NUTRITIONAL VALUE AND FUNCTIONAL PROPERTIES OF *MORINGA OLEIFERA* LEAF PROTEIN CONCENTRATE

Miss Annelise Ida Halafihi

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คุณค่าทางโภชนาการและสมบัติเชิงหน้าที่ของโปรตีนเข้มข้นใบมะรุม

น.ส.แอนเนลลิส ไอดา ฮาลฟิฮิ

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By	Miss Annelise Ida Halafihi
Field of Study	Food Science and Technology
Thesis Advisor	Assistant Professor KIATTISAK DUANGMAL, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF SCIENCE
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Assistant Professor SIRIMA PUANGPRAPHANT, Ph.D.)

..... Thesis Advisor
(Assistant Professor KIATTISAK DUANGMAL, Ph.D.)

..... Examiner
(Assistant Professor NATTIDA CHOTECHUANG, Ph.D.)

..... Examiner
(Dr. SARISA SURIYARAK, Dr.rer.nat.)

..... External Examiner
(Associate Professor Jirawat Yongsawatdigul, Ph.D.)

แอนเนลลิส ไอดา ฮาลฟิฮิ : คุณค่าทางโภชนาการและสมบัติเชิงหน้าที่ของโปรตีนเข้มข้นใบมะรุม. (NUTRITIONAL VALUE AND FUNCTIONAL PROPERTIES OF *MORINGA OLEIFERA* LEAF PROTEIN CONCENTRATE) อ.ที่ปรึกษาหลัก : เกียรติศักดิ์ ดวงมาลย์

ด้วยใบมะรุม (*Moringa oleifera*) สามารถเป็นแหล่งโปรตีนที่มีราคาไม่แพง การใช้โปรตีนเข้มข้นจากใบพืชมีข้อดีในด้านปริมาณโปรตีนที่สูงกว่าและสามารถใช้ประโยชน์ได้หลากหลายมากกว่าการใช้ใบพืช ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อเตรียมโปรตีนเข้มข้นจากใบมะรุมแห้ง (MoLPC) และศึกษาสมบัติทางเคมีกายภาพ สมบัติเชิงหน้าที่ของโปรตีนเข้มข้นจากใบมะรุมแห้ง รวมถึงคุณค่าทางโภชนาการของแพนเค้กที่มีการเติม MoLPC เทียบกับแพนเค้กที่ไม่มีการเติม MoLPC ในการทดลองศึกษาใบมะรุมสองสายพันธุ์ ได้แก่ มะรุมไทย (TMo) และมะรุมอินเดีย (IMo) จากจังหวัดน่าน เตรียมตัวอย่างโปรตีนเข้มข้นจากใบมะรุมแต่ละสายพันธุ์โดยอาศัยหลักการละลายโปรตีนด้วยสารละลายด่างที่ pH 9 จากนั้นใช้การตกตะกอนด้วยกรดที่ค่า pI (pH 4.5) และแปรผลของการให้ความร้อนต่อขั้นตอนการตกตะกอนโปรตีนที่ค่า pI โดยเทียบกระบวนการให้ความร้อนในช่วงเวลาที่กำหนด(อุณหภูมิ 55^0C 20 นาที) กับการตกตะกอนตามปกติที่อุณหภูมิห้อง จากนั้นปรับค่า pH ของโปรตีนที่สกัดได้ให้มีค่า 7.0 ก่อนนำไปทำแห้งด้วยวิธีการทำแห้งแบบแช่เยือกแข็ง ผลการทดลองพบว่าโปรตีนเข้มข้นจากใบมะรุมไทยมีปริมาณโปรตีนสูงกว่าใบมะรุมอินเดียอย่างมีนัยสำคัญ (61.48 ± 1.04%) และมีผลผลิตของโปรตีนเข้มข้นที่สกัดได้ 4.50 ± 0.13% การให้ความร้อนในขั้นตอนการตกตะกอนโปรตีนมีอิทธิพลอย่างมีนัยสำคัญต่อองค์ประกอบทางเคมีของตัวอย่างโปรตีนเข้มข้น ในขณะที่สายพันธุ์มะรุมมีอิทธิพลต่อผลผลิตของโปรตีนเข้มข้นที่ได้และปริมาณโปรตีนหยาบเท่านั้น ทุกตัวอย่างของโปรตีนเข้มข้น MoLPC มีความสามารถในการละลายต่ำสุดที่ pH 3.5 ในขณะที่ pH 6 และสูงกว่าความสามารถในการละลายโปรตีนจะสูงขึ้น ความร้อนมีผลทำให้ความสามารถในการละลายของ MoLPC ลดลงอย่างมีนัยสำคัญ ตัวอย่างโปรตีนเข้มข้นจากใบมะรุมอินเดียแสดงสมบัติการทำให้เกิดอิมัลชันสูงอย่างมีนัยสำคัญในช่วง pH 6, 7 และ 8 ในขณะที่ตัวอย่างโปรตีนเข้มข้นจากใบมะรุมไทยมีสมบัติในการทำให้เกิดอิมัลชันได้ดีขึ้นเมื่อได้รับความร้อนในขั้นตอนการตกตะกอน และพบว่าค่าความไม่ชอบน้ำที่บริเวณผิวสัมผัส (surface hydrophobicity) มีความสัมพันธ์กับสมบัติการทำให้เกิดอิมัลชันของโปรตีนเข้มข้น MoLPC เมื่อศึกษาคุณค่าทางโภชนาการของแพนเค้กที่มีการเติม MoLPC 15% เพื่อทดแทนแป้งผสมสำหรับทำแพนเค้ก พบว่าแพนเค้กที่มีการเติม MoLPC มีปริมาณโปรตีนหยาบเพิ่มขึ้น (18.7 ± 1.2%) เส้นใยหยาบเพิ่มขึ้น (12.6 ± 1.3%) และคาร์โบไฮเดรตลดลง (48.1 ± 3.8%) เมื่อเทียบกับค่าดังกล่าวในตัวอย่างควบคุมอย่างมีนัยสำคัญ การวิเคราะห์ลักษณะทางเนื้อสัมผัสของแพนเค้กพบว่าแพนเค้กยังสามารถรักษาความคืนตัว (springiness) และความเกาะติด (cohesiveness) ของแพนเค้กได้ถึงแม้จะใช้ MoLPC ทดแทนแป้งสาลีในปริมาณ 15% อย่างไรก็ตามความแข็ง (hardness) ของเนื้อสัมผัสแพนเค้กเพิ่มขึ้นอย่างมีนัยสำคัญจากการเติม MoLPC ที่ปริมาณ 15% โดยข้อมูลจากการศึกษานี้มีความสำคัญต่อศักยภาพการพัฒนาและการประยุกต์ใช้โปรตีนเข้มข้นจากใบมะรุมในผลิตภัณฑ์อาหารชนิดต่าง ๆ

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Annelise Ida Halafihi : NUTRITIONAL VALUE AND FUNCTIONAL PROPERTIES OF *MORINGA OLEIFERA* LEAF PROTEIN CONCENTRATE. Advisor: Asst. Prof. Dr. KIATTISAK DUANGMAL, Ph.D.

Moringa oleifera leaf is considered an inexpensive source of protein. As leaf protein concentrate (LPC), protein content offered is higher and easier for use than in leaf. Therefore, this study aimed to prepare moringa leaf protein concentrate (MoLPC) from dried moringa leaves, compare their physico-chemical and functional properties and determine enhanced nutritional value of pancakes fortified with MoLPC compared to unfortified pancake. Using two varieties (Thai (TMo) and Indian (IMo) moringa from Nan area), LPC from each variety was prepared by alkali solubilization (pH 9) followed by acid-precipitation at pH 4.5. Effects of heat on the protein precipitation was studied by pI precipitation undergoing an additional heating period (55^0C, 20min) or regular precipitation at room temperature. MoLPC pellet was then adjusted to pH 7.0 before subjected to freeze drying. ThaiLPC-Heat showed significantly higher protein content (61.48 ± 1.04%) and yield of mass MoLPC extraction (4.50 ± 0.13%). Heating significantly influences proximate compositions of MoLPC whereas variety influences yield of mass MoLPC extraction and crude protein content only. Solubility showed pH 3.5 to give minimum solubility for all MoLPC. At pH of 6 and higher, higher protein solubility for all MoLPC occurred. Heat had significant influence on the decreased solubility of MoLPC. IndianLPC-RT showed significant high emulsifying properties across pH 6, 7 and 8 whereas ThaiLPC was found to have improved emulsifying properties when heated at precipitation step. Results of surface hydrophobicity correlated with emulsifying properties of MoLPC. ThaiLPC-Heat was chosen to replace 5% to 15% of pancake mix. The MoLPC substitution showed nutritional enhancement of protein content increasing compared to the control. Increased crude protein content (18.7 ± 1.2%), increased crude fiber (12.6 ± 1.3%) and decreased carbohydrate (48.1 ± 3.8%) significantly occurred at MP15%. Texture profile analysis of the pancakes showed that springiness and cohesiveness of pancakes are maintained even with substitution of MoLPC at 15%. However, the hardness of the pancake textures was significantly influenced with replacement of pancake-mix with MoLPC even at 15%. Data obtained from this study is important for the development and implementation of *Moringa oleifera* leaf protein concentrate for its potential use in food.

Field of Study:	Food Science and Technology	Student's Signature .....
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Chapter I

1.1. Introduction

Malnutrition worldwide can often be seen in two forms either as being undernourished or overweighed which both results from an unbalanced diet. One of the main causes for this is the inadequate supply or lack of access to the right amount of nutrients necessary for the functions of the body. Protein is an essential nutrient to all life-forms, without it, one will succumb to an inadequate immunity system. Nowadays, there is an increasing demand for healthier, affordable, and sustainable alternatives of protein. Animal proteins has always been the most consumed source amongst consumers, but its ability to suffice the growing population demands has its limitation in supply and can also contribute to other environmental impacts in its process. Moreover, diverse preferences in terms of protein sources according to vegetarian consumers and health awareness issues arise.

Common and popular sources of proteins from animals are running scarce and are related to its fast impact on non-communicable diseases (NCD's) due to its high calorific gains. Consumers' attention has begun to diversify towards plant foods as a potential source of protein since it offers additional health benefits of lower calorific gains, high fiber content, available bioactive compounds and phenolics in comparison to animal foods. The quality of proteins from a source can depend on its high crude content, nutritional composition of amino acids available, digestibility and bioavailability and additional functional properties in food. Popular examples of plant foods with high protein contents include cereal grains and seeds, legumes such as soybeans and nuts have been favored due to its high amino acid contents and its ability to closely mimic functional properties from animal proteins. These sources have been utilized by many food industries in developing various food products such as tofu and other bakery goods. However, aside from consumer acceptance in terms of unfamiliar flavors such as beany flavor in soybean products, potential risks of allergens from these sources are often cautioned to those who may be affected. In addition, accessing these protein sources is a challenge for some countries. Pacific regions do not have these

sources of food locally available nor the appropriate climate conditions to cultivate it and thus have to rely on importation for access. This promotes an increasing dependency on imported food and most heavily on meat products, which provide risks for high volatile prices (Santamaría-Fernández & Lübeck, 2020). It is then vital to consider locally available sources of protein from plants as leaf protein concentrate in developments of enhanced nutritional foods. This provides sustainability of protein source, reduces dependency on food imports and contributes to the reduction of non-communicable disease and improve the health and eating habits in consumers. Consequently, alternatives such as the leaves from *Moringa oleifera* are being suggested to close these gaps.

Moringa oleifera under the family Moringaceae is a multi-purpose plant with reported claims of its leaves being considered as a cheap and abundant possible source of protein. It is the most widely distributed species under its category from a total of 13 species of the genera Moringa. Dubbed by common names like “miracle tree” or “tree of life” which is attributed to its utilization for medicinal and therapeutic benefits. This perennial tree is widely cultivated in tropical and subtropical parts of the world. The plant is said to be native in subcontinent areas of India, Asia, Africa and the Middle East. It has also been utilized for many purposes such as food, fertilizer, source of biogas, fodder, fuel wood, medicine and therapeutic uses (Sahay *et al*., 2017). In India it is commonly used as a vegetable in soups, weaning food for infants, cooked and eaten like spinach. It is described to be a famine food due to its tolerance of harsh conditions of drought and needing fewer water supplies than other plants for its growth. This makes it favorable and easier for cultivation and expansion in tropical and sub-tropical countries. Loamy and sandy soil, temperatures 25-35℃ and rainfall of 250-300cm is favorable for its growth (Chhikara *et al*., 2020).

Moringa leaves contain protein content (dry basis) in fresh leaves which is approximated to 6.7% whereas in dried leaves, is where its high crude protein is claimed from, which varies in the range of 26.8-30.29% (Chhikara *et al*., 2020). Studies have showed protein as one of the major nutrients with high content in moringa leaf. This is considered an advantage given that plant sources of high protein with low fat contents, is an approach towards reducing high calorific food in the diet which can cause health

risks of non-cardiovascular diseases. The potential of moringa leaf as a high protein source can however be limited by the presence of other fiber constituents, anti-nutrients, non-protein materials such as tannins, saponins, and phytates. These plant cell wall materials can restrict the full availability of protein from leaves through complex formations when directly consumed as leaves. But in the form of leaf protein concentrate (LPC), there is an enhanced nutritional value in the crude protein content. LPC is considered a sustainable and low health-risk source that can reduce environmental impacts in its production compared to animal products (Santamaría-Fernández & Lübeck, 2020). However, different methods and conditions are used in extracting LPC from different sources of leaves and hence can have different effects on the compositions and functionality of LPC.

1.2. Hypothesis

This study hypothesized that:

- Dissimilarities in contents of nutritional composition in *Moringa oleifera* leaf are due to the different variety of the plant.
- Functional property of the moringa leaf protein concentrate (MoLPC) is largely influenced by the method of extraction and environmental conditions used such as temperature.
- Fortification of pancake with MoLPC would maintain product texture but enhance the nutritional value of the pancake especially with protein content

1.3. Objectives

The aim of this study is to:

- Prepare MoLPC from dried moringa leaves using two varieties of *Moringa oleifera*: Thai moringa and Indian moringa and compare their physico-chemical and functional properties
- Determine any enhanced nutritional value of pancakes fortified with MoLPC compared to its control.

1.4. Scope of study

This study focused on:

- Extraction of LPC in *Moringa oleifera* using two varieties:
    - Thai variety obtained from Nan province
    - Indian variety obtained from Nan province.
- Method of LPC extraction used was alkali extraction under room temperature with acid precipitation. Acid precipitation step of MoLPC was done under two conditions:
    - Acid precipitation under room temperature (-RT) only for 1hr
    - Acid precipitation under room temperature with additional heating period at 55℃for 20mins (-Heat).
- Studying chemical and functional properties of MoLPC
- Fortification of pancake with MoLPC by replacing pancake mix (PWM) with a portion of MoLPC and study physico-chemical properties of fortified pancake compared to its control (unfortified pancake)

1.5. Expected output

The information obtained in this study is significant for further improving the use of Moringa leaves high alternative protein sources in food such as its' implementation as leaf protein concentrate in pancake for enhanced nutritional value.

