analysis was run at 25 ^{o}C and average values of 3 measurements of each sample were generated and each sample was prepared in triplicate.

3.14 Measurement of size particles

The size particle distribution or the z-average diameter of all the protein isolates was determined by a dynamic light scattering technique using a zetasizer Nano-ZS (model: ZEN3600) instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The protein samples were dispersed in distilled water (0.5 % w/v) and diluted further in distilled water to obtain an appropriate concentration index in the zetasizer and to obtain polydispersity index (PDI) below 0.5 which indicates accurate measurement.

3.15 Structural properties analysis

3.15.1 Fourier transform infrared spectroscopic analysis (FTIR)

The okara protein isolate samples were measured in a Perkin-Elmer Spectrum 100 FTIR spectrophotometer at room temperature by placing the samples on the crystal cell and the cell was clamped into the pin hole of the FTIR spectrophotometer. The spectra from the samples in the range of 600 – 4000 cm^{-1} wavenumbers, averaged from 16 scans at a resolution of 4 cm^{-1} were automatically recorded against a background spectrum from the clean empty cell at room

temperature. The amide 1 spectrum was deconvoluted and then curve fitted at 100% Gaussian to calculate the percentages of the secondary structures in it using WIRE 4.0 software.

3.15.2 Scanning electron microscopy (SEM)

The structural morphology of the different protein isolates was evaluated using a scanning electron microscope (FEI QUANTA 600 FEG SEM), at an accelerating voltage of 20.0 kV. The samples were first administered on a Leit Adhesive Carbon Tabs placed on Aluminium SEM Pin stub and then gold coated using a gold sputter coater (model: Edwards S150B) before scanned with the FEI Quanta 600 FEG SEM instrument.

3.15.3 Differential scanning calorimetric (DSC) analysis of the samples

Samples of OPI and CSP were analysed as is using a Q2000 DSC (TA instruments, New Castle, DE). Samples were weighed and hermetically sealed in aluminium pans. A sealed pan containing nothing was used as a reference. The sample was run in a nitrogen atmosphere flushed at 50 mL/min. The instrument was equilibrated at 20 ^{o}C before the sample was heated at 10 ^{o}C/min over a temperature range of 20 ^{o}C to 250 ^{o}C. The peak/denaturation temperature (T_d) and enthalpy (ΔH) were calculated using Advantage/Universal Analysis software (Version 4.5A TA Instruments).

3.16 Materials used for emulsion preparation

The okara protein isolate (OPI) used was processed in the laboratory according to the method developed which was reported in the previous chapters (Chapter 3 and 4). The commercial soybean protein isolate used was Bodybuilding Warehouse brand. Olive oil used was purchased from a local retailer (Co-op, Reading, UK). Distilled water was used. Plamil egg-free mayo was purchased from a local store in Reading, UK.

3.17 Emulsion production procedure

Highly-concentrated oil-in-water (o/w) emulsion emulsion samples were prepared with olive oil, distilled water and protein isolates. Based on initial trial experiments, 57 % (w/w)

olive oil was constantly used while protein isolates and water concentrations varied. Protein isolates varied from 1-6 % (w/w) for OPI and 1-4 % (w/w) for CSP (CSP instability sets in at 4 % concentration). The protein was weighed out and mixed with distilled water to obtain the required concentration. The protein solution was vortexed for 30 min and homogenised using Ultra Turrax T25 S7 (IKA-Labortechnik, Germany) at 12,500 rpm speed while olive oil was added slowly into the mixture. Based on Codex alimentarius (2000), xanthan gum was added as stabiliser at 1g/kg, to evaluate its effect on particle size, texture and flow properties in selected samples.

3.18 Particle size determination

The droplet size of mayonnaise-like emulsions was determined within 3 hours of preparation using a Mastersizer S laser light scattering instrument (Malvern Instruments Ltd., Worcestershire, UK) equipped with a Hydro sample handling unit. Distilled water was used as the dispersant in the sample handling unit. The mayonnaise-like emulsion samples were dispersed in 1 % (w/v) SDS solution before introducing into the sample handling chamber. The obscuration was brought between 10-20 % by sample addition. The droplet size was reported as surface average diameter, ($d_{3,2}$), which is mathematically expressed as:

$$d_{3,2} = \Sigma_{i=1}\, n_i.d_i^3/\Sigma_{i=1} n_i.d_i^2 \qquad (5.1)$$

Where n_i is the number of droplets of diameter, d_i;

d_i is the droplet diameter.

3.19 Texture analysis (Spreadability)

The texture profile analysis of the mayonnaise-like emulsions was carried out using the TAXT2 texture analyser (Stable Micro Systems, Godalming, Surrey, UK). The parameters determined included firmness (g), work of shear (g.s), stickiness (g), work of adhesion (g.s). The instrument was calibrated for force prior to start of the experiment with empty female cone and a load cell of 1 kg. The height was calibrated, and the instrument was set to operate at a

return distance of 25 mm, return speed of 10 mm/sec and contact force of 5 g. Samples were filled into a female cone and levelled, ensuring no air pocket existed and then placed under the matching upper male cone probe before running the test. The test was performed in triplicate and mean values were calculated.

3.20 Dynamic rheological properties

The dynamic rheological measurements were carried out using a Modular Compact Rheometer (MCR 102, Anton paar, Austria). The measurements were carried out using a 25 mm parallel plate set to 1 mm gap. Samples were loaded on the base geometry with a spatula and the plate met the sample at a specified position. The excess samples were completely trimmed off using the spatula. The oscillatory measurement was carried out at least in triplicate and new sample was used for every measurement.

Flow curve: The curve of shear rate against shear stress was used to determine the flow properties of the emulsions. The values for n and k were obtained from plots of log shear stress versus log shear rate, according to the power law equation:

$$\tau = K\Upsilon^{n} \quad (5.2)$$

$$\text{Log } \tau = \log k + n \log \Upsilon \quad (5.3)$$

Where τ is the shear stress (Pa); Υ is the shear rate (s^{-1}); n is the flow behaviour index; and k is the consistency index ($Pa.s^{n}$). The casson model (Eq 5.3) is a structure-based model and was used to characterise the parameters that relate to the structural network of the formed emulsions, namely the casson yield stress and casson plastic viscosity, by plotting the square root of shear rate, $(\Upsilon)^{0.5}$ against the square root of shear stress, $\tau^{0.5}$ with slope, K_c and intercept, K_{oc}. The casson yield stress is calculated as the square of the intercept, $\tau_{0c} = (K_{oc})^2$ and the casson plastic viscosity as the square of the slope, $\eta_{ca} = (K_c)^2$

$$\tau^{0.5} = k_{0c} + k_c (\Upsilon)^{0.5} \quad (5.4)$$

Strain sweep: This was performed in the range of 0.1 – 100 % at a fixed frequency of 1 Hz, to establish the linear viscoelastic region (LVR) of the mayonnaise-like emulsions. The limit of linearity was detected when the dynamic rheological properties, storage, G' modulus and loss, G" modulus, change rapidly from their almost constant values. Hence, a fixed strain was selected to be 0.5 % and used to run the dynamic tests.

Frequency sweep: This is a dynamic test to determine the storage (G') and loss (G") moduli as a function of frequency (Hz) at a specified temperature. The samples were subjected to oscillatory shear at a frequency range of 0.1 to 10 Hz, shear strain of 0.5 % and temperature of 23 ^{o}C.

3.21 Confocal laser scanning microscopy (CLSM) measurement

The fluorescent dyes used were 20 µL of 0.2 % (w/v) rhodamine B in PEG 200 (polyethylene glycol) and 20 µL of 0.2 % (w/v) nile red in PEG200, which were applied sequentially onto a concave microscope slide and mixed thoroughly. A drop of emulsion was subsequently applied on top of the dyes and then covered with a cover slip. The slides were imaged with a Nikon A1R confocal microscope unit embedded with NIS-Elements software, using a 100 *x* oil immersion objective. The excitation wavelengths used for rhodamine B and Nile red were 488 nm and 561 nm respectively. The image obtained was analysed using ImageJ (Natl. Inst. Of Health, Bethesda, Md., USA) to determine the droplet average size, circularity, percentage area and total area. A total of 5 images were analysed for each sample.

3.22 pH measurement

The pH of the o/w emulsions were determined after being dispersed in distilled water using a SevenEasy pH meter (Mettler Toledo, UK) at room temperature (20 ^{o}C). The instrument was calibrated with buffer standard solutions of pH 7 and 4. At least triplicate measurements were obtained.

3.23 Statistical analysis

All experiments were done in triplicate and the values represented are their means. Statistical analysis was carried out by ANOVA using IBM SPSS statistics version 25 (SPSS Inc, Chicago, USA) and mean compared with Tukey's HSD test at $P<0.05$ level of significance.

Chapter 4 - Alkaline extraction of protein from soybean residue (okara)

Abstract

Soybean residue (okara) is a low-value by-product of soymilk manufacturing, rich in protein, with valuable functional properties for the food industry. The development of a process for okara protein extraction would lead to its valorisation, in line with the second-generation incentive of conversion of waste to wealth. This study took a different approach of conventional soybean protein extraction by using alkaline phosphate buffers (0.1 M) at pH 9, 10, 11 and 12 as the extraction media and investigating the protein yield, chemical composition, structural and functional properties of the extracts. Buffers with high pH values increased the protein yield, with pH 12 having 36 % (w/w) protein yield. To this end, phosphate buffer (pH 12) was selected for subsequent optimisation of the protein recovery. The extraction medium with pH 12 led to production of okara protein isolate (OPI) with the best solubility (80 %, w/v). The zeta potential (-38.77) of the OPI from pH 12 was the highest as well as its emulsion capacity (66 % v/v). The commercial soybean protein (CSP) showed the least protein solubility and the highest surface hydrophobicity. The differences observed in the protein solubility and surface hydrophobicity between CSP and OPI, could be attributed to the differences in their structures as depicted by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) results.

Keywords: Soybean residue (okara), soy protein, protein, extraction, alkaline phosphate buffer

4.1 Introduction

Soybean residue (okara) is a by-product generated during soymilk production, with low commercial value, as it is either discarded or used as animal feed. A small amount of okara is put into food use but is not regarded as a common practice to any particular area of the world (Spring, 2005). Okara is a cheap plant source of proteins (26.8 %-37.5 % w/w), fat (8.5 %-23.2 % w/w) and carbohydrates (37.9 %-52.9 % w/w)) (Ma et al., 1997; Surel & Couplet, 2005;

Vishwanathan et al., 2011). Proteins identified in soybean include storage proteins (70-80 %), protease inhibitors (Bowman-Birk and Kunitz inhibitors) which comprise about 8-10 % of soybean proteins (Miroljub & Slađana, 2004), whereas enzymes (lipoxygenase and lactate dehydrogenase) and other storage proteins (e.g., lectin) account for the remaining 10 %. The storage proteins in soybean are globulins which are classified into 2S, 7S (Glycinin), 11S (Beta-conglycinin), and 15S, based on their sedimentation properties (Liu, 1997). According to Kinsella, (1979), these 2S, 7S, 11S and 15S account for 8 %, 35 %, 52 % and 5 % (w/w) of the total protein content of soybean seed, respectively. Among these four identifiable fractions of soy globulin, 11S glycinin and 7S β-conglycinin are the two major ones and they possess different structures and functional properties. *β*-Conglycinin is a cysteine-rich glycoprotein which comprises of three major subunits, ȧ, α, β with molecular weight of 58 kDa, 57 kDa and 42 kDa, respectively (Thanh & Shibasaki, 1978), that are linked together by strong hydrophobic and hydrogen bonds without disulfide bond to form a trimer (Mujoo et al., 2003; Tezuka et al., 2004; Utsumi & Kinsella, 1985).

Proteins in soybean and its by-product (okara) contain essential amino acids that are important for human nutrition (Lönnerdal 1994; Ma et al., 1997), due to their high digestibility (91 % to 96 % w/v) which is similar to that of milk (FAO/WHO/UNU, 1985; Öste 1991). For this reason, soybean proteins can be used in milk substitutes, which are particularly attractive to the part of the population that suffers from lactose intolerance as the milk from soybean contains no lactose (USDA, 2015a; Hajirostamloo, 2009; Shakeel et al., 2015). In addition, soybean proteins are believed to confer some health benefits which include lowering of plasma cholesterol and reducing the risk of cardiovascular diseases (Chao, 2008) because of their content of isoflavones and antioxidants. The quality protein in okara can be made available as flour, protein concentrate and protein isolate, which are the three major protein ingredients. Among all protein ingredients, soy protein isolate has attracted much interest, as it contains the

highest content of quality protein, with techno-functional properties such as emulsifying properties, foaming properties, water holding capacity and fat holding capacity and gelation properties. These properties of soy protein isolates enable their application in the food industry and could be dependent upon the extraction method used. Soybean protein isolate has been applied in several food products such as baked goods (cookies and biscuits), mayonnaise, pasta, sausages, infant formulas and meat, due to its emulsion stabilisation, gelation, fat and water absorption properties (all regarded as functional properties) and as protein enrichment. Therefore, the investigation of extraction techniques of the inherent proteins in okara would lead to its valorisation which is in line with the second-generation incentive of conversion of waste to wealth. Previous research studies have reported the use of aqueous-alkaline conditions by adjustment of the pH of the slurry with NaOH solution, enzymatic-assisted extraction, or more recently, ultrasonication to extract proteins in soybean flakes (Ma et al., 1997; Fischer et al., 2001; Karki et al., 2010); Vishwanathan et al., 2011); only aqueous-alkaline with NaOH has been exploited for protein extraction in okara (Ma et al., 1997; Vishwanathan et al., 2011). The effect of an extraction method on the functional properties of protein isolate should always be taken into consideration since method of extraction can change the protein conformation which would subsequently affect the functionality of the protein (L'Hocine et al., 2006). However, some factors that can affect protein extractability from soy products include, the nature of the extractant, the temperature, the pH, the agitation speed and the extraction time (Kasai & Ikehara, 2005; Mason et al., 1996). Protein in okara is not readily extractable compared to soybean flour or soybean flakes. This has been attributed to the effect of elevated heat treatment used during soymilk production that caused the proteins in the intact cotyledon cells to be aggregated (Preece et al., 2015a). Previous research evaluated effect of temperature to enhance extraction of protein in okara at pH 9.0 for 30 mins but 14.1 % (w/w) and 53.4 % (w/w) of protein was recovered when extraction was done at 25 ^{o}C and 80 ^{o}C respectively. But

since the latter temperature of extraction is close to the denaturation temperatures, 82 ^{o}C and 68 ^{o}C, of the two major soybean proteins, glycinin and β-conglycinin respectively (Riblett et al., 2001), the method may cause cause denaturation of the proteins that may negatively affect their functionality (Ma et al., 1997). However, the effect of pH on okara protein extraction, as well as the nutritional composition and functional and structural properties of the obtained extracts has not been investigated yet. Hence, the aim of this study was to develop a method that could enhance protein extraction from okara. The effect of this method and pH on the nutritional, structural and functional properties would be investigated with the view of identifying industrial application.

4.2 Results and Discussion

4.2.1 Chemical composition of okara flour

The chemical composition of both original okara and defatted okara flour samples was investigated and the results are presented in **Tables 4.1** and **4.2**. Protein contents of undefatted and defatted okara were 37.7 % and 42.5 % (w/w) respectively, comparable with the protein contents of undefatted (34.7 %, w/w) and defatted okara (44.2 %, w/w) reported by Vishwanathan et al. (2011). In contrast, Ma et al. (1997) reported only 26.8 % (w/w) of protein in defatted okara samples. The reason for this variation could reside in the processing that okara samples had undergone as the okara used by Ma et al. (1997) was obtained from a commercial soymilk facility and more protein might have entered into the milk during the pressing step, probably done with the aid of a machine rather than hand pressing which is usually the case with laboratory soymilk processing, like in this present research. The fat content of original okara (17.48 %, w/w) fell within the range of 9 % -22 % (w/w) that has been reported by other researchers (Ma et al., 1997; Vishwanathan et al., 2011). The total carbohydrate of defatted okara (37.14 %, w/w) was higher than the value of total carbohydrate content (31.59 %, w/w) reported by Vishwanathan et al. (2011). By comparing the macromolecules' composition of

original and defatted okara, significant differences ($P < 0.05$) were seen in the values of protein, carbohydrate and fat (**Table 4.1**). This shows that defatting prior to protein and carbohydrate extraction is essential, since oil bodies or spherosomes surround the protein bodies (Preece et al., 2015) and their removal prior to protein extraction would expose the protein bodies to the extraction medium and facilitate their solubilisation. The acid soluble lignin (ASL) was 2.68 % (w/w) in undefatted okara and 2.14 % (w/w) in defatted okara which are comparable with the value (3.7 %, w/w) reported by Brillouet & Carré (1983) for soybean cotyledon. The range of lignin in soybean seed coat (2.45 %-4.04 %, w/w) reported by Mullin & Xu (2001) is equally low. This shows that soybeans and their okara cell wall material are poorly lignified. ASL in defatted okara appeared slightly lower than that in undefatted okara. This could be attributed to the fact that, from a chemical point of view, lignin consists of cross-linked phenol polymers and some could be lost in the process of defatting with hexane (Naczk et al., 1986). The acid insoluble lignin (AIL) recorded was generally low, but comparable with the Klason lignin content (0.016 %) in soybean meal reported by Knudsen (1997).

Table 4. 1: Chemical composition of raw okara and defatted okara flour (g/100g dry matter)

Sample	Moisture	Protein	Fat	Ash	Carbohydrates	Lignin
Okara flour	4.0±0.37	37.8±0.52*	17.5±0.06*	1.9±0.03	31.3±0.01*	2.7±0.35
Defatted okara flour	6.2±0.06	42.5±0.32	3.8±0.08	2.1±0.03	37.14±0.22	2.1±0.32

The asterisk* shows significant difference ($p<0.05$) between the two means in the same column as determined by Tukey HSD test. Results are mean ± standard deviation of triplicate values.

Table 4.2 presents the monomeric sugar composition of okara samples. The presence of arabinose (5.13 %, w/w), galactose (13.41 %, w/w) and galacturonic acid (2.72 %, w/w) observed for defatted okara indicate the presence of arabinans, galactans, and specifically, the arabinogalactan side chains of rhamnogalacturonan (RG) (Dekker & Richards 1976; Huisman et al., 1996). These sugars are indicative of pectic polysaccharides in okara (Yamaguchi et al., 1996) consisting of linear galacturonans (GN) and branched rhamnogalacturonans (RG)

(Mccleary & Matheson 1986; Matsuhashi et al., 1993). Hence, defatted okara could be a potential source for arabinogalactan extraction.

Table 4. 2: Monomeric sugar contents of okara and defatted okara (g/100g of total carbohydrate).

Components	Undefatted okara	Defatted okara
Fucose	1.75±0.012	1.81±0.003
Rhamnose	0.78^{a}±0.037	1.05^{b}±0.013
Arabinose	4.37^{a}±0.07	5.13^{b}±0.2
Galactose	11.80^{a}±0.02	13.41^{b}±0.20
Mannose	1.21^{a}±0.15	2.38^{b}±0.12
Glucose	9.12^{a}±0.08	10.64^{b}±0.24
Galacturonic acid	2.27^{a}±0.01	2.72^{b}±0.19

Values in the same row with different letters are significantly different ($p<0.05$) according to Tukey HSD test. Results are mean ± SD of triplicate readings.

Moreover, the presence of glucose could be attributed to the presence of cellulose or xyloglucans. Xyloglucans represent a type of hemicelluloses found in most legumes (Huisman et al., 1996; Huisman et al., 1998). The monomeric sugar (fucose, rhamnose, arabinose, galactose, mannose, glucose) values obtained in this study are comparable with the values for defatted okara reported by Mateos-Aparicio et al. (2010).

4.2.2 Effect of alkaline pH on protein extraction yield

The next step of the experimental procedure involved the protein extraction from defatted okara. Aqueous extractions at varying pH values were employed, in order to investigate the influence of pH on protein recovery from okara. A range of alkaline pH was studied (pH 8-12) by employing either 0.1 M phosphate buffer or 0.1 M carbonate buffer. The protein recovery during aqueous extractions of defatted okara at different pH values and buffers is shown in **Fig. 4.1**. The protein yield obtained with carbonate buffer ranged from 5.42 % (w/w) at pH 9 to 21.48 % (w/w) at pH 11. Protein recovery in carbonate buffer showed a progressive increase from pH 9 to pH 11, indicating that carbonate buffer has a strong buffer capacity from pH 9.5 to pH 11.1 with pKa at pH 10.33. Protein recovery in phosphate buffer (**Fig. 4.1**) ranged from 3.5 % (w/w) at pH 8 to 35.89 % (w/w) at pH 12. The low protein

recovery at pH values of 8 to 11 indicated that phosphate buffer has weak buffer capacity within the range of pH 8 -11 while the significant increase ($p<0.05$) in the recovered protein (35.89%, w/w) with phosphate buffer pH12 agreed with the fact that phosphate buffer has a strong buffer capacity around pH 12, having its pKa at pH 12.33. These findings are also in agreement with the report of other researchers indicating that high pH can increase protein solubility and extractability in okara (Ma et al., 1997). The isoelectric point (pI) of soybean proteins is around pH 4.5 and in order to achieve high solubility, the pH of the extraction conditions should be kept away from the pI, leading to higher charges of protein molecules, strong electrostatic repulsions and strong water-protein interactions (Van Megen, 1974; Phillips et al., 1994). The highest yield (35.89 %, w/w) was obtained with 0.1 M phosphate buffer at pH 12. Karki et al. (2010) reported lower protein yields of about 27 % (w/w), using aqueous-alkaline extraction at pH of 9. The low extractability of soybean protein by conventional methods could be as a result of the complex nature of soybean proteins. The major soybean proteins co-exist with other proteins and non-protein components which result into protein-protein interactions or protein-carbohydrate interactions that may hinder protein solubility in aqueous alkaline media. Repeated extractions with fresh media has been shown to enhance the protein extraction yield (Vishwanathan et al., 2011) and may assist in overcoming issues related to solvent saturation. Phosphate buffer was selected for further studies, having strong buffer capacity at pH 12 with the highest protein extractability.

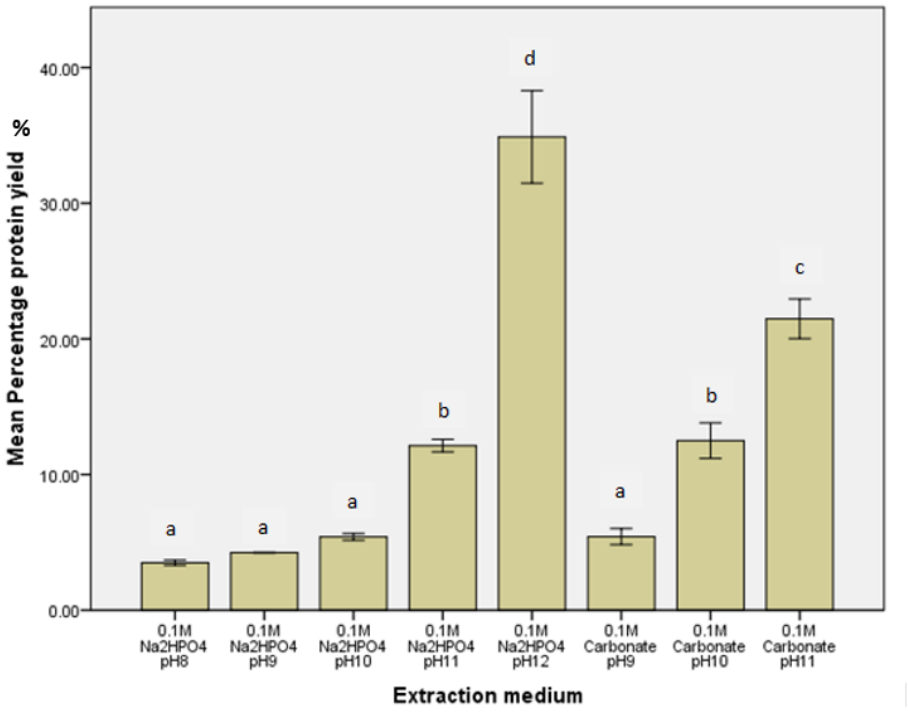


Fig.4. 1: Protein recovery (g/100g of protein in defatted okara flour) achieved during extraction of defatted okara at different buffers and pH values.
Significant difference exists between bars with different letters as determined by Tukey HSD test ($p<0.05$). Values are mean ± SD of triplicate readings.

4.2.3 SDS PAGE and RP-HPLC profiles of okara protein isolates (OPI) and CSP

Fig.4.2 presents the SDS pattern of the proteins extracted with phosphate and carbonate buffers at different pH and **Fig.4.3** presents the RP-HPLC profile of the OPI and CSP. Both carbonate (pH 9-11) and phosphate (pH 8-12) buffers extracted proteins within the range of the two major soybean protein subunits, namely the acidic (AS) (34 kDa-44 kDa) and basic (BS) (20 kDa) of 11 S glycinin, and the three subunits, α (57 kDa), ȧ (58 kDa) and β (42 kDa), of 7S β-conglycinin (Marcone et al., 1998). The same protein bands were noted irrespective of the buffers used for protein extraction. Aggregated protein was observed at the top of the lane for protein sample extracted with 0.1 M phosphate buffer pH 12, which indicates more denaturation of the proteins and subsequent protein-protein association or interaction other than disulphide linkage because reducing agent was used in the SDS-PAGE analysis (Ma et al., 1997; Pochan et al., 2003; Cox et al., 2005; Roberts, 2007).

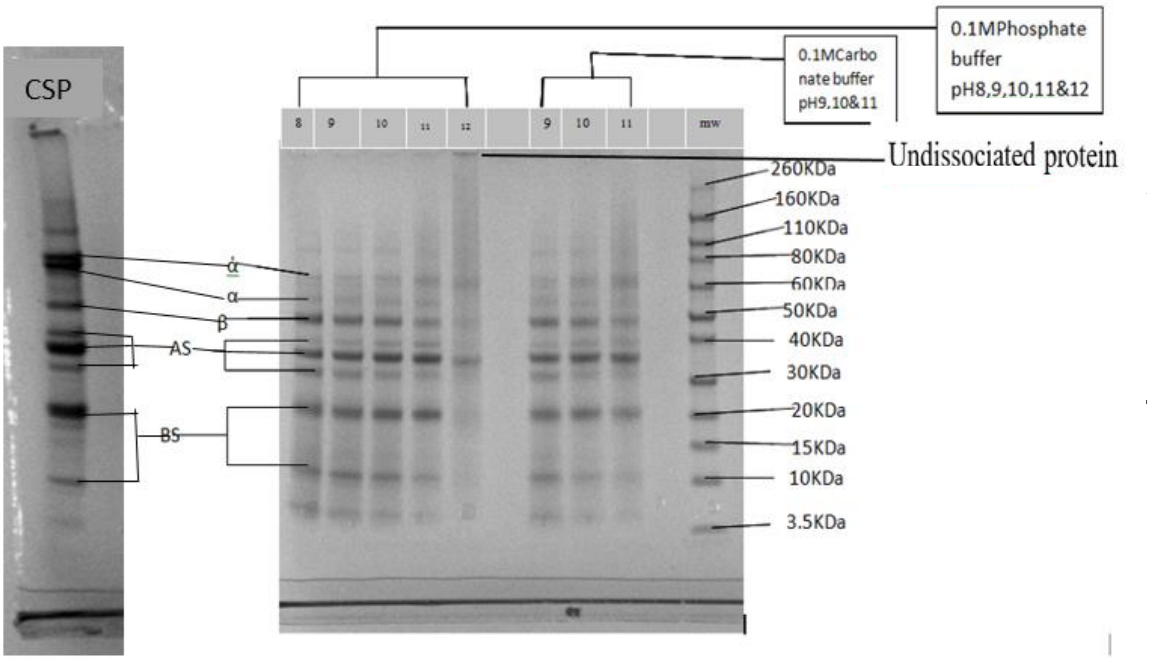


Fig.4. 2: SDS pattern of okara protein extracts from different extraction buffers.
Lanes 8, 9, 10, 11 and 12 represent 0.1 M phosphate pH 8, 9, 10, 11, and 12, while Lanes 9, 10, 11 represent 0.1 M carbonate buffer pH 9, 10, and 11. Lane MW represents the molecular weight. AS is acidic subunit of 11S Glycinin; BS is basic subunit of 11S Glycinin; ά, α, β are three subunits of 7S β-conglycinin.

The profile of the proteins was determined by RP-HPLC (**Fig.4.3**). RP-HPLC is an important technique used for analysis of peptides and proteins. Its separation mechanism is based on the hydrophobic binding affinity of the solute molecule carried in the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase (Aguilar, 2004; Gilar et al., 2005). However, proteins are separated in order of their increasing hydrophobicity. According to literature, the 11 S (glycinin) fraction of soybean proteins are less hydrophobic and are eluted first while the 7 S (β-conglycinin) fraction is more hydrophobic with greater retention time (Riblett et al., 2001; Mujoo et al., 2003). The elution profiles of the OPI and CSP were similar, suggesting that they both contain similar proteins; this is in agreement with our SDS-PAGE results. Although, the proportion of the subunits deferred with different protein isolates which is revealed in their peak areas as shown in **Table 4.3**.

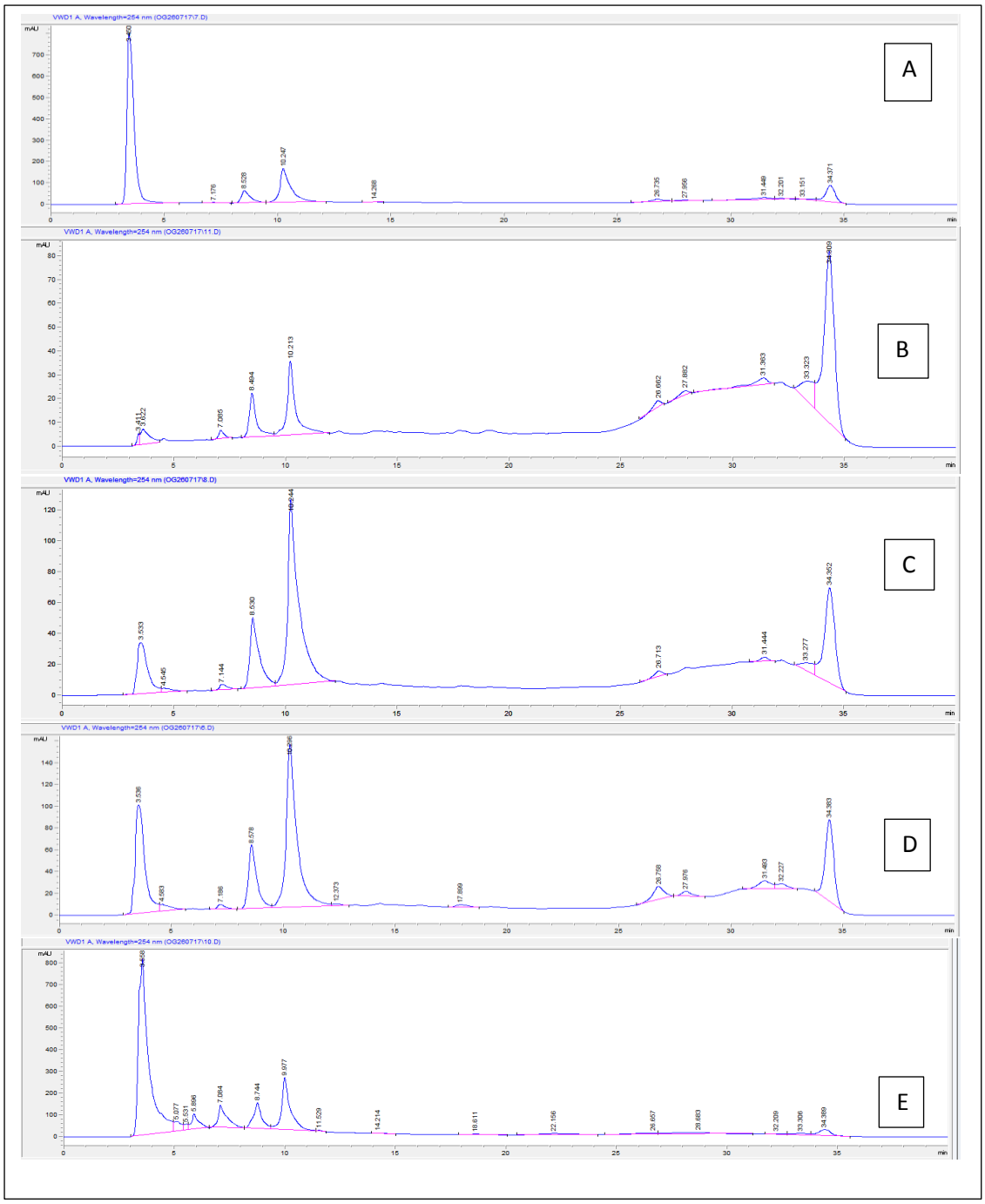


Fig.4. 3: RP-HPLC profile of okara protein isolates and CSP.

