Melatonin Content in Food Materials and Its Application to Prepare High Melatonin Pasteurized Milk

Jintana Sangsopha

A Thesis Submitted in Partial Fulfillment of Requirements for
degree of Doctor of Philosophy in Food Technology (International Program)
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The examining committee has unanimously approved this Thesis, submitted by Miss Jintana Sangsopha , as a partial fulfillment of the requirements for the Doctor of Philosophy Food Technology (International Program) at Mahasarakham University

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TITLE Melatonin Content in Food Materials and Its Application to Prepare High Melatonin Pasteurized Milk

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ABSTRACT

Milk is a functional food that contains many nutrients and bioactive compounds essential to human health. Consumer demand for milk products has recently increased resulting in high competition in types and pricing. Production of functional milk adds value and increases competitive marketing. Products containing high antioxidants improve relaxation and sleep quality but have not been extensively produced. Functional milk high in melatonin has recently attracted interest. Natural milk is low in melatonin and tryptophan compared to edible plants. Mulberry leaf tea and edible grains were analyzed for melatonin and tryptophan as a precursor to produce a pasteurized milk product with high melatonin content and antioxidant activity. Edible grains studied included five rice, three corn, four legumes, and two oilseed cultivars. Highest melatonin concentrations were observed in white sesame, sunflower seed and soybean (75.24, 67.45 and 56.49 ng/g dry weight (dw), respectively). Highest free tryptophan was detected in soybean, red bean and mung bean (2.62, 1.53 and 0.85 µg/g dw, respectively). Total phenolic content was highest in sunflower and white sesame seeds (30.86 and 21.42 mg GAE/g dw). Antioxidant activity evaluated using DPPH, FRAP and ABTS assays showed sunflower seed extract as strongest followed by white sesame. Results indicated that white sesame, sunflower, and soybean were good sources of melatonin, free tryptophan and antioxidants. Soybean was selected and incorporated into pasteurized milk as it contained high concentrations of melatonin, free tryptophan and antioxidants. Soybean was also easy to incorporate in milk at a reasonable price. Mulberry leaves were dried using solar energy, a hot air oven, and freeze drying to compare melatonin content. Results indicated no significant difference in melatonin content between leaves dried by solar energy and the freeze drying technique.

To develop pasteurized milk high in melatonin content, nine treatments were developed by mixing raw milk with mulberry leaf tea and soymilk powder. Results revealed that milk quality, milk composition, bioactive components, and antioxidant activities significantly increased ($p<0.05$). Highest parameter values were observed in pasteurized milk added with 6% mulberry leaf tea, 6% soymilk powder, and 85.80% milk. For the shelf life study, melatonin, free tryptophan, and total phenolic content were not significantly different and slightly reduced during storage. Sensory

evaluation of pasteurized milk comprising 4% mulberry leaf tea, 4% soymilk powder, and 89.80% milk yielded the highest overall liking score of 7 by consumer testing.

To obtain a higher quality pasteurized milk product in terms of milk standard, mulberry leaf tea, soymilk powder, and raw milk were optimized using mixture design. A total of eleven treatment results revealed that the optimum quality of pasteurized milk contained 3.90% soymilk powder, 4.50% mulberry leaf tea and 89.40% raw milk.

A clinical study of pasteurized milk in healthy volunteers was conducted. Urinary 6-sulfatoxymelatonin (aMT6-s) was determined as an indicator of melatonin levels in the human body after milk consumption. Urinary antioxidant was performed using ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC). Results indicated that after consumption of high melatonin pasteurized milk, including pasteurized milk mulberry leaf tea (PMM) and high melatonin pasteurized milk mulberry leaf tea mixed with soybean powder (PMMS), urinary aMT6-s and urinary antioxidant of FRAP and TEAC increased compared with the baseline.

Overall results indicated that both mulberry leaves and soybean are good sources of nutrients, bioactive compounds, and antioxidants. Supplementation of both ingredients in milk improved melatonin, free tryptophan, phenolic content and antioxidant activity. Therefore, milk supplemented with mulberry leaf tea and soymilk powder is beneficial as a health-promoting functional food. This product showed potential for use in assisting relaxation and sleep quality.

Keyword : Melatonin, Free tryptophan, Antioxidant, Response surface methodology, Pasteurized milk, Urinary 6-sulfatoxymelatonin (aMT6-s)

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CHAPTER 1
INTRODUCTION

1.1 Background

Functional food consumption has potential for rapid expansion with increasing marketing opportunities. A survey by Euromonitor International reported growth of 6.5% in 2011 for global sales value of health beneficial products, with predicted annual growth of 7.2% by 2017 to over 1 trillion US dollars (Hudson, 2012). In Thailand, growth of the functional food market share was predicted at 6.0% from 2015 to 2017, with the highest as the health food sector growth at 60% followed by natural products with 30%, specific groups at 8%, and others at 2% (Kasikorn Research Center, 2015).

Milk is a functional food containing many different nutrients and bioactive compounds that are essential for human health (Alyaqoubi et al., 2014). Global milk production showed an increase of 1.24% to 499.81 million tons (MTs) in 2016 from 493.69 MTs in 2015. In Thailand, milk production increased by 2.50% to 1.11 MTs in 2016 compared with 1.08 MTs in 2015 (Office of Agricultural Economics, 2017). This increase reflected consumption demand and resulted in higher competition regarding the types and pricing of milk products. Global milk consumption demand increased by 1.19% to 182.29 MTs in 2016 compared with 180.14 MTs in 2015. Milk consumption in Thailand rose by 3.03% to 1.08 MTs in 2016 compared with 1.05 MTs in 2015. (Office of Agricultural Economics, 2017). With this growing trend of milk market value, Thailand has potential in the beverage industry, with the capability of competing on the global stage. Thus, the country must remain competitive through continuous innovation and production of new functional products that can respond to consumer demand for healthier food options. Numerous milk products have been developed with high functional properties, such as low fat and low sugar that are also high in bioactive compounds (Corbo et al., 2014). One way to add value and boost competitive milk pricing and marketing involves producing functional milk. To add value in milk, we need to develop a new product with health benefits to improve sleep disorder that has high bioactive compound content and antioxidant activity. However, such a product has

still not been extensively produced and studied. Therefore, the properties of functional milk containing melatonin and high antioxidant were investigated.

Melatonin (*N*-acetyl-5-methoxytryptamine), an important bioactive hormone is present in raw milk in low concentrations at 4.0-56.4 pg/mL (Milagres et al, 2014; Setyaningsih et al., 2015; Valtonen et al., 2005). Daily 0.5 L milk consumption (melatonin 5-20 ng) did not increase melatonin concentration in the blood but offered benefits by increasing daytime activity of some elderly subjects and might also improve sleep quality (Valtonen et al. 2005). Low melatonin concentration may not be sufficient to promote relaxation and sleep quality, requiring improvement or supplementation from other sources. Melatonin has been detected in various edible plants with especially high concentrations in herbs (Chen et al., 2003) and edible grains (Manchester et al., 2000). In Thailand, melatonin has been reported in tropical fruits (Johns et al., 2013) and mulberry leaves (Pothinuch & Tongchitpakdee, 2011). However, numerous food plants in Thailand have not been studied for melatonin and its precursor, the amino acid tryptophan.

This study collaborated with a milk factory to produce a new pasteurized milk flavor with high nutrition and bioactive compounds. Pasteurized milk with high melatonin content remains understudied. Our objectives were to determine melatonin and free tryptophan contents in common edible grains and mulberry leaves sourced in Thailand. A suitable rich source of melatonin and free tryptophan was prepared and applied to improve the health-promoting properties of pasteurized milk with high melatonin content in a clinical study.

1.2 Objectives of the research

1.2.1 To determine melatonin and free tryptophan content in edible grains, mulberry leaf tea and raw milk.

1.2.2 To develop pasteurized milk with high melatonin content.

1.2.3 To evaluate melatonin concentration in urine following clinical study methodology.

1.3 Expected outcomes

1.3.1 Quantify contents of melatonin and free tryptophan in the raw materials.

1.3.2 Develop pasteurized milk with high melatonin content and investigate milk quality and composition, bioactive content, antioxidant activity, consumer acceptability and shelf life stability.

1.3.3 Undertake a clinical study of high melatonin pasteurized milk.

1.4 Research hypotheses

1.4.1 Different raw materials have diverse levels of melatonin and free tryptophan contents.

1.4.2 Different melatonin contents of pasteurized milk affect quality and chemical compositions, bioactive compounds, antioxidant activity, and consumer acceptability.

1.4.3 High melatonin pasteurized milk consumption affects the melatonin levels of individuals.

1.5 Scope of the research

Objective 1: To determine melatonin and free tryptophan content in edible grains, mulberry leaf tea and raw milk.

1.5.1 Raw materials

Sixteen raw materials were purchased from local markets and supermarkets in Maha Sarakham Province, Thailand. Materials listed below were harvested in 2015-2016, except for dried mulberry leaves and cow's milk.

1.5.1.1 Rice grains:

1) Khao dok mali 105 (*Oryza sativa* L.)
2) Red dok mali (*Oryza sativa* L.)
3) Riceberry (*Oryza sativa* L.)
4) Black glutinous (*Oryza sativa* L. var. glutinosa)
5) Glutinous RD6 (*Oryza sativa* L. var. glutinosa)

1.5.1.2 Corn grains:

1) Sweet corn (*Zea mays saccharata*)
2) Waxy corn (*Zea mays ceratina*)
3) Waxy berry corn (*Zea mays* L.)

1.5.1.3 Legumes:

1) Soybean [*Glycine max* (L.) Merrill]

2) Peanut (*Arachis hypogaea* L.)

3) Mung bean [*Vigna radiata* (L.) Wilczek]

4) Red bean (*Phaseolus vulgaris* L.)

1.5.1.4 Oilseeds:

1) Sesame (*Sesamum indicum* L.)

2) Sunflower (*Helianthus annuus* L.)

1.5.1.5 Mulberry leaves (*Morus alba*)

1.5.1.6 Raw milk

1.5.2 Sample preparation

1.5.2.1 Good quality raw milk was collected from milk storage tanks of Kokko Milk Factory, Maha Sarakham Province after raw milk delivery between 07.30 and 09.00 a.m., supplied by local dairy farms in the Maha Sarakham area, and stored in clean packages at 4±2°C until required for analysis.

1.5.2.2 Dried grain samples were ground with grinding halted every 30 s to avoid excessively heating the samples. Ground samples were sieved through mesh no. 20.

1.5.2.3 Mulberry leaf tea was produced using three different drying methods. Mulberry leaves were cleaned, chopped (1.5 x 1.5 cm^2) and roasted at 50±2°C for 30 min. Rolled leaves were dried using a hot air oven, freeze drying and solar drying.

The three drying methods were compared to determine the most suitable the yielded high melatonin and free tryptophan content in mulberry leaf tea.

The method to produce high melatonin and free tryptophan content must consider process economics that could be applied on a large scale in milk factories. All ground samples were kept in zip-lock plastic bags to restrict exposure to light and air, and stored at -20°C prior to extraction.

1.5.3 Sample analysis

Melatonin, free tryptophan, total phenolic content and antioxidant activity were determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric-reducing antioxidant power assay (FRAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

Objective 2: To develop pasteurized milk with high melatonin content.

1.5.4 Pasteurized milk development

Pasteurized milk with high melatonin content was generated by adding different quantities of ingredients included raw milk, mulberry leaf tea and selected grain with remaining ingredients as sugar and food grade color. The mixtures were pasteurized and the quality, chemical composition, bioactive contents, antioxidant activity, sensory and shelf life of pasteurized milk samples were assessed.

1.5.5 The most acceptable pasteurized milk by sensory evaluation was formulated using mixture design. The optimized condition was obtained by testing the response functions as quality, composition, bioactive contents, antioxidant activity and sensory evaluation by considering model significant differences ($p < 0.05$), lack-of-fit ($p > 0.05$) and $R^2 > 0.8$ for all response functions.

Objective 3: To evaluate melatonin concentration in urine following clinical study methodology.

1.5.6 The accepted pasteurized milk isolated as per 1.5.4 was tested using clinical study methods. Healthy participants consumed food for their evening meal (baseline). Urine samples were collected overnight with washout period for one week during consumption. At the next visit, after food consumption, participants consumed pasteurized milk samples and then followed the same procedure of urine collection.

1.5.7 Urinary 6-sulfatoxymelatonin (aMT6-s) and urinary antioxidant capacity were determined after healthy volunteers consumed pasteurized milk samples.

1.6 Definitions

1.6.1 A functional beverage is a drinking product that is non-alcoholic and includes in its formulation ingredients such as herbs, vitamins, minerals, amino acids or additional raw fruit or vegetables (Corbo et al., 2014).

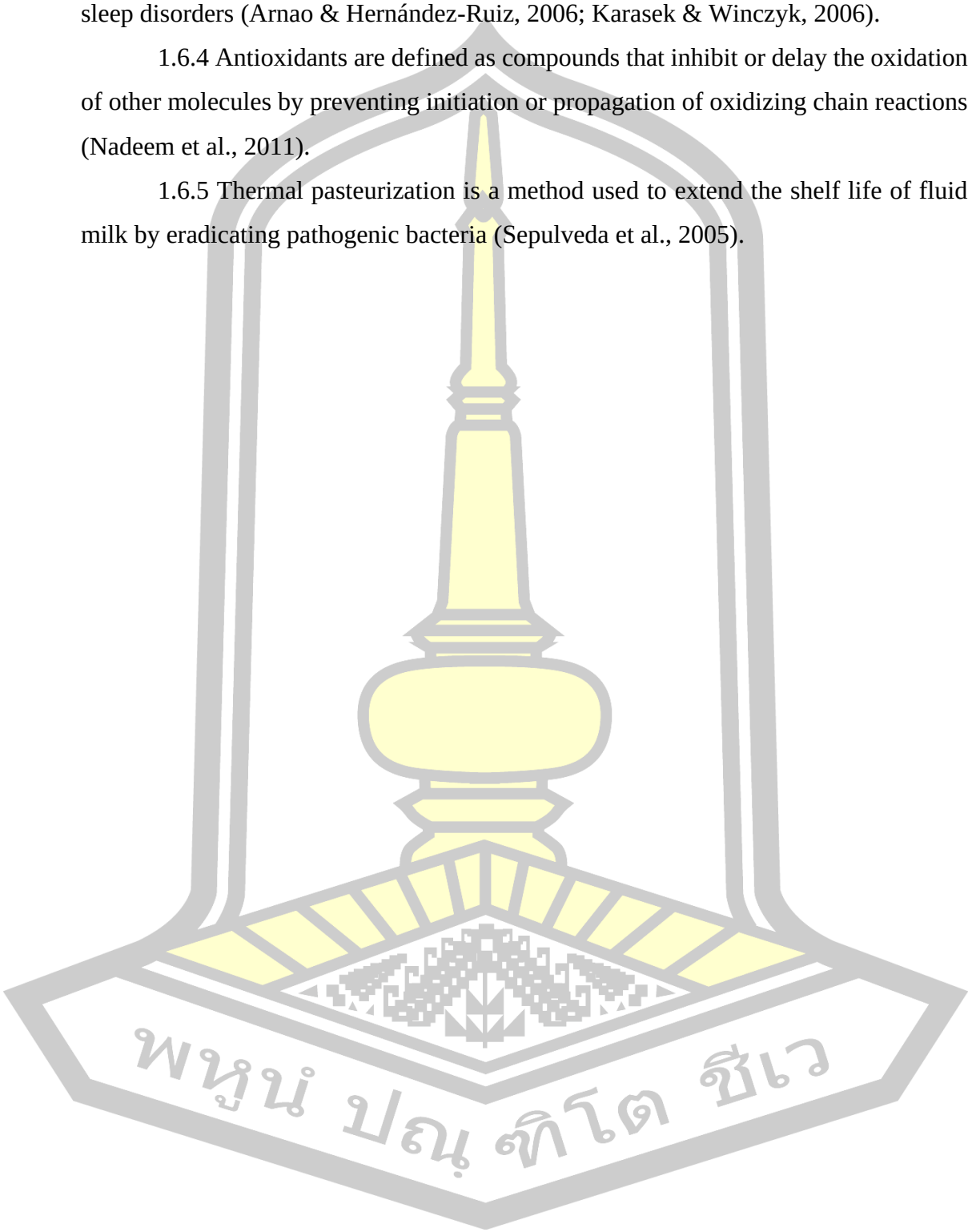
1.6.2 A functional food is a food given an additional function (often one related to health-promotion or disease prevention) by adding new ingredients or more of existing ingredients (Corbo et al., 2014).

1.6.3 Melatonin (*N*-acetyl-5-methoxytryptamine) is an indoleamine synthesized from the essential amino acid tryptophan which metabolizes via serotonin.

Melatonin secretion is related to the characteristics of circadian rhythms, jet lag and sleep disorders (Arnao & Hernández-Ruiz, 2006; Karasek & Winczyk, 2006).

1.6.4 Antioxidants are defined as compounds that inhibit or delay the oxidation of other molecules by preventing initiation or propagation of oxidizing chain reactions (Nadeem et al., 2011).

1.6.5 Thermal pasteurization is a method used to extend the shelf life of fluid milk by eradicating pathogenic bacteria (Sepulveda et al., 2005).



CHAPTER 2
LITERATURE REVIEW

2.1 Functional Food

The global health and wellness market grew continuously from 2007 to 2017 with a sales increase of 6.5% in 2011. The biggest sales increase was in fortified/functional natural health products at 7%. This growth was fuelled by the development of new markets, with China and Brazil contributing US$15 billion in 2011. Global health and wellness sales growth continued at 7.2% up to 2017 with a total value of US$1 trillion (Figure 1).

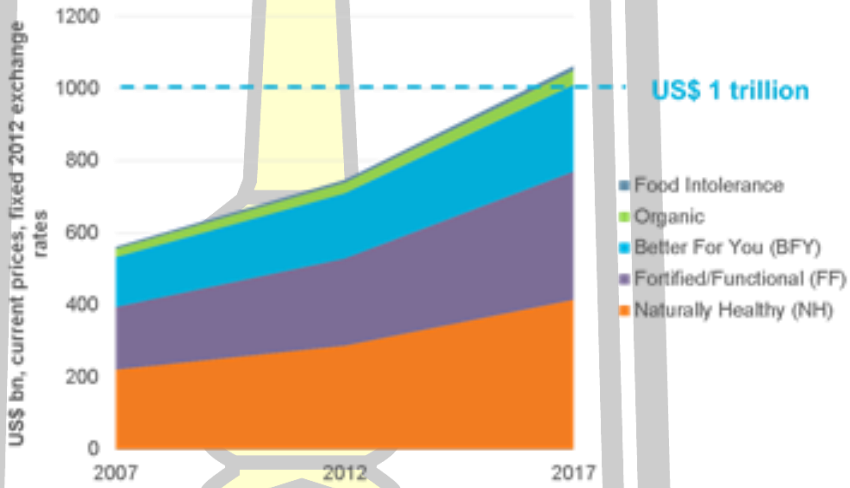


Figure 1 Euromonitor International; Global Health and Wellness 2007-2017

Source: Hudson (2012)

A survey by Euromonitor International found that the market value of food and drink products showed highest growth in China, Brazil and the United States, respectively. Thailand was ranked 20th (Figure 2) (Hudson, 2012).

With the growing trend of global health food markets, Thailand has potential for the development of health food products. Health food market growth in Thailand was 6.0% in 2017 and valued at 161 billion baht. The health food market comprises functional food at 60%, followed by natural products at 30% and health foods at 8%.

In 2013, the Thai beverage market was valued at 4.3 to 4.4 billion baht with a slight increase compared to 2012. The market value of functional beverage in 2013 was 5.0-5.3 million baht with average annual expansion of 13-15% (Kasikorn Research Center, 2015).

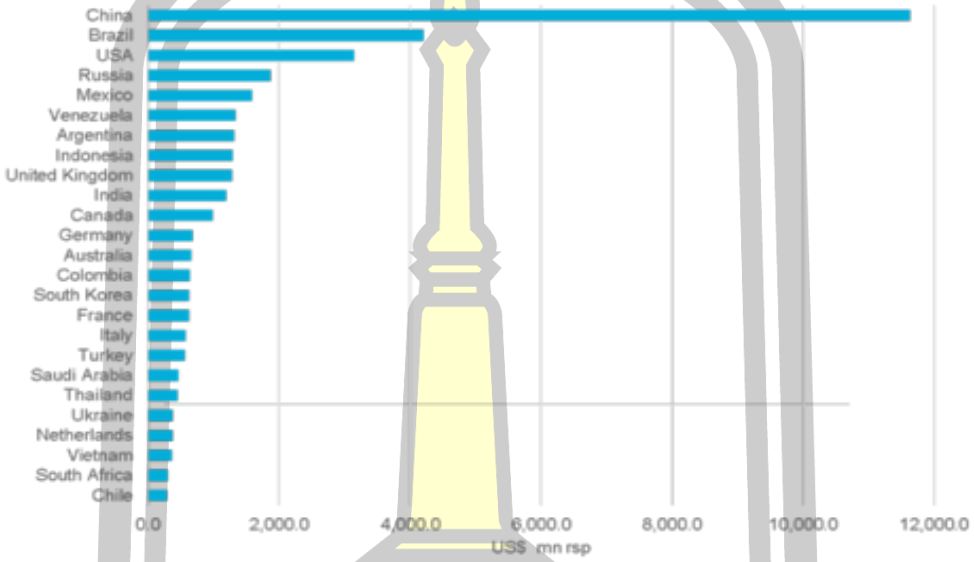


Figure 2 Euromonitor International; Top 30 Health and Wellness Growth Markets 2011-2012

Source: Hudson (2012)

2.2 Milk

Milk is a functional drink that is widely consumed around the world. Milk is produced from mammals such as cows, sheep, camels, or goats (Alyaqoubi et al., 2014; Macpherson & Smyth, 1985; Zulueta et al., 2009). Nutritional value of milk is high due to the well-balanced nutrients. Milk composition varies among animal species and breeds within the same species, and also from one dairy to another depending on the period of lactation and diet (Table 1).

Table 1 Composition of milk from different mammals in g/100 g

Species	Water	Protein	Fat	Lactose	Ash
Cow	87.2	3.5	3.7	4.9	0.72
Sheep	82.7	5.5	6.4	4.7	0.92
Goat	86.5	3.6	4.0	5.1	0.82
Camel	87.7	3.5	3.4	4.7	0.71

Source: Guetouache et al. (2014)

Milk provides essential nutrients and is an important source of dietary energy, high-quality proteins and fats. Milk intake can make a significant contribution to the required nutrient intake of minerals and vitamins such as calcium, phosphorus, riboflavin (vitamin B12), vitamin B12 (cobalamin) and pantothenic acid (vitamin B5) (Bath & Bath, 2011; Fredeen, 1996). In general, cow's milk is less rich in lactose, fat and proteins compared to goat's milk but has similar mineral content, while both are rich sources of phenolic compounds and demonstrate antioxidant properties (Table 2).

Table 2 Composition of milk and goat milk per 100 g of milk

Components/Species	Cow	Goat	References
Proteins	3.5	5.5	(Guetouache et al., 2014)
Casein	2.8	2.3	
Fat	3.9	3.38	
Lactose	0.9-4.9	4.4-4.7	
Ash	0.90	0.5-0.8	
TPC (mg/100 g fw)	477.6	460.0-544.8	
FRAP (mg/100 g fw)	398.8	386.0-481.8	(Alyaqoubi et al., 2015)
DPPH (%)	60.8	59.6-67.4	

In 2016, the global milk market showed increased production of 1.24% compared to 2015. Thailand's milk production also increased by 2.50% (Office of Agricultural Economics, 2017). Global milk consumption increased by 1.19% in 2016 from 2015 with a 1.05% increase in Thailand. With the rise in consumer demand,

several new products with high functional properties have been developed and produced. To control the quality of milk products, heat treatment is utilized to destroy microbials and increase shelf life.

Unpasteurized milk can contain dangerous microorganisms such as *Staphylococci*, *Coliform*, *Salmonella*, and *Escherichia coli* that cause outbreaks of many foodborne illnesses (Varga, 2007). Usually, raw or 'untreated' milk has a short shelf life. To extend shelf life, the two commonly used heat treatment processes are pasteurization and sterilization (Tamime, 2009). These processes reduce pathogenic and spoilage microorganisms but also inactivate enzymes and initiate chemical reactions and physical changes. Heating has an adverse effect on the nutritional value and appearance of milk as color, flavor and odor. To decrease microbiological activity and increase shelf life, heat treatment of milk can be processed as follows:

1) Low Temperature Long Time (LTLT), where the holding temperature is around 63°C for 30 min.

2) High Temperature Short Time (HTST), where the holding temperature is around 72°C for at least 15 sec.

These processes do not kill all pathogenic microorganisms in the milk but there is no significant reduction of nutrition, and product appearance is similar to that of raw milk (Sepulveda et al., 2005).

3) Ultra high temperature treatment (UHT) is also used, where the holding temperature is at least 135-150°C for 2-3 sec. The flavor and color are better than that of pasteurized milk. Both pasteurized and UHT treated milk should not contain pathogenic bacteria but if the milk is not processed properly there may be a high microbial load. Post-treatment contamination in containers that are not properly sterilized can also cause contamination.

4) Sterilization uses high heat of at least 100°C, commonly 115-120°C for 20-30 min. This can destroy all microbial and enzymatic contents but results in loss of nutrients and the appearance of the milk changes drastically (Tamime, 2009).

2.3 Melatonin

Melatonin or *N*-acetyl-5-methoxytryptamine is an indoleamine. It is synthesized from an essential amino acid, tryptophan as a by-product of metabolism via

serotonin (Lerner et al., 1958). The chemical formula is $C_{13}H_{16}N_2O_2$ with molecular weight of 232.28 g/mol. Water solubility of melatonin is 2 g/L at 20°C and in ethanol 5 g/L at 50°C. Melatonin is an off-white or yellowish powder with melting point ranging from 116-118°C (National Center For Biotechnology Information, n.d.). Absorbance of excitation and emission wavelengths occurs at 280 nm and 345 nm (Arnao & Hernández-Ruiz, 2007). The chemical structure of melatonin is shown in Figure 3.

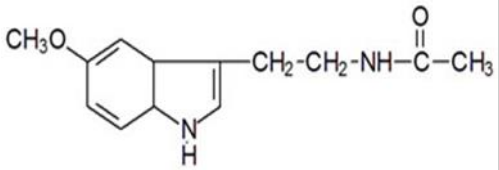


Figure 3 Chemical structure of melatonin
Source: Gómez-Moreno et al. (2010)

2.3.1 Melatonin in animals

Melatonin is well known as an animal hormone. It was first discovered in the bovine pineal gland and is found in both vertebrate and non-vertebrate tissues (Lerner et al., 1958). Melatonin is also found in bacteria, protozoa, fungi and mainly in edible mono- and dicotyledon plant families (Arnao & Hernández-Ruiz, 2006; Hardeland & Poeggeler, 2003). In humans, melatonin is generally produced in the pineal gland, retina, gastrointestinal tract and other organs (Triantafillidis & Triantafillidis, 2009). Melatonin secretion usually occurs at night or in darkness and drops during the day or in bright conditions. Functions of melatonin include regulation of the circadian rhythm and alleviation of jet lag and sleep disorders. Melatonin also enhances immune response, reproductive function, and ameliorates age-related diseases as a potential radical scavenger.

2.3.1.1 Melatonin synthesis in animals

In animals, melatonin is synthesized from tryptophan via 5-hydroxytryptophan, serotonin, and N-acetylserotonin using the four serial enzymes tryptophan-5-hydroxylase, 5-tryptophan decarboxylase, aryalkylamine N-

acetyltransferase (AANAT) and hydroksyindole-o-methyltransferase (HIOMT) (Figure 4).

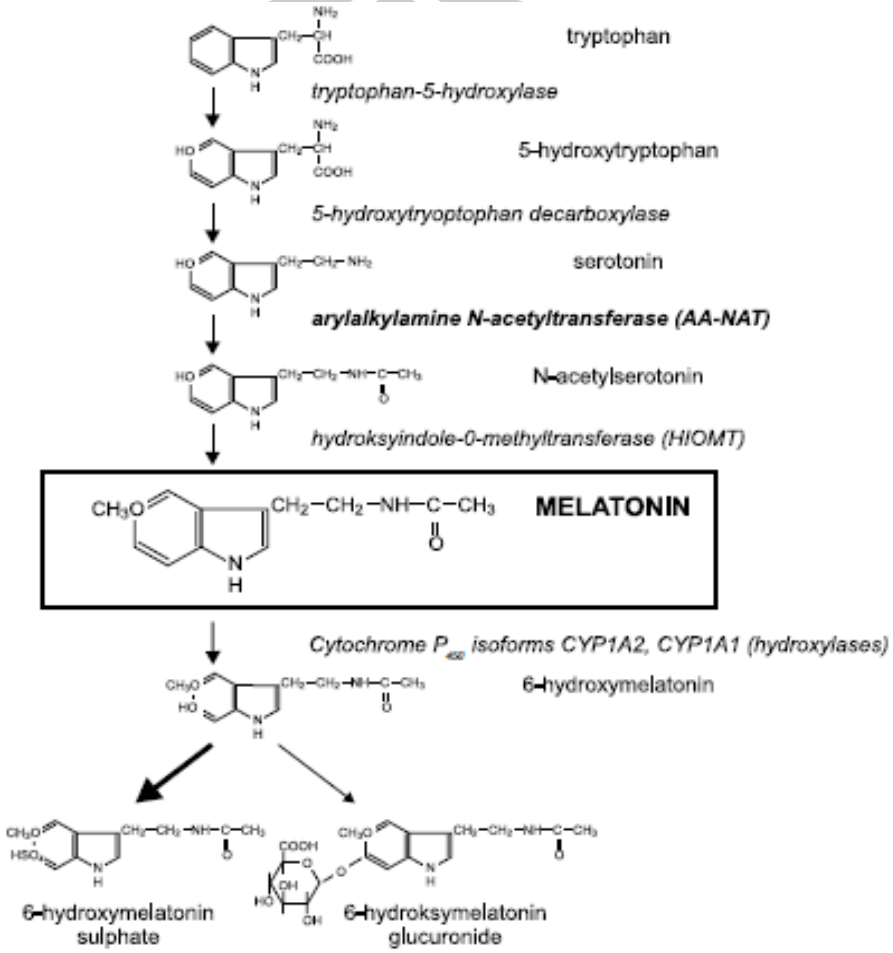


Figure 4 Pathway of melatonin synthesis in animals

Source: Karasek and Winczyk (2006)

2.3.1.2 Melatonin secretion

Melatonin regulates the circadian rhythm or sleep-wake cycle. It is produced in the pineal gland during dark conditions (80% synthesized at night). During the day, the level of melatonin in serum is low, ranging from 10-20 pg/mL but significantly increases at night to 80-120 pg/mL. The secretion peaks between 24:00 and 03:00 h. Onset of secretion occurs around 21:00-22:00 h with offset at 07:00-09:00 h (Figure 5).

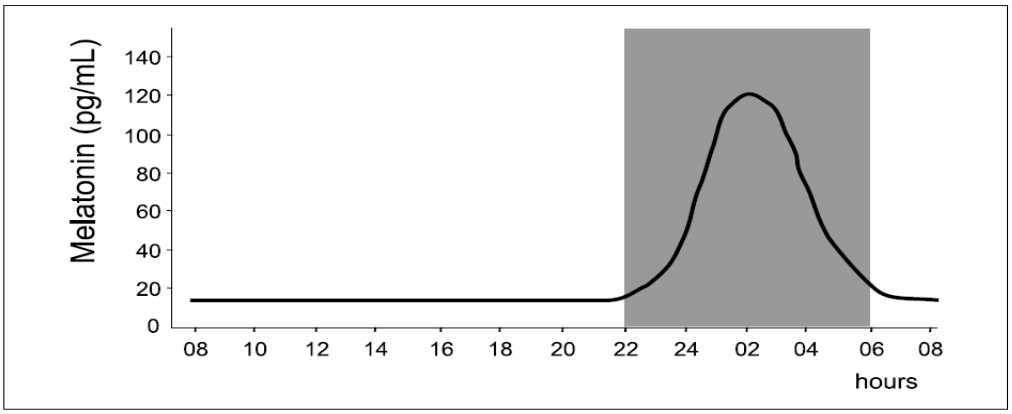


Figure 5 Circadian profiles of serum melatonin concentrations in humans; gray area = period of darkness

Source: Karasek and Winczyk (2006)

Melatonin secretion appears around 6-8 weeks after birth and is abundant at around 21-24 weeks (5-6 months) of infancy. The secretion reaches high levels between the ages of 4 and 7 and diminishes gradually at around 70 years (Figure 6).

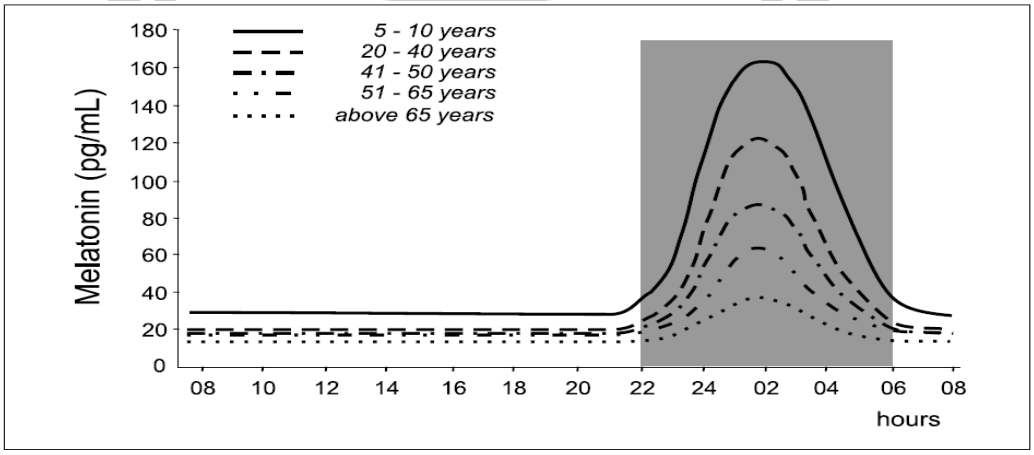


Figure 6 Circadian profiles of serum melatonin concentrations in humans at various ages; gray area = period of darkness

Source: Karasek and Winczyk (2006)

2.3.1.3 Melatonin catalysis

Melatonin is catalyzed at 90% by the hepatic P450 monooxygenases and after conjugation with 6-hydroxymelatonin results as a derivative of sulfate (60-70%)

or glucuronide (20-30%) that is released as a urinary metabolite 6-sulfatoxymelatonin (Hardeland et al., 2006).

2.3.1.4 Stability of melatonin

Melatonin solution at pH 1.2, 2, 4, 7.4, 10 and 12 stored at 20°C and 37°C does not degrade for the first 2 days. After that, melatonin starts declining gradually, not exceeding 30% at 20°C and 29% at 37°C until day 21 (Daya et al., 2001). Melatonin was prepared in aqueous solutions ranging from 1.0-113.0 μg/mL using a sterile technique and placed into pyrogen-free glass vacuum vials. The samples were then stored at room temperature, 4°C and -70°C. Melatonin was more stable as an aqueous solution for a period of 6 months (Cavallo & Hassan, 1995). Subsequently, melatonin stored at -32°C, 4°C, 25°C, 50°C in light and darkness without exposure to air appeared stable from the start until day 6 and then showed no significant decrease until day 13. At 25°C in light exposure to air, melatonin started decreasing from day 6 up 43% and showed maximum degradation on day 15 at 68%. Melatonin exposed to darkness decreased up to 41% by day 6 and then intensely decreased to 66% by day 15 (Moussaoui & Bendriss, 2014). Results indicated that melatonin stored in light or darkness without exposure to air had less degradation. Oxygen exposure in light or dark conditions increased degradation.

2.3.2 Melatonin in plants

Melatonin was first detected in plant tissues in 1995 and has been found in numerous plant families in leaves, roots, shoots, fruits, and seeds. Melatonin content in plants depends on several factors such as cultivars, species, growth conditions and environmental stresses (Feng et al., 2014). The function of melatonin in plants is to enhance growth and regulate circadian rhythm and antioxidant activity (Arnao & Hernández-Ruiz, 2006).

2.3.2.1 Melatonin synthesis in plants

In plants, tryptophan conversion catalyzed by tryptophan decarboxylase (TDC) converts to tryptamine. Tryptamine conversion catalyzed by tryptamine-5-hydroxylase (TP5H) converts to serotonin. Serotonin conversion catalyzed by serotonin

N-acetyltransferase (SNAT) converts to N-acetylserotonin. Finally, N-acetylserotonin conversion is catalyzed by acetylserotonin N-methyltransferase (ASMT) and melatonin is obtained (Figure 7).

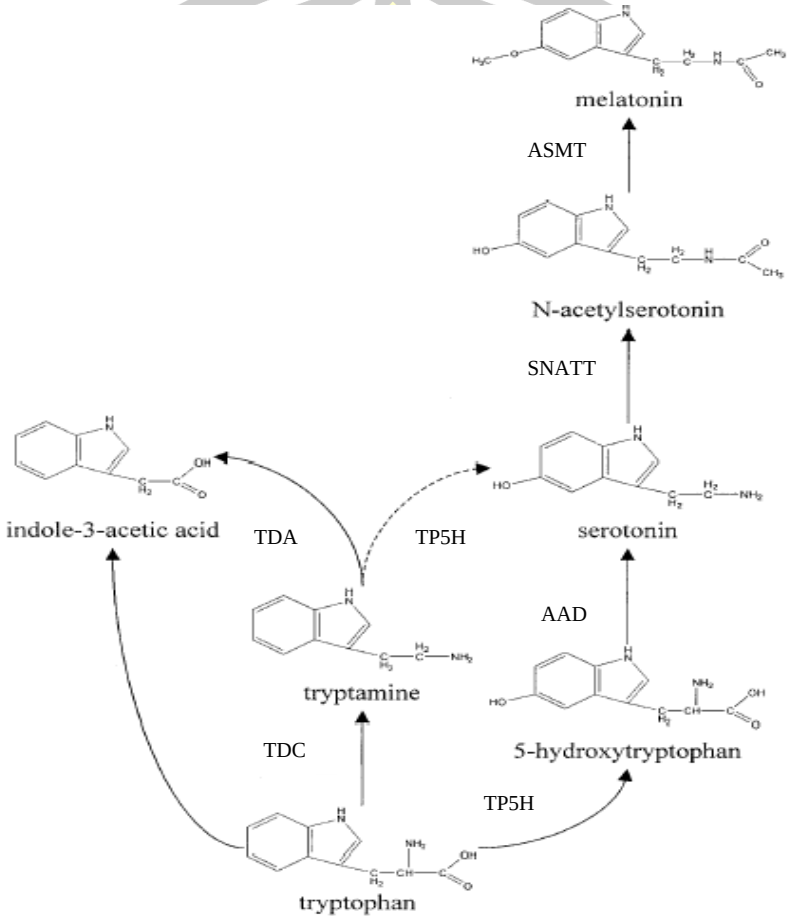


Figure 7 Pathway of IAA and melatonin in plants

Source: Murch and Saxena (2002)

Following the same metabolic pathway, tryptophan and tryptamine are precursors of indoleacetic acid (IAA) and melatonin synthetis IAA is a plant hormone with function similar to melatonin (Murch & Saxena, 2002). Murch et al. (2000) studied melatonin and serotonin biosynthesis from tryptophan precursor in St. John's wort (*Hypericum perforatum* L. cv. Anthos). They reported that tryptophan increased 7-fold in the higher radiolabel over 60 min of incubation. The 14C tryptophan was incorporated into indoleacetic acid (IAA) and melatonin, both on exposure to low light stimuli (6 µmol $m^{-2}S^{-1}$) and supplementation with light (40 µmol $m^{-2}S^{-1}$). IAA

significantly increased after 30 min in both cases. Melatonin showed significant increase at 60 min under supplemented light but no change in low light. These results indicated that melatonin and IAA could be found in St. John's wort plantlets and accumulation of radiolabel of tryptophan had a potential role in plant growth on exposure to light.

2.3.2.2 Melatonin concentration in plants tissues

Melatonin presence has been reported in different plant species and in several plant tissues (Table 3). Highest melatonin concentration was found in herbs followed by seeds and fruits. Concentration of melatonin in 108 Chinese herbs ranged widely from 10-3,000 ng/g dry weight (dw); 64 herbs exceeded 10 ng/g dw and 43 herbs exceeded 100 ng/g dw. Highest concentration was found in chantui (*Periostracum cicadae*) (3,771 ng/g dw) (Chen et al., 2003). For seeds, highest contents were found in white mustard (*Brassica hirta*) (189 ng/g dry weight) (Manchester et al., 2000) and in fruits, Burlat cherry (*Prunus avium* L.) (0.22 ng/g dry weight) (González-Gómez et al., 2009). High melatonin contents in herbs were channeled toward medicinal purposes for therapeutic action to slow down aging and treat other diseases which occurred due to radicals.