Chapter II

LITERATURE REVIEW

2.1. *Moringa oleifera*

Increase of malnutrition due to unbalanced diet worldwide has motivated consumers to consider healthy alternatives of plant-based food with low calorific gain and high in nutritional content such as protein. Moringa leaves has been attempted as a mean to fight malnutrition in some tropical and sub-tropical countries (Fuglie, 2001). Studies on the leaves of *Moringa oleifera* have mentioned it to contain rich amounts of nutrients in protein, minerals and vitamins, but also essential phytochemicals that gives it the potential to be anti-fungal (Foidl *et al*., 2001; Sánchez-Machado *et al*., 2010), antioxidant, anti-inflammatory, inhibit platelet aggregation, antimicrobial, anti-tumor (Asiedu-Gyekye *et al*., 2014; Benhammouche *et al*., 2020; Chhikara *et al*., 2020; Gopalakrishnan *et al*., 2016; Moyo *et al*., 2011; Rawdkuen, 2020).

2.1.1 Nutritional Compositions of *Moringa oleifera* leaf

Crude protein content in fresh moringa leaves can be approximated to 6.7% whereas in dried leaves, crude protein can vary in the range of 23.6-30.29% (db.) (Benhammouche *et al*., 2020; Chhikara *et al*., 2020; Foidl *et al*., 2001). Proximate compositions of some plant proteins are shown in Table 2.1 below compared with some studies on *Moringa oleifera* leaf. Dried moringa leaf study by Benhammouche *et al*. (2020) shows sufficient crude protein content with lower content in lipids compared to other seed protein and other leaf protein sources.

A study done by Foidl *et al*. (2001), showed crude protein in moringa leaf (25.1% db.) slightly higher than some fresh forage such as alfalfa and clover. Benhammouche *et al*. (2020) mentioned the crude protein in tea leaves (17-19%), olive leaves (7-12.9%) to be less than moringa leaf crude protein. Rani and Arumugam (2017) stated that moringa leaf protein (27.1% db.) is quite comparable to milk protein content as it mentioned cow, buffalo, goat and sheep milk to have the following crude proteins content; 3.4%, 4.4%, 4.1%, 6.3%, respectively.

Moringa is recommended as a good vegetable for pregnant and lactating women as it contains calcium higher than milk, higher magnesium than eggs, more iron than spinach. It has been reported to meet the iron and calcium requirements for pregnant and lactating women (Chhikara *et al*., 2020). It is a good source of vitamin A, B complex, C as well as other essential minerals such as potassium, phosphorus, zinc, fiber and phytonutrients (Foidl *et al*., 2001; Fuglie, 2001).

Table 2. 1 Proximate Compositions of plant proteins (db%)

Raw Material	Moisture Content (%)	Crude Protein (%)	Crude Fat (%)	Ash (%)	Crude Fiber (%)	Carbohydrates (%)	Reference
Fresh Moringa leaves	75	6.7	1.7	0.8	0.9	13.4	Dhakar *et al.* (2011)
Dried Moringa leaf	9.1	23.6	6.8	7.2	24.9	28.4	Benhammouche *et al*. (2020)
Defatted Moringa leaves	9.8	24.1	0	7.8	24.3	34.0	Benhammouche *et al*. (2020)
Tea (*C. sinensis*) leaves	5.43	14.32	0.99	5.02	49.36	74.24	Rubab *et al*. (2020)
Amaranth Leaves	-	22.8	7.26	7.14	7.93	54.87	Ngugi *et al.* (2017)
Black Sesame Seeds	-	25.06	43.57	4.73	-	3.24	Cheng, Liao, *et al*. (2021)
Peanut	3.53	29.59	46.35	2.27	5.20	13.06	de Oliveira Sousa *et al*. (2011)
Soyabean seed	8.13	39.24	30.31	4.61	6.84	5.08	Ogbemudia (2018)

Table 2.2 shows some studies on amino acids compositions of *Moringa oleifera* leaf. Moringa leaf contains most essential amino acids where leucine appears to be the most dominant essential amino acid and methionine as a limiting amino acid. This is common in most green foliage, but when compared with amino acid requirements for infants from FAO/WHO, the amino acid values of moringa leaves are quite capable of meeting the requirement threshold.

Foidl *et al*. (2001) stated the organic matter digestibility of moringa leaves unextracted as 74% which shows that moringa leaves has the potential to be used as an important source of protein and for industrial production.

Table 2.2 Essential Amino Acids from 3 studies with FAO/WHO amino acid requirements for infants

Essential Amino Acids (g/100g of protein)	Sánchez-Machado *et al*. (2010)	Moyo *et al*. (2011)	Rani and Arumugam (2017)	FAO/WHO requirements for Infants. Joint *et al* (2007)
His	3.12	2.3	2.26	2
Ile	3.9	3.9	3	3.2
Leu	7.8	6.5	7.2	6.6
Lys	6.8	5.4	4.9	5.7
Met	0.6	0.98	1.3	NA
Phe	3.9	5.4	5.1	NA
Thr	3.5	4.5	4.4	3.1
Trp	-	1.6	1.6	0.85
Val	2.1	4.7	3.9	4.3
Met + Cys	-	1	-	2.8

2.1.2 Thai variety and Indian variety of *Moringa oleifera*

Nutritional contents in moringa leaf can vary due to factors such as soil with different nutrients available, the climate and weather of that environment, harvesting season, maturity stage, the fertilizer used, different methods of handling but insignificantly affected by variety (Peddi *et al*., 2018). Two varieties of *Moringa oleifera* are commonly used in Thailand, a Thai variety and that of an Indian variety. Different cultivars of the 'Indian' *Moringa oleifera* are plentiful that the identity of the 'Indian' variety in Thailand is not assertive. Nevertheless, Wangcharoen (2013) reported on the antioxidant activities between two local Thai varieties 'Num Phrae' and 'Ang Thong' obtained from different locations of Thailand (Amphoe Mueanga, Phrae province and Amphoe Pamok, Ang Thong province, respectively) and one Indian variety known as PKM-1 (Bangkok) but were all grown in Chiang Mai province and harvested. The Thai variety of moringa are described as soft and small leaves and less bitter taste in comparison to the Indian variety. PKM-1 (Peryakulam-1) is mentioned in several studies of moringa in India which we may assume be the only Indian variety that exist here in Thailand. "Varieties and Ecotypes of *Moringa oleifera*", (2021) described

PKM-1 as an annual type of variety which is the product of plant breeding research in India. It is characterized as a dwarf variety that was developed for its high adaptability to varied soil and climatic conditions as well for its high yield of leaf production. Its leaves were described as wide and dark green on upper side and pale green on lower side. This variety is suitable to be planted for large scale productions.

2.2 Leaf Protein Concentrate

Since the growing population worldwide is increasing, malnutrition due to protein deficiency consequently arises. Globally, there is a necessity to look beyond animal proteins in search of alternative proteins hence the chosen field on leaf protein concentrate. Animal sources of protein can possess high glycemic index aside from having high level and good quality protein. Such food gives rise to blood glucose levels in the body which is not favorable for those with diabetes. This is to show, that some of these foods consumed in high amounts due to claimed high protein content as a benefit, but on the other hand, it is increasing the risk for non-communicable diseases such as cardiovascular, diabetes, hypertension and so on. As each year passes, these common sources of protein become more limited in abundance with time. Reported studies acknowledged plant-based proteins from legumes, cereals, seeds, almonds, nuts and leaves (Sá *et al*., 2020) as potential replacements. Considering protein per hectare, plant leaves as a source are without a doubt more abundant (Akeson & Stahmann, 1966; Fiorentini & Galoppini, 1983) requires lesser cost compared to animal protein and other plant-based protein. Maintaining leaves requires less water supply, it has less ethical and environmental impacts when processed. Not only that, but in terms of cultural practices and some religions, plant-based nutrients are more accepted especially by vegetarians and vegans. Even though they do not consume animal products, protein is indispensable hence the vitality for plant-based protein for their community. Leaves as a source can come with benefits such as low-calorific gains and with some studies mentioning the balanced amino acids and good protein digestibility discovered. This approach may be able to help in lowering the negative effects of cardiovascular diseases due to the consumption of animal products.

2.2.1 Extraction Method of leaf protein concentrate

Leaves on average can have crude protein content in the range of 20-30% (Nagy *et al*., 1978). Unfortunately, its digestibility is limited due to the presence of other fiber constituents, tannins, saponins, phytates etc. They restrict the full availability of protein from the leaves by forming complexes which avoids the protein being in an available form for digestion. So, the focus on the sole extraction of proteins to be isolated from these restrictions have been recognized and studied. Various methods developed includes heat coagulation, alkali-acid extraction, the use of organic solvents, enzymatic activity, fermentation, ultrafiltration methods with modern technology (Aluko, 2004). Basically, the methods usually involve two main steps, (1) extraction, in which the proteins are extracted from other components of the raw material as much as possible. This involves using the physical and chemical properties of the proteins such as its solubility, pH, ionic strength, hydrophobicity/hydrophilicity by adjusting the environment the raw material is in, hence isolating the protein. Once it is extracted (usually as the soluble supernatant), it is then (2) precipitated in which the proteins are coagulated into semi-solid material that is later dried to a specific moisture content.

Each method comes with certain advantages and disadvantages in regard to the effects it has on the protein acquired. Heat coagulation at 60℃ - 95℃ for instance is the most simples conventional method, however, irreversible denaturation of the protein's native structure often occurs which can mostly affect other properties of the protein (Betschart & Kinsella, 1974). Optimization studies on leaf protein concentrates have shown temperatures with optimum yield achieved for sugar-beet leaves as 54.25℃ (Akyüz & Ersus, 2021), protein yield from tea leaves still showed increasing trend at 50℃ (Shen, Xiangyang, *et al*., 2008) whereas temperatures more than 60℃ in moringa leaves can decrease protein yield (Cheng, Shu, *et al.*, 2021). Heat is always associated with unfavorable denaturation of proteins but has been suggested to improve some functions of proteins such as its emulsification properties (Voutsinas *et al*., 1983). This may require the use of a mild temperature for the extraction and precipitation of proteins.

Alakali-acid extraction is a relatively simple, low-energy and at low cost conventional method for extraction of proteins (Lu *et al*., 2019). Studies has compared its efficiency with the conventional thermocoagulation method suggesting alkali-acid extraction to

cause less denaturation and efficient in extraction yield and crude protein content (Betschart & Kinsella, 1974). As prior mentioned, it is more commercially available to conduct in terms of available equipment compared to the other advanced methods of ultrafiltration and time friendly.

The simple principle of this method utilizes alkali solution, usually sodium hydroxide, to extract the proteins from the leaves. Plant proteins are known to mostly be insoluble in water due to hydrophobic groups and di-sulphide bonds between the molecules of proteins (Akyüz & Ersus, 2021). The alkali solution increases the solubility of the plant proteins by dissociating hydrogen break hydrogen bonds. It is also mentioned by Betschart and Kinsella (1974) the attachments of Na^+ (from NaOH alkali solution) to the protein increases the affinity of the proteins to water and hence cause dispersal which is seen as its ability to be soluble at higher pH. At this point, cell walls are broken enabling the leach out of the soluble proteins. The pH of extraction can be further increased up to 12, however, it has been suggested that higher than pH 9 causes irreversible denaturation due to high alkalinity and that pH 7-8 is most preferable in most plants (Betschart & Kinsella, 1974). Once, the supernatant solution is obtained through steps of filtration and centrifugation, the pH of that solution is again adjusted to its isoelectric point with an acidic solution usually hydrochloric acid. This is known as acid precipitation. The isoelectric point is where the net charge of the proteins becomes zero and the solubility of the proteins are at its lowest. At this stage, the proteins neither repels nor attract due to charge, however, they associate according to the hydrophobic side chains present in the protein. Acid precipitation is mostly used to achieve unfractionated proteins where green and white proteins are both present (Santamaría-Fernández & Lübeck, 2020) and that pI of most plant proteins are around the range of 3.0-5.0.

2.2.2 Nutritional Quality

In plant leaves, two distinct type of proteins extracted exists and are mentioned to be chloroplastic proteins (green proteins) and cytoplasmic proteins (white proteins) (Santamaría-Fernández & Lübeck, 2020). Chloroplastic proteins is where the leafy or herbal taste comes from if used in food. It is also said to contain most insoluble proteins and it accounts for about 80% of total protein. As for the cytoplasmic proteins, it

contains most of the soluble proteins and accounts for about 20% of total proteins. The protein RuBisCO, usually involved in the photosynthesis process in the leaf, has been mentioned to be the main protein in the cytoplasmic fraction of the leaf protein. It is also said to contain high amounts of lysine (Tamayo Tenorio *et al*., 2016) which is a limited amino acid in some cereals (Sá *et al*., 2020). This is considered beneficial in terms of supplementing or mixing with protein products that has low levels of limiting amino acids such as lysine in cereals contributing to overall benefits of plant-base protein.

A study by Nagy *et al*. (1978) on sources of proteins from 60 tropical and sub-tropical plants outlined their crude protein content to be in the range of 20-60% with bamboo leaves having least of 20.1% and coriander leaves having 60.8% of crude protein from dry matter (db.). Majority of these plant leaves' crude proteins are in the range of 20-30%. Those greater than 20% of crude protein is considered potential sources for protein concentrates extraction (Nagy *et al*., 1978). Protein concentrates and isolates contain the enhanced protein content of extracts. Aluko (2004) reported protein concentrates to be in the range of at least 65% protein content and at least 90% of protein content is in protein isolates from most protein sources. However, protein concentrate from leaves can vary in the range of at least 40% to 70% as shown in most studies depending on the method of extraction used. For example, alfalfa LPC extracted with Tris-buffer (51% crude protein), alfalfa LPC extracted with NaOH (59.8% protein content) (Wang & Kinsella, 1976). Cassava leaves under heat coagulation (42.92%) (Castellanos *et al*., 1994), *E. dulcis* LPC (56.88%) (Pandey & Srivastava, 1991), soybean LPC from alkali extraction and acid precipitation (70%) (Betschart & Kinsella, 1974).