A: OPI extracted with buffer pH9; B: OPI extracted with buffer pH10; C: OPI extracted with buffer pH11; D: OPI extracted with buffer pH12; E : CSP. All the proteins were standardised to 6 mg/mL.

Table 4. 3: Areas of protein subunits in the alkaline extracted OPI at different pH and CSP identified with RP-HPLC

	CSP	pH9	pH10	pH11	pH12
Retention time (min) of protein subunits	**mean %Area**	**mean %Area**	**mean %Area**	**mean %Area**	**mean %Area**
3.4-3.5	NA	62.1±0.78	25.5±3.54	13.2±1.48	1.2±0.31
3.6-3.7	57.4±0.28	NA	NA	NA	4.7±0.86
4.5-4.8	NA	NA	1.7±0.30	1.2±0.21	NA
4.9-5.4	2.3±0.14	NA	NA	NA	NA
5.5-5.7	0.7±0.01	NA	NA	NA	NA
5.8-6.0	3.6±0.01	NA	NA	NA	NA
7.0-7.3	6.4±0.18	0.3±0.05	0.9±0.05	1.0±0.01	1.5±0.05
8.5-8.7	6.4±0.06	5.4±0.09	11.5±0.09	15.0±1.29	8.9±0.06
10.0-10.4	14.4±0.32	18.4±0.03	38.9±0.16	46.1±0.54	19.4±1.41
11.0-11.5	0.2±0.00	NA	NA	NA	NA
12.3-12.6	NA	NA	0.4±0.01	NA	NA
14.0- 14.3	0.2±0.00	0.2±0.01	NA	NA	NA
17.5-18.0	NA	NA	0.7±0.02	NA	NA
18.5-19.0	0.4±0.01	NA	NA	NA	NA
19.5-20.0	NA	NA	NA	NA	NA
22.0-22.2	1.4±0.05	NA	NA	NA	NA
26.5-27.0	1.1±0.05	1.8±0.01	3.5±0.14	1.2±0.12	1.8±0.06
27.5-28.0	NA	0.9±0.01	1.1±0.06	NA	1.4±0.14
28.5-29.0	2.4±0.02	NA	NA	NA	NA
31.4-31.7	NA	2.4±0.05	2.5±0.15	0.8±0.02	3.3±0.18
32.0-32.5	0.3±0.01	0.6±0.06	1.2±0.01	NA	NA
33.0-33.5	0.8±0.01	0.8±0.03	NA	2.5±0.07	8.2±0.43
34.3-34.5	2.4±0.02	8.3±0.40	15.8±0.72	22.9±1.45	53.6±2.45

NA means not available.

4.2.4 Characterisation of okara protein isolates

The chemical composition of okara protein isolates are presented in **Table 4.4**. The CSP and OPIs at pH 9, 10, 11 and 12 contained 88.8 %, 82.73 %, 82.98 %, 84.61 % and 86.15 % (w/w) protein, respectively. The CSP had the lowest fat (0.06 %, w/w) and ash content (2.01 %, w/w). Ash content is the inorganic residue mainly minerals present in the food sample. Food preparation processes which include soaking and washing could lead to loss of some of these minerals into the water for the washing of the food product (Fabbri & Crosby, 2016). Therefore, the low ash content might be as a result of different method used to prepare the protein isolates, it could be that during purification process, the ash in the CSP was lost during the washing

process to purify the proteins (Fabbri & Crosby, 2016) and probably the washing was done more than the number of times the OPI in this research was subjected to. This is an assumption of the possible step that could lead to mineral loss, since the procedure for preparation of the CSP is not disclosed. The variation in the composition might also be due to genetic or environmental changes from where the seeds were grown. The fat and carbohydrate contents recorded for all the OPI were lower than the fat content (4.1 %, w/w) and carbohydrate content (7.5 %, w/w) of crude OPI reported by Chan & Ma (1999). The protein contents of the OPI at different pH were higher than 77.8 % (w/w) as reported by Chan & Ma (1999) and they compared with the values obtained for other legumes such as mung bean protein isolate, black bean protein isolate and Bambara groundnut protein isolate (Kudre et al., 2013). Therefore, all obtained OPI could potentially be used in food applications as protein enrichment ingredients.

Table 4. 4: Chemical composition of the okara protein isolates (OPI) extracted at various pH values (g/100g OPI)

Sample	Moisture	Ash	Fat	Protein	Carbohydrates
CSP	5.6^{a}±0.2	2.0^{a}±0.1	0.6±0.03	88.1^{b}±0.7	3.8^{e}±0.02
pH 9	6.6^{bc}±0.1	5.6^{d}±0.2	1.4±3.4	82.7^{a}±1.6	3.6^{d}±0.01
pH 10	6.8^{c}±0.2	5.5^{cd}±0.3	0.9±3.8	83.0^{a}±2.5	3.5^{c}±0.03
pH 11	6.3^{b}±0.1	5.0^{bc}±0.2	0.7±2.5	84.6^{ab}±2.1	3.4^{b}±0.01
pH 12	5.4^{a}±0.1	4.9^{b}±0.3	0.7±2.0	86.1^{ab}±0.4	3.3^{a}±0.01

Results are mean ± SD of triplicate values. pH 9, 10, 11, and 12 represent the different pH of the medium used during the extraction of the OPIs. Values in the same column with different superscripts are significantly different ($p<0.05$) according to Tukey HSD test.

4.2.5 Amino acid composition of okara protein isolate

The amino acid composition and profile of okara protein isolates (OPI) are shown in **Fig.4.4** below. All OPI contained most of the essential amino acids such as valine, leucine, isoleucine, threonine, tyrosine, phenylalanine, and histidine above the FAO scoring pattern (FAO/WHO, 1991). Based on the results, lysine decreased with increase in the pH of extraction medium and this agrees with the findings reported by Finley & Kohler (1979). This is attributed to possible chemical reactions of amino acids (Lysine and cystine) in alkaline medium which tends to increase with increase in the pH of the medium. Like in other legumes,

tryptophan and methionine were the limiting amino acids while glutamic acid and aspartic acid were the most predominant amino acids in all isolates. In general, all OPI contained sufficient amounts of both essential and non-essential amino acids (**Fig.4.4**) and this could compare favourably with the CSP, hence, could be used as a source of quality protein.

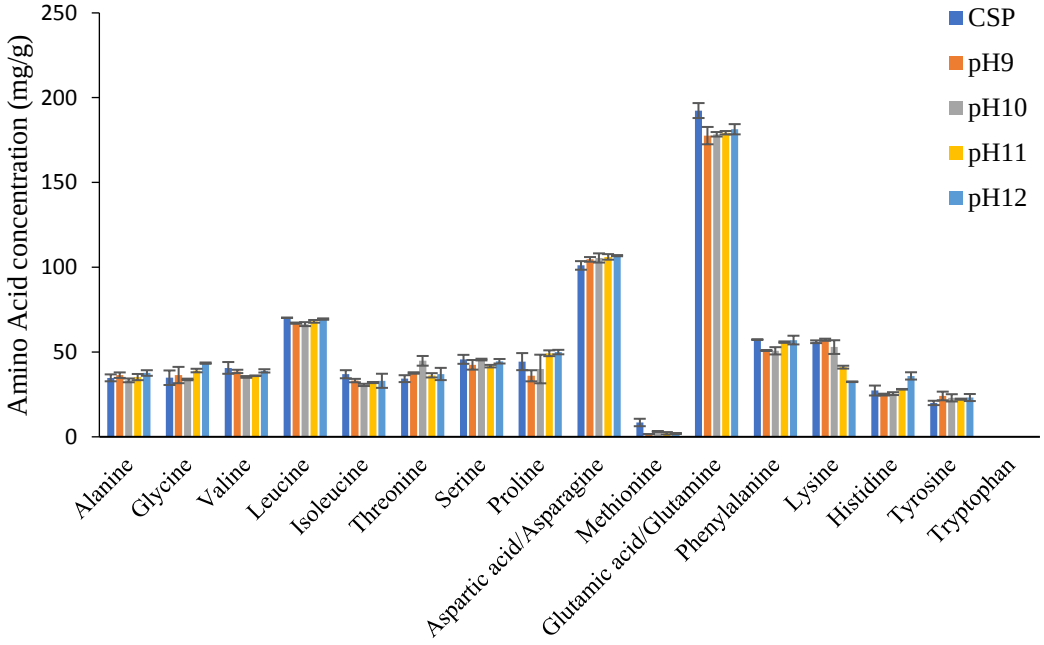


Fig.4. 4: Amino acid composition of CSP (Commercial soy protein isolate) and OPI extracted at different pH values with phosphate buffer.
Data are mean of triplicate determinations.

4.2.6 Surface hydrophobicity

The surface hydrophobicity (So) of the commercial protein isolate (CSP) and different okara protein isolates is presented in **Fig.4.5**. The commercial protein isolate had much higher surface hydrophobicity (519) than the okara protein isolates (151 – 297). The difference in surface hydrophobicity indicates modification in the protein conformation probably due to different processing conditions employed to derive the different protein isolates (Wagner et al., 2000). It is evident that the increase in the pH of extraction medium increased the surface hydrophobicity, with pH 11 leading into the isolate with the highest surface hydrophobicity (280). This would suggest that pH 11 OPI might have more partially unfolded proteins

compared to pH 12 protein isolates, owing to the fact that it might not have denatured as much as pH 12 protein isolates. This agrees with the report that ANS fluorescence probe has stronger affinity binding exposed hydrophobic surfaces in partially unfolded proteins than native or completely unfolded proteins (Pallares et al., 2004). The okara protein isolate produced in this current research using pH 12 extraction medium had 240 surface hydrophobicity, indicating higher number of exposed hydrophobic ends on the protein structure compared to the range (125 and 150) reported by Ma et al. (1997) for okara protein isolate extracted at pH 9 and at 25^{o}C and 80^{o}C respectively.

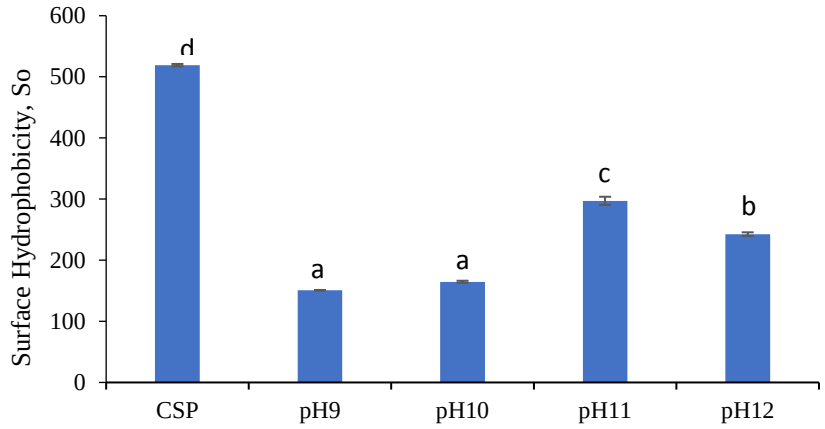


Fig.4. 5: Surface hydrophobicity of OPI.
Means and standard deviation are from duplicate determinations.
Significant difference ($p < 0.05$) as determined by Tukey HSD test.

4.2.7 Sulfhydryl contents of okara protein isolates

Fig.4.6 shows the free sulfhydryl (FSH), total sulfhydryl (TSH) and disulphide bond (SS) contents of commercial soybean protein isolate (CSP) and okara protein isolates (OPI) extracted at various pH values. The CSP had 93.5 µM/g of FSH, 117.1 µM/g of TSH and 11.8 µM/g of SS. The free sulfhydryl (FSH) content in the okara protein isolates ranged from 89.4 µM/g at pH 12 to 102.2 µM/g in the case of pH 9. It can be deduced that the pH of extraction medium affected the free and total sulfhydryl content as well as the disulphide content of the protein isolates. Additionally, OPI of pH 12 had FSH, TSH and SS values close to those of the

CSP. The disulphide bond of CSP (11.8 µM/g) could compare with the disulphide bond content of native soy protein isolate (18.4 µM/g) reported by Zhang et al. (2016). Most of the sulfhydryl content is attributed to glycinin (11 S), since glycinin contains higher concentrations of sulphur-containing amino acids, such as methionine and cysteine, than other soy protein subunits (Kim et al., 2005). Specifically, there are two –SH groups and 20 S–S bonds per molecule in 11S (glycinin); while, there are no –SH groups but two S–S bonds per molecule in 7S (β-conglycinin) (Kim et al., 2005).

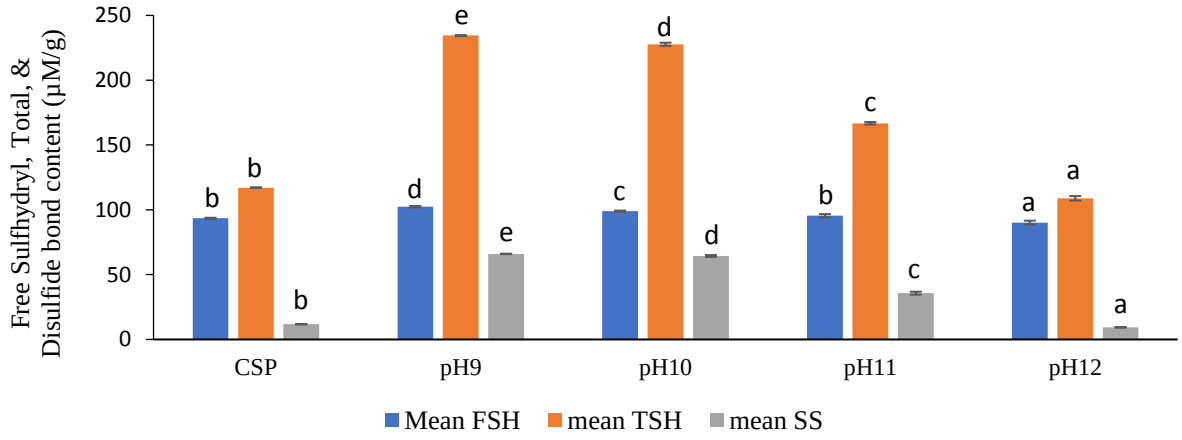


Fig.4. 6: Free sulfhydryl (FSH), total sulfhydryl (TSH) and disulphide bond (SS) contents of OPI from various extraction pH values and CSP.

Means and standard deviation are from duplicate determinations. CSP: Commercial Soy Protein Isolate. Significant difference ($p < 0.05$) as determined by Tukey HSD test.

4.2.8 Functional properties of okara protein isolates

4.2.8.1 Protein solubility of okara protein isolates

The protein solubility of the OPI and CSP samples based on pH is shown in **Fig.4.7**. Protein solubility is an important parameter to be considered in food applications, as it influences most of the functional properties where dissolution in a liquid is required such as water absorption capacity, emulsion capacity and foaming capacity. The solubility of a protein is affected by many factors including processing methods and storage conditions (extrinsic

factors) and some intrinsic factors such as the structure of the proteins and type of amino acid composition. The solubility of all OPI and the CSP samples followed a U-shaped curve in pH values ranging from 1.5 to 10 (**Fig.4.7**) with the lowest solubility near pH 4. The latter is in agreement with the findings reported by Ma et al. (1997) and Kudre et al. (2013) on okara protein isolates and some legumes (mung bean, black bean, and Bambara groundnut protein isolates) respectively. CSP had the lowest solubility at pH above 4 while OPI samples extracted at pH 9 and 10 had the lowest solubility at pH below 4. The low solubility of CSP can be positively correlated with its high surface hydrophobicity (**Fig.4.5**) which agrees with the findings of Wagner & Anon (1990) and Wagner et al. (2000). However, protein functionality can be affected by the extraction conditions employed such as temperature, and pH as a result of their effect on the structural conformation of the proteins (L'Hocine et al., 2006). Some degrees of dissociation, aggregation or denaturation of the protein structural conformation could arise because of the different temperature and pH of the extraction process (Wagner et al., 1996; L'Hocine et al., 2006). However, these differences in the degree of association or dissociation and presence of different protein subunits in OPI (**Fig.4.2**), would have contributed to their different solubility behaviour at different pH. OPI extracted at pH 12 exhibited the highest solubility compared to the rest, suggesting that the method used would be suitable for producing isolate with high solubility. This is a desired attribute in food applications.

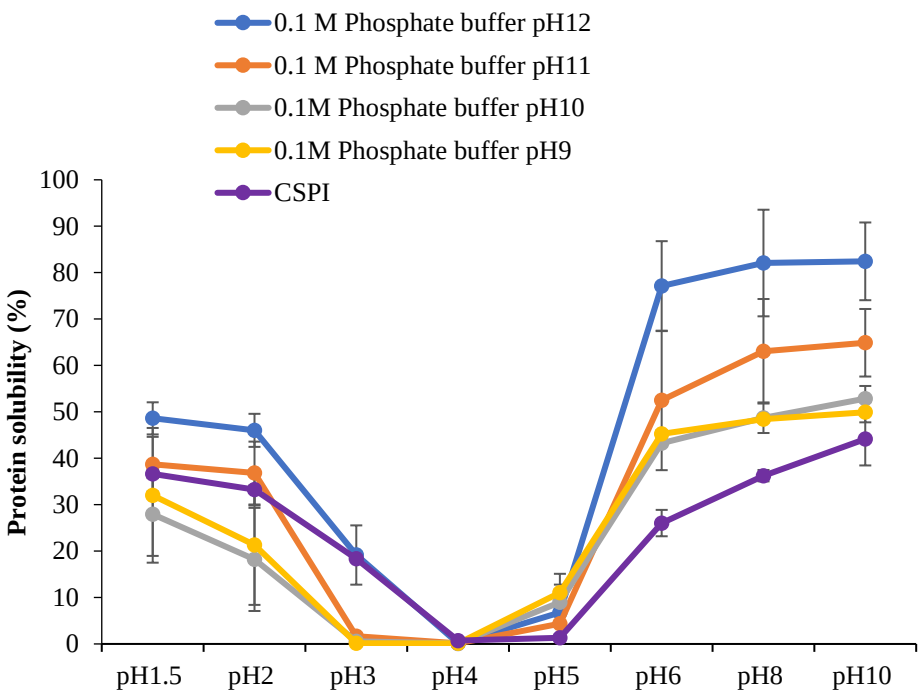


Fig.4. 7: Protein solubility curves of CSP and OPI samples extracted using phosphate buffer at different pH.
Each data point represents mean of a triplicate determination.

4.2.8.2 Emulsion properties of okara protein isolates (OPI)

The emulsion capacity and stability of okara protein isolates (OPI) and commercial protein isolate (CSP) is shown in **Fig.4.8** below. The emulsion capacity and stability tests were performed at pH 7 which is away from the isoelectric point of soybean major proteins, (4-5) (Kinsella, 1979). OPI extracted at pH 12 had the highest emulsion capacity (66%, v/v) and stability (62.5 %, v/v), most likely as a result of its highest solubility functionality (**Fig.4.7**) combined with its good surface hydrophobicity (**Fig.4.5**) properties. Therefore, high protein solubility and high surface hydrophobicity are important parameters required by emulsifiers to obtain stable emulsions (Hettiarachchy et al., 1996; Aoki et al., 2006). Adequate protein solubility is important because protein must first be dissolved before it can be adsorbed at the interface between water and oil to reduce the interfacial or surface tension that leads to the formation and stabilization of emulsion. Hence, to achieve sufficient emulsifying functionality,

protein (or emulsifier) must have high surface hydrophobicity (to get attached to the oil) and high solubility (to get attached to the water phase) at the same time. Meanwhile, during the formation of an emulsion, the mixing action could cause the native protein structure to unfold, exposing the hydrophobic region to the oil and the hydrophilic region to the water, which lowers the surface tension between the water and oil and the rate or capacity at which this is formed is influenced by the protein structure (Hill, 1996). The structure of protein is one of the factors affecting protein solubility. All OPI samples and CSP had emulsion capacity and stability higher than 50 % (v/v) and this agrees with the emulsion capacity (56.3 %, v/v) and stability (55.6 %, v/v) of commercial soy protein isolate (Supro 610) as reported by (Wu, 2001).

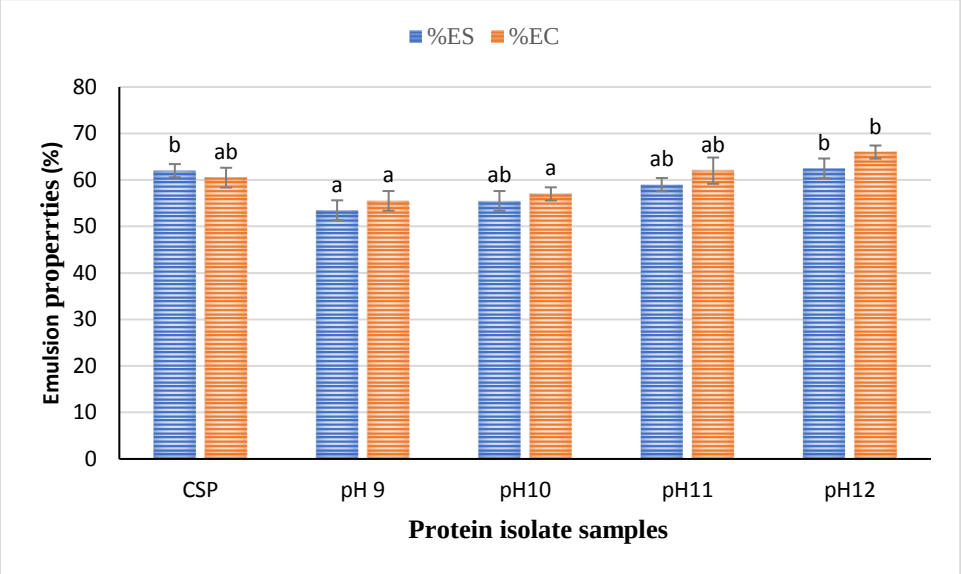


Fig.4. 8: Emulsion properties of CSP and OPI samples extracted at different pH of 0.1 M phosphate buffer.

ES: Emulsion stability; EC: Emulsion capacity. Means and standard deviation are from duplicate determinations. Different alphabets on the bars of the same colour signifies significant difference ($p < 0.05$) as determined by Tukey HSD test.

4.2.8.3 Foaming properties of okara protein isolates (OPI)

The foaming properties (capacity and stability) of OPI and CSP are shown in **Fig.4.9** below. Soluble proteins form the most stable foams by dissolving and diffusing to the air/water interface to decrease the surface tension in order to form strong films while increasing both the

viscous and elastic properties of the liquid phase (Kinsella, 1981). From the results below, it can be seen that OPI extracted at pH 12 formed the most stable foam (35 %, v/v) ($p<0.05$) followed by CSP (23 %, v/v) and OPI at pH 11 (22.5 %, v/v). The least stable foam was formed by pH 9 (7.5 %, v/v) and pH 10 (7.0 %, v/v) OPI, while there was no significant difference ($p>0.05$) between the two of them (OPI at pH 9 and pH 10). The best stability of OPI from pH 12 could be correlated with its high solubility. This is because highly soluble proteins form strong films as opposed to the weak films formed by partially soluble proteins. Foam capacity measures the volume of foam formed after whipping and this seemed to decrease with increase in the pH of the extraction medium. CSP had the highest foaming capacity (76.5 %, v/v) followed by OPI at pH 9 with 73 % (v/v). OPI from pH 9 and 10 showed the least foam stability, probably because of their low surface hydrophobicity and solubility, which might have affected their ability to reduce the interfacial tension and subsequent formation of strong or stabilizing film around the formed bubbles. These two factors (surface hydrophobicity and solubility) exhibit crucial role in the reduction of the interfacial tension and the formation of stable emulsions and foams. Nevertheless, comparing the result from this study with those of Kempka et al. (2014), the foaming capacity of all the OPI in this study were higher than the range 25 % to 43.3 % (v/v) reported by Kempka et al. (2014).

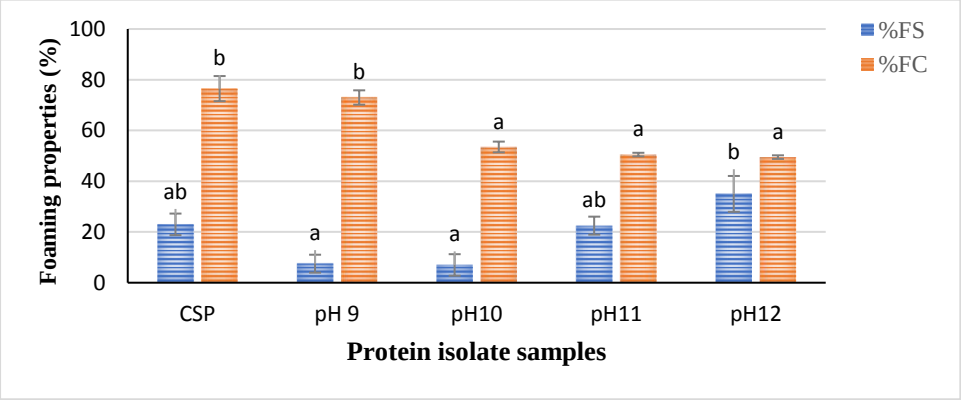


Fig.4. 9: Foaming properties of CSP and OPI extracted at different pH of 0.1M Phosphate buffer.

FS: Foaming stability; FC: Foaming capacity. The foaming properties were determined at pH 7. Means and standard deviation are from duplicate determinations. Different alphabets on the bars of the same colour signifies significant difference ($p < 0.05$) as determined by Tukey HSD test.

4.2.8.4 Water absorption capacity (WAC) and oil absorption capacity (OAC)

The results of oil and water absorption capacity of the CSP and OPI extracted at different pH are shown in the **Fig.4.10** below. Oil absorption is a physical property indicating the ability of a material to entrap oil into its matrix. In the case of proteins, this ability is affected by several factors which include protein concentration, the number of non-polar sites on the protein and protein-lipid interactions. In general, less soluble and hydrophobic proteins have a high oil binding capacity. It was found that CSP had the highest oil absorption capacity (3.3 mL/g), but not significantly different ($p>0.05$) from the values obtained by OPI at pH 9 (3.15 mL/g) and pH 10 (3.2 mL/g). OPI extracted at pH 12 had the least OAC (2.25 mL/g) followed by OPI extracted at pH 11 (2.5 mL/g). Hence, increase in the pH of extraction medium led to a decrease in the OAC. Kempka et al. (2014) reported OAC values within the range of 1.0 mL/g to 2.4 mL/g, where crude soy protein isolate with the least solubility exhibited the highest oil absorption capacity of 2.4 mL/g at pH 7. In terms of water absorption capacity, it seems from **Fig.4.10**, that WAC decreased as the pH of the extraction medium increased. These findings suggest that despite the fact that WAC is dependent on protein concentration, protein solubility as a functional property affects WAC more profoundly. Findings by Kempka et al., (2014) and Wagner & Anon (1990) supported the fact that low protein solubility leads to high WAC.

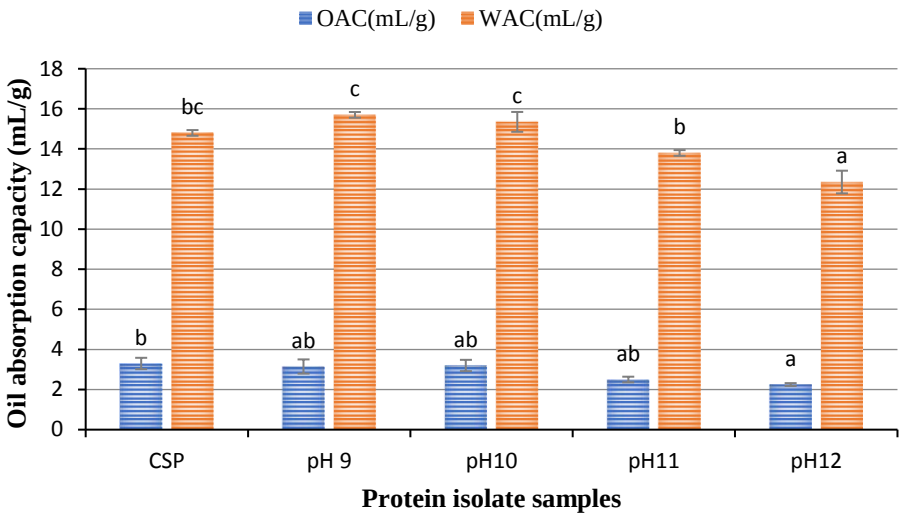


Fig.4. 10: Water holding capacity (WAC) and oil absorption capacity (OAC) of CSP and OPI extracted at different pH of 0.1 M phosphate buffer.

Means and standard deviation are from duplicate determinations. Different alphabets on the bars of the same colour signifies significant difference ($p < 0.05$) as determined by Tukey HSD test.

4.2.9 Zeta potential and particle size of okara protein isolate (OPI)

The zeta-(ζ-) potential or, in other words, the electrostatic force between the particles of the OPI and CSP in water is presented in **Fig.4.11** while the particle distribution is presented in **Fig.4.12**. This investigation was carried out in order to evaluate whether the extraction conditions (pH value) affected the charge (repulsion/attraction) between the protein particles in water. Zeta-(ζ-) potential is an important property that determines the stability of suspension, emulsion and dispersion by proteins (Jachimska et al., 2008). The ζ-potential was carried out at pH 7, away from the isoelectric pH (4.5) of soy proteins and because neutral pH values are encountered in most food applications. The CSP had the lowest ζ-potential value of -27.73 mV, comparable with the zeta value (-28 mV) obtained by Zhang et al. (2016) for the native soy protein isolate. OPI extracted at pH 12 had the highest ζ-potential value of -38.77 mV and was significantly different ($p<0.05$) from the ones extracted at pH 9 (-34.23 mV), and the CSP. This OPI extracted at pH 12 showed slight significant difference when compared with OPI extracted at pH 10 (-37.20 mV) and pH 11 (-36.50 mV). This high value of the OPI at pH 12 could have

impacted to its high emulsion stability, since emulsion with low magnitude of ζ-potential is prone to coagulation or flocculation more easily than emulsions with high ζ- potential value further away from zero like -30 mV (Lu & Gao, 2010), and thus, predispose it as a very good emulsifier in formulation of mayonnaise-like emulsions and salad dressings.

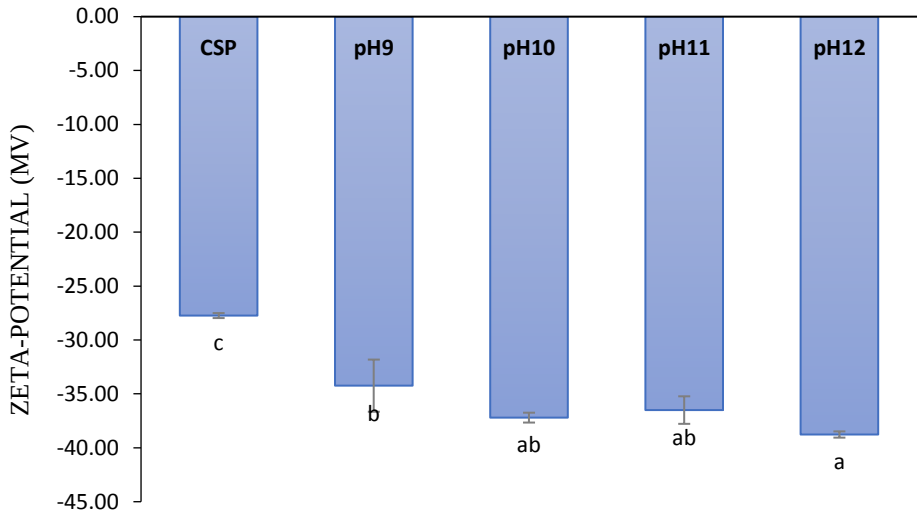


Fig.4. 11: Zeta- (ζ-) potentials of CSP and OPI extracted at different pH of 0.1 M phosphate buffer.