Table 3 Melatonin concentration in plant tissues

Plant	Scientific name	Melatonin (ng/g)	Reference
Root			
Japanese radish	*Brassica campestris*	0.66	Hattori et al. (1995)
Carrot	*Paucus carota*	0.06	(24 edible plants)
Ginger	*Zingiber officinale*	0.06	
Shoots			Hattori et al. (1995)
Japanese ashitaba	*Angelica keiskei*	0.62	(24 edible plants)
Japanese butterbur	*Patasites japonius*	0.05	
Asparagus	*Asparagus officinalis*	0.01	
Bulbs			Hattori et al. (1995)
Welsh onion	*Allium fistulosum*	0.09	(24 edible plants)
Onion	*Allium cepa*	0.03	
Leaf			
Chungiku	*Chrysanthemum coronarium*	0.42	Hattori et al. (1995)
Cabbage	*Brassica oleraceah*	0.11	(24 edible plants)
Chinese cabbage	*Raphanus sativus*	0.11	
Seeds			
White mustard	*Brassica hirta*	189.00	Manchesteret al. (2000)
Wolf berry	*Lycium barbarum*	103.00	(15 seeds of edible
Black mustard	*Brassica nigra*	129.00	plants)
Chinese herbs			
Chantui	*Periostracum cicadae*	3,771.00	Chen et al. (2003)
Diding	*Viola philipica Cav.*	2,368.00	(108 Chinese herbs)
Gouteng	*Uncaria rhynchophylla*	2,460.00	
Fruits			
Burlat cherry	*Prunus avium* L.	0.22	González-Gómez et al. (2009)
Pico Negro cherry	*Prunus avium* L.	0.12	(8 Sweet cherries)
Sweetheart cherry	*Prunus avium* L.	0.06	

2.3.2.3 Melatonin content in plants

Amount of melatonin is influenced by various factors such as plant type, cultivars, various stage of growth, location, growth condition and different analytical techniques for melatonin determination (Baghurst & Coghill, 2006; Feng et al., 2014; Hardeland et al., 2006; Manchester et al., 2000). Many researchers have presented the effect of various factors on melatonin content in plants (Table 4).

Table 4 Several factors on melatonin content in plants

Factor	Source	Report	Reference
Effect of growth location and condition	Tomato plants (*Lycopersicon esculentum* Mill. var. Cherry)	- Melatonin concentration of tomato plants grown in an open field was higher than in pots and in vitro. - The leaves had higher concentration than roots and stems. - Melatonin was synthesized well in leaves and/or roots and transported to every plant tissue including flowers, fruits and seed.	Arnao and Hernández-Ruiz (2013)
Effect of cultivars and leaf ages	Mulberry (*Morus* spp.) leaves	- Tip positions were selected at 1 to 3 on the top downward, young leaves from position 4 to 6 and old leaves from position 7 to 10. - Tips had highest melatonin concentration (384.4 ng/g dw) followed by young leaves and old leaves. - Difference in amount of melatonin concentration was attributed to several factors including cultivar, leaf age and area of growth.	Pothinuch and Tongchitpakdee (2011)
Effect of cultivars	Six pepper (C. *annuum* L.) and seven tomato (*Solanum lycopersicum* Mill.) cultivars.	- Melatonin concentration in all cultivars of red pepper fruit ranged from 4.48-11.90 ng/g fresh weight (fw), (31.01-93.40 ng/g dw), highest in Barranca and lowest in F26 cultivar. - Red tomato fruits ranged from 0.64-14.77 ng/g fw, (7.47-249.98 ng/g dw), highest in Ciliegia and lowest in Optima cultivar. - Melatonin increased from green to mature red fruits.	Riga et al. (2014)

Table 4 Several factors on melatonin content in plants (continued)

Factor	Source	Report	Reference
Effect of growth stage	Red pepper and sweet pepper	- At the cotyledon growth stage (15-17 days) of red pepper and sweet pepper, melatonin concentrations were higher than the seedling, flowering and fruit stages. - Melatonin in leaves and roots of plants decreased at the flowering stage and then increased when fruits and seeds matured.	Korkmaz et al. (2014)
	Tomato (*Solanum lycopersicum* L.)	- Melatonin in red tomato was higher than in mature green tomato. - Melatonin concentrations in pericarp and locula tissue of tomato gradually decreased from day10 to day 26 of fruit stage (mature green fruits) and then increased to the highest in mature red fruit (34 days).	Okazaki and Ezura (2009)
Effect of harvest time	Tomatoes (*Lycopersicon esculentum*) and Strawberries (*Fragaria ananassa*)	- Melatonin concentration in tomatoes ranged from 4.11 ng/g to 114.52 ng/g fw in Catalina cultivar and Marbone cultivar. - Melatonin concentrations in strawberries were 11.26 ng/g fw in Festival cultivar (harvest, 2009) and 1.38 ng/g fw, (harvest, 2010), respectively.	Stürtz et al., (2011)
	Sweet cherry (*Prunus avium* L.)	- Melatonin concentrations in sweet cherry varied according to harvest times. Burlat cultivar was 22.4 ng/g fw and Pico Negro and Sweetheart cultivars were 11.5 and 6.0 ng/g fw at 37 and 33 days after harvest.	González-Gómez et al. (2009)

Table 4 Several factors on melatonin content in plants (continued)

Factor	Source	Report	Reference
Effect of harvest time	Montmorency and Balaton Tart Cherries (*Prunus cerasus*)	- Melatonin concentration in Montmorency cherries (13.46 ng/g) was higher than in Balaton cherries (2.06 ng/g). - No difference was found between the early harvest, mid-harvest and late harvest in Montmorency orchards 1 and 2 and between Montmorency cherry trees.	Burkhardt et al. (2001)
Effect of sample preparation	Mulberry (*Morus* spp.) leaves	- Sample prepared using ultrasonication combined with solid phase extraction (ultrasonic/SPE) gave increased efficiency than liquid–liquid extraction (homogenization/LLE). - The fibrous nature of plant residues adhered to the homogenizer. - The loss in LLE step was due to the amphiphilic properties of the melatonin molecule that could not completely move to the organic phase.	Pothinuch and Tongchitpakdee (2011)
	Feverfew (*Tanacetum parthenium* L.)	- Melatonin contents of freeze-dried and oven-dried leaves of feverfew showed losses of up to 15% and 30%, respectively.	Murch et al. (1997)
Results reported	Six red pepper (*C. annuum* L.) and seven tomato (*Solanum lycopersicum* Mill.) cultivars	- Melatonin concentration of six red pepper cultivars ranged from 4.48-11.90 ng/g fw or 31.01-93.40 ng/g dw. Seven tomato cultivars ranged from 0.64-14.77 ng/g fw or 7.47-249.98 ng/g dw. - The dry weight unit was a reliable measurement when compared to fresh weight because water content in fresh tissue was higher than dry tissue resulting in low concentration estimation.	Riga et al. (2014)

2.3.2.4 Functions of melatonin in plants

The relation between melatonin and vascular plants has been studied almost exclusively from a phytochemical viewpoint. The possible roles of melatonin in plants are presented in Figure 8.

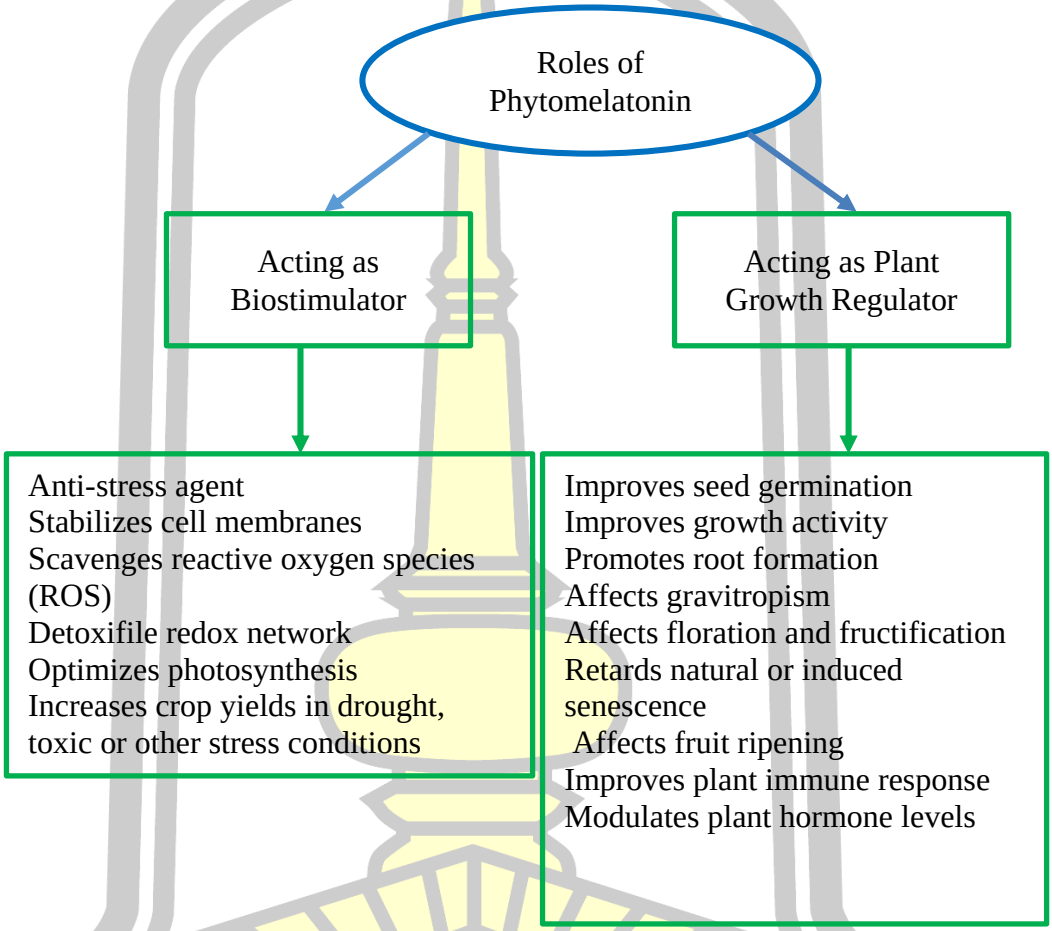


Figure 8 Roles of phytomelatonin in plant physiology
Source: Arnao and Hernández-Ruiz (2018)

Melatonin structure has indolic derivates similar to auxin in plants, therefore both may perform similar roles. Auxin is a plant hormone, commonly present as an indole acetic acid (IAA) derivative and its role is to regulate plant growth. Therefore,

both melatonin and IAA regulate circadian rhythms, seasonal photoperiodic regulation and also radical scavenging (Hernández-Ruiz et al., 2004).

1) Role of melatonin in reproductive development and circadian rhythms

Tan et al. (2007) determined and identified the circadian rhythm of melatonin and N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) in water hyacinths [*Eichhornia crassipes* (Mart) *Solms*] using LC/MS/MS analysis. Water hyacinth grown in pond water under sunlight ranged 10,000-15,000 $\mu W/cm^2$, (29.5-31.5°C) in daytime and was compared with growth under no artificial light (24.5-26.5°C). Leaves and flowers were collected. Measurements started at 16:00 h and were taken every 4 h for 24 h. Melatonin and AFMK peaked at around 20.00 h with highest levels under sunlight (306 ng/g) and lowest at 08.00 h (4 ng/g) in darkness. AFMK levels were 20 ng/g at 20:00 h and a low level of 2.0 ng/g at 08:00 h. Results showed that both compounds were highest during late evening in the light phase due to its related photosynthesis process or photo-protection from light exposure. During the photosynthesis process, free radicals are generated by reactive oxygen species (ROS) at high levels of antioxidants including melatonin and AFMK as free radical scavengers with efficiency against radicals by UV exposure. Therefore, water hyacinth has an antioxidant capacity with high tolerance to pollutants or oxidative stress.

2) Role of melatonin in cell protection

One of the important roles of melatonin in plants is its cell protection properties against damage by free radicals as an important aspect of indoleamine. Manchester et al. (2000) reported melatonin content in 15 edible seeds as ng/g unit. Melatonin in seeds at high levels is an antioxidative defense mechanism for germs and reproductive tissues and also protection from environmental assaults. An interesting experimental approach was adopted by Murch and Saxena (2002). They studied the possible protective role of melatonin during flower development of *Hypericum perforatum* L. A profile of indole levels (IAA, serotonin and melatonin) throughout flower development indicated that serotonin and melatonin presented higher concentrations at given stages, and that higher melatonin level coincided with maximum regeneration potential of isolated anthers. The authors suggested that

melatonin could act as a stress-protecting agent, providing an adaptive mechanism to ensure reproduction.

3) Role of melatonin in vegetative development

There are numerous hypotheses concerning the role of melatonin as a hormone agent. They demonstrate that melatonin is a growth promoter, as the first physiological role. One study investigated an auxin-induced root and shoot in vitro culture of St. John's wort (*Hypericum perforatum)*. The culture medium included six inhibitors of indoleamin and auxin regeneration with IAA supplementation. Results indicated that amounts of root and shoot increased with average root regeneration at 8-10 roots per stem that occurred after 5-7 days and shoot regeneration at 25-35 days (Murch et al., 2001). Hernández-Ruiz et al. (2004) studied growth-stimulating compounds present in lupin (*Lupinus albus* L.) tissues using melatonin. They found that optimum concentrations for growth-promoting activity in lupin hypocotyls were 10 µM melatonin and 100 µM IAA. De-rooted hypocotyls were optimized in 10 µM of both hormones.

2.4 Antioxidant property of melatonin

Melatonin is a potential free radical scavenger (Figure 9). It plays an important role in antioxidant activity of a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical, peroxynitrite anion, singlet oxygen, and nitric oxide. Melatonin helps to stimulate several antioxidative enzymes including glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and superoxide dismutase. Conversely, it inhibits prooxidative enzymes and nitric oxide synthase.

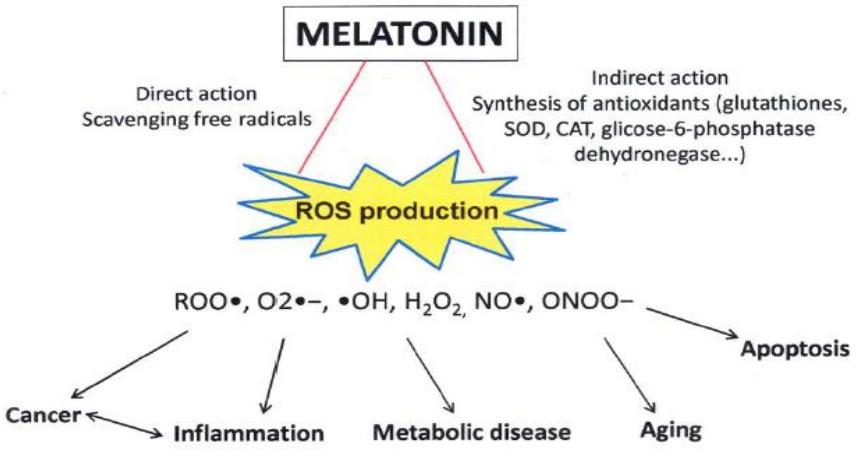


Figure 9 Actions of melatonin as a natural antioxidant on ROS generation and potential applications in some human diseases

Source: de Almeida Chuffa et al. (2013)

Antioxidant mechanisms of melatonin and its metabolites; [cyclic-3-hydroxymelatonin (cyclic-3OHM), *N*(1)-acetyl-*N*(2)-formyl-5 methoxykynuramine) (AFMK), *N*(1)-acetyl-5-methoxykynuramine (AMK) and 6-hydroxymelatonin (6-OHmel)] are presented through one electron transfer (Figure 10). They are efficient scavenging radicals that can mitigate many ailments such as cancer and neurodegenerative diseases, regulate circadian rhythms, improve sleep disorders and the immune system through anti-flamatory properties and control aging-related diseases. Research indicated that melatonin is a potential antioxidant scavenger. Antioxidant properties of melatonin are shown in Table 5.

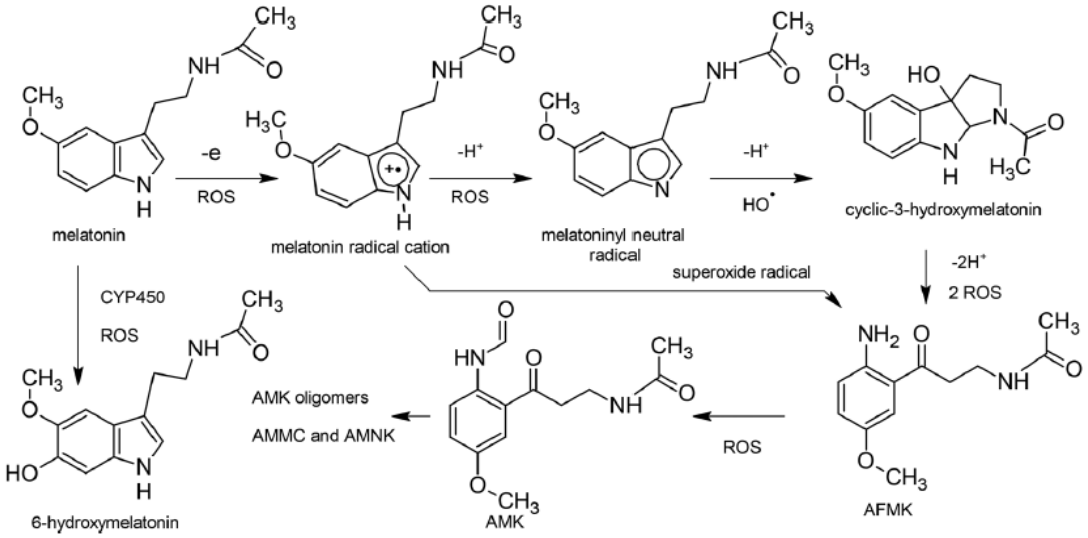


Figure 10 Transformation of melatonin by antioxidant activity

Source: Johns and Platts (2014)

The ability of melatonin for radical scavenging depends on radical species and increases the activities of antioxidative enzymes. Degradation products of melatonin (AFMK, AMK and cyclic 3-hydroxymelatonin) are highly effective to radical scavengers (Korkmaz et al., 2011; Reiter et al., 2010). Aguilera et al. (2015) evaluated a variety of antioxidants consisting of indolamine, polyphenols and common antioxidants at the same concentration of 300 µM on kinetic reactions of DPPH radical scavenging (Figure 11). They found that ascorbic acid was completely reacted within 5 min similar to Trolox, whereas fast radical inhibition was found in polyphenols, quercetin and gallic acid but still slower than ascorbic acid. Lowest radical scavenging was found in p-coumaric and melatonin with less than 10% DPPH inhibition, whereas 70% DPPH inhibition was observed in polyphenols up to a steady state at approximately 60 min. Different reaction times to reach the steady state depended on the chemical nature of the antioxidant (Anissi et al., 2014). Trolox and melatonin show similar radical scavenging behavior but they consist of different important functional groups. The absence of hydroxyl groups in indoleamine makes it less effective in

trapping peroxyl radicals in vitro, whereas it shows ability to scavenge peroxyl in vivo (Reiter et al., 2003).

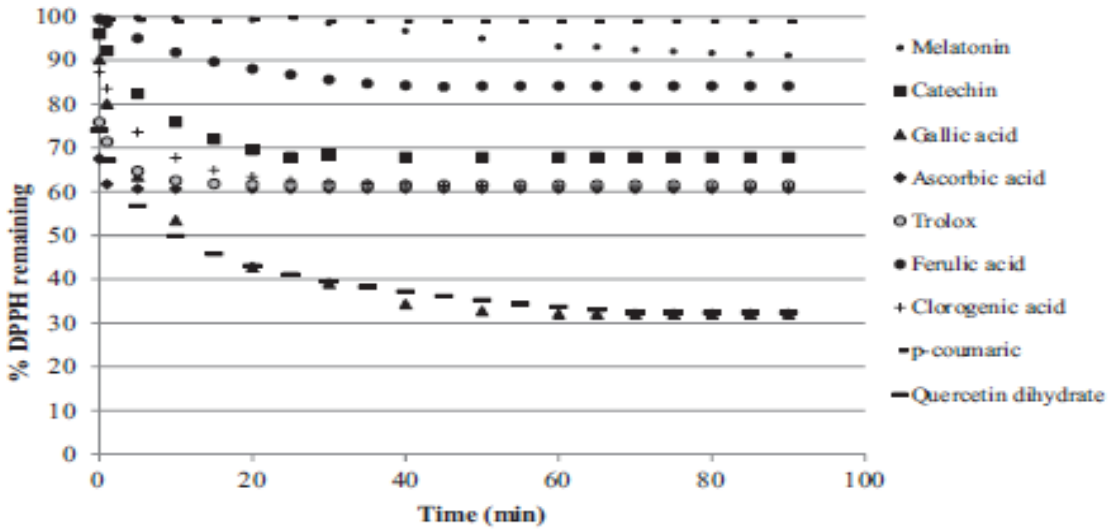


Figure 11 Different antioxidants at 300 µM

Source: Aguilera et al. (2015)

Table 5 Antioxidant properties of melatonin

Source	Report	Reference
Cereals (corn, milo, beans and rice)	- Normal food containing four cereals or melatonin-rich food (3.5 ng/g). - Chicks fed melatonin-rich food showed significant melatonin increase in plasma ($p<0.01$).	Hattori et al. (1995)
Melatonin	- Melatonin protection of human erythrocytes against hemolysis induced by 2,2' azo-bis (2-amidinopropane) dihydrochloride (AAPH) at 37°C versus other antioxidants. - Antioxidant efficiency of antioxidants, melatonin was higher than vitamin E, ascorbic acid and glutathione. - The 50% inhibition of cell lysis (I_{50}) was shown by 85, 190, 230 and 400 pM of melatonin, Trolox, ascorbic acid and glutathione, respectively.	Pieri et al. (1995)
Walnut	- Melatonin levels in rodents fed walnut were 3-fold higher than in the control group at 38.0 pg/mL and 11.5 pg/mL, respectively. - Serum melanin of rats fed walnuts showed significantly increased TEAC and FRAP values.	Reiter et al. (2005)
Seven seeds germinated (alfafa, lentis, mung bean, onion, broccoli, red cabbage, and radish)	- The germination process improved melatonin concentration by 3-fold in lentil and alfalfa, 2.5-fold in red cabbage and 2-fold in radish. - Bioactive compounds and melatonin significantly increased after germination. - Germination gave highest levels of antioxidants by DPPH, FRAP and ORAC measured in all seeds. Results indicated that melatonin had potential antioxidant capacity.	Aguilera et al. (2015)

2.5 Availability of melatonin

Dietary supplements are famous and widely produced in many countries. These products are popular and claimed to contain the necessary nutrients for diet. Consumers are willing to pay high prices for dietary supplements that they believe are beneficial for their health. Definitions of dietary supplements depend on individual countries. The USA uses the term “dietary supplements” whereas “food supplements” is used in the European Union (EU). EU regulations that appear on the label have not been prescribed by the Food and Drug Administration (FDA). In the USA, melatonin dosage is allowed at up to 10 mg, while in the EU it is less than 2 mg/unit with higher amounts classified as drugs. Oral administration of melatonin at 1 mg up to 1 g daily for 30 days had no adverse effects (Arnao & Hernández-Ruiz, 2018).

2.6 Melatonin dietary intake

Melatonin has been detected in several plants that can be directly consumed without food processing such as vegetables and fruit. By contrast, other plants have to be processed for preservation and added value. After processing or until consumer consumption, melatonin is easily degraded. Recent research revealing melatonin concentration in edible plants and food products, and melatonin concentration transfer to blood after food intake is shown in Table 6.

Table 6 Melatonin content in food products

Product	Report	Reference
Beer	- Melatonin concentrations were considered in eighteen brands of beer from the Spanish market. - Highest melatonin concentrations were observed in higher alcohol beers. - Valt-Damn beer had the highest melatonin concentration (169.7 pg/g) with alcoholic concentration of 7.2%. - Serum melatonin concentration and total antioxidant status (TAS) of seven healthy volunteers significantly increased after Valt-Damn beer intake.	Maldonado et al. (2009)
Wine	- Melatonin and melatonin isomer in eight wines produced in different grape cultivars were determined. - Highest melatonin concentrations were found in Tempranillo (129.5 ng/mL) and Jaen Tinto (32.6 ng/Ml) but could not be detected in Petit Verdot or Syrah samples.	Rodriguez-Naranjo et al. (2011)
Juice	- Pomegranate juice lacked melatonin but occurred during the winemaking process. - Melatonin content at the alcoholic fermentation stage (first 4 days) increased up to 7.37, 8.78 and 4.16 ng/mL in Wonderful, Coupage, and Mollar de Elche wine (Wonderful mixed with Mollar de Elche) varieties, respectively. - At the end of alcoholic fermentation (20 days), melatonin degraded 90% in Mollar de Elche wine (0.54 ng/mL), less than 25% in Wonderful wine (5.50 ng/mL) and less than 67% in Coupage wine (2.91 ng/mL). - Melatonin was synthesized by yeast during alcoholic fermentation to generate biogenic amines in wines. Furthermore, degradation of melatonin was metabolized by S. *cerevisiae* to other methoxylated indoles.	Mena et al. (2012)

Table 6 Melatonin content in food products (continued)

Product	Report	Reference
Juice	- Cherry juice supplements significantly increased time in bed, total sleep time and sleep efficiency, while decreasing napping time.	Howatson et al. (2012)
Oils	- Melatonin concentration in extra virgin olive oil was higher than refined olive and sunflower oil. - Extra virgin olive oils registered designations of origin (D.O.) and ranged at 71-119 pg/mL. Refined olive oil was between 53 and 75 pg/mL. Higher melatonin concentration was found in oil without heat treatment. - Melatonin content in olive oils may be concluded as combinations of phytochemicals that enhance health benefits by synergistic effects.	de la Puerta et al. (2007)
Vegetables	- Vegetable intake was significantly associated with urinary 6-sulfatoxymelatonin levels. - Mean urinary aMT6-s of women with highest quartile of vegetable intake was 15.9% higher than the lowest quartile after controlling covariates including age, total energy, body mass index, alcohol intake, menopausal status, and day length. - Association between vegetable intake and aMT6 levels was related to the melatonin content in vegetables.	Nagata et al. (2005)

Table 6 Melatonin content in food products (continued)

Product	Report	Reference
Fruits	- Highest melatonin concentrations were observed in mango (699 pg/g) followed by pineapple (302 pg/g), papaya (241 pg/g), orange (150 pg/g) and banana (9 pg/g). Melatonin was not detected in makmao. - After the healthy volunteers consumed each fruit, concentrations of urinary 6-sulfatoxymelatonin (aMT6-s) significantly increased with highest levels in banana (266% increase), pineapple (180% increase), orange (49% increase) and no significant increase in makmao and papaya. - Banana, pineapple and orange consumption increased aMT6 in urine up to 2-fold. This concentration may not be high enough for treatment of disease but has health promotion benefits for age-related melatonin reduction and enhanced sleep disorder.	Johns et al. (2013)
Fruits	- Melatonin levels were orange (150 pg/g), pineapple (302 pg/g) and banana (8.9 pg/g). - Serum melatonin levels significantly increased after consuming fruit with highest at 120 min and baseline at 180 min. - Antioxidant capacity significantly increased by 7-14% with FRAP assay and 6-9% by ORAC assay. - Results indicated that tropical fruit consumption enhanced melatonin concentration in serum and improved antioxidant capacity.	Sae-Teaw et al. (2013)

2.7 Food analysis methods for melatonin

Melatonin was discovered in higher plants and is well known as a bioactive compound that exerts a strong oxidant capacity. Recently, many methods have been used for melatonin qualification and identification. However, it is difficult to analyze melatonin in food at very low concentrations. Thus, analytical methods must be sensitive since the melatonin molecule has amphipathic properties and extraction to obtain accurate results is difficult. Melatonin reacts with molecules in food and an adequate matrix is required for adequate sensitivity and specificity (Garcia-Parrilla et al., 2009). Efficient analytical methods for melatonin analysis with optimized extraction methods should be used to confirm melatonin concentrations in food. Several methods have been used such as immunological techniques, radioimmunoassay (RIA) and enzyme-immunoassay (EIA) (de la Puerta et al., 2007); chromatographic techniques: GC-MS (González-Gómez et al., 2009), HPLC-MS/MS (Rodriguez-Naranjo et al., 2011), chemiluminescent techniques, HPLC-FD (Garcia-Parrilla et al., 2009) and HPLC-ECD (Reiter et al., 2005).

Comparison of different methods used for melatonin analysis (Table 7) showed that tomato extraction by ether using HPLC-FD had high concentrate at 1.0685 fw, (Pape & Lüning, 2006). Chromatographic methods have been the most widely used separation techniques in this area in recent years. HPLC techniques are more economical and time efficient when derivatization of the sample is not required before analysis. Most HPLC methods reviewed used reverse phase columns (e.g. RP18 or RP8) for melatonin separation. Fluorescence detectors (FD) were found to be sensitive and versatile to quantify melatonin in food samples and also gave low limits of detection and quantification (Garcia-Parrilla et al., 2009). However, the variation of melatonin concentrate depends on several factors such as sample type, stage of growth, location, growth condition and sample prepare and analytical techniques (Feng et al., 2014; Hardeland et al., 2006; Manchester, 2000).

Table 7 Comparison of melatonin in tomato determined by different methods

Sample	Extraction solvent	Analytical method	Melatonin (ng/g)	Reference
Tomato	Acetone-water-glycerol, tricine, NaOH pH 8	GC–MS RIA	0.002 0.008-0.016	Van Tassel et al. (2001)
	Ether	HPLC-FD ELISA	1.0685 (fw) 0.9821	Pape and Lüning (2006)

Techniques used for melatonin extraction depend on the study objective such as pressurized liquid extraction (Setyaningsih et al., 2015), microwave-assisted (Setyaningsih et al., 2012), homogenization combined with liquid–liquid extraction and ultrasonic with solid phase extraction (Pothinuch & Tongchitpakdee, 2011), incubator shaking overnight (Aguilera et al., 2015) and homogenization and centrifugation (Manchester et al., 2000; Reiter et al., 2005) (Table 8). Whatever the limitation of the equipment, the most appropriate, convenient and possible technique is chosen for the study. Therefore, similar types of extraction and estimation are used for melatonin analysis.

Table 8 Methods used for melatonin analysis

Sample	Preparation	Solvent/Ratio	Extract				Filter/Dry/Analyze	Reference
			Ultrasonicate	Incubator Shake	Homogenization	Centrifugation		
Germinated seed	Mortar	Methanol 2 g:10 mL (1:5)	-	16 h in darkness	-	4,200 rpm, 15 min	Filter, evaporated under vacuum and Nitrogen gas/ ELISA	Aguilera et al. (2015)
Seeds	Mortar	Ethanol 0.1 g :30 mL (1:30)	-	-	No time detail	10,000g at 4°C, 10 min	Evaporated under vacuum/ HPLC-ECD	Manchester et al. (2000)
Walnut	Mortar	Methanol 1 g:15 mL (1:15)	-	-	No time detail	10,000g at 4°C, 30 min	Evaporated under vacuum/ HPLC-ECD	Reiter et al. (2005)
Mulberry	Freeze-dried and ground	Methanol 2 g:3 mL (1:6.5)	-	-	2 min on ice	10,000g at 4°C, 3 min	Filter, evaporated under vacuum/ HPLC-FD	Pothinuch and Tongchitpakdee (2011)
Mulberry	Freeze-dried and ground	Methanol 2 g:13 mL (1:6.5)	30 min on ice	-	-	10,000g at 4°C, 30 min	Filter, evaporated under vacuum/ HPLC-FD	
Rice grain	Milled	Ethyl acetate in Methanol 2.5 g:22.5 mL (1:10)	Microwave- assisted				Evaporate/ HPLC-FD	Setyaningsih et al. (2012)
Rice grain	Milled	Ethyl acetate 2.5 g:60 mL (1:24)	Pressurized liquid extraction				Evaporate/ HPLC-FD	Setyaningsih et al. (2015)

CHAPTER 3
METHODOLOGY

Melatonin and free tryptophan contents in grains and mulberry leaf tea were determined. Suitable sources rich in melatonin and free tryptophan of grains and mulberry leaf tea were selected and prepared to supplement pasteurized milk. The quality, chemical composition, bioactive contents, antioxidant activity and sensory were evaluated. The most acceptable pasteurized milk treatment was selected for the shelf-life study. To achieve high melatonin pasteurized milk and with accepted quality, an experimental mixture design was used to model the response surface and optimize levels of soymilk powder, mulberry leaf tea, and raw milk. Pasteurized milk with the highest score of overall liking was then subjected to a clinical study. The experimental design is presented in this chapter as follows:

3.1 Experimental plan
3.2 Instruments and equipment
3.3 Chemicals and reagents
3.4 Survey and requirement of the milk factory
3.5 Materials
3.6 Methods
3.7 Statistical analysis

3.1 Experimental plan

The experimental plan was divided into three phases as follows:

Phase I: To determine melatonin and free tryptophan content in edible grains, mulberry leaf tea and raw milk.

Phase II: To develop pasteurized milk with high melatonin content.

Phase III: To evaluate melatonin concentration in urine following clinical study methodology.

All experimental designs are summarized in Figures 12-14.

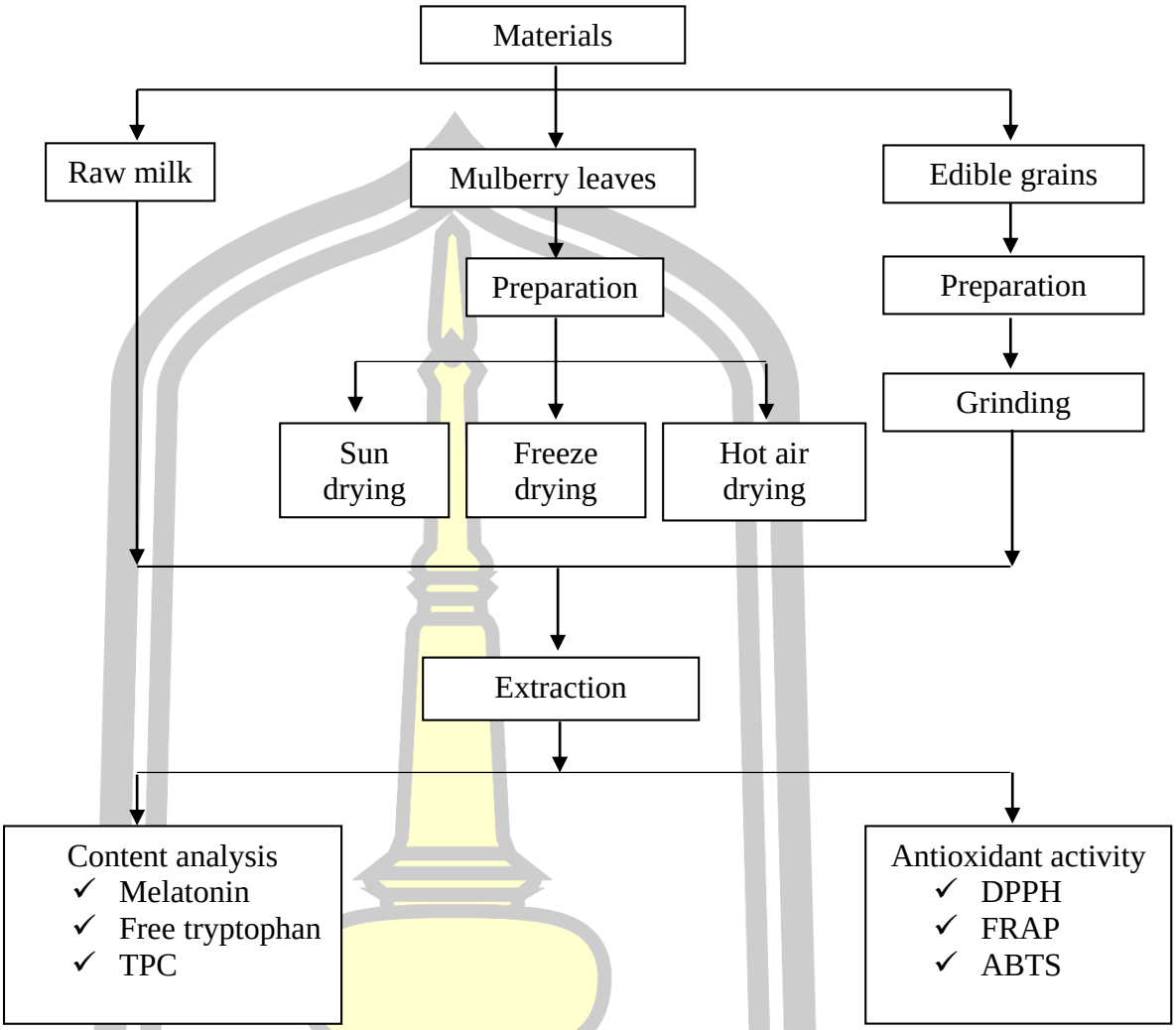


Figure 12 Phase I study framework

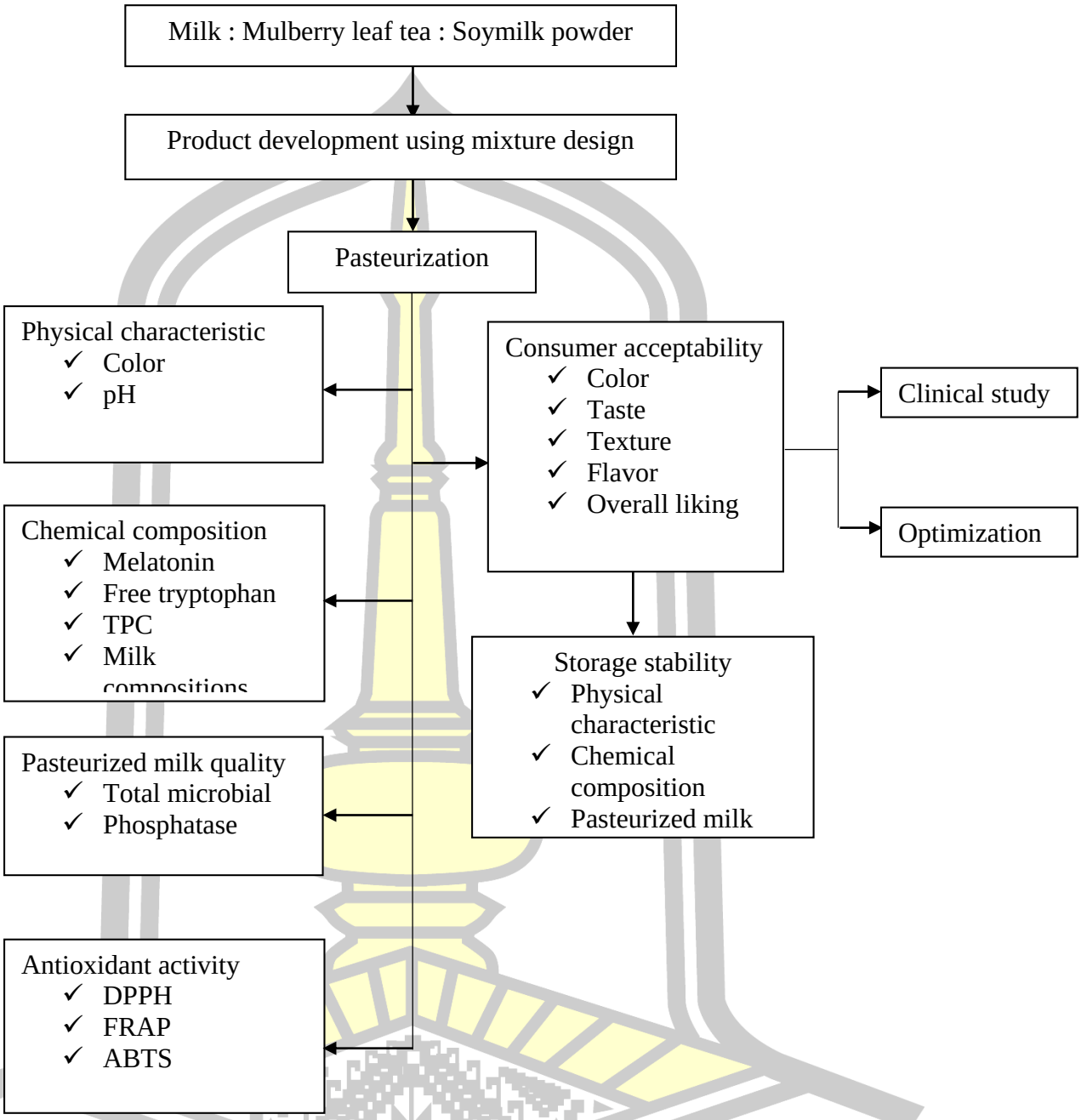
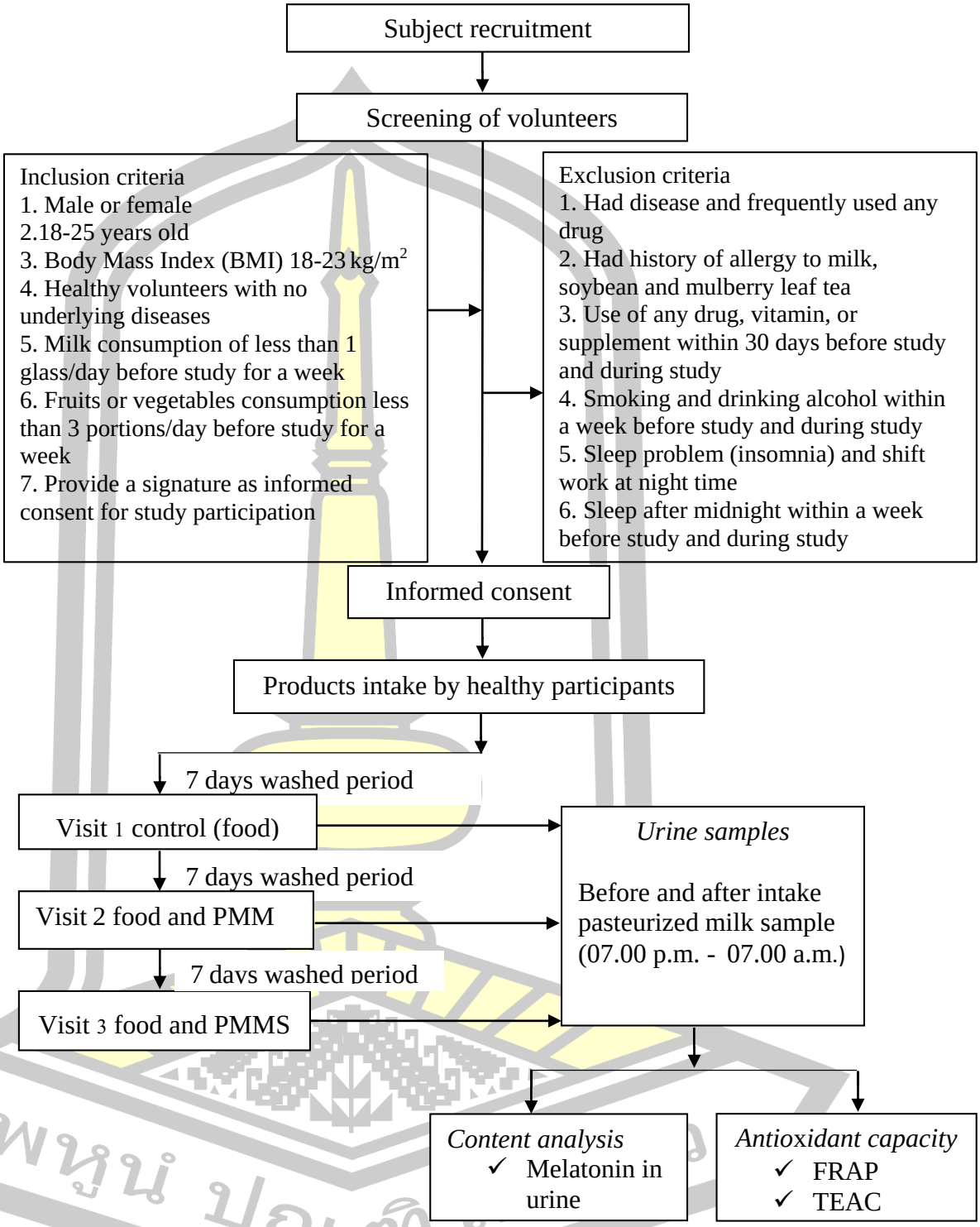