Table 2. 3 Comparison of essential amino acids in different leaves with FAO/WHO amino acid requirements for infants and adults

Essential Amino Acids (g/100g of protein)	Aquatic Weed *(E. dulcis)* (Pandey & Srivastava, 1991)	Water Hyacinth Leaf (Adeyemi & Osubor, 2016)	Cassava-PCU (Castellanos, Altamirano, & Moretti, 1994)	Cassava- PCT (Castellanos, Altamirano, & Moretti, 1994)	Tea LPC (Shen, Wang, Wang, Wu, & Chen, 2008)	Requirement for Infants (Joint *et al.*, 2007)	Requirement for Adults (Joint *et al.*, 2007)
His	3.7	1.1	2.42	2.7	1.96	2	1.5
Ile	3.12	2.29	3.9	4.15	3.98	3.2	3
Leu	5.68	5.01	NA	NA	7.78	6.6	5.9
Lys	4.5	3.72	5.05	4.9	5.62	5.7	4.5
Met	1.32	1.34	1.2	3.5	1.12	NA	1.6
Phe	3.89	3.67	4.8	4.1	4.46	NA	NA
Thr	3.33	2.6	4.15	3.6	3.83	3.1	2.3
Trp	NA	NA	0.7	0.55	NA	0.85	0.6
Val	4.87	2.81	4.98	4.1	4.63	4.3	3.9
Met + Cys	2.05	2.06	1.75	3.9	1.92	2.8	2.2
Digestibility (%)	67.87	NA	85	80	NA		
Protein Content (%)	56.88	NA	43	42	NA		
Chemical Amino Acid score	NA	NA	50	40	NA		
Method of Extraction	Heat Coagulate	Ethanol Extract	Ultrafiltration	Thermo-coagulate-acid precipitate	NA		
PER	1.22	NA	1.81	1.6	NA		

It is assumed that the protein yield, protein content and nutritional qualities in the leaves as well from extracted concentrates (protein etc), do not just depend on the characteristics of the plant. There are other numerous factors that can affect the nutritional content such as environmental factors like the soil fertility of an area, climate change, fertilizers used, harvesting seasons as well as the processing methods used to obtain the concentrates (Fiorentini & Galoppini, 1983). Different levels of amino acid composition in LPC reported by Nagy *et al*. (1978) implied that most of these LPC gave lower levels of lysine, tryptophan as well as the sulfhydryl containing amino acid. These differences in amino acids can be seen in Table 2.3 below comparing amino acids from various studies (Adeyemi & Osubor, 2016; Castellanos *et al*., 1994; Pandey & Srivastava, 1991; Shen, Wang, *et al*., 2008) and from different sources. Compared with the amino acid requirement for infants and adults from FAO/WHO (Joint *et al*., 2007), the amino acids are quite comparable and seems to meet the requirements.

Amino acid scores of studies on whole leaf protein concentrate from plants such as alfalfa is comparable (99%) with FAO requirements (100%), hen's egg (100%), cow's milk (100%) (Fiorentini & Galoppini, 1983) indicating the potential quality of amino acids in leaf protein concentrates. Other nutrients such as fiber and fat can be quite low in leaf protein concentrates since they are separated from the protein through pre-treatment processes especially to prevent its effects on the bioavailability of the protein from leaves. Minerals has been reported not to be affected that much during extraction of leaf protein concentrate in which they add as additional health benefits to the other properties of leaf protein concentrates.

2.2.3 Physico-chemical and Functional properties of LPC

Aside from the good nutritional aspects of protein concentrates, its physico-chemical and functional properties are desirable especially in the food industry for the enhancement of food. Some studies on soybean LPC (Betschart & Kinsella, 1974), alfalfa LPC (Hadidi *et al*., 2020; Lamsal *et al*., 2005, 2007; Miller *et al*., 1975), tea LPC (Shen, Xiangyang, *et al*., 2008), tobacco LPC (Teng & Wang, 2012), sugar-beet LPC (Akyüz & Ersus, 2021; Martin *et al*., 2019) were conducted to investigate the physico-chemical and functional properties of leaf protein concentrates. Out of these studies, alfalfa leaf seems to be the most studied. According to Lamsal *et al.* (2007), the functional properties of protein are affected by the preparation methods to extract the proteins. Functionality of proteins can be disintegrated when prepared under high thermal conditions or extreme solvent conditions. LPC prepared from soybean under heat coagulation (LPC-H) and alkali-acid precipitation (LPC-IP) method showed solubility of the heat-coagulated protein to be insoluble in the range of pH 1.5-11 (Betschart & Kinsella, 1974). LPC-IP on the other hand had high solubility at pH 2 and 10. Solubility profiles of many LPC prepared under moderate conditions shows high solubility at pHs lower than 4 and higher than and at alkaline pH in the range of 7-10. Protein concentrates and isolates extracted under the right conditions yields protein with high solubility which results in good water and oil holding capacity (Fiorentini & Galoppini, 1983), as well as other functional properties such as emulsification, foaming and gelling. Rubisco protein isolate extracted from sugar-beet leaves was reported with high solubility at pH lower than 4 and higher than 5.5 (Martin *et al*., 2019). Martin *et*

al. (2019) reported its foaming and emulsification capacity to be more stable and similar to whey protein isolate and soybean isolate respectively at pH 4 and 7. Concentration of rubisco protein isolate at 50% was able to provide a self-supporting gelling network without any mixture of whey isolate (Martin *et al*., 2019). In alfalfa leaves, ultra-filtrated proteins gave good water and oil holding capacity but low emulsification and foaming capacity than its proteins produced by acid-precipitated and resolubilized method (Lamsal *et al*., 2007). Lamsal *et al*. (2007) suggests that the gelling activity of alfalfa proteins can be softer gel.

2.2.4 Applications and Uses of LPC

Throughout past reviews and studies related on numerous plant leaf protein concentrates (Santamaría-Fernández *et al*., 2018) such as alfalfa (Wang & Kinsella, 1976), amaranth (Santamaría-Fernández *et al*., 2017), sugar-beet leaves (Tamayo Tenorio *et al*., 2016), cassava (Castellanos *et al*., 1994), *Moringa oleifera* (Benhammouche *et al*., 2021), certain applications are recommended for the further use of these products.

In the food industry, the traditional use mentioned for the leaf protein concentrates are as animal feed. Due to the fact that protein concentrates from leaves can have herbal sensory characteristics that would be undesirable to the human taste, this was proposed (Santamaría-Fernández *et al*., 2017). Not only does it provide high supply, less cost and energy for its industrial production, it has been studied years before to reveal the possible and good nutritional quality presented by these protein concentrates through amino acid scores and digestibility scores compared with other animal feeds. On that note, researchers have also recommended the positive potentials of leaf protein concentrates for human consumption because of its good quality nutrients of protein, amino acids and minerals. Used as food dietary supplements, functional food ingredients or food fortification, several studies have been done so in this field with different plant sources. In terms of developing steps, leaf protein concentrates for animal feed are already in industrial production, there is not much yet expansion for those designed for human consumption (Fiorentini & Galoppini, 1983).

Other applications for leaf protein concentrates mentioned is that it is useful for enzyme production such as cellulosic enzymes which are usually extracted from micro-organisms (Tamayo Tenorio *et al*., 2016). This will require less bio-energy input into the production processes. Additional applications as bio-based chemicals are its utilization for thin films and coatings and its innovative developments for cosmetic products (Tamayo Tenorio *et al*., 2016).

2.3 Related studies to *Moringa oleifera* leaf protein concentrates

2.3.1 Extraction methods of moringa leaf protein concentrate

Given the vast richness in nutrients and health benefits of moringa leaf mentioned in various studies, there is a compelling need to further the investigation towards protein concentrates extracted from it. With crude protein on average in the range of 26-29% (Chhikara *et al*., 2020; Foidl *et al*., 2001), it can be further enhanced to increase the amount of protein by extracting from other fiber constituents, phytates, saponins and non-protein components that can prevent the digestibility and bioavailability of protein in moringa leaves. Throughout the research for extraction methods, it is clear that not many studies have been conducted on moringa leaf protein concentrate, hence there is room for more.

Table 2.4 represents 6 studies on moringa leaf concentrate and have used various methods for the extraction of protein from the leaves. Aside from nutritional components and functionality of proteins, the yield and protein content of concentrates are desirable in determining the right extraction method. The differences in protein content and yield is greatly affected by different methods and conditions used as well as other factors involved such as environmental conditions. The most common methods use is the alkali extraction/acid precipitation method. Rawdkuen (2020) as well as (Ahmed & Supervisor, 2016) used the same method of extraction as well as similar conditions. The only difference is that the precipitation of protein by Rawdkuen (2020) was left overnight and it gave a protein concentrate with protein content of 77.4% which is greater than the 38.02% of protein content by (Ahmed & Supervisor, 2016).

Table 2. 4 Extraction methods of moringa leaf protein concentrates yields & crude protein contents

Method	Conditions	Yield	Crude Protein Content	References
Heat Coagulation	• Green-juice coagulates by steam between 80-90℃	-	39.13mg/100g	Sodamode *et al.* (2013)
Extraction buffers	• 0.1M NaOH • 50mM NaCl • 20mM $CaCl_2$ • 100mM NaH_2PO_4	70mg/ml 84mg/ml 111mg/ml 94.5mg/ml	37.5% 42% 55.5% 47.25%	Falode (2015)
Alkali Extraction/Acid Precipitation	• Extraction pH = 9 with 1M NaOH • Extraction time = 1hr • Solvent to solid = 20:1 • Precipitate pH = 4.5 and stir for 30mins before resolubilize at pH 7	-	38.02%	Ahmed and Supervisor (2016)
Enzymatic Extraction	• pH = 5.5 • E/S ratio = 1:20 • Incubation Temp= 30℃ • Incubation Time = 30mins • Enzyme Concentration = 6 FBG	14.2%	55.7%	Benhammouche *et al*. (2020)
Alkali Extraction/Acid Precipitation	• Extraction pH = 9 • Solvent to solid = 20:1 • Extraction time = 1hr • Precipitate = 4.5 and left overnight before resolubilize at pH 7	-	77.44%	Rawdkuen (2020)
Solvent extraction assisted by ultrasound-microwave extraction(UMAE)	• 0.15M Tris-HCl • UMAE	6.9% 8.2%	-	Cheng, Shu, *et al.* (2021)

Time given for extraction and precipitation can plan a critical role in letting protein molecules diffuse out of cell wall materials as well as having time to coagulate due to hydrophobic association thus significantly increasing its content. Benhammouche *et al*. (2020) on the other hand claims the alkaline-acid precipitation method unfavorable due to low environmental sustainability, low digestibility, and nutritional value. The study produced protein concentrate from defatted moringa leaves which shows lower protein content compared to Rawdkuen (2020). This may be since subsequent to the enzymatic extraction, alkaline extraction was also done but at pH 11. Extracting proteins at high alkaline pH than 9 can cause detrimental effect to the protein structures and its contents (Betschart & Kinsella, 1974). Heat coagulation extraction of protein is most convenient

and easiest method for extraction of protein. Heating can provoke the aggregation of proteins by opening hydrophobic sites hence cause irreversible denaturation with intense heating(Santamaría-Fernández & Lübeck, 2020). This disintegrates the functional properties with very low solubility (Betschart & Kinsella, 1974). But with mild temperature irreversible denaturation can be avoided and the functionality of the protein can be improved.

Other methods such as extraction buffers by Falode (2015), shows calcium chloride buffer to be more superior and effective in extracting protein concentrate from moringa leaf than the other buffers used whereas sodium hydroxide shows least protein content in its concentrate. Nowadays, new and advance techniques such as that from Cheng *et al.* (2021) are developed in order to give a highly efficient and faster extraction of protein as well as environmental protection. The study compared between solvent extraction with Tris-HCl solution and assisted ultrasonic-microwave extraction (UMAE). It resulted in concentrates from the UMAE giving a more increased yield than just using the traditional solvent extraction.

2.3.2 Nutrition and Chemical Composition

From Table 2.4 it can be shown that protein content in leaf protein concentrates from moringa can be in the range of 30%-77% depending on the conditions used. It has been reported to still maintain a level of high ash (~6%) exposing high amounts of minerals still maintained (Ca, Mg, Mn and Na) (Sodamode *et al*., 2013). Compositions of protein in MoLPC can be made up of albumin (3.1%), globulins (0.3%), prolamin (2.2%), glutelin (3.5%) of the whole leaf protein as studied by Teixeira *et al*. (2014).

Amino acid profile of *Moringa oleifera* leaf concentrate shows to contain all essential amino acids as displayed in Table 2.5. Most abundant amino acids usually shown in the studies are those of glutamic acid, glycine, aspartic, leucine, arginine, cysteine and threonine. In terms of essential amino acids, high amounts of leucine, threonine, phenylalanine, valine and lysine usually dominate. The limiting essential amino acids are usually those of methionine, histidine and tryptophan which is common in most green plants. The daily intake requirement for methionine amino acid by Joint *et al*. (2007) is 1.6% (16mg/ 1g protein). Study by Benhammouche *et al*. (2020) on *Moringa oleifera* defatted leaves (db.) determined about 1.54% of methionine, and 1.44% for its

protein concentrate (by enzymatic extraction). This correlated well with a study by Rani and Arumugam (2017) and the review by Sahay *et al*. (2017)with the methionine content of 1.29% and 1.66% respectively. Other studies by Moyo *et al*. (2011) and Chhikara *et al*. (2020) presented very different values that are very low at 0.297% and 0.004% for methionine levels in *Moringa oleifera* leaves.

This indicates the variation of amino acid levels among the plant of *Moringa oleifera* leaves at different environments and time. However, as stated by these studies, the values of these limiting amino acids are still comparable with the FAO/WHO standard amounts and requires careful recognition to be good potential sources for protein and amino acids. Another study by Lin *et al*. (2019) on bioactive peptides acquired from *Moringa oleifera* leaf concluded that amino acids such as tyrosine, cysteine, histidine and methionine in the leaves were attributed for the exceptional anti-oxidant scavenging properties of the leaves. Leucine, isoleucine, proline, phenylalanine and valine are hydrophobic amino acids which presents *Moringa oleifera* the potential for good functional properties and be incorporated in food (Lin *et al*., 2019).

The quality of protein depends on the contents of the amino acids as well as the bioavailability of the protein to be digested in the human body. Teixeira *et al*. (2014) did not agree well with most studies on *Moringa oleifera* leaf protein by stating that its leaf has a high amount of crude protein but most of it attributed to insoluble protein. It also performed an in-vitro digestibility test on the defatted leaf flour in which it resulted in low value of 31.8% in comparison to casein protein from milk supporting its conclusion. Benhammouche *et al*. (2020) deviates from Teixeira *et al*. (2014) in terms of digestibility with a crude protein of 24.1% (db.) of the defatted leaves (a small increase from the crude protein of the dried moringa leaves only of 23.6%), a digestibility score of 64.75% was obtained. Benhammouche *et al*. (2020) optimized the nutritional value of the *Moringa oleifera* leaf protein concentrate by enzymatic extraction using Viscoenzyme. L. It resulted in leaf protein concentrate with 55.7% of the protein content and an increasing digestibility value of 99.86% in which the study concluded with *Moringa oleifera* leaf protein concentrate to be of good nutritional value to be considered a protein source. The digestibility score for the leaf protein concentrate by Benhammouche *et al*. (2020) is higher than Rawdkuen (2020) who had a

digestibility score of 75.54% for its moringa leaf protein concentrate (not defatted) by alkaline-acid extraction.