Data are mean of triplicate values. Different alphabets on the bars signifies significant difference ($p < 0.05$) as determined by Tukey HSD test.

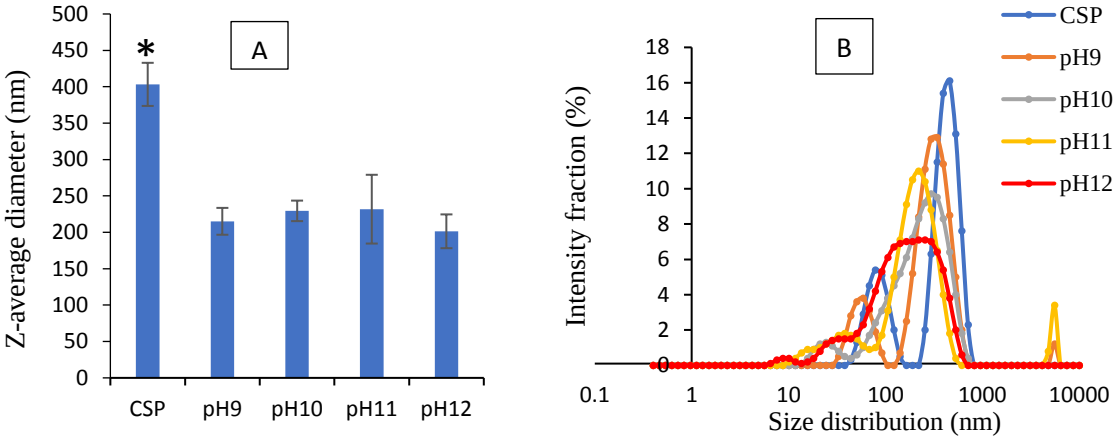


Fig.4. 12: Z-average diameter of CSP and OPI extracted at different pH of 0.1 M phosphate buffer.

Data are mean of triplicate values. A is Z-Average diameter chart; B is curve of Intensity fraction against size distribution. CSP: Commercial soy protein isolate; pH9: OPI extracted with phosphate buffer pH9; pH10: OPI extracted with phosphate buffer pH10; pH11: OPI extracted with phosphate buffer pH11; pH12: OPI extracted with phosphate buffer pH12. The asterisk* shows significant difference ($p<0.05$) between the other protein isolates as determined by Tukey HSD test.

The protein particle size indicated as the z-average diameter of the OPI and CSP is given in **Fig.4.12A** while **Fig.4.12B** shows the plot of intensity averaged to get the cumulant size which is the z-average. The mean diameter of the CSP was about 400 nm followed by OPI extracted at pH 11 with ~230 nm, and OPI extracted at pH 10 had ~ 229 nm particle size; the least particle size of ~200 nm was obtained by the OPI extracted at pH 12. The lowest particle size of OPI extracted at pH 12 would have contributed to its high zeta-potential (**Fig.4.11**). Based on the results of the particle size distribution as shown in **Fig.4.12B**, OPIs had more uniform particle size compared to CSP, although all the samples do not exhibit unimodal size particle distribution. This might be as a result of possibility of formation of aggregate (protein-protein interaction). Among the OPIs, OPI from pH 12 had broader curve and thus, more uniform particle size and this would have contributed to its better solubility and emulsion stability properties. With this information, the OPI generally, could form better emulsions than the CSP and thus, could be useful ingredients in food applications that require dissolution, dispersion and emulsion formation, as in the case of mayonnaise and liquid drink formulations.

4.2.10 Structural properties of okara protein isolates (OPI)

4.2.10.1 Scanning electron microscopy (SEM) of okara protein isolates

SEM pictures depicting the structural morphology of the defatted okara flour, CSP, and okara protein isolate samples are given in **Fig.4.13**. All OPI samples showed flaky plate like structures which appeared larger with increase in pH of extraction medium. The CSP had sphere-like structure, totally different from okara protein isolates. This might have contributed to the low protein solubility and high surface hydrophobicity that CSP exhibited. The defatted okara flour had more compacted morphology indicating the presence of other components such as carbohydrates and lipids in higher amounts compared to the protein isolates. The physical morphology of OPIs is similar to that of walnut protein isolate (Mao & Hua 2012; Hu et al. 2013). SEM images revealed that OPI extracted at pH 11 and 12 appeared thicker than OPI

extracted at pH 9 and 10 and this suggests more uniform particle size and structure for pH 11 and 12 OPIs (see **Fig.4.12B**). This might have contributed to the high protein solubility they had compared to OPI extracted at pH 9 and 10 (see **Fig.4.7**).

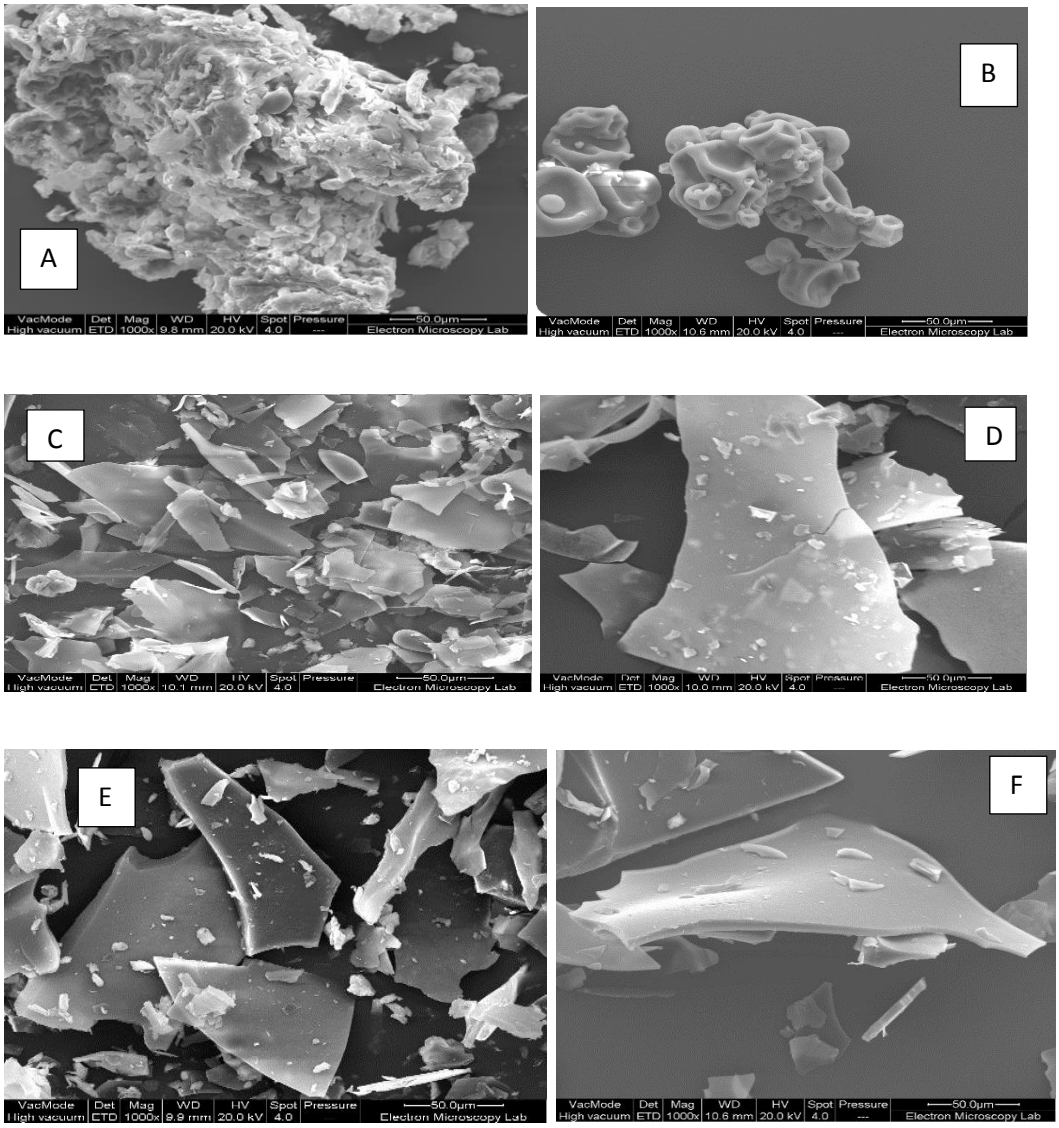


Fig.4. 13: Scanning electron microscope images of (1000 *x* magnification, 50.0 µm) of protein samples.

A:defatted okara flour; B:CSP; C:OPI extracted at pH 9; D:OPI extracted at pH 10; E:OPI extracted at pH 11; F:OPI extracted at pH 12.

4.2.10.2 Fourier transform infrared (FTIR) spectroscopic analysis

The FTIR spectra of all OPI, CSP and defatted okara flour are presented in **Fig.4.14**, while the Fourier self deconvoluted (FSD) or second derivatives of amide I spectra for all the protein isolates are presented in **Fig.4.15** respectively. The bands assigned to the different secondary structural components of the amide I spectra are shown in appendix II and the percentages of the different secondary structural components are given in **Table 4.5**. FTIR is a technique used to estimate the content of secondary structure of a protein. Proteins have different amounts of structural components (α-helix, β-sheet, random coil, turn and some side chains) and structure of proteins could affect the functions they perform in a food system. The range of the spectra that is most sensitive to slight changes in the protein structure is that of 1600 nm – 1700 nm frequency band and it is referred to as the amide I band and are associated to greater extent with C=O stretching vibration (70 – 85 %) and to a lesser extent with C-N groups (10-20%). To analyse this amide I band and evaluate any change in the type and amounts of the secondary structural components of the protein in question, the original spectra (**Fig.4.14**) must be deconvoluted and the Fourier self deconvoluted or the second derivative spectra (**Fig.4.15**) obtained must be curve fitted (Byler & Susi, 1986). Secondary structures are continuous structures kept in place by hydrogen bonding between the peptide bonds. The main secondary structures are the α-helix and β-sheet structure. Side chains are not considered as secondary structure because they are not involved in the hydrogen bonding, but they do determine the type and stability of the secondary structure of a protein (Pelley, 2007).

Looking at the FSD curve of amide I spectra of all the samples, CSP had a distinct different wave pattern from 1600 nm- 1640 nm frequency; pH 12 had two defined waves at 1632 nm and 1637 nm; pH11 had a rough surface wave at 1635 nm while pH 10 and pH 9 OPI had a smooth surface wave at 1635 nm. Moreover, the result of the percentage of the secondary structural component (**Table 4.5**) showed that pH 12 OPI had the highest β-sheet (38.8 %)

while others including the CSP had β-sheet ranging from 31.0 % to 32.7 %. On the other hand, pH 12 OPI showed a lower α-helix and Turn by about 2 % - 3 % compared to other OPI. This shift in the bands observed suggests, there might be a change in hydrogen bonding (Krimm & Bandekar, 1986) or occurrence of partial unfolding of the protein structure (Allain et al., 1999). Additionally, the lower the content of the side chain, the higher the β-sheet content. The changes observed in the structure of the protein samples might have contributed to the differences that occurred in the protein solubility (**Fig.4.7**), since structural conformation is one of the factors that affect protein solubility. There was a minor difference in the random coil of all the samples based on the extraction pH. This suggests that the unordered conformation that occurred in the proteins was not due to changes in the pH of the extraction medium, but due to the fact that the protein concentration that could lead to aggregate formation was low (Jiang et al., 2014). However, the OPI and CSP samples obtained in this research showed higher amount of β-sheet than α-helix and β-turn, similar to other plant globulins such as buckwheat and rice globulins (Choi & Ma, 2005; Ellepola et al., 2005).

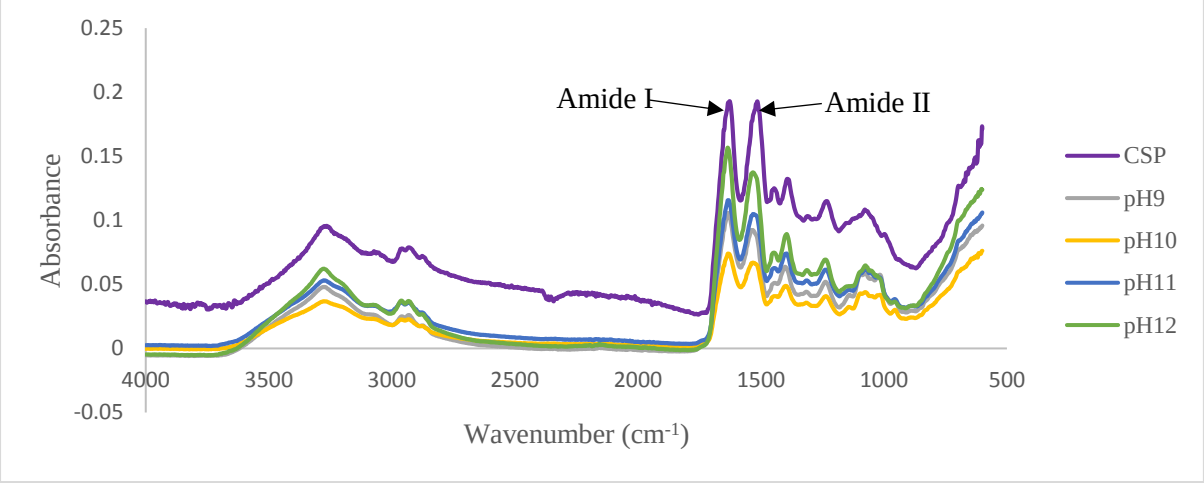


Fig.4. 14: FTIR spectra of the CSP, OPI and defatted okara flour

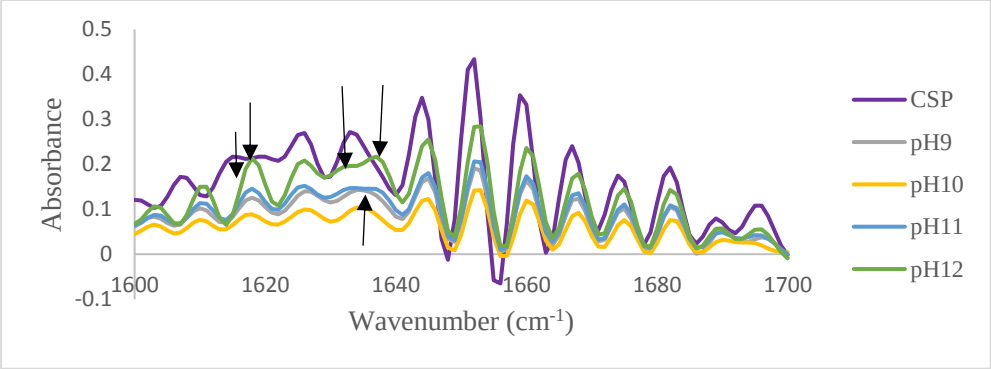


Fig.4. 15: Fourier Self Deconvoluted (FSD) spectra of amide 1 of CSP, OPI and okara flour.

Table 4. 5:Percentage content (%) of the secondary structure including the side chains of the different protein isolates after curvefitting using Raman WIRE software.

Sample	β-sheet	α-helix	Random coil	Turn	Side chain
pH 9	32.7	22.9	11.0	21.3	12.0
pH 10	31.2	23.2	11.0	21.7	12.9
pH 11	32.6	23.0	10.9	21.3	12.1
pH 12	38.8	21.4	10.5	18.9	10.5
CSP	31.0	25.8	11.3	19.2	12.7

4.2.10.3 Thermal Characteristics of OPI

Differential scanning calorimetry (DSC) was used to evaluate the thermal properties of the proteins in OPI and CSP. The denaturation temperature and enthalpy values calculated are shown in **Table 4.6**. In this study, all the endothermic peaks fell within the major endothermic peak range (0 to 180 ^{o}C) as observed by other researchers for soy proteins, rice and corn gluten meal (Tang et al., 2006; Ellepola & Ma, 2006; Di Gioia et al., 1999); except for CSP and undefatted okara flour (UOF) that had a third peak at 187.1 °C and 187.4 °C respectively. OPI from pH 9, 11, and 12 extractions had only one major endothermic peak at 153.6 ^{o}C, 156.4 °C and 148.9 °C respectively. The occurrence of only one peak could be due to the ability of the proteins (especially 7 S) to dissociate to 11 S globulin (Koshiyama, 1972; Utsumi et al., 1987; Lakemond et al., 2000). Although, these endothermic peaks are above the range of denaturation

temperatures reported in literature for β-conglycinin and glycinin when conventional method for DSC analysis was used, they agreed with the modulated DSC determinations (Tang et al., 2007). The occurrence of major endothermic peaks are attributed to loss of residual water (Di Gioia et al., 1999), while the occurrence of the minor endothermic peaks (180 to 240 °C) is attributed to loss of protein mobility because of loss of immobilized water. Hence, these minor endothermic peaks are referred to as relaxation endotherm. The enthalpy changes (ΔH) of OPI from pH 9, 11 and 12 were higher than the enthalpy changes for OPI from pH 10. The enthalpy change observed in these proteins could be associated with the loss of residual water because of disruption of the hydrogen bonds holding the water to the protein molecules. Therefore, the stronger the interactions between the water and the proteins, the higher the temperature at which they could be released which is often referred to as denaturation temperature. They are reflected by the Td_peak1 and Td_peak2 in this study.

Table 4. 6: Thermal characteristics of OPI

Sample	**Denaturation temperature, Td**			**Enthalpy Change, ΔH**		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
CSP	134.2	156.1	187.1	3.17	5.99	7.57
pH9	NA	153.6	NA	NA	13.6	NA
pH10	135.5	168.1	NA	3.25	11.9	NA
pH11	NA	156.4	NA	NA	15.1	NA
pH12	NA	148.9	NA	NA	12.0	NA
UOF	135	156.3	187.4	2.1	3.31	9.62

NA means not available.

4.3 Conclusion

Extraction of okara protein using 0.1 M phosphate buffer at pH 12 can be used as a modified conventional method to release proteins in okara (soybean residue). The optimum pH value was identified as that of 12 for maximum protein extraction yield (34.9%, w/w). The extracted isolate at pH 12 had the highest protein solubility of 83 % (w/v) at pH 10. All OPIs had emulsion capacity and stability ranging from 53.5 % to 66.0 % (v/v), which compared favourably with the emulsion capacity (60.5 %) and emulsion stability (62.0 %) of the CSP.

The surface hydrophobicity of the CSP was significantly ($p<0.05$) higher than the values obtained by all the OPI, and this could be correlated to the distinct differences in their structures as depicted with FTIR and SEM. This study revealed that extraction of protein from okara at 60 ^{o}C using 0.1 M phosphate buffer pH 12 has the potential to enhance protein recovery from okara and impact desirable properties to the isolated protein. Hence, should be used if any of the functional properties such as the protein solubility, emulsion properties, foaming properties and zeta potential, is required to be modified for their application in a food system. Based on the superior functional properties showed by the OPI extracted with phosphate buffer pH 12, the soluble aggregates formed by this OPI (pH 12) as revealed by the SDS-PAGE analysis is a desirable one. Thus, making the method a promising one for production of isolate with desirable functional properties such as protein solubility, emulsification properties and foaming properties. OPIs contained essential amino acids higher than the FAO scoring pattern except the lysine, which is the limiting amino acids for legumes, whereas the OPI extracted at pH 12 had the highest protein content of 88.8 % (w/w). This suggests their suitability as an ingredient for protein enriched food products.

Chapter 5 - Optimisation of protein extraction from soybean residue by ultrasonication

Abstract

This study focused on the optimisation of okara protein extraction via ultrasonication and on the evaluation of the functional and structural properties of protein isolates. The use of ultrasonication power at 10μmpp for 50 mins resulted in more than 100 % increase in the protein recovery when compared with the recovery from conventional alkaline extraction (control). The purity of protein recovered with ultrasonication was 85.9 % (w/w), comparable with the value (86.2 %) obtained with conventional alkaline extraction method. The nutritional quality of the ultrasonication derived OPI as shown by the amino acid composition did not differ considerably compared with the control. Although the control had the best protein solubility, it exhibited lower emulsion and foaming properties. This was attributed to structural differences resulting from ultrasonication application as depicted by Fourier transform infra-red (FTIR) spectroscopy. Moreover, the ultrasonication-extracted OPI had higher value of zeta potential and sulfhydryl contents which could have contributed to its higher emulsion and foaming properties.

Keywords: Okara, ultrasonication, extraction, soy protein, functional properties, structural properties.

5.1 Introduction

Plant-based sources of protein are considered more sustainable for the increasing global population compared to animal protein (Pimentel & Pimentel, 2003). Among all vegetable sources of protein, soybeans have gained much popularity because they are the only source of vegetable protein that contains all eight essential amino acids, that also exhibit a digestibility comparable to that of cow's milk, meat and egg protein (Endres, 2001; Soderberg, 2013). Soybean seeds are principally used to produce soymilk and tofu. Soymilk is increasingly becoming more popular by the general public, although, it is mostly consumed by lactose intolerant individuals, and those conscious of cholesterol. Soymilk global demand is estimated

to rise from 13.48 billion litres in 2015 to 16.29 billion litres in 2018 (Statista, 2018) while in the UK, the sale volume rose to 85.7 million litres in 2016 (AHDB, 2017). The main market drive for soymilk production is its association with health benefits, especially following the FDA (1999) health claim approval on soy protein effectiveness in reduction of coronary heart disease risk. Consequently, the accumulation of soymilk by-product (okara) is expected to increase; it is estimated that for every 1000 litre of soymilk produced via commercial process (the soya technology systems process) or traditional process (cold extracted and no treatment to remove off-flavour), 250 kg or 398 kg of okara is generated respectively (Gavin & Wettstein, 1990). Okara contains notable amounts of protein (26.8 %-37.5 % w/w) (Ma et al., 1997; Surel & Couplet, 2005; Vishwanathan et al., 2011). However, as it is scarcely utilised, it has little market value. Recently, okara has caught the interest of some researchers for its potential application in the food industry, as a raw material for soy protein isolate extraction, potential incorporation in beef burger production, cookies, and sausage formulation. Soybean based protein isolates are reported to demonstrate useful functional properties, that would enable their application in a variety of food systems (Singh et al., 2008). Therefore, okara would gain an added value if it could be used as a feedstock for commercial production of protein isolates and this approach could, in turn, minimise waste and agree with the cradle-to-cradle concept for sustainability.

Previous studies have evaluated the extraction of proteins in soybean flakes or soybean meal in aqueous-alkaline (NaOH) conditions (conventional method), via enzymatic routes or through the use of ultrasonication-assisted method (Ma et al., 1997; Fischer et al., 2001; Karki et al., 2009; Karki et al., 2010; Vishwanathan et al., 2011). Until now, protein extraction from okara has only been carried out using aqueous-alkaline (NaOH). According to Ma et al. (1997), okara protein extraction in aqueous-alkaline (NaOH) conditions at 25°C resulted in low protein recovery (14.1%, w/w); when temperature was elevated 80 ^{o}C, 53.4 % (w/w) of okara proteins

were extracted. However, the latter extraction conditions may cause protein denaturation and aggregation as the denaturation temperatures of the two major soybean proteins, (glycinin and β-conglycinin) are approximately 82 ^{o}C and 68 ^{o}C, respectively (Riblett et al., 2001). It is also expected that most of the soluble proteins are removed during soymilk production, leaving the residue (okara) with mostly water insoluble proteins. Heating at elevated temperature of about 80 ^{o}C is an indispensable process step during soymilk production to get final product with desirable quality (Nik et al., 2009), but it leaves the residue (okara) with aggregated proteins in the intact cotyledon cells that are not easily extracted (Preece et al., 2015b). Moreover, protein fractions that have been extracted under high temperature conditions could have low solubility and decreased thermal stability (Ma et al., 1997). Ultrasonication technology has recently attracted much research interest as a technique to assist extraction processes from a variety of raw materials; examples represent the extraction of phenolic compounds from coconut shell powder (Rodrigues & Pinto, 2007), hesperidin from penggan (*Citrus reticulate*) peel (Ma et al., 2008), polyphenols, amino acids and caffeine from green tea (Xia et al., 2006), protein from soybean/soybean meal, rice, rapeseed meal and wheat germ (Moulton & Wang, 1982; Karki et al., 2010; Zhang et al., 2018; Yagoub et al., 2017; Zhu et al., 2009). Ultrasonication allows a sustainable extraction process by increasing extraction efficiency, reducing solvent utilisation, number of unit operation, extraction time, energy utilisation and environmental impact (Yagoub et al., 2017; Chemat et al., 2017). The mechanism of extraction by ultrasonication is based on the cavitation phenomenon which leads to particle or cell disintegration (Khanal et al., 2007). Cavitation occurs when movement of the sound wave in the medium causes bubbles to form, grow and collapse systematically, with consequent generation of negative pressure that exerts strong impact on the solid surface which leads to cell disintegration (Luque-Garcıa & De Castro, 2003). The disintegration of cell walls by cavitation exposes the hidden compounds in the cells to the extracting medium, hence

promoting higher extraction at shorter time (Mason et al., 1996). However, this effect can alter the native conformational structure of proteins with resultant change in their functional properties (D Julian McClements, 1995). Based on this fact, ultrasonication has been employed not only for extraction processes but also for enhancement of functional properties of extracted ingredients. On the same note, ultrasonication has been applied to enhance the antioxidant activity of flavonoids (Ashokkumar et al., 2008) but was not beneficial in terms of preserving the bioactivity of phenols (Wan et al., 2005).

According to previous studies, ultrasonication-assisted alkaline extraction is a promising approach for protein extraction (Moulton & Wang, 1982; Karki et al., 2010; Zhang et al., 2018; Yagoub et al., 2017; Zhu et al., 2009). Therefore, the aim of the study was to develop and optimise okara protein extraction process via ultrasonication by using 0.1 M phosphate buffer as the extraction medium. Ultrasonication acts by disrupting the cell wall structure and it is hypothesized that this type of treatment could enhance the release of proteins that are located in the protein bodies of palisade-like cells in soybean cotyledon within a short time and possibly, preserve the functional properties of the protein isolates.

5.2 Results and Discussion

5.2.1 Ultrasonication-assisted protein extraction from soybean residue

Okara proteins were extracted via ultrasonication by utilising alkaline phosphate buffer as the extraction medium. For control purposes, extractions were also carried out in water bath using alkaline phosphate buffer only. In both cases, the pH value of the extraction media was 12 and the processes were carried out at 60^{o}C with recirculating water bath connected to the extraction vessel for ultrasonication process to maintain the temperature at 60 ^{o}C throughout the experiment. **Fig.5.1** shows protein recovery from okara using different extraction conditions and **Fig.5.2** presents the kinetics of the protein recovery using ultrasonication method at three different amplitudes (5, 10, and 15 μmpp, peak to peak amplitude in μm,

representing low, medium and high intensities). It was noted that ultrasonication improved the protein extraction yield by more than 2-fold in the case of medium and high intensities (10 µmpp and 15 µmpp), whereas the low intensity (5 µmpp) yielded about 66 % (w/w) improvement with respect to the control (conventional aqueous alkaline method).

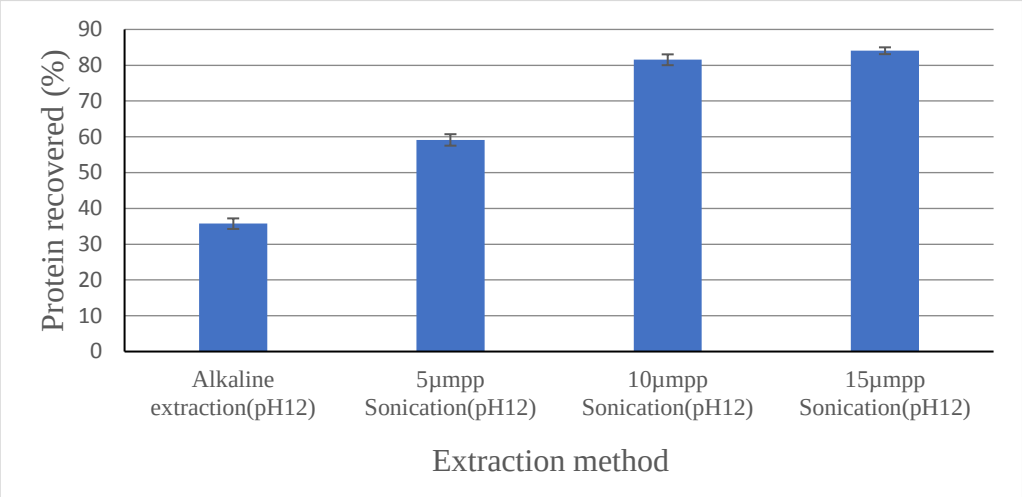


Fig.5. 1: Protein recovery from different extraction conditions.
Extraction conditions: (1) Alkaline extraction (pH12, 60 min); (2) Ultrasonication method (5µmpp amplitude, pH12, 50 min); (3) Ultrasonication method (10 µmpp amplitude, pH12, 50 min); (4) Ultrasonication method (15 µmpp amplitude, pH12, 50 min). (n=3).

Under all ultrasonication conditions protein recovery from okara was enhanced, indicating that this treatment caused disruption of the cell wall and enabled more protein bodies to be in contact with the extraction liquid and ultimately be solubilised. The result showed that increase in ultrasonication intensity increased the cavitation effects which resulted to increase in the amount of proteins that were released. The rate of the protein recovery at the three levels of amplitudes applied as shown in **Fig.5.2**, revealed that at high amplitude (15 µmpp) the rate was not considerably higher than that at medium amplitude, and specifically after 35 min of extraction. Moreover, there was difficulty maintaining the temperature at 60 ^{o}C at the high amplitude due to excess heat dissipated. Hence, further studies were carried on under low and medium amplitude for 50 min of extraction time.

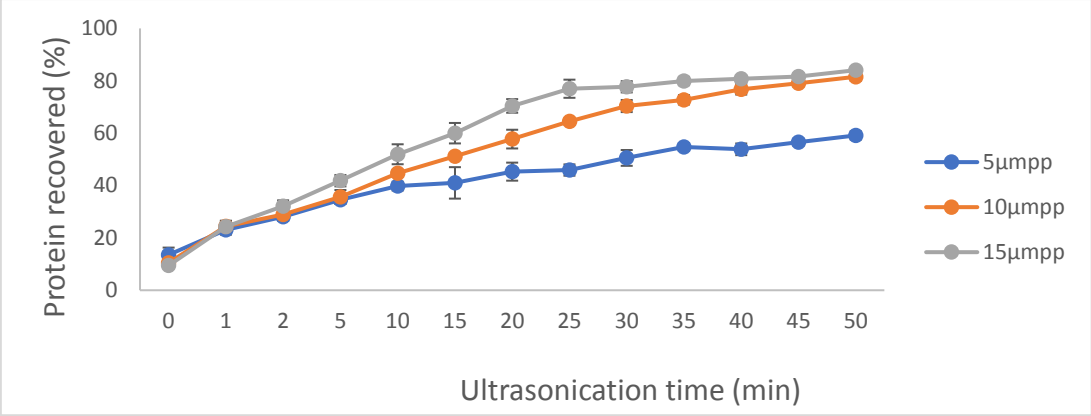


Fig.5. 2: Ultrasonication kinetics for protein recovery from okara at different amplitudes.
Data ± mean of triplicate determinations.

5.1.1 Okara protein profile by reversed phase-high performance liquid chromatography (RP-HPLC)

RP-HPLC analysis of okara protein isolates and CSP was carried out in order to compare the profile of the obtained extracts (**Fig.5.3**). The area of the protein subunits in the different protein isolates are presented in **Table 5.1**. Samples for RP-HPLC analysis for all okara protein isolates (OPIs) and CSP were standardised to 6 mg/mL. The pattern of the spectrum of commercial soybean protein (CSP) isolate was similar with those of okara protein isolates (OPIs), suggesting that they contained similar proteins. Some subunits found in the CSP were not present in the OPI and vice versa. Moreover, ultrasonication had effect on the protein subunits in the OPI by changing the percentage area as well as the time of elution.