Figure 13 Phase II study framework



Remark; PMM refers pasteurized milk mulberry leaf tea and PMMS refers pasteurized milk mulberry leaf tea mixed soymilk powder

Figure 14 Phase III study framework

3.2 Instruments and equipment

Table 9 Instruments and equipment used

List	Instrument/Equipment	Model/Source
1	Blender machine	MX-900M, Panasonic
2	Rotary shaker	LSI-1005R, Lab Tech, Korea
3	Hot air oven	Binder, Germany
4	Libra S12 UV-vis spectrophotometer	Biochrom, Cambridge, UK
5	Muffle furnace	S1202PID, Thailand
6	Chroma meter	Minolta CR-300, Japan
7	Rotary evaporator	Buchi R-114, Switzerland
8	Centrifuge	Universal 320R, Germany
9	Shaking incubator	LSI-1005R, LabTech, Korea
10	pH meter	Mettler Toledo FiveEASY™Plus, FEP20 Switzerland
11	Analytical balance	Presica 25A, Switzerland
12	High performance liquid chromatography (HPLC)	Prominance HPLC, Shimadzu, Japan (Fluorescence detector)
13	C18 column (5µm, 4.6 mm x 150 mm)	Agilent Zorbax SB-C18, Agilent Technologies, CA, USA
14	Vortex mixer	Harmony, Japan
15	Freeze drying machine	Heto PowerDry PL3000, Czech Republic
16	Ultrasonic milk analyzer	Milkotronic , Stara Zagora, Bulgaria
17	Coffee grinder	Cuisinart, DBM-8HK, USA
18	Evaporator centrifuge	Savant SC210A SpeedVac Plus, USA
19	Household blender	QH-900B1, Joyoung Co., Ltd., Hangzhou, China
20	Microplate reader	Anthos ELISA reader; Labtec Instruments, Salzburg, Austria with ADAP 1.6 software
21	Other equipment	-

3.3 Chemicals and reagents

Table 10 Chemicals and reagents used

List	Chemical/reagent	Type/Grade	Source
1	Melatonin standard (≥99.5%)	HPLC	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
2	L-Tryptophan standard (≥98%)	HPLC	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
3	Acetonitrile	HPLC	BDH (Poole, UK)
4	Methanol	HPLC	BDH (Poole, UK)
5	Ethanol	HPLC	BDH (Poole, UK)
6	Methanol	Analytical	BDH (Poole, UK)
7	Ethanol	Analytical	BDH (Poole, UK)
8	Dichloromethane	Analytical	BDH (Poole, UK)
9	6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)	Analytical Analytical	Fluka Chemical Co. (Buchs, Switzerland)
10	1,1-diphenyl-2-picrylhydrazyl (DPPH)	Analytical	Fluka Chemical Co. (Buchs, Switzerland)
11	2,4,6-tripyridyl-s-triazine (TPTZ)	Analytical	Fluka Chemical Co. (Buchs, Switzerland)
12	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	Analytical	Fluka Chemical Co. (Buchs, Switzerland)
13	Gallic acid	Analytical Standard	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
14	α-tocopherol (vitamin E)	Analytical Standard	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
15	L-ascorbic acid (vitamin C)	Analytical Standard	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
16	Butylated hydroxyanisole (BHA)	Analytical	Fluka Chemical Co. USA

Table 10 Chemicals and reagents used (continued)

List	Chemical/reagent	Type/Grade	Source
17	Folin-Ciocalteu's reagent	Analytical	Fluka Chemical Co., USA
18	Glacial acetic acid	Analytical	RCI Labscan Ltd., Thailand
19	Melatonin-sulfate urine ELISA	Analytical	RE54031, BL International GMBH, Hamburg, Germany
20	Iron (III) chloride 6 hydrate ($FeCl_3.6H_2O$)	Analytical	BDH (Poole, UK)
21	Potassium persulfate ($K_2S_2O_8$)	Analytical	Ajax Finechem Pty Ltd, Australia and New Zealand
22	Ferrous sulfate heptahydrate ($Fe\ SO_4.7H_2O$)	Analytical	Ajax Finechem Pty Ltd, Australia and New Zealand
23	Ferric chloride hexahydrate ($FeCl_3.6H_2O$)	Analytical	LobaChemie Pvt Ltd, India
24	Sulfuric acid (H_2SO_4)	Analytical	Qrec, New Zealand
25	Hydrochloric acid (HCl)	Analytical	Carlo Erba, Thailand
26	Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	Analytical	Ajax Finechem Pty Ltd, Australia and New Zealand
27	Sodium carbonate anhydrous (Na_2CO_3)	Analytical	Ajax Finechem Pty Ltd, Australia and New Zealand
28	Sodium dihydrogen phosphate (NaH_2PO_4)	Analytical	BDH Prolabo, UK
29	Disodium hydrogen phosphate (Na_2HPO_4)	Analytical	Merck, Germany
30	Maltodextrin (DE10)	Food grade	K. science Center & Medical
31	Plate count agar	Standard methods agar for laboratory	HiMedia Laboratories Pvt Ltd, India
32	Peptone water	Standard methods agar for laboratory	HiMedia Laboratories Pvt Ltd, India
33	Other reagents	Analytical	-

3.4 Survey and requirement of milk factory

In recent times, many products have been produced using milk as a base such as yogurt, ice cream, and cheese. Interestingly, the products with high melatonin and antioxidants, improving relaxation, and sleep quality still has not been developed to its full potential. This study was done in collaboration with Kokko milk industrial, Maha Sarakham province to produce a functional drink by adding value to milk for developing a pasteurized milk product with beneficial properties. Kokko milk factory is a pasteurized milk producer for pre-elementary and primary school and also sells at several food and drink shops. To improve growth and sustainability, they need to develop a new product for consumers and popularize the marketing channels. Pasteurized milk with mulberry leaf tea flavor was considered to be produce as a new product due to mulberry leaf is rich in melatonin and phenolic content. However, the ratio of ingredients to be used affects the product quality hence this needs to be optimized and enhanced by supplementation with other ingredients for improving the melatonin and antioxidants in the product. However, before production, the requirement of Kokko milk factory must be elucidated through meetings and discussions. The present study was formulated as per requirements conducted the requirement of Kokko milk factory as:

1. A functional product with high functional value to promote relaxation and sleep quality.

2. Mulberry leaves were to be used for supplementation in the product because the factory wanted to encourage farmers in Borabue district and other areas in Maha Sarakam Province to cultivate mulberry trees and thereby increase their income.

3. Melatonin rich materials be used for improving the functional value by supplementing in pasteurized milk with mulberry leaf tea. This material used should be easily available and must support the farmers growing grain materials.

4. The production process must be feasible and convenient.

5. Production cost must be minimized.

6. The product should have good sensory characteristics.

7. New option for consumers to increase consumption of milk.

3.5 Materials

A total sixteen materials were collected as follows:

1. Raw milk was supplied by Maha Sarakham Kokko milk factory. Good quality raw milk was collected from milk storage tanks of Kokko Milk Factory, Maha Sarakham Province, after raw milk delivery between 07.30 and 09.00 a.m. supplied by local dairy farms in the Maha Sarakham area.

2. Mulberry (*Morus* spp.) leaves, Buriram 60 cultivar were harvested from Borabua district, Maha Sarakham, Thailand. The leaves were collected from the top to down at leaf positions 4-10.

3. Grain materials

3.1 Rice grains (*Oryza sativa* L.) were purchased from a local market in Maha Sarakham Province, Thailand and harvested in 2016. Different pigmented rice cultivars were used. Samples were dehulled using a milling machine to obtain brown rice. For RD-6, the bran was removed to obtain white rice. Rice samples consisted of five cultivars as follows:

3.1.1 Khao dok mali 105 (*Oryza sativa* L.)

3.1.2 Red dok mali (*Oryza sativa* L.)

3.1.3 Riceberry (*Oryza sativa* L.)

3.1.4 Black glutinous (*Oryza sativa* L. var. glutinosa)

3.1.5 Glutinous RD-6 (*Oryza sativa* L. var. glutinosa)

3.2 Corn grains included the pigmented waxy berry corn (purple color) was purchased from a local market in Khon Kaen Province, Thailand. Sweet corn (yellow color) and waxy corn (white color) were purchased from a local market in Vapee-Pathum district, Maha Sarakham Province. Harvest time of sweet corn was 75-80 days and for the others was 60-65 days, all are in year 2016. Samples included three cultivars as:

3.2.1 Sweet corn (*Zea mays saccharata*)

3.2.2 Waxy corn (*Zea mays ceratina*)

3.2.3 Waxy berry corn (*Zea mays* L.)

Sweet corn, a F1 hybrid top sweet 1320 cultivar, as a variety of corn with high sugar content, has good quality grain confirmed by yellow color, uniformity and grain pods mounted full in the endpoint. Different pigmented waxy corn was also used including white color, F1 hybrid top white 365 and dark purple or F1 hybrid waxy corn cultivars. Fresh kernels were manually removed and then dried using a freeze dryer.

3.3 Legume samples were purchased from local supermarkets in Maha Sarakham Province, Thailand. All samples were guaranteed by GMP and HACCP standard certification. All samples were peeled, with a shelf life of 4-6 months stored at room temperature and consisted of:

3.3.1 Soybean (*Glycine max* (L.) Merrill)
3.3.2 Peanut (*Arachis hypogaea* L.)
3.3.3 Mung bean (*Vigna radiata* (L.) Wilczek)
3.3.4 Red bean (*Phaseolus vulgaris* L.)

3.4 Oilseeds were purchased from local supermarkets in Maha Sarakham Province, Thailand with guaranteed by GMP and HACCP standard certification with shelf life of 4-6 months and consisted of:

3.4.1 Sesame seed (*Sesamum indicum* L.)
3.4.2 Sunflower seed (*Helianthus annuus* L.)

3.6 Methods

Phase I: To determine melatonin and free tryptophan content in edible grains, mulberry leaf tea and raw milk.

3.6.1 Sample preparation

3.6.1.1 Milk preparation

Good quality raw milk was collected after milk inspection. The sample was kept in a clean package and stored at 4°C until required for initial quality and chemical compositions, total plate count, melatonin, and free tryptophan analysis.

3.6.1.2 Mulberry leaf tea preparation

Mulberry leaves were hygienically prepared by cleaning and chopping into small pieces (1.5 x 1.5 cm^2) before roasting with mild heating at 50±2°C for 30 min to stop oxidation by polyphenol oxidase enzyme catalysis. A rolling process was conducted to break down of cell wall components and compounds coating the surface of leaves. After that, rolled leaves were dried using three different methods including solar dried for 6-7 h until the final moisture content approximately 10% according to the process of Kokko milk factory, freeze drying, and hot air oven at 120°C for 20 min (Pothinuch & Tongchitpakdee, 2011). After that, dried mulberry leaf tea was packed in a ziplock airtight plastic bag and stored at -20°C prior to analysis. Dried mulberry leaf tea containing high melatonin and free tryptophan obtained from different drying method was selected to use for fortifying into pasteurized milk in phase II along with grain selected from previous section.

3.6.1.3 Grain preparation

The samples (20 g) were ground using a coffee grinder (Cuisinart, DBM-8HK, USA). The grinding process was halted every 30 s to avoid excessive heating of the samples. Ground samples were sieved through mesh no. 20 (841 μm aperture) and kept in a zip-lock plastic bag to restrict exposure to light and air before storage at -20°C prior to extraction.

3.6.2 Extraction and analysis of melatonin and free tryptophan content

Melatonin and free tryptophan content were determined according to the method of Sae-Teaw et al. (2013) with some modifications. Free tryptophan is a precursor of protein and melatonin synthesis. It is found in proteins as a constituent of amino acids and also as a free form. Both are important for availability in the body, especially the free or non-protein-bound form which is the main substance related to melatonin biosynthesis. Samples (20 g) were dissolved in 100 mL of ethanol and mixed using a shaking incubator (Daihan LabTech, LSI-1005R, Korea) at 150 rpm and room temperature for 16 h. Mixed samples were then filtered through Whatman (No.1) paper and then filtrated. The samples were evaporated using a vacuum rotary evaporator

(Buchi R-114, Switzerland) at 45°C. Dry samples were eluted with 2.5 mL each of deionized water and dichloromethane. Next, a sample of the extract (100 µL) was pipetted into a test tube and 1 mL each of dichloromethane and deionized water were added and sonicated for 10 min. A further 2 mL of dichloromethane was added, mixed, and the dichloromethane layer was separated into a new test tube using a pasteurized glass pipette. The extracted dichloromethane layer was dried using an evaporator centrifuge (Savant SC210A SpeedVac Plus, USA). Then, 1 mL of HPLC-grade ethanol was added and mixed for 30 s using a vortex mixer (Harmony, VTX-3000L, Japan). Melatonin and free tryptophan content were analyzed using an HPLC-FLD (Prominance HPLC, Shimadzu, Japan) equipped with a binary LC-20AD pumping system using an RF-20Axs detector at 290/330 nm excitation/emission for fluorescence detection. An analytical RP-C18 column (4.6 x 250 mm, 5µm), (GL Science, Japan) was used as a stationary phase. The mobile phase consisting of 50 mM phosphate buffer at pH 7.2 (A) and (B) acetonitrile was computer program-controlled for gradient elution as follows: (time, solvent B), (0.0 min, 0%), (5.00 min, 35%), (12.00 min, 40%), (20.00 min, 45 %), (25.00 min, 50%), (30.00 min, 0%), and then stopped at 40 min with a flow rate of 1.0 mL/min and 20 µL injection volume. The accuracy (% recovery) and precision (repeatability and reproducibility, %RSD) of melatonin and free tryptophan content were determined following FDA guidance for Industry Bioanalytical Method Validation (Food and Drug Administration, 2001). Accuracy of standards recovery should be in the range 85-115% with % RSD < 15%. Values were calculated as: Recovery (%) = Recovered conc./Injected conc. x 100, and

RSD (%) = Standard deviation/Mean x100

3.6.3 Antioxidant capacity

3.6.3.1 Extraction for antioxidant analysis

Ground samples (2 g) were extracted with 20 mL of 80% methanol (analytical grade), mixed by shaking in an incubator at 150 rpm at room temperature for 16 h, filtered through Whatman (No.1) and then evaporated using a vacuum rotary evaporator at 45±2°C. Dried samples were eluted with 5 mL of 80% methanol and

stored at -20°C until required for antioxidant analysis using DPPH, ABTS and FRAP assays. Several standards used for antioxidant equivalent were Trolox, melatonin, BHA, vitamins E and C.

3.6.3.2 Free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

Stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging was evaluated according to the method of Brand-Williams et al. (1995). Extracted samples (50 µL) were added to 1.95 mL of DPPH radical in methanol solution at 6 x 10^{-5} mol/L. Samples were shaken vigorously using a vortex mixer, left for 30 min and then measured at 517 nm. Results were expressed as mg antioxidant standard equivalents/g sample.

3.6.3.3 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

ABTS assay was performed according to the method of Re et al. (1999). ABTS reagent was prepared by mixing 7.0 mM ABTS and 2.45 mM $K_2S_2O_8$ in ratio 1:1 v/v. The resulting ABTS radical cation ($ABTS^{\bullet+}$) solution was allowed to react in the dark at room temperature for 12-16 h, and then 1 mL was diluted with methanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. Extracted samples of 50 µL and 1 mL $ABTS^{\bullet+}$ solution were added and left for 6 min in the dark at room temperature. The reaction was measured at 734 nm and expressed as mg antioxidant standard equivalents/g sample.

3.6.3.4 Ferric-reducing antioxidant power (FRAP) assay

FRAP assay was performed following the method of Benzie and Szeto (1999). Working FRAP reagent was prepared in acetate buffer 0.3 M (pH 3.6), 10 mM of 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM of HCl and 20 mM of $FeCl_3.6H_2O$ in ratio 10:1:1 v/v/v and warmed at 37°C for 30 min. Then, 0.3 mL of the extracted sample and 1.7 mL of FRAP reagent were added into a test tube, mixed, kept for 60 min and measured at 593 nm. FRAP values were calculated as antioxidant standard equivalents/g sample.

3.6.3.5 Total phenolic content (TPC)

Total phenolic content was determined according to the method of Singleton et al. (1999). The extracted sample (0.2 mL) was mixed with 1 mL of Folin-Ciocalteu reagent at 1:10 with water, then 0.8 mL of sodium carbonate (7.5% w/v) was added and the mixture was kept in the dark at room temperature for 2 h. Absorbance at 750 nm was measured using a Libra S12 UV-vis spectrophotometer (Biochrom, Cambridge, UK). Results were expressed as mg gallic acid equivalents/g sample.

Phase II: To develop pasteurized milk with high melatonin content.

3.6.4 Soymilk powder preparation

Suitable material from phase I was selected and prepared for pasteurized milk production. Soybeans were selected and prepared as soymilk powder before adding to milk following Jiang et al. (2013) with some modifications. Briefly, whole soybeans were cleaned and then soaked in water (1:3 w/v) for 8 h. After rinsing, soaked soybeans were blended in two stages, each using a 1:1 w/v ratio of soaked soybeans to water for one minute at the lowest speed (No.1) using a household blender (QH-900B1, Joyoung Co., Ltd., Hangzhou, China). The soymilk slurry was filtered through two layers of cotton cloth, 1% of maltodextrin (DE10) food grade was added as an anticaking agent, and the mixture was dried using a freeze dry machine (Heto PowerDry PL3000, Czech Republic). Dried powder was packed in a ziplock plastic bag and stored at -20°C prior to use.

3.6.5 Pasteurized milk formulation

Grains with high melatonin content, free tryptophan and suitable physical appearance for improving functional milk were selected to develop high melatonin pasteurized milk. Here, soybeans and mulberry leaves were selected and prepared as soymilk powder and mulberry leaf tea. Mulberry leaf tea provided an acceptable green color; therefore, we expected the consumer to readily accept the high melatonin pasteurized milk.

A preliminary study determined 12% as a maximum added level of soymilk powder and mulberry leaf tea (sun dried). Therefore, nine milk formulations were generated by adding different quantities of ingredients (Table 11). The three main ingredients used for pasteurized milk formulation included raw milk (85.80-93.80% RM), mulberry leaf tea (2-6% MLT) and soymilk powder (2-6% SMP), with the remaining ingredients as sugar (2%) and food grade color (0.2%).

Table 11 Pasteurized milk treatments

Treatment	Formulation (%)			Sugar	Color
	SMP (X_1)	MLT (X_2)	RM (X_3)		
1	2.00	2.00	93.80	2.00	0.20
2	2.00	4.00	91.80	2.00	0.20
3	2.00	6.00	89.80	2.00	0.20
4	4.00	2.00	91.80	2.00	0.20
5	4.00	4.00	89.80	2.00	0.20
6	4.00	6.00	87.80	2.00	0.20
7	6.00	2.00	89.80	2.00	0.20
8	6.00	4.00	87.80	2.00	0.20
9	6.00	6.00	85.80	2.00	0.20

SMP (soymilk powder) = X1, MLT (mulberry leaf tea) = X2, RM (raw milk) = X3.

3.6.6 Pasteurized milk process

Pasteurized milk was prepared by brewing mulberry leaves in approximately one-quarter of the whole portion of raw milk at 73±2°C for 30 min. The brew was then filtered twice through cheesecloth, prior to mixing with the remaining three-quarters of milk, and soymilk powder and the remaining ingredients were added. The mixture was subjected to pasteurization at 65°C for 30 min, cooled to 4°C and then stored in sterilized bottles at 4°C.

3.6.7 Analysis of pasteurized milk

3.6.7.1 Physical analysis

1) Color value

The color value was measured using a Minolta Chroma Meter CR-300 (Konica Minolta, Japan). Parameters were expressed as color values of L*, a* and b* where L* from 0 to 100 represents darkness to lightness, +a* and -a* represent red and green, and +b* and -b* represent yellow and blue, respectively.

2) pH value

The pH values of pasteurized milk samples were measured by using a pH meter (Mettler Toledo, USA).

3.6.7.2 Content analysis

1) Pasteurized milk compositions consisting of fat, solid not fat (SNF), lactose and protein were determined using an Ultrasonic milk analyzer (Milkotronic, Stara Zagora, Bulgaria) and total solids (TS) were calculated by SNF and fat content.

2) Melatonin and free tryptophan content

Pasteurized milk samples were extracted following the method of Alyaqoubi et al. (2014) with minor modification. The extraction solvent was prepared with 1 N HCl in 95% ethanol in the ratio 15:85 v/v. Briefly, 10 mL of milk samples were dissolved with 40 mL of extraction solvent and placed in a shaking incubator at 150 rpm at room temperature for 4 h. Extracted samples were centrifuged at 4,500 rpm at 4°C for 20 min.

Extracted samples were purified to assess melatonin and free tryptophan contents using C18 solid phase extraction (SPE) cartridges (Waters, Milford, MA, USA) according to the method of Pothinuch & Tongchitpakdee (2011) and Cao et al. (2006) with some modifications. Cartridges were activated by adding 10mL of methanol and then 10 mL of deionized water, followed by the extracted sample. The cartridges were washed with 10 mL of 5% methanol, and 80% methanol was used to elute the retained compound in the cartridge. Extracted samples were filtrated through a nylon syringe filter (0.2 μm) (Whatman, USA) and then analyzed using a Shimadzu 20ADS liquid chromatograph; SIL-20AC HT autosampler; CTO-

20AC column oven coupled with an LC-MS 8030 (Shimadzu, Japan). Chromatographic separation of each sample was performed using an InertSustain® C18 column (2.1 x 150 mm i.d., 3 μm) (GL Sciences Inc., Japan) with the mobile phase consisting of 0.45%formic acid (A) and acetonitrile (B). Elution gradient was controlled asmobile phase B (0.0 min, 20%) (5.00 min, 50%) (6.00 min, 100%) (9.00 min, 20%) and (10.00 min, 0%) with flow rate set at 0.25 mL/min, column temperature 30 °C and injected volume 2 μL. The MS/MS system consisted of a triple quadrupole mass spectrometer with an electrospray ionization (ESI) setting. Nitrogen (N) was used as the drying gas at a flow rate of 15 L/min and nebulizing gas at 3 L/min, interface temperature 350 °C, desolvation line (DL) temperature 250 °C and temperature of the heat block 400 °C. Melatonin and free tryptophan were identified using multiple reaction monitoring (MRM) with positive ion mode. Qualitative identification of precursor ions was achieved by scanning through argon gas, and product ions were created by collision-induced dissociation (CID). Transitions for melatonin were determined at m/z 233.2→174.2, Q1 pre-bias at -20.0, collision energy (CE) at -16.0 and Q3 pre-bias at -19.0. Free tryptophan was scanned at m/z 205.00→188.00, Q1 pre-bias at -30.0, CE at -12.0 and Q3 pre-bias at -22.0 with collision-induced dissociation gas at 230 kPa and interface voltage at 4.5 kV.

3.6.7.3 Pasteurized milk quality

1) Phosphatase test

Alkaline phosphatase (ALP) was determined using an alkaline phosphatase test kit (Phosphatesmo MI) to control the quality of pasteurized milk conditions as sufficient or not to destroy the phosphatase enzymes. ALP paper test strips were dipped in pasteurized milk samples and then incubated at 36°C. If the paper color was unchanged from white to yellow then the milk was completely pasteurized.

2) Standard plate count (SPC)

The total microbial count was determined according to the method of Saxena and Rai (2013). Pasteurized milk samples were serially diluted in peptone water. Appropriate dilution was spread on an agar plate and incubated at 37°C for 48 h. The microbial colony was counted (accepted colony forming units on a plate must

be less than 250), and the microbial count was expressed in colony forming unit per milliliter (cfu/mL).

3.6.7.4 Antioxidant capacity

1) Extraction for antioxidant analyses

Pasteurized milk samples were extracted following the method described in section 3.6.7.2 (2) with some modifications.

2) Free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay followed the method described in section 3.6.3.2.

3) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay followed the method described in section 3.6.3.3.

4) Ferric-reducing antioxidant power (FRAP) assay followed the method described in section 3.6.3.4.

5) Total phenolic content (TPC) followed the method described in section 3.6.3.5.

3.6.8 Sensory evaluation

The study was reviewed and approved by the Ethics Committee for Research Involving Human Subjects, Mahasarakham University, certificate of approval number 048/2016. Thirty untrained panelists comprising students of the Faculty of Technology, Mahasarakham University, Thailand were enrolled and all provided signed informed consent. Panelists were familiar with consuming milk, had no allergy to milk, soybean and mulberry leaf tea and followed the rules of the study. Pasteurized milk samples were served. Drinking water and biscuits were given to avoid carryover effects between samples. Different attributes of pasteurized milk samples were evaluated as color, flavor, taste, texture and overall liking using a 9-point hedonic scale following the method of Marsanasco et al. (2015) and Sawale et al. (2015). The liking score of each attribute was represented as a number between 1 and 9 as follows:

9 = like extremely	8 = like very much	7 = like moderately
6 = like slightly	5 = neither like nor dislike	4 = dislike slightly
3 = dislike moderately	2 = dislike very much	1 = dislike extremely

3.6.9 Study of shelf life

Pasteurized milk with the highest score of overall liking was selected for the stability study. The sample was stored at 4°C and sampling procedures to determine the quality and other parameters were conducted every two days for eight days.

3.6.9.1 Physical analysis

1) Color value evaluation was as described in section 3.6.7.1 (1).

2) pH value evaluation was as described in section 3.6.7.1 (2).

3.6.9.2 Content analysis

1) Pasteurized milk composition analysis was described in section 3.6.7.2 (1).

2) Melatonin and free tryptophan content extraction and analysis were described in section 3.6.7.2 (2).

3.6.9.3 Pasteurized milk quality analysis

1) Phosphatase test was described in section 3.6.7.3 (1).

2) Standard plate count (SPC) followed methodology described in section 3.6.7.3 (2).

3.6.9.4 Antioxidant capacity

1) For free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay see section 3.6.3.2 for details.

2) For 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay see section 3.6.3.3 for details.

3) For ferric-reducing antioxidant power (FRAP) assay see section 3.6.3.4 for details.

4) For total phenolic content (TPC) see section 3.6.3.5 for details.

3.6.10 Optimization of pasteurized milk

The purpose of this study was to produce a pasteurized milk with high quality control as per the Notification of the Ministry of Public Health (2013) regulations (NO.351). Ranges of three ingredients were obtained from previous studies and considered by the highest sensory score of overall liking as section 3.6.8, consisting of:

X_1=soymilk powder (SMP) ranging from 3.50-4.50%,

X_2= mulberry leaf tea (MLT) ranging from 3.50-4.50%

X_3=raw milk (RM) ranging from 88.80-90.80%.

Estimated components of X1 + X2 + X3 = 97.80% with fixed basic formulations of sugar (2.00%) and food grade color (0.20%). Pasteurized milk was obtained for all 11 treatments consisting of two center points as shown in Table 12.

Table 12 Total formulation of three mixed ingredients using mixture design

Treatment	Formulation (%)			Sugar	Color
	SMP (X_1)	MLT (X_2)	RM (X_3)		
1	4.50	4.00	89.30	2.00	0.20
2	4.50	3.50	89.80	2.00	0.20
3	4.50	4.50	88.80	2.00	0.20
4	4.00	4.50	89.30	2.00	0.20
5	4.00	4.00	89.80	2.00	0.20
6	4.00	4.00	89.80	2.00	0.20
7	4.00	4.00	89.80	2.00	0.20
8	4.00	3.50	90.30	2.00	0.20
9	3.50	4.50	89.80	2.00	0.20
10	3.50	3.50	90.80	2.00	0.20
11	3.50	4.00	90.30	2.00	0.20

3.6.11 The pasteurized milk process

The pasteurized milk process was explained in section 3.6.6.

3.6.11.1 Physical analysis

1) Color value was explained in section 3.6.7.1 (1).

2) pH value followed details in section 3.6.7.1 (2).

3.6.11.2 Content analysis

1) Pasteurized milk compositions followed section 3.6.7.2 (1).

2) Melatonin and free tryptophan contents were determined as described in section 3.6.7.2 (2).

3.6.11.3 Pasteurized milk quality

1) The phosphatase test was described in section 3.6.7.3 (1).

2) Standard plate count method was described in section 3.6.7.3 (2).

3.6.11.4 Antioxidant capacity

1) Extraction for antioxidant analyses was described in section 3.6.7.2 (2).

2) Free radical scavenging activity (DPPH) was described in section 3.6.3.2.

3) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was described in section 3.6.3.3.

4) Ferric-reducing antioxidant power assay (FRAP) was described in section 3.6.3.4.

5) Total phenolic content (TPC) was described in section 3.6.3.5.

3.6.12 Sensory evaluation

Sensory evaluation was described in section 3.6.8.

Phase III (objective 3): To evaluate melatonin concentration in urine following clinical study methodology

3.6.13 Pasteurized milk sample preparation for clinical study

Two different pasteurized milk samples were produced and studied consisting of pasteurized milk mulberry leaf tea (PMM) based on the formula of Kokko Milk Factory and pasteurized milk mulberry leaf tea mixed soymilk powder (PMMS). PMM sample was prepared by adding 0.2% MLT in milk whereas the PMMS sample was added MLT and SMP each 3.5% in milk. Fixed ingredients were mixed of both formulas including 2.0% of sugar and 0.2% food grade color. PM samples were pasteurized using high temperature short time (HTST) and the pasteurized was

processed supporting by Kokko Milk Factory. Our primary study reported that fresh milk contained melatonin at 0.03 ng/mL, while melatonin in PMM was 0.34 ng/mL and in PMMS 1.03 ng/mL.

3.6.14 Experimental design and participants

The experimental study was conducted at the Faculty of Pharmaceutical Sciences and Melatonin Research Group, Khon Kaen University, Thailand. The study was reviewed and approved by Khon Kaen University Ethics Committee for Human Research, certificate of approval number HE602125. An open trial with crossover design was used to examine the association of urinary 6-sulfatoxymelatonin (aMT6s) with pasteurized milk containing high melatonin content regarding consumption. aMT6s is a conjugation between sulfate and primary metabolite of melatonin. Here, it was used as an indicator of total melatonin in urine circulated during the time until the last urine void. Thirty healthy volunteers were enrolled and all provided their signature of informed consent. Participants were both male and female (18-25 years old), body mass index (BMI) 18-23 kg/m^2. They had no underlying disease, did not use any drugs, vitamins, minerals or food supplements for a month before the study, abstained from smoking and alcohol, had milk consumption of less than a glass/day, fruits and vegetable intake less than three portions/day and slept before midnight for a week before the study. They had no allergy to milk, soybean or mulberry leaf tea, no sleep problem and did not work at night time.

3.6.15 Urine collection

Participants followed the stipulated rules before and during the study. On the first visit, participants were requested to consume only rice, fried pork and soup (baseline) for their evening meal; three crackers and a bottle of flavored drinking syrup were provided. Urine samples were collected overnight between 07.00 p.m. and 07.00 a.m. the next morning with a washout period for a week during consumption. On the next visit, 30 min after food ingestion, participants consumed 200 mL of PMM and then followed the same procedure of urine collection and washout period. For the last visit,

the same procedure was repeated but participants consumed 200 mL of PMMS. All urine samples were delivered the same day and kept at -20°C prior to determining aMT6-s using Melatonin-sulfate urine ELISA (RE54031, IBL International GmbH, Hamburg, Germany) and urinary antioxidant activity. Total aMT6-s produced was calculated with creatinine and adjusted with the total volume of urine collected.

3.6.16 Melatonin-sulfate urine ELISA test procedure

Melatonin-sulfate urine ELISA (RE54031, IBL International GmbH, Hamburg, Germany) showed detection limits of 1.0 ng/mL with a mean recovery of 105.8% (91-122%). Intra-assay and inter-assay precisions were 5.8-204 ng/mL and 12.4-220 ng/mL, respectively. Measurements were carried out according to the manufacturer's instructions (IBL).

3.6.16.1 Preparation of concentrated components

Concentrated wash buffer components were prepared by diluting 50 mL into 1L of bidistilled water and concentrated enzyme conjugate was prepared by diluting 200 µL with 8 mL of assay buffer.

3.6.16.2 Dilution of standards, controls and urine samples

Ten microliters of each standard, control and urine sample were pipetted into polystyrene and 500 µL of assay buffer was added and mixed using a vortex.

3.6.16.3 Test procedure

Fifty microliters of each diluted standard, control, and sample were pipetted into each well. Then, 50 µL of freshly prepared enzyme conjugate and 50 µL of melatonin sulfate antiserum were added. The wells were covered with adhesive foil and incubated for 2 h at 18-25°C on an orbital shaker set at 500 rpm. Incubated solutions were then discarded by washing the well plate with 250 µL of diluted wash buffer (4 times). One hundred microliters of TMB substrate solution was pipetted into each well using an eight-channel micropipette to avoid air bubbles and incubated and shaken as an above condition for 30 min. Then, 100 µL of TMB stop solution was pipetted into each well to stop the substrate reaction, before measuring optical density with a

photometer at 450 nm using a plate reader (Anthos ELISA reader; Labtec Instruments, Salzburg, Austria) with ADAP 1.6 software.

3.6.17 Urinary antioxidant activity

3.6.17.1 Trolox equivalent antioxidant capacity (TEAC)

ECA assay was evalusted following the method of Re et al. (1999). ABTS radical cation was described in section 3.6.3.3. Urine sample (50 µL) was reacted with 150 µL of ABTS radical cation, left for 6 min before measuring at 734 nm and results were reported as mM TE.

3.6.17.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was evaluated following the method of Benzie and Szeto (1999). FRAP reagent preparation was described in section 3.6.3.4. Urine sample (50 µL) and 150 µL of FRAP reagent were added, mixed and then left for 15 min. The reaction was measured at 593 nm using a microplate reader and results were reported as mM $FeSO_4$.

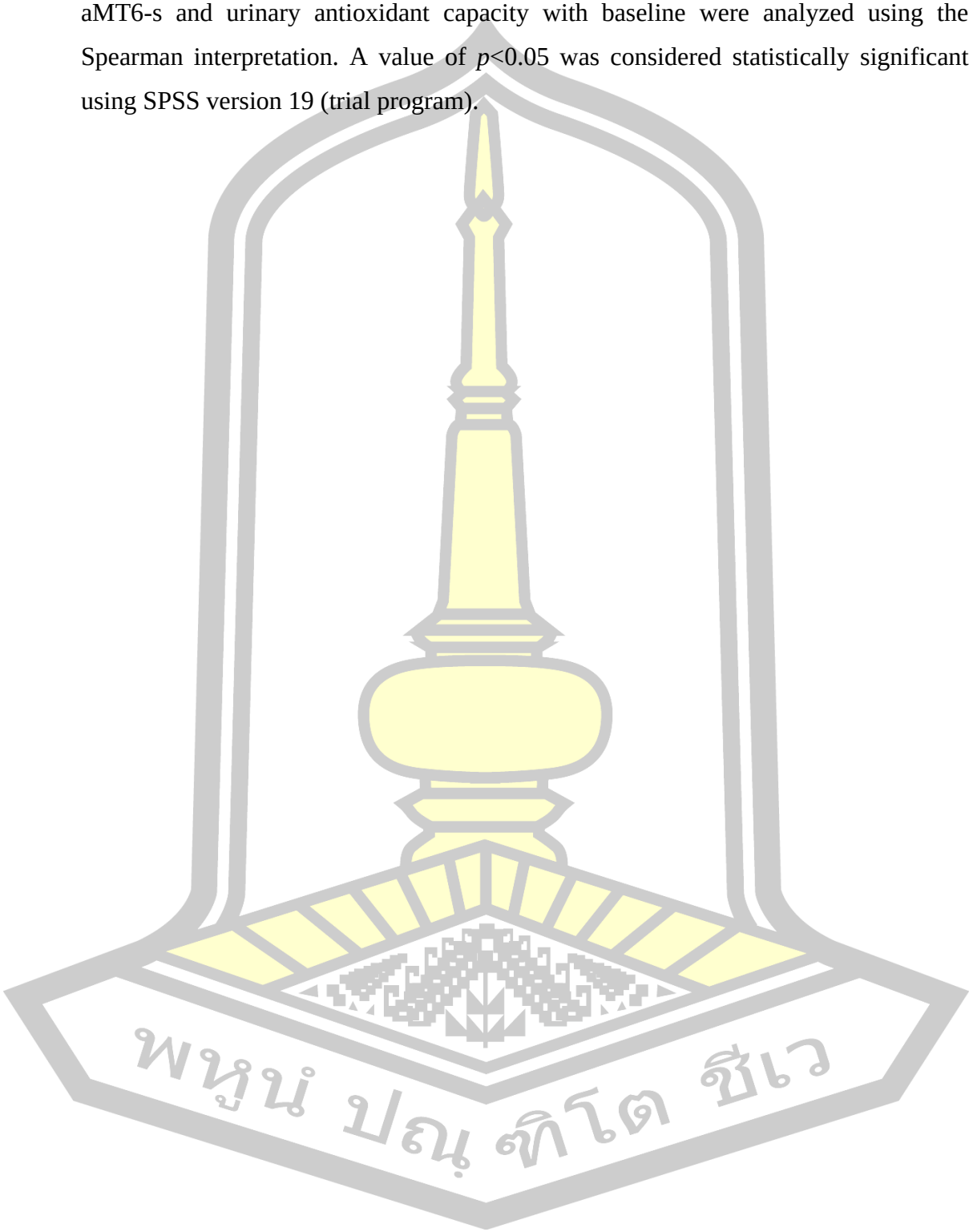
3.6.18 Sensory evaluation

The sensory of PM samples were evaluated using a 9-point hedonic scale described in section 3.6.8.

3.7 Statistical analysis

Results were expressed as means and standard deviations performed from triplicate determinations of each treatment for all experiments. A statistical program was employed for data analyses including F-test (One-way ANOVA in a completely randomized design) and multiple comparisons tests using Duncan Multiple Range Tests to test for significant differences between treatments. The sensory score was evaluated using two-way ANOVA in RBD. Data on response surface methodology (RSM) were collected using Design-Expert Version 7.0 (trial program). Data of urinary 6-sulfatoxymelatonin (aMT6-s) and antioxidant capacity were presented as mean ± SE. Differences between urinary aMT6-s and urinary antioxidant capacity versus baseline

were analyzed using the Wilcoxon signed-rank test. Correlations between urinary aMT6-s and urinary antioxidant capacity with baseline were analyzed using the Spearman interpretation. A value of $p<0.05$ was considered statistically significant using SPSS version 19 (trial program).



CHAPTER 4
RESULTS AND DISCUSSION

Results were reported with discussion as follows:

4.1 Symbols used for data analysis and to express the results

The symbols used for data analysis and to express the results were as follows:

$\overline{X}$ = Mean

SD = Standard deviation

SE = Standard Error

df = Degrees of freedom

F = F-distribution

p = Probability

n = Number of replications

4.2 Results were expressed as follows

Phase I: To determine melatonin and tryptophan content in edible grains, mulberry leaf tea and raw milk.

1.1 Validation method of melatonin and free tryptophan contents

1.2 Melatonin content

1.3 Free tryptophan content

1.4 Total phenolic content (TPC)

1.5 Antioxidant activity

1.6 Correlation of melatonin content and individual antioxidant assays

Phase II (objective 2): To develop pasteurized milk with high melatonin content.