Table 2. 5 Essential Amino Acids in moringa leaf protein concentrates

Essential Amino Acids (g/100g of protein)	Benhammouche *et al.* (2021)	Rawdkuen (2020)	Requirements for Infants (Joint *et al.*, 2007)	Requirements for Adults (Joint *et al.*, 2007)
His	1.37	1.907	2	1.5
Ile	3.61	2.871	3.2	3
Leu	5.89	6.714	6.6	5.9
Lys	5.05	3.791	5.7	4.5
Met	1.44	1.318	-	1.6
Phe	5.04	3.592	-	-
Thr	6.86	3.037	3.1	2.3
Trp	2.24	1.21	0.85	0.6
Val	5.31	1.86	4.3	3.9
Met + Cys	9.56	2.21	2.8	2.2
Digestibility%	99.8	75.53		
PDCAAS	91.41	-		

2.4 Physico-chemical properties and functional properties of *Moringa oleifera* Leaf protein concentrate

In addition to the desirable effects protein plays in food, are its physico-chemical and functional properties. So far, the most studied properties in leaf protein concentrate from moringa leaves are those of protein solubility, water and oil holding capacities, emulsifications and foaming capacities.

Authors have concluded that these properties are pH-dependent among others (Ahmed & Supervisor, 2016; Rawdkuen, 2020) and were investigated throughout their studies. In reference to Table 2.6, protein solubility reported by Rawdkuen (2020) showed maximum solubility of the protein concentrate at pH 9 and low at pH 4 . Ahmed and Supervisor (2016) on the other hand showed a different profile where the highest solubility is at pH 7 and low for pH 2, 3 and 9. For foaming capacity and emulsion capacity, MoLPC from Rawdkuen (2020) shows high capacities at acidic and alkaline pH, whereas its low capacities at alkaline pH. Ahmed and Supervisor (2016) showed high capacities of its foaming and emulsification at alkaline pH and low at acidic pH. The differences in these properties can be attributed to the difference in balance of

hydrophobic and hydrophilic amino acids. The study by Rawdkuen (2020) showed in its amino acid profiles that non-hydrophobic amino acids outweighs the hydrophobic amino acids. Each amino acid is unique to have its own isoelectric point and high solubility pH and by changing the external factors of its environment, their interactions overall will determine their behavior and the capacity of their functional property(Ahmed & Supervisor, 2016). Both studies reported good water holding capacity (2.31-5.82g water/ g protein) and oil holding capacity (3.55 – 3.87g oil/ g protein) given that the values are quite comparable with WHC and OHC from alfalfa protein isolates (Wang & Kinsella, 1976). The color of the MoLPC is described to possess a darker color (low-lightness) which can be due to the presence of some phenolic compounds as well as the reaction of chlorophyll pigments in the green plants during extraction with the acid (Rawdkuen, 2020).

Table 2. 6 Some functional properties in moringa LPC with corresponding pH

Functional Properties	**Rawdkuen (2020)**		**Ahmed *et al.* (2016)**	
	High	Low	High	Low
Solubility	pH 9	pH 4	pH7	pH2, 3, 9
Foaming capacity	pH 4, 10	pH 8	pH 11	pH 4
Emulsifying Capacity	pH3 , 10	pH 12	pH 11	pH 4

2.5 Use of moringa and moringa LPC in foods

2.5.1 General Use of Moringa leaf as Food

Fresh young *Moringa oleifera* leaves in parts of India are use cooked as a vegetable in soups with pumpkin, potato. Other countries drink it as tea, by just brewing in hot water. This is a special food supplement for pregnant women and lactating mothers (Rani & Arumugam, 2017). The meal is also used for those who are suffering from osteoporosis and bone fracture. During off seasons, the dried leaves are powdered and stored. The leaf powder is also used in the mixing of juice and beverages. The incorporation of moringa into the diet was reported to enable lactating mothers to produce more milk during pregnancy, their babies having higher healthy birth weights. In addition, it was able to cure tapeworms, control diabetes and high blood pressures (Fuglie, 2001).

2.5.2 Use of Moringa and Moringa LPC as food fortification

Food fortificant as defined by Oyeyinka and Oyeyinka (2018) should not improve nutritional value at the expense of sensory properties. Studies on food fortification with *Moringa oleifera* leaf powder are mostly done from parts of India, sub-Saharan Africa, Asia and Middle East. This is because, most of these parts face malnutrition due to protein deficiency as well as the famine food plant being native to these parts. These studies concluded that each food fortified with *Moringa oleifera* powder increases in contents of protein, dietary fiber, ash and phytochemical nutrients such as antioxidants.

Moringa oleifera leaf powder has been used for fortifying cake in some studies (Kolawole *et al*., 2013) and that 8% of the moringa leaf addition to wheat flour (300g) and other ingredients showed an increase in moisture, protein, crude fiber and total ash with a decrease in crude fat and carbohydrates. This was also the accepted level of the *Moringa oleifera* leaf powder in terms of sensory qualities. Kolawole *et al*. (2013) concluded with the importance of utilizing fiber rich plant food to help in traffic movement through intestinal tract and lower cholesterol in blood.

Chocolate fortified with *Moringa oleifera* leaf powder (Atef & Aziz, 2014) reported the enhanced nutritional value and sensory characteristics by 15% addition of the powder. Cookies fortified with *Moringa oleifera* leaf by Olabode *et al*. (2015) reported how there was no significant difference between fortified cookies without eggs and milk and the control which had no moringa leaf powder but contained milk and egg (balanced contents). This indicates *Moringa oleifera* leaf powder having comparable protein with egg and milk. A review in 2018 (Oyeyinka & Oyeyinka, 2018) on *Moringa oleifera* as a food fortificant was reported on stiff dough ‘Amala’, a type of stable food made in parts of Africa with yam. Its fortification with *Moringa oleifera* enhanced some nutritional qualities however, there was a reduction of its swelling and pasting which is an important aspect of the food in terms of storage purposes. They reported that above 2.5% addition of moringa gave unfavorable sensory characteristics. Same observation in terms of swelling was observed for cereal gruel. Micro-graphs of *Moringa oleifera* leaf fortified wheat cookies displayed the moringa leaf powder covering the starch granules of the wheat flour which can explain the viscosity reduction in ‘amala’ and

cereal gruel. It concluded that viscosity was decreased despite the increase in nutritional contents. With bread, the sensory qualities were mostly affected in terms of color and taste being unfavorable with more than 5% addition of the *Moringa oleifera* powder but with increased protein and fiber content. Oyeyinka and Oyeyinka (2018) reported the necessary need for technological techniques that can separate the chlorophyll and phytochemicals responsible for the unfavorable sensory qualities. Other foods like yoghurts and cheese also took part in being fortified with *Moringa oleifera* leaf powder. However, with increased nutritional value in high concentrations of leaf powder, the sensory characteristics were unfavorable. It was then recommended that the addition of fruits and additives can help mask the color and herbal taste of moringa fortified food. The existence of studies on moringa leaf protein concentrate being fortified into food is difficult to find which just proves that there is room for investigation in this aspect.

Information obtained from the above studies is important for the development of appropriate food systems that could implement the efficiency of high protein sources such as *Moringa oleifera* leaves and be stabilized for a more improved diet.

Chapter III

MATERIALS AND METHODS

Materials

Moringa oleifera leaves

Two varieties of moringa leaves were obtained: Thai moringa (TMo) and Indian moringa (IMo) from Nan area.

Pancake Ingredients

All ingredients were bought from Tesco Lotus supermarket.

Uncle Barn's Pancake Waffle Mix (R&B Food Supply Co., Ltd, Bangkok)

Hygienic Healthy Hens fresh eggs (Betagro Group Co., Ltd, Bangkok)

Orchid creamy unsalted butter (Food Com Co., Ltd, Bangkok)

Mazola Corn oil (ACH Food Co., Ltd, Mississauga, Canada)

Drinking water

Chemicals Reagents

All chemicals used in this experiment were analytical grade.

8-Anilino-1-naphthalenesulfonic acid ammonium salt (Sigma-Aldrich, Switzerland)

Albumin fraction V (Merck, Germany)

Anhydrous copper sulfate (Ajax Finechem Co., Ltd, New Zealand)

Anhydrous sodium carbonate (Lons Chemie Pvt Ltd., India)

Boric acid (Ajax Finechem Co., Ltd, New Zealand)

di-Sodium hydrogen phosphate (Elago Enterprises Pty Ltd, Australia)

Ethanol (Qrec Chemicals, New Zealand)

Folin-Ciocalteu's phenol reagent (Merck, Germany)

Hydrochloric acid (Qrec Chemicals, New Zealand)

Kjeblet catalyst (Oskon Co. Ltd, Thailand)

Methyl red indicator (Merck, Germany)

Petroleum Ether (Qrec Chemicals, New Zealand)

Reagent grade concentrated. sulfuric acid (Qrec Chemicals, New Zealand)

Sodium di-hydrogen phosphate (Elago Enterprises Pty Ltd, Australia)

Sodium dodecyl sulphate (Ajax Finechem Co., Ltd, New Zealand)

Sodium hydroxide (Ajax Finechem Co., Ltd, New Zealand)

Laboratory Equipment

Centrifuge (Model6000, Kubota Corporation, Japan.)

Colorimeter (Chroma meter CR-400, Konica Minolta Sensing, Inc., Japan)

Digestion Unit (K-424) (Buchi, Switzerland)

Distillation Unit (K-324) (Buchi, Switzerland)

Drying oven (Memmert UN 30 plus, Germany)

Fluorescence spectrophotometer (Jasco FP-6200, Japan)

Viscometer (Fungilab Premium series, Barcelona, Spain)

Homogenizer (IKA®T25 Digital Ultra Turrax, Guangzhou, China)

Induction Cooker (IF-404, Thailand)

Kitchen Aid Mixer (5KPPM5, Michigan, USA)

Milling grinder (Type2200, Yongkang Zhaoshen Electric Co., Ltd. Yongkang, China)

Muffle furnace (CWF 1200, Scientific Promotion Co. Ltd, Thailand)

Seven compact pH meter (Mettler Toledo Co. Ltd, Victoria, Australia)

Soxhlet extractor (Gerhardt, Germany)

TAXT2i texture analyzer (Stable Micro systems Co; Ltd, Godalming, UK)

Tray Dryer Oven (Thermotec2000, Auckland, New Zealand)

Ultraviolet spectrophotometer (Evolution One, Thailand)

Water bath (SW 23, Germany)

Methodology

3.1 Preparation of *Moringa oleifera* leaf powder

The leaves of both varieties, Thai and Indian, were harvested and dried in a tray dryer at 40-45 ℃ until moisture content in leaves reached approximately 6.0%. The dried leaves were grounded and sieved using a sieve with mesh size 80 micron and sealed in polyethylene plastic bags then stored in a dark place under ambient temperature until further experiment.

3.2 Extraction of *Moringa oleifera* leaf protein concentrate (MoLPC)

As a preliminary work for the extraction of *Moringa oleifera* leaf protein concentrate, an optimization study of alkaline extraction at pH 8.5, 9 and 10 at room temperature using Indian variety were monitored due to previous studies of *Moringa oleifera* and various leaf protein concentrates. This enabled the selection of the pH condition for the study according to pH with highest yield and less denatured protein concentrate as recommended by the literatures.

The alkali-acid extraction process was performed according to the method done by Rawdkuen (2020) with some modification. In a 1:10 (w/v) ratio, moringa leaf powder was suspended in distilled water and then 1 M of NaOH was used to adjust the pH of the solution to 9, stirring for 1hr. The suspension was regularly checked every 15mins to re-adjust the solution to pH 9. The solution was then centrifuged for 10 mins at 9000*g*

and resulted in the brownish supernatant liquid as the soluble protein. The supernatant was then acid-precipitated by adjusting the pH with 1M of HCl to its isoelectric pH of 4.5. It was then left standing to precipitate for 1hr. Suspension was then centrifuged at 9000*g* for 10 mins and the pellet was suspended in an amount of distilled water and re-solubilize with 1M NaOH at pH 7. The resolubilized MoLPC at pH 7, was then freeze dried, vacuum packed and stored at -35 ± 2 ℃ for further analysis. Two variables were used in this procedure, the variety of leaves (Thai and Indian) and applying heat (precipitated protein during pI precipitation was exposed to heat in a water bath at 55℃ for another 20 mins) or no heat for pI precipitation. As a result, 4 MoLPC samples were obtained.

3.3 Determination of MoLPC extraction yield and Chemical Properties

3.3.1 MoLPC extraction yield

A mass balance of the protein extraction process was conducted and used for calculation of the yield of LPC extracted according to Eq.1. Yield% was obtained as wet basis and converted to dry basis using moisture content of sample.

$$Yield\% = \frac{weight\ of\ MoLPC\ (g) \times 100}{weight\ of\ Leaf\ powder(g)} \qquad (Eq1)$$

3.3.2 Chemical Properties

Determination of proximate compositions

Moisture, crude protein, crude fat, crude fiber, ash and available carbohydrates were determined according to AOAC (1995). Crude protein determined using Kjeldahl method and calculated with conversion factor of 6.25. All values were obtained as wet basis values and converted to dry basis.

Surface Hydrophobicity (H_0)

Surface hydrophobicity was determined by the hydrophobicity fluorescence probe method using 1-anilino-8-nephtalene sulfate (ANS) as described by Krasaechol *et al*. (2008) with some modifications. A stock solution of each MoLPC (1000µg/ml) in 0.1M sodium phosphate buffer of desired pH (6, 7 and 8) was used to serially dilute into final MoLPC concentrations from 25, 50, 100, 150, 200 and 250µg/ml in the

appropriate 0.1M phosphate buffers of pH 6, 7 and 8. 20µL of 8mM ANS in 0.1M phosphate buffer of pH 7.0 was added to 4ml of each dilution and its fluorescence intensity was measured with a fluorescence spectrophotometer (Jasco FP-6200, Analytical Lab Science Co. Ltd, Thailand). Excitation and emission wavelength of the fluorescence was measured at 390nm and 470nm respectively. From each MoLPC, its fluorescence intensity was plotted against the dilution concentrations in which its gradient was used to calculate the surface hydrophobicity.

3.4 Determination of MoLPC functional properties

3.4.1 Protein solubility

Protein solubility was determined according to the method described in Krasaechol *et al*. (2008) with some modifications. MoLPC was dispersed in deionized water at a ratio of 1:1(w/v) and adjusted to the following desired pH of 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10 with 0.1 M of HCl or NaOH. Solution was then stirred with a magnetic bar for 1 hr. Suspension was transferred to a 50 ml conical tube and centrifuged at 9000*g* for 10 mins. Supernatant obtained was used to determine the protein concentration by Lowry assay (Bollag & Edelstein, 1991). Protein solubility (%) was then calculated as the amount of protein in the supernatant in regard to the total crude protein determined by the Kjeldahl method.