Table 5. 1: Areas of protein subunits in the OPI and CSP samples identified with RP-HPLC

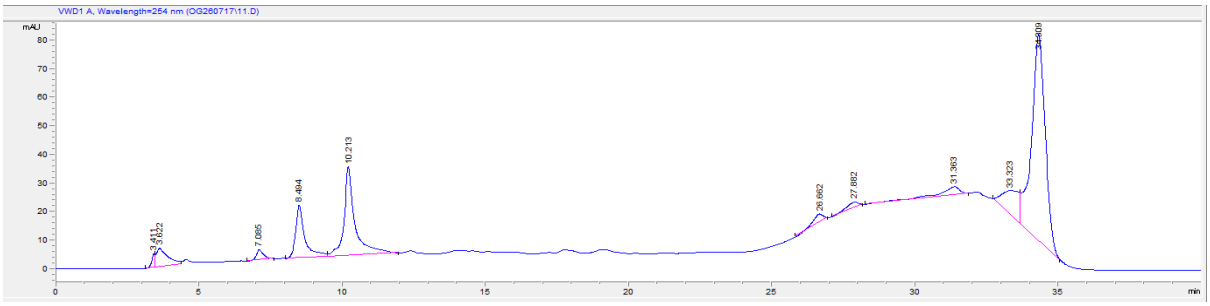
	CSP	pH12	5µm	10µm
Retention time (min)	**mean %Area**	**mean %Area**	**mean %Area**	**mean %Area**
3.4-3.5	NA	1.2±0.31	NA	8.5±0.06
3.6-3.7	57.4±0.28	4.7±0.86	14.8±0.38	6.8±0.24
4.5-4.8	NA	NA	2.0±0.09	1.2±0.15
4.9-5.4	2.3±0.14	NA	1.8±0.04	NA
5.5-5.7	0.7±0.01	NA	NA	NA
5.8-6.0	3.6±0.01	NA	NA	NA
7.0-7.3	6.4±0.18	1.5±0.05	2.6±0.05	1.9±0.06
8.5-8.7	6.4±0.06	8.9±0.06	6.4±0.13	4.2±0.05
10.0-10.4	14.4±0.32	19.4±1.41	6.1±0.06	5.3±0.09
11.0-11.5	0.2±0.00	NA	NA	NA
12.3-12.6	NA	NA	1.4±0.06	NA
14.0-14.3	0.2±0.00	NA	NA	NA
17.5-18.0	NA	NA	2.9±0.07	3.3±0.06
18.5-19.0	0.4±0.01	NA	NA	NA
19.5-20.0	NA	NA	4.1±0.01	4.3±0.15
22.0-22.2	1.4±0.05	NA	NA	NA
26.5-27.0	1.1±0.05	1.8±0.06	NA	NA
27.5-28.0	NA	1.4±0.14	9.4±0.14	NA
28.5-29.0	2.4±0.02	NA	NA	NA
31.4-31.7	NA	3.3±0.18	3.9±0.02	NA
32.0-32.5	0.3±0.01	NA	NA	NA
33.0-33.5	0.8±0.01	8.2±0.43	NA	6.1±0.15
34.3-34.5	2.4±0.02	53.6±2.45	45.8±0.36	58.9±0.17

NA means not available.

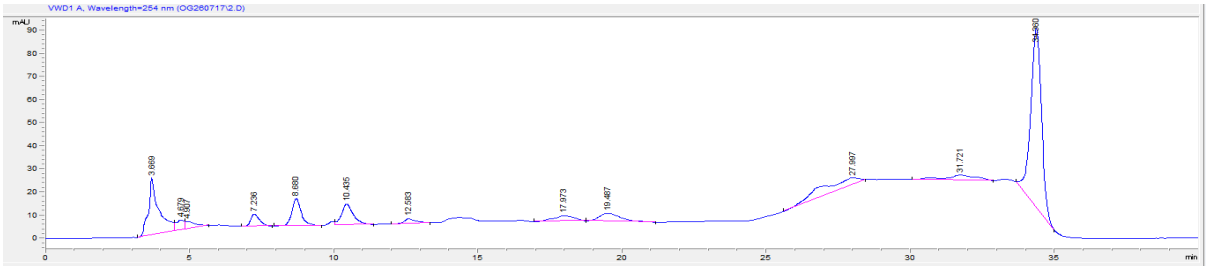
The proportion of the protein subunits in the OPI differed with each other based on the percentage area of the eluted peaks. This suggests some sorts of structural changes that resulted to partial dissociation of the two major globulins (Lakemond et al., 2000). This shows that alkaline extracted OPI may contain different amounts of 11S and 7S protein with respect to ultrasonication derived OPIs and the CSP. However, the proportion of these 11S and 7S in soy protein ingredients could influence their functionality (Lakemond et al., 2000). This study provides useful information that ultrasonication can be used to tailor proteins to perform a desired function. According to literature, the 11 S (glycinin) fraction of soybean proteins are

less hydrophobic and are eluted first while the 7 S (β-conglycinin) fraction is more hydrophobic with greater retention time (Riblett et al., 2001; Mujoo et al., 2003).

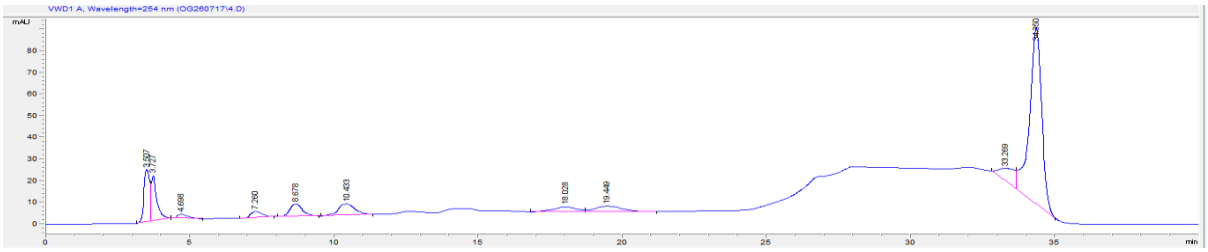
A. Alkaline extraction (6 mg/mL)



B. 5 µmpp sonication (6 mg/mL)



C. 10 µmpp sonication (6 mg/mL)



D. CSP (6 mg/mL)

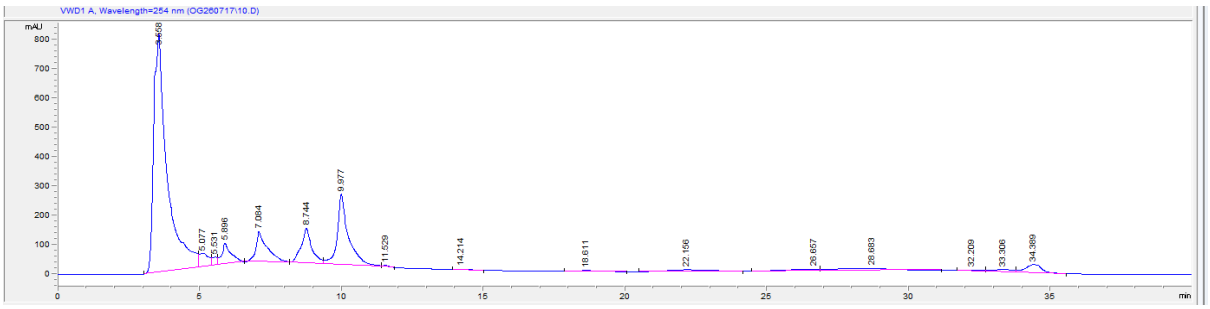


Fig.5. 3: RP-HPLC profile of okara protein isolates and CSP.
A: OPI extracted with phosphate buffer only (6 mg/mL); C: OPI extracted with ultrasonication (5 µmpp) (6 mg/mL); D: OPI extracted with ultrasonication (10 µmpp) (6 mg/mL); D: CSP (6 mg/mL).

5.1.2 Compositional analysis of okara protein isolates

The chemical composition of okara protein isolates (OPIs) is given in **Table 5.2**. OPIs extracted using conventional aqueous alkaline method had 86.2 % (w/w) protein content, whereas ultrasonication-derived OPIs (50 min) had 85.0 % (w/w) (5µmpp) and 85.9 % (w/w) protein content (10µmpp), respectively. The slight reduction in protein purity between the samples was not significantly different ($p>0.05$), suggesting that they are comparable and that ultrasonication did not affect the purity of the protein. The protein contents of all the OPIs obtained in this study were higher than the protein contents of OPI extracted using conventional method at 80^{o}C and 25^{o}C respectively, as reported by Ma et al. (1997). However, the protein contents of OPIs realised from this study could be compared with those of mung bean (87.83%), black bean (88.21%) and Bambara groundnut (85.21%) isolates reported by Kudre et al. (2013). In terms of carbohydrate content of the protein isolates, there was a small and statistically insignificant increase ($p> 0.05$) in their content in the ultrasonicated extracts (3.5 %, w/w), compared to 3.3% (w/w) in the non-ultrasonicated ones. The monomeric sugars that made up the total carbohydrate in all the okara protein isolates are glucose, rhamnose, arabinose, fucose, galactose, mannose which indicate presence of cellulosic material and pectic polysaccharide in okara. Ultrasonication caused a slight decrease in the ash content which could be attributed to the cavitation during sonication process. The cavitation effect might have loosened some minerals attached to proteins which were lost during the washing steps after protein precipitation. The difference between the ash content of the OPIs in this study and that of CSP (**Table 5.2**) might be attributed to separation method used to prepare the different isolates or the soil composition where the original seeds were grown.

Table 5. 2: Composition of okara protein isolates (OPI) (g/100g OPI)

Samples	Moisture	Ash	Fat	Protein	Carbohydrates
CSP	5.7±0.3	2.0±0.4[a]	0.1±0.04	88.8±0.7	3.8±0.1
OPI 1	5.4±0.4	4.9±1.0[b]	0.1±0.01	86.2±1.3	3.3±0.1
OPI 2	6.4±0.4	4.0±0.4[ab]	0.25±0.1	85.1±1.3	3.4±0.1
OPI 3	6.0±0.3	3.7±0.3[ab]	0.15±0.1	85.9±1.4	3.5±0.2

Values within the same column with different letters are significantly different ($p<0.05$) from each other according to Tukey HSD test. Mean = SD of duplicate values. CSP: Commercial soy protein isolate; OPI 1: Okara protein isolate extracted in water bath; OPI 2: okara protein isolate extracted using ultrasonication at 5 μmpp; OPI 3: okara protein isolate extracted using ultrasonication at 10 μmpp.

5.2.4 Amino acid composition

The amino acid composition of okara protein isolates (OPIs) and CSP are shown in **Fig.5.4**. The amino acid composition recorded for the OPIs did not differ considerably from each other. All OPI samples contained most of the essential amino acids such as valine, leucine, isoleucine, threonine, tyrosine, phenylalanine, and histidine above the FAO scoring pattern (FAO/WHO, 1991). The total amino acids in the conventional aqueous alkaline OPI, ultrasonication method at 5 μmpp and ultrasonication method at 10 μmpp were 792.9, 784.5, and 786.7 mg/mL respectively. Like other legumes, tryptophan and methionine are the limiting amino acids while glutamic acid and aspartic acid are the most predominant amino acids in all the isolates determined. This agrees with the findings of Kudre et al. (2013) on amino acids composition of mung bean, black bean Bambara groundnut protein isolates.

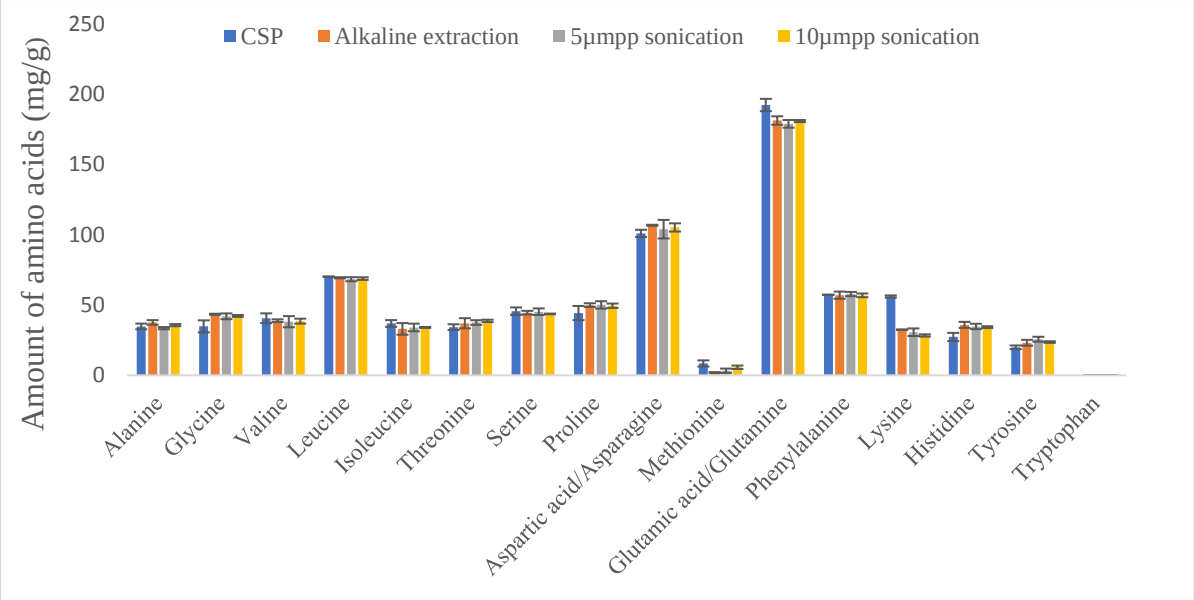


Fig.5. 4: Amino acid composition of OPI extracted at pH12 without ultrasonication, and pH 12 with ultrasonication at 5 µm and 10 µm intensity for 50 min and CSP.
CSP: Commercial soy protein isolate. Data are mean of duplicate determinations.

5.2.5 Effect of ultrasonication on the surface hydrophobicity of okara protein isolates (OPI)

The surface hydrophobicity (So) of commercial soy protein isolate (CSP) and different OPIs extracted using conventional aqueous alkaline method, and ultrasonication method at two amplitudes (5µmpp and 10µmpp), is presented in **Fig.5.5**. The surface hydrophobicity of the commercial soy protein isolate (CSP) was 518.8 and that of aqueous alkaline extracted OPI was 241.9; while OPI extracted using ultrasonication method gave 118.5 (5µmpp) and 141.7 (10µmpp). From the result, commercial soy protein isolate had much higher surface hydrophobicity (518.8) than the okara protein isolates (118.5-241.9), which could be that the different processing methods used to prepare the protein isolates, caused the CSP to have more partially unfolded proteins with more of the hydrophobic ends exposed (Wagner et al., 2000). Moreover, conventional method derived OPI had higher surface hydrophobicity than ultrasonication method derived OPI; this could be attributed to possible formation of complexes/aggregates resulting from cavitation effect during ultrasonication process (Tang et

al., 2009; Jiang et al., 2014). The result also showed that application of ultrasonication at lower amplitude of 5µmpp caused a higher reduction in the surface hydrophobicity than when sonicated at amplitude of 10µmpp. It could be that more cavitation effect that occurred with 10µmpp amplitude, led to more partially unfolded proteins to remain on the surface, causing it to have more surface hydrophobicity than OPI extracted at lower (5µmpp) amplitude. This agrees with the fact that ANS fluorescence probe has stronger affinity binding exposed hydrophobic surfaces in partially unfolded proteins than native or completely unfolded protein (Pallares et al., 2004). However, the values of surface hydrophobicity obtained for the ultrasonication method derived protein isolates (118.5 for 5µmpp and 141.7 for 10µmpp) could compare favourably with the range (122 – 150) reported by Ma et al. (1997).

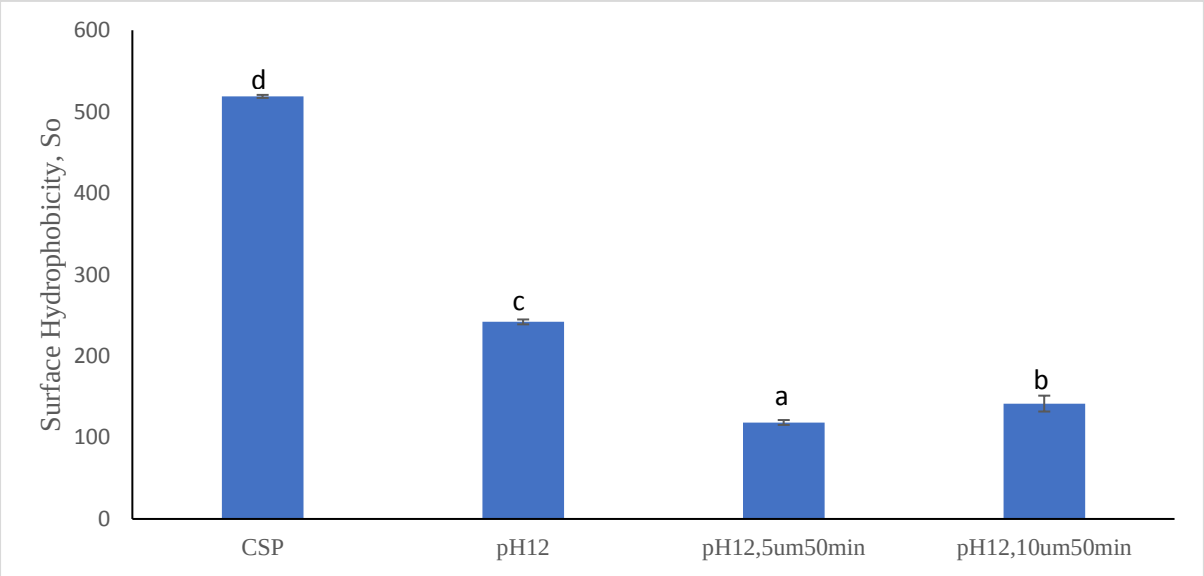


Fig.5. 5: Surface hydrophobicity of OPI.
Means and standard deviation are from triplicate determinations.
Significant difference ($p < 0.05$) is as determined by Tukey HSD test.

5.2.6 Effect of ultrasonication on the sulfhydryl content of okara protein isolate (OPI)

The free sulfhydryl (FSH), total sulfhydryl (TSH) and disulphide bond (S-S) contents of CSP and different OPIs extracted using conventional aqueous alkaline method, and ultrasonication method at two amplitudes (5µmpp and 10µmpp) are presented in **Fig.5.6** below.

The result showed that the FSH content increased with increase in the sonication amplitude. Comparing CSP and OPI samples, CSP had 93.5 µM/g of FSH while OPI derived from conventional aqueous alkaline method had 90.2 µM/g of FSH, and OPIs derived from ultrasonication method at 5 µmpp and 10 µmpp had 92.6 and 94.2 µM/g of FSH respectively. The values of FSH for all the protein isolates compared well with each other except OPI derived from conventional aqueous alkaline method that had FSH significantly different ($p<0.05$) from the rest statistically. The increase in sulfhydryl contents and S-S bonds because of ultrasonication could be attributed to structural changes that might lead to dissociation from one form of sedimentation coefficient to another (such as from 11S glycinin to 7S β-conglycinin or vice versa). However, this could be referred to as cavitation induced conformation changes. Previous research has confirmed the possible shift from one sedimentation coefficient of soy globulins to another especially between the two major globulins (7S and 11S) caused by environmental factors such as ionic strength and pH (Koshiyama, 1972; Utsumi et al., 1987; Lakemond et al., 2000). Specifically, there are two –SH groups and 20 S–S bonds per molecule in 11S (glycinin); while, there are no –SH groups but two S–S bonds per molecule in 7S (β-conglycinin) (Kim & Wicker, 2005).

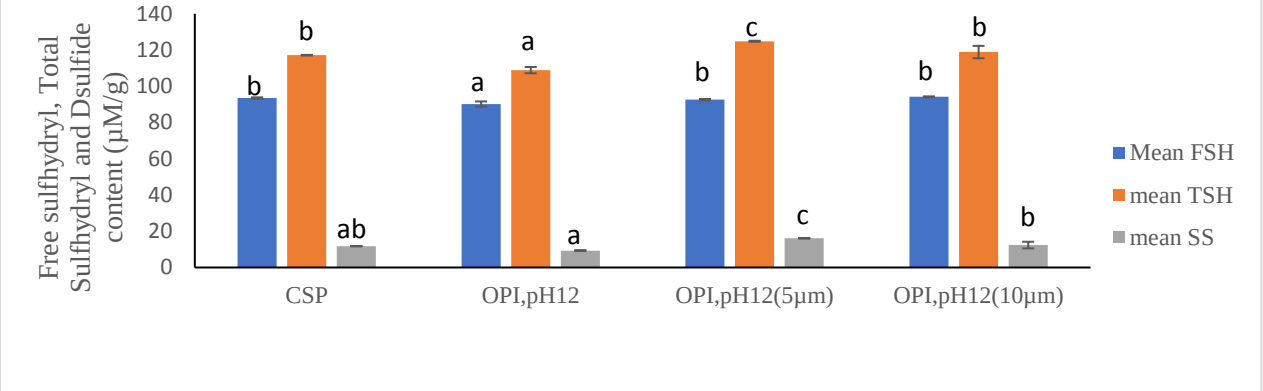


Fig.5. 6: Free sulfhydryl (FSH), total sulfhydryl (TSH) and disulphide bond (SS) contents of OPI extracted without sonication at pH 12 and with sonication at 5 µmpp and 10 µmpp amplitude and CSP.

Means and standard deviation are from triplicate determinations. CSP: Commercial Soy Protein Isolate; Means with different letters on the top of the bars of the same colour were significantly different ($p<0.05$).

5.2.7 Effect of ultrasonication on the zeta-potential of okara protein isolate (OPI)

Zeta- (ζ-) potential is the potential difference that exist between the surface of a solid particle and the liquid where the particle is immersed. An emulsion can be electrically stable if it has high ζ- potential (either positive or negative), provided it is further away from zero point and unstable (tendency to coagulate or flocculate easily) if it has low zeta potential (Lu & Gao, 2010). The pH of the medium is the most important factor that affect the zeta potential. The ζ-potential was carried out at pH 7, being away from the isoelectric pH (pH 4) of soy proteins and the pH for most food application. The values of the zeta potential of the CSP and OPIs from conventional aqueous alkaline extraction method and ultrasonication method are presented in **Fig.5.7**. CSP had the lowest zeta value of -27.73 mV but this value is comparable to the zeta value (-28 mV) obtained by Zhang et al. (2016) for the native soy protein isolate. These values are lower than zeta potential of all the OPIs, and thus, would form lesser stable emulsion or dispersion. From **Fig.5.7**, ultrasonication improved the ζ- potential of the OPI from -38.8 (obtained with alkaline extraction) to -44.1 mV (with 5µmpp) or 42.8 mV (with 10µmpp amplitude). This could be attributed to possible changes in the structural conformation of the proteins as a result of cavitation effects. RP-HPLC chromatogram revealed that ultrasonication caused some differences in the proportion of the protein subunits in the OPI (see **Table 5.1** for percentage area of the protein subunits in the OPI). The improvement indicates that ultrasonication derived OPI would form emulsions and dispersions with longer stability, which is a desired property in beverage formulations, salad dressings and mayonnaise formulations.

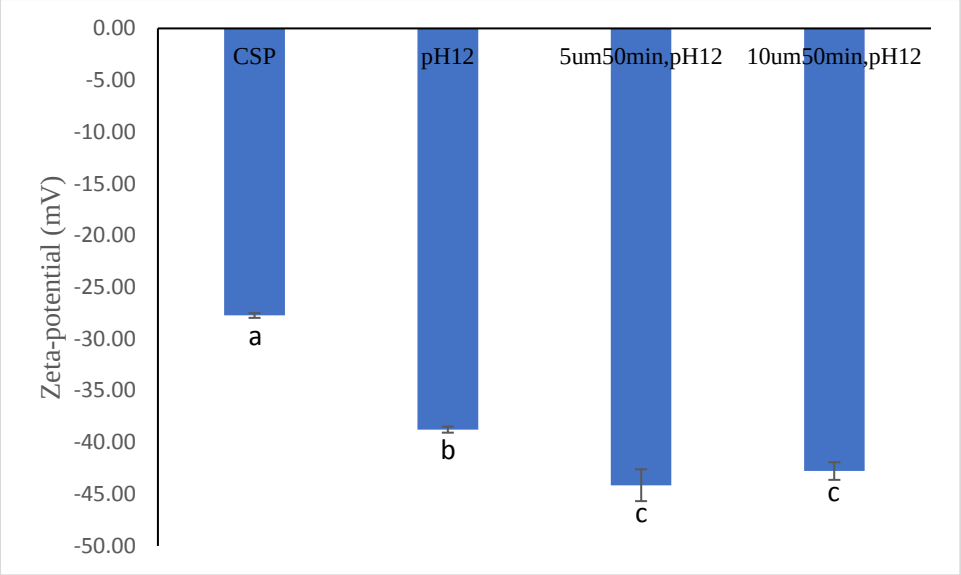


Fig.5. 7: Chart of zeta-(ζ-) potential of Commercial soy protein isolate (CSP), OPI from conventional aqueous alkaline method (pH12), OPI from ultrasonication method (5µmpp,pH12), OPI from ultrasonication method (10µmpp,pH12).
Mean±SD of triplicate results. Chart with different alphabet is significantly different ($p<0.05$), according to Tukey HSD.

5.2.8 Effect of ultrasonication on the particle size of okara protein isolate (OPI)

The size particles of CSP, OPI from conventional alkaline extraction and OPI from ultrasonication method at 5µmpp and 10µmpp amplitudes determined using nano-sizer were presented in **Fig.5.8A** and the size distribution by intensity given in **Fig.5.8B**. The CSP had the highest size particles (403.3 nm) which is significantly different ($p<0.05$) from the size particles (ranging from 201.1 nm – 222.4 nm) of all the OPI. The higher size particles of CSP would have contributed to its lower zeta-potential when compared with the OPIs. The size particles of the OPI are not significantly different ($p>0.05$) from each other irrespective of the different extraction methods applied. But sonication at low amplitude of 5 µmpp did not have any effect on the particle size while sonication at 10 µmpp increased the particle size from 201.4 nm (for the control) to 222.4 nm. This might be linked with the formation of soluble complex/aggregates due to cavitation effects (Yagoub et al., 2017). Based on **Fig.5.8B**, OPIs had more uniform particle size compared to CSP. Among the OPIs, the control (OPI from

alkaline extraction) had broader curve and thus, more uniform particle size and this would have contributed to its better solubility properties. With this information, the OPI generally, could form better emulsion than CSP and thus, are useful ingredient in food applications that require dissolution, dispersion and emulsion formation, as in the case of mayonnaise and beverage formulation.

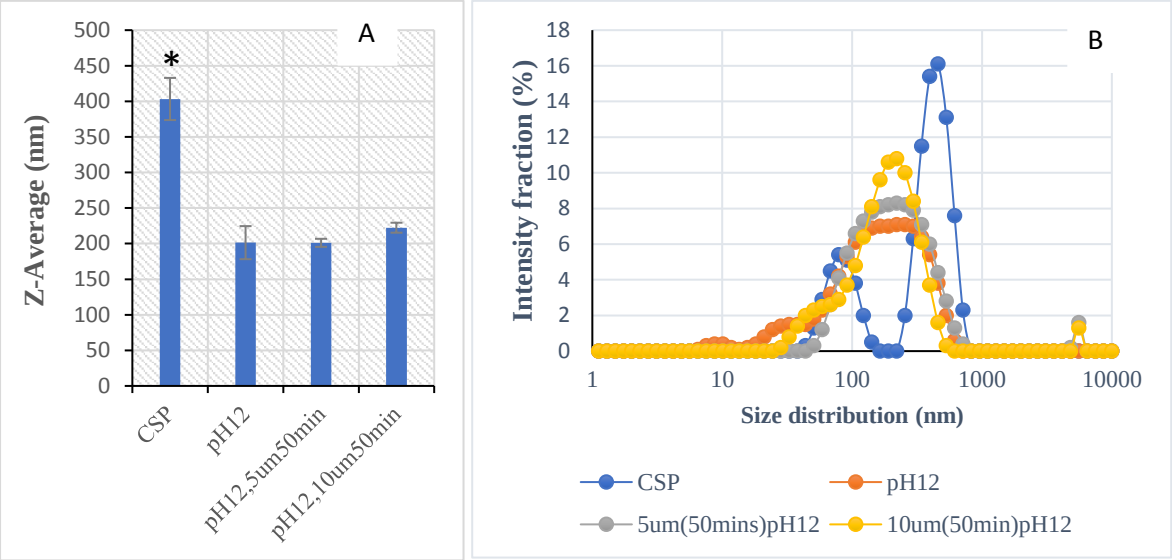


Fig.5. 8 A: Chart for Z-average diameter or cumulant size of the CSP and OPI. B: Size distribution by intensity of the CSP, and OPIs.

CSP: Commercial soy protein isolate; pH12: OPI from conventional alkaline extraction method; 5µmpp(50min) pH12: OPI from ultrasonication method at 5 µmpp amplitude; 10µmpp(50min) pH12: OPI from ultrasonication method at 10µmpp amplitude. Chart with different alphabet is significantly different ($p<0.05$), according to Tukey HSD.

5.2.9 Functional properties of OPI extracted using sonicated-assisted protein extraction method

5.2.9.1 Effect of ultrasonication on protein solubility of isolates

Protein solubility is a measure of the amount of protein that is dissolved in a solvent or liquid in relation to the total amount of protein present in the liquid and solid phase. It is a significant property for food and pharmaceutical applications as it could affect other functional properties of the protein such as emulsifying properties, foaming properties, water holding capacities, fat holding capacities, and even gelation properties. The protein solubility of okara

protein extracts is shown in **Fig.5.9** below. The solubility patterns of all OPI and CSP samples exhibited a U-shaped curve around pH values of 1.5 to 10 which agrees with the pattern of solubility curve obtained by other researchers (Ma et al., 1997; Kudre et al., 2013). From the solubility curve, OPI samples and CSP exhibited more solubility at alkaline pH than acidic pH. This is in line with the report made by Kudre et al. (2013), that the difference in solubility of protein isolates at different pH could be attributed to differences in their degree of association or dissociation of protein molecules which might have occurred due to effect of different extraction methods. The CSP had the lowest protein solubility above and below pH 4 and this can be correlated to the highest surface hydrophobicity it exhibited which agrees with the findings of (Wagner & Anon, 1990), that commercial soy protein isolates tend to have low solubility and high surface hydrophobicity as a result of the effect of denaturation. Despite that OPI from conventional aqueous alkaline method had more surface hydrophobicity (**Fig.5.5**) compared to OPI obtained from ultrasonication-assisted method, it exhibited the highest protein solubility below pH 4 and above pH 5 than OPIs from ultrasonication method; this might be attributed to some sorts of structural changes caused during ultrasonication process. The higher protein solubility obtained with all the OPIs compared to the CSP, would predispose them as a promising ingredient in food application.

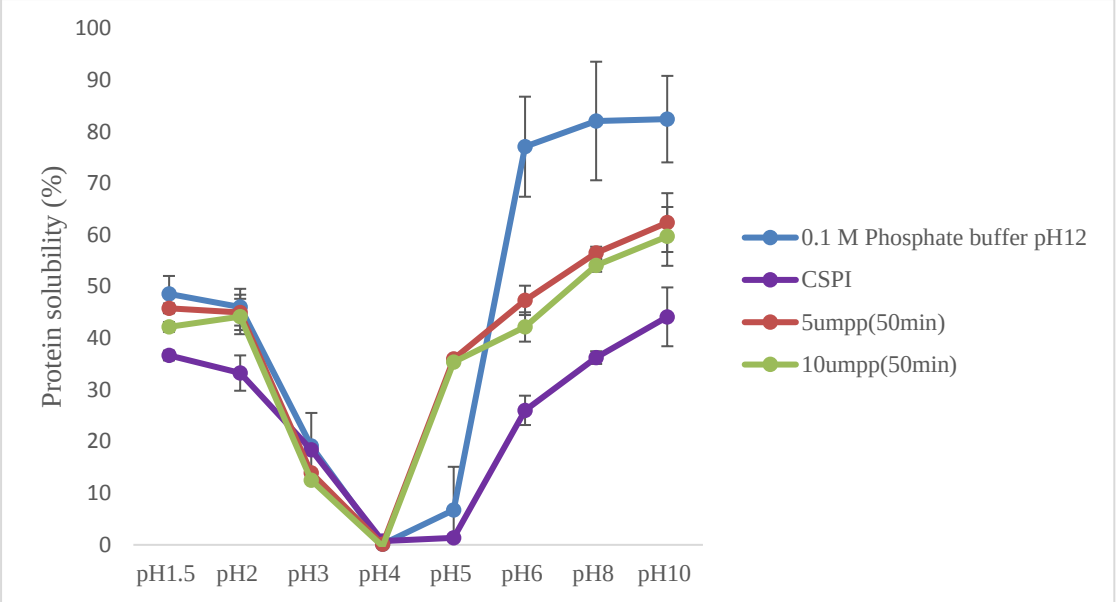


Fig.5. 9: Protein solubility curves of CSP (commercial soy protein isolate) and OPI extracted using conventional alkaline method and ultrasonication-assisted alkaline method at pH 12.
Each data point represents mean of a triplicate determination.