2.1 Composition of pasteurized milk treatments

2.2 Quality of pasteurized milk treatments

2.3 Melatonin, free tryptophan and total phenolic content (TPC) of pasteurized milk treatments

2.4 Antioxidant activity of pasteurized milk treatments

2.5 Sensory evaluation

2.6 Shelf life study

2.7 Optimization of pasteurized milk using mixture design

2.7.1 Composition of pasteurized milk treatments

2.7.2 Quality of pasteurized milk treatments

2.7.3 Melatonin, free tryptophan and total phenolic content (TPC)

2.7.4 Antioxidant activity of pasteurized milk treatments

2.7.5 Sensory evaluation

2.7.6 Shelf life study

Phase III (objective 3): To evaluate melatonin concentration in urine following clinical study methodology.

3.1 Urinary 6-sulfatoxymelatonin (aMT6s)

3.2 Urinary antioxidant activity

3.3 Sensory evaluation

4.3 Results and discussion

4.3.1 Determination of melatonin and free tryptophan content in edible grains, mulberry leaf tea and raw milk (Phase I)

4.3.1.1 Validation method of melatonin and free tryptophan content

Accuracy and precision were determined by validating method parameters (Table 13). Linear equations and correlation curves of melatonin standard were evaluated using concentrations ranging from 0.002-0.125 µg/mL (0.008 to 0.538 mM), and free tryptophan standards ranging from 0.010-0.625 µg/mL (0.048 to 3.060 mM). Correlation coefficients for the calibration curves of melatonin and free tryptophan were 0.9992 and 0.9993. Precision was evaluated using low standard concentrations (melatonin; 0.002 µg/mL and free tryptophan 0.010 µg/mL) and high standard concentrations (melatonin; 0.125 µg/mL and free tryptophan 0.625 µg/mL). Repeatability for seven replications and reproducibility (inter-day study) were measured over three days. Calibration curve of the standard was calculated based on a lower limit of quantification (LLOQ) and an upper limit of quantification (ULOQ)

representing lowest and highest expected concentrations, and recovery was reported. Results showed that relative standard deviation (%RSD) of repeatability of melatonin concentration at 0.002 µg/mL was 14.5% with 13.2% at 0.125 µg/mL, while for free tryptophan concentration at 0.010 µg/mL it was 2.9% with 10.2% at 0.625 µg/mL. Moreover, LLOQ and ULOQ of melatonin at lowest and highest concentrations were 0.002 µg/mL and 0.006 µg/mL, whereas free tryptophan recorded 0.01 and 0.03 µg/mL, respectively. Percentage recoveries of melatonin at 0.004 µg/mL, 0.016 µg/mL and 0.063 µg/mL were 92.4, 85.6 and 89.5%, respectively, while % recoveries of free tryptophan at 0.020, 0.078 and 0.313 µg/mL were 110.6, 111.8 and 99.0%, respectively.

Table 13 Validation method parameters of melatonin and free tryptophan standards

Parameter	Melatonin standard	Tryptophan standard
Correlation curve (r^2)	0.9992	0.9993
Range of linearity (µg/mL)	0.002-0.125	0.010-0.625
Repeatability (% RSD of lowest and highest concentration, n = 7)	14.5, 13.2	2.9, 10.2
Inter-day precision (% RSD of lowest and highest concentration, different days = 3)	12.1, 9.4	8.2, 1.8
LLOQ / ULOQ (µg/mL)	0.002, 0.006	0.01, 0.03
Recovery (%)	85.6-92.4	99.0-111.8

Typical HPLC chromatograms of the standards and samples are shown in Figure 15. Retention times of free tryptophan and melatonin were roughly at 9.6 min and 13.3 min, respectively.

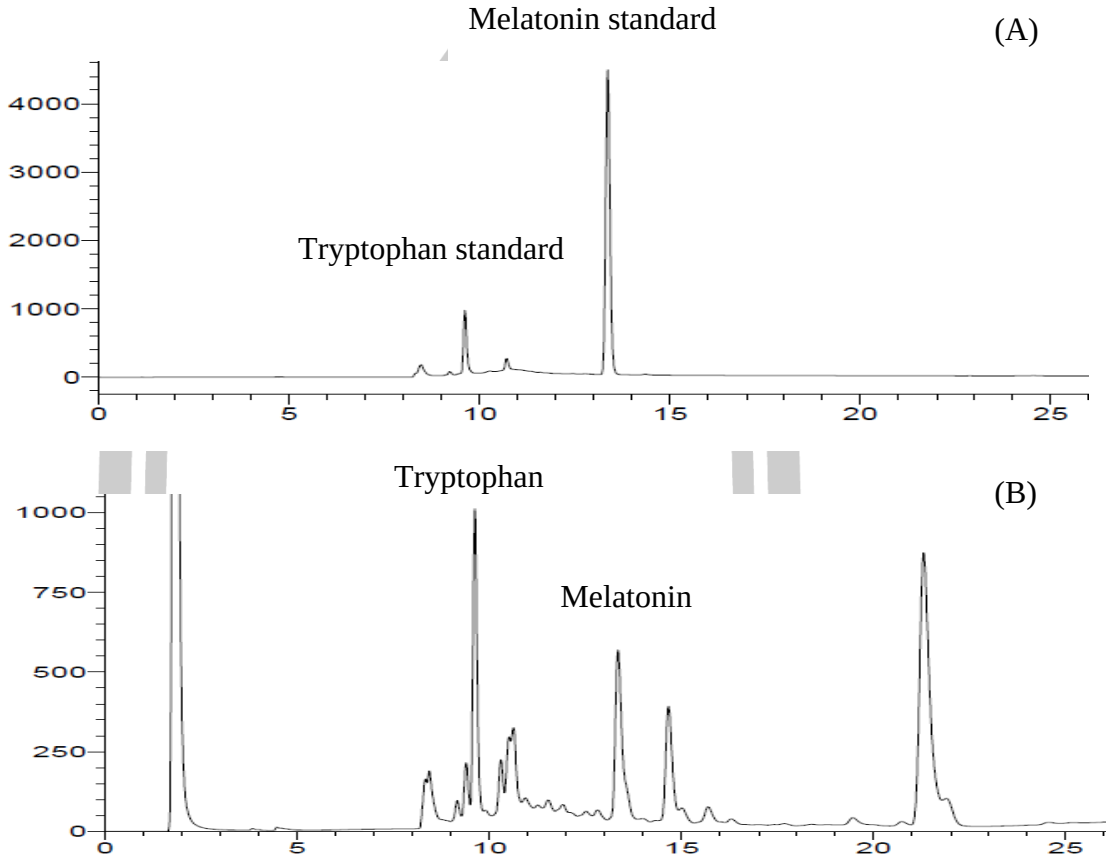


Figure 15 HPLC chromatograms

(A) melatonin (31.25 ng/mL) and free tryptophan standards (156.25 ng/mL), and (B) melatonin and free tryptophan in soybean extract at concentration of 400 mg/mL.

4.3.1.2 Melatonin content

Melatonin contents of grains are shown in Table 14. High concentrations of melatonin were recorded in oilseeds and legumes with highest in white sesame (75.24 ng/g dw) followed by sunflower seed (67.45 ng/g dw) and soybean (56.49 ng/g dw) and lowest in red rice (17.50 ng/g dw). For corn and rice cultivars, highest concentrations were shown in sweet corn and riceberry rice at 34.88 and 36.26 ng/g dw. Our results indicated that melatonin contents were higher in pigmented seeds that

contained oil since germ and bran layers contain high levels of lipids that are easily oxidized by free radicals. After synthesis in plants, melatonin is transported to those tissues for cell protection against oxidative damage; this enhances survival and protects the next plant generation (Arnao & Hernández-Ruiz, 2013). During plant growth, melatonin content in the roots decreases as it is transported to protect new organs from the harsh environment, whereas during flowering, in mature fruits and especially in seeds, melatonin content increases again (Korkmaz et al., 2014). However, variations of melatonin content in seeds are affected by a number of factors including cultivars, harvested time, location and growth conditions (Arnao & Hernández-Ruiz, 2013; Pothinuch & Tongchitpakdee, 2011).

In soymilk powder, melatonin was 46.40 ng/g dw. In raw milk, melatonin was detected at a concentration of 0.03 ng/mL. Milagres et al. (2014) reported melatonin concentration in milk at 02:00 a.m. as 0.039 ng/mL, higher than concentration at 03:00 p.m. as 0.004 ng /mL.

Melatonin in fresh mulberry leaves was found to be 51.57 ng/g dw and mulberry leaf tea produced with different drying methods contained melatonin ranging from 31.57-42.15 ng/g dw (Table 15). Highest melatonin concentration was found in mulberry leaves dried using a freeze dryer (ML-F) which showed no significant difference with that solar drying (ML-S) (4 0 .1 8 ng/g dw). Pothinuch and Tongchitpakdee (2011) reported that melatonin concentration of mulberry leaf cultivars ranged from 40.8-279.6 ng/g dw while melatonin contents in mulberry green tea and black tea brews were 46.5 and 40.6 ng/g dw. Here, melatonin slightly decreased in mulberry leaves dried using a freeze dryer and solar methods compared to a hot air oven. This can be explained by the high oven temperature which destroyed the melatonin structure. Melatonin degradation through heat treatment was supported by results of Moussaoui and Bendriss (2014). They found that melatonin solution stored at 50°C was noticeably degraded than solutions stored at lower temperature, while (de la Puerta et al. (2007) reported melatonin concentration in olive oil without the heat-treatment process as higher than oil extracted using heat.

4.3.1.3 Free tryptophan content

Free tryptophan concentration in seed cultivars ranged from 0.14-2.62 µg/g dw (Table 14). Highest concentration was found in soybean (2.62 µg/g dw) followed by red bean (1.53 µg/g dw) and mung bean (0.85 µg/g dw), with the lowest level in waxy corn (0.14 µg/g dw). High free tryptophan levels in legumes also make them a good source of melatonin, especially soybean and red bean. High free tryptophan content was also found in pigmented rice and oilseeds. Comai et al. (2007a, 2007b) reported that free tryptophan concentration of cereal flours ranged from 3.0-77.9 µg/g dw, highest in spelt seed and lowest in rice, whereas corn recorded 6.6 µg/g dw and legumes ranged from 22.4-582.0 µg/g dw, the highest being chick-peas and lowest in groundnuts.

Free tryptophan in soybean powder was 3.81 µg/g dw. Free tryptophan in milk was 0.19 µg/mL. This result was supported by Biasiolo et al. (1995) who reported that free tryptophan in cow's milk (UHT) was 0.35 µg/g.

Free tryptophan in fresh mulberry leaves was 0.21 µg/g dw and mulberry leaf tea ranged from 0.08-0.12 µg/g dw (Table 15). The highest value was found in freeze dried mulberry leaves and lowest in leaves dried in a hot air oven. These results were supported by Jyothi et al. (2018) who reported that total free amino acid in tender, medium and coarse leaves of four mulberry leaf cultivars ranging from 9.28-15.33µg/g, 9.31-14.36 µg/g and 8.12-13.62 µg/g, respectively.

Differences of tryptophan concentration between our results and previous research stem from diverse extraction and purification conditions, and also disparities in the dietary sources. Barium hydroxide ($Ba(OH)_2$) and heating at 130°C for about 8 h were previously employed to break down and hydrolyze proteins to release free amino acids; however, our samples were not treated at high temperature and long digestion to screen the relationship between melatonin and free tryptophan content. Total tryptophan analysis results were recorded at high concentrations of milligrams per gram, whereas extraction at low temperature measured only free tryptophan not incorporated into proteins in the microgram per gram range (Comai et al., 2007a). Thus,

differences in amounts of free amino acid and tryptophan depend on the material source, condition and extraction method used.

4.3.1.4 Total phenolic content (TPC)

Phenolic compounds represent potential radical inhibitors and are common in plants especially in seeds, fruits and vegetables. We found highest TPC present in sunflower seeds (30.86 mg GAE/g dw), followed by white sesame (21.42 mg GAE/g dw) and peanut (16.12 mg GAE/g dw), whereas the lowest level was recorded in glutinous rice (RD6) (0.44 mg GAE/g dw) (Table 14). In rice cultivars, TPC ranged from 0.44-6.48 mg GAE/g dw with the highest being pigmented black glutinous rice and the lowest glutinous rice (RD6). In corn cultivars, TPC ranged from 2.28-5.26 mg GAE/g dw with the highest in waxy berry corn (purple) and the lowest in waxy corn (white). In legumes, TPC varied from 10.14-16.12 mg GAE/g dw, with the highest in peanut and lowest in mung bean, whereas red bean and soybean, phenolic contents were not significantly different (11.84 and 11.48 mg GAE/g dw). These results concurred with previous studies; TPC of six sunflower seed hybrids ranged from 31.6-36.1 mg GAE/g dw (Nadeem et al., 2011), thirteen pigmented rice varieties with black and red pericarp ranged from 0.8-6.9 mg GAE/g dw (Sompong, et al., 2011), five pigmented corn cultivars ranged from 2.4-3.2 mg GAE/g dw, lowest in red and highest in high carotenoid cultivar (de la Puerta et al., 2007).

In legumes, corn and rice seed endosperm is a major source of chemical components containing important bioactive compounds such as flavonoids, anthocyanins, and carotenoids (Ramos-Escudero et al., 2012). These are present as pigments, especially in the bran layer and are good sources of oil. Flavonoids and anthocyanins can dissolve in higher polarity polar solvents. Pigmentations in corn and rice cultivars derived from high levels of anthocyanins react with Folin-Ciocalteu reagent similar to polyphenols. High quantities of phenolic compounds are detected in oilseeds which contain high levels of lipids and oils that readily undergo oxidation and hydrolysis, especially under the action of ultraviolet light.

TPC of soymilk powder was 4.00 mg GAE/g dw. In milk was 0.10 mg GAE/mL in accordance with Vázquez et al. (2015) who determined TPC in ten samples

of cow's milk at 0.05 mg GAE/mL whereas goat, sheep and human milk were 0.07, 0.17, and 0.08 mg GAE/mL, respectively.

TPC of mulberry leaves was 32.87 mg GAE/g dw and mulberry leaf tea ranged from 19.07-30.36 mg GAE/g dw with highest and lowest contents found in mulberry leaves dried using a freeze dryer and hot air oven, respectively (Table 15). This result agreed with Wanyo et al. (2011) who reported TPC in fresh mulberry, commercial mulberry leaf tea and mulberry leaf tea dried using far-infrared-hot air (FIR-HA) as 16, 29, and 51 mg GAE/g dw, respectively whereas Iqbal et al. (2012) reported TPC of dried mulberry leaves ranging from 16.21-24.37 mg GAE/g dw.

Table 14 Melatonin, free tryptophan and total phenolic contents in grain cultivars and raw milk

Sample	Melatonin (ng/g dw)	Free tryptophan (µg/g dw)	TPC (mg GAE/g dw)
Red dawk mali rice	17.50±0.40^{f}	0.35±0.01^{e}	3.26±0.02^{hi}
Khao dawk mali 105	18.56±0.57^{f}	0.20±0.00^{gh}	1.47±0.04^{jk}
Black glutinous rice	21.15±0.65^{f}	0.18±0.01^{gh}	6.48±0.09^{f}
Glutinous (RD 6)	21.84±1.52^{f}	0.15±0.01^{h}	0.44±0.02^{k}
Waxy corn	27.04±0.12^{e}	0.14±0.01^{h}	2.28±0.03^{ij}
Wax berry corn	27.97±0.12^{e}	0.23±0.01^{fg}	5.26±0.11^{g}
Mung bean	30.25±0.03^{e}	0.85±0.01^{c}	10.14±0.63^{e}
Sweet corn	34.88±1.02^{d}	0.17±0.01^{gh}	4.21±0.07^{gh}
Riceberry rice	36.26±0.80^{d}	0.74±0.03^{d}	4.31±0.05^{gh}
Peanut	39.43±1.61^{d}	0.21±0.01^{gh}	16.12±1.43^{c}
Red bean	54.79±0.79^{c}	1.53±0.03^{b}	11.84±1.35^{d}
Soybean	56.49±1.45^{c}	2.62±0.14^{a}	11.48±0.49^{d}
Sunflower	67.45±0.96^{b}	0.30±0.03^{ef}	30.86±1.18^{a}
White sesame	75.24±9.66^{a}	0.36±0.02^{e}	21.42±0.36^{b}
Soymilk powder	46.40±4.45	3.81±0.16	4.00±0.20
Raw milk*	0.03±0.003	0.19±0.04	0.10±0.01

Raw milk (*) expressed per mL, dw refers to dry weight. Mean values within a column represent mean ± standard deviation of replicate experiments (n = 3). Different letters indicate significant difference (p<0.05).

Table 15 Melatonin, free tryptophan and total phenolic contents in mulberry leaf

Sample	Melatonin (ng/g dw)	Free tryptophan (µg/g dw)	TPC (mg GAE/g dw)
Fresh mulberry leaves (ML)	51.57±2.41[a]	0.21±0.10[a]	32.87±1.82[a]
ML-Freeze drying (F)	42.15±3.89[b]	0.12±0.01[b]	30.36±1.64[b]
ML-Solar drying (S)	40.18±2.39[b]	0.10±0.00[c]	28.05±1.10[b]
ML-Hot air oven drying (H)	31.57±0.26[c]	0.08±0.00[d]	19.07±0.70[c]

ML means mulberry leaves, dw refers to dry weight. Mean values within a column represent mean ± standard deviation of replicate experiments (n = 3). Different letters indicate significant difference (p<0.05).

4.3.1.5 Antioxidant activity

DPPH, FRAP and ABTS assays of seed extracts are shown in Tables 16-18. Highest antioxidant activity equivalent with standards of Trolox (TE), vitamin E (VE), vitamin C (VC), BHA and melatonin (Mel) were found in sunflower seeds, followed by sesame seeds and black glutinous rice, respectively whereas lowest scavenging efficiency activity was found in glutinous rice (RD6). Scavenging efficiency values of DPPH assay ranged from 0.57-4.18 TE/g dw, 1.41-13.08 mg VE/g dw, 0.50-4.60 mg VC/g dw, 0.72-5.68 mg BHA/g dw and 3.04-192.13 mg Mel/g dw. Scavenging values of FRAP assay ranged from 0.29-2.99 TE/g dw, 0.57-5.69 mg VE/g dw, 0.82-8.38 mg VC/g dw, 0.33-1.87 mg BHA/g dw and 12.54-121.23 mg Mel/g dw. Values for ABTS assay were 1.09-11.75 TE/g dw, 1.53-17.70 mg VE/g dw, 0.72-8.12 mg VC/g dw, 0.38-3.46 mg BHA/g dw and 9.66-240.91 mg Mel/g dw. These results agreed with Al-Bishri and Nabil Danial (2013) who reported that antioxidants of thirteen medicinal seeds, with sunflower and flax seeds showed the highest antioxidant efficiency with the lowest found in basil using DPPH assay. Here, powerful antioxidant activity along with high radical scavenging was also observed in oilseeds, pigmented rice and corns. Daiponmak et al. (2014) recorded the highest DPPH and ABTS radical scavenging activities in purple and red rice with lowest in white rice, whereas Sompong et al. (2011) reported that red rice showed highest antioxidant activity using FRAP

assay followed by black rice. Žilić et al. (2012) reported that pigmented maize genotypes showed higher antioxidant activity using ABTS assay, with highest in orange and lowest in white cultivars. We found that oilseeds showed high antioxidant activity probably due to high amounts of phenolic compounds which play a key role as biological antioxidants.

Antioxidant activity of soymilk powder and milk equivalent with standards of Trolox (TE), vitamin E (VE), vitamin C (VC), BHA and melatonin (Mel) using DPPH, FRAP and ABTS assays were reported as shown in Table 16-18.

Antioxidant activity was affected by different drying methods. Mulberry leaves dried using freeze drying showed highest antioxidant activity followed by solar drying and hot air oven, respectively (Table 19).

Here, mulberry leaves dried using freeze drying and solar drying exhibited high levels of TPC and antioxidant activity as both methods excluded high temperature treatment. Therefore, components of leaf structure and bioactive compound molecules were not destroyed, with greater retention of biological compounds than those dried using a hot air oven. However, drying at low temperature required longer time for the same moisture content.

Bioactive compounds such as phenolics, anthocyanins, tocopherols, tocotrienols and γ-oryzanols provide protection against oxidation of these sensitive molecules. Melatonin is soluble in both high and low polarity environments and can provide additional protection to the antioxidants mentioned above that are soluble only in lipophilic (tocopherols, tocotrienols, γ-oryzanols) or hydrophilic (phenolics, anthocyanins) environments (Milczarek et al., 2010).

Table 16 Scavenging efficiency of grain and milk extracts with various standards using DPPH assay

Sample	DPPH (mg standard equivalent/g dw)				
	TE	VE	VC	BHA	Mel
Glutinous (RD 6)	0.57±0.01^{l}	1.41±0.03^{l}	0.50±0.01^{l}	0.72±0.02^{l}	3.04±0.14^{h}
Mung bean	0.62±0.02^{kl}	1.56±0.05 ^{kl}	0.55±0.02^{kl}	0.79±0.02^{kl}	3.80±0.25^{h}
Waxy corn	0.65±0.02^{jk}	1.65±0.05^{jk}	0.58±0.02^{jk}	0.83±0.02^{jk}	4.36±0.26^{h}
Khao dawk mali 105	0.71±0.03^{j}	1.83±0.10^{j}	0.64±0.04^{j}	0.91±0.04 ^{j}	5.45±0.64^{gh}
Soybean	0.79±0.03^{i}	2.08±0.02^{i}	0.73±0.01^{i}	1.02±0.01^{i}	7.29±0.18^{gh}
Red bean	0.93±0.03^{h}	2.51±0.09^{h}	0.88±0.03^{h}	1.21±0.04^{h}	11.15±0.95^{g}
Sweet corn	1.14±0.02^{g}	3.19±0.07^{g}	1.12±0.02^{g}	1.50±0.03^{g}	19.15±0.94^{f}
Wax berry corn	1.47±0.03^{f}	4.33±0.09^{f}	1.52±0.03^{f}	1.96±0.03^{f}	37.98±1.87^{e}
Peanut	1.51±0.03^{ef}	4.45±0.08^{ef}	1.56±0.03^{ef}	2.01±0.03^{ef}	40.45±1.73^{de}
Riceberry rice	1.56±0.03^{de}	4.62±0.08^{de}	1.62±0.03^{de}	2.08±0.03^{de}	43.92±1.71^{de}
Red dawk mali rice	1.59±0.03^{d}	4.73±0.09^{d}	1.66±0.03^{d}	2.13±0.04^{d}	46.32±2.02^{d}
Black glutinous rice	1.88±0.03^{c}	5.77±0.09^{c}	2.03±0.03^{c}	2.54±0.04^{c}	72.66±2.49^{c}
White sesame	2.12±0.05^{b}	6.65±0.19^{b}	2.34±0.07^{b}	2.88±0.08^{b}	99.84±6.47^{b}
Sunflower	4.18±0.08^{a}	13.08±0.33^{a}	4.60±0.11^{a}	5.68±0.13^{a}	192.13±10.74^{a}
Soymilk powder	1.18±0.02	2.98±0.05	6.11±0.10	4.91±0.07	56.84±2.11
Raw milk*	0.15±0.00	0.41±0.01	0.17±0.01	0.21±0.01	2.20±0.16

Raw milk (*) is expressed per mL, dw refers to dry weight. Mean values within a column are represented as mean ± standard deviation of replicate experiments (n = 3), and different letters indicate significant difference (p<0.05).

Table 17 Scavenging efficiency of grain and milk extracts with various standards using FRAP assay

Sample	FRAP (mg standard equivalent/g dw)				
	TE	VE	VC	BHA	Mel
Glutinous (RD 6)	0.29±0.01^{h}	0.57±0.02^{h}	0.82±0.03^{h}	0.33±0.01^{h}	12.54±0.47^{h}
Khao dawk mali 105	0.58±0.03^{g}	1.15±0.04^{g}	1.63±0.07^{g}	0.36±0.02^{g}	23.20±0.98^{g}
Mung bean	0.58±0.03^{g}	1.15±0.04^{g}	1.64±0.07^{g}	0.37±0.01^{g}	23.35±0.99^{g}
Red bean	0.59±0.01^{g}	1.16±0.02^{g}	1.65±0.03^{g}	0.37±0.01^{g}	23.53±0.40^{g}
Red dawk mali rice	0.60±0.02^{fg}	1.18±0.04^{fg}	1.69±0.06^{fg}	0.38±0.02^{fg}	24.15±0.91^{fg}
Waxy corn	0.63±0.04^{ef}	1.24±0.07^{ef}	1.78±0.01^{ef}	0.40±0.03^{ef}	25.52±0.74^{ef}
Riceberry rice	0.64±0.04^{e}	1.25±0.06^{e}	1.81±0.10^{e}	0.41±0.02^{e}	25.94±1.47^{e}
Sweet corn	0.66±0.01^{e}	1.27±0.01^{e}	1.84±0.02^{e}	0.41±0.01^{e}	26.49±0.20^{e}
Soybean	0.77±0.01^{d}	1.46±0.02^{d}	2.16±0.03^{d}	0.48±0.01^{d}	31.22±0.39^{d}
Wax berry corn	0.83±0.02^{c}	1.56±0.03^{c}	2.32±0.05^{c}	0.52±0.01^{c}	33.77±0.73^{c}
Black glutinous rice	0.83±0.02^{c}	1.56±0.03^{c}	2.32±0.04^{c}	0.52±0.01^{c}	33.78±0.63^{c}
Peanut	0.86±0.02^{c}	1.60±0.03^{c}	2.40±0.05^{c}	0.54±0.01^{c}	35.02±0.70^{c}
White sesame	1.65±0.01^{b}	3.10±0.02^{b}	4.63±0.03^{b}	1.03±0.01^{b}	67.36±0.39^{b}
Sunflower	2.99±0.04^{a}	5.69±0.07^{a}	8.38±0.11^{a}	1.87±0.03 ^{a}	121.23±1.75^{a}
Soymilk powder	0.83±0.01	1.56±0.02	2.40±0.03	0.54±0.01	35.61±0.04
Raw milk*	0.13±0.00	0.27±0.01	0.40±0.01	0.09±0.00	5.71±0.17

Raw milk (*) is expressed per mL, dw refers to dry weight. Mean values within a column are represented as mean ± standard deviation of replicate experiments (n = 3), and different letters indicate significant difference (p<0.05).

Table 18 Scavenging efficiency of grain and milk extracts using ABTS assay

Sample	ABTS (mg standard equivalent/g dw)				
	TE	VE	VC	BHA	Mel
Glutinous (RD 6)	1.09±0.01^{j}	1.53±0.02^{jk}	0.72±0.01^{jk}	0.38±0.01^{j}	9.66±0.16^{i}
Khao dawk mali 105	1.16±0.02 ^{j}	1.63±0.02^{j}	0.76±0.01^{j}	0.40±0.01^{j}	10.60±0.17^{i}
Mung bean	1.16±0.01^{j}	1.63±0.02^{j}	0.77±0.01^{j}	0.41±0.01^{j}	10.60±0.16^{i}
Red bean	1.81±0.03^{i}	1.34±0.02^{k}	0.63±0.01^{k}	0.34±0.00^{k}	8.01±0.13^{i}
Red dawk mali rice	2.17±0.02^{h}	3.13±0.03^{h}	1.45±0.02^{h}	0.71±0.01^{h}	27.05±0.40^{g}
Waxy corn	2.28±0.01^{h}	2.69±0.03^{i}	1.25±0.01^{i}	0.62±0.01^{i}	21.72±0.27^{h}
Riceberry rice	3.16±0.03^{g}	3.41±0.11^{g}	1.58±0.05^{g}	0.77±0.02^{g}	30.65±1.44^{g}
Sweet corn	3.35±0.02^{f}	4.93±0.02f	2.28±0.01^{f}	1.05±0.01^{f}	51.95±0.35^{f}
Soybean	3.56±0.02^{e}	5.24±0.02^{e}	2.42±0.01^{e}	1.11±0.01^{e}	56.80±0.35^{e}
Wax berry corn	3.75±0.01^{d}	5.53±0.02^{d}	2.56±0.01^{d}	1.16±0.01^{d}	61.41±0.22^{c}
Black glutinous rice	4.84±0.05^{c}	7.23±0.08^{c}	3.33±0.04^{c}	1.46±0.02^{c}	90.13±1.44^{c}
Peanut	4.90±0.03^{c}	7.31±0.04^{c}	3.37±0.02^{c}	1.47±0.01^{c}	91.61±0.67^{c}
White sesame	6.22±0.07^{b}	9.39±0.12^{b}	4.30±0.06^{b}	1.82±0.02^{b}	131.17±2.42^{b}
Sunflower	11.75±0.26^{a}	17.70±0.41^{a}	8.12±0.18^{a}	3.46±0.07^{a}	240.94±7.94^{a}
Soymilk powder	4.71 ±0.11	3.19±0.08	6.88 ±0.17	2.21±0.05	68.36±2.36
Raw milk*	0.24±0.02	0.33±0.02	0.16 ±0.01	0.11±0.001	1.60±0.16

Raw milk (*) is expressed per mL, dw refers to dry weight. Mean values within a column are represented as mean ± standard deviation of replicate experiments (n = 3), and different letters indicate significant difference (p<0.05).

Table 19 Scavenging efficiency of mulberry leaf extracts with various standards using DPPH, FRAP and ABTS assays

Assay	Sample	DPPH (mg standard equivalent/g dw)				
		TE	VE	VC	BHA	Mel
DPPH	Fresh mulberry leaves	5.04±0.14^{a}	18.39±0.59^{a}	6.45±0.21^{a}	7.16±0.20^{a}	189.99±13.87^{a}
	ML-Freeze drying	4.90±0.15^{ab}	17.68±0.64^{ab}	6.21±0.22^{ab}	6.95±0.22^{ab}	165.11±13.38^{b}
	ML-Solar drying	4.75±0.12^{bc}	16.95±0.52^{bc}	5.95±0.18^{bc}	6.70±0.18^{bc}	145.75±10.29^{bc}
	ML-Hot air oven	4.55±0.13^{d}	16.24±0.55^{d}	5.70±0.19^{d}	6.42±0.19^{d}	140.00±10.94^{c}
FRAP	Fresh mulberry leaves	0.60±0.01^{a}	1.07±0.01^{a}	1.67±0.01^{a}	0.37±0.00^{a}	24.79±0.23^{a}
	ML-Freeze drying	0.57±0.01^{b}	1.03±0.01^{b}	1.58±0.02^{b}	0.35±0.00^{b}	23.45±0.29^{b}
	ML-Solar drying	0.51±0.01^{c}	0.95±0.02^{c}	1.44±0.03^{c}	0.32±0.01^{c}	21.14±0.44^{c}
	ML-Hot air oven	0.32±0.02^{d}	0.62±0.02^{d}	0.89±0.03^{d}	0.20±0.01^{d}	12.66±0.48^{d}
ABTS	Fresh mulberry leaves	3.75±0.15^{a}	5.54±0.24^{a}	2.56±0.11^{a}	1.16±0.04^{a}	60.71±3.72^{a}
	ML-Freeze drying	3.51±0.13^{b}	5.15±0.20^{b}	2.38±0.10^{b}	1.10±0.04^{b}	53.76±3.04^{b}
	ML-Solar drying	2.83±0.12^{c}	4.11±0.18^{c}	1.91±0.10^{c}	0.91±0.03^{c}	38.49±2.42^{c}
	ML-Hot air oven	2.06±0.02^{d}	2.95±0.03^{d}	1.38±0.02^{d}	0.68±0.05^{d}	24.36±0.03^{d}

ML means mulberry leaves, dw refers to dry weight. Mean values within a column are represented as mean ± standard deviation of replicate experiments (n = 3), and different letters of each standard indicate significant difference (p<0.05).

4.3.1.6 Correlation between melatonin and individual antioxidant assays

Correlation coefficients between melatonin and antioxidant assays among seed extracts are shown in Table 20. Results indicated that higher melatonin content positively correlated with free tryptophan (r^2 = 0.539, p< 0.05) and TPC (r^2 = 0.829, p< 0.01). There was no significant correlation of melatonin content with DPPH (r^2 = 0.295) or ABTS (r^2 = 0.437). Melatonin, though a potent antioxidant (Tan *et al*., 2000), showed little reactivity with DPPH due to steric reasons and the presence of many other antioxidants in the samples may mask the correlation; however, significant correlation was found with FRAP assay (r^2 = 0.578, p<0.05). TPC showed significant

correlation with free tryptophan (r^2 = 0.574, p <0.05), DPPH (r^2 = 0.543, p<0.05), ABTS (r^2 = 0.572, p<0.05) and FRAP (r^2 = 0.713, p<0.01). These results indicated that the seeds contained various bioactive compounds with powerful broad radical type scavenging activity.

Table 20 Correlation between melatonin, TPC, free tryptophan content and antioxidant assays of grain extracts

Correlation	Melatonin	Free tryptophan	TPC	DPPH	ABTS	FRAP
Melatonin	1.000	0.539*	0.829**	0.295	0.437	0.578*
TPC	-	0.574*	1.000	0.543*	0.572*	0.713**

(*) correlation is significant at p<0.05; (**) correlation is significant at p<0.01. Values are expressed as mean ± standard deviation of replicate experiments (n = 3) using Spearman's rank correlation test.

Selection of materials to develop pasteurized milk with high melatonin content in Phase I considered those with high melatonin, free tryptophan, total phenolic content and antioxidant activity which were suitable for application in pasteurized milk products (Tables 21-22). Fresh mulberry leaf presented highest melatonin and antioxidants but this material was not appropriate for adding in milk because its properties were different from tea. To improve the quality of mulberry leaf, the tea was produced using different drying methods. Mulberry leaf tea dried using solar energy was selected as it contained high melatonin content with antioxidant activity. There was no significant difference between freeze dried and solar drying methods but the latter could be performed at lower cost. However, adding high levels of mulberry leaf tea may influence product appearance, smell and color. Therefore, limited used was considered and other materials were sourced to increase melatonin content in pasteurized milk.

Previous studies indicated that seeds contained high melatonin content (Manchester et al., 2000). Here, fourteen seed cultivars were tested to determine melatonin, free tryptophan and antioxidants. The best was selected to fortify pasteurized

milk mulberry leaf tea to improve melatonin content and antioxidant activity. Soybean showed high melatonin, antioxidant activity and highest free tryptophan content and contained high protein, phenolics and isoflavones (Tyug, Prasad, & Ismail, 2010). Soybean was also cheaper than sunflower and sesame seeds. The physical appearance of soymilk powder was similar to milk color with a savory smell. Therefore, we expected the consumer to readily accept soymilk because it is popular and widely consumed for health benefits.

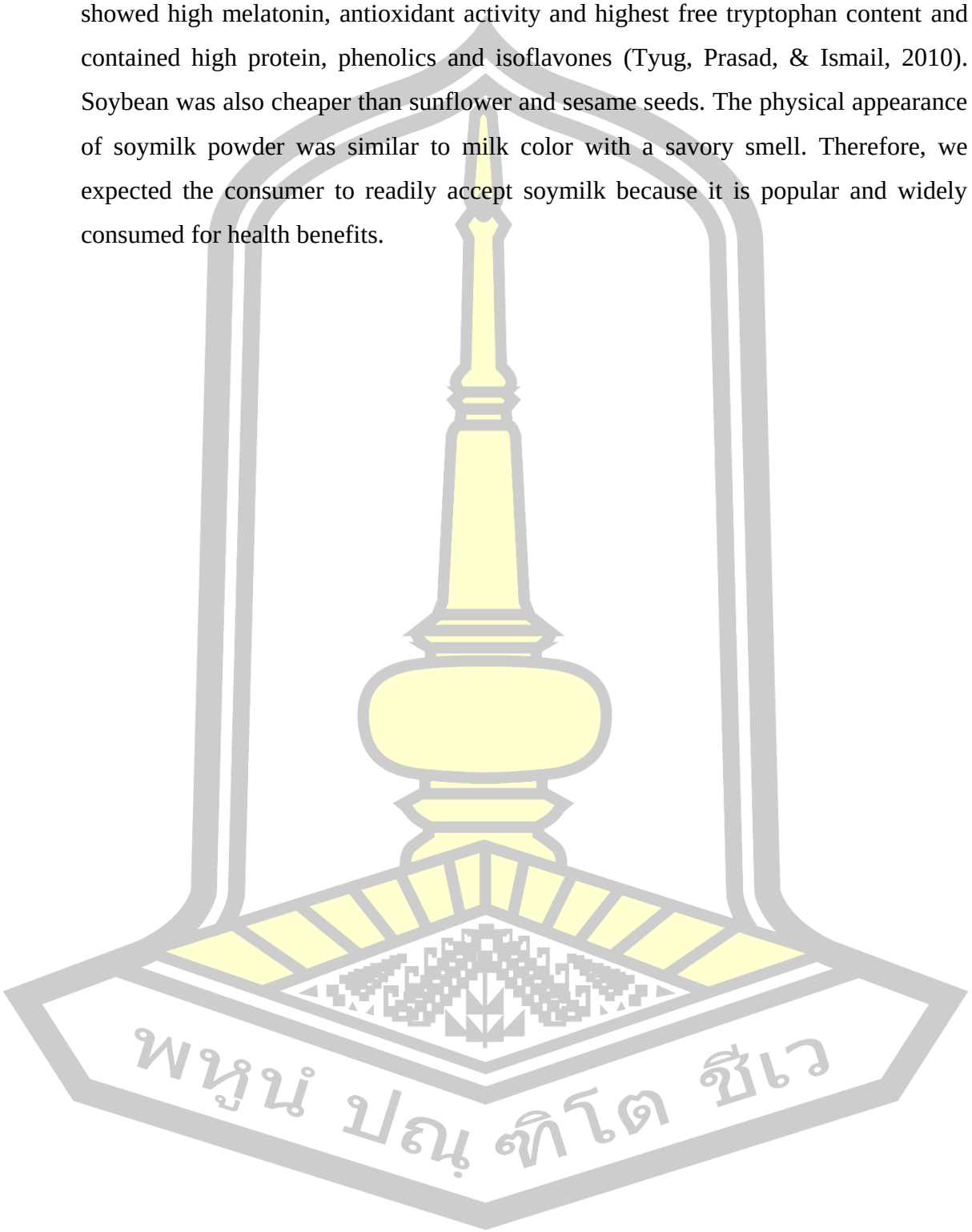


Table 21 Comparison of the top four grain materials for application in pasteurized milk with high melatonin content

Material	Advantages	Limits	Cost (baht/kg)
White sesame	- Melatonin (75.24 ng/g dw), free tryptophan (0.36 µg/g dw) and TPC (21.42 mg GAE/g dw) - Application for snack bar and beverages - Light white color, easy to find and high in nutrients and bioactive compounds	- Difficult to prepare as a fine powder and problems in the process used as difficult to dissolve and high oil - Unique smell - Expensive	210.0
Sunflower	- Melatonin (67.45 ng/g dw), free tryptophan (0.30 µg/g dw) and TPC (30.86 mg GAE/g dw) - Application for roasted cereal snack products - Light brown color, easy to find and high in nutrients and bioactive compounds	- Difficult to prepare as a fine powder and problems in the process used as difficult to dissolve and high oil - Most expensive - Unique smell	312.5
Soybean	- Melatonin (56.49 ng/g dw), free tryptophan (2.62 µg/g dw) and TPC (11.48 mg GAE/g dw) - Application for soymilk powder and widely consumed as fresh soymilk - Light yellow color, easy to find and widely consumed and high in nutrients and bioactive compounds	- Easy to prepare and dissolve - Very cheap - Unique smell	42.0
Red bean	- Melatonin (54.79 ng/g dw) free tryptophan (1.53µg/g dw) and TPC (11.84 mg GAE/g dw) - Application for snacks and desserts - Light white color, easy to find and widely consumed and high in nutrients especially protein, amino acid and minerals	- Easy to prepare and dissolve - Medium price - Unique smell	84.0

Table 22 Comparison of mulberry leaf tea drying methods for application in pasteurized milk with high melatonin content

Material	Advantages	Limits	Cost (baht/batch of drying)
Mulberry leaf tea - Freeze drying	- High of bioactive compounds contents, melatonin (42.15 ng/g dw), free tryptophan (0.12 µg/g dw) and TPC (30.36 mg GAE/g dw) - Bright green color more than solar drying - Easy to find the material - High efficiency drying method and hygienic	- High level use affected smell, color, appearance and taste - Expensive	2,880
Mulberry leaf tea - Solar drying	- High of bioactive compounds contents, melatonin (40.18 ng/g dw), free tryptophan (0.10 µg/g dw) and TPC (28.05 mg GAE/g dw) - Light green color - Easy to find material - Original drying method	- High level use affected smell, color, appearance and taste - No cost	No cost

Remark; cost considered only the equipment used.

4.3.2 Development of pasteurized milk with high melatonin content (Phase II)

4.3.2.1 Composition of pasteurized milk treatments

Nine different pasteurized milk treatments were formulated and their composition were evaluated (Table 23). Fat ranged from 3.89-4.64%, solid not fat (SNF) 9.85-11.67%, lactose 5.72-6.00%, protein 4.32-5.59% and total solids (TS) 13.82-16.31%. Highest composition was found in treatment 9 consisting of milk (85.80%), mulberry leaf tea (6.00%) and soymilk powder (6.00%), whereas the lowest composition was treatment 1 consisting of milk (93.80%), mulberry leaf tea (2.00%) and soymilk powder (2.00%) which showed no significant difference with treatment 2. These results indicated that high levels of protein, fat, SNF and TS in pasteurized milk treatments resulted from addition of large amounts of soymilk powder and mulberry leaf tea. Soybean is abundant in protein, carbohydrate and oil content; it improves nutrients and the physiochemical properties of milk. Kpodo et al. (2013) found that protein, fat, carbohydrate and total solids content increased with increasing soy, peanut and cow milk in the formulation.