3.4.2 Emulsification Properties

Preparing Emulsion

Preparation of emulsion for all emulsifying properties (emulsifying capacity, stability index and activity index) were performed according to the method described by Hall (2011) with some modifications. Emulsifying properties for MoLPC was tested at pH 6, 7 and 8 at room temperature 25±1℃. LPC powders were immersed in 0.1M sodium phosphate buffer solutions of appropriate pH. The emulsion of the samples was prepared with 0.4g of MoLPC in 20ml (0.02g/ml) of buffer solution at desired pH. The solution was stirred for 15mins before adding 10ml of corn oil (density= 0.908g/ml) and then homogenized at 22000 rpm for 1min.

Emulsifying Capacity (EC)

EC was determined according to the method recorded by Hall (2011) with some modifications. After 30secs of preparing the emulsion as stated above in section 3.4.2, corn oil was released from a burette at a rate of 0.1ml/sec after 30secs of emulsion preparation. The mixture was homogenized while measuring the conductivity at the same time. EC was indicated with the sudden drop of conductivity specifying the occurrence of a phase inversion. The amount of oil (g) before phase inversion was recorded as oil per 20ml of protein solution (0.02g/ml). This was used to calculate EC as grams of oil per grams of crude protein (g oil/g protein) as determined by Kjeldahl method.

Emulsifying Activity Index (EAI)

Determination of EAI was performed according to the turbidimetric method by Krasaechol *et al.* (2008) with some modifications. Aliquots of the emulsion as prepared above in section 3.4.2 (0.1ml) in 10ml volumetric flask was made up to the mark with 0.1% of SDS in distill water. 1ml of this 0.01g/ml solution was then added with 4ml of 0.1% SDS to make a diluted concentration of 0.002g/ml. The absorption of the 0.002g/ml diluted emulsion was measured immediately after homogenization at 500nm with a 1cm pathlength cuvette.

Emulsifying Stability Index (ESI)

Determination of ESI was performed according to the turbidimetric method by Krasaechol *et al.* (2008) as described under emulsifying activity index. Absorption of the emulsion (as described above in section 3.4.2) diluted (0.002g/ml) for ESI was measured at 500nm on the 0^{th} minute and 10^{th} minute interval immediately after homogenization.

$$\text{Turbidity (T)} = \frac{2.303 \times A_{500} \times F}{l} \quad (Eq.2)$$

$$\text{EAI } (m^2/g) = \frac{2 \times T}{\emptyset \times C} \quad (Eq.3)$$

$$\text{ESI (mins)} = \frac{T \times \Delta t}{\Delta T} \quad (Eq.4)$$

Where: A_{500} = sample absorbance, F = dilution factor, l = cuvette pathlength (cm), Φ = oil volume fraction, C = weight pf protein per unit volume of aqueous phase, t = time (mins).

3.5 Formulation and preparation of MoLPC pancake

3.5.1 Preliminary study

Proportion of each ingredient for the formulation of the pancake was performed according to the instructions given by "Uncle Barn's Pancake Waffle Mix" on the package. Percentage replacement of the pancake mix (PWM) with ThaiLPC-heat was prepared according to the formulation shown in Table 3.1

As preliminary work on pancake formulation, 20% replacement of PWM with MoLPC compared with 15% replacement was first commenced in making of pancake batter. This determined a threshold for maximum replacement of PWM with MoLPC in which MP20% gave a viscous batter that was hard and unfavorable to manage into induction cooker for cooking of pancake. Hence, three formulations for fortified moringa pancake (MP%) was chosen (MP5%, MP10%, MP15%) in addition to the control (MP0%). In Table 3.1, MP5% refers to replacing 5% of the total amount of PWM in the control with MoLPC powder and therefore applied to other formulations.

Eggs were beaten first for 1.5mins with a mixer (Kitchen Aid, MA) at high speed at dial 10. Melted butter and water was added next and then the speed of the mixer was decreased down to dial 2 for 2mins. PWM was mixed with MoLPC sample before adding into the rest of the batter. Mixing of batter at dial 2 continued on for an extra 2 mins until the batter was seen to be completely homogenized and not much lumps seen. A pan was heated on an induction cooker (IF-404) at 120-140℃ for 2mins before adding a small amount of butter to grease the pan. A spherical mould of 7.5cm diameter was placed in the pan before pouring in about 40ml of pancake batter. The pancake was cooked for about 2.67mins which showed pancake to be completely cooked before flipping to the other side and cooked for 1.5min. Pancake samples were then taken for further analysis.

Table 3. 1 Fortified pancake formulation

Ingredients	Formulation			
	MP0% (Control)	MP5%	MP10%	MP15%
PWM (%)	38.4	36.48	34.56	32.64
ThaiLPC-heat (%)	0	1.92	3.84	5.76
Butter (%)	6.91	6.91	6.91	6.91
Egg (%)	16.28	16.28	16.28	16.28
Water (%)	38.4	38.4	38.4	38.4

PWM = Pancake-Waffle Mix
ThaiLPC-heat = Thai leaf protein concentrate-heat (MoLPC powder use for replacement of PWM)

3.5.2 Determination of Pancake Batter Viscosity

Pancake batter viscosity was measured using Fungilab Premium Viscometer at room temperature (25±1℃) immediately after beating and mixing of batter. Batter of pancake was made three times for the measurement of viscosity. The viscosity was measured with a spindle probe No. R4 at 20rpm and the results were recorded as centipoises and converted to $N.s.m^{-2}$.

3.6 Determination of Pancake Properties

3.6.1 Chemical Properties

Determination of proximate compositions of pancakes

Moisture, crude protein, crude fat, crude fiber, ash and available carbohydrates were determined as stated above in section 3.3.2.

3.6.2 Physical properties

Color Analysis

Color analysis of the pancakes were measured according to the method by Rawdkuen (2020) with some modifications. A colorimeter (Chroma meter CR-400) was used to measure the color of the pancakes. Pancakes were cut horizontally in half to reveal the color of the inner texture which shows more clearer the color contributed to by the addition of MoLPC. The color was measured as L* which expresses the lightness of the pancake (0 = dark, 100=lightness), a* which expresses the redness to greenness of the pancake (Green = negative values, Red = positive values) and b* which

expresses blueness to yellowness color (Blue = negative values, Yellow = positive values). Prior to measuring the color, the colorimeter was initially calibrated using a standard white plate with the specifications: L* = 97.54, a* = -0.02, b* = 2.17. Each pancake formulation was measured 3 times. Hue and chroma of the fortified pancakes were calculated according to Mclellan *et al*. (1995)

Bulk Density

Bulk density of pancake samples was performed according to the displacement method by Wang and Kinsella (1976) with slight modification. Weight of pancake was recorded (g). Using white sesame seeds to measure the total volume (with 1000ml measuring cylinder) of a box container by filling the sesame seeds into the box until it was full (900ml = 900cm^3). A layer of sesame seeds was layered at the bottom of the box container, the pancake was then placed on top before pouring in the rest of the sesame seeds until the container was full and then remove the excess sesame seeds. The volume was calculated by the difference between total volume of sesame seeds in the box container without the pancake and the volume of sesame seeds in the box container with the pancake. Bulk density was recorded as (g/cm^3). Bulk density for each pancake formulation was measured three times.

Texture Profile Analysis (TPA)

Texture profile analysis was performed according to the method by Shih *et al*. (2006) with slight modifications. A TAXT2i texture analyzer (Stable Micro systems Co; Ltd, Godalming, UK) was used for the analysis of pancake in which it was measured over a 30min period after cooking. An acrylic cylinder probe (P/100) was used to mimic the double bite compression of the pancake by setting conditions to a test speed of 10mm/sec and strain of 50%. “Exponent” software was used for the measuring of the TPA parameters of hardness, springiness, and cohesiveness of the pancake texture. TPA for each pancake formulation was measured six times to obtain the TPA parameters.

3.7 Statistical Analysis

Experiments were carried out with completely randomized design. Statistical analysis was determined using SPSS software for Windows (version 22). Significance

of the values was assessed using 95% confidence interval. The comparison of means was done using Tukey's test at $p<0.05$ significant difference.

Chapter IV

RESULTS AND DISCUSSION

4.1 Extraction of *Moringa oleifera* leaf protein concentrate (MoLPC)

4.1.1 Preliminary work on MoLPC extraction yield

Preliminary work done for the extraction of *Moringa oleifera* leaf protein concentrate, was an optimization study of alkaline extraction at pH 8.5, 9 and 10 using Indian moringa leaf at room temperature were monitored due to previous studies of *Moringa oleifera* and various leaf protein concentrates. The results in Table 4.1 showed pH 8.5 to be significantly lower in mass yield than pH 9 and 10 even though the values do not show such a big variance. pH 9 and pH 10 showed no significant difference even though pH 10 gave the highest mass yield of 3.11%. Betschart and Kinsella (1973) has recommended the best range for protein extraction at the range of pH 7.5 – 8 for soybean LPC due to the high protein solubility and less risk of denaturation. However, in most MoLPC studies, pH 9 was utilized as pH of extraction and has given satisfactory results of functional properties and nutritional content attributes (Ahmed & Supervisor, 2016; Benhammouche *et al*., 2021; Rawdkuen, 2020). Therefore, in this study, pH 9 was chosen as it shows to be significantly higher in yield than pH 8.5 and that less adverse denaturation of proteins may occur at this pH in comparison to pH 10.

Table 4. 1 Optimization results on MoLPC extraction yield – Preliminary workd (db.)

	Ph		
	8.5	9.0	10.0
Yield (%)	1.8 ± 0.4^{b}	2.9 ± 0.3^{a}	3.11 ± 0.4^{a}

Values presented as mean ± standard deviation (n=3)

Different letters across rows shows mean values that were significantly different at $p < 0.05$ using Tukey mean comparison.

4.2 Determination of MoLPC extraction yield and Chemical properties

4.2.1 MoLPC extraction yield

MoLPC extraction yield was determined through a mass balance of the protein extraction process. The yield of MoLPC was calculated as the mass of MoLPC (g) from the mass of moringa leaf powder (g) on a wet basis and then converted to dry basis. Table 4.2 showed that LPC from Thai variety gave significantly higher MoLPC yield (3.16%-4.59% db) than the Indian variety (2.00%-2.47%), however, the marginal difference was not that much. The yield in this study showed to be lower in comparison to the MoLPC mass yield (6.14%) extracted by Benhammouche *et al*. (2021) through enzyme assisted extraction. In terms of protein yield, the current study possessed 4.33%-5.90% (db) for Indian-LPC and 6.60%-9.73% (db) for Thai-LPC. Protein yield acquired by Benhammouche *et al.* (2021) showed higher value of 14.2% with the carbohydrase Viscozyme L. This suggests that some proteins in the current study may have still been stuck within the cell wall fibers of the leaves during extraction (formula for protein yield calculation is displayed in Appendix A).

In terms of heating condition during acid precipitation, LPC yield without heating gave lower values in both varieties but not significant for the Indian variety. This showed that moringa variety had more significant effect on the yield rather than the heating conditions during precipitation. The differences in yield could be due to the different harvesting times, plant age, cultivation and breeding of the moringa variety (Kaszás *et al*., 2020). Its chemical content can affect levels of LPC extraction in moringa leaves. For instance, a study of wild types of moringa and its domestic types by Chodur *et al*. (2018). Though the study was based on how the bitter taste in the wild types is more than the domestic moringa leaf due to the different composition of glucosinolates, their results showed protein content in the wild type to be lower (26.28%) than the domesticated moringa leaf (30.24%). Furthermore, the effect of heat on the yield may not be significant since acid-precipitation was more efficient in the coagulation of the proteins rather than the mild heat used at 55℃. As discussed by Santamaría-Fernández and Lübeck (2020), acid precipitation technique utilizes the isoelectric pH of protein where the net charge of the proteins are zero. This promotes the lowest solubility of

proteins at this point as they aggregate by hydrophobic association as water molecules are leeched out. Heating on the other hand can give high extraction yield at extreme level of 80℃ but can cause irreversible denaturation of proteins hence decreasing its solubility (Betschart & Kinsella, 1974). Thermal treatment can cause protein aggregation by disrupting hydrogen bonds or form cross-linking of di-sulfide bonds making them have lower solubility (Cheng, Shu, *et al*., 2021). Since the current study uses moderate level of 55℃ of heating during precipitation, the role of heat may only serve a partial denaturation on the protein structure

The yield of MoLPC mass extracted was relatively small compared to the yield of protein from ultrasound-microwave assisted extraction of MoLPC by Cheng, Shu, *et al*. (2021)(85mg protein per 1g of leaf powder) and by Rawdkuen (2020) (77.44% extractable protein content) suggesting higher MoLPC mass yield. This could be attributed to the differences in the methods as well as the solute to solvent ratio used in these two studies in which they suggested ratio higher than the current study. According to Cheng, Shu, *et al*. (2021), the effect of solvent to solid ratio on protein extraction can affect the driving force of mass diffusion by increasing the solvent to solid ratio hence increasing protein yield and thus protein content. Conditions used in the current study such as solute to solvent ratio of extraction, the time of alkali extraction within one hour could have limited the maximum extraction of MoLPC yield for the current study as it may appear from protein contents in Table 4.3 that maximum protein extraction may not have been reached.

Table 4. 2 Mass extraction yield of moringa leaf protein concentrate per 100g of moringa leaf powder on a wet and dry basis

Condition	Indian variety		Thai variety	
	Heat	No-Heat	Heat	No-Heat
Yield (db%)	2.47 ± 0.20^{bc}	2.00 ± 0.20^{c}	4.59 ± 0.13^{a}	3.16 ± 0.47^{b}
Yield (wb%)	2.36 ± 0.19^{bc}	1.91 ± 0.19^{c}	4.39 ± 0.12^{a}	2.97 ± 0.44^{b}

Values presented as mean ± standard deviation (n=2)
Different letters across rows shows mean values that were significantly different at $p < 0.05$ using Tukey mean comparison.

4.2.2 Chemical Properties

Determination of proximate compositions

Proximate compositions of moringa leaf and its leaf protein concentrates are summarized in Table 4.3 for comparison purposes. Table 4.3 showed that crude protein from dried Thai moringa leaf powder (29.01%) is significantly higher than dried Indian moringa leaf powder (23.93%). Thai moringa leaf in the current study has higher crude protein than the values of moringa leaves (23.6%) and moringa defatted leaves (24.1%) studied by Benhammouche *et al*. (2020). Crude protein of Indian moringa leaf powder seems to align with the values from Benhammouche *et al*. (2020). It is quite comparable with crude protein in amaranth leaves (22.84% db.) studied by Ngugi *et al*. (2017).