5.2.9.2 Emulsion properties of okara protein isolates (OPI)

The emulsion capacity and stability of okara protein isolates (OPI) and commercial protein isolate (CSP) is shown in **Fig.5.10** below. The emulsion capacity and stability tests were performed at pH 7 which is away from the isoelectric point of soybean major proteins, (4-5) (Kinsella, 1979). There was no significant increase ($p>0.05$) in the emulsion stability of samples and its range rose from 62 % for CSP to 72 % (v/v) for OPI from 10 µmpp amplitude. Both samples derived via ultrasonication method had the highest emulsion stability, 71.5 % (v/v) (for 5 µmpp amplitude) and 72 % (v/v) (for 10 µmpp) amplitude) and this correlated with the result of the zeta potential (**Fig.5.7**). Moreover, ultrasonication-assisted OPI exhibited higher emulsion capacities (67.5 % and 72 % (v/v)) when compared with that of control (conventional method derived OPI) (66 %, v/v), though not significantly different ($p>0.05$). The emulsion capacity of the CSP was lower ($p<0.05$) than emulsion capacity of all the OPIs. This might be attributed to the low solubility (**Fig.5.9**) it exhibited despite that it had the highest surface hydrophobicity (**Fig.5.5**) when compared with the OPIs. With this information, it can

be deduced that high protein solubility is required for a protein to effectively form an emulsion. Meanwhile, during the formation of emulsion, the mixing action could cause the native protein structure to unfold, exposing the hydrophobic region to the oil and hydrophilic region to the water, which lowers the surface tension between the water and oil and the rate or capacity at which this is formed is influenced by the protein structure (Hill, 1996).

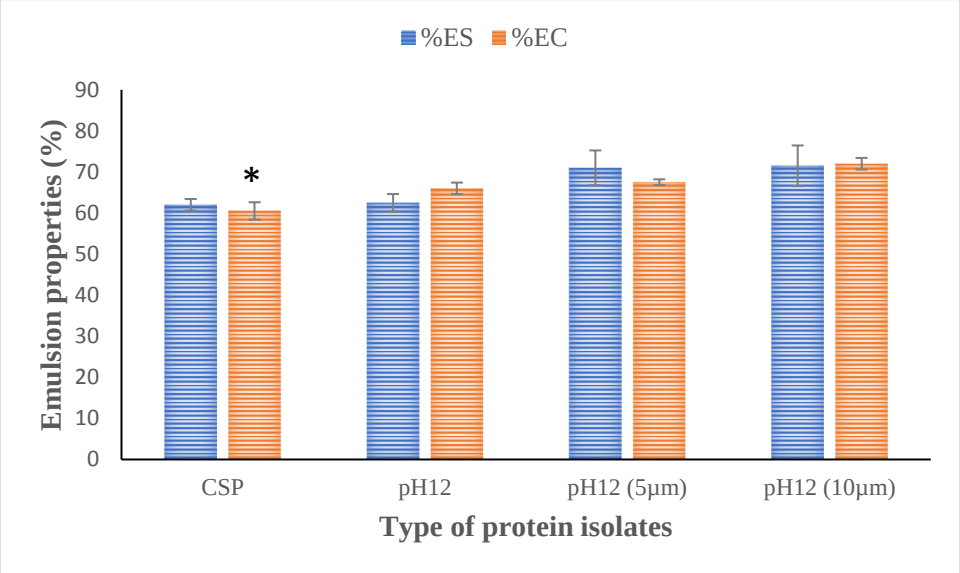


Fig.5. 10: Emulsion properties of CSP and OPI samples.
CSP: Commercial soy protein isolate; pH12: OPI from conventional alkaline extraction method; 5µmpp(50min) pH12: OPI from ultrasonication method at 5 µmpp amplitude; 10µmpp(50min) pH12: OPI from ultrasonication method at 10µmpp amplitude. ES: Emulsion stability; EC: Emulsion capacity. Means and standard deviation are from duplicate determinations. Significant difference ($p < 0.05$) as determined by Tukey HSD test. The asterisk * shows significant difference between means represented with chart of the same colour.

5.2.9.3 Foaming properties of okara protein isolates (OPI)

The foaming properties (capacity and stability) of OPI and CSP are shown in **Fig.5.11** below. The foam was produced within 1 min. The CSP had the highest foaming capacity (76.5 %, v/v) compared to the OPIs that had foaming capacity ranging from 49.5 % to 70.5 % (v/v). The use of ultrasonication at amplitude of 10 µmpp improved ($p<0.05$) the foamability of OPI from 49.5 % (v/v) obtained with OPI from conventional alkaline method to 70.5 % (v/v). This high foamability might be attributed to the rate at which the proteins are transported to the interface (within the time studied). More so, protein penetration and adsorption into the surface

layer due to their ability to unfold at the surface are other factors that may contribute to high foamability. However, having excellent foamability does not lead to formation of a stable foam. In this study, CSP with the highest foaming capacity (76.5 %, v/v) produced the least foaming stability (23 %, v/v) while the OPI from ultrasonication method at 10 µmpp amplitude produced the highest foaming stability (69 %, v/v). The high foaming stability showed by the OPI from ultrasonication method (10 µmpp amplitude), might be attributed to the bulk viscosity of the protein solution. It has been demonstrated that increase in solution viscosity decreases liquid drainage after formation of foam and thus, leads to high foam stability (Maeda et al., 1991; Kumagai et al., 1991).

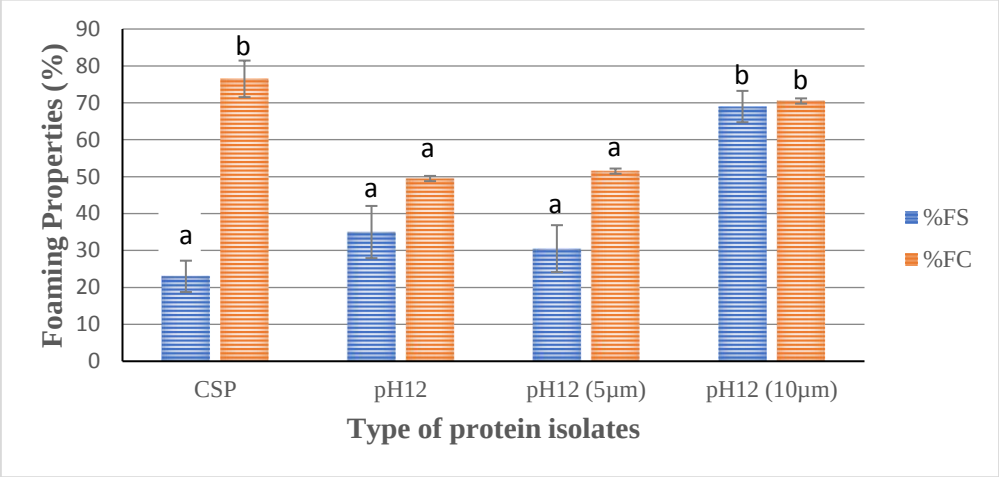


Fig.5. 11: Foaming properties of CSP and OPI samples.
CSP: Commercial soy protein isolate; pH12: OPI from conventional alkaline extraction method; 5µmpp(50min) pH12: OPI from ultrasonication method at 5 µmpp amplitude; 10µmpp(50min) pH12: OPI from ultrasonication method at 10µmpp amplitude. FS: Foaming stability; FC: Foaming capacity. Means and standard deviation are from duplicate determinations. Significant difference ($p < 0.05$) as determined by Tukey HSD test.

5.2.9.4 Water absorption capacity (WAC) and oil absorption capacity (OAC) of okara protein extracts

The results of oil and water absorption capacity of the CSP and OPI are presented in **Fig.5.12** below. Ultrasonication improved the OAC ($p<0.05$) from 2.25 mL/g (for the control) to 3.6 mL/g and 4.35 mL/g for the OPI from ultrasonication at 5 µmpp and 10 µmpp respectively. The OAC from CSP was not significantly different ($p>0.05$) from the OAC obtained with the ultrasonication derived samples. This agrees with the fact that less soluble

and hydrophobic proteins have a high oil binding capacity. This result (**Fig.5.12**) showed that ultrasonication improved the WAC (from 12.35 mL/g to 18.42 mL/g), with significant difference ($p<0.05$) seen when ultrasonicated at 10 µmpp amplitude. Considering the WAC, there was no significant difference between the result of CSP and OPI. Although, OPI derived with conventional alkaline method had the least WAC, and this could be because of its high protein solubility, which supports the findings of Kempka et al. (2014) and Wagner & Anon (1990), that low protein solubility leads to high WAC.

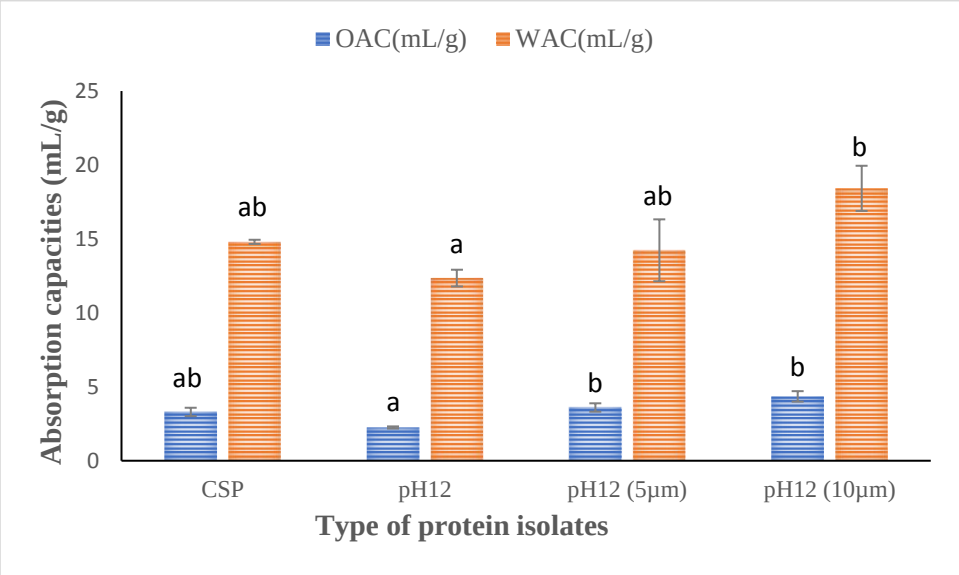


Fig.5. 12: Oil absorption capacity (OAC) and water absorption capacity (WAC) of CSP and OPI samples.

CSP: Commercial soy protein isolate; pH12: OPI from conventional alkaline extraction method; 5µmpp(50min) pH12: OPI from ultrasonication method at 5 µmpp amplitude; 10µmpp(50min) pH12: OPI from ultrasonication method at 10µmpp amplitude. Means and standard deviation are from duplicate determinations. Significant difference ($p < 0.05$) as determined by Tukey HSD test.

5.2.10 Effect of ultrasonication extraction on structural properties of okara protein isolates (OPI)

5.2.10.1 Fourier transform infrared (FTIR) spectroscopic analysis of okara protein isolates

The FTIR spectra of the OPI and CSP samples are presented in **Fig.5.13**. The original and Fourier self deconvoluted (FSD) of amide I spectra for all the protein isolates are presented

in **Figs.5.14** and **5.15**, respectively. The percentages of the different secondary structural components are given in **Table 5.3**.

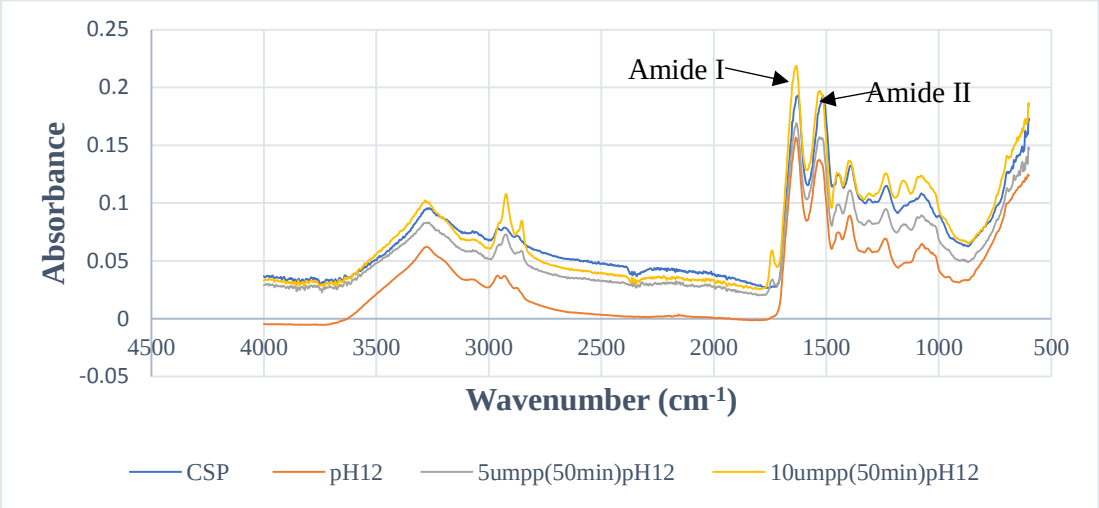


Fig.5. 13: FTIR spectra of OPI extracted with water bath and ultrasonication using buffer pH 12.

FTIR is a technique used to estimate the content of secondary structure of a protein. Proteins have different amounts of structural components (α-helix, β-sheet, random coil, turn and some side chains). Secondary structures are continuous structures kept in place by hydrogen bonding between the peptide bonds. The main secondary structures are the α-helix and β-sheet structure. Side chains are not considered as secondary structure because they are not involved in the hydrogen bonding, but they do determine the type and stability of the secondary structure of a protein (Pelley, 2007). Therefore, they are included in the percentage calculation of the contents of the secondary structure. The use of ultrasonication as the extraction process caused the β-sheet, random coil and side chain contents to become slightly decreased, while α-helix and β-turn increased. The reduction in the random coil and side chain that occurred because of the use of ultrasonication method to extract okara proteins, suggested that the proteins reordered slightly to give rise to more α-helix structure. Similarly, subjecting soybean protein isolate (SPI) to high power ultrasonication (20 kHz, 400 W and 600 W) for 30 min resulted to an increase in the α-helix components and a decrease in the β-sheet and random coil

components of the SPI secondary structure (H. Hu et al., 2013). Also, treatment of whey protein concentrate (WPC) with ultrasonication (20 kHz, 450 W) caused the α-helix contents to increase with a concomitant decrease in the β-sheet contents (Chandrapala et al., 2011). From the results of this current study, the content of the β-sheet of the OPI samples showed a positive correlation with protein solubility, as the OPI with the highest β-sheet content had the highest protein solubility.

Table 5. 3: Percentage content of the secondary structure including the side chains of the different protein isolates (%) after curvefitting with 100% Guassian band shape in WIRE 4.0 software

Sample (%)	β-sheet	α-helix	Random coil	Turn	Side chain
CSP	31.0	25.8	11.3	19.2	12.7
pH 12	38.8	21.4	10.5	18.9	10.5
5µmpp-pH12	37.7	22.7	9.4	20.7	9.4
10µmpp-pH12	38.2	23.0	9.8	19.5	9.4

Looking at the FSD spectra, distinct shift in the band around 1636 cm^{-1} and 1697 cm^{-1} occurred when OPI from conventional method (control) is compared with OPI from ultrasonication method. These shifts or differences between the control and ultrasonication derived OPI could be the cause of the differences observed in some of their functional properties such as solubility, emulsion properties, foaming properties, zeta potential as well as sulfhydryl contents. More so, CSP had a shift in the bands around 1640, 1615, 1613, 1693 and 1696 cm^{-1} when compared with the OPI samples. These shifts or differences in the structure of the CSP might have contributed to the differences in the surface hydrophobicity and protein solubility showed by the CSP when compared with the OPI samples.

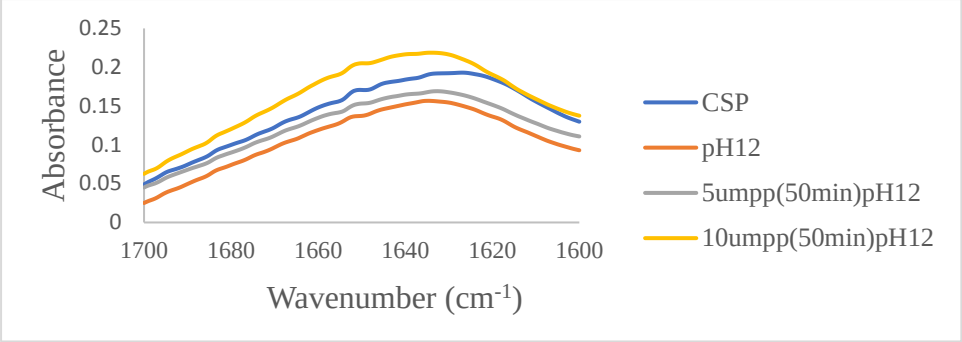


Fig.5. 14: Original amide I spectra of the OPI extracted with water bath and ultrasonication using buffer pH 12

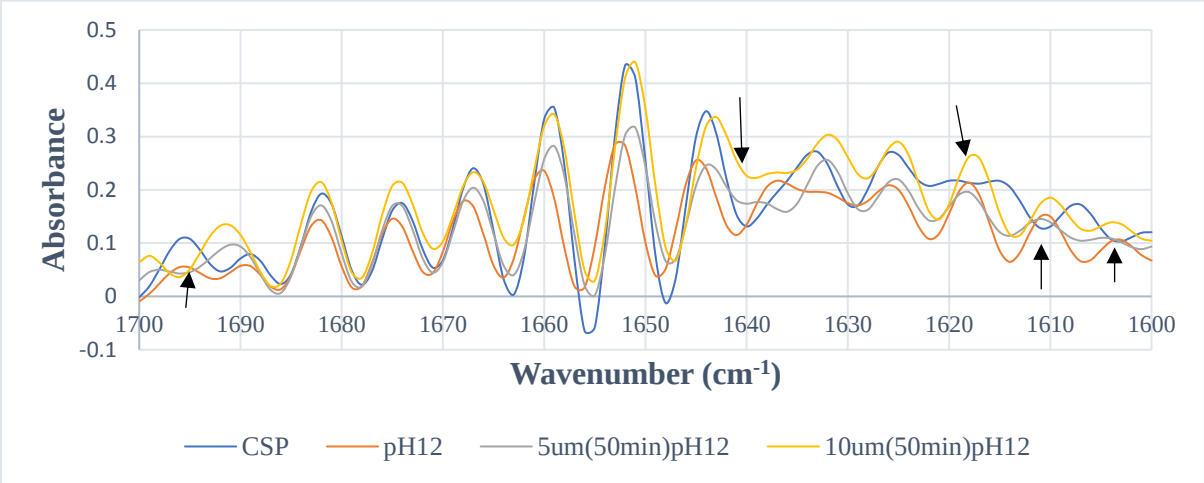


Fig.5. 15: Fourier Self Deconvoluted (FSD) of OPI extracted with water bath and ultrasonicated-assisted extraction at pH 12.

5.2.10.2 Scanning electron microscopy (SEM) of okara protein isolates

SEM images depicting the microstructure of aqueous alkaline extracted OPI, ultrasonication extracted OPI and the CSP are shown in **Fig.5.16** below. There was no difference in the structure of the OPI samples, they both showed flaky plate-like structures which is similar to that of walnut protein isolate and soy protein isolate reported by Mao & Hua (2012); and Hu et al. (2013) respectively. The structure of the OPI from aqueous alkaline method appeared thicker than the ones from ultrasonication method. This agrees with the findings of Hu et al. (2013), that although, the structure of the protein isolates may appear thicker or larger in the dry state, the actual size in dispersion is small which usually leads to

high protein solubility. However, the thick flaky plate-like structure showed by the aqueous alkaline extracted OPI, suggests that it had more uniform particle size (**Fig.5.8**). The CSP had sphere-like hollow structure, totally different from the structure of okara protein isolates. This difference in the structure, might have contributed to the large particle size (**Fig.5.8**), least protein solubility (**Fig.5.9**) and highest surface hydrophobicity (**Fig.5.5**), exhibited by the CSP.

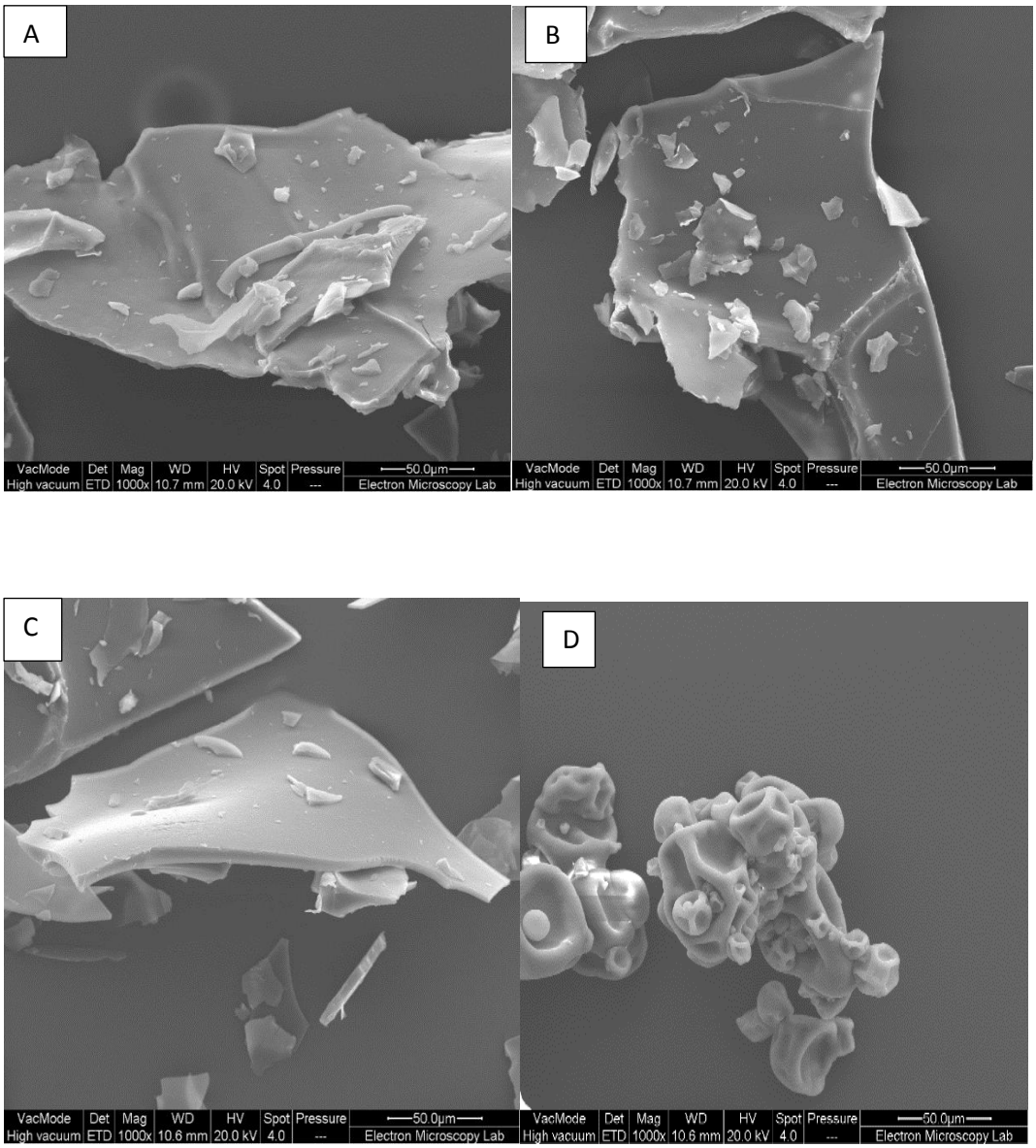


Fig.5. 16: Scanning electron microscope pictures (1000 X magnification, 50.0 µm).
A: OPI from ultasonication method (5µmpp,50min, pH12); B: OPI from ultasonication method (10µmpp,50min,pH12); C: OPI from conventional method at (60 min, pH12); D: Commercial Soy protein isolate (CSP).

5.2.10.3 Thermal Characteristics of OPI

The effect of ultrasonication method of protein extraction on the thermal properties of OPI was evaluated using DSC and the result obtained is reported in **Table 5.4**. OPI produced using conventional alkaline method (control) showed 1 peak at a temperature of 148.9 ^{o}C, while OPI produced using ultrasonication method had two peaks. The disappearnce of one of the denaturation temperation, Td1 in the control OPI could be attributed to existence of stronger protein-protein interaction than protein-water interaction; which resulted to a shift to one of the globulins coefficient of sedimentation and its subsequent structural stabilization (German et al., 1982) when heated up during DSC analysis. It would be necessary to do further studies to confirm the behaviour of these globulins (7S and 11S) by fractionating them and evaluating their thermal properties separately. OPI derived from ultrasonication method had both Td1 and Td2 with Td2 appearing at higher temperatures (158.4 ^{o}C for 5 µmpp amplitude and 168.5 ^{o}C for 10 µmpp amplitude). This suggests that they have stronger chemical bonding between the protein molecules and water molecules. In this study, all the endothermic peaks fell within the major endothermic peak range (0 to 180 ^{o}C) as observed by other researchers for soy proteins, rice and corn gluten meal (Tang et al., 2006; Ellepola & Ma, 2006; Di Gioia et al., 1999); except for CSP and undefatted okara flour (UOF) that had a third peak at 187.1 °C and 187.4 °C respectively.

Table 5. 4: Thermal properties of OPI and CSP

Sample	Denaturation temperature,Td (^{o}C)			Enthalpy,ΔH (J/g)		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
CSP	134.2	156.1	187.1	3.17	5.99	7.57
pH12	-	148.9	-	-	12.0	-
5µmpp	131.2	158.4	-	3.03	6.14	-
10µmpp	131.1	168.5	-	2.6	5..09	-
UOF	135	156.3	187.4	2.1	3.31	9.62

UOF: Undefatted okara flour; CSP: Commercial soybean protein isolate.

5.3 Conclusions

The yield of okara protein was optimised by using ultasonication alkaline phosphate buffer method. Approximately, 81 % (w/w) protein recovery was achieved with ultrasonication method when 10 µmpp amplitude was used. Evaluation of the functional properties showed that ultrasonication yielded OPI with improved ($p<0.05$) zeta potential, emulsion capacity, foaming stability, foaming capacity, WAC, and OAC. DSC analysis showed OPI from ultrasonication had better thermal properties with more stable structure during heating at temperature aboves 100 ^{o}C than the control method. Hence, ultrasonication is a promising procedure to release most of the proteins in okara (soybean residue) with improved functional properties, hence, making okara an alternative source of commercial protein isolate. Further studies can be carried out to fractionate the proteins and evaluate their chemical, functional and structural properties separately.

Chapter 6 - Application of okara protein isolate (OPI) as emulsifier in oil-in-water emulsions

Abstract

The performance of okara protein isolate (OPI) as emulsifier in highly concentrated oil-in-water (o/w) emulsion was investigated and compared with commercial plamil egg free mayo (PEM) and commercial soybean protein isolate (CSP). The flow curves (shear stress, shear rate, and apparent viscosities data), textural properties, rheological properties (storage modulus, G' and loss modulus, G"), droplet average size and confocal laser scanning microscopy were used to characterise the emulsion produced. The effect of addition of xanthan gum to enhance the quality of the emulsion based on the above-mentioned parameters was also evaluated. The result showed that ultrasonication (US) impacted positively on the rheological properties, since the emulsion made with OPI from ultrasonication method had the highest values of storage modulus (G') compared to emulsions with alkaline extracted OPI, CSP and PEM. US-extracted OPI produced emulsions had the highest casson plastic viscosity (0.58 Pa.s), followed by alkaline extracted OPI (0.21 Pa.s) and then PEM (0.12 Pa.s). This suggests that US-extracted OPI provides the most reliable structural network compared to the other emulsions. Although the addition of xanthan gum improved ($p<0.05$) all the textural properties in emulsions stabilised with alkaline extracted OPI, in the case of US-extracted OPI, xanthan gum only improved the firmness and work of shear of the emulsions. Overall, OPI samples as emulsifiers produced highly concentrated o/w emulsions with better quality than PEM even in the absence of xanthan gum addition.

Keywords: Okara protein isolates, emulsifier, emulsion, xanthan gum, rheology

6.1 Introduction

Food emulsion is defined as a mixture of two (or more) immiscible liquids in which one liquid is dispersed as droplets in the other liquid referred to as continuous phase by the help of an emulsifier under a mechanical shear (McClements, 2016). In the food industry, there are various low molecular weight synthetic emulsifiers, including monoglycerides, sucrose

esters, polyglycerol esters, and natural emulsifiers such as proteins and polysaccharides. Proteins are of great importance for the food industry, as they are not only used to enrich the nutritional quality of food products, but are also used for their functional properties, including emulsion stabilization. Proteins function as emulsifiers in various food products, mainly because they possess both hydrophobic and hydrophilic groups that adsorb at oil-water or water-oil interface and lower the interfacial tension (Bos & Van Vliet, 2001). The formation of interfacial layer around the fat globule induces electrostatic, structural and energy barriers that prevent destabilization process from setting in. The use of proteins as emulsifiers is becoming more popular since they are natural, nutritive, widely available and potentially cheap. Since in recent years people are becoming more conscious of their health and the demand for foods with "clean labels" is high, this has driven food producers towards the use of natural alternative ingredients in food formulations. For instance, in the production of spreads, replacement of egg yolk with plant protein is becoming increasingly attractive because of the health concerns associated with frequent egg yolk consumption which include cardiovascular diseases and obesity. Moreover, protein from soy would be a promising alternative since it has a health claim for reduction of the risk of coronary heart disease (Chao, 2008) and contain no cholesterol (Hajirostamloo, 2009; Shakeel et al., 2015).

The two major components of soybean protein are glycinin (11S) with molecular mass of approximately 350 kDa and β-conglycinin (7S) with molecular mass of approximately 150-180 kDa (Boye et al., 2010). The proportion at which they occur in a soy protein ingredient affects its functionality. Soy proteins like other proteins are made up of amino acid chains containing both lipophilic and hydrophilic groups which enable their interaction with fat and water. During emulsion stabilization with globular proteins (eg. soy proteins), proteins first get adsorbed to the oil droplet and form a charged layer around fat globules which leads to mutual repulsion to prevent collision; this adsorption is dependent on the amount of hydrophobic

groups within the protein and how easily the protein structure can unfold to expose these groups (Evans et al., 2013; Chen et al., 2014). Soybean proteins have demonstrated their functionality as emulsifiers in oil in water emulsions but their functionality is limited due to their large size (Chen et al., 2014). Moreover, because of the larger size of soy protein molecules and the level of coverage of the layer formed around the oil droplet, some small oil droplets may be bridged via soy protein molecules to form large oil droplets. Hence, the surface-active property of soybean protein can be improved by physical (heating and pressure), chemical and enzymatic methods (Chen et al., 2014). These types of modification are usually carried out directly on protein isolates and could be time consuming. Moreover, it has been reported that the protein extraction method can affect the functionality of soybean protein by changing its structural conformation (Arrese et al., 1991; Sorgentini et al., 1995; Achouri et al., 2012). Ultrasound is a promising processing technology that can be used to aid extraction process in the food industry, offering advantages such as reduction in extraction time and solvent use, more effective energy utilisation and improvement of product quality (Li et al., 2013; Chemat et al., 2017). Ultrasound technology has been used to aid extraction processes of several components such as phenolic compounds from coconut shell powder (Rodrigues & Pinto, 2007), protein from soybean flakes, hesperidin from penggan (*Citrus reticulate*) peel (Ma et al., 2008; Karki et al., 2010). Ultrasound has been specifically used to modify some functional properties of soy protein isolate by applying it directly on soy protein isolate suspension (Jambrak et al., 2009; Arzeni et al., 2012). Moreover, when used as a pretreatment on soy flakes slurry prior to alkaline protein extraction to enhance protein yield (Karki et al., 2009), ultrasound was found to cause some changes in the functional properties of the soy protein isolates generated. The emulsification properties was decreased in the ultrasonication pretreated samples (Karki et al., 2009). Scarce information is available on the effect of ultrasound extracted soy protein isolates as emulsifiers in the formulation of highly concentrated oil-in-water (o/w) emulsions.