Table 23 Composition of nine pasteurized milk treatments

Treatment	%SMP	%MLT	%RM	pH	%Fat	%SNF	%Lactose	%Protein	%TS
Raw milk	-	-	100.00	6.72±0.01	3.35±0.02	8.37±0.01	5.38±0.03	3.05±0.09	11.72±0.03
1	2.00	2.00	93.80	6.85±0.01^{cd}	3.89±0.08^{c}	9.93±0.07^{c}	5.81±0.02^{b}	4.32±0.03^{f}	13.82±0.15^{d}
2	2.00	4.00	91.80	6.76±0.03^{e}	3.94±0.12^{c}	9.97±0.11^{c}	5.72±0.04^{b}	4.48±0.04^{de}	13.91±0.08^{d}
3	2.00	6.00	89.80	6.85±0.02^{cd}	4.01±0.10^{c}	9.85±0.13^{c}	5.96±0.12^{a}	4.35±0.04^{ef}	13.87±0.13^{d}
4	4.00	2.00	91.80	6.89±0.02^{ab}	4.19±0.08^{b}	10.33±0.08^{b}	5.81±0.05^{b}	4.50±0.05^{d}	14.52±0.16^{c}
5	4.00	4.00	89.80	6.83±0.02^{d}	4.26±0.07^{b}	10.38±0.09^{b}	5.82±0.02^{b}	4.87±0.03^{c}	14.64±0.16^{c}
6	4.00	6.00	87.80	6.85±0.01^{cd}	4.31±0.06^{b}	10.47±0.10^{b}	5.95.±0.09^{a}	4.76±0.23^{c}	14.78±0.14^{c}
7	6.00	2.00	89.80	6.91±0.02^{a}	4.53±0.08^{a}	11.52±0.07^{a}	6.07±0.07^{a}	5.27±0.06 ^{b}	16.04±0.15^{b}
8	6.00	4.00	87.80	6.90 ±0.02^{a}	4.60±0.08^{a}	11.64±0.02^{a}	5.98±0.05^{a}	5.49±0.04^{a}	16.24±0.10^{ab}
9	6.00	6.00	85.80	6.87±0.02^{bc}	4.64±0.09^{a}	11.67±0.12^{a}	6.00±0.08^{a}	5.59±0.02^{a}	16.31±0.20^{a}

Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n =3) and different letters indicate significant difference (p<0.05). SNF refers to solid not fat, TS refers to total solids.

4.3.2.2 Quality of pasteurized milk treatments

Quality of nine pasteurized milk treatments were measured, the results are shown in Table 24. pH value of nine different pasteurized milk treatments as ranging from 6.76-6.91, temperature of pasteurized milk after process ranged 4-5°C and phosphatase was not detected. Color parameter results of all treatments indicated that lightness (L*) value was highest in treatments with least amounts of mulberry leaf tea added, similar to green (a*) and yellow (b*) color values. Lightness decreased when mulberry leaf tea increased while addition of soymilk powder enhanced the yellow color. Our results indicated that the green color appeared due to chlorophyll pigment release by the leaf. However, color of the leaves changed while the tea brewed and affected their physiochemical properties. The dark green color resulted from roasting and drying processes. After the tea boiled, chlorophyll was released as the waxy constituents of the leaves melted and the pigments dispersed. These results concurred with Shokery et al. (2017) reported that L* values of yogurt decreased with addition of extracted leaves while a* values increased and became redder with addition of green tea and greenish with addition of moringa leaves. Sawale et al. (2015) found that decreasing lightness and increasing yellow and redness in milk infused with herbs were due to anthocyanins in the herbs.

Milk spoilage from microbial contamination decreases shelf life and impacts on smell and taste. However, usage of suitable heat treatment can destroy microbes and have a negligible effect on milk properties. Microbial count is an index used for confirmation of milk safety. Microbial numbers after pasteurization ranged from 30-50 cfu/mL. Our results were supported by Gnan et al. (2013) who reported total microbial count of cow milk after pasteurization as $7.9x10^2$ cfu/mL at day 1 of storage time. Banik et al. (2015) found that pasteurized milk samples selected from different locations had microbial counts ranging from 0 to $1.8 x10^3$ cfu/mL. Lower microbial counts can be achieved by monitoring the milk source, pasteurization and usage of aseptic techniques during the handling of equipment and surface area.

Table 24 Quality of nine pasteurized milk treatments

Treatment	%SMP	%MLT	%RM	pH	Temp.	Phosphatase test	Colony (cfu/mL) (ns)	Color values		
								L*	a*	b*
Raw milk	-	-	-	6.72±0.01	5.6±0.1	nd	1.67 x10^5	82.35±0.19	-4.47±0.04	3.57±0.02
1	2.00	2.00	93.80	6.85±0.01^{cd}	4.8±0.2^{c}	nd	3.00 x 10	74.72±028^{a}	-14.86±0.38^{e}	22.63±0.30^{a}
2	2.00	4.00	91.80	6.76±0.03^{e}	5.3±0.2^{a}	nd	3.67 x 10	66.15±0.59^{e}	-11.20±0.33^{c}	19.20±0.32^{d}
3	2.00	6.00	89.80	6.85±0.02^{cd}	5.4±0.2^{a}	nd	4.33 x 10	61.88±0.18^{g}	-9.87±0.24^{a}	18.04±0.23^{f}
4	4.00	2.00	91.80	6.89±0.02^{ab}	5.0±0.2^{bc}	nd	3.00 x 10	73.05±0.19^{b}	-14.45±0.12^{d}	22.36±0.16^{ab}
5	4.00	4.00	89.80	6.83±0.02^{d}	5.3±0.2^{a}	nd	3.33 x 10	66.79±0.07^{e}	-11.04±0.04^{c}	19.75±0.03^{c}
6	4.00	6.00	87.80	6.85±0.01^{cd}	4.9±0.1^{c}	nd	4.00 x10	64.37±0.43^{f}	-9.77±0.04^{a}	18.77±0.76^{e}
7	6.00	2.00	89.80	6.91±0.02^{a}	5.1±0.2^{abc}	nd	5.00 x10	72.09±0.13^{c}	-14.10±0.23^{d}	22.01±0.28^{b}
8	6.00	4.00	87.80	6.90 ±0.02^{a}	4.8 ±0.2^{c}	nd	3.00 x10	69.14±0.58^{d}	-10.41±0.19^{b}	19.65±0.34^{c}
9	6.00	6.00	85.80	6.87±0.02^{bc}	5.2±0.1^{ab}	nd	3.67 x 10	65.04±0.64^{f}	-9.52±0.09^{a}	18.57±0.18 ^{e}

L* refers to light (+) and black (-), a* refers to red (+) and green (-), b* refers to yellow (+) and blue (-) whereas nd means not detect and ns means no significant difference. Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n =3) and different letters indicate significant difference (p<0.05).

4.3.2.3 Melatonin, free tryptophan and total phenolic content (TPC) of pasteurized milk treatments

Melatonin, free tryptophan and TPC in pasteurized milk with different concentrations of ingredients were significantly different (p<0.05) for all treatments (Table 25). Highest concentrations were found in treatment 9 with each 6.00% mulberry leaf tea and soymilk powder, whereas lowest concentrations were found in treatment 1 with 2.00% of each ingredient. Highest concentrations of melatonin, free tryptophan and TPC were 4.34 ng/mL, 0.47 µg/mL and 117.99 mg GAE/100 mL, respectively. Previous studies regarding melatonin content in beverages reported that variation of melatonin concentration in red wines ranged from 0-129.5 ng/mL (Rodriguez-Naranjo et al., 2011), orange juice ranged from 3.15-21.80 ng/mL (Fernández-Pachõn et al., 2014) with 0.5 ng/mL in grape juice (Mercolini et al., 2012). Gad and El-Salam (2010) found that phenol contents increased with increasing amounts of green tea and rosemary blended with skim milk. Our results indicated that high concentrations of melatonin, free tryptophan and TPC were associated with levels of ingredients used and concentration of the compounds. Increasing mulberry leaf tea and soymilk powder in milk improved melatonin, free tryptophan and TPC contents. Moreover, loss of compounds may occur during the pasteurization process, possibly due to heat treatment, filtration and hominization processes. Melatonin concentration with no heat treatment or chemical processing was higher than untreated products (de la Puerta et al., 2007). Loss of melatonin during homogenization process combined with a liquid-liquid extraction procedure was higher than using an ultrasonic technique combined with solid phase extraction (Pothinuch & Tongchitpakdee, 2011).

Table 25 Melatonin, free tryptophan and total phenolic content (TPC) of pasteurized milk treatments

Treatment	%SMP	%MLT	%RM	Melatonin (ng/mL)	Free tryptophan (µg/mL)	TPC (mg GAE/100 mL)
1	2.00	2.00	93.80	0.53±0.08^{g}	0.04±0.00^{f}	48.85±2.10^{h}
2	2.00	4.00	91.80	0.99±0.49^{f}	0.06±0.00^{f}	60.07±3.40^{f}
3	2.00	6.00	89.80	1.11±0.01^{f}	0.07±0.00^{f}	92.33±1.91^{c}
4	4.00	2.00	91.80	1.44±0.11^{e}	0.15±0.00^{e}	54.69±3.00^{g}
5	4.00	4.00	89.80	1.60±0.06^{e}	0.26±0.02^{d}	69.77±2.92^{e}
6	4.00	6.00	87.80	1.99±0.09^{d}	0.34±0.03^{c}	105.09±3.58^{b}
7	6.00	2.00	89.80	2.70±0.14^{c}	0.37±0.03^{bc}	62.99±1.60^{f}
8	6.00	4.00	87.80	3.26±0.10^{b}	0.41±0.03^{b}	77.83±2.92^{d}
9	6.00	6.00	85.80	4.34±0.37^{a}	0.47±0.03^{a}	117.99±3.95^{a}

Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n = 3) and different letters indicate significant difference (p<0.05).

4.3.2.4 Antioxidant activity of pasteurized milk treatments

Antioxidant activity was reported as equivalents with TE, VE, VC, BHA and Mel standards using DPPH, FRAP and ABTS assays with significant difference (p<0.05) for all treatments (Tables 26-28). Antioxidant equivalents represent antioxidant capacity of bioactive compounds in the sample compared to the standard. Results were in accordance with the trend of melatonin, free tryptophan and TPC. Highest antioxidant activity equivalents for all standards were found in treatment 9 and lowest activity was found in treatment 1. Our results cannot compare antioxidant capacity between antioxidant standards as each standard has a different structure and molecules that can be exerted on the radical species. However, high values between treatments of each standard represent high antioxidant capacity in sample equivalents with the individual standard. Results indicated that standards equivalent values markedly increased with increasing amounts of soymilk powder and mulberry leaf tea

supplemented in milk. Both ingredients improved the antioxidant capacity as they contain numerous bioactive compounds especially polyphenols, flavonoids and melatonin. Phenol structure bonds were broken during heat treatment and a complex was released with the protein in milk (Gad & El-Salam, 2010). Milk consists of diverse compounds including vitamin C, vitamin A, α-tocopherol and phenolics (Khan et al., 2017). These exhibit strong antioxidant properties and can neutralize radicals through hydrogen atom transfer (HAT) and electron transfer (ET) by reducing the Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. However, antioxidant efficiency depends on the molecules of the antioxidant compounds and mechanisms of the radical species.

Table 26 Antioxidant activity of pasteurized milk treatments using DPPH assay

Treatment	%SMP	%MLT	%RM	DPPH (mg standard equivalent/mL)				
				TE	VE	VC	BHA	Mel
1	2.00	2.00	93.80	0.33±0.01^{f}	1.05±0.04^{f}	0.37±0.02^{f}	0.45±0.01^{f}	15.33±.1.30^{f}
2	2.00	4.00	91.80	0.44±0.04^{e}	1.46±0.14^{e}	0.51±0.05^{e}	0.61±0.05^{e}	32.39±3.67^{e}
3	2.00	6.00	89.80	0.49±0.02^{cd}	1.64±0.08^{d}	0.57±0.03^{d}	0.68±0.03^{d}	42.11±4.26^{d}
4	4.00	2.00	91.80	0.45±0.02^{e}	1.47±0.06^{e}	0.52±0.03^{e}	0.62±0.03^{e}	33.16±3.37^{e}
5	4.00	4.00	89.80	0.50±0.02^{cd}	1.68±0.05^{cd}	059±0.02^{cd}	0.69±0.02^{cd}	44.31±3.23^{cd}
6	4.00	6.00	87.80	0.52±0.02^{c}	1.77±0.08^{c}	0.62±0.02^{c}	0.73±0.03^{c}	50.17±4.87^{c}
7	6.00	2.00	89.80	0.48±0.01^{d}	1.62±0.03^{d}	0.57±0.01^{d}	0.67±0.01^{d}	40.67±1.53^{d}
8	6.00	4.00	87.80	056±0.01^{b}	1.91±0.06^{b}	0.67±0.02^{b}	0.78±0.02^{b}	59.16±3.87^{b}
9	6.00	6.00	85.80	0.60±0.01^{a}	2.08±0.05^{a}	0.73±0.02^{a}	0.84±0.02^{a}	72.42±3.93^{a}

Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n = 3) and different letters indicate significant difference (p<0.05).

Table 27 Antioxidant activity of pasteurized milk treatments using FRAP assay

Treatment	%SMP	%MLT	%RM	FRAP (mg standard equivalent/mL)				
				TE	VE	VC	BHA	Mel
1	2.00	2.00	93.80	0.36±0.02^{g}	0.71±0.03^{g}	1.11±0.04^{g}	0.24±0.01^{f}	15.90±.0.61^{g}
2	2.00	4.00	91.80	0.45±0.02^{e}	0.86±0.03^{e}	1.35±0.06^{e}	0.30±0.01^{d}	20.09±0.98^{e}
3	2.00	6.00	89.80	0.50±0.01^{d}	0.94±0.02^{cd}	1.50±0.03^{cd}	0.33±0.01^{bc}	22.45±0.44^{cd}
4	4.00	2.00	91.80	0.42±0.01^{f}	0.81±0.02^{f}	1.25±0.03^{f}	0.28±0.01^{e}	18.54±0.37^{f}
5	4.00	4.00	89.80	0.50±0.02^{cd}	0.93±0.04^{d}	1.48±0.06^{d}	0.33±0.01^{c}	22.13±0.99^{d}
6	4.00	6.00	87.80	0.53±0.01^{bc}	0.98±0.02^{bc}	1.57±0.03^{bc}	0.35±0.01^{b}	23.69±0.49^{bc}
7	6.00	2.00	89.80	0.48±0.02^{d}	0.91±0.03^{de}	1.43±0.05^{de}	0.32±0.01^{cd}	21.43±0.81^{d}
8	6.00	4.00	87.80	0.55±0.02^{b}	1.02±0.04^{ab}	1.65±0.07^{ab}	0.37±0.02^{a}	24.87±1.13^{ab}
9	6.00	6.00	85.80	0.58±0.02^{a}	1.06±0.03^{a}	1.72±0.05^{a}	0.39±0.01^{a}	26.04±0.74^{a}

Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n = 3) and different letters indicate significant difference (p<0.05).

Table 28 Antioxidant activity of pasteurized milk treatments using ABTS assay

Treatment	%SMP	%MLT	%RM	ABTS (mg standard equivalent/mL)				
				TE	VE	VC	BHA	Mel
1	2.00	2.00	93.80	0.48±0.03^{g}	0.65±0.04^{g}	0.31±0.02^{g}	0.21±0.01^{g}	3.13±.0.29^{g}
2	2.00	4.00	91.80	0.68±0.01^{e}	0.93±0.01^{e}	0.44±0.00^{e}	0.30±0.00^{e}	5.24±0.03^{e}
3	2.00	6.00	89.80	0.79±0.04^{c}	1.10±0.06^{c}	0.52±0.03^{c}	0.35±0.02^{c}	6.66±0.53^{c}
4	4.00	2.00	91.80	0.61±0.01^{f}	0.84±0.01^{f}	0.40±0.00^{f}	0.27±0.01^{f}	4.53±0.04^{f}
5	4.00	4.00	89.80	0.72±0.04^{de}	1.00±0.05^{de}	0.47±0.02^{de}	0.32±0.02^{de}	5.81±0.37^{de}
6	4.00	6.00	87.80	0.88±0.02^{b}	1.23±0.02^{b}	0.58±0.01^{b}	0.39±0.01^{b}	7.78±0.20^{b}
7	6.00	2.00	89.80	0.76±0.03^{cd}	1.05±0.05^{cd}	0.50±0.02^{cd}	0.34±0.02^{cd}	6.25±0.39^{cd}
8	6.00	4.00	87.80	0.92±0.03^{b}	1.28±0.05^{b}	0.60±0.02^{b}	0.41±0.02^{b}	8.31±0.49^{b}
9	6.00	6.00	85.80	1.09±0.04^{a}	1.54±0.06^{a}	0.72±0.03^{a}	0.49±0.02^{a}	10.74±0.58^{a}

Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n = 3) and different letters indicate significant difference (p<0.05).

4.3.2.5 Sensory evaluation

Sensory score was evaluated using a 9-point hedonic scale with five different attributes including color, flavor, taste, texture and overall liking. The best one was selected as the highest score of overall liking. Results showed that all treatments were significantly different (p<0.05) in all attributes (Figure 16). The highest scores of each attribute were found in treatment 5 as mulberry leaf tea (4.00%), soymilk powder (4.00%) and milk (89.80%), whereas the lowest were found in treatment 9 as soymilk powder (6.00%), mulberry leaf tea (6.00%) and milk (85.50%) with no significant difference to treatment 8. The panelists recorded score 7 as like moderately for treatment 5 while they mostly liked treatments containing 2.00% and 4.00% of soymilk powder (1, 2, 3, 4 and 6). The panelists answered that they neither liked nor disliked treatments 7, 8, and 9 with increased levels of soymilk powder at 6.00%. Results indicated that increasing the amount of mulberry leaf tea and soymilk powder affected the appearance of the product and consumer acceptability. Previous studies by Sawale et al. (2015) reported that adding 0.2-0.5% of herb (*Pueraria tuberosa*) in milk resulted in a significant drop in color and appearance, flavor, mouthfeel and overall acceptability. Shokery et al. (2017) found that addition of different plant extracts (green tea and moringa leaves) to yogurt affected the appearance of the product. Thus, supplementation of different materials or levels of ingredients in milk based products influenced product characteristics and consumer satisfaction. The pasteurized milk treatment with the highest score of overall liking was chosen to study shelf life at different storage times.

Figure 16 Sensory score of pasteurized milk treatment using a 9-point hedonic scale Different letters on the top of columns express significant differences ($p<0.05$). For the sensory score, 1= dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8= like very much, and 9= like extremely.

4.3.2.6 Shelf life study

1) Composition of the accepted pasteurized milk

Accepted pasteurized milk (treatment 5) [mulberry leaf tea (4.00%), soymilk powder (4.00%) and milk (89.80%)] was prepared and kept in sterilized bottles and refrigerated at 4°C. Sampling was performed every two days to evaluate milk composition, quality, bioactive compounds and antioxidant activity with results shown in Table 29. Pasteurized milk compositions ranged from 4.31-4.34% for fat, 10.59-10.63% for SNF, 5.70-5.76% for lactose, 4.75-4.79% for protein and 14.91-14.97% for TS. Compositions of pasteurized milk during storage for 8 days showed no significant variation differences. These results concurred with Brodziak et al. (2017) who found that basic components such as fat, protein, lactose and dry matter of opened drinking milk showed no significant differences during refrigerated storage for 7 days.

Table 29 Composition of the accepted pasteurized milk (treatment 5) at different storage times

Day	%Fat (ns)	%SNF (ns)	%Lactose (ns)	%Protein (ns)	%TS (ns)
0	4.34±0.05	10.62±0.14	5.75±0.13	4.77±0.13	14.96±0.18
2	4.34±0.10	10.63±0.06	5.76±0.08	4.79±0.08	14.97±0.15
4	4.33±0.07	10.59±0.10	5.73±0.09	4.76±0.09	14.92±0.18
6	4.31±0.09	10.60±0.05	5.70±0.09	4.77±0.10	14.91±0.14
8	4.32±0.08	10.61±0.10	5.71±0.11	4.75±0.07	14.93±0.17

SNF refers to solid not fat, TS refers to total solids and ns means no significant difference.

2) Quality of the accepted pasteurized milk

Pasteurized milk qualities are shown in Table 30. pH value of pasteurized milk was not significantly difference during storage (pH, 6.70-6.75). Color parameters of lightness (L*) slightly decreased similar with b* value (yellow color),

whereas a* (green color) slightly increased over the 8 days storage period. Changes in color were due to increase in microbial count in accordance with Paul-Sadhu (2016) who found that bacterial count correlated with color values while pH values did not significantly decrease. Increasing bacterial count slightly influenced color parameters L*, -a*, and b* over 30 days of storage time due to bacterial consumption of oxygen for growth. Popov-Raljić et al. (2008) observed that L* and b* values of UHT milk stored at ambient temperature decreased while a* value increased from negative to positive over storage for 90 days. Our results indicated that L* and b* values decreased inversely to a* value due to the mixed ingredients. Green color was obtained from the pigments of mulberry leaf tea and yellow color from soymilk powder. Thus, variations in color can be influenced by several factors including pigments of the ingredients, survival of bacterial population, milk composition and heat treatment processes.

Total microbial count significantly increased (p<0.05) throughout 8 days of storage from 3.9x10 to 9.8x10^3 cfu/mL but the count was still within acceptable limits of 1x10^5 cfu/mL (Notification of the Ministry of Public Health (No. 351), B.E., 2013). Gnan et al. (2013) reported that total microbial count in pasteurized milk increased from day 1 (7.9x10^2 cfu/mL) to day 7 (9.0x10^3 cfu/mL). Pasteurized milk stored at 2°C and 5°C was not immune to contamination by microbial growth but showed delayed contamination (Paul-Sadhu, 2016). However, differences in total microbial populations detected depend on total microbial counts in materials, hygiene, aseptic techniques and processes used.

Table 30 Quality of the accepted pasteurized milk (treatment 5) at different storage times

Day	pH (ns)	Colony (cfu/mL)	Color values L* (ns)	a* (ns)	b* (ns)
0	6.74±0.05	3.89 x10^{a}	67.05±0.06	-10.86±0.07	20.00±0.07
2	6.75±0.05	2.78 x10^{2b}	67.03±0.23	-10.83±0.06	19.95±0.01
4	6.74±0.05	1.69 x10^{3c}	67.00±0.02	-10.84±0.01	19.92±0.04
6	6.72±0.09	5.32 x10^{3d}	67.02±0.07	-10.82±0.05	19.96±0.06
8	6.70±0.10	9.78 x10^{3d}	66.97±0.07	-10.79±0.07	19.94±0.07

L* refers to light (+) and black (-), a* refers to red (+) and green (-), b* refers to yellow (+) and blue (-) and ns means no significant difference. Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n = 3) and different letters indicate significant difference (p<0.05).

3) Melatonin, free tryptophan and total phenolic content (TPC) of the accepted pasteurized milk

Melatonin, free tryptophan and TPC of the accepted pasteurized milk were not significantly different in terms of storage time (Table 31). All compounds gradually decreased from the beginning to the last day of storage. Melatonin ranged from 1.55-1.83 ng/mL and free tryptophan ranged from 0.27-030 µg/mL and TPC ranged from 62.67-67.17 mg GAE/100 mL. Degradation of all compounds resulted from pasteurization as the samples were kept in closed bottles which delayed oxidation by interaction of reactive oxygen species. In addition, storing pasteurized milk at low temperature (4°C) suppresses microbial growth which affects milk quality and bioactive compounds. Previous studies determined that melatonin stored in aqueous solution in pyrogen-free glass vacuums at room temperature, 4°C and -70°C was more stable over 6 months (Cavallo & Hassan, 1995). Moussaoui and Bendriss (2014) indicated that melatonin was stable over 6 days and did not significantly decrease until day 13 on exposure to light and dark conditions at 32°C, 4°C, 25°C and 50°C with no

air. They concluded that oxygen exposure combined with other factors resulted in high degradation.

Table 31 Melatonin, free tryptophan and total phenolic content (TPC) of the accepted pasteurized milk at different storage times

Day	Melatonin (ng/mL) (ns)	Free tryptophan (µg/mL) (ns)	TPC (mg GAE/100 mL) (ns)
0	1.83±0.18	0.28±0.02	67.17±2.26
2	1.79±0.07	0.29±0.02	65.16±1.70
4	1.66±0.11	0.30±0.03	66.65±1.76
6	1.69±0.04	0.26±0.01	64.69±1.13
8	1.55±0.11	0.28±0.01	62.67±2.42

Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n = 3) and ns means no significant difference.

4) Antioxidant activity of the accepted pasteurized milk treatment

Antioxidant activity of the accepted pasteurized milk treatment is shown in Table 32. TE and Mel standards were used to estimate the antioxidant equivalent capacity of compounds which can dissolve both polar and nonpolar phases. Antioxidant activity was not significantly different and slightly reduced over 8 days of storage for all assays. Our results agreed with Ryan and Petit (2010) who demonstrated that antioxidant capacity of FRAP did not significantly decrease during storage of whole milk for 14 days, whereas a significant decrease was found in semi-skimmed milk stored for 7 days and skimmed milk stored for 14 days. Stability of antioxidant activity in pasteurized milk treatment during storage time was due to both soymilk powder and mulberry leaf tea addition as good sources of bioactive compounds. Soybean contains significant polyphenols including isoflavone analogs such as daidzin, genistin, daidzein and genistein (Xu & Chang, 2009), whereas mulberry leaf tea is rich

in polyphenolic compounds such as chlorogenic acid, rutin, isoquercitrin and quercetin 3-(6-malonylglucoside) (Katsube et al. 2009). Moreover, lipid-soluble antioxidants as vitamins E, vitamin A and carotenoids were present in milk (Khan et al., 2017). All these bioactive substances effectively inhibit lipid peroxidation and peroxyl/superoxide which causes cell damage and many diseases (Chen et al. 2003).

Table 32 Antioxidant activity using DPPH, FRAP and ABTS assays of the accepted pasteurized milk at different storage times

Day	DPPH		FRAP		ABTS	
	(mg TE/mL) (ns)	(mg Mel/mL) (ns)	(mg TE/mL) (ns)	(mg Mel/mL) (ns)	(mg TE/mL) (ns)	(mg Mel/mL) (ns)
0	0.51±0.01	47.63±2.69	0.74±0.02	21.96±0.61	0.49±0.01	5.98±0.23
2	0.50±0.01	45.69±1.72	0.72±0.01	22.36±0.93	0.50±0.02	5.72±0.07
4	0.49±0.02	43.20±3.09	0.73±0.03	21.14±1.25	0.48±0.03	5.87±0.36
6	0.51±0.01	47.53±3.96	0.72±0.01	21.66±1.08	0.49±0.02	5.72±0.06
8	0.49±0.01	43.20±3.16	0.70±0.03	20.96±0.96	0.47±0.02	5.32±0.02

Mean values within a column are expressed as mean ± standard deviation of replicate experiments (n = 3) and ns is not significant.

4.3.2.7 Optimization of pasteurized milk using mixture design

Ratios of mulberry leaf tea, soymilk powder and raw milk were evaluated and optimized based on the responses functions of the product. More information concerning the optimized ratio the ingredients were required to obtain a product with high quality. The quality of pasteurized milk is controlled as per the Notification of the (Notification of the Ministry of Public Health (No. 351), B.E., 2013). Parameters of milk quality control standards are shown in Table 33.

Table 33 Specific standards of flavored milk

Composition	Standard control
Temp	≤8 °C
%Fat	≥3.0
%Solid not fat (SNF)	≥7.7
%Protein	≥2.6
Standard plate count	≤50,000
Phosphatase enzyme	Not detected

To evaluate melatonin, free tryptophan, milk composition, antioxidant activity and product acceptability, pasteurized milk with mulberry leaf tea and soymilk powder was formulated by user-defined mixture design. Ingredients were obtained from previous studies as mentioned in section 4.3.2. The accepted treatment 5 with soymilk powder 4.00%, mulberry leaf tea 4.00% and milk 89.80% was used for further study. Lower and upper limits of soymilk powder and mulberry leaf tea used to generate the design were 3.50 and 4.50% for each with raw milk at 88.88-90.80%. This ranges use were considered by the appearance and the bioactive content needed, and sensory study. Adding low amount of ingredients, melatonin and other compounds was decreased whereas adding high amount have effected on the product appearance.

X1=soymilk powder (SMP) ranging from 3.50-4.50%

X2= mulberry leaf tea (MLT) ranging from 3.50-4.50%

X3=raw milk (RM) ranging from 88.80-90.80%

All eleven treatments with consisting of two center points were obtained.

The equation of the predictive model for linear, quadratic and special cubic term is shown as follows:

$$Y=\beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{123}X_1X_2X_3$$

where β_1, β_2, and β_3 represent regression coefficients of the linear model, β_{12}, β_{13}, and β_{23} represent the quadratic model and β_{123} is a special cubic model.

Y represents the response function of estimated regression coefficients.

X_1, X_2 and X_3 express levels of the dependent variables.

1) Composition and quality of pasteurized milk treatments using mixture design.

Results showed that treatment temperature after processing ranged from 4.6-6.1°C and pH ranged from 6.50-6.67. Pasteurized milk compositions consisted of protein (4.65-4.91%), fat (4.18-4.32%), solid not fat (SNF) (10.07-10.42%), total solids (TS) (14.26-14.72%) and lactose (5.61-5.69%). Total microbial count ranged from 1.67-6.67x10 cfu/mL. Color parameters indicated that L* values of all treatments ranged from 63.68-67.43, a* values ranged from (-9.01 to -11.64) and b* values ranged from 18.41-20.85 as shown in Table 34-35. In our study, the highest compositions were found in treatment 3 with 4.50% soymilk powder and mulberry leaf tea and 88.80% raw milk and not significantly different with treatment 1. Lowest values were found in treatment 10 with no significant differences with treatments 9 and 11 for the lowest ratio of soymilk powder (3.50%) added. pH and temperature of pasteurized milk treatments after processing varied with total microbial count less than $1x10^2$ cfu/mL in all treatments. Appearance of pasteurized milk samples showed an increase in lightness (L*) and greenness (a*) for treatments containing low ratios of mulberry leaf tea and soymilk powder and high ratios of raw milk, whereas yellowness (b*) appeared in treatments with increased ratios of soymilk powder.

Table 34 Composition and quality of pasteurized milk treatments using mixture design

Treatment	Formula (%)			Composition (%)				
	SMP	MLT	RM	Fat	SNF	Lactose (ns)	Protein	TS
1	4.50	4.00	89.30	4.32±0.04^{a}	10.40±0.08^{ab}	5.68±0.08	4.88±0.05^{ab}	14.72±0.06^{a}
2	4.50	3.50	89.80	4.31±0.02^{ab}	10.39±0.09^{abc}	5.66±0.05	4.90±0.08^{ab}	14.70±0.10^{a}
3	4.50	4.50	88.80	4.29±0.07^{abc}	10.42±0.09^{a}	5.69±0.02	4.91±0.08^{a}	14.71±0.12^{a}
4	4.00	4.50	89.30	4.26±0.06^{abcd}	10.26±0.08^{bcd}	5.65±0.04	4.80±0.05^{abc}	14.52±0.14^{b}
5	4.00	4.00	89.80	4.23±0.06^{abcd}	10.25±0.07^{de}	5.61±0.11	4.78±0.09^{bcd}	14.48±0.11^{bc}
6	4.00	4.00	89.80	4.22±0.02^{abcd}	10.23±0.06^{de}	5.64±0.04	4.77±0.08^{bcd}	14.45±0.07^{bc}
7	4.00	4.00	89.80	4.23±0.08^{abcd}	10.22±0.11^{de}	5.62±0.05	4.75±0.09^{cd}	14.45±0.11^{bc}
8	4.00	3.50	90.30	4.22±0.08 ^{abcd}	10.11±0.10^{de}	5.65±0.08	4.74±0.09^{cd}	14.33±0.03^{bcd}
9	3.50	4.50	89.80	4.20±0.05^{cd}	10.12±0.06^{de}	5.68±0.06	4.67±0.03^{cd}	14.32±0.09^{d}
10	3.50	3.50	90.80	4.18±0.03^{d}	10.07±0.09^{e}	5.67±0.07	4.65±0.04^{d}	14.25±0.12^{cd}
11	3.50	4.00	90.30	4.20±0.05^{bcd}	10.10±0.08^{de}	5.66±0.06	4.67±0.06^{d}	14.30±0.12^{cd}

SMP is soymilk powder, MLT is mulberry leaf tea and RM is raw milk. SNF refers to solid not fat, TS refers to total solids and ns means no significant difference.

Table 35 Composition and quality of pasteurized milk treatments using mixture design

Treatment	Formula (%)			pH	Temp.	Colony	Color values		
	SMP	MLT	RM			(cfu/mL)	L*	a*	b*
1	4.50	4.00	89.30	6.65±0.03^{ab}	5.60	4.00x10^{abc}	63.68±0.16^{e}	-11.04±0.08^{de}	20.66±0.10^{ab}
2	4.50	3.50	89.80	6.67±0.02^{a}	4.90	4.33x10^{abc}	64.07±0.37^{e}	-11.09±0.24^{def}	20.85±0.12^{a}
3	4.50	4.50	88.80	6.50±0.03^{e}	5.10	6.67x10^{a}	63.70±0.21^{e}	-9.88±0.26^{b}	20.12±0.15^{bcd}
4	4.00	4.50	89.30	6.65±0.01^{ab}	4.80	6.00x10^{ab}	65.94±0.68^{cd}	-9.01±0.30^{a}	18.41±0.60^{f}
5	4.00	4.00	89.80	6.51±0.01^{d}	5.40	4.67x10^{abc}	65.55±0.35^{d}	-10.92±0.17^{de}	19.81±0.28^{cd}
6	4.00	4.00	89.80	6.54±0.01^{cd}	6.10	3.00x10^{bc}	65.87±0.19^{d}	-10.29±0.08^{bc}	19.77±0.14^{d}
7	4.00	4.00	89.80	6.57±0.02^{c}	4.70	3.00x10^{bc}	66.77±0.42^{b}	-10.62±0.10^{cd}	19.18±0.57^{e}
8	4.00	3.50	90.30	6.53±0.02^{de}	5.50	5.67x10^{ab}	65.49±0.12^{d}	-10.00±0.07^{b}	18.99±0.33^{e}
9	3.50	4.50	89.80	6.57±0.02^{c}	4.90	3.33x10^{bc}	66.52±0.05^{bc}	-11.24±0.11^{efg}	20.40±0.08^{abc}
10	3.50	3.50	90.80	6.64±0.02^{b}	4.60	1.67x10^{c}	67.43±0.03^{a}	-11.56±0.60^{fg}	20.00±0.41^{cd}
11	3.50	4.00	90.30	6.67±0.02^{a}	5.40	5.33x10^{ab}	66.84±0.69^{ab}	-11.64±0.34^{g}	19.03±0.34 ^{a}

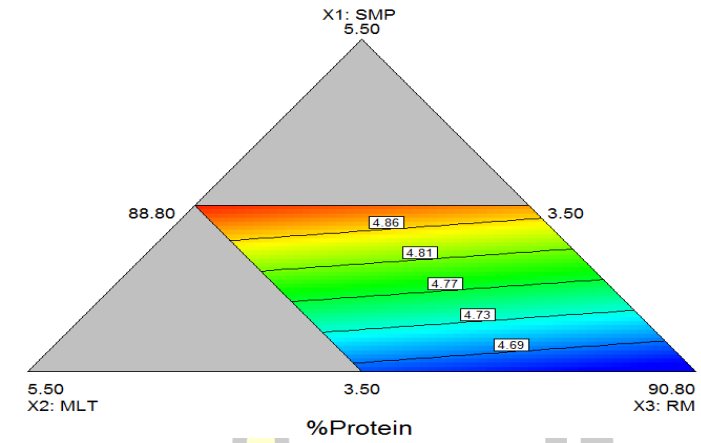
SMP is soymilk powder, MLT is mulberry leaf tea and RM is raw milk. L* refers to light (+) and black (-), a* refers to red (+) and green (-), b* refers to yellow (+) and blue (-) and ns means no significant difference.

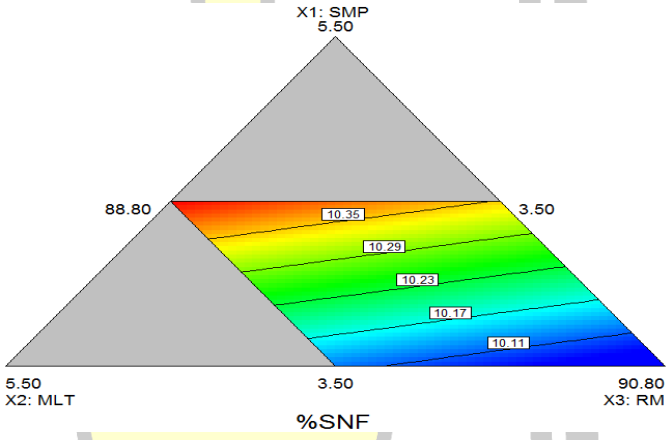
Models and equation predictions of pasteurized milk composition and quality are shown in Table 36. Mathematical models for the composition of pasteurized milk treatments including protein, fat, solid not fat and total solids were significant (p<0.05) in linear terms. Lack of fit was not significant (p>0.05) and regression coefficients (R^2) presented more than 0.8 of all responses whereas lactose presented as a quadratic model. Mathematical models were not used to predict pH, temperature, microbial number (total plate count) and color of b* value as the probability value was higher than 0.05 (p>0.05), whereas L* and a* were significant linear and quadratic models (p<0.05). Highest positive coefficients for protein, fat, solid not fat, total solids and lactose were found in soymilk powder followed by mulberry leaf tea and milk treatment. Therefore, the component with the highest influence was soymilk powder. This can be explained because soybeans are a good source of protein, fat and carbohydrate content; they can improve both nutrient and physiochemical properties of pasteurized milk. Although, mulberry leaves contain a lower concentration of fat than soybean they are still an important source of protein and bioactive compounds. Thus, increasing the chemical components of all pasteurized milk samples related to the amount of ingredients added. Kpodo et al. (2013) studied soy-peanut-cow milk formulations and found that variations of these ingredients influenced the chemical composition and physicochemical properties of soy-peanut-cow milk. Increasing soy, peanut and cow milk ratios also increased protein content, whereas fat content was influenced by increasing peanut. Carbohydrate content and pH values increased with increase in soy milk content. Contour plots of component ratios are shown in Figure 17.