Variety of MoLPC seemed to have a much more influence on the difference in crude protein from Thai-LPC being significantly higher than Indian-LPC shown in Table 4.3. Different variety resulting from different cultivation and breeding methods can significantly affect protein content of leaves (Stevens *et al.*, 2015). Compared with LPC values from other studies, protein content of Thai-LPC is lower compared to soybean LPC (70.98%) (Betschart & Kinsella, 1974) but slightly higher than alfalfa LPC (59.8%) (Wang & Kinsella, 1976) and LPC from cassava (50.0%) (Ferri Coldebella *et al*., 2013). For the effect of heating, the difference in crude protein content in Indian moringa showed to be significant but in Thai moringa, the difference is not as significant showed by Table 4.3. Similarly, the significance of differences in crude protein within each variety corresponds to the difference in significance of its yields of extraction. This indicates a correlative relationship between yield of extraction and its crude protein content in MoLPC.

Table 4.3 for the current study showed crude protein content of MoLPC within the range of 51.82%-61.48% (d.b). MoLPC study by Rawdkuen (2020) gave higher crude protein content of 77.44% for their samples. This can be attributed to the higher solute-to-solvent (as prior mentioned for the yield of MoLPC) (deionized water) ratio of 1:20 (w/v) and longer isoelectric precipitation of moringa LPC at pH 4.5 which was done overnight under room temperature. In optimization studies of sugar-beet LPC (Akyüz & Ersus, 2021), tea leaves (Shen, Xiangyang, *et al*., 2008), time is seen as a factor that

increase yield of LPC extraction and hence crude protein content. They suggest how yields of extraction can only increase up to a certain point of time and then level off or decrease. Sugar-beet LPC gave a maximum yield time of 81mins, and tea leaves gave 4hrs as the maximum time to achieve maximum yields of LPC. This provides an indication there is still a potential of increasing yield and crude protein from MoLPC in the current study if precipitation time was prolonged for more than 1hr. Another possible cause for lower yield and protein yield extracted in the current study is due to solubility of proteins during extractions in which most of the insoluble proteins also known as cytoplasmic proteins were not fully solubilized for extraction (Fiorentini & Galoppini, 1983) due to the mild heat used.

However, the enhanced crude protein content of MoLPC analyzed from the current study at its current conditions (1g of MoLPC = 0.5182g – 0.6148g of crude protein content) shows an improvement towards satisfying the average protein requirement from FAO/WHO (Food & Agriculture Organization, 2007) required for adults and pregnant women (0.83g protein/kg weight/day). This means that an average person of 50kg requires about 41.5g of protein per day, in which about 67.5g – 80g of MoLPC from the current study can provide the required protein per day for an average 50kg person.

The differences between the two leaf powders in terms of crude fat, crude fiber, ash, and carbohydrates, were not significant. For LPC, variety showed no significant effect on the differences of proximate values. But the incorporation of heating on the precipitation step showed a significant effect on crude fat values for both Thai-LPC and Indian-LPC. During protein precipitation, heat can assist the break-up of lipo-protein complexes and separate proteins by precipitating it into the pellet whereas lipids end up in the supernatant (Betschart & Kinsella, 1974).

Crude fiber for Indian moringa leaf (40.0%) was not significantly different from Thai moringa leaf (46.9%). MoLPC also showed no significant difference which could suggest the two varieties to be in close in maturity stage. As plants increase in maturity, amount of total fiber increases due to possible lignification of cell wall constituents of the leaves (Punna & Paruchuri, 2004). Table 4.3 showed presence of crude fiber for MoLPC which was attributed to the presence of other substances co-precipitating with

the proteins during precipitation. Heating showed a significant effect on LPC within the Indian moringa where IndianLPC-heat has lower crude fiber compared to IndianLPC-NoHeat. Similarly to crude fat, heat may have the possibly in broken the binding complexes formed by protein with fiber. In turn, these proteins were released in free forms as protein concentrates thus lowering fiber content of the leaf protein concentrate. Significantly, this effect of heat on the fiber content of Indian-LPC corresponds to its significant effect on its protein content. As for the Thai-LPC, effect of heat on the fiber content was not as significant on the differences of its values, hence may also correspond to that of its crude protein values. Reported values of fibers in dried moringa leaves (18.7%) by Chhikara *et al*. (2020) and Dhakar *et al.* (2011) (19.2%) showed fiber in the current study to be higher. Reported values of fiber content in MoLPC study by Ahmed and Supervisor (2016) gave a close similar value of 13.94% to the fiber content of MoLPC in the current study.

The presence of carbohydrates in the current study can be attributed to the presence of dietary fiber composed of water-soluble fibers (Dhingra *et al*., 2012). This type of fiber is not included in crude fiber since crude fiber determination is usually referred to the remaining fiber after alkali and acid treatments and those are usually lignin and hemicellulose in plants (Dhingra *et al*., 2012).

Table 4. 3 Proximate compositions of dried moringa leaf and moringa leaf protein concentrate (db.)

Condition	Indian variety			Thai variety		
	Leaf Powder	Leaf Protein Concentrate		Leaf Powder	Leaf Protein Concentrate	
		Heat	No-Heat		Heat	No-Heat
Crude Protein (%)	23.93±0.45^{e}	57.19±0.88^{b}	51.82±1.73^{c}	29.01±0.03^{d}	61.48±1.04^{a}	60.57±0.88^{ab}
Crude Fat (%)	7.22±0.48^{ab}	2.88±0.02^{c}	5.06±0.90^{b}	8.54±1.21^{a}	3.52±0.56^{c}	6.03±0.13^{b}
Crude Fiber (%)	40.0±23.1^{a}	9.28±0.07^{c}	14.20±0.16^{b}	46.96±15.50^{a}	11.4±1.30^{bc}	12.30±1.28^{bc}
Ash (%)	0.73±0.08^{a}	0.05±6.51^{a}	0.07±0.61^{a}	0.21±0.08^{a}	0.01±0.04^{a}	0.05±0.03^{a}
Carbohydrates (%)*	28.8±23.9^{a}	29.07±1.08^{a}	29.34±1.70^{a}	19.74±15.45^{a}	22.62±2.53^{a}	20.94±0.60^{a}

Values presented as mean ± standard deviation (n=2)
*Estimated by difference using replicated values at wet basis (100 – Moisture + Protein + Ash + Crude fiber + crude fat) and then converted to dry basis.
Different letters across rows shows mean values that were significantly different at $p < 0.05$ using Tukey mean comparison.

Determination of surface hydrophobicity

Figure 4.1 showed IndianLPC-RT to give significantly high surface hydrophobicity of 729.2 and 600.1 at pH 7 and 8 respectively, whereas ThaiLPC-RT gives significantly high surface hydrophobicity of 760.2 at pH 6. Across pH 6, 7 and 8, IndianLPC-Heat showed significantly lowest surface hydrophobicity. It's also showed that the surface hydrophobicity values across pH 6, 7 and 8 for all MoLPC are significantly different.

Within the MoLPC variety, a specific trend was seen for each across pH 6, 7 and 8. At pH 7, both IndianLPC-RT and IndianLPC-Heat gave highest values of surface hydrophobicity than at pH 6 and pH 8. For ThaiLPC, both its MoLPC gives a decreasing trend of surface hydrophobicity with increasing pH. This showed that pH affects the surface hydrophobicity of both varieties differently. This may be due to the interactions of surface charges of the proteins present as the pH changes.

On the other hand, heat also showed a significant influence on the surface hydrophobicity for the LPC of both varieties. Surface hydrophobicity of IndianLPC was decreased when heated, whereas there is an increase in surface hydrophobicity for ThaiLPC.

Different occurrence of surface hydrophobicity may give a small indication of what type of amino acid residues in the protein may be present in each variety and how they are affected by pH and heat. Surface hydrophobicity has been mentioned in some studies to negatively correlate with protein solubility due to the moderate exposure of hydrophobic amino acid groups of native proteins that are buried in the interior (Voutsinas *et al*., 1983). With the increasing pH (pH6 – 8), the protein tends to be more electronegative. This results in corresponding charged amino acids gradually repelling thus inducing a moderate unfolding of protein molecule to expose hydrophobic sites out of the interior. The different response of both varieties in surface hydrophobicity indicates different amount or different amino acid groups present in each LPC variety. In relation to its solubility, its negative correlative relationship can be seen clearly on the native protein for ThaiLPC where at pH 6-8, protein solubility increases. By the effects of heat on protein surface hydrophobicity, the increased surface hydrophobicity given by ThaiLPC-heat can be attributed to the disappearance of native proteins which had maximum solubility at the range close to and at pH 7 for ThaiLPC-RT. This suggests that these proteins could have aggregated contributing to lower solubility, hence increasing surface hydrophobicity. The decreasing surface hydrophobicity given by IndianLPC-Heat, can be attributed to the fact that even though its solubility for all pH decreased overall after heating, its maximum solubility for IndianLPC-Heat was still retained at pH 6 and ranges close to this. This suggests that unfolding of proteins to expose hydrophobic sites for IndianLPC native proteins may have not been as high compared to ThaiLPC. Overall, the interaction of these two properties, surface hydrophobicity and protein solubility has an effect on functional properties of proteins such as emulsifying properties. More details on that will be further explained in the emulsifying section.

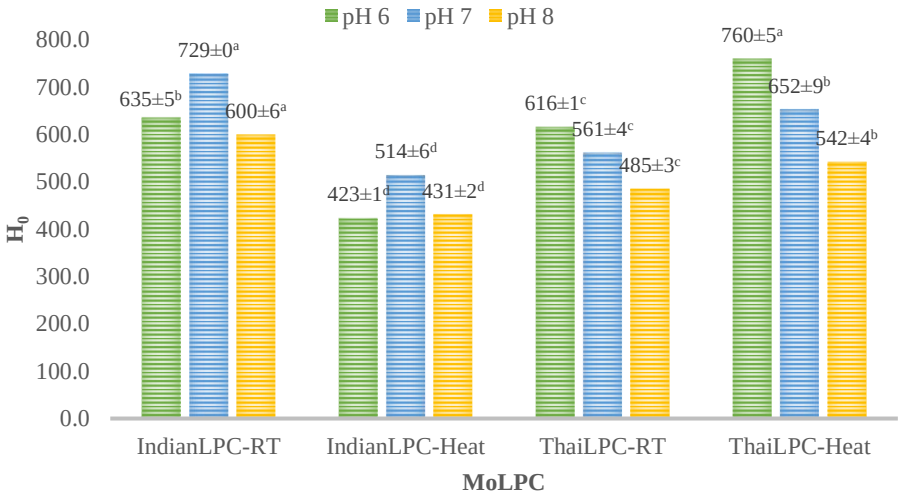

Figure 4. 1 Surface Hydrophobicity of MoLPC at pH 6, 7 and 8. Mean±SD with same letters on the same colored bars represent surface hydrophobicity that are not significantly different on same pH at $p<0.05$.

4.3 Determination of MoLPC functional properties

4.3.1 Determination of protein solubility

Protein solubility of MoLPC was determined using Lowry method with Folin-ciocalteu reagent. Figure 4.2 showed protein solubility to be pH dependent, where it was lower around acidic pH (3-4) close to the isoelectric point and higher at alkaline pH (8 - 10). This trend was seen to be within range with other LPC studies in alfalfa leaves (Hadidi *et al*., 2020; Lamsal *et al*., 2007; Wang & Kinsella, 1976) and soybean leaves (Betschart & Kinsella, 1974). The lowest protein solubility was seen at pH 3.5 for all MoLPC samples with a similar pattern of MoLPC-Heat having lower solubility than their MoLPC-RT counterparts. At pH 3.5, ThaiLPC-RT gave a significantly minimum protein solubility of 42.04% followed by IndianLPC-RT (36.25%) not significantly different from ThaiLPC-heat (34.74%) and lastly to IndianLPC-heat (31.93%). Rawdkuen (2020) reported pH 4 whereas Ahmed and Supervisor (2016) reported pH 3 to yield minimum solubility for their respective MoLPC. Differences of these reports is accounted for selectivity of pH to be investigated for protein solubility, in which both studies did not include pH 3.5 and Rawdkuen (2020) did not include pH 3 in their investigation. Minimum solubility of MoLPC in the current study at pH 3.5 showed to

be similar with the pH of minimum solubility of soybean LPC (Betschart & Kinsella, 1974).

Occurrence of lowest protein solubility at acidic pH especially at pH 3.5 showed that it is at this range that isoelectric pH for MoLPC exist. Isoelectric pH for proteins is where the negative electrostatic charge in the protein balances out the positive electrostatic charges. This gives an overall net charge of zero and causes proteins to interact with each other due to their hydrophobic affiliations and aggregate, thus decreasing solubility as water is leeched out of the protein molecule (Santamaría-Fernández & Lübeck, 2020).

The highest protein solubility for both IMoLPC-RT and TMoLPC-RT was at pH 7 and higher as there was no significant difference in protein solubility among that pH range for both MoLPC. This falls in range with the values reported by Rawdkuen (2020) (pH 10 and 12) and Ahmed and Supervisor (2016) (pH7) for MoLPC. Similarly, it is at this range that maximum protein solubility for other LPC such as soybean LPC (pH 11) (Betschart & Kinsella, 1974) and alfalfa LPC (pH 9) (Lamsal *et al*., 2007) was achieved. So, in more a precise manner, IMoLPC-RT gave maximum solubility at pH 9 and 6 whereas TMoLPC-RT gave maximum solubility at pH 7. Maximum protein solubility for both IMoLPC-heat and TMoLPC-heat occurred at alkaline pH range, specifically at pH 6 and pH 10 respectively.

The occurrence of maximum solubility of MoLPC in alkaline pH and its minimum solubility at low acidic pH may give an indication of the electrostatic nature of proteins to be highly electronegative that it requires lower pH of 3.5 to balance out its electronegative charges.

On another note, heating the respective MoLPC varieties showed a significant decrease of its protein solubility compared to MoLPC without heat. An important phenomenon to note in Figure 4.2 was the disappearance of native proteins for each MoLPC variety at the pH of their given maximum solubility when it was precipitated at room temperature. To be clearer, ThaiLPC no longer had maximum solubility at pH 7 but has shifted to pH 10 after heating. IndianLPC on the other hand, no longer had maximum solubility at pH 9 but still retains its maximum solubility at pH 6 after heating. This is

important in terms of relation to surface hydrophobicity and its effects on emulsifying properties of MoLPC proteins. This happened due to the presence of heat during protein extraction process which can provoke the coagulation of proteins by opening hydrophobic sites and cause protein denaturation decreasing the solubility property of proteins. Irreversible denaturation is caused by extreme high heat temperature of approximately 80℃ which can cause drastic effects on the solubility of soybean LPC (Betschart and Kinsella (1974). Minimal heating in the current study, was sufficient to cause reversible denaturation which increases protein to protein interactions due to exposed hydrophobic sites. Concurrently, unfractionated LPC contain chloroplastic proteins which are also known to be lipoproteins that are insoluble and easily coagulated at 50-60℃ than the more soluble cytoplasmic proteins (Betschart & Kinsella, 1974). Their affiliation with lipids can be accounted by the high proportion of hydrophobic amino acids in chloroplastic proteins. Thus, at extraction method, heating can increase the collection of chloroplastic proteins in the pellet hence contributing to lower solubility due to high proportion of exposed hydrophobic sites.