Therefore, in this study, we hypothesise based on the properties of okara protein isolate reported in the previous chapters (Chapters 3 and 4) that okara protein isolate (OPI) extracted in sodium phosphate buffer at pH 12 with conventional method and with ultrasonication could be used as emulsifier to stabilise a highly concentrated o/w emulsion. Hence, the aim of this study is to evaluate the effect of ultrasonication and alkaline method protein extracts as emulsifier to stabilise a highly concentrated o/w emulsion (mayonnaise-like emulsion). The rheological properties (storage modulus, G' and loss modulus, G''), flow properties (using power law parameters and casson law parameters), textural properties, average droplet size based on the confocal scanning microscopic image analysis were used to characterise the formed emulsions. Also, the effect of xanthan gum addition was evaluated and the rheological, flow and textural properties and average droplet size were used to characterise the formed emulsions.

6.2 Results and Discussion

6.2.1 Effect of protein concentrations on emulsion flow properties

The flow curves of the concentrated o/w emulsions containing different concentrations of CSP, alkaline extracted OPI, and US-extracted OPI as emulsifiers are shown in **Fig.6.1**, **Fig.6.2** and **Fig.6.3** respectively. Additionally, **Table 6.1** reveals the power law model parameters and the viscosities at initial and final shear rates for the emulsions made with different protein concentrations. Plamil egg free mayo was used as a reference sample in order to compare the flow behaviour of the highly concentrated o/w emulsions produced with okara protein isolates and commercial soybean protein isolate.

Table 6. 1: Power law model parameters and viscosities at initial and final shear rates for the different concentrations of the protein isolates.

Sample	Protein Conc, %, w/w	$\eta_{100S^{-1}}$	$\eta_{0.1S^{-1}}$	Model Parameters		
CSP				n	k	R^2
	1.0	$0.26^{a}\pm0.28$	$79^{a}\pm17.44$	$0.17^{b}\pm0.02$	$1.03^{ab}\pm0.10$	0.974
	2.0	$0.37^{b}\pm0.01$	$148^{ab}\pm27.06$	$0.17^{b}\pm0.03$	$1.16^{b}\pm0.01$	0.934
	2.5	$0.54^{c}\pm0.01$	$281^{bc}\pm7.39$	$0.16^{b}\pm0.00$	$1.38^{c}\pm0.03$	0.948
	3.0	$0.71^{d}\pm0.03$	$387^{c}\pm49.30$	$0.13^{ab}\pm0.02$	$1.57^{d}\pm0.07$	0.930
	4.0	$0.24^{a}\pm0.01$	$167^{ab}\pm27.88$	$0.23^{c}\pm0.02$	$0.90^{a}\pm0.05$	0.934
	PEM	$2.72^{e}\pm0.07$	$2006^{d}\pm138$	$0.09^{a}\pm0.00$	$2.25^{e}\pm0.03$	0.973
OPI_1				n	k	R^2
	1.0	$0.20^{a}\pm0.00$	$0.77^{a}\pm0.40$	$0.80^{e}\pm0.01$	$-0.16^{a}\pm0.01$	0.967
	2.0	$0.31^{a}\pm0.01$	$12^{a}\pm1.40$	$0.49^{d}\pm0.01$	$0.49^{b}\pm0.03$	0.997
	2.5	$0.45^{a}\pm0.00$	$51^{a}\pm4.54$	$0.31^{c}\pm0.01$	$0.96^{c}\pm0.02$	0.985
	3.0	$0.63^{a}\pm0.02$	$159^{a}\pm15.54$	$0.19^{b}\pm0.01$	$1.31^{d}\pm0.03$	0.948
	4.0	$1.29^{b}\pm0.02$	$559^{b}\pm2.30$	$0.11^{a}\pm0.00$	$1.82^{e}\pm0.01$	0.940
	5.0	$2.87^{c}\pm0.15$	$1569^{c}\pm84.5$	$0.10^{a}\pm0.03$	$2.21^{f}\pm0.03$	0.899
	6.0	$2.57^{c}\pm0.63$	$8633^{e}\pm358$	$0.12^{a}\pm0.00$	$2.13^{f}\pm0.11$	0.839
	PEM	$2.72^{c}\pm0.07$	$2006^{d}\pm138$	$0.09^{a}\pm0.00$	$2.25^{f}\pm0.03$	0.973
OPI_2				n	k	R^2
	1.0	$0.19^{a}\pm0.01$	$0.97^{a}\pm0.30$	$0.73^{g}\pm0.01$	$-0.11^{a}\pm0.03$	0.990
	2.0	$0.35^{ab}\pm0.01$	$25^{a}\pm1.10$	$0.38^{f}\pm0.00$	$0.71^{b}\pm0.02$	0.987
	2.5	$0.50^{ab}\pm0.00$	$54^{a}\pm0.29$	$0.32^{e}\pm0.00$	$0.98^{c}\pm0.00$	0.980
	3.0	$0.63^{b}\pm0.01$	$72^{a}\pm1.76$	$0.32^{e}\pm0.01$	$1.10^{d}\pm0.01$	0.981
	4.0	$1.61^{c}\pm0.06$	$375^{b}\pm19.7$	$0.21^{d}\pm0.01$	$1.75^{e}\pm0.03$	0.978
	5.0	$3.35^{e}\pm0.21$	$981^{c}\pm18.6$	$0.17^{c}\pm0.00$	$2.14^{f}\pm0.05$	0.971
	6.0	$7.82^{f}\pm0.24$	$3658^{e}\pm189$	$0.12^{b}\pm0.00$	$2.65^{h}\pm0.07$	0.916
	PEM	$2.72^{d}\pm0.07$	$2006^{d}\pm138$	$0.09^{a}\pm0.00$	$2.25^{g}\pm0.03$	0.973

K= Consistency index ($Pa.s^n$); n=Flow behaviour index; R^2= Coefficient of determination; $\eta_{100S^{-1}}$= Viscosity at shear rate of $100S^{-1}$; $\eta_{0.1S^{-1}}$= Viscosity at share rate of $0.1S^{-1;}$ CSP=commercial soybean protein isolate; PEM=Plamil egg-free mayo; OPI_1= okara protein isolate extracted using conventional method; OPI_2 = okara protein isolate extracted using ultrasonication method. Data on the same column with different letters are significantly different ($p<0.05$) according to Tukey HSD test.

From **Table 6.1**, it is evident that all samples exhibited shear thinning behaviour (flow behaviour indices n, less than 1). **Table 6.1** also showed that the flow behaviour index, and consistency index, k, of PEM were significantly different ($p<0.05$) from those of the emulsions stabilised with CSP. Emulsions containing 3 % (w/w) CSP had the lowest ($p<0.05$) flow behaviour index, (n=0.126) compared to all other concentrations of CSP used, indicating that the particular emulsions exhibited a shear thinning (pseudoplastic) behaviour at this CSP concentration. These particular emulsions had also the highest ($p<0.05$) value for consistency index (K=1.568 $Pa.s^n$), meaning that they were more viscous, potentially had formed a better

three-dimensional network and were more uniformly packed, compared to other emulsions stabilised with CSP. Interestingly, the emulsion stabilised with 4 % CSP (see **Table 6.1** and **Fig.6.1**) lost its consistency with an increase in its flow behaviour index and exhibited almost the same flow curve as emulsion stabilised with 1 % CSP (**Fig.6.1** and **Table 6.1**). Since a stable emulsion could not be formed with CSP above 3 % protein concentration, it means that CSP had a smaller surface coverage compared to the surface coverage of OPI samples. Surface coverage is described as surface load, which corresponds to the mass of emulsifier per unit surface area at saturation (McClements, 2016; Ozturk & Mcclements, 2016). The amount of emulsifier required to stabilize an emulsion is directly proportional to the surface load (Ozturk & Mcclements, 2016). With this information, 3 % (w/w) protein concentration was selected for CSP as the minimum amount of emulsifier required to produce a kinetically stable emulsion.

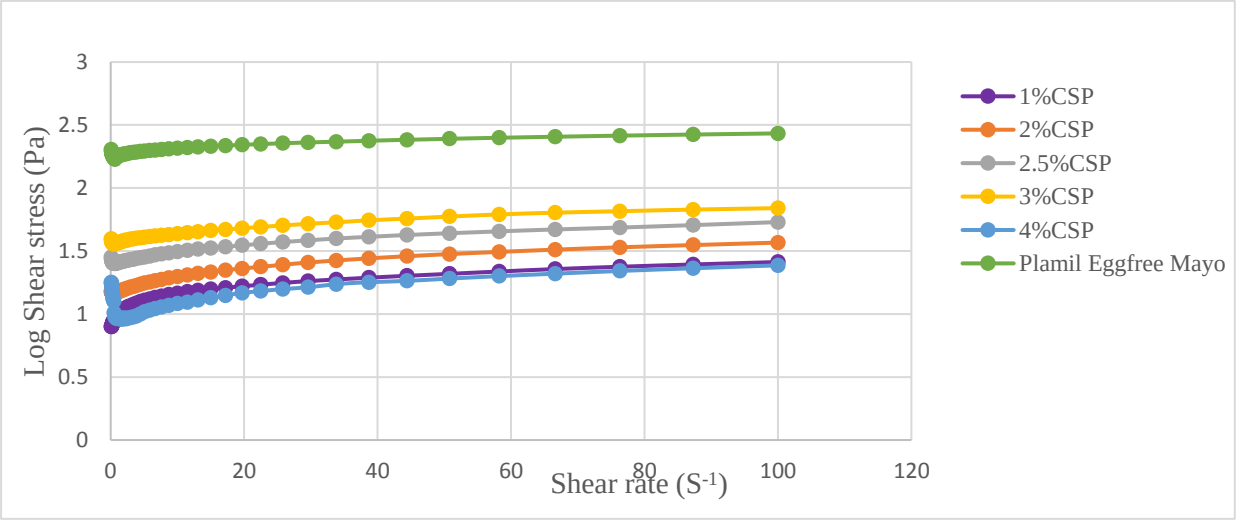


Fig.6. 1: Flow curve of the o/w emulsion, with commercial Soybean Protein Isolate (CSP) as emulsifier.

Comparing the power law parameters for all the emulsions stabilised with different concentrations of OPI from conventional method, there was a decrease in the flow behaviour index values when increasing the amounts of protein concentration until reaching 5 % (w/w) (**Table 6.1**). Also, in emulsions stabilised with alkaline extracted OPI, an increase in the consistency indices was noted, when protein concentration increased until at 5 % (w/w) protein

concentration. The consistency index of the emulsion at 5 % (w/w) protein concentration was 2.205 Pa.s^n whereas at 6 % (w/w) it was decreased to 2.129 Pa.s^n. At initial shear rate (0.1 s^{-1}), the viscosity of the emulsion made with 6 % (w/w) alkaline extracted OPI was significantly higher ($p<0.05$) (**Table 6.1** and **Fig.6.2**) than all other concentrations; however, at final shear rate (100 s^{-1}), 5 % (w/w) concentration exhibited the highest viscosity. This suggested that the three-dimensional network structure formed by the 5 % protein is more stress stable compared to the 6 % w/w protein concentration. Hence, 5 % w/w of alkaline extracted OPI was selected as the optimum amount required as emulsifier for the formation of a kinetically stable emulsion.

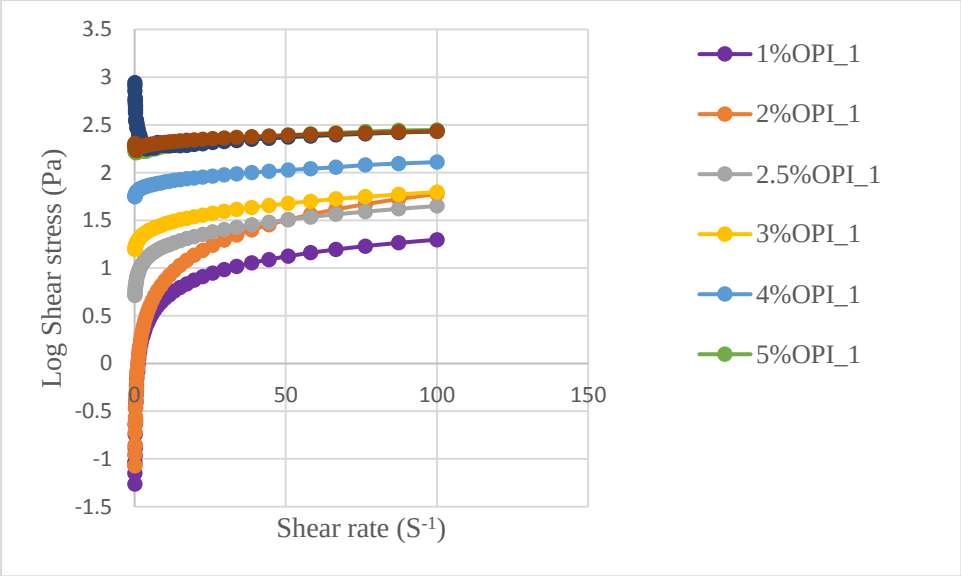


Fig.6. 2: Flow curve of the o/w emulsion containing okara protein isolates (OPI_1) from conventional alkaline phosphate buffer protein extraction method as emulsifier.

For US-extracted OPI, the flow behaviour indices decreased while the consistency indices increased with increase in OPI concentrations as expected. The 5 % (w/w) concentration was selected based on the coefficient of determination, R^2 which suggested that it fitted the model better than the 6 % (w/w) concentration of US-extracted OPI (**Table 6.1**). Moreover, the emulsion stabilised with 6 % US-extracted OPI was too viscous compared to the other concentrations and PEM (the reference sample) by having the highest yield stress

(**Fig.6.3**) as well as maintaining the significantly high viscosity at both the initial and final shear rates (**Table 6.1**).

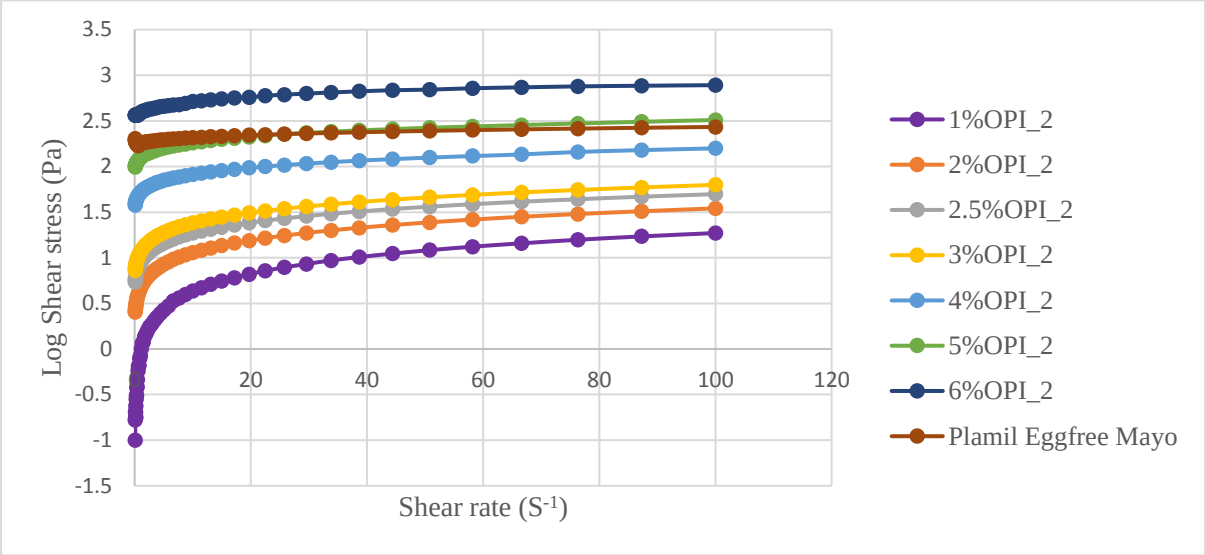


Fig.6. 3: Flow curve of the o/w emulsion containing okara protein isolates (OPI_2) from ultrasonication assisted alkaline phosphate buffer protein extraction method as emulsifier.

6.2.2 Effect of protein concentrations on emulsion droplet size

Fig.6.4 shows the average droplet size of emulsions, formed with different protein concentrations as emulsifier, as determined by light scattering using Melvin Mastersizer S. The concentration of the different emulsifiers that can be used to obtain the minimum droplet size was deduced from the analysis shown in **Fig.6.4**. Droplet size is very crucial in the formation of a kinetic stable emulsion. There was a decrease in the droplet size as the protein concentration increased (**Fig.6.4**) for all protein samples until a certain stage, where more protein addition resulted in no further changes in droplet size. During emulsion formation, proteins form a thick adsorption layer at oil/water interface, which lowers the interfacial tension at the interface and in turn causes disruption of the droplet size (Mcclements, 2004). Once the protein multilayer becomes saturated, more addition of protein would rather cause more droplets attraction, which leads to formation of larger droplet size and subsequent emulsion instability due to flocculation and/or creaming (Dickinson & Golding, 1997; Sánchez & Patino,

2005). Different emulsifiers have different maximum concentration levels in which they can best stabilize emulsions. In other words, emulsifiers have different surface coverage, often referred to as surface load. For instance, egg yolk powder was found to best stabilize emulsion at 3.2 % (w/w) concentration with minimum droplet size obtained (Moros et al., 2002), while sodium caseinate was found to be 2 % (w/w) (Dickinson & Golding, 1997; Sánchez & Patino, 2005). Therefore, for the proteins evaluated in this study, 5 % (w/w) was the threshold of concentration level for okara proteins while 3 % (w/w) was the limit for CSP, in order to achieve minimum droplets size and kinetically stable emulsions.

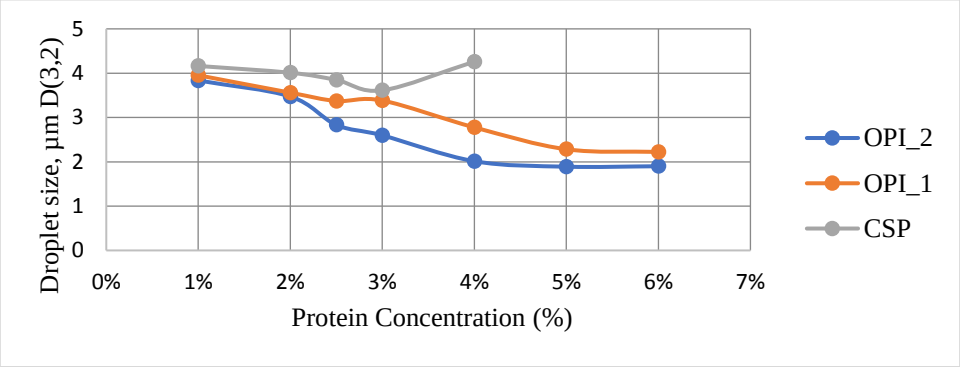


Fig.6. 4: Effect of protein isolate concentration on the sauter mean diameter of the o/w emulsions.

Data is mean of triplicate measurements. CSP: Emulsion made with commercial soybean protein isolate; OPI_1: Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method. OPI_2: Emulsion made with okara protein isolate from ultrasonication assisted alkaline phosphate buffer extraction method.

6.2.3 Spreadability (textural) properties of emulsions

Spreadability is a textural characteristic, important for foods like mayonnaise that can be spread on another food to make them palatable or offer a soft feel to the mouth. Spreadability is used to describe how fast a product can be spread on another product and parameters such as firmness, work of shear, stickiness, and work of adhesion were used to characterise the spreadability ability of the concentrated o/w emulsion produced. Firmness and work of shear of the emulsion samples are presented in **Fig. 6.5** and the stickiness and work of adhesion are presented in **Fig.6.6**. Firmness (g) of a concentrated emulsion system can be defined as the

maximum resistance displayed by it when a maximum force was applied on it. Stickiness (g) of a concentrated emulsion system is taken to be the maximum force it exhibited to overcome any attractive forces that exist between its surface and the surface of the probe that comes in contact with it. Work of shear (g.s) is the force applied for the shearing action on the emulsion product. Work of adhesion (g.s) is defined as the force used to withdraw the probe from the test sample (concentrated emulsion). Based on **Figs.6.5** and **6.6**, CSP had the lowest ($p<0.05$) firmness and stickiness properties and the addition of 0.1 % xanthan gum, increased slightly ($p<0.05$) these properties. This showed that CSP could not resist as much force as the OPI samples and this implies poorer emulsion stability when compared with the OPI samples and PEM. It also suggested that the emulsion stabilised with CSP formed a structural network that was less viscous and less rigid compared to the emulsions stabilised with OPI samples and the reference sample (PEM). The protein multilayer and the membrane formed between the CSP protein and oil droplets might have lower protective barrier, considering that the maximum amount of CSP protein required to form stable emulsion, was lower than the amount for OPI. Moreover, CSP had a different structure from the OPI as reported in the previous chapters (Chapter 3 and 4) which was revealed by the scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) results, as well as a lesser zeta potential value and these could contribute to the lesser amount of protein required to stabilize emulsions.

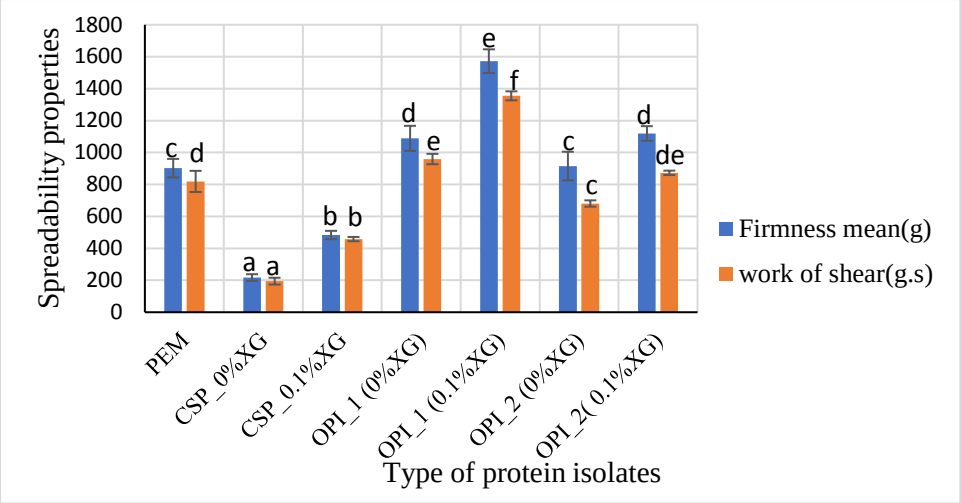


Fig.6. 5: Effect of addition of xanthan gum on the spreadability properties of the emulsions.
Data presented is mean ± SD of triplicate measurements. PEM: Plamil egg free mayo; PEM: Plamil egg free mayo; CSP_0%XG: Emulsion made with commercial soybean protein isolate without XG; CSP_0.1%XG: Emulsion made with commercial soybean protein isolate with XG; OPI_1(0%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; OPI_1(0.1%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; OPI_2(0%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; OPI_2(0.1%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum. Means with different letters on the top of the bars of the same colour were significantly different ($p<0.05$) according to Tukey HSD test.

Overall, addition of xanthan gum, improved the spreadability properties and this is probably due to the resultant high viscosity that occurred. High viscosity could enhance emulsion stability by causing a decrease in droplet mobility and prevention of subsequent interactions between the droplets (Mcclements, 2004). All spreadability parameters for emulsions containing alkaline extracted OPI were significantly higher ($p<0.05$) than PEM. Irrespective of the presence of xanthan gum, the stickiness of the emulsion containing US-extracted OPI compared favourably ($p>0.05$) with PEM, whereas the firmness was higher than ($p<0.05$) the firmness of the PEM when xanthan gum was added. Comparing only the OPI samples, emulsions containing alkaline extracted OPI had higher firmness and stickiness compared to US-extracted OPI. Moreover, alkaline extracted OPI exhibited higher solubility properties which might have enabled them to adsorb and unfold more rapidly on the interface and establish a highly viscous system with more solid-like characteristics than the US-extracted OPI. The two OPI had different structural properties as shown by the Fourier transform infrared

spectroscopy (FTIR) results reported in the previous chapter (Chapter 5). The spreadability result showed that OPI samples could form concentrated emulsions that had comparable or better textural properties than egg-free mayo, at 5 % level of concentration. They could achieve that by forming a three-dimensional network with the interacting molecules that impacted them the solid-like or mayonnaise-like characteristics.

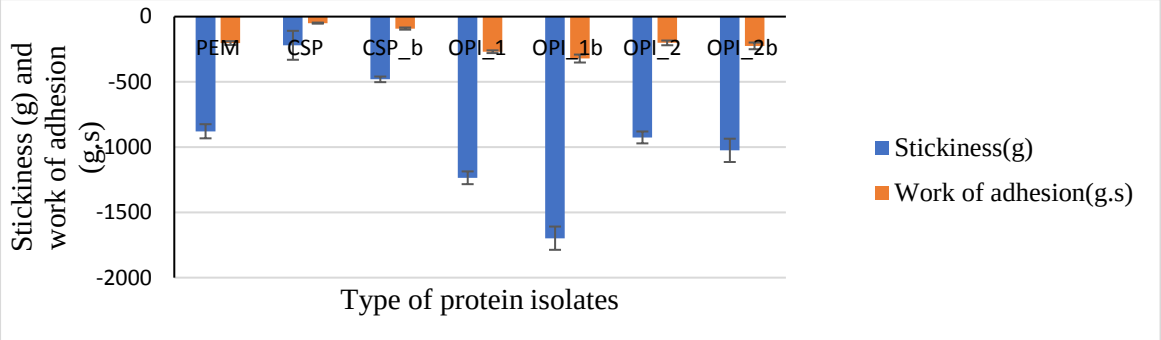


Fig.6. 6: Effect of addition of xanthan gum on the spreadability properties of the emulsions.
Data presented is mean ± SD of triplicate measurements. PEM: Plamil egg free mayo; CSP: Emulsion made with commercial soybean protein isolate without XG; CSP_b: Emulsion made with commercial soybean protein isolate with XG; OPI_1: Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; OPI_1b: Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; OPI_2: Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; OPI_2b: Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum. Means with different letters on the top of the bars of the same colour were significantly different ($p<0.05$) according to Tukey HSD test.

6.2.4 Flow curve characteristics of the emulsions

The flow curves of shear rate against shear stress of prepared emulsions and commercial sample (PEM) are shown in **Fig.6.7** below and the power law parameters and viscosities are given in **Table 6.2**. All samples showed shear-thinning (non-newtonian) behaviour, having n (flow behaviour index) less than 1. The addition of xanthan gum decreased the non-newtonian behaviour of the emulsions containing OPI samples, although not in a significantly different ($p>0.05$) manner. The consistency index for emulsions containing OPI samples were similar ($p>0.05$) whereas the addition of 0.1 % (w/w) xanthan gum did not cause any significant differences ($p>0.05$). Hence, this suggests that at 5 % OPI concentrations, the emulsion formation does not require the addition of a thickener to enhance its emulsification efficiency.

On the other hand, xanthan gum addition to CSP emulsions improved the consistency index and its non-newtonian (pseudoplastic) behaviour (see **Table 6.2** for the flow behaviour index) compared to the CSP stabilised emulsion without xanthan gum. As a shear thinning fluid, the viscosities of all samples decreased with increase in the shear rate (see **Table 6.2** for viscosity at 0.1 s^{-1} and 100 s^{-1} shear rates). The reduction in the viscosity of the emulsion at increased shearing is attributed to the disaggregation or deformation of the established network (McClements, 1999; McClements, 2016).

Table 6. 2: Power law model parameters

Emulsion Samples	**XG (w/w%)**	**$\eta_{100S^{-1}}$(Pa. s)**	**$\eta_{0.1S^{-1}}$ (Pa. s)**	**Power law model parameters**		
				n	K (Pa.S^n)	R^2
CSP	0	0.7^{a}±0.03	423^{a}±69.0	0.13^{a}±0.02	1.55^{a}±0.04	0.932
	0.1	1.4^{b}±0.04	976^{b}±192	0.09^{a}±0.00	1.95^{b}±0.01	0.880
OPI_1	0	2.9^{c}±0.16	1569^{c}±84.5	0.10^{a}±0.03	2.21^{c}±0.03	0.899
	0.1	2.6^{c}±0.25	2095^{d}±45.0	0.12^{a}±0.01	2.17^{c}±0.08	0.894
OPI_2	0	3.4^{d}±0.21	981^{b}±18.6	0.17^{b}±0.00	2.14^{c}±0.05	0.971
	0.1	3.9^{e}±0.05	1247^{b}±67.0	0.20^{b}±0.01	2.15^{c}±0.03	0.980
PEM		2.7^{c}±0.07	2006^{d}±138.6	0.09^{a}±0.00	2.25^{c}±0.03	0.973

Data presented is mean ± SD of triplicate measurements. PEM: Plamil egg free mayo; CSP: Emulsion made with commercial soybean protein isolate; OPI_1: Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method; OPI_2: Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method. XG: Xanthan gum. Means on the same column with different superscript are significantly different ($P<0.05$) according to Tukey HSD test.

The emulsion stabilised with alkaline extracted OPI and XG showed rapid deformation of its established network. This is because it had the highest viscosity at initial shear rate which got reduced rapidly causing it to have the lowest viscosity when compared with the other OPI stabilised emulsions at the end of the shearing action. This could be that the type of interaction that existed at the interface of the emulsion stabilised with alkaline extracted OPI and XG was different from the interaction at the interface of the emulsion made with OPI alone. The efficiency of emulsifiers to maintain the stability of an emulsion also depends on the conformation they assume at the interface that enables the structure to withstand an applied stress (Dickinson & Matsumura, 1991; Lefèvre & Subirade, 2003). Moreover, emulsion with

high viscosity is meant to be more stable but without a strong protective barrier, the stability would not be enhanced (Kinyanjui et al., 2003).

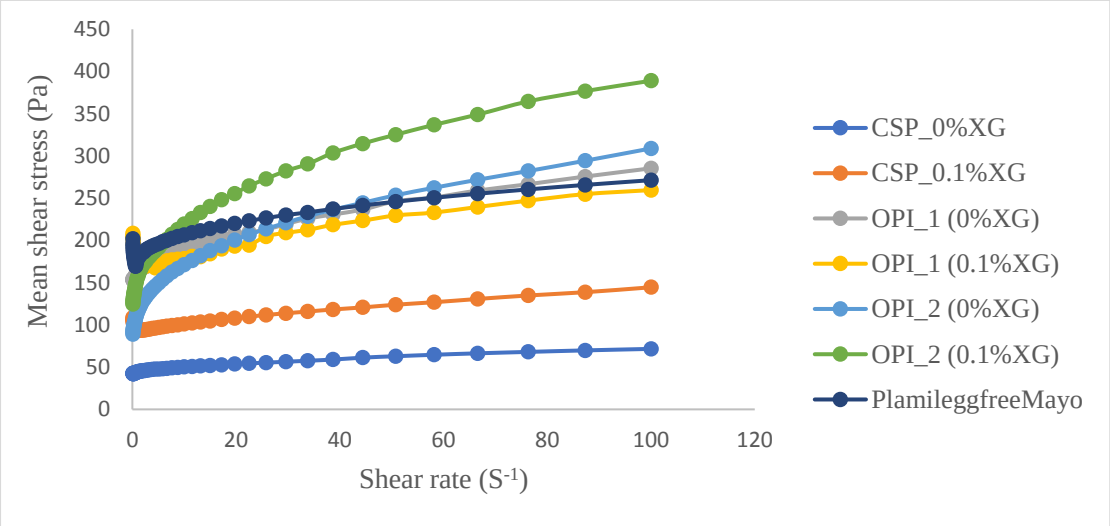


Fig.6. 7: Flow curve of the o/w emulsion emulsified with or without xanthan gum.
PEM: Plamil egg free mayo; CSP_0%XG: Emulsion made with commercial soybean protein isolate without XG; CSP_0.1%XG: Emulsion made with commercial soybean protein isolate with XG; OPI_1(0%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; OPI_1(0.1%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; OPI_2(0%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; OPI_2(0.1%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum.