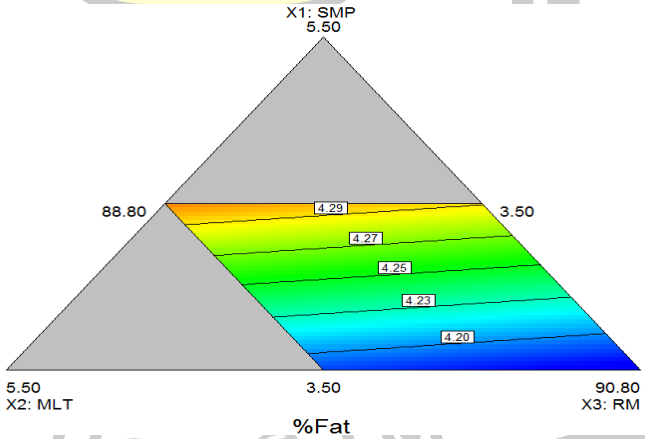
Table 36 Model predictions of the composition and quality of pasteurized milk treatments

Response	Prediction equation	R^2	*p*-value	Lack-of-fit
Composition (%) and quality				
Protein	$0.27X_1+0.07X_2+0.04X_3$	0.9784	<0.0001	0.5886
Fat	$0.15X_1+0.05X_2+0.04X_3$	0.8848	0.0002	0.1136
Solid not fat	$0.40X_1+0.16X_2+0.09X_3$	0.9301	<0.0001	0.1200
Total solids	$0.55X_1+0.21X_2+0.13X_3$	0.9370	<0.0001	0.0840
Lactose	$13.92X_1+5.54X_2+0.10X_3-0.20X_1X_2-0.15X_1X_3-0.06X_2X_3$	0.8485	0.0409	0.6696
pH	$-6.91X_1-23.76X_2-0.03X_3+0.13X_1X_2+0.09X_1X_3+0.27X_2X_3$	0.8154	0.0644	0.1167
Total plate count	$14.77X_1+13.66X_2-0.78X_3$	0.2953	0.2466	0.3064
L*	$-2.30X_1+0.53X_2+0.81X_3$	0.8650	0.0003	0.7299
a*	$-398.95X_1+260.22X_2-0.27X_3+2.37X_1X_2+4.41X_1X_3-2.93X_2X_3$	0.9116	0.0114	0.4823
b*	$+351.44X_1-61.48X_2+0.50X_3-4.24X_1X_2-3.85X_1X_3+0.74X_2X_3$	0.6117	0.3151	0.2118

X1 = SMP = soymilk powder X2 = MLT = mulberry leaf tea X3 = RM = raw milk

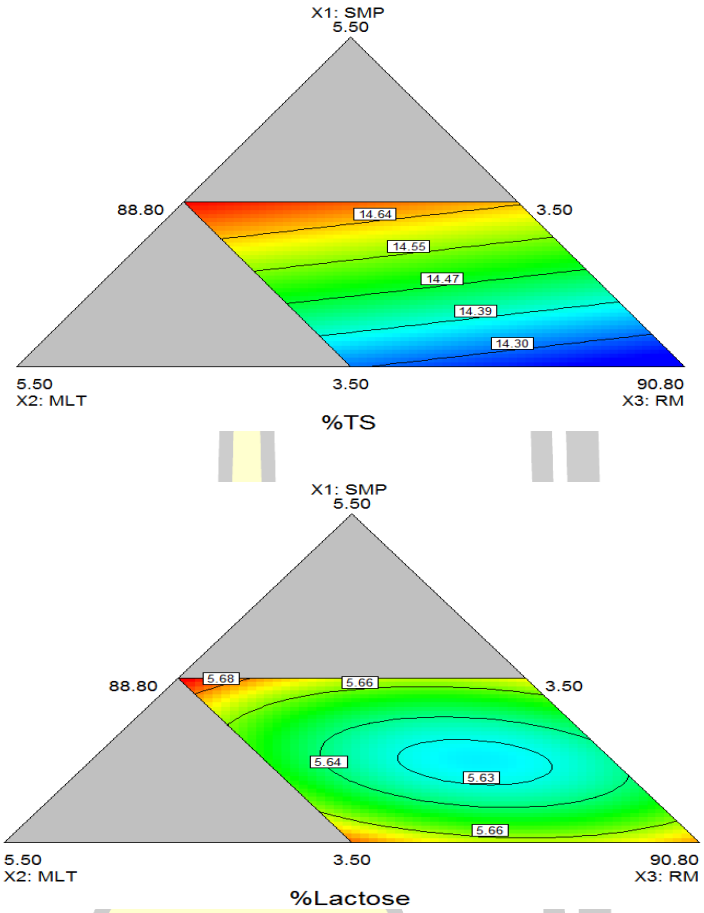






X1 = SMP =Soymilk powder X2 = MLT = Mulberry leaves tea X3 = RM = Raw milk

Figure 17 Contour plots for components of pasteurized milk treatments



X1 = SMP =Soymilk powder X2 = MLT = Mulberry leaves tea X3 = RM = Raw milk

Figure 17 Contour plots for components of pasteurized milk treatments (continued)

2) Melatonin, free tryptophan, total phenolic content (TPC) and antioxidant activity of pasteurized milk treatments using mixture design

Melatonin contents of all eleven treatments ranged from 1.03-1.93 ng/mL, free tryptophan ranged from 0.20-0.30 µg/mL, and TPC ranged from 67.02-73.30 GAE/100 mL. Antioxidant activity reported as equivalent with the Trolox standard (TE) showed that all treatments ranged from 0.47-0.54 mg TE/mL of DPPH, 0.63-0.82 mg TE/mL of ABTS, and 0.42-0.54 mg TE/mL of FRAP assay. The equivalent melatonin standard (Mel) of all treatments ranged from 38.82-54.24 mg

Mel/mL of DPPH, 4.72-7.08 mg Mel/mL of ABTS and 18.61-24.12 mg Mel/mL of FRAP assay (Table 37-38).

Table 37 Melatonin, free tryptophan and total phenolic content (TPC) of pasteurized milk treatments using mixture design

Treatment	% SMP (X1)	% MLT (X2)	% RM (X3)	Melatonin (ng/mL)	Free Tryptophan (µg/mL)	TPC (mg/100 mL)
1	4.50	4.00	89.30	1.61 ± 0.15^{b}	0.30 ± 0.02^{ab}	0.68 ± 0.02^{ab}
2	4.50	3.50	89.80	1.70 ± 0.06^{b}	0.29 ± 0.02^{abc}	0.68 ± 0.03^{ab}
3	4.50	4.50	88.80	1.93 ± 0.01^{a}	0.27 ± 0.01^{bcd}	0.73 ± 0.03^{a}
4	4.00	4.50	89.30	1.61 ± 0.08^{b}	0.26 ± 0.02^{cde}	0.72 ± 0.04^{ab}
5	4.00	4.00	89.80	1.32 ± 0.12^{cd}	0.28 ± 0.01^{abcd}	0.71 ± 0.03^{ab}
6	4.00	4.00	89.80	1.38 ± 0.05^{c}	0.29 ± 0.01^{ab}	0.71 ± 0.04^{ab}
7	4.00	4.00	89.80	1.37 ± 0.11^{c}	0.30 ± 0.01^{a}	0.70 ± 0.03^{ab}
8	4.00	3.50	90.30	1.16 ± 0.09^{ef}	0.26 ± 0.01^{cde}	0.68 ± 0.02^{b}
9	3.50	4.50	89.80	1.18 ± 0.04^{de}	0.24 ± 0.01^{e}	0.72 ± 0.01^{ab}
10	3.50	3.50	90.80	1.03 ± 0.03^{f}	0.20 ± 0.01^{f}	0.67 ± 0.03^{a}
11	3.50	4.00	90.30	1.20 ± 0.09^{de}	0.26 ± 0.01^{de}	0.70 ± 0.02^{ab}

SMP is soymilk powder, MLT is mulberry leaf tea and RM is raw milk.

Table 38 Antioxidant activity of pasteurized milk treatments using mixture design

Treatment	% SMP (X1)	% MLT (X2)	% RM (X3)	mg TE equivalent/mL			mg Mel equivalent/mL		
				DPPH	ABTS	FRAP	DPPH	ABTS	FRAP
1	4.50	4.00	89.30	0.52 ± 0.01^{ab}	0.80 ± 0.01^{ab}	0.51 ± 0.02^{ab}	50.65 ± 2.82^{abc}	6.77 ± 0.14^{ab}	22.97 ± 0.85^{ab}
2	4.50	3.50	89.80	0.51 ± 0.01^{bc}	0.72 ± 0.01^{de}	0.49 ± 0.02^{bc}	45.96 ± 1.20^{cde}	5.79 ± 0.16^{de}	21.70 ± 0.82^{bc}
3	4.50	4.50	88.80	0.54 ± 0.01^{a}	0.82 ± 0.04^{a}	0.54 ± 0.03^{a}	54.24 ± 3.75^{a}	7.08 ± 0.49^{a}	24.12 ± 1.50^{a}
4	4.00	4.50	89.30	0.53 ± 0.01^{ab}	0.78 ± 0.03^{bc}	0.52 ± 0.02^{ab}	51.39 ± 2.25^{ab}	6.47 ± 0.32^{bc}	23.23 ± 0.71^{ab}
5	4.00	4.00	89.80	0.51 ± 0.04^{abc}	0.72 ± 0.02^{de}	0.50 ± 0.01^{bc}	47.76 ± 1.10^{bcd}	5.80 ± 0.19^{de}	22.41 ± 0.61^{bc}
6	4.00	4.00	89.80	0.52 ± 0.01^{abc}	0.73 ± 0.01^{de}	0.49 ± 0.01^{bc}	49.32 ± 2.87^{bc}	5.84 ± 0.08^{de}	21.75 ± 0.65^{bc}
7	4.00	4.00	89.80	0.51 ± 0.01^{abc}	0.74 ± 0.02^{cd}	0.49 ± 0.02^{bc}	46.48 ± 3.14^{cde}	5.96 ± 0.25^{cd}	21.96 ± 0.86^{bc}
8	4.00	3.50	90.30	0.49 ± 0.01^{cd}	0.67 ± 0.03^{fg}	0.44 ± 0.01^{de}	42.05 ± 2.29^{ef}	5.17 ± 0.33^{fg}	19.62 ± 0.34^{de}
9	3.50	4.50	89.80	0.50 ± 0.01^{bcd}	0.76 ± 0.01^{bcd}	0.51 ± 0.01^{ab}	43.90 ± 2.25^{de}	6.25 ± 0.16^{cd}	22.64 ± 0.35^{ab}
10	3.50	3.50	90.80	0.47 ± 0.01^{d}	0.63 ± 0.04^{g}	0.42 ± 0.02^{e}	38.82 ± 1.64^{f}	4.72 ± 0.44^{g}	18.61 ± 1.00^{e}
11	3.50	4.00	90.30	0.49 ± 0.02^{cd}	0.69 ± 0.03^{ef}	0.47 ± 0.02^{cd}	42.05 ± 3.65^{ef}	5.39 ± 0.33^{ef}	20.93 ± 0.92^{cd}

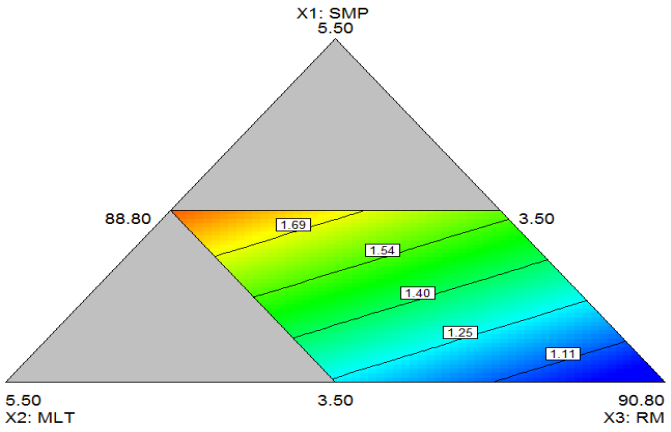
SMP is soymilk powder, MLT is mulberry leaf tea and RM is raw milk.

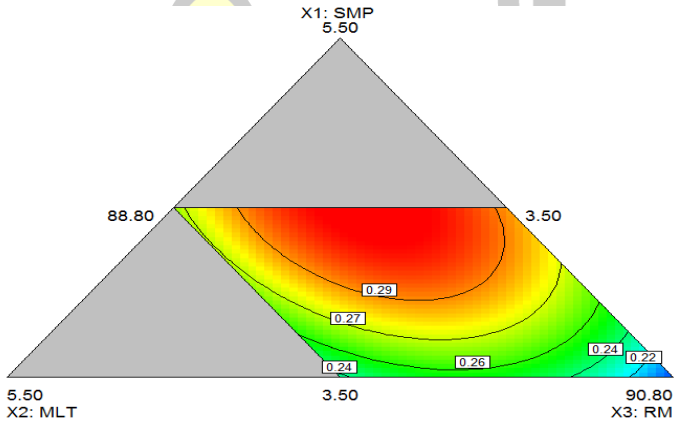
Analysis of variance of mathematical models for melatonin, free tryptophan, total phenolic component (TPC) and antioxidant activity of pasteurized milk treatments were significant (p<0.05) in linear and quadratic terms with lack of fit was not significant (p>0.05) and the regression coefficients (R^2) presented more than 0.8 of all responses (Table 39). Increase in melatonin and free tryptophan content was largely contributed by soymilk powder whereas mulberry leaf tea also increased melatonin content. Regarding TPC and antioxidant activity, high positive coefficients were shown by mulberry leaf tea and soymilk powder. Results can be explained because increases in melatonin, free tryptophan, TPC and antioxidant activity were influenced by addition of soymilk powder and mulberry leaf tea which both contain high compositions of these bioactive components. All ingredients were good sources of nutrients and bioactive compounds which display antioxidant properties (Iqbal et al., 2012; Tyug et al., 2010). Contour plots showing the responses of the proportions are depicted in Figure 18-19.

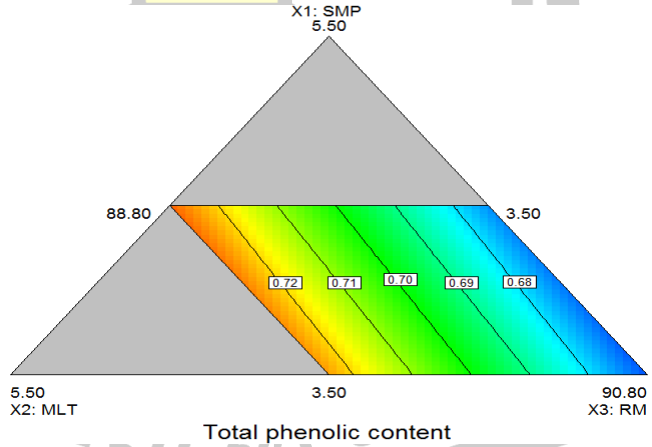
Table 39 Model predictions of melatonin, free tryptophan, total phenolic content (TPC) and antioxidant activity of pasteurized milk treatments

Response	Prediction equation	R^2	p-value	Lack-of-fit
Bioactive component				
Melatonin (ng/mL)	$0.59X_1+0.26X_2-0.02X_3$	0.9046	<0.0001	0.0834
Free tryptophan (µg/mL)	$-4.87X_1-9.60X_2-0.03X_3+0.12X_1X_2+0.06X_1X_3+0.11X_2X_3$	0.9392	0.0046	0.7292
TPC (mg GAE/mL)	$8.64E\text{-}003X_1+0.05X_2+5.06E\text{-}003X_3$	0.8331	0.0008	0.2385
Antioxidant activity (mg TE/mL)				
DPPH	$0.04X_1+0.03X_2+2.44E\text{-}003X_3$	0.9255	<0.0001	0.5860
ABTS	$0.09X_1+0.11X_2-8.51E\text{-}004X_3$	0.9628	<0.0001	0.2099
FRAP	$0.05X_1+0.07X_2+1.89E\text{-}0.04X_3$	0.9423	<0.0001	0.3961

X1 = SMP = soymilk powder X2 = MLT = mulberry leaf tea X3 = RM = raw milk

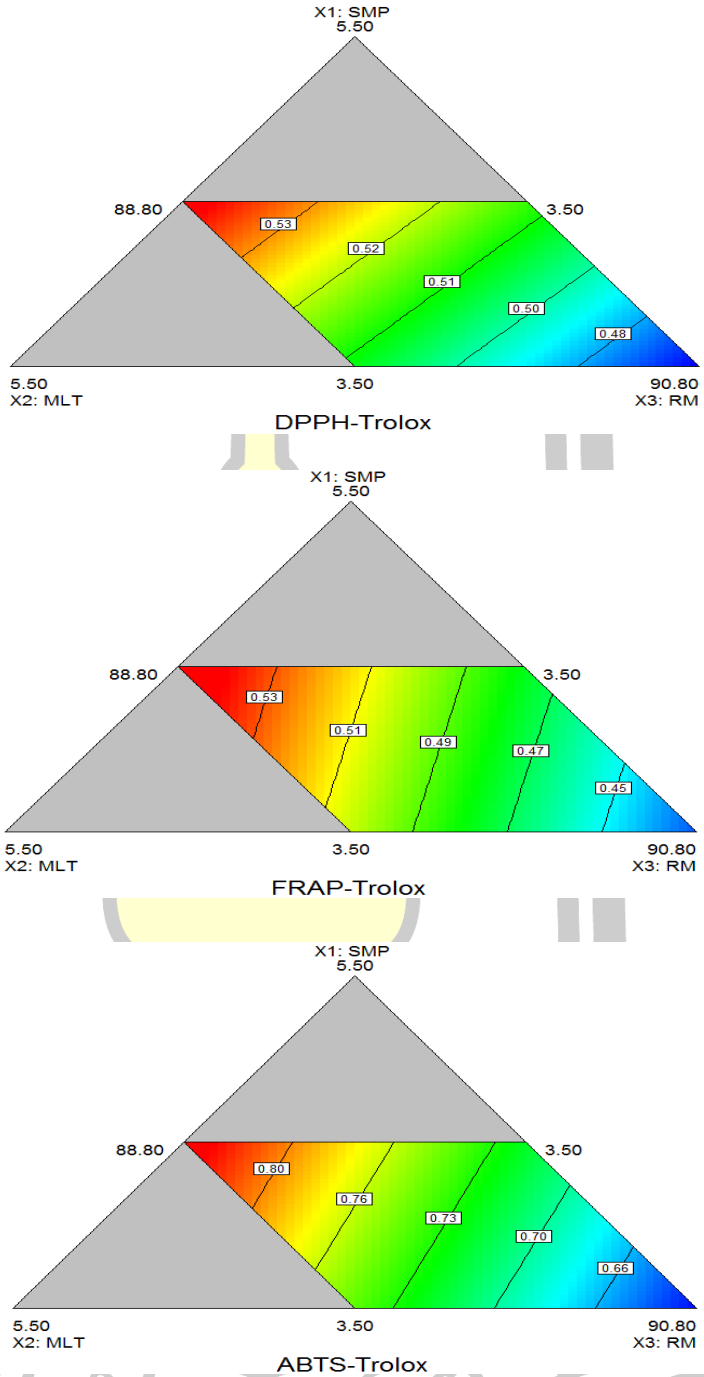






X1 = SMP =Soymilk powder X2 = MLT = Mulberry leaves tea X3 = RM = Raw milk

Figure 18 Contour plots for melatonin, free tryptophan and total phenolic contents



X1 = SMP =Soymilk powder X2 = MLT = Mulberry leaves tea X3 = RM = Raw milk

Figure 19 Contour plots for antioxidant activity of the pasteurized milk treatments

3) Sensory evaluation

Sensory evaluation of all eleven pasteurized milk treatments using a 9-point hedonic scale was tested for five different attributes with results shown in Figure 20. Highest sensory scores on color, flavor, taste, texture and overall liking were observed in treatment 10 which comprised 3.50% soymilk powder, 3.50% mulberry leaf tea and 90.80% raw milk and showed no significant difference with treatments 8, 9 and 11. Low scores were found in treatments containing higher ratios of soymilk powder and mulberry leaf tea as treatments 2, 3 and 4.

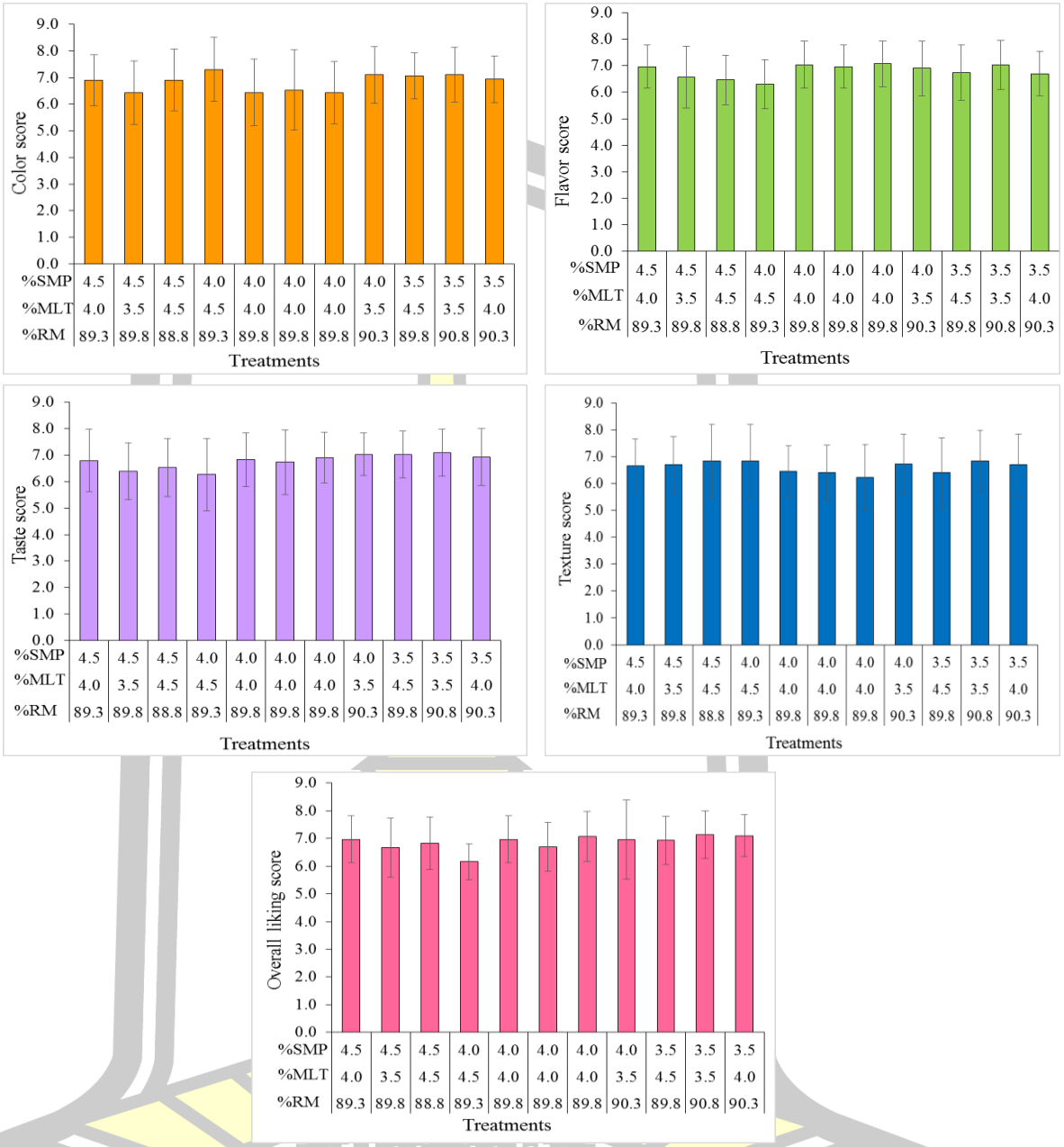


Figure 20 Sensory evaluation of eleven pasteurized milk treatments using mixture design

Numbers 1 to 9 were presented for consumer acceptability in each attribute of the milk product, where 1= dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8= like very much, and 9= like extremely.

The mathematical model of analysis of variance (ANOVA) and regression coefficients (R^2) gave no significant in all model term for color, flavor, taste, texture and overall liking (Table 40). Values of each attribute represented a probability of more than 0.05 (p>0.05). Lack of fit of the model was significant (p<0.05) with R^2 less than 0.7 for all attributes. These results explain why using the response surfaces was not appropriate for prediction and optimization. Rebouças et al. (2016) indicated that response surfaces could not be used to analyze sensory evaluation as the variance of his model showed a significant lack of fit (p<0.05); thus, this model could not be used for prediction. Mixture proportions of soymilk powder (3.50-4.50%), mulberry leaf tea (3.50-4.50%) and raw milk (88.8-90.80%) had no effect on consumer satisfaction when judging color, flavor, taste, texture and overall liking. There were no significant differences in ratios of the components added and consumers were unable to distinguish any differences.

Table 40 Model prediction for sensory evaluation of pasteurized milk treatments

Response	Prediction equation	R^2	p-value	Lack of fit
Color	$13.23X_1+115.50X_2+0.39X_3-0.97X_1X_2-0.17X_1X_3-1.30X_2X_3$	0.5663	0.3884	0.0229
Flavor	$-14.27X_1-98.26X_2-0.08X_3+1.43X_1X_2+0.15X_1X_3+1.08X_2X_3$	0.6372	0.2757	0.0316
Taste	$-0.35X_1-0.14X_2+0.10X_3$	0.5088	0.0582	0.1113
Texture	$34.73X_1+70.49X_2+0.36X_3-0.65X_1X_2-0.41X_1X_3-0.81X_2X_3$	0.6171	0.3066	0.2490
Overall liking	$-355.89X_1-523.56X_2-1.50X_3+114.15X_1X_2+4.15X_1X_3+6.02X_2X_3-1.27X_1X_2\ X_3$	0.6797	0.3842	0.2957

X1 = SMP = soymilk powder, X2 = MLT = mulberry leaf tea and X3 = RM = raw milk.

4) Optimized formulation of pasteurized milk treatments

Although results of sensory evaluation could not predict and optimize ratios of components added to pasteurized milk treatments for consumer acceptability, other responses such as milk composition, bioactive compounds and antioxidant properties could be estimated. To estimate the optimum criteria for response values of pasteurized milk product, specification of the flavored pasteurized milk standard was followed. Optimization was considered based on minimization of fat, solid not fat, total solids and lactose combined with maximization of protein, melatonin, free tryptophan, total phenolic content and antioxidant activity. Optimum qualities of pasteurized milk with mulberry leaf tea and soymilk powder were suggested, with desirability selected from a formulation of 3.90% soymilk powder, 4.50% mulberry leaf tea and 89.40% raw milk (Figure 21). Response functions using optimization showed 4.24% of fat, 10.25% of solid not fat, 14.50% of total solids, 4.78% of protein, 71.66 mg GAE/100 mL of TPC, 1.49 ng/mL of melatonin and 0.25 µg/mL of free tryptophan. Antioxidant capacity equivalents with Trolox were presented as 0.52, 0.77 and 0.52 mg TE equivalent/mL for DPPH, ABTS and FRAP assays, respectively. To confirm model adequacy, optimum proportions were verified for five replicates. The predicted optimized values are compared with verified results in Table 41.

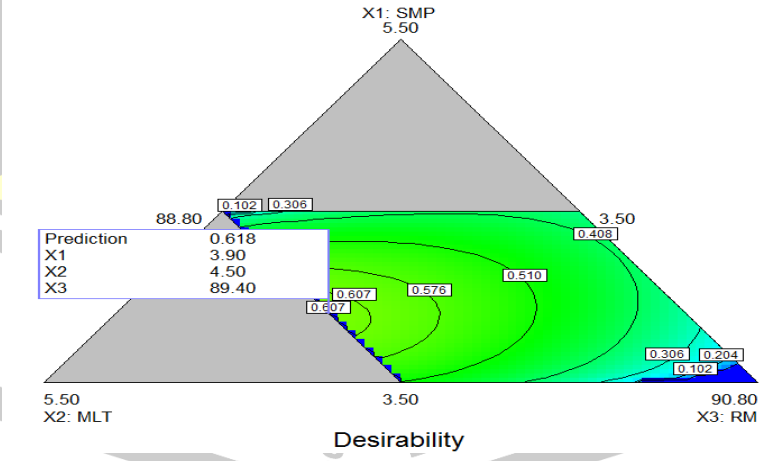


Figure 21 Contour plots of optimization

Table 41 Verification of optimum experimental proportions compared with predicted values

Response	Experimental value	Predicted value	%CV
Composition (%)			
Protein	4.87±0.07	4.77	1.47
Fat	4.31±0.06	4.24	1.16
Solid not fat	10.32±0.11	10.24	0.55
Total solids	14.63±0.12	14.48	0.73
Bioactive component			
Melatonin (ng/mL)	1.46±0.11	1.49	1.44
Free tryptophan (µg/mL)	0.23±0.02	0.26	8.66
TPC (mg GAE/mL)	0.73±0.04	0.72	0.98
Antioxidant activity (mg TE/mL)			
DPPH	0.50±0.02	0.52	2.77
ABTS	0.80±0.03	0.78	1.79
FRAP	0.53±0.01	0.52	1.35

4.3.3 Clinical study (Phase III)

4.3.3.1 Demographic data

A crossover design was used to examine aMT6-s as an indicator of circulating total melatonin in urine after high melatonin pasteurized milk consumption. According to the literature, aMT6-s is a conjugation between sulfate and primary metabolite of melatonin. Urine was collected as a non-invasive technique to determine melatonin levels in the body; 50-80% of urinary metabolized aMT6-s excreted overnight consists of both endogenous and exogenous melatonin from food sources. The study enrolled 28 healthy volunteers (7 male and 21 female participants). Mean age was 20.6, weight 53.9 kg, height 163.2 cm and body mass index (BMI) 20.1 kg/m^2 (Table 42). All participants had no underlying disease, did not take drugs, vitamins, minerals or food supplements and abstained from smoking, alcohol, tea and coffee before and during the study. They were instructed to consume milk at less than a glass/day or fruits and vegetables at less than three portions/day. They had no allergy to milk, soybean or mulberry leaf tea, no sleep problem, did not work at night time and slept before midnight before and during the study.

Table 42 Basic characteristic of healthy volunteers

Basic characteristic	Healthy volunteers		
	Male	Female	Total
Healthy volunteers (n)	7	21	28
Age (years)	20.14 ±0.90	20.71 ±0.64	20.57 ±0.74
Weight (kg)	62.43±7.55	51.00±4.06	53.86±7.09
Height (cm)	175.00±5.66	159.28±5.28	163.21±8.71
Body mass index (kg/m^2)	20.32±1.49	20.09±1.01	20.14±1.12

Mean values within a column are expressed as mean ± standard deviation

4.3.3.2 Chemical composition of raw milk and pasteurized milk samples

Chemical composition and quality of raw milk and pasteurized milk samples are shown in Table 43-44. The inspected raw milk consisted of fat (3.51%),

solids not fat (NSF) (8.23%), lactose (5.52%), protein (2.94%), total solids (TS) (11.74%) and total plate count (TPC) ($1.1x10^5$ cfu/mL). The appearance of raw milk presented as whiteness (L*) with slight yellowness (b*) and less greenness (a*) value. Pasteurized milk supplemented with mulberry leaf tea (PMM) and pasteurized milk mulberry leaf tea mixed with soymilk powder (PMMS) contained fat (3.44%, 4.12%), NSF (8.18%,10.06%), lactose (5.59%, 5.70%), protein (3.01%, 4.69%), (TS) (11.62%, 14.18%) and TPC (8.3x10 cfu/mL, 9.7x10 cfu/mL). Color appearance of PMM and PMMS showed decreased whiteness with increasing yellowness and greenness. This indicated that adding MLT and SMP in milk increased the chemical components, especially in PMMS and influenced milk appearance.

Table 43 The quality of raw milk and pasteurized milk samples

Treatment	%SMP	%MLT	%RM	pH	TPC (cfu/mL)	Color value		
						L*	a*	b*
Raw milk	-	-	100.0	6.77±0.04	$1.1x10^5$	82.52±0.29	-3.33±0.19	8.47±0.12
PMM	-	0.8	97.0	6.85±0.01	8.3 x10	79.82±0.22	-15.86±0.38	23.63±0.30
PMMS	3.50	3.50	90.8	6.70±0.05	9.7x10	67.49±0.20	-11.59±0.25	20.05±0.24

SMP is soymilk powder, MLT is mulberry leaf tea and RM is raw milk. L*; (+) white and (-) Black, a*; (+) red and (-), green, b*; (+) yellow and (-) blue and values expressed as mean ± standard deviation of replicate experiments (n = 3).

Table 44 Chemical composition of raw milk and pasteurized milk samples

Treatment	%SMP	%MLT	%RM	%Fat	%SNF	%Lactose	%Protein	%TS
Raw milk	-	-	100.0	3.51±0.04	8.23±0.02	5.52±0.03	2.94±0.02	11.74±0.04
PMM	-	0.8	97.0	3.44±0.13	8.18±0.11	5.59±0.08	3.01±0.09	11.62±0.06
PMMS	3.50	3.50	90.8	4.12±0.04	10.06±0.10	5.70±0.05	4.69±0.08	14.18±0.07

SMP is soymilk powder, MLT is mulberry leaf tea and RM is raw milk. SNF refers to solids not fat, TS refers to total solids and values expressed as mean ± standard deviation of replicate experiments (n = 3).

4.3.3.3 Associate of urinary 6-sulfatoxymelatonin (aMT6-s)

This study represents the first report on levels of aMT6-s and urinary antioxidant capacity after human consumption of high melatonin pasteurized milk samples (PMM and PMMS) against the baseline. Levels of aMT6-s after PMM and PMMS consumption are shown in Table 45. Results indicated that aMT6-s levels significantly increased (p<0.05) after consuming PMM, whereas they did not significantly increase after consuming PMMS versus the baseline. Level of aMT6-s was 7.61 µg aMT6-s at baseline and after consuming PMM was 12.00 µg aMT6-s (57.69% increase from baseline, p = 0.015). The aMT6-s of PMMS consumption was 9.99 µg aMT6-s (31.27% increase from baseline, p = 0.139). There was no significant difference between PMM and PMMS consumption (12.00 versus 9.99 µg aMT6-s, p = 0.172; data not shown). Results indicated that high melatonin pasteurized milk (PMM and PMMS) consumption increased melatonin content in urine. A previous study reported normative data on total amount of 24 h aMT6-s of healthy subjects with age range 20-35 at 7.5-58 µg (average 36.8 µg aMT6-s) and amount decreased in older subjects (Mahlberg et al., 2006). Here, we reported 12 h adjusted melatonin overnight, which may not represent total aMT6-s for 24 h. Significantly, aMT6-s levels increased after consuming either PM versus baseline, whereas comparison between PM samples found that aMT6-s levels of PMMS were lower than for PMM consumption. Thus, adding higher amounts of mulberry leaf tea (MLT) and soymilk powder (SMP) in milk were not fully effective in increasing melatonin levels because several factors affect melatonin circulation after PMMS ingestion. Reducing melatonin content may be influenced by physiochemicals in soymilk powder occurring during the pasteurization process. Soymilk powder contains high carbohydrate, fiber and protein but low fat and ash compared to soyhusk powder as reported by Tyug et al. (2010). Therefore, these components may partially dissolve, making the milk thicker and hampering filtration during the pasteurizing process. Soymilk powder also adhered to equipment such as filters, storage tanks, tubes and the heat plate exchanger of the process line resulting in melatonin reduction. Pothinuch and Tongchitpakdee (2011) mentioned that loss of melatonin occurred during the homogenization step because the fibrous nature of plants

adhered to the homogenizer probe. Similarly, Manchester et al. (2000) noted loss of melatonin in seeds due to adherence to the fibers and equipment used for extraction. Moreover, after consuming PM, melatonin may be distributed and absorbed in plasma; its metabolization rate before excretion as urine depends on the biological mechanism of individual bodies. Intake of food rich in melatonin showed contrasting results for melatonin levels. This finding was supported by Johns et al. (2013) who reported that aMT6-s levels after consuming high melatonin fruits were lower than after consumption of low melatonin fruits, possibly influenced by differences in metabolism and/or absorption rates of melatonin in individual participants and also different fruit types, fruit preparation, and fruit texture/combination. With regard to diverse vegetable color, green and yellow vegetable consumption was significantly associated with aMT6-s levels (p<0.05), whereas other vegetables consumed showed borderline significance at p>0.05 (Nagata, et al., 2005). Here, consumption of PMM and PMMS increased exogenous melatonin in the body, with health-promoting anti-oxidative processes. This finding concurred with (Howatson et al., 2012) who reported that consuming tart cherry juice contained melatonin elevated exogenous melatonin in urine and improved the quality of sleep.

Table 45 Levels of aMT6-s after PM samples consumption

Sample type	N	Median	Interquartile	Mean ± SE	[a]Mean difference (%)	[b]p-value
Baseline	28	5.99	2.52-10.74	7.61±1.28	-	-
PMM	28	12.31	5.17-15.92	12.00±1.63	57.69 % increase	0.015
PMMS	28	4.97	2.24-15.94	9.99±2.01	31.27% increase	0.139

[a] Difference of means compared with baseline. [b] Means were analyzed using the Wilcoxon signed rank test.

4.3.3.4 Urinary antioxidant capacity

Results of urinary antioxidant capacity after PMM and PMMS consumption using TEAC and FRAP assays significantly increased (p<0.05). TEAC increased 19.05% for PMM (0.50 mM TE) and 54.76% for PMMS (0.65 mM TE)

consumption versus the baseline (0.42 mM TE), while FRAP value increased 37.04% for PMM (1.11 mM $FeSO_4$) and 86.42% after PMMS (1.51 mM $FeSO_4$) consumption versus the baseline (0.81 mM $FeSO_4$). Urinary antioxidant capacity of both assays showed significant differences between PMM and PMMS consumption. Radical scavenging efficiency of PMMS was greater than PMM for both assays. Results indicated increased antioxidant capacity from adding mulberry leaf tea (MLT) and soymilk powder (SMP) in milk (RM). MLT, SMP and RM contain a variety of bioactive compounds such as vitamins, flavonoids (TFC), carotenoids, isoflavone and phenolic compounds (TPC) as presented by Khan et al. (2017) and Xu and Chang (2009). These compounds have water-soluble and fat-soluble antioxidant properties. They can dissolve in both hydrophilic and lipophilic phases and easily permeate plasma. Therefore, consuming foods containing a variety of bioactive compounds including melatonin may increase total antioxidant capacity. Tyug et al. (2010) reported that isoflavone and TPC in soymilk powder potentially inhibited free radicals using TEAC, FRAP and β-carotene bleaching assay, while aMT6-s levels and total antioxidant capacity in urine increased after intake of Jerte Valley cherry cultivars and nutraceutical products from Jerte Valley cherries (Garrido et al., 2009; Garrido et al., 2010). Serum melatonin and total antioxidant status (TAS) increased after intake of beer (Maldonado et al., 2009). Urinary antioxidant activities using TEAC and FRAP assays of participants after PMM and PMMS consumption versus the baseline are shown as Figure 22 and Figure 23.

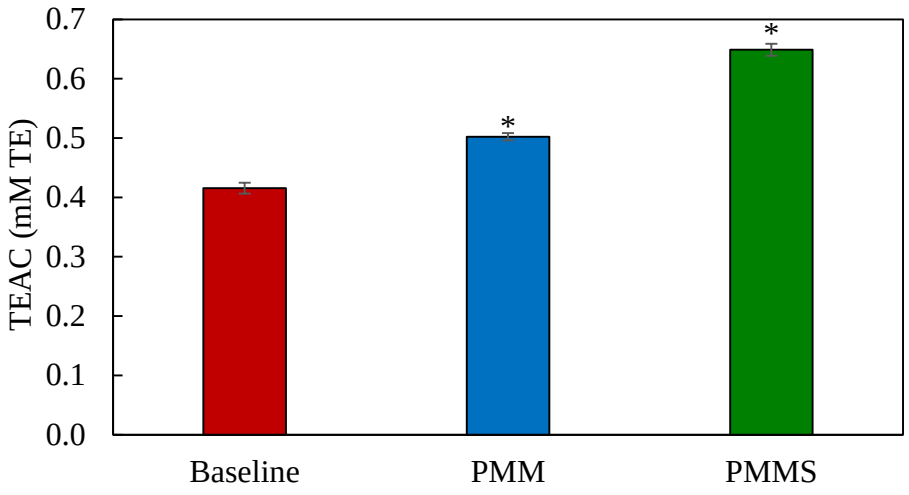


Figure 22 Urinary antioxidant activity using TEAC (mM TE) after consuming PMM and PMMS versus baseline

* Significant difference versus baseline at $p < 0.05$.

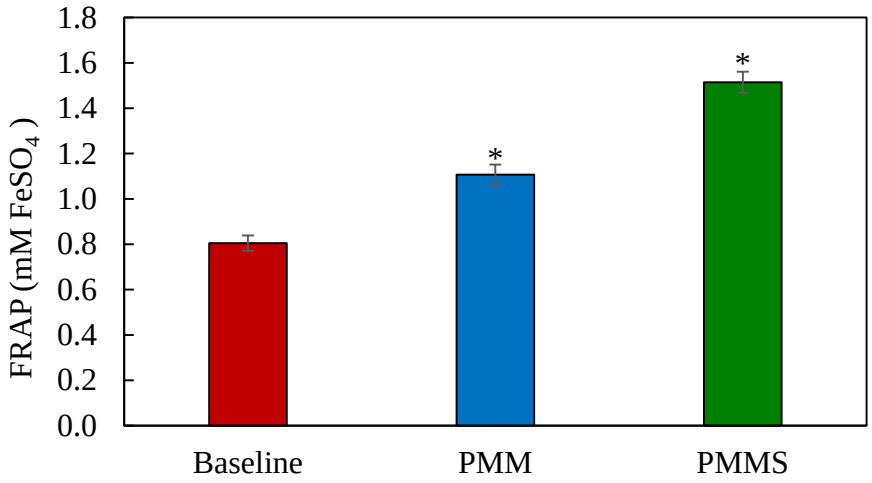


Figure 23 Urinary antioxidant activity using FRAP (mM $FeSO_4$) after consuming PMM and PMMS versus baseline

* Significant difference versus baseline at $p < 0.05$.

4.3.3.5 Correlation between urinary aMT6-s and urinary antioxidant activity

Correlation between urinary aMT6-s and urinary antioxidant activity using TEAC and FRAP assays after PMM and PMMS consumption are shown in Table 46. Results indicated a significant correlation between aMT6-s and urinary antioxidant capacity. The correlation coefficient between aMT6-s and TEAC of PMM was 0.703 ($p<0.01$), while PMMS was 0.621 ($p<0.01$). Correlation coefficient between aMT6-s and FRAP of PMM was 0.549 ($p<0.01$), while PMMS was 0.733 ($p<0.01$). There was a slight correlation of aMT6-s with both antioxidant assays at baseline. However, results of antioxidant capacity may not reflect the total antioxidant status of melatonin but respond to the total bioactive antioxidants due to the variety of antioxidant substances distributed in MLT, SMP and RM which can contribute to the total antioxidant status. Moreover, assays used for determining antioxidant capacity are related to electron transfer as a direct reaction measure, whereas the antioxidant action of melatonin is both direct and indirect to inhibit oxidation (Reiter et al., 2003). Our results were supported by Reiter et al. (2005). They noted that increasing antioxidant capacity using TEAC and FRAP assays did not relate to serum melatonin of rats fed walnuts because of the variety of antioxidants in walnuts such as ω-3 fatty acids, vitamin E and polyphenols that contribute to total antioxidant capacity in serum. Grape juice consumption increased circulation of urinary melatonin. Increasing antioxidant capacity may be influenced by polyphenol binding with the lipid fraction in serum and excreted in urine as reported by González-Flores et al. (2012).

Table 46 Correlation between aMT6-s and urinary antioxidant activity using TEAC and FRAP assays

Correlation	TEAC	FRAP
aMT6-s (Baseline)	Baseline	
	-0.067	0.027
aMT6-s (PMM)	PMM	
	0.703**	0.621**
aMT6-s (PMMS)	PMMS	
	0.549**	0.733**

** correlated significance at $p<0.01$. Values expressed using Spearman's rank correlation test.

4.3.4.6 Sensory evaluation

In this study, sensory evaluations of color, flavor, taste, texture and overall liking were evaluated using a 9-point hedonic scale and compared between PMM and PMMS. Results showed no significant differences in all attributes between PMM and PMMS. Participants recorded higher scores on color (7.9) and flavor (7.5) for PMM, whereas they gave higher scores on taste (7.6), texture (7.0) and overall liking (7.9) for PMMS. Overall acceptability was considered using overall liking. Results showed that participants accepted both PM samples with no significant difference at 'like moderately' to 'like very much' as shown in Figure 24.