Figure 4.2 showed higher protein solubility for the native proteins of both moringa varieties at pH 6, 7 and 9. Possible contributions to the high solubility can be attributed to the final pH of MoLPC pellet during extraction process which was its re-solubilization at pH 7 before freeze drying to achieve MoLPC powder. In addition, was the use of Lowry assay by the current study in which possible presence of some phenolic compounds in the LPC could react with Folin-Ciocalteau and give positive result for protein determination as shown in Figure 4.2 (Winters & Minchin, 2005).

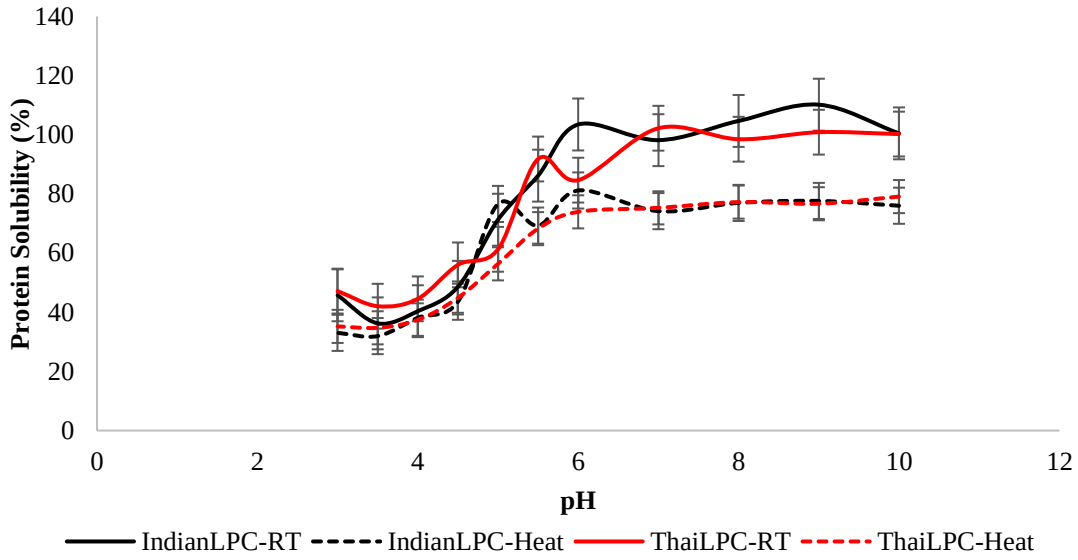

Figure 4. 2 Protein Solubility of Moringa leaf protein concentrate at pH range of 3 – 10. Error bars indicating standard deviation range of protein solubility for each pH at $p<0.05$

4.3.2 Determination of emulsifying properties

Emulsifying properties of MoLPC was determined from emulsions of protein solution made with corn oil. Emulsifying capacity (EC), emulsifying stability (ES) and emulsifying activity results of MoLPC is displayed in Table 4.4

IndianLPC-RT showed significantly high emulsifying capacity at all pH6, 7 and 8, followed by IndianLPC-heat and ThaiLPC-heat who are not significantly different and followed by ThaiLPC-RT showing lowest EC at all pH. The vary in range of EC between pH for all MoLPC was seen to not be too wide which can suggest that within these pH range, pH has no direct effect on emulsifying capacity of MoLPC. However, the additional heating of MoLPC during precipitation step for both Indian and Thai moringa contrastingly shows significant influence on EC of MoLPC. EC of IndianLPC, showed lower values from its heated counterpart. On the other hand, for ThaiLPC, heating showed improvement of EC for ThaiLPC native proteins as the ThaiLPC-heat samples have high EC on all three pHs than ThaiLPC-RT. IndianLPC showed emulsifying stability index (ESI) to be more stable at pH 8 where ThaiLPC-Heat showed significantly high stability at pH 6. Again, stable emulsification of ThaiLPC

showed improvement at all three pH values when heated. Whereas stable emulsification for native proteins of Indian variety was more improved at pH 7 and 8 with heating condition, but not at pH 6. Differences of ES for all MoLPC at pH 7 were shown to not be too significant but also lower than the other two pH. From pH 6 – 8, emulsifying activity index (EAI) for all MoLPC increased. IndianLPC-RT showed to have significant high values at all pH whereas ThaiLPC-RT was showed to have least EAI. ThaiLPC-heat showed no significant difference in EAI from IndianLPC-heat but gave lower EAI values than IndianLPC-heat. EAI of native proteins for ThaiLPC showed to be slightly lower than its heated counterpart (ThaiLPC-heat) but not significant at pH 6. Low emulsion activity shows stability of small oil droplet sizes which is considered good indicators of emulsion formation capacity (Famuwagun *et al*., 2020).

Good emulsifying properties can depend on degree of unfolding in protein, heating of proteins can enable the unfolding or opening of hydrophobic sites which increase emulsifying capacity of proteins (Wang & Kinsella, 1976). ThaiLPC in this case corresponds to this behaviour. IndianLPC on the other hand, mostly correspond oppositely which could suggest proteins in IndianLPC affected differently by heat either by slightly denaturing native proteins and affect its emulsifying property negatively. As prior mentioned in section 4.2.2 under surface hydrophobicity, around the range of pH 6 for IndianLPC-Heat, maximum solubility of proteins occurs, which can suggest a lower balance of surface hydrophobicity for that protein, hence an imbalance of hydrophilic-hydrophobic interactions which also contributes to the low emulsifying properties (Omana *et al*., 2010).

Furthermore, this correlates well with the study by Voutsinas *et al*. (1983) in which they discover, that the effect of heat on functional properties of proteins was not uniform. Effect of heat resulted in 4 different group of response from the various proteins they studied and two of the responses were similar to the responses on emulsifying properties in the current study. They showed β-lactoglobulin, pea, canola and casein to have adverse effects on their emulsifying properties, whereas ovalbumin and gelatin gave improved emulsifying properties upon heating.

The study by Voutsinas *et al*. (1983) suggested that emulsifying properties of proteins can be much more predicted by surface hydrophobicity rather than the solubility. This

supports the increased surface hydrophobicity for ThaiLPC-Heat corresponding to its improved emulsifying properties and the decreased surface hydrophobicity for IndianLPC-RT and its decreased emulsifying properties shown by Figure 4.1 and Table 4.4 respectively. Improvement of functional property is due to unfolding of molecule to expose hydrophobic amino acid groups, thus making protein more amphiphilic and capable to orient at an oil-water interface (Voutsinas *et al*., 1983).

Compared to other LPC studies, IndianLPC-RT seems to level with alfalfa proteins (EC of 242ml oil/ g of crude protein) studied by Lamsal *et al*. (2007) which was compared with egg white proteins (159 ml oil/ g of crude protein). Alkali extracted alfalfa proteins studied by Wang and Kinsella (1976) gave higher EC values of 384 ml oil/ g of protein. They stated that sources with high EC has the potential to be good emulsifying stabilizers in meat-like emulsions at around pH 6.0 (Wang & Kinsella, 1976).

On that note, this information was considered for the selection of one MoLPC to be used in the fortification of pancake. The pH of pancake batter was measured to be around the range of 6.7±0.2. The selection criteria required that MoLPC gives satisfactory levels of emulsifying properties but at the same time contains high nutritional value of crude protein content. ThaiLPC-heat was selected to fit these criteria best as even though it showed to have inferior emulsifying properties than IndianLPC, some of its values are not significantly different or are in proximity and has highest crude protein content.

Table 4. 4 Emulsifying properties of MoLPC

MoLPC	Emulsifying Capacity (g oil/ g of crude protein)			Emulsifying Stability (mins)			Emulsifying Activity (m^2 of oil interface/ g of crude protein)		
	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8
IndianLPC-RT	243.3±8.1^{a}	257.9±5.7^{a}	252.0±3.6^{a}	58±14.0^{b}	23±0.7^{bc}	119±17.0^{a}	82.1±3.3^{a}	96.7±1.0^{a}	108.9±1.9^{a}
IndianLPC-heat	222.6±0.6^{b}	233.61±4.6^{b}	225.8±3.1^{b}	16±1.0^{c}	27±3.0^{a}	208±72.0^{a}	63.2±1.6^{b}	65.0±1.9^{c}	64.9±0.2^{c}
ThaiLPC-RT	188.7±4.0^{c}	196.5±5.0^{d}	173.1±7.3^{c}	20±0.4^{c}	21±0.3^{c}	17±0.4^{b}	56.8±1.0^{c}	63.0±3.4^{c}	67.1±0.5^{c}
ThaiLPC-heat	211.6±3.7^{b}	213.6±1.3^{c}	214.3±12.6^{b}	86±4.0^{a}	26±1.0^{ab}	23±1.0^{b}	58.4±0.2^{bc}	79.7±1.0^{b}	82.2±2.4^{b}

Values presented as mean ± standard deviation (n=3)
Different letters down the column for each emulsifying property shows mean values that are significantly different at $p < 0.05$ using Tukey mean comparison.

4.4 Formulation and preparation of MoLPC pancake

4.4.1 Preliminary work on pancake formulation

As preliminary work on pancake formulation, MP20% replacement of pancake-mix (PWM) with MoLPC compared with MP15% replacement was first commenced in making of pancake batter. This determined a threshold for maximum replacement of PWM with MoLPC in which MP20% gave a viscous batter of 35.4N.s.m^{-2} (measured with R4 spindle at 5rpm) which was difficult and unfavorable to manage into induction cooker for cooking of pancake. Hence, three formulations for fortified moringa pancake was chosen (MP5%, MP10%, MP15%) in addition to the control (MP0%).

4.4.2 Determination of Pancake Batter Viscosity

Batter viscosity of pancakes was measured using a Fungilab Viscometer at room temperature. Results for the viscosity of batter are shown in Table 4.5

Batter viscosity of the pancakes shows significantly different values for all formulations. As the amount MoLPC fortification increases, so does the viscosity of the batter with MP15% giving significantly highest value of 9.7% in comparison to the control. This indicates the ability of proteins in MoLPC to absorb more water in the batter which increases the viscosity of the batter.

Table 4. 5 Viscosity pancake batter

	Pancake Formulation			
	MP0% (Control)	MP5%	MP10%	MP15%
Batter Viscosity (N.s.m^{-2})	1.4±0.1^{d}	3.3±0.4^{c}	8.2±0.6^{b}	9.7±0.4^{a}

Values presented as mean ± standard deviation (n=3)
Spindle R4 used at 20rpm
Different letters across the row shows mean values that are significantly different at $p < 0.05$ using Tukey mean comparison

4.5 Determination of Pancake Properties

4.5.1 Chemical Properties

Determination of proximate compositions for pancake

Proximate composition of fortified pancakes with MoLPC (ThaiLPC-heat) is shown in Table 4.6 with the following analysis done using AOAC (1995) methods.

Table 4.6 showed that crude protein content of the pancakes positively correlates with increasing amount of MoLPC replacing the PWM as compared to the control (13.1%). MP15% showed its protein content (18.7%) to be significantly higher and different from the control. MP5% and MP10% showed their crude protein content (14.1% and 14.6% respectively) to be higher than the control but not significantly different from the control.

Other proximate composition such as crude fat for the pancakes showed a lower value from the control at MP5% (12.7%) and higher values at MP10% (14.6%) and MP15% (16.6%) in which the differences are not significant at $p<0.05$.

Crude fiber of pancakes showed a similar increasing trend as the crude fat with the addition of MoLPC. Significant difference of the values from control was showed at 15% replacement of pancake-mix with MoLPC. The increasing of fiber content in food is important as it helps in regulating traffic of food through intestinal tract and the lowering of high cholesterol in the blood (Kolawole *et al*., 2013).

Ash contents for all pancakes showed no significant difference from the control but showed MP5% and MP10% to contain slightly highest values of ash contents.

Carbohydrates on the other hand was shown to decrease with each MoLPC fortification and MP15% was seen to have the significantly lowest value compared to the Control pancake. The decrease of carbohydrates is seen to be largely influenced by the replacement of PWM with the MoLPC. This is because PWM is the main contributor for most of the carbohydrates present in the pancakes.

There are not many fortification studies using leaf protein concentrate from moringa leaf but a study in the fortification of white and brown bread with moringa leaf powder by Govender and Siwela (2020) showed the increasing trend of crude protein content

in bread as moringa leaf powder is used to replace the flour ingredient. Compared to the current study, their control (no moringa leaf powder) contained a crude protein content of 13.68% (d.b). At 5% replacement of flour with moringa leaf powder, the increase of protein content was about 2% whereas in the current study, the 5% replacement of pancake-mix with MoLPC gave about 7.63% increase of protein content. This is calculated approximation of the potential of enhanced nutritional level of protein content in food by using MoLPC compared to the moringa leaf powder. Similar trends of increased crude protein content, increased crude fiber and decreased carbohydrates were seen in other fortification studies with the increased incorporation of moringa leaf powder in cake (Kolawole *et al*., 2013) and chocolate (Atef & Aziz, 2014) and with decreased fat content.

Each pancake cooked from the formulation above was approximated to be 40g in weight. This means that for 1 pancake, at MP0%, MP5%, MP10%, MP15%, it contains crude protein content of about 5.24g, 5.64g, 5.84g and 7.48g respectively. So for an adult with an average weight of 50kg (average protein requirement per kg/day = 0.83g protein/kg/day (Food & Agriculture Organization, 2007), at least five MP15% (200g batter) can suffice their daily protein requirement whereas with MP0%, at least seven pancakes (280g batter) can satisfy their daily protein requirement. For children at the age group of 3yrs with an average weight of 14.6kg (average protein requirement per kg/day = 0.90g protein/kg/day (Food & Agriculture Organization, 2007) would require a daily intake of 13.1g protein which seems to be satisfied by at least two MP5% pancakes (~80g batter). 100g batter of MP15% can suffice the daily protein requirement for children age 3-6 yrs (0.87-0.90g protein/kg/day (Food & Agriculture Organization, 2007).