The casson model was used to further interpret the rheological data of the o/w emulsions produced, by plotting the square root of shear rate, Υ against the square root of shear stress, τ. The square of the slope, K_c of the equation is calculated as the casson plastic viscosity, η_{ca} while the square of the intercept, K_{0c} is calculated as the casson yield stress, τ_{0c} and the data obtained are presented in **Table 6.3**. Casson model is a structure-based model and it is suggested as reliable model to evaluate the yield stress of a non-newtonian fluid (Servais et al., 2004). Based on the values of the coefficient of determination, R^2, all the samples fitted the model well (R^2 = 0.9075 – 0.9976). The casson model revealed that all emulsions had casson yield stress, suggesting that all protein emulsifiers formed a three-dimensional network with resultant solid-like characteristics. However, casson yield stress values for all the emulsion

systems differed, agrees fact that indicates that globular proteins have different surface activity that leads to formation of systems with different viscosity and rigidity. PEM had the highest casson yield stress whereas the highest plastic viscosity was obtained by the emulsions stabilised with US-extracted OPI (**Table 6.3**). This suggested that casson plastic viscosity offers a better indication of the stability of food emulsion than casson yield stress. Moreover, **Fig.6.7** revealed that emulsions with alkaline extracted OPI with and without xanthan gum, exhibited increased shear stress with increase in shear rate and also had the highest viscosity value at the highest shear rate of 100 S^{-1} (**Table 6.2**). The highest viscoelasticity exhibited by the emulsion stabilised with US-extracted OPI could be attributed to the possibility of cross-linking formation of disulphide bonds by the adsorbed proteins at the interface and/or in the bulk of the system (Dickinson & Matsumura, 1991).

Table 6. 3: Casson model parameters and pH of the emulsion samples

		Casson Model Parameters		
Emulsion Samples	**pH**	**Casson yield stress,τ_{0c} (Pa)**	**Casson Plastic Viscosity,η_{ca} (Pa.s)**	**R2**
PEM	3.67	174	0.12	0.991
CSP_0%XG	6.9	41.7	0.04	0.998
CSP_0.1%XG	7.10	86.3	0.07	0.992
OPI_1 (0%XG)	7.33	151	0.21	0.999
OPI_1 (0.1%XG)	7.52	154	0.10	0.908
OPI_2 (0%XG)	7.40	109	0.58	0.983
OPI_2 (0.1%XG)	7.46	144	0.69	0.990

PEM: Plamil egg free mayo; CSP_0%XG: Emulsion made with commercial soybean protein isolate without XG; CSP_0.1%XG: Emulsion made with commercial soybean protein isolate with XG; OPI_1(0%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; OPI_1(0.1%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; OPI_2(0%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; OPI_2(0.1%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum.

6.2.5 Dynamic rheological properties of the emulsions

The result of the dynamic rheological properties, storage modulus, G' and loss modulus, G" are presented in **Figs. 6.8** and **6.9** below. G' is the magnitude of the energy stored in the

product, that is recoverable per cycle of deformation and G" is a measure of the energy lost as viscous dissipation per cycle of deformation. This implies that G" is zero for a perfectly solid material that stores all its energy. In this case also, stress and strain would be in phase. However, for a non-elastic material (fluid), all the energy is lost as heat, hence, G' is zero and the stress and the strain remain out of phase by 90^{o}. It is known that for a specific food, the magnitude of the G' and G" is influenced by frequency of oscillation, temperature and extent of deformation which is the strain. Therefore, in this experiment, temperature and strain were kept constant while the effect of frequency was evaluated. The result showed that for all samples, the magnitude of both G' and G" increased with increase in the frequency of oscillation, and the value of G' (1000 Pa – 2500 Pa) for all OPI samples remained higher than the value of G" (150 Pa- 300 Pa) throughout the frequency range evaluated. This suggests that o/w emulsion samples behaved more like solid and referred to as viscoelastic solid materials, meaning that any deformation they encounter within this condition would be recoverable. This behaviour also showed that there is a strong interaction in the emulsion systems. (Çiftçi et al., 2008). This is in agreement with the dynamic rheological properties of other spreadable products such as sesame paste, pistachio butter, and mayonnaise-like emulsions (Çiftçi et al., 2008; Bengoechea et al., 2009; Emadzadeh et al., 2013). The o/w emulsion stabilised with OPI samples, with and without XG, showed higher G' and G" values than CSP which positions them as a better viscoelastic material than the commercial one. The addition of 0.1 % XG was found to increase the viscoelastic properties of all samples. Interestingly, the loss modulus, G" (**Fig.6.9**) of the emulsion stabilised with mixture of alkaline extracted OPI and XG was higher than the G" of the emulsion stabilised with US-extracted OPI only throughout the frequency tested (0.1-10 Hz), whereas the later had higher G'. This implies that alkaline and XG stabilised emulsion lost more energy as viscous dissipation per cycle of deformation than the emulsion stabilised with US-extracted OPI. This could be used to explain the reason the emulsion

stabilised with mixture of alkaline extracted OPI and XG showed lower casson plastic viscosity (0.099 Pa.s) than emulsion stabilised with only US-extracted OPI, even though the former had higher casson yield stress (see **Table 6.3** for Casson model parameters). This suggests that there could be some differences in the way that OPI proteins self-aggregate or rearrange or interact with other molecules when adsorbed at the interface to form a three-dimensional network (Dickinson & Matsumura, 1991; Lefèvre & Subirade, 2003; Rickert et al., 2004). This could be attributed to the possibility of formation of disulphide bond linked network by the US-extracted OPI at the interface, owing to its higher sulfhydryl content (see Chapter 5 for sulfhydryl contents of the US-extracted OPI and the alkaline extracted OPI) than alkaline extracted OPI. This implies that US-extracted OPI emulsions can withstand stress and handling better than alkaline extracted OPI and even PEM, the reference, commercial egg-free mayo sample.

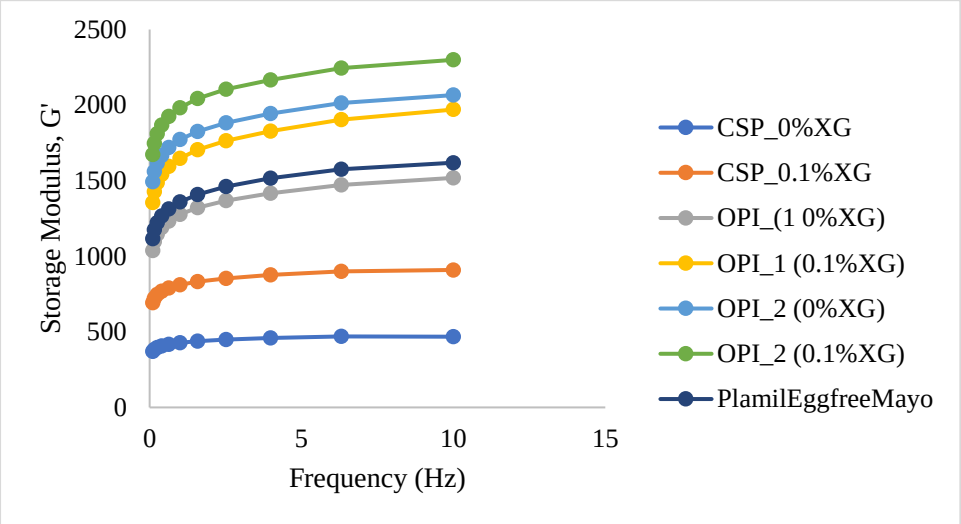


Fig.6. 8: Storage modulus of the o/w emulsions.
CSP_0%XG: Emulsion made with commercial soybean protein isolate without XG; CSP_0.1%XG: Emulsion made with commercial soybean protein isolate with XG; OPI_1(0%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; OPI_1(0.1%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; OPI_2(0%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; OPI_2(0.1%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum.

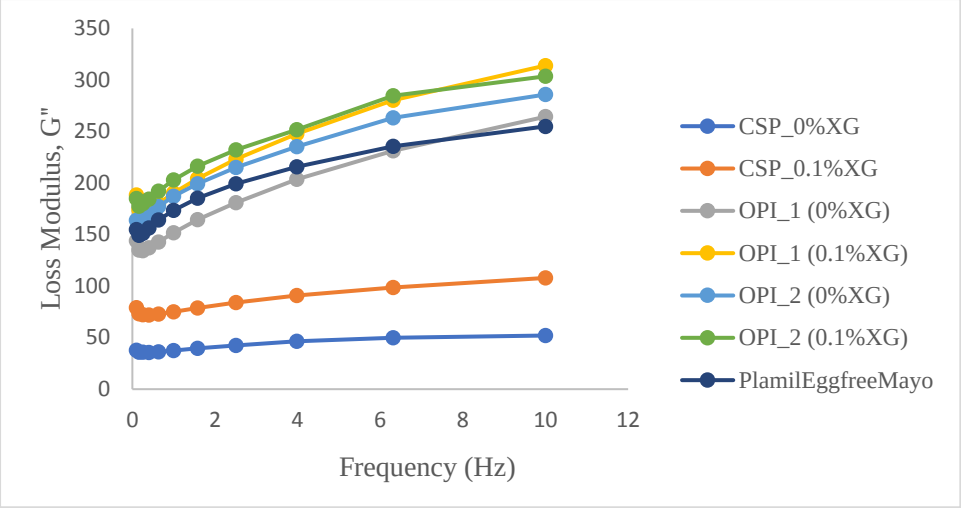


Fig.6. 9: Loss modulus of the o/w emulsions.

CSP_0%XG: Emulsion made with commercial soybean protein isolate without XG; CSP_0.1%XG: Emulsion made with commercial soybean protein isolate with XG; OPI_1(0%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; OPI_1(0.1%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; OPI_2(0%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; OPI_2(0.1%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum.

6.2.6 Confocal laser scanning microscopy (CLSM) and image analysis of fat droplets

The microstructural images of o/w emulsions captured using confocal laser scanning microscopy (CLSM) are presented in **Fig.6.10** while the results of the image analysis carried out using ImageJ are presented in **Table 6.4**. Two dyes were used to stain each sample to enhance the visibility of its components and they were selected based on their functions (rhodamine B for protein staining and nile red for staining fat). The fat droplets were shown in green while the continuous phase (comprising of the protein, water and xanthan gum) that surrounded the fat droplets appeared in red (**Fig.6.10**). Emulsions made with OPI had the smallest droplet size (ranging from 1.69 µm to 3.13 µm), while CSP had an average droplet size of 40.97 µm without 0.1 % XG and 23.84 µm with 0.1 % XG. This large droplet size of CSP emulsions could have contributed to the low rheological values they exhibited (**Figs.6.8**, **6.9**). The results also showed a negative correlation between the average size of the fat droplet and their total number (recorded as droplet count) (**Table 6.4**).

Table 6. 4: Mean values of fat droplets image analysis

Samples	Average size(μm)	Total area(μm^2)	%Area	Circularity	Droplet count
PEM	9.80^{a}±1.2	251^{a}±30.2	28.6^{a}±3.4	0.9^{ab}±0.02	26.2^{a}±1.8
CSP_0%XG	40.97^{c}±11.6	1188^{b}±190	30.5^{ab}±4.9	0.76^{a}±0.06	30.0^{a}±13.9
CSP_0.1%XG	23.84^{b}±5.5	1634^{bc}±275	40.9^{abc}±6.9	0.77^{a}±0.05	68.8^{a}±6.7
OPI_1(0%XG)	3.13^{a}±0.8	1721^{c}±265	43.2^{bc}±6.7	0.84^{ab}±0.04	564^{b}±75.8
OPI_1(0.1%XG)	2.00^{a}±0.7	1452^{bc}±291	37.3^{abc}±7.5	0.84^{ab}±0.02	771^{bc}±179
OPI_2(0%XG)	2.89^{a}±0.9	1878^{c}±350	48.2^{c}±8.9	0.83^{ab}±0.03	643^{bc}±190
OPI_2(0.1%XG)	1.69^{a}±0.7	1194^{bc}±208	29.2^{ab}±5.9	0.87^{b}±0.05	878^{c}±239

Data is mean ± SD. Mean on the same column with different superscript is significantly different ($p<0.05$) according to Tukey HSD test. PEM: Plamil egg free mayo; CSP_0%XG: Emulsion made with commercial soybean protein isolate without XG; CSP_0.1%XG: Emulsion made with commercial soybean protein isolate with XG; OPI_1(0%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; OPI_1(0.1%XG): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; OPI_2(0%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; OPI_2(0.1%XG): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum.

The formation of a larger number of droplet count is attributed to the effect of the emulsifier on droplet size reduction or disruption as it covers a larger interfacial area (Bengoechea et al., 2009). The emulsion stabilised with mixture of US-extracted OPI samples and 0.1 % XG had the smallest droplet size and the largest droplet count which supported their superior rheological properties (**Fig.6.8** and **Fig.6.9**). The circularity for the OPI samples were similar ($p>0.05$) to those of PEM, and greater than those of CSP suggesting the droplets had distinct spherical shapes, that could contribute to their well-defined network structure that gave rise to their good textural and viscoelastic properties. The percentage area covered by the droplet was below 50 % for all the samples, which indicated more coverage by the dispersion phase and subsequent larger interfacial area stabilization.

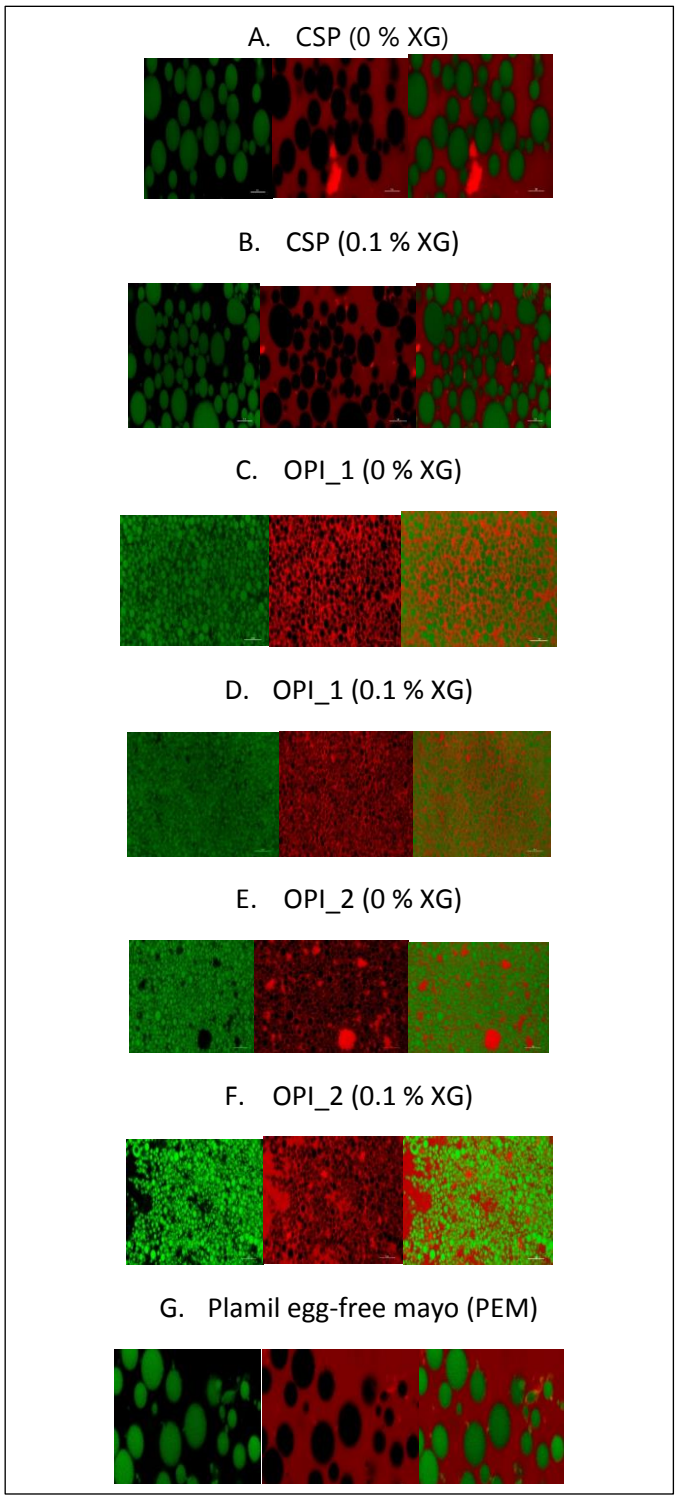


Fig.6. 10: Confocal laser scanning microscopy images of o/w emulsions stained with Rhodamine B and Nile red.

Scale bar: 10 µm. A (CSP_0%XG): Emulsion made with commercial soybean protein isolate without XG; B (CSP_0.1%XG): Emulsion made with commercial soybean protein isolate with XG; C (OPI_1(0%XG)): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method without XG; D (OPI_1(0.1%XG)): Emulsion made with okara protein isolate from conventional alkaline phosphate buffer extraction method with XG; E (OPI_2(0%XG)): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method without XG; F (OPI_2(0.1%XG)): Emulsion made with okara protein isolate from sonication assisted alkaline phosphate buffer extraction method with XG; XG: Xanthan gum; G: Plamil egg-free mayo.

6.3 Conclusion

From this research, OPI demonstrated ability to emulsify 57 % (w/w) of olive oil to produce concentrated kinetic stable o/w emulsion. This was shown in the properties (rheological, flow, textural, and microstructural) characterised which were better than the CSP and the reference sample (PEM). However, the information derived from this research on the texture, rheology and microstructure (especially the droplet size and packing) of the emulsion systems stabilised with OPI samples, revealed they can be applied to a range of food products and obtain desired characteristics. Considering that US-extracted OPI produced emulsion with the best rheological properties with the lowest droplet size, it could find applications in foods as emulsifier in the formulation of mayonnaise-like emulsions, in soy-based salad dressings, or within diary alternative products such as frozen dessert, sour cream, and cheese-like products.

Chapter 7 - General discussion and recommendations

People nowadays are more aware of the effect of their diet and lifestyle on their health. As a result, the demand for functional food products that can help in decreasing the risk of certain diseases, such as coronary heart disease, diabetes and obesity which are leading causes of morbidity and mortality. Moreover, there is a clear need to identify and develop sustainable solutions for the generation of such functional ingredients. To this end a key research priority is to minimise waste and maximise resource utilisation across the food supply chain. This project fits well with this concept as it aimed to investigate the valorisation of okara, the soybean residue produced as by-product during soymilk production. Okara is produced in increasingly large amounts worldwide, due to the increased popularity of soymilk. The fact that okara consists of high amounts of proteins, makes it a suitable material for the production of functional proteins, such as soy protein concentrates and soy protein isolates, which currently are primarily produced from soy flour. The demand for soy proteins is high because of their good nutritional values and also because they have been associated with a range of health benefits, strengthened also by the 1999 FDA claim stating that 25 grams of soy protein a day may reduce the risk of coronary heart disease. In addition to the nutritional and health benefits associated with soy protein consumption, soy proteins have important contributions towards the organoleptic and physical properties of foods including water and oil absorption capacities, as well as foaming, emulsification and gelation properties. As such, soy protein has been incorporated in a variety of food products including bakery, dairy and meat products, salad dressings and infant formulas.

7.1 Summary of the findings

In the first work package (Chapter 3), the research focused on identifying the optimum pH for soy protein extraction using phosphate buffer as the extraction medium, with the aim not to adversely affect the nutritional and functional properties of the isolated proteins. Although there are some studies on protein extraction from soybean and/or okara using

conventional alkaline extraction where NaOH is used to bring the pH of the soy/okara slurry to the required pH, this research took a different approach by employing 0.1 M phosphate buffer as the extraction medium. Moreover, this study investigated the effect of pH on the nutritional, functional and structural properties of the isolates in much more depth than in previous studies. This extraction approach used is simple and proved to be an effective method to improve protein extraction and functionality by causing favourable modification of the structural conformation (at pH 12). It was shown that the optimum pH for soy protein extraction from okara was pH 12, giving considerably higher yields than pH 9 – 11. Besides the higher extraction yield obtained at pH 12 compared to extractions at pH 9-11, the protein had better physicochemical properties including protein solubility, emulsion capacity and stability compared to CSP and the other OPIs extracted at pH 9-11. Moreover, at pH 12 the sample had the highest zeta potential value, significantly higher than that of CSP. The high zeta potential means that the protein particles had sufficient electrostatic repulsion to maintain stability and this is a good property required in making suspensions and emulsions. This type of approach which enabled the extraction of protein isolates with desirable property would be adopted by ingredients production companies, especially those producing ingredients for emulsion stabilisation. Another desirable property exhibited by the OPI extracted at pH 12 is its high protein solubility. One would think that use of the extreme pH of 12 would result to a protein isolate of poor solubility but the opposite was observed, probably due to use of buffer rather than the usual direct application of NaOH. Solubility is a very important characteristic for food applications because it influences properties such as emulsion stability, foam stability, as well as water and oil capacity absorption. The high solubility of OPI at pH 12 would enable it to find applications in systems requiring protein dissolution, such as beverages. The high zeta potential would predispose it to be used as an emulsifier to stabilise emulsions. According to the electrophoresis result of the protein profile, the OPI samples extracted at different pH had

the same protein subunits of 7S and 11S proteins as the commercial protein isolate (CSP) sample but the intensity of the bands for the CSP was higher. However, the proportion of these two major proteins (7S and 11S) influences the functional properties of soy protein ingredients because the two proteins possess different structures and consequently, different functional properties. Overall, the amino acid composition of the OPI samples compared favourably with the CSP. Lysine was the only amino acid that its concentration decreased with increasing the pH of the extraction medium, but this may not be a great concern as it can easily be enriched. Interestingly, the protein content/purity of the OPI extracted at pH 12 was the highest when compared with the OPIs extracted at pH 9, 10, and 11. Structural modification did occur during extraction at pH 12 which conferred it was indicated by the higher β-sheet content compared to the CSP and the other OPIs at pH 9-11. Moreover, Fourier self deconvoluted spectra of amide I revealed that OPI at pH 12 had two peaks between 1630 and 1640 cm^{-1} wavelength whereas the other OPIs at pH 9-11 and the CSP had only one peak within that range of wavelength. From this finding it could be suggested that the protein solubility is directly related to the β-sheet content of the protein. Hence, the improved functional properties exhibited by the OPI extracted at pH 12 could be attributed to this particular structural modification.

Another key finding of this work is that protein extraction from okara could be enhanced by the ultrasonication-assisted alkaline phosphate method (Chapter 4). Although, the alkaline phosphate buffer at pH 12 produced the highest yield of extracted protein when compared with the pH 9-11 results from this work as well as relevant literature, this was still less than 50 % recovery of the total protein in okara. The extraction of protein in okara using the conventional alkaline method is less effective generally because the protein in okara has been denatured during soy milk processing which involves high temperature treatment. The denaturation may cause self-aggregation of the protein in okara and result in increase in the size of the protein (Cox et al., 2005; Roberts, 2007), thus reducing their surface area. Hence,

ultrasonication which is a green method that uses cavitation phenomenon to disintegrate the cells, was hypothesised to enhance the release of proteins at the optimum pH of extraction (pH 12) by disrupting the cell wall of the cotyledon and dissociating the aggregated proteins. It was also hypothesised that by causing dissociation of the aggregated proteins, it could lead to structural changes and consequently modify the protein functionality. Interestingly, the findings agreed with the hypothesis. There was more than 2-fold increase in the protein recovery when ultrasonication was applied at 10 μmpp amplitude for 50 mins, compared to the recovery obtained using the conventional alkaline phosphate buffer method at the optimum pH. Overall, the amino acid composition of the OPI samples from the conventional alkaline phosphate buffer method and the ultrasonication method compared well with each other and with CSP. The result of the Fourier transform infrared spectroscopy (FTIR) revealed a reduction in the random coil content and an increase in the α-helix, suggesting that ultrasound caused the proteins to reorder giving rise to more α-helical structure. In addition, there was a shift in the bands between 1630 and 1640 cm^{-1} and another shift in the bands between 1688 and 1700 cm^{-1}. The modifications in the structure of the OPI by the ultrasonication-assisted alkaline phosphate buffer method impacted positively to the functional properties, as it improved the foaming properties (foamability and foam stability), the oil and water binding capacities, and the emulsification properties, and had higher zeta potential, when compared with the OPI extracted with the conventional alkaline phosphate buffer method. All these functional properties were better than those of CSP. Taking these improved functional properties into account, OPI derived from the ultrasonication method could find applications in food either as emulsifier in the formulation of mayonnaise-like emulsions, in soy-based salad dressings, or within diary alternative products such as frozen dessert, sour cream, cheese-like products. The improved foaming properties in particular could predispose OPI for applications in products such as dry and liquid coffee whiteners, liquid whipped toppings, pre-whipped

toppings and as alternative to sodium caseinate for uses in toppings. Such applications already have a big market in Japan and China. Moreover, the improved oil and water binding capacity together with the emulsification properties would make the OPI extracted by the ultrasonication method find applications in the manufacture of meats, bread, pasta and other bakery products. OPI would be most preferred than any synthetic surfactant or binding agent, being a natural ingredient with some physiological effects associated with it. In recent years there have been several products in the market containing soy products ranging from meat-free products (e.g. tofurky, Gardein); imitation cheese (e.g. Tyne chease at yumbles); vegetable toppings (e.g. parmesan grated topping). By developing a green and simple approach that can effectively extract the majority of the proteins from okara as well as positively modify their functional properties would add significant value to okara and make its commercial exploitation possible. Subsequently, this approach could potentially be used for the extraction of proteins from other plant sources.

The next work package focused on evaluating the emulsification properties of the extracted OPI samples from the conventional alkaline method and the ultrasonication-assisted alkaline phosphate buffer method, as well as CSP, in a highly concentrated oil-in-water emulsion model system (Chapter 5). The rheological, spreadability, droplet size and flow properties were used to characterise the emulsions formed. The result showed that ultrasonication impacted positively on the rheological properties, since the emulsion made with OPI from ultrasonication method produced much greater values of the storage modulus (G') than the loss modulus, (G") when compared with the systems emulsified with OPI from the conventional alkaline phosphate buffer method, CSP and the plamil egg-free mayo (PEM). Overall, the OPI in this study showed better rheological properties than the CSP. The spreadability properties of the OPI was comparable with that of the reference sample PEM, but better than the values obtained for CSP. This also implies OPI can be successfully used in

dairy-free spreads, imitation cheese and cream. The Casson model, a structure-based model, was successfully applied to the mayonnaise-like emulsion in this current research and was used to evaluate the yield stress and Casson plastic viscosity. The OPI extracted using the ultrasonication method also produced the emulsion with the highest value of Casson plastic viscosity (0.58 Pa.s), followed by OPI from the conventional method (0.21 Pa.s) and then the PEM (0.12 Pa.s). This suggests that the OPI from the ultrasonication method would make an emulsion with a strong structural network, as also indicated by the Casson model, which showed the emulsion could withstand stress better than the other emulsions tested. This means that handling and transportation would not affect its viscosity as it would affect other emulsions stabilised with OPI from conventional alkaline phosphate buffer and CSP. The average droplet size of the emulsion emulsified with OPI from the ultrasonication method (with and without xanthan gum) was the lowest (1.69 μm with xanthan gum and 2.89 μm without xanthan gum) followed by the emulsion emulsified with OPI from conventional alkaline phosphate buffer method (2.00 μm with xanthan gum and 3.13 μm without xanthan gum). The reduction in the droplet size with the addition of xanthan gum (stabiliser) is most likely due to the effect of the stabiliser in increasing further the viscosity of the emulsion which subsequently decreased the movement of the droplet and caused further droplet size reduction. Overall, the results of the rheological properties showed that OPI could form a viscoelastic material which can serve as a concentrated base for making imitation spreads, cheese and even cream. This suggests that the emulsions with the two OPI samples would have longer stability due to a reduced rate of droplet aggregation than the emulsions made with CSP. The possibility of emulsifying up to 57 % w/w of olive oil using OPI at 6 % (w/w) concentration to form highly concentrated stable o/w emulsion at pH 7 was achieved. On the other hand, CSP could not form stable emulsion at above 3 % protein concentration, suggesting a small surface coverage for CSP. Moreover, at 3 % protein concentration, CSP although, emulsified 57 % olive oil to form kinetic stable

emulsion system, the system was not concentrated. This was obvious by the big droplet sizes displayed by confocal laser scanning microscopy (see Chapter 5 for confocal laser scanning microscopy images). This amount of oil emulsified is high compared to other studies that used plant proteins as emulsifiers. It is also higher than the amount of oil in commercial egg-free mayo emulsified with soy protein and thickener. The pH 7 was evaluated in this study because it is far from the isoelectric point of soy protein. The extraction method utilising ultrasonication to extract proteins from okara in 0.1 M phosphate buffer pH 12 is novel, green and effective in extracting almost all the proteins in okara and favourably modifying the structural conformation of the OPI. This shows that okara can be used as alternative raw material to produce soy protein ingredients which is in high demand currently for application in several food productions.

7.2 Recommendations

Further studies are recommended to translate the findings of this research and support the implementation of this technology and the use of okara proteins in food products. A pilot study of the ultrasonication-assisted extraction method (in batch or continuous mode) is recommended to compare the yield with the values obtained in the laboratory. A larger scale of 1000 litre is recommended to be used first using the parameters that gave the highest yield in this current research before it could be further optimised. Overall, the cost effectiveness of the method needs also to be assessed, as well as the energy required to operate the system. The latter could be conducted by connecting a wattmeter to the power supply to measure the power consumed during ultrasonication; alternatively it could be calculated using the method described by Karki et al., (2010). Generating the energy data would help in the determination of the overall cost effectiveness of the process. It is also recommended to establish the exact proportion of the two major soybean proteins (Glycinin, 11S and β-conglycinin, 7S) in the OPI samples since the functional properties of soy protein ingredients could be influenced by their

ratio. Assessment of potential health benefits of OPI extracted by the ultrasonication method targeting the risk of coronary heart disease is recommended to be carried out through a human clinical trial. The risk of coronary heart disease is targeted because of the FDA claim (FDA, 1999; Henkel, 2000; Chao, 2008) for soy protein to reduce this particular risk. Another recommendation for further work is to evaluate the rheological properties at low pH values, i.e. 4.0 and 3.0, since some food products are prepared at low pH. Moreover, at least one-month storage studies are recommended to be carried out on the model emulsions made at pH 7, 4 and 3, to evaluate their kinetic stability at these pH values. Another important parameter to monitor during storage is microbial contamination. Mayonnaise is meant to have a pH of 4.0 primarily to avoid risk of salmonellosis. This is because salmonellosis has been associated with mayonnaise emulsified with egg; the latter has been confirmed to be the most likely source of *Salmonella* in mayonnaise (J. S. Garcia, 2009). Since OPI is a plant protein, this risk is negligible, however it is essential to confirm it experimentally. In addition to the okara proteins, it is recommended to develop a valorisation strategy for harnessing the polysaccharide present in okara which are expected to be more concentrated in the okara residue after protein extraction. The monomeric sugars in okara (see Chapter 3) indicated the presence of arabinogalactan, pectic polysaccharides, cellulose, and hemicellulose. These molecules constitute the dietary fibre, which depending on its structure and properties, when consumed could exert some physiological effects in human. These could include lowering the risk of coronary heart disease, lowering the serum cholesterol concentration, lowering the levels of cardiovascular disease risk factors, and improving gastrointestinal function and glycaemic control. If such an additional approach is developed, okara could then become a very important source of proteins and dietary fibre, which can be implemented as functional and nutritional and potentially healthy ingredients in a range of food products.