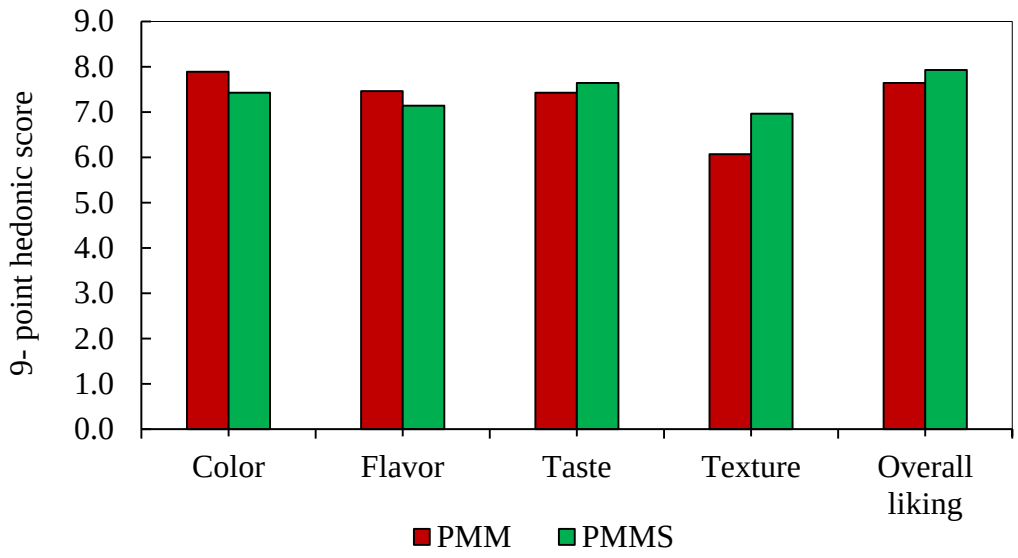


Figure 24 Sensory score of PMM and PMMS using a 9-point hedonic scale where number 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = like extremely.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

5.1.1 Determination of melatonin and tryptophan content in edible grains, mulberry leaf tea, and raw milk

Highest melatonin concentrations were found in white sesame, sunflower seed, and soybean while the highest concentrations of free tryptophan were detected in soybean, red bean, and mung bean. Total phenolic content was highest in sunflower and white sesame seeds. Evaluation using DPPH, FRAP and ABTS assays indicated sunflower seed as the strongest antioxidant activity followed by white sesame seeds.

Mulberry leaf tea was prepared using different drying methods. Freeze-dried leaves recorded the highest bioactive contents and antioxidant activity using DPPH, FRAP and ABTS assay. Melatonin content in leaves dried using solar energy showed no significant difference with the freeze drying method.

Both grains and mulberry leaf tea were good sources of melatonin, tryptophan, and antioxidants. The selection of materials for pasteurized milk production depended on several factors including high bioactive contents, appearance, cost, source, and production process. Soybean and mulberry leaf tea dried using solar energy were selected to produce pasteurized milk with high melatonin content.

5.1.2 Development of pasteurized milk with high melatonin content

Mulberry leaf tea and soymilk powder were supplemented in milk. Results showed that all parameters as milk qualities, milk compositions, bioactive components, antioxidants, and sensory evaluation significantly increased (p<0.05) with increasing amounts of both ingredients. Melatonin, free tryptophan, and total phenolic content did not significantly decrease during storage. Sensory evaluation gave the highest score of overall liking for treatment 5, consisting of mulberry leaf tea (4.00%), soymilk powder (4.00%) and milk (89.80%).

Pasteurized milk components were optimized using mixture design. The mathematical model showed significance for composition and bioactive components

(p<0.05) but not for milk quality and sensory evaluation. Optimum desirability of pasteurized milk with mulberry leaf tea and soymilk powder was suggested at the formulation of 3.90% soymilk powder, 4.50% mulberry leaf tea and 89.40% raw milk. Verification of optimum proportions showed that experimental values of chemical compositions, bioactive components, and antioxidant activity agreed with model predictions. Optimum PM contained melatonin (1.49 ng/mL), free tryptophan (0.26 µg/mL), and total phenolic content (0.72 mg GAE/mL) with high antioxidant activity when assayed by DPPH, ABTS and FRAP. Results suggested that mixture design RSM has the potential to optimize SMP, MLT and RM levels to obtain PM with increased amounts of bioactive compounds and high melatonin content.

Supplementation of both mulberry leaf tea and soymilk powder in milk improved functional properties and antioxidant activity. However, adding high amounts of both ingredients adversely affected product appearance as color, flavor, and texture.

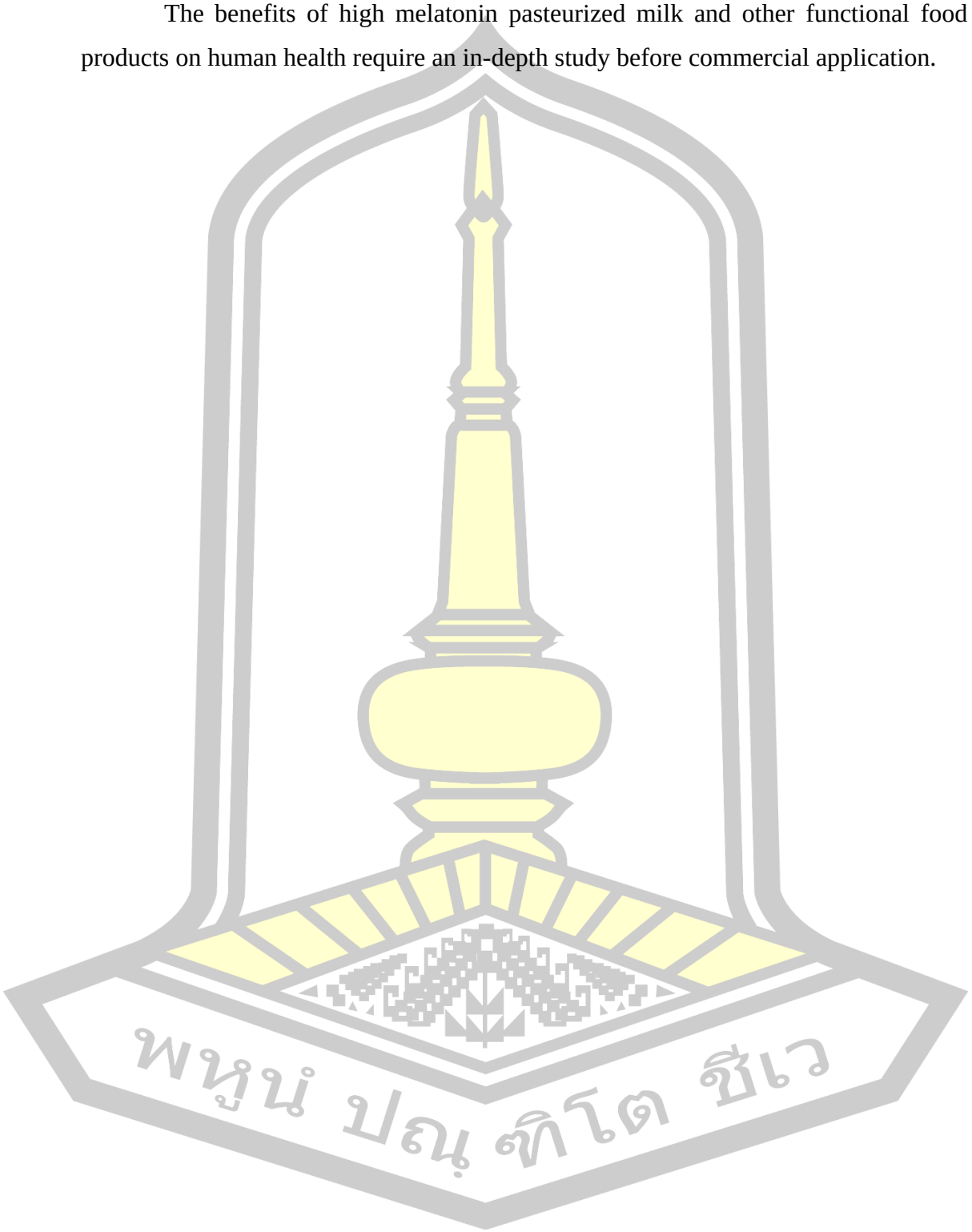
5.1.3 Clinical study of pasteurized milk

Consumption of pasteurized milk mulberry leaf tea (PMM) and pasteurized milk mulberry leaf tea mixed with soymilk powder (PMMS) improved melatonin levels in the human body, increased melatonin levels in urine (aMT6-s), and also increased urinary antioxidant capacity compared with the baseline. There were significant correlations between aMT6-s and urinary antioxidant capacity of both pasteurized milk samples; however, increasing urinary antioxidant capacity using FRAP and TEAC may also be influenced by other bioactive compounds or total antioxidants in the pasteurized milk samples which impact on the functional added ingredients (mulberry leaf tea and soymilk powder).

This study indicated that high melatonin pasteurized milk produced by adding mulberry leaf tea and soymilk powder effectively enhanced exogenous melatonin and antioxidant capacity in the body. In the future, rich melatonin ingredients can be supplemented for developing other high melatonin functional food.

5.2 Recommendations

The benefits of high melatonin pasteurized milk and other functional food products on human health require an in-depth study before commercial application.



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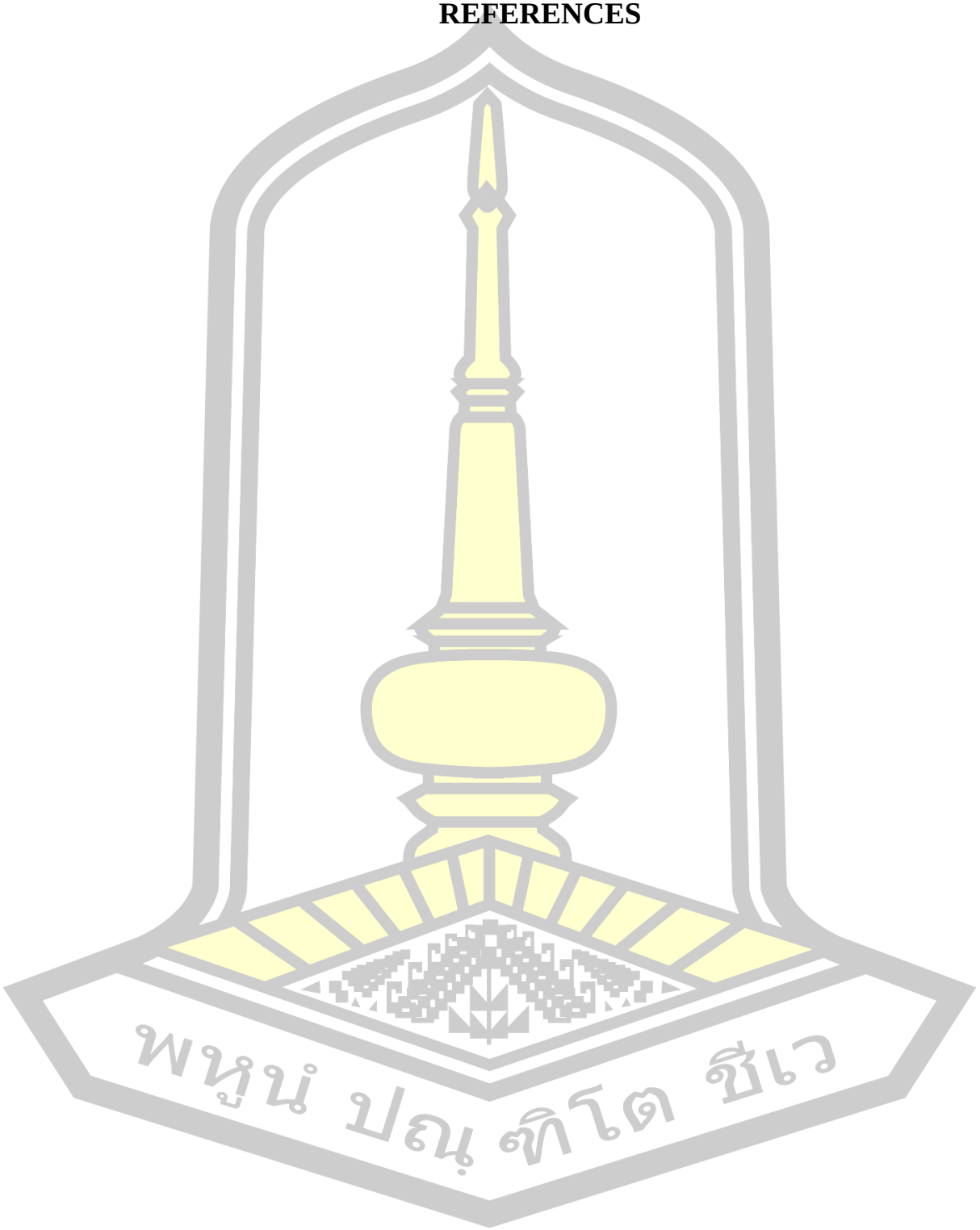

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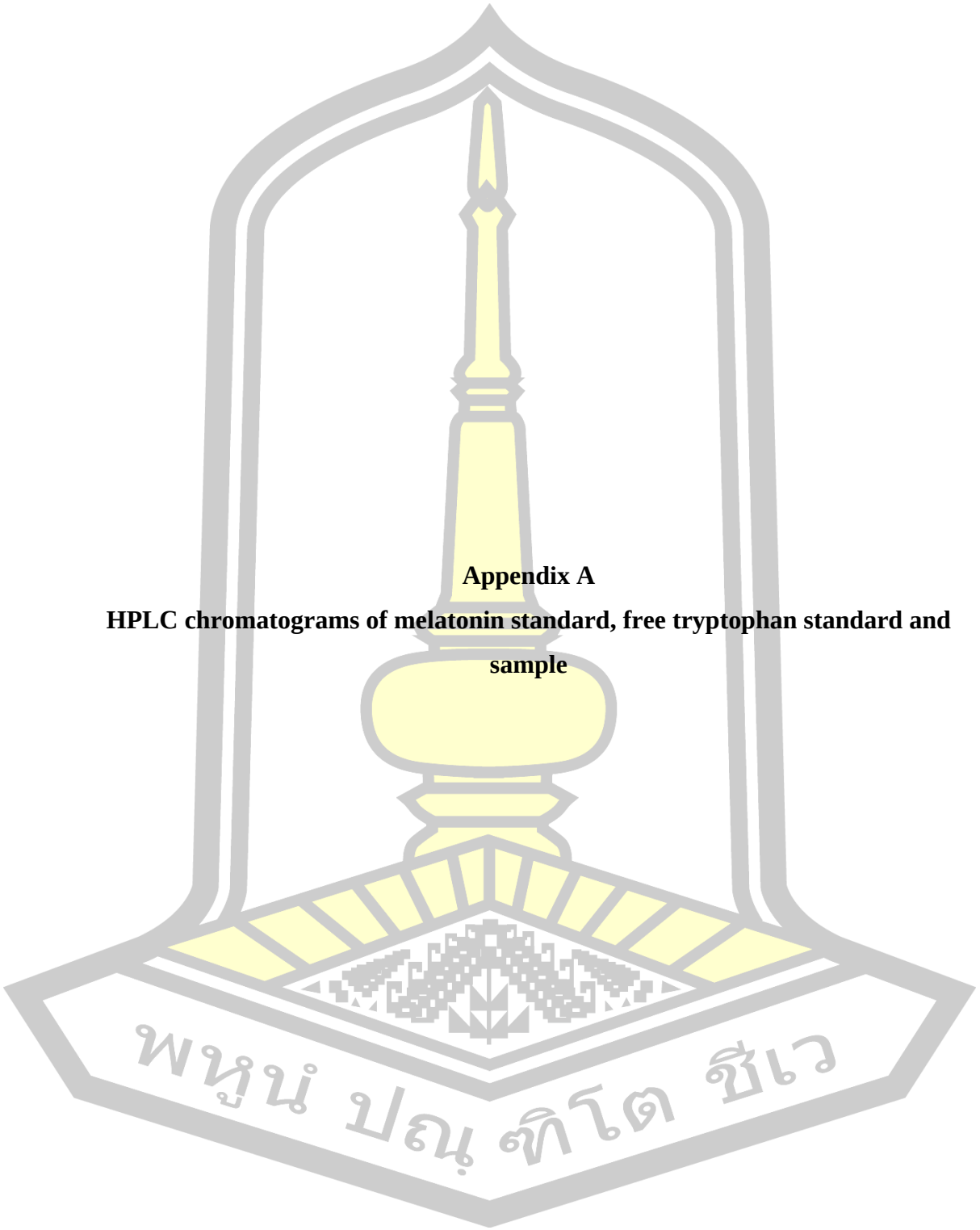
APPENDICES



Appendix A

HPLC chromatograms of melatonin standard, free tryptophan standard and sample

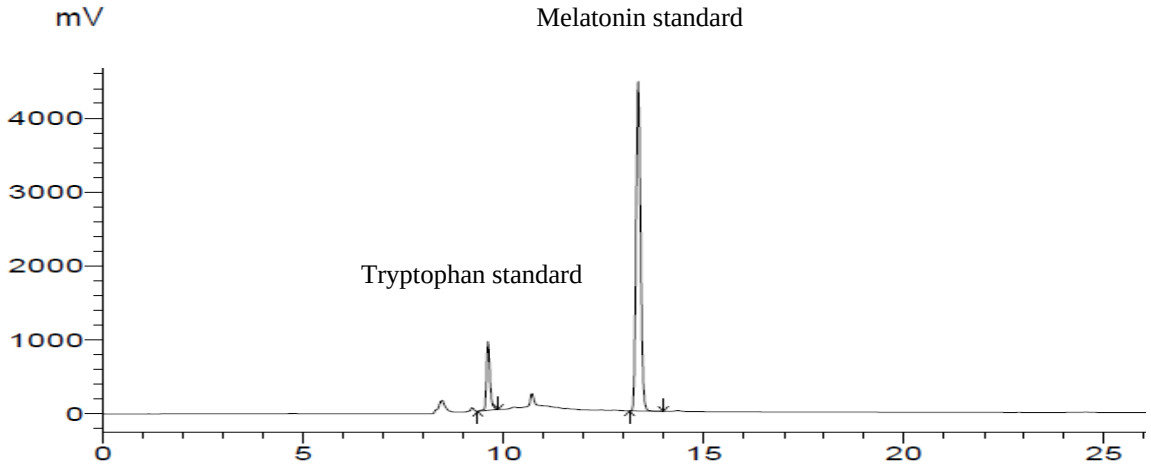


Figure 25 HPLC chromatograms; melatonin (31.25ng/mL) and free tryptophan standards (156.25 ng/mL)

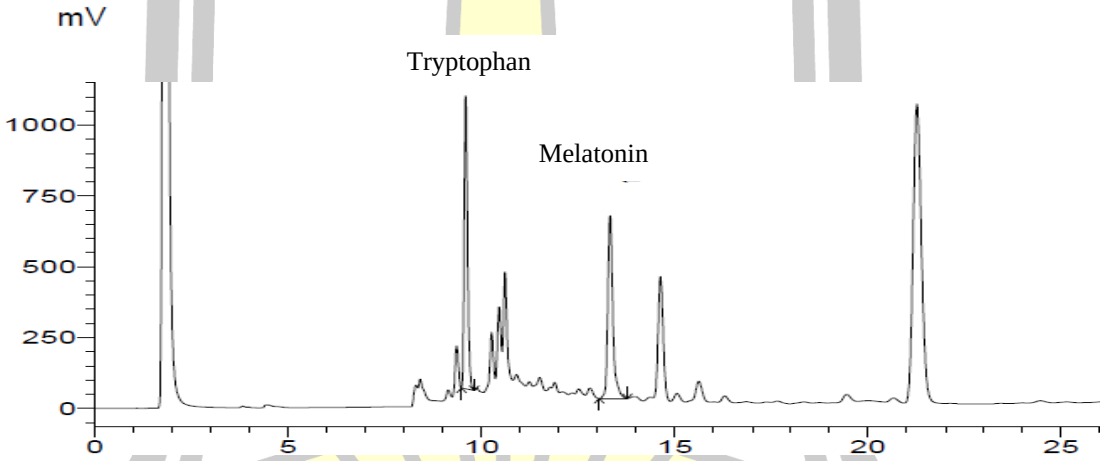
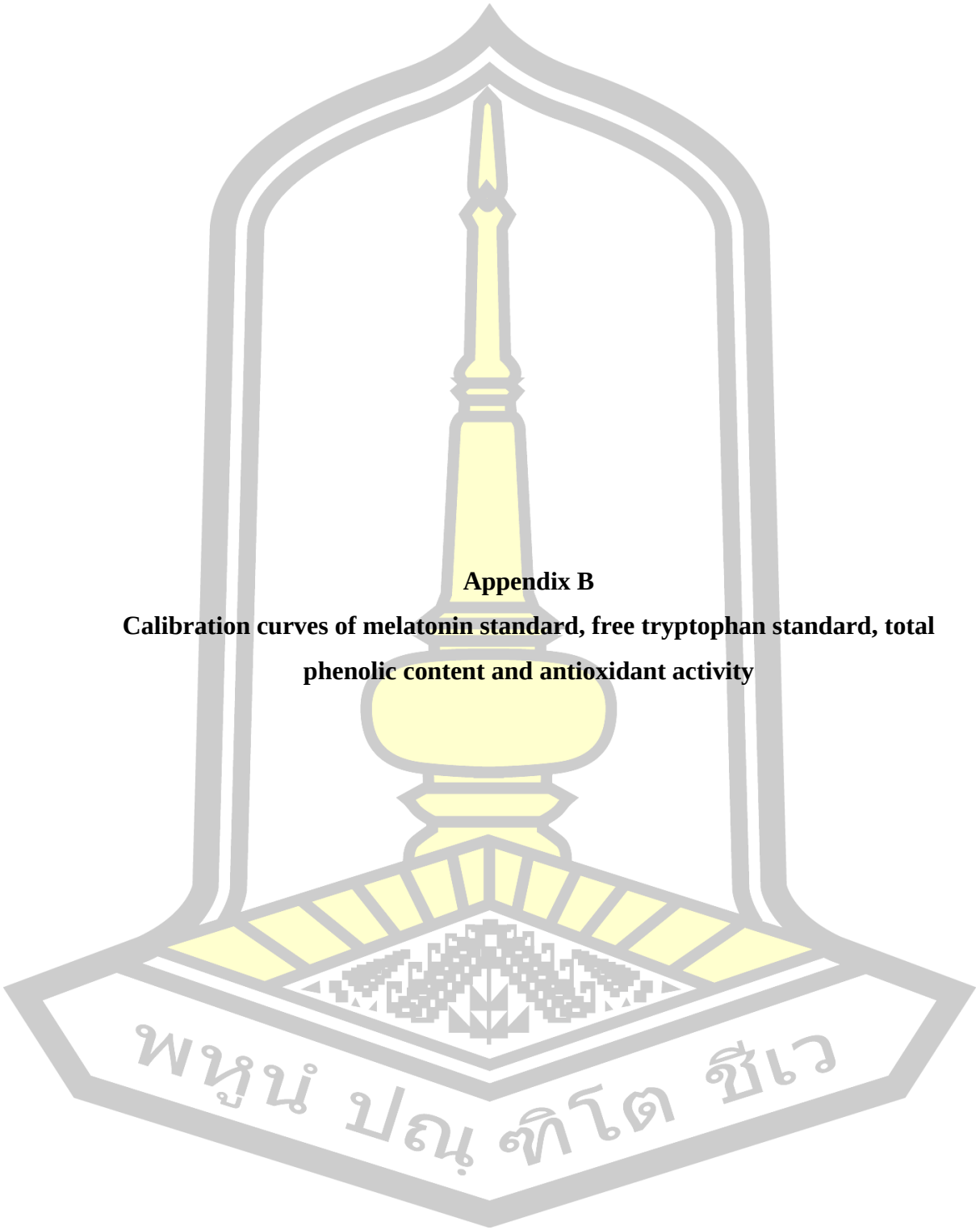


Figure 26 HPLC chromatograms of melatonin and free tryptophan in red bean extract (400 ng/mL)



Appendix B

Calibration curves of melatonin standard, free tryptophan standard, total phenolic content and antioxidant activity

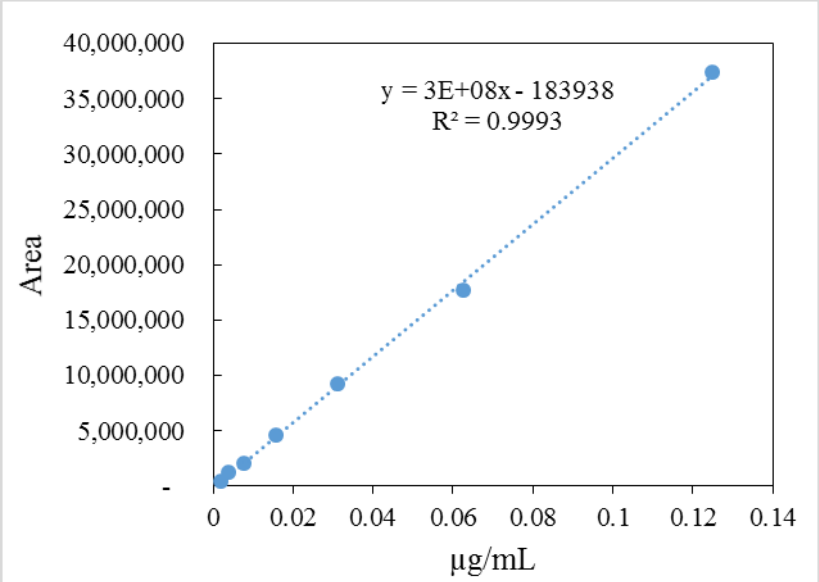


Figure 27 Calibration curve of melatonin standard

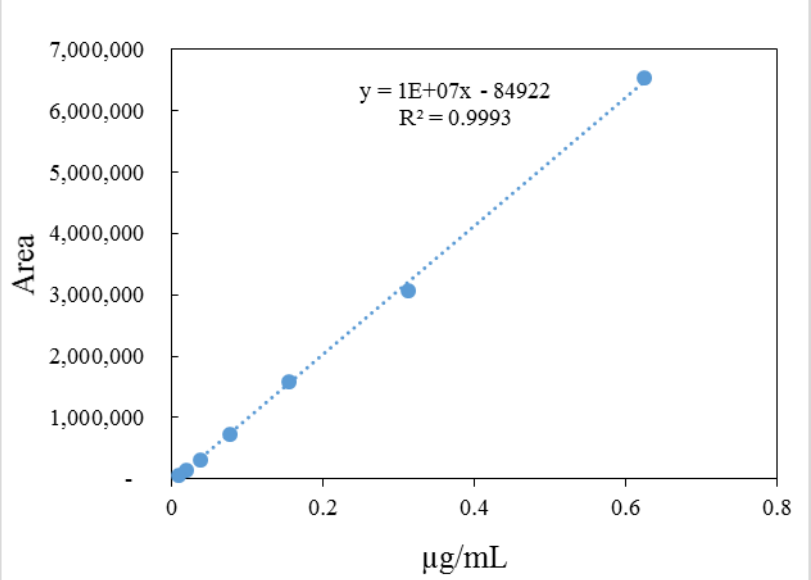


Figure 28 Calibration curve of free tryptophan standard

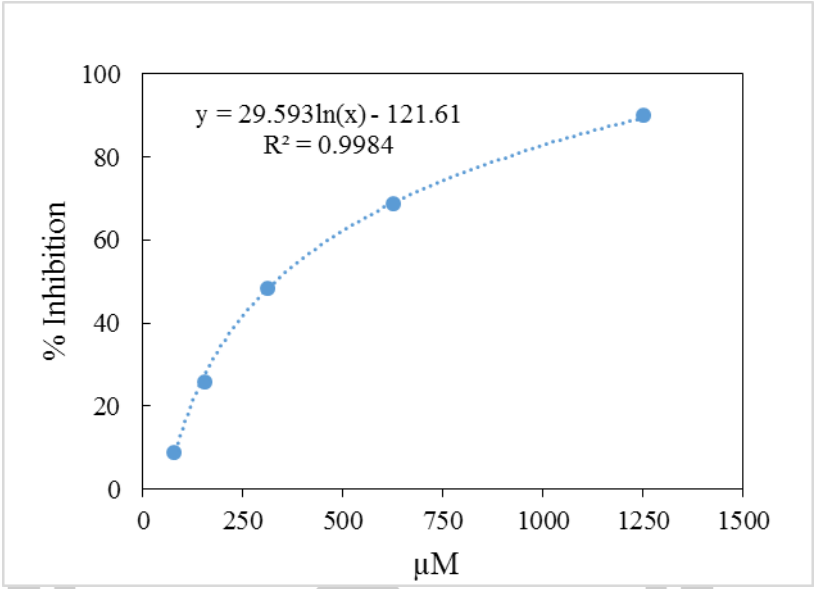


Figure 29 Calibration curve of Trolox (TE) standard using DPPH assay

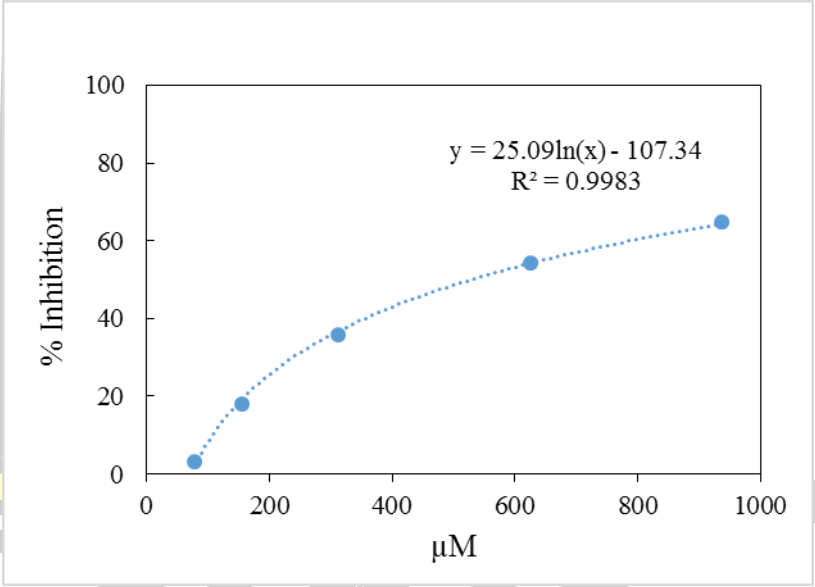


Figure 30 Calibration curve of α-tocopherol (vitamin E; VE) standard using DPPH assay

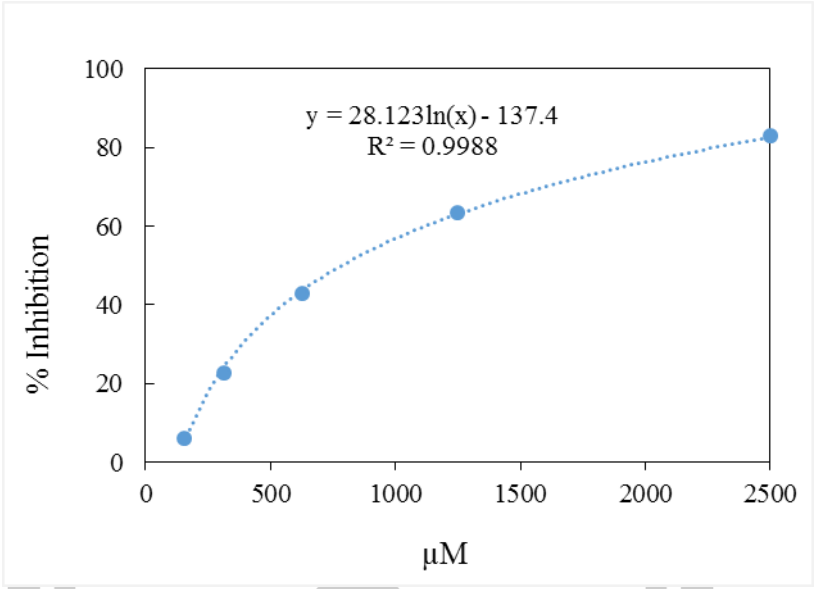


Figure 31 Calibration curve of butylated hydroxyanisole (BHA) standard using DPPH assay

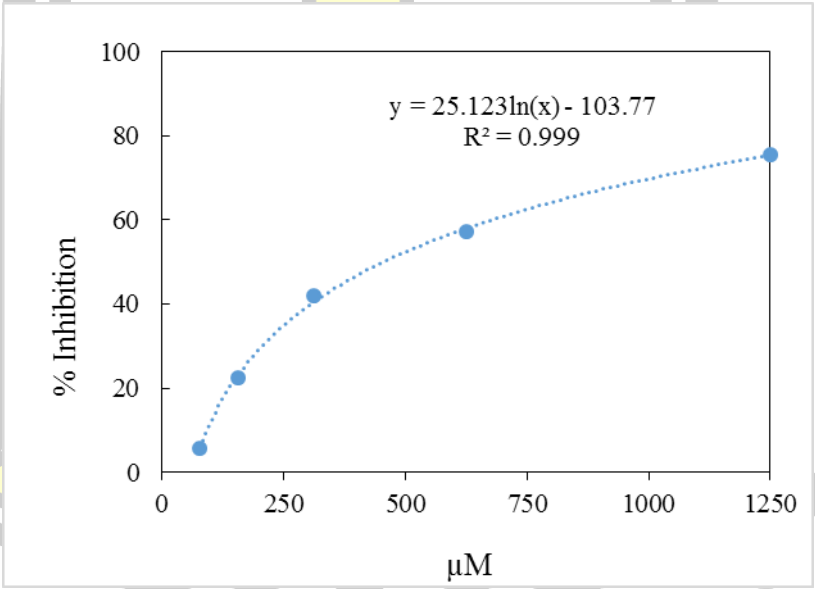


Figure 32 Calibration curve of L-ascorbic acid (Vitamin C; VC) standard using DPPH assay

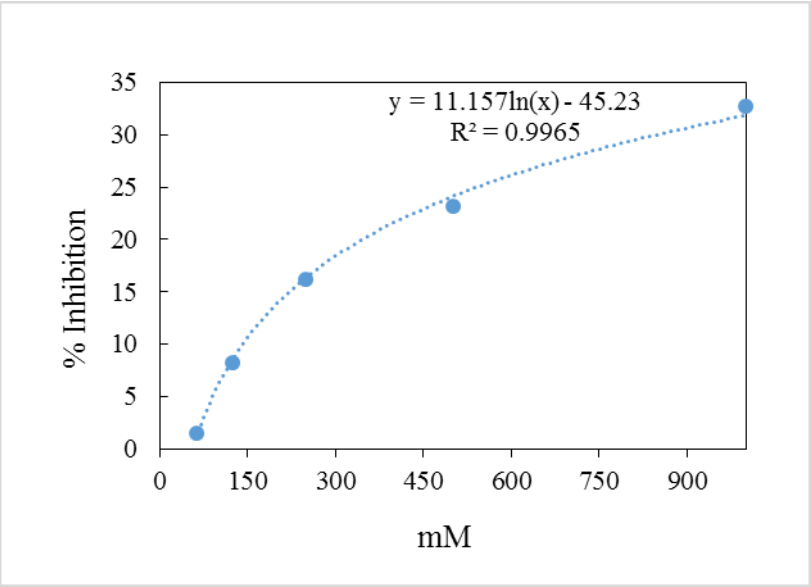


Figure 33 Calibration curve of melatonin standard (Mel) using DPPH assay

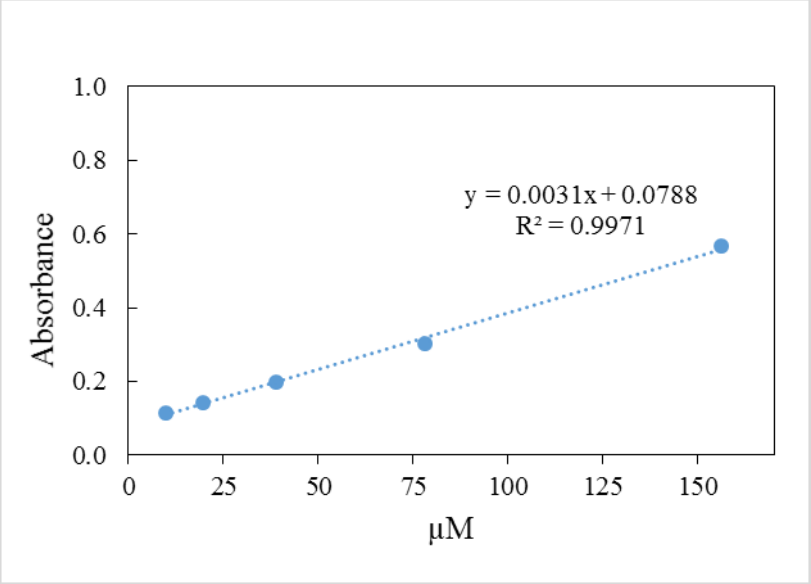


Figure 34 Calibration curve of Trolox standard (TE) using FRAP assay

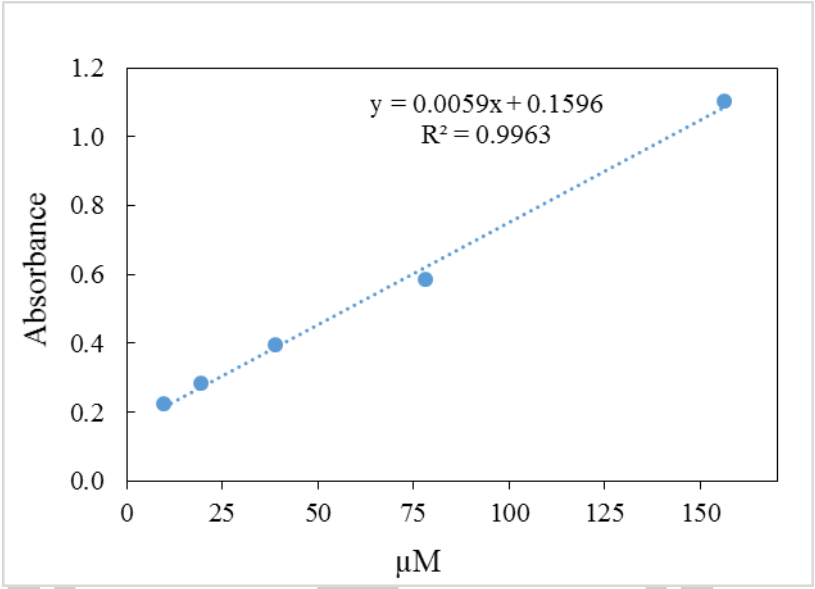


Figure 35 Calibration curve of α-tocopherol (vitamin E; VE) standard using FRAP assay

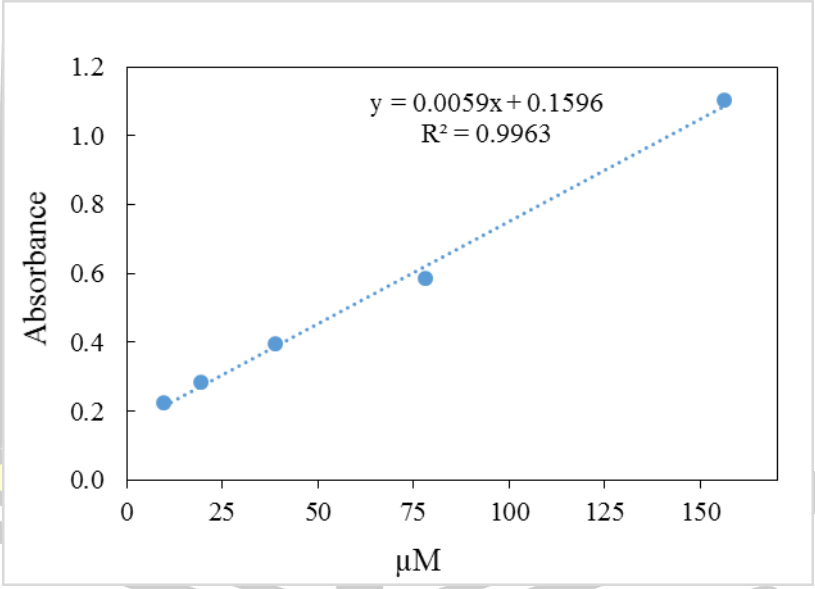


Figure 36 Calibration curve of butylated hydroxyanisole (BHA) standard using FRAP assay