Table 4. 6 Proximate composition of pancake at dry basis

Proximate Composition	Pancake Formulation			
	Control (MP0%)	A (MP5%)	B (MP10%)	C (MP15%)
Crude Protein (%)	13.1±0.1[b]	14.1±0.3[b]	14.6±1.2[b]	18.7±1.2[a]
Crude Fat (%)	13.2±0.2[a]	12.7±0.9[a]	14.6±1.7[a]	16.6±1.0[a]
Crude Fiber (%)	7.5±0.3[b]	8.4±0.0[b]	9.9±0.9[ab]	12.6±1.3[a]
Ash (%)	3.5±0.0[a]	3.7±0.2[a]	3.7±0.0[a]	3.5±0.1[a]
Carbohydrates (%)	62.7±2.7[a]	61.1±1.0[a]	57.6±0.4[ab]	48.1±3.8[b]

Values presented as mean ± standard deviation (n=2)
*Estimated by difference using replicated values at wet basis (100 – Moisture + Protein + Ash + Crude fiber + crude fat) and then converted to dry basis.
Different letters across rows shows mean values are that significantly different at $p < 0.05$ using Tukey mean comparison.

4.5.2 Physical properties

Color Analysis and Bulk density of pancake

Results for physical properties of pancake regarding color analysis of its' inner texture, viscosity of batter and bulk density of cooked pancake is displayed in Table 4.7. Figure 4.3 displays a visual of the fortified pancakes compared to the control. Color in food is an important attribute that might influence the acceptance of a product. Color analysis of the pancakes showed attributes of lightness (L*) to be significantly different for all pancakes. Lightness of pancake in decreasing order was highest at MP0% and lowest for MP15%. Values indicating the closeness of pancake to red color is shown by (a*) and the hue angle of the pancake. Red intensity of foritified pancakes starts increasing with addition of MoLPC as the hue angle migrates from yellow hue (81.2^0) at MP5% to red hue (67.3^0) for MP15%. The value (b*) in the pancakes showed more yellowness in the pancakes where MP0% shows high yellowness intensity and decreased as additions of MoLPC were done. In terms of chroma, the intensity of the hue as MoLPC was added, significantly decreases.

Decreasing lightness of pancake with addition of MoLPC is due to the dark intense color that MoLPC powder possesses. Possible complexation of proteins with phenolic compounds in MoLPC when being extracted under alkaline conditions can result in the development of its dark green colors (Rawdkuen, 2020). In addition, the occurrence of Maillard reaction during cooking of pancakes as amino acids in proteins can form

complexes with simple sugars available giving a distinct brown color which could contribute to the decreasing lightness of pancakes as the addition of MoLPC increases.

Bulk density of the pancakes indicates the increase in mass given the amount of volume it is retaining. Table 4.7 showed a slight increase as the pancakes are fortified with increasing amount of MoLPC. The significantly different value of bulk density from the Control is achieved with MP10% (0.5g/cm^3). The increase of bulk density corresponds well to the texture of the pancake in terms of hardness by indicating a tightly packed network of proteins interacting with other components in the pancake batter. The physical analysis of the pancake sample shows the slight effects of protein from MoLPC on the texture and physical attributes of the pancake samples.

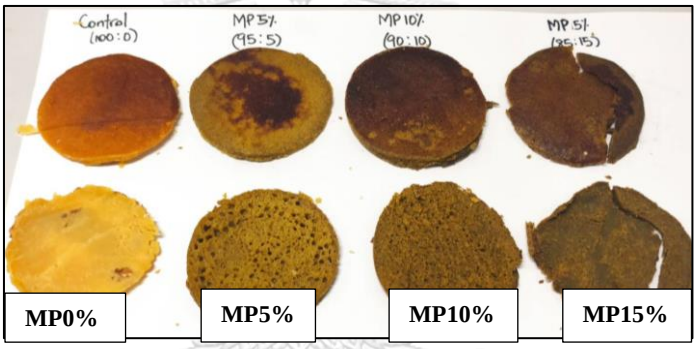

Figure 4. 3 Color of fortified pancakes and control

Table 4. 7 Physical properties of pancake

Physical Properties	Pancake Formulation			
	MP0% (Control)	MP5%	MP10%	MP15%
Color Analysis				
L*	67.4±0.2^a	50.0±09^b	41.1±1.4^c	35.6±0.5^d
a*	3.5±0.2^a	2.4±0.3^b	2.9±0.0^{ab}	3.2±0.0^{ab}
b*	28.7±0.4^a	16.1±3.1^b	9.5±0.0^{bc}	7.8±0.2^c
Hue (^{0})	82.9±0.4^a	81.2±0.9^a	72.9±0.1^b	67.3±1.0^c
Chroma	28.9±0.5^a	16.3±2.0^b	9.9±0.0^b	8.4±0.2^b
Bulk Density (g/cm^3)	0.4±0.0^b	0.5±0.1^{ab}	0.5±0.1^a	0.6±0.02^a

Values presented as mean ± standard deviation (n=3)
Different letters across the row shows mean values that are significantly different at $p < 0.05$ using Tukey mean comparison.

Determination of Texture Profile Analysis for pancake

Texture profile analysis of pancake attributes relating to hardness, springiness and cohesiveness shown in Table 4.8 together with other physical properties.

Hardness of the pancake was displayed as the peak-force necessary to attain a 50% deformation of the pancake's texture (g-force/mm). Table 4.8 showed increasing values of hardness in the pancakes with the increased replacement of PWM with MoLPC. MP15% is shown by Table 4.8 to be significantly higher in hardness value compared to control. This indicates the increased firmness of the pancake texture with the increased MoLPC. The increase in hardness can be explained with the increase of protein content and a decrease of water absorption by the gluten from PWM due to replacement of PWM. With the increased protein, a network can be established with the polysaccharides available to replace the decrease of gluten (Sun *et al*., 2019). It could also be attributed to the increase in fiber content as mentioned by Govender and Siwela (2020) in the increased hardness for bread. Springiness of the pancake indicates the elasticity of the pancake as it tends to recover from the first and second compression test. Table 4.8 showed that no significant difference among the springiness for all pancake and it remains unchanged from the Control up to MP15%. Cohesiveness of the pancake indicates internal resistance of the pancake structure after first and second compression. Table 4.8 showed that the Control maintains higher cohesiveness (0.8) of the pancake structure than the moringa fortified pancake. At MP5%, there is an abrupt lowering of the pancake cohesiveness which then builds up with increased MoLPC into the pancake. This build-up of cohesiveness with the increased MoLPC can be attributed to the increase of protein in the pancake structure interacting and forming a network with other compounds in the pancake such as available starch, fiber and carbohydrates in absence of water after cooking. This network relates the firmness of the pancake structure and having that internal resistance to the compression test. The abrupt change of cohesiveness from the Control to MP5% can be explained with the Control having a stable structure due to the full availability of gluten network in the pancake. With the replacement of 5% PWM with MoLPC in the MP5% pancake may have caused unstable protein network which was not strong enough to have an internal resistance against the compression. But with the increased addition of MoLPC, there is also an increase

towards a stable protein network hence increasing the internal resistance against the compression test. On the other hand, Table 4.8 shows that the difference amongst the values of cohesiveness is not significant.

Table 4. 8 Texture Profile Analysis of fortified pancake

Texture Analysis	Pancake Formula			
	MP0% (Control)	**MP5%**	**MP10%**	**MP15%**
Hardness (g)	6489.5±160.6^{b}	8034.6±1550.9^{b}	8457.9±1712.9^{b}	11095.5±961.4^{a}
Springiness	1.0±0.1^{a}	1.0±0.1^{a}	1.1±0.1^{a}	1.0±0.04^{a}
Cohesiveness	0.8±0.0^{a}	0.4±0.3^{a}	0.5±0.2^{a}	0.7±0.01^{a}

Values presented as mean ± standard deviation (n=6)
Different letters across the row shows mean values that are significantly different at $p < 0.05$ using Tukey mean comparison.

Chapter V

5.1 Summary and Conclusion

This study considered *Moringa oleifera* leaf to have a sufficient amount of crude protein. When extracted as a leaf protein concentrate, the crude protein was enhanced to a greater value of content for all 4 MoLPC used in this study. This makes it easier for its utilization in food ingredients as food supplements that could improve nutritional value. The current study found that variety of *Moringa oleifera* may have a significant influence on the differences of MoLPC yield of extraction and the crude protein content. Heat on the other hand, showed to increase crude protein for both MoLPC variety, however, the significant increase was only seen for IndianLPC.

Protein solubility of all MoLPC showed minimum solubility at pH 3.5 suggesting this as pH for MoLPC proteins to achieve a net overall charge of zero. Maximum solubility for MoLPC occurred at pH 6 and above. The addition of mild heating in the extraction of proteins showed greater influence on the functional properties of MoLPC by having inter-related effects on their solubility, surface hydrophobicity and emulsifying properties. Heated MoLPC showed to decrease solubility of both MoLPC varieties. However, its effects on emulsifying properties of MoLPC was not uniform as it showed to improve the emulsifying properties of ThaiLPC but decrease emulsifying properties for IndianLPC. This was related to the disappearance of highly soluble native proteins of ThaiLPC at pH 7 and the new maximum solubility of IndianLPC at pH 6 when heated. The ability of ThaiLPC-heat to give improved emulsifying properties at pH 6-8 suggests its potential as a good emulsifying stabilizer for meat products or other food at this range.

The fortification of ThaiLPC-Heat in pancake gave an enhanced nutritional value in the crude protein and crude fiber of the pancake samples with MP15% (crude protein showing a significant difference from the unfortified pancake. The slight increase of crude fat from MP10% and MP15% showed no significant difference from the control. Physical attributes of the fortified pancakes showed significant increase of batter viscosity from the control at the replacement of at least 5% pancake mix with MoLPC.

Similarly, the color of the cooked pancakes showed to decrease in lightness (L*) and yellow intensity (b*) than the control pancake but tends towards red color intensity (a*) with increasing replacement. Bulk density of the cooked pancakes showed slight significant increase at MP10% from the control. In terms of texture profile analysis, the pancakes showed that springiness and cohesiveness of pancakes are maintained even with addition of MoLPC, however, the hardness of the pancake textures is significantly influenced with every replacement of pancake-mix with MoLPC. When adjusted and compared with protein requirement recommended by FAO/WHO, the nutritional content of protein in MoLPC fortified pancakes can sufficiently satisfy adults, pregnant mothers. The information gained from this study can be further used for more exploration and further improving the implementation of moringa leaves high alternative protein sources in food

5.2 Recommendations for future work

This study serves as a foundation in studying the potential of leaf protein concentrate from edible leaves such as *Moringa oleifera* to be utilized for enhanced nutritional value and be implemented in developments of plant-based food applications. It is recommended that:

(1) Other potential functional properties such as water holding and viscosity of MoLPC should be further studied to increase the range of functional potential given by MoLPC in food.

(2), further in-depth studies of the quality of proteins that exist in MoLPC such as available amino acids, digestibility scores and types of protein should be further researched to determine further concrete evidence of its value.

(3) Lastly, more research is encouraged in food fortification studies by using MoLPC in other different food system such as milk-based beverages to provide a variety of information on implementation of MoLPC in food.

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Appendix A
Chemical Analysis Method

1. Protein Yield

$$\%Protein\ Yield = \frac{Crude\ protein\ of\ MoLPC(g)\ per\ 1g\ leaf\ powder}{Crude\ protein\ per\ 1g\ leaf\ powder} \times 100$$

2. Proximate Composition

2.1 Moisture Content by Oven drying method

- Dried in 105℃ for 5hrs or until constant weight.
- Calculated by:

$$\%Moisture = \frac{w2 - w3}{w2 - w1} \times 100$$

Where w1 = initial weight of empty aluminum pan,

w2= weight of aluminum pan + sample before drying,

w3= final weight of aluminum pan + sample after drying.

2.2 Protein Content by Kjeldahl Method

- Digestion with concentrated sulfuric acid for 1hr
- Distillate with 35% NaOH into 4% boric acid
- Titrate with 0.1M of hydrochloric acid
- Calculate by:

$$\%Nitrogen = \frac{0.14 \times v}{w1} \times 100$$

$$\%Protein = \%Nitrogen \times F$$

Where w1 = weight of sample (g),

v = volume of 0.1M HCl used in titration,

F = conversion factor of 6.25

2.3 Crude Fat content by Soxtec method

- Reflux condensation with petroleum ether
- Extraction with rotary evaporator at 60℃
- Dry round bottom flask in oven at 105℃ for 5hrs or until constant weight
- Calculate by:

$$Crude\ Fat\% = \frac{W2 - W1}{W3} \times 100$$

Where w1 = weight of empty flask (g), w2 = weight of flask + fat (g), weight of food taken (g)

2.4 Crude fiber

- Boiled in 1.25% in sulfuric acid solution
- Boiled in 1.25% sodium hydroxide
- Washings with distill water
- Dried in oven at 105℃ for 3hrs or until constant weight
- Burnt in muffle furnace at 550℃ for 2hrs
- Calculate by:

$$Crude\ Fiber\% = \frac{W1 - W2}{Ws} \times 100$$

Where Ws= weight of sample before boilings,
W1 = weight of crucible with fiber,
W2= weight of crucible with ash

2.5 Ash

- Combustion of sample in muffle furnace at 550℃ for 4hrs or until white grey ash is achieved
- Calculate by:

$$Ash\% = \frac{W3 - W1}{W2 - W1} \times 100$$

Where W1 = weight of empty crucible,
W2= weight of crucible + sample before ashing,

W3 = weight of crucible + ash

2.6 Carbohydrates

- Calculated by difference:

$$Carbohydrates\% = 100 - (Moisture\% + Protein\% + Ash\% + Crude\ Fiber + Crude\ Fat)$$

2.7 Conversion from Wet basis to Dry basis

- Proximate values obtained as wet basis were converted to dry basis based upon the following formula:

$$Dry\ basis\% = Wet\ basis\% \times \frac{100}{100 - Moisture\%}$$

Where Wet basis% = proximate value obtained in wet basis

3. Lowry Assay

- **Solution A, 100ml**

  0.5 g $CuSI_4$. 5 H_2O

  1g $Na_3C_6H_5O$. $2H_2O$

  Add distilled water to 100ml

- **Solution B, 1L**

  20g Na_2CO_3

  4g NaOH

  Add distilled water to 1L

- **Solution C, 51ml**

  1ml Solution A

  50ml Solution B

- **Solution D, 20ml**

  10ml Folin-Ciocalteu phenol reagent

  10ml Distilled water

VITA

NAME	Annelise Ida Halafihi
DATE OF BIRTH	11 March 1994
PLACE OF BIRTH	Copenhagen, Denmark
INSTITUTIONS ATTENDED	Bachelor of Science (Chemistry), University of Auckland, New Zealand Pursuing a M.Sc (Food Science & Technology), Department of Food Technology, Faculty of Science, Chulalongkorn University
HOME ADDRESS	Rangnam Apartment, 521/3-4, Soi Sriayuthaya 2-4, Sriayuthaya Rd, Thanon Prayatai, Rachatvee, Bangkok, 10400.
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