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Appendices

Appendix I: Differential scanning calorimetry (DSC) Chromatogram

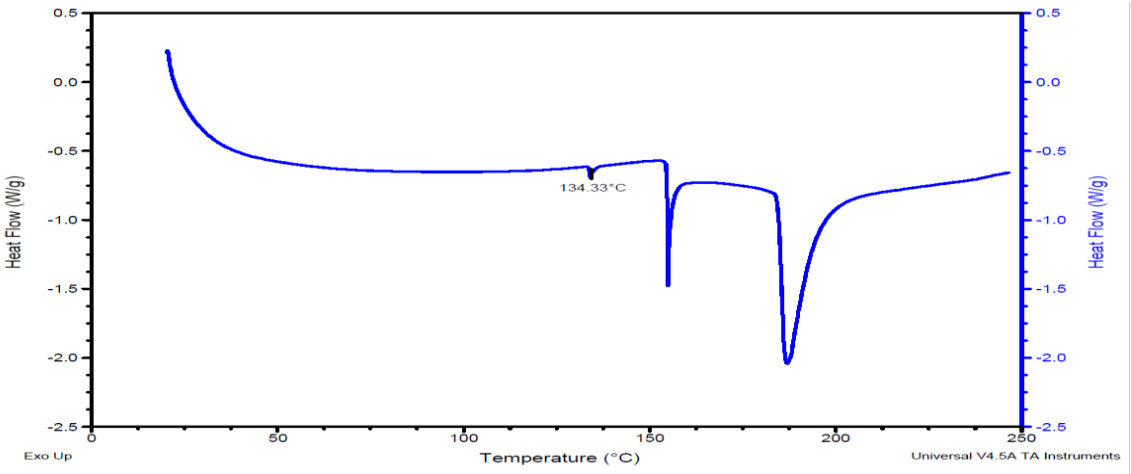

Fig.A1.1: DSC chromatogram for UOF (undefatted okara flour)

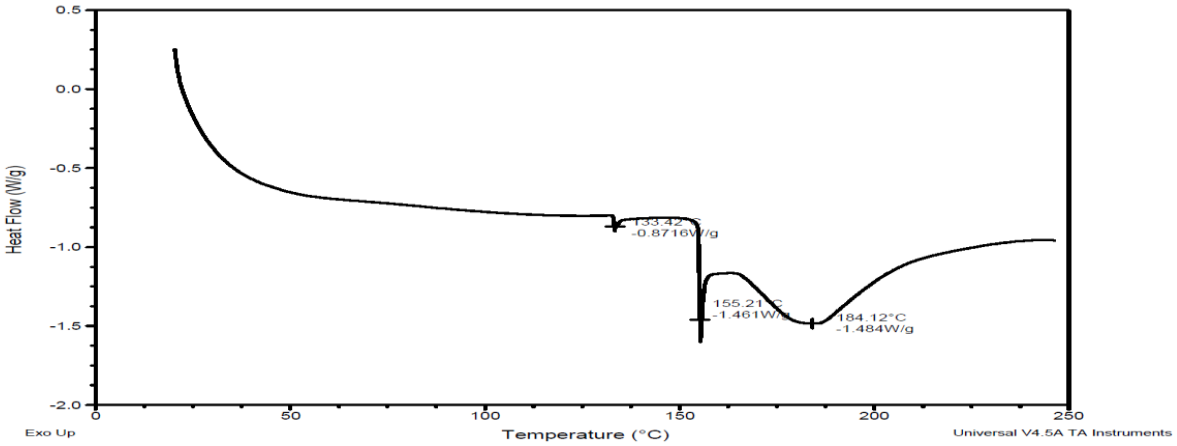

Fig.A1.2: DSC chromatogram for CSP (Commercial soy protein isolate)

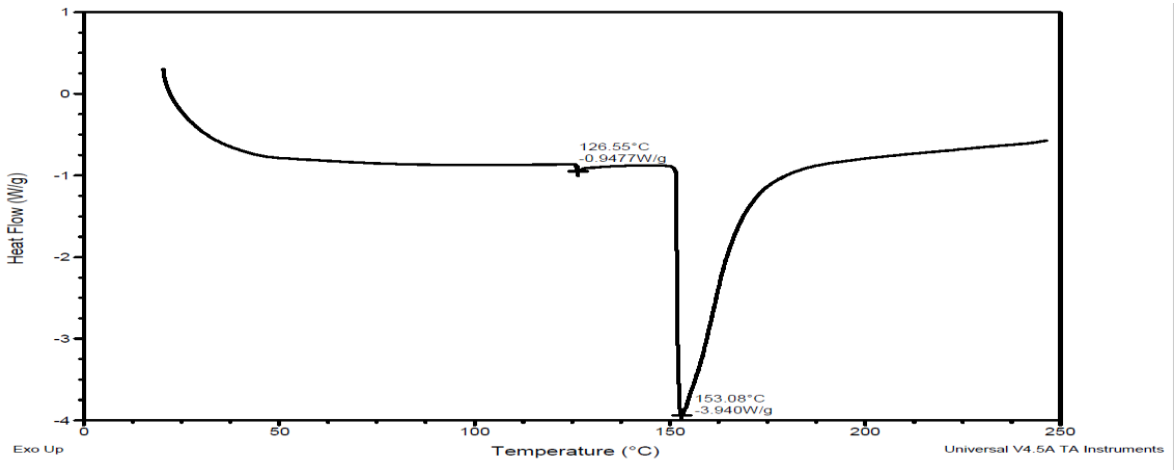

Fig.A1.3: DSC chromatogram for the OPI extracted using alkaline phosphate buffer pH 9.

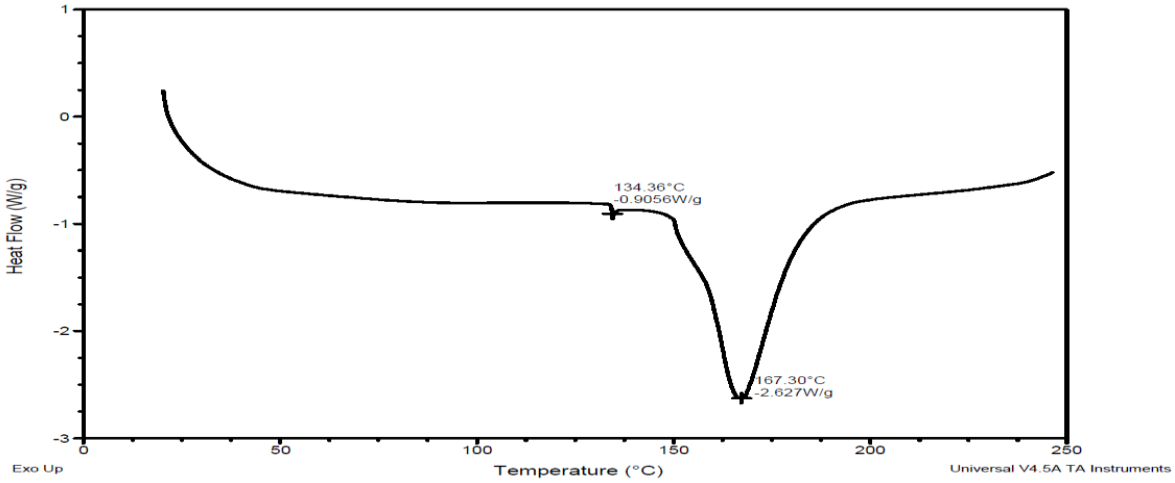

Fig.A1.4: DSC chromatogram for the OPI extracted using alkaline phosphate buffer pH10.

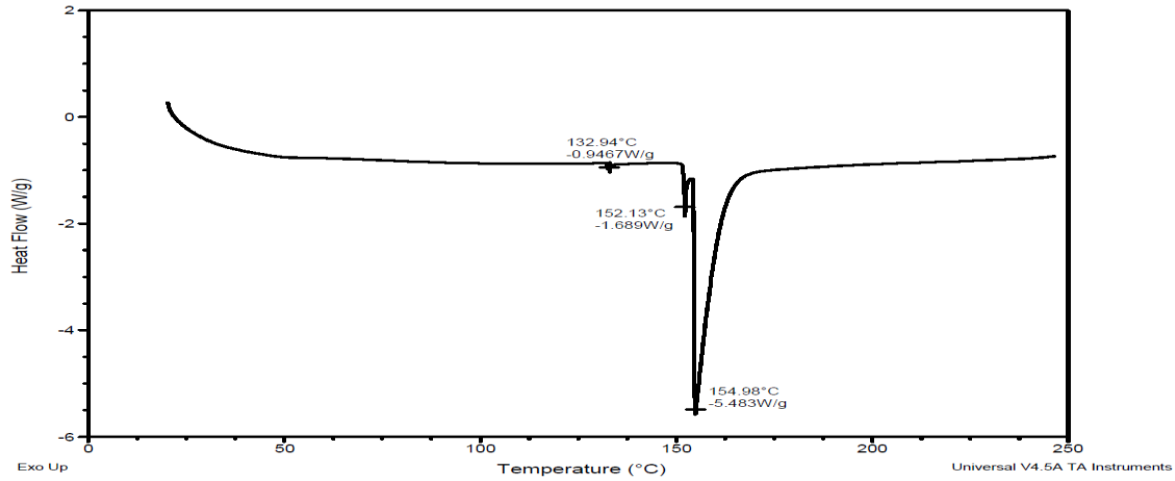

Fig.A1.5: DSC chromatogram for the OPI extracted using alkaline phosphate buffer pH11.

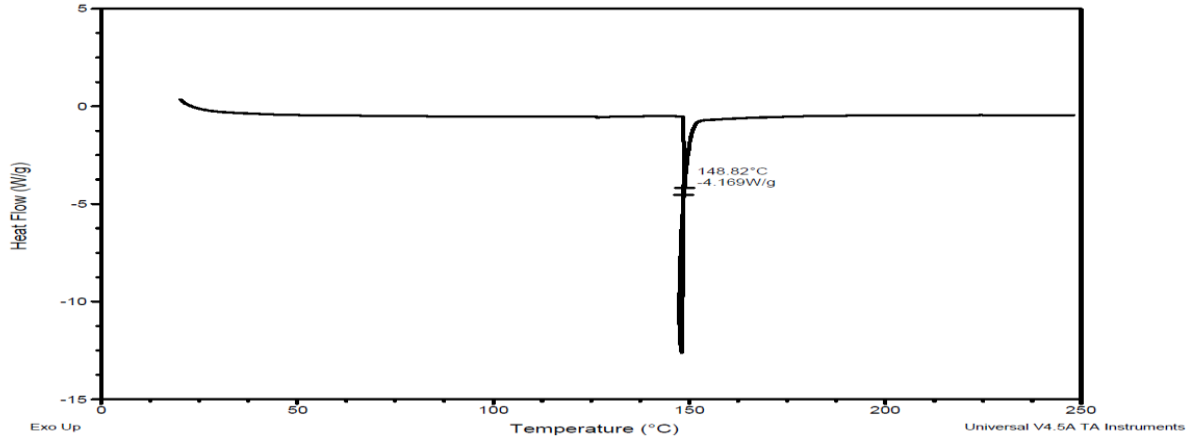

Fig.A1.6: DSC chromatogram for the OPI extracted using alkaline phosphate buffer pH12.

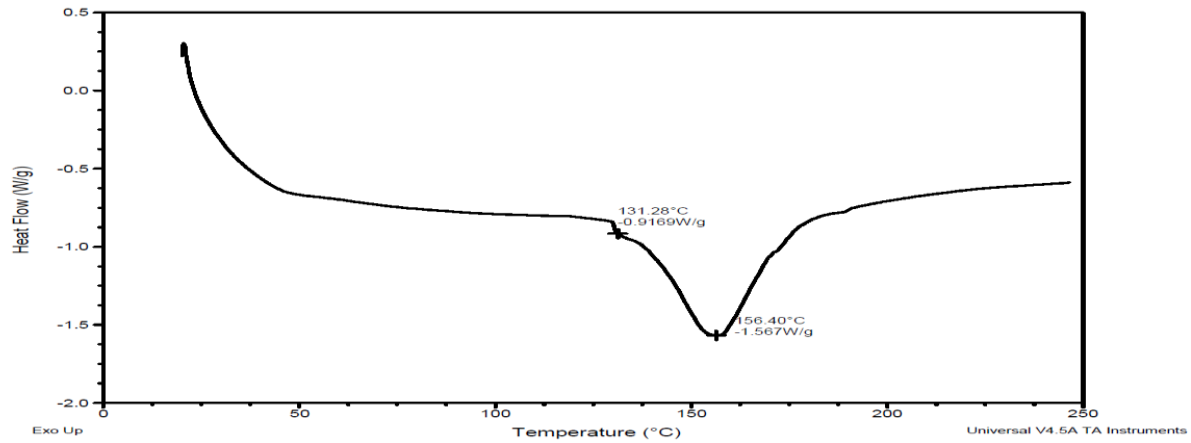

Fig.A1.7: DSC chromatogram for ultrasonication extracted OPI (5µm, 50min).

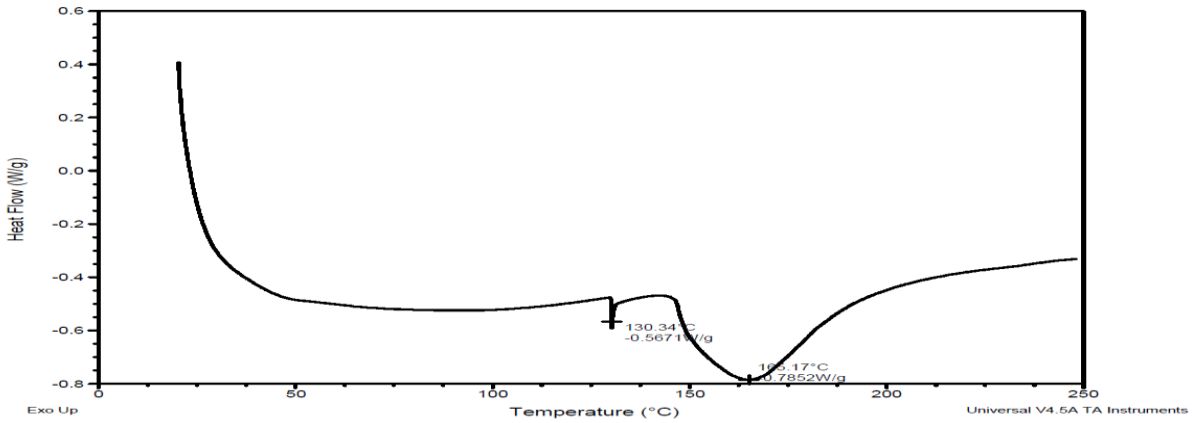

Fig.A1.8: DSC chromatogram for ultrasonication extracted OPI (10µm, 50min).

Appendix II: Images of the emulsions stabilised by OPI and CSP

Fig.A2.1: Emulsion stabilised with Ultrasonication derived OPI at different concentrations.

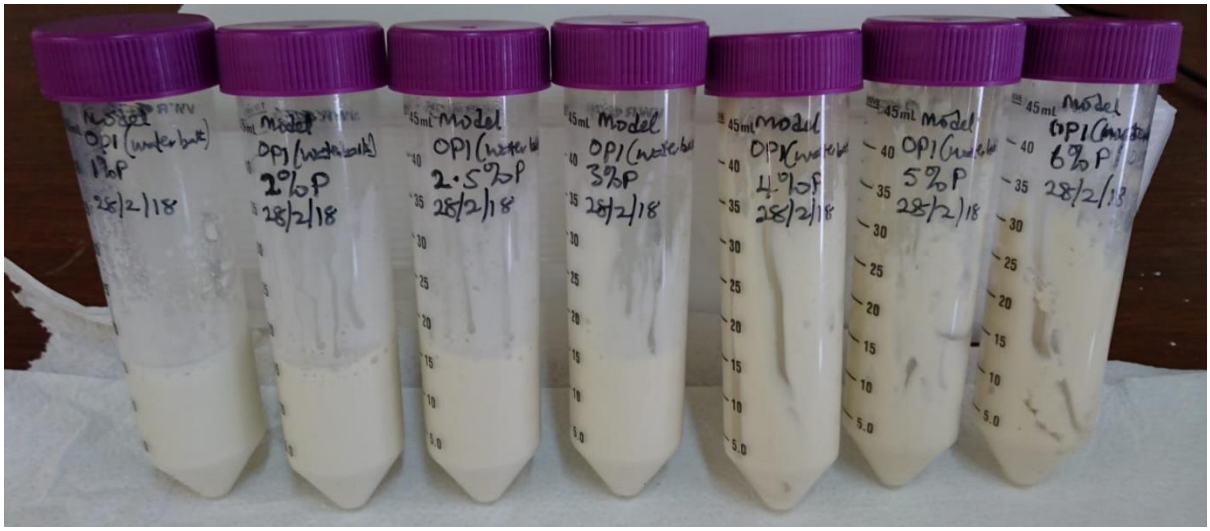

Fig.A2.2: Emulsion stabilised with aqueous alkaline extracted OPI at different concentrations.

Fig.A2.3: Emulsion stabilised with CSP (commercial soy protein isolate). A: Emulsion containing 0 % XG; B: Emulsion containing 0.1 % XG. XG: Xanthan gum.

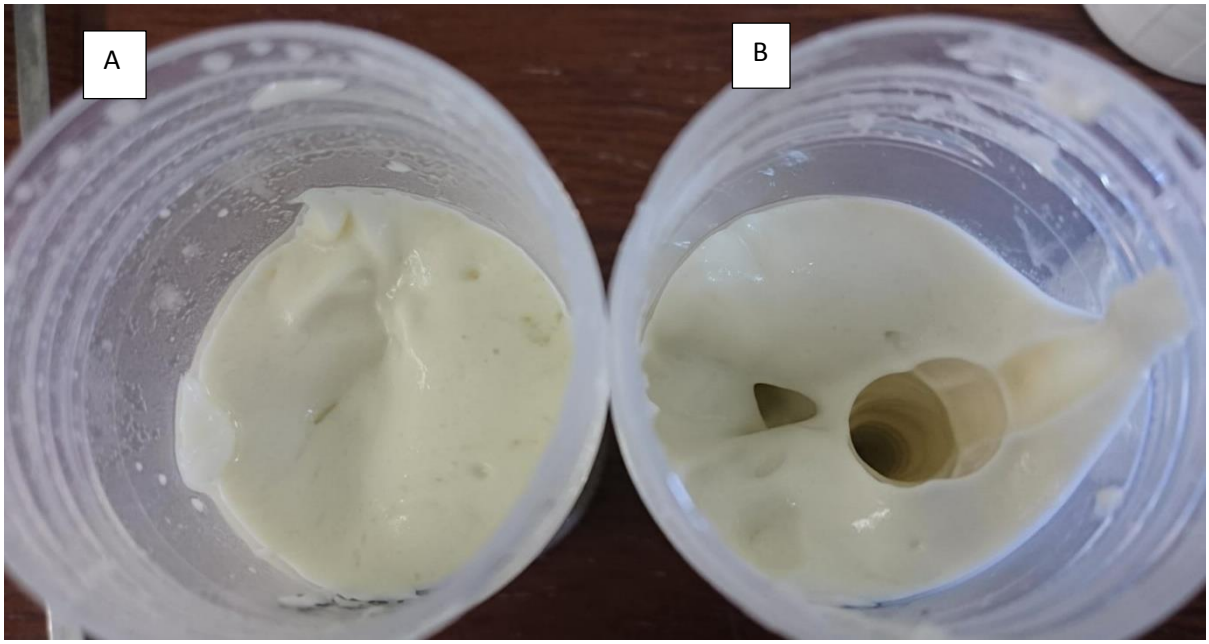

Fig.A2.4: Inner view of the emulsion stabilised with CSP (commercial soy protein isolate). A: Emulsion containing 0 % XG; B: Emulsion containing 0.1 % XG. XG: Xanthan gum.

Fig.A2.5: Emulsion stabilised with Ultrasonication extracted OPI (okara protein isolate). A: Emulsion containing 0 % XG; B: Emulsion containing 0.1 % XG. XG: Xanthan gum.

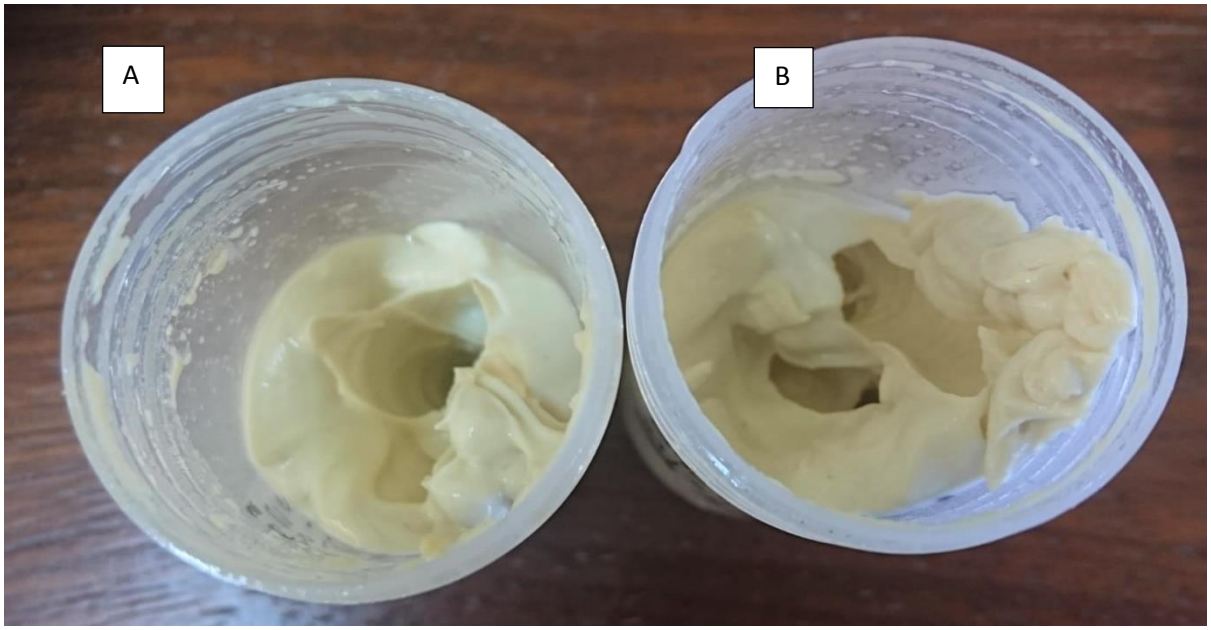

Fig.A2.6: Inner view of the emulsion stabilised with ultrasonication extracted OPI (okara protein isolate). A: Emulsion containing 0 % XG; B: Emulsion containing 0.1 % XG. XG: Xanthan gum.

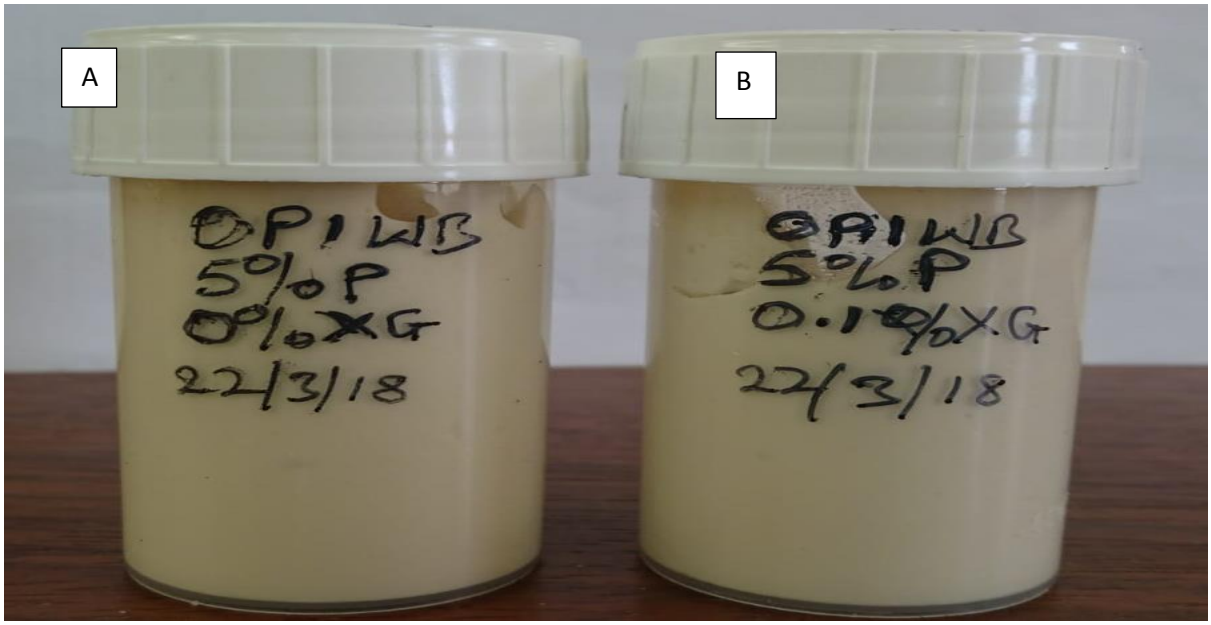

Fig.A2.7: Emulsion stabilised with aqueous alkaline extracted OPI (okara protein isolate). A: Emulsion containing 0 % XG; B: Emulsion containing 0.1 % XG. XG: Xanthan gum.

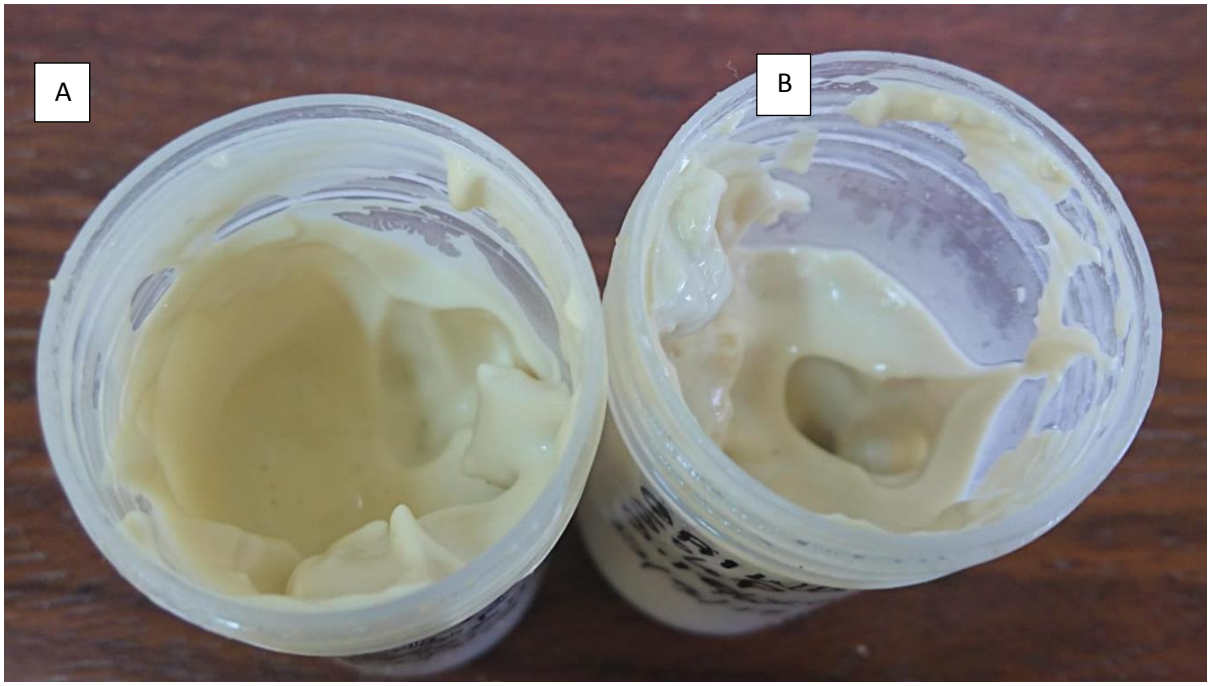

Fig.A2.8: Inner view of the emulsion stabilised with aqueous alkaline extracted OPI (okara protein isolate). A: Emulsion containing 0 % XG; B: Emulsion containing 0.1 % XG. XG: Xanthan gum.

Overview of completed training activities

Conferences/workshops/seminars:

- FABS Seminar, Department of Food and Nutritional Sciences, University of Reading, 10th July 2015 (oral presentation);
- SCI Young Research in Agri-Food 2016: Food Quality and Sustainability from Plough to Plate, University of Reading,12th May 2016 (poster presentation);
- Workshop for Joint BBSRC-NIBB Industrial Biotechnology Skills, Manchester MacDonald Hotel, Manchester, 18-19th May 2016;
- 10th Global Summit on Food Safety, Processing & Technology, San Antonio, Texas 5th December 2016 (oral presentation);
- FNS Seminar, Department of Food and Nutritional Sciences, University of Reading, 8th March 2017 (oral presentation);
- The Grain, cereal and bakery waste valorisation, University of Reading, Whiteknights, 7th June 2017 (poster presentation);
- The 19th Gums and stabilisers for the food industry conference: Hydrocolloid multifunctionality. Seminaris Campus Hotel, Berlin, Germany, 27-30th June 2017 (poster presentation);
- Workshop on Process Design Tools: SuperPro Designer, organized by Foodwastenet at University of Reading, Whiteknights, (3-4 July 2017).

Preparing to teach course:

- Introduction to teaching and learning, 4th November 2015;
- Marking and feedback, wed 4th November 2015;
- laboratory demonstrating and leading small groups, wed 27 January 2016;
- large group presentation skills, Thursday 28 January 2016;
- Project supervision, Thursday 28 January 2016.

Level 2 award in Food safety for manufacturing, 14 December 2016.

Young Entrepreneurs scheme competition and workshop, YES 2017.

Graduate school Reading Researcher Development Programme (RRDP) Training courses:

University of Reading

Graduate School
University of Reading
Old Whiteknights House
Reading RG6 6AH
phone +44 (0)118 3785063
fax +44 (0)118 3784252
email gradschool@reading.ac.uk

26 November 2018

GRADUATE SCHOOL
READING RESEARCHER DEVELOPMENT PROGRAMME
CERTIFICATE OF ATTENDANCE

Student **Ogemdi Florence Eze** (22828197) attended the following Graduate School training sessions at the University of Reading:

Doctoral Research Conference - registration opens at 13.00 2017/06/20

Self-Management: Managing academic pressure 2017/05/18

Effective CVs How to impress both employers within and outside HE 2017/05/03

Exploring skills in influence and leadership 2017/04/24

Career Journeys: From PhD to Higher Education Planning & Strategy 2017/03/22

How to summarise your research in 3 minutes 2016/11/23

An essential guide to critical academic writing 2015/12/02

Statistical methods for research online e-learning course - an introduction 2015/11/20

How to get published - 2015/11/12

Open access for research publications 2015/11/11

Managing your research project 2015/10/27

Doctoral Research Conference – registration from 13.00, 2015/06/18

You and your supervisor, 2015/05/12

What are you waiting for? A guide to understanding and avoiding procrastination, 2015/05/19

How to avoid plagiarism, 2015/05/19

Presentation skills 2015/03/26

How to write a literature review 2015/03/10

Quality assurance in research 2015/03/03

Ensuring confirmation of registration 2015/02/25

MATLAB 2014/11/04

Basic statistics refresher 2014/10/29

Preparing Posters 2014/11/25

Fairbrother Lecture 2017/03/14

Managing SPSS for Windows 2015/06/02

Sourcing information for a literature review – information retrieval 2014/11/06

How to write a paper 2014/11/24

RRDP - Getting your first post-doc position 2018/02/12

Professor Dianne Berry
Dean of Postgraduate Research Studies

UNIVERSITY OF
READING
GRADUATE SCHOOL

Preparing to Teach
Certificate of Attendance

Ogemdi Florence Eze

has attended the core sessions of the 2015-16

Preparing to Teach Programme

Professor Dianne Berry OBE

Dean of Postgraduate Research Studies

Unleash your ingenuity YES
YOUNG ENTREPRENEURS SCHEME

This is to certify that

Ogemdi Eze

participated in the YES 2017 Competitions

Professor Simon Mosey
on behalf of the Organising Group

BBSRC
Nottingham University Business School
Haydn Green Institute for Innovation and Entrepreneurship
NERC SCIENCE OF THE ENVIRONMENT
MRC Medical Research Council
P&G
gsk
syngenta
POTTER CLARKSON
STEVENAGE BIOSCIENCE CATALYST

RSPH
ROYAL SOCIETY FOR PUBLIC HEALTH
VISION, VOICE AND PRACTICE

This is to certify that

Ogemdi Florence Eze

Has been awarded the

Level 2 Award in Food Safety for Manufacturing
500/5371/1

PASS

Date of Award
14 December 2016

Richard Burton
Head of Qualifications

Ofqual

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