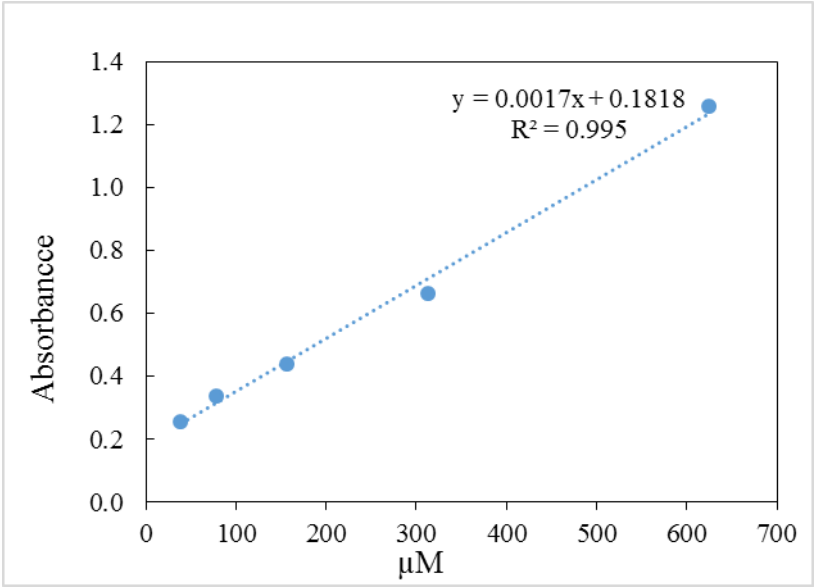


Figure 37 Calibration curve of L-ascorbic acid (Vitamin C; VC) standard using FRAP assay

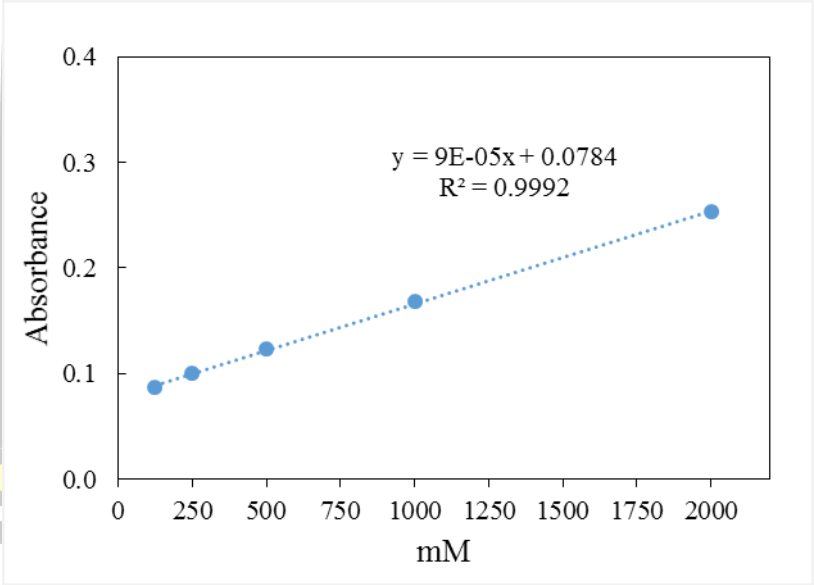


Figure 38 Calibration curve of melatonin standard (Mel) using FRAP assay

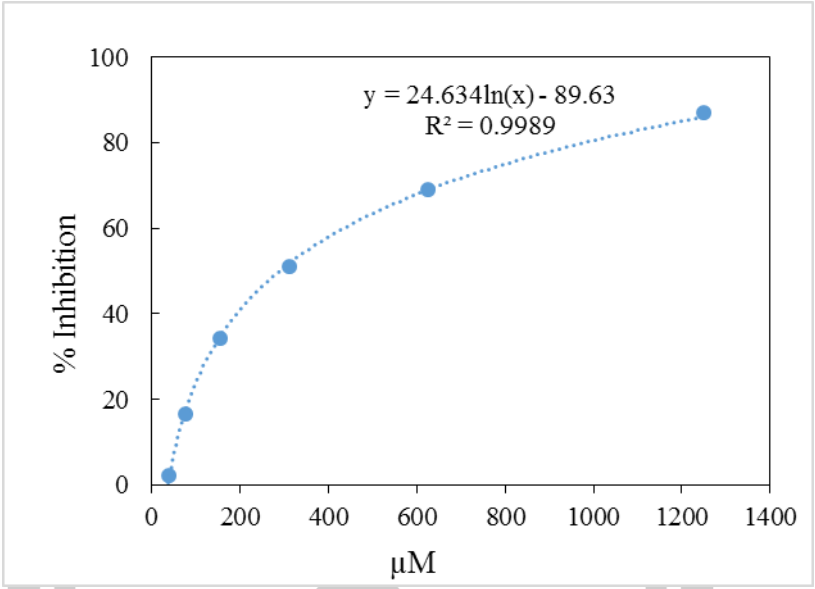


Figure 39 Calibration curve of Trolox standard (TE) using ABTS assay

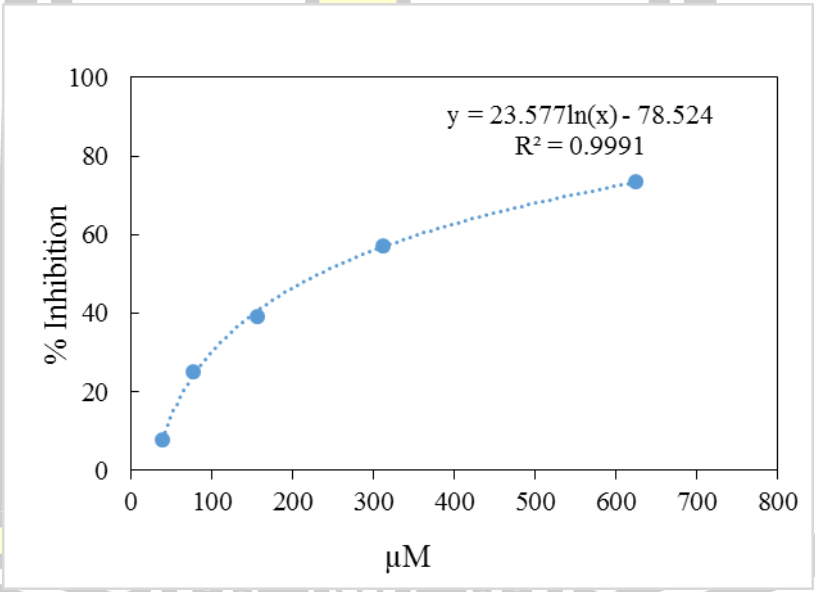


Figure 40 Calibration curve of α-tocopherol (vitamin E; VE) standard using ABTS assay

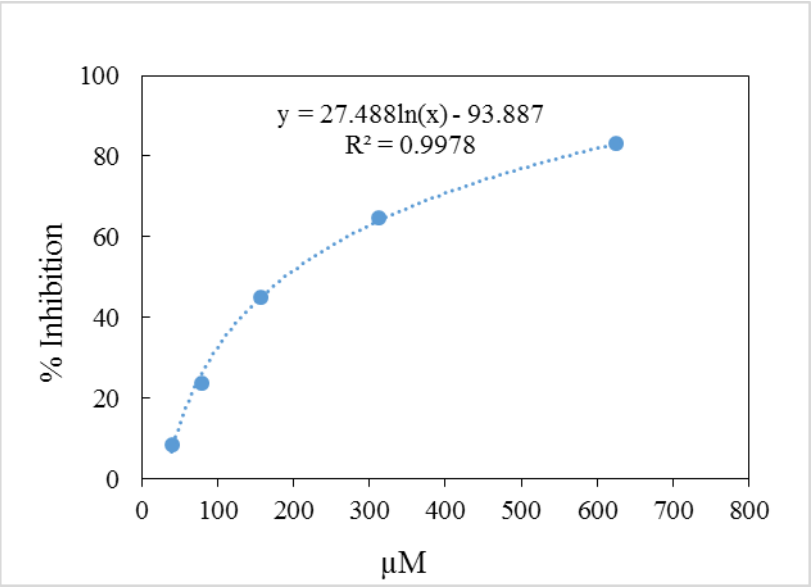


Figure 41 Calibration curve of butylated hydroxyanisole (BHA) standard using ABTS assay

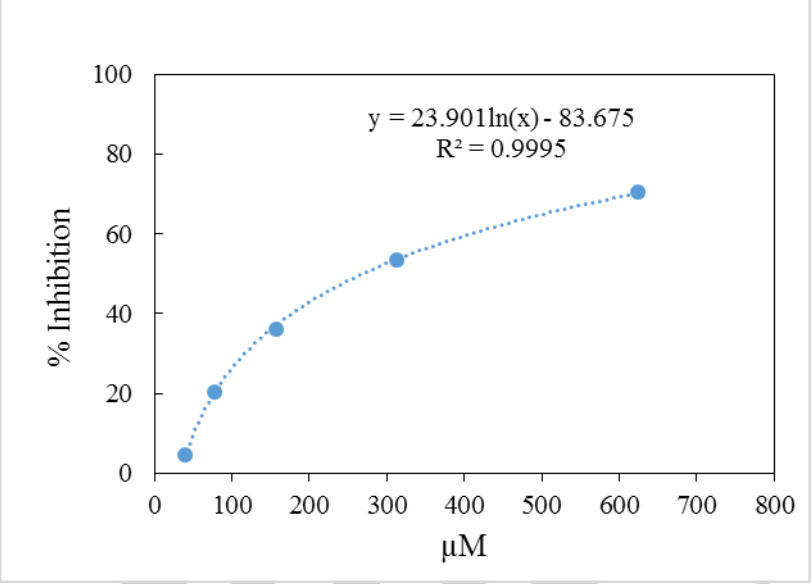


Figure 42 Calibration curve of of L-ascorbic acid (Vitamin C; VC) standard using ABTS assay

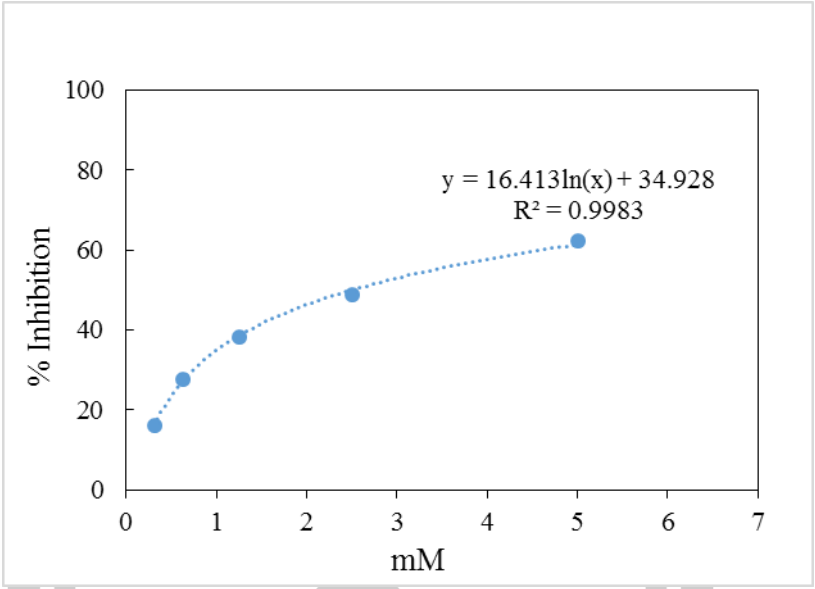


Figure 43 Calibration curve of melatonin standard (Mel) using ABTS assay

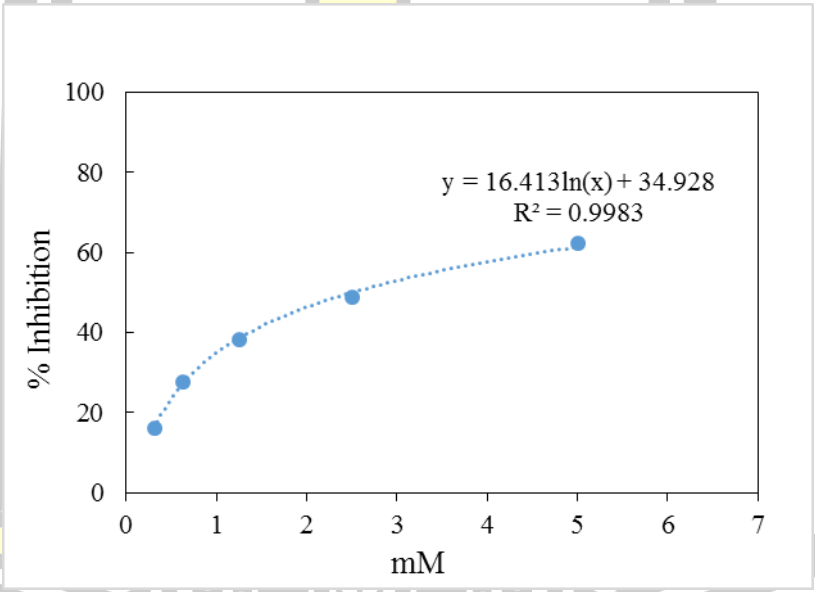


Figure 44 Calibration curve of total phenolic content (TPC)

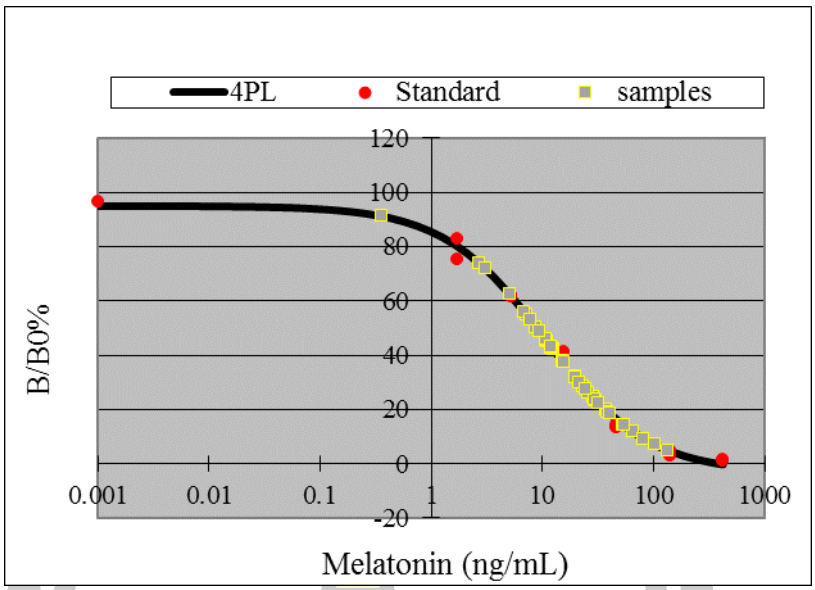


Figure 45 Calibration curve of melatonin of visit 1-2

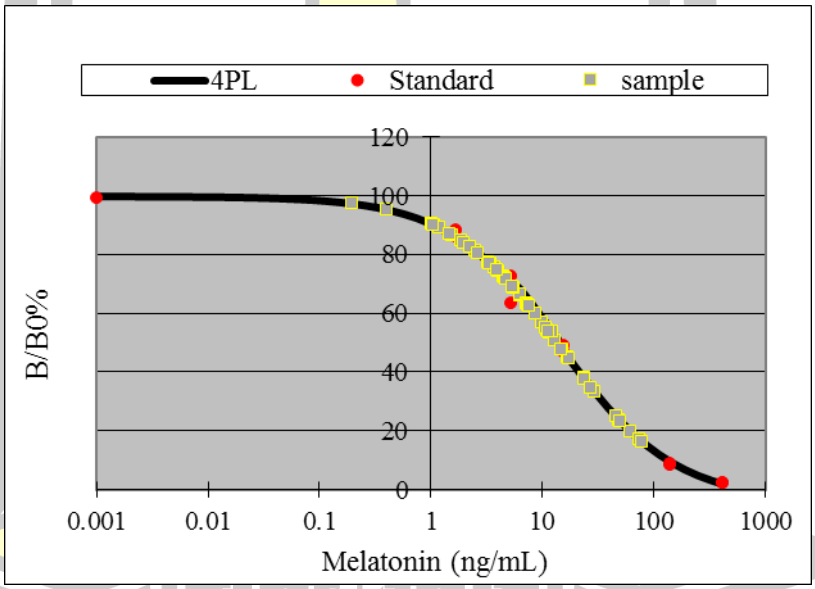
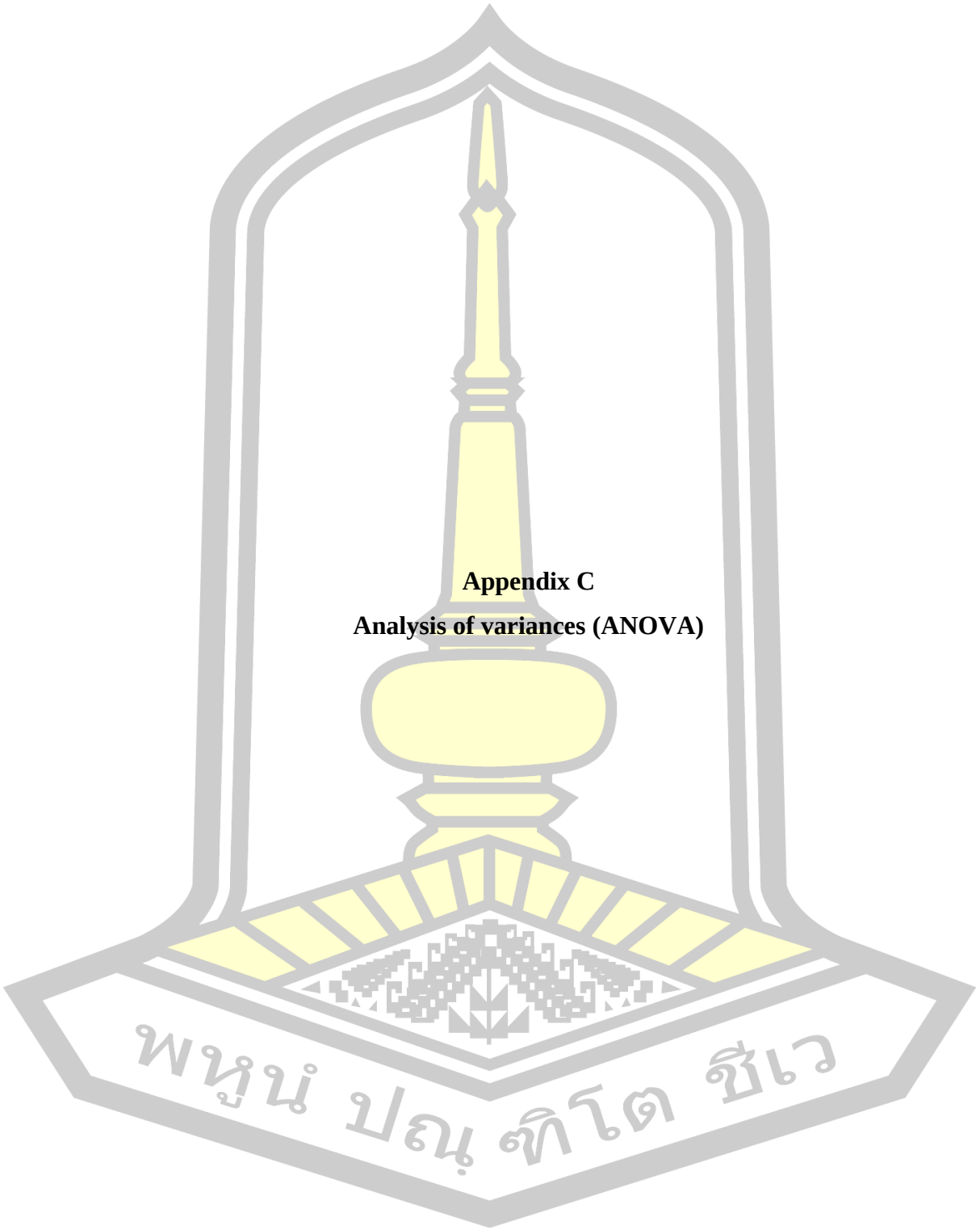


Figure 46 Calibration curve of melatonin of visit 3



Appendix C

Analysis of variances (ANOVA)

Table 47 Analysis of variance of melatonin, free tryptophan and total phenolic content of grain extracts

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Melatonin	Between Groups	13548.942	13	1042.226	139.748	0.000*
	Within Groups	208.820	28	7.458		
	Total	13757.763	41			
Free tryptophan	Between Groups	19.328	13	1.487	862.429	0.000*
	Within Groups	0.048	28	0.002		
	Total	19.377	41			
Total phenolic	Between Groups	2913.113	13	224.086	518.740	0.000*
	Within Groups	12.095	28	0.432		
	Total	2925.209	41			

*Significant difference (p<0.05)

Table 48 Analysis of variance of melatonin, free tryptophan and total phenolic content of mulberry leaf tea using different drying method

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Melatonin	Between Groups	606.561	3	202.187	30.354	0.000*
	Within Groups	53.287	8	6.661		
	Total	659.848	1			
Free tryptophan	Between Groups	0.029	3	295.000	50.104	0.000*
	Within Groups	0.000	8	0.000		
	Total	0.030	11			
Total phenolic	Between Groups	324.899	3	108.300	56.338	0.000*
	Within Groups	15.379	8	1.922		
	Total	340.277	1			

*Significant difference (p<0.05)

Table 49 Analysis of variance of antioxidant activity of grain extracts with the variety standard using DPPH assay

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
TE	Between Groups	34.619	13	2.663	2310.870	0.000*
	Within Groups	0.032	28	0.001		
	Total	34.651	41			
VE	Between Groups	369.153	13	28.396	1898.215	0.000*
	Within Groups	0.419	28	0.015		
	Total	369.572	41			
BHA	Between Groups	65.698	13	5.054	2148.336	0.000*
	Within Groups	0.066	28	0.002		
	Total	65.764	41			
VC	Between Groups	45.566	13	3.505	1911.847	0.000*
	Within Groups	0.051	28	0.002		
	Total	45.617	41			
Mel	Between Groups	105823.839	13	8140.295	636.050	0.000*
	Within Groups	358.350	28	12.798		
	Total	106182.189	41			

*Significant difference ($p<0.05$)

Table 50 Analysis of variance of antioxidant activity of grain extracts with the variety standard using FRAP assay

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
TE	Between Groups	17.720	13	1.363	2436.107	0.000*
	Within Groups	0.016	28	0.001		
	Total	17.735	41			
VE	Between Groups	63.184	13	4.860	3600.239	0.000*
	Within Groups	0.038	28	0.001		
	Total	63.222	41			
BHA	Between Groups	6.656	13	0.512	2239.929	0.000*
	Within Groups	0.006	28	0.000		
	Total	6.662	41			
VC	Between Groups	139.152	13	10.704	2660.161	0.000*
	Within Groups	0.113	28	0.004		
	Total	139.264	41			
Mel	Between Groups	29259.390	13	2250.722	2399.375	0.000*
	Within Groups	26.265	28	0.938		
	Total	29285.656	41			

*Significant difference ($p<0.05$)

Table 51 Analysis of variance of antioxidant activity of grain extracts with the variety standard using ABTS assay

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Trolox	Between Groups	305.419	13	23.494	4191.754	0.000*
	Within Groups	0.157	28	0.006		
	Total	305.576	41			
VE	Between Groups	750.513	13	57.732	3961.985	0.000*
	Within Groups	0.408	28	0.015		
	Total	750.921	41			
BHA	Between Groups	26.697	13	2.054	5103.579	0.000*
	Within Groups	0.011	28	0.000		
	Total	26.708	41			
VC	Between Groups	157.066	13	12.082	4105.533	0.000*
	Within Groups	0.082	28	0.003		
	Total	157.148	41			
Mel	Between Groups	159960.127	13	12304.625	2325.617	0.000*
	Within Groups	148.145	28	5.291		
	Total	160108.273	41			

*Significant difference ($p<0.05$)

Table 52 Analysis of variance of antioxidant activity of mulberry leaf tea using different drying method by DPPH, FRAP and ABTS assays

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
DPPH	Between Groups	0.394	3	0.131	7.328	0.011*
	Within Groups	0.143	8	0.018		
	Total	0.537	11			
ABTS	Between Groups	5.222	3	1.741	125.763	0.000*
	Within Groups	0.111	8	0.014		
	Total	5.333	11			
FRAP	Between Groups	0.145	3	0.048	641.311	0.000*
	Within Groups	0.001	8	0.000		
	Total	0.146	11			

*Significant difference ($p<0.05$)

Table 53 Analysis of variance of correlation between melatonin (Mel), free tryptophan total phenolic content (TPC) and antioxidant assays of grain extracts

	Source of variable	Mel	Free tryptophan	TPC	DPPH	ABTS	FRAP
Mel	Correlation Coefficient	1.000	0.539*	0.829**	0.295	0.437	0.578*
	Sig. (2-tailed)	.	0.047	0.000	0.306	0.118	0.030
	N	14	14	14	14	14	14

Data analysed using Spearman's rho

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

Table 54 Analysis of variance of correlation of between antioxidant activity assays among grain extracts

Source of variable		DPPH	ABTS	FRAP
DPPH	Correlation Coefficient	1.000	0.902**	0.790**
	Sig. (2-tailed)	.	0.000	0.001
	N	14	14	14
ABTS	Correlation Coefficient	0.902**	1.000	0.867**
	Sig. (2-tailed)	0.000	.	0.000
	N	14	14	14
FRAP	Correlation Coefficient	0.790**	0.867**	1.000
	Sig. (2-tailed)	0.001	0.000	.
	N	14	14	14

Data analysed using Spearman's rho, * Correlation is significant at the 0.05 level (2-tailed), ** Correlation is significant at the 0.01 level (2-tailed)

Table 55 Analysis of variance of the composition of nine pasteurized milk treatments

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Fat	Between Groups	1.936	8	0.242	33.278	0.000*
	Within Groups	0.131	18	0.007		
	Total	2.066	26			
Solid not fat	Between Groups	13.785	8	1.723	202.543	0.000*
	Within Groups	0.153	18	0.009		
	Total	13.938	26			
Lactose	Between Groups	0.310	8	0.039	8.001	0.000*
	Within Groups	0.087	18	0.005		
	Total	0.397	26			
Protein	Between Groups	5.812	8	0.727	105.412	0.000*
	Within Groups	0.124	18	0.007		
	Total	5.937	26			
Total solids	Between Groups	25.679	8	3.210	153.473	0.000*
	Within Groups	0.376	18	0.021		
	Total	26.055	26			

*Significant difference (p<0.05)

Table 56 Analysis of variance of quality of nine pasteurized milk treatments

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
pH	Between Groups	0.048	8	0.006	18.968	0.000*
	Within Groups	0.006	18	0.000		
	Total	0.054	26			
Temp.	Between Groups	1.156	8	0.145	6.614	0.000*
	Within Groups	0.393	18	0.022		
	Total	1.550	26			
Total plate count	Between Groups	1133.333	8	141.667	0.781	0.625*
	Within Groups	3266.667	18	181.481		
	Total	4400.000	26			
L*	Between Groups	458.121	8	57.265	356.356	0.000*
	Within Groups	2.893	18	0.161		
	Total	461.013	26			
a*	Between Groups	112.475	8	14.059	296.056	0.000*
	Within Groups	0.855	18	0.047		
	Total	113.330	26			
b*	Between Groups	73.860	8	9.233	163.945	0.000
	Within Groups	1.014	18	0.056		
	Total	74.874	26			

*Significant difference ($p<0.05$)

Table 57 Analysis of variance of melatonin, free tryptophan and total phenolic content (TPC) of pasteurized milk treatments

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Melatonin	Between Groups	35.850	8	4.481	210.459	0.000*
	Within Groups	0.383	18	0.021		
	Total	36.233	26			
Free tryptophan	Between Groups	0.666	8	0.083	178.264	0.000*
	Within Groups	0.008	18	0.000		
	Total	0.674	26			
TPC	Between Groups	13586.106	8	1698.263	199.319	0.000*
	Within Groups	153.366	18	8.520		
	Total	13739.472	26			

*Significant difference (p<0.05)

Table 58 Analysis of variance of antioxidant activity of pasteurized milk treatments using DPPH assay

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
DPPH-TE	Between Groups	0.136	8	0.017	53.844	0.000*
	Within Groups	0.006	18	0.000		
	Total	0.141	26			
DPPH-VC	Between Groups	0.256	8	0.032	50.918	0.000*
	Within Groups	0.011	18	0.001		
	Total	0.268	26			
DPPH-VE	Between Groups	2.100	8	0.262	53.364	0.000*
	Within Groups	0.089	18	0.005		
	Total	2.188	26			
DPPH-BHA	Between Groups	0.291	8	0.036	51.406	0.000*
	Within Groups	0.013	18	0.001		
	Total	0.304	26			
DPPH-Mel	Between Groups	6480.469	8	810.059	51.104	0.000*
	Within Groups	285.319	18	15.851		
	Total	6765.788	26			

*Significant difference (p<0.05)

Table 59 Analysis of variance of antioxidant activity of pasteurized milk treatments using FRAP assay

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
FRAP-TE	Between Groups	0.110	8	0.014	53.254	0.000*
	Within Groups	0.005	18	0.000		
	Total	0.115	26			
FRAP-VC	Between Groups	0.948	8	0.119	51.039	0.000*
	Within Groups	0.042	18	0.002		
	Total	0.990	26			
FRAP-VE	Between Groups	0.289	8	0.036	47.755	0.000*
	Within Groups	0.014	18	0.001		
	Total	0.302	26			
FRAP-BHA	Between Groups	0.050	8	0.006	54.145	0.000*
	Within Groups	0.002	18	0.000		
	Total	0.052	26			
FRAP-MEL	Between Groups	239.448	8	29.931	50.214	0.000*
	Within Groups	10.729	18	0.596		
	Total	250.177	26			

*Significant difference (p<0.05)

Table 60 Analysis of variance of antioxidant activity of pasteurized milk treatments using ABTS assay

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
ABTS-Trolox	Between Groups	0.769	8	0.096	106.778	0.000*
	Within Groups	0.016	18	0.001		
	Total	0.785	26			
ABTS-VC	Between Groups	0.348	8	0.043	122.214	0.000*
	Within Groups	0.006	18	0.000		
	Total	0.354	26			
ABTS-VE	Between Groups	1.620	8	0.202	108.896	0.000*
	Within Groups	0.033	18	0.002		
	Total	1.653	26			
ABTS-BHA	Between Groups	0.165	8	0.021	105.113	0.000*
	Within Groups	0.004	18	0.000		
	Total	0.169	26			
ABTS-Mel	Between Groups	120.836	8	15.105	106.526	0.000*
	Within Groups	2.552	18	0.142		
	Total	123.389	26			

*Significant difference ($p<0.05$)

Table 61 Analysis of variance of sensory score of pasteurized milk treatment using a 9-point hedonic scale

	Source of variable	Type III Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Color	Corrected Model	380.900[a]	37	10.295	5.686	0.000*
	Intercept	9684.033	1	9684.033	5348.427	0.000*
	Formulas	172.600	8	21.575	11.916	0.000*
	Block	208.300	29	7.183	3.967	0.000*
	Error	420.067	232	1.811		
	Total	10485.000	270			
	Corrected Total	800.967	269			
	a. R Squared = 0.476 (Adjusted R Squared = 0.392)					
Flavor	Corrected Model	125.759[a]	37	3.399	2.320	0.000*
	Intercept	10817.337	1	10817.337	7383.333	0.000*
	Formulas	66.996	29	2.310	1.577	0.036*
	Block	58.763	8	7.345	5.014	0.000*
	Error	339.904	232	1.465		
	Total	11283.000	270			
	Corrected Total	465.663	269			
	a. R Squared = 0.270 (Adjusted R Squared = 0.154)					
Texture	Corrected Model	181.793[a]	37	4.913	3.683	0.000*
	Intercept	10880.726	1	10880.726	8156.638	0.000*
	Formulas	101.719	29	3.508	2.629	0.000*
	Block	80.074	8	10.009	7.503	0.000*
	Error	309.481	232	1.334		
	Total	11372.000	270			
	Corrected Total	491.274	269			
	a. R Squared = 0.370 (Adjusted R Squared = 0.270)					

*Significant difference ($p<0.05$)

Table 61 Analysis of variance of sensory score of pasteurized milk treatment using a 9-point hedonic scale (continued)

Source of variable		Type III Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Taste	Corrected Model	246.481[a]	37	6.662	3.611	0.000*
	Intercept	9961.481	1	9961.481	5399.214	0.000*
	Block	109.630	29	3.780	2.049	0.002*
	Formulas	136.852	8	17.106	9.272	0.000*
	Error	428.037	232	1.845		
	Total	10636.000	270			
	Corrected Total	674.519	269			
	a. R Squared = 0.365 (Adjusted R Squared = 0.264)					
Overall liking	Corrected Model	224.444[a]	37	6.066	3.960	0.000*
	Intercept	10528.133	1	10528.133	6872.184	0.000*
	Block	94.311	29	3.252	2.123	0.001*
	Formulas	130.133	8	16.267	10.618	0.000*
	Error	355.422	232	1.532		
	Total	11108.000	270			
	Corrected Total	579.867	269			
	a. R Squared = 0.387 (Adjusted R Squared = 0.289)					

Table 62 Analysis of variance of composition of the accepted pasteurized milk (treatment 5) at different storage times

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
Fat	Between Groups	0.002	4	0.001	0.083	0.986
	Within Groups	0.063	10	0.006		
	Total	0.065	14			
Solid not fat	Between Groups	0.003	4	0.001	0.095	0.982
	Within Groups	0.091	10	0.009		
	Total	0.094	14			
Lactose	Between Groups	0.007	4	0.002	0.168	0.950
	Within Groups	0.097	10	0.010		
	Total	0.104	14			
Protein	Between Groups	0.002	4	0.000	0.052	0.994
	Within Groups	0.090	10	0.009		
	Total	0.092	14			
Total solids	Between Groups	0.008	4	0.002	0.078	0.987
	Within Groups	0.270	10	0.027		
	Total	0.279	14			

*Significant difference ($p<0.05$)

Table 63 Quality of the accepted pasteurized milk (treatment 5) at different storage times

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
pH	Between Groups	0.004	4	0.001	0.194	0.936
	Within Groups	0.058	10	0.006		
	Total	0.062	14			
TPC count	Between Groups	2.050E8	4	51256479.783	88.836	0.000*
	Within Groups	5769772.274	10	576977.227		
	Total	2.108E8	14			
L*	Between Groups	0.011	4	0.003	0.201	0.932
	Within Groups	0.136	10	0.014		
	Total	0.147	14			

*Significant difference *($p<0.05$)*

Table 64 Analysis of variance of quality of the accepted pasteurized milk (treatment 5) at different storage times (continued)

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
a*	Between Groups	0.009	4	0.002	0.687	0.617
	Within Groups	0.033	10	0.003		
	Total	0.042	14			
b*	Between Groups	0.012	4	0.003	0.958	0.471
	Within Groups	0.030	10	0.003		
	Total	0.042	14			

*Significant difference ($p<0.05$)

Table 65 Analysis of variance of melatonin, free tryptophan and total phenolic content (TPC) of the accepted pasteurized milk at different storage times

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
TPC	Between Groups	37.843	4	9.461	2.595	0.101
	Within Groups	36.461	10	3.646		
	Total	74.304	14			
Melatonin	Between Groups	0.149	4	0.037	2.870	0.080
	Within Groups	0.129	10	0.013		
	Total	0.278	14			
Free tryptophan	Between Groups	0.003	4	0.001	2.745	0.089
	Within Groups	0.003	10	0.000		
	Total	0.007	14			

*Significant difference ($p<0.05$)

Table 66 Analysis of variance of antioxidant activity using DPPH, FRAP and ABTS assays of the accepted pasteurized milk at different storage times

Source of variable		Sum of Squares	df	Mean Square	F	Sig. (*p*-value)
DPPH-TE	Between Groups	0.001	4	0.000	3.439	0.052
	Within Groups	0.001	10	0.000		
	Total	0.002	14			
FRAP-TE	Between Groups	0.002	4	0.000	1.032	0.437
	Within Groups	0.004	10	0.000		
	Total	0.006	14			
ABTS-TE	Between Groups	0.002	4	0.001	1.081	0.416
	Within Groups	0.005	10	0.000		
	Total	0.007	14			
DPPH-Mel	Between Groups	58.291	4	14.573	1.680	0.230
	Within Groups	86.744	10	8.674		
	Total	145.035	14			
FRAP-Mel	Between Groups	4.027	4	1.007	1.031	0.438
	Within Groups	9.764	10	0.976		
	Total	13.791	14			
ABTS-Mel	Between Groups	0.000	4	0.000	1.081	0.416
	Within Groups	0.001	10	0.000		
	Total	0.001	14			

*Significant difference ($p<0.05$)

Table 67 Analysis of variance of color of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.57	5	0.11	1.31	0.3884	not significant
Linear Mixture	0.19	2	0.096	1.10	0.4009	
AB	0.071	1	0.071	0.82	0.4079	
AC	4.491E-003	1	4.491E-003	0.052	0.8293	
BC	0.27	1	0.27	3.08	0.1394	
Residual	0.44	5	0.087			
Lack of Fit	0.43	3	0.14	42.84	0.0229	significant
Pure Error	6.667E-003	2	3.333E-003			
Cor Total	1.00	10				
The model to Fit						
R-Squared	0.5663					
Adj R-Squared	0.1326					
C.V. %	4.32					

Table 68 Analysis of variance of flavor of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.43	5	0.086	1.76	0.2757	not significant
Linear Mixture	0.20	2	0.10	2.07	0.2211	
AB	0.15	1	0.15	3.14	0.1366	
AC	3.439E-003	1	3.439E-003	0.070	0.8016	
BC	0.18	1	0.18	3.77	0.1097	
Residual	0.24	5	0.049			
Lack of Fit	0.24	3	0.080	30.82	0.0316	significant
Pure Error	5.185E-003	2	2.593E-003			
Cor Total	0.68	10				
The model to Fit						
R-Squared	0.6372					
Adj R-Squared	0.2744					
C.V. %	3.26					

Table 69 Analysis of variance of taste of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.38	2	0.19	4.14	0.0582	not significant
Linear Mixture	0.38	2	0.19	4.14	0.0582	
Residual	0.36	8	0.046			
Lack of Fit	0.35	6	0.058	8.31	0.1113	not significant
Pure Error	0.014	2	7.037E-003			
Cor Total	0.74	10				
The model to Fit						
R-Squared	0.5088					
Adj R-Squared	0.3860					
C.V. %	3.15					

Table 70 Analysis of variance of texture of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.27	5	0.054	1.61	0.3066	not significant
Linear Mixture	0.019	2	9.259E-003	0.28	0.7679	
AB	0.032	1	0.032	0.95	0.3744	
AC	0.026	1	0.026	0.79	0.4151	
BC	0.10	1	0.10	3.10	0.1385	
Residual	0.17	5	0.033			
Lack of Fit	0.14	3	0.046	3.17	0.2490	not significant
Pure Error	0.029	2	0.014			
Cor Total	0.43	10				
The model to Fit						
R-Squared	0.6171					
Adj R-Squared	0.2342					
C.V. %	2.76					

Table 71 Analysis of variance of overall liking of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.52	6	0.086	1.41	0.3842	not significant
Linear Mixture	0.20	2	0.099	1.62	0.3044	
AB	0.075	1	0.075	1.23	0.3297	
AC	6.478E-003	1	6.478E-003	0.11	0.7604	
BC	0.20	1	0.20	3.35	0.1410	
ABC	0.067	1	0.067	1.10	0.3534	
Residual	0.24	4	0.061			
Lack of Fit	0.17	2	0.086	2.38	0.2957	not significant
Pure Error	0.072	2	0.036			
Cor Total	0.76	10				
The model to Fit						
R-Squared	0.6797					
Adj R-Squared	0.1994					
C.V. %	3.59					

Table 72 Analysis of variance of %fat of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.019	2	9.393E-003	30.73	0.0002	significant
Linear Mixture	0.019	2	9.393E-003	30.73	0.0002	
Residual	2.445E-003	8	3.056E-004			
Lack of Fit	2.349E-003	6	3.915E-004	8.13	0.1136	not significant
Pure Error	9.630E-005	2	4.815E-005			
Cor Total	0.021	10				
The model to Fit						
R-Squared	0.8848					
Adj R-Squared	0.8560					
C.V. %	0.41					

Table 73Analysis of variance of %solid not fat of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.15	2	0.074	53.25	< 0.0001	significant
Linear Mixture	0.15	2	0.074	53.25	< 0.0001	
Residual	0.011	8	1.398E-003			
Lack of Fit	0.011	6	1.786E-003	7.66	0.1200	not significant
Pure Error	4.667E-004	2	2.333E-004			
Cor Total	0.16	10				
The model to Fit						
R-Squared	0.9301					
Adj R-Squared	0.9127					
C.V. %	0.37					

Table 74 Analysis of variance of %lactose of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	5.390E-003	5	1.078E-003	5.41	0.0437	significant
Linear Mixture	2.519E-004	2	1.259E-004	0.63	0.5691	
AB	1.977E-003	1	1.977E-003	9.93	0.0254	
AC	3.373E-003	1	3.373E-003	16.94	0.0092	
BC	4.386E-004	1	4.386E-004	2.20	0.1979	
Residual	9.957E-004	5	1.991E-004			
Lack of Fit	4.401E-004	3	1.467E-004	0.53	0.7061	not significant
Pure Error	5.556E-004	2	2.778E-004			
Cor Total	6.386E-003	10				
The model to Fit						
R-Squared	0.8441					
Adj R-Squared	0.6882					
C.V. %	0.25					

Table 75 Analysis of variance of %protein of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.082	2	0.041	180.87	< 0.0001	significant
Linear Mixture	0.082	2	0.041	180.87	< 0.0001	
Residual	1.821E-003	8	2.276E-004			
Lack of Fit	1.354E-003	6	2.257E-004	0.97	0.5886	not significant
Pure Error	4.667E-004	2	2.333E-004			
Cor Total	0.084	10				
The model to Fit						
R-Squared	0.9784					
Adj R-Squared	0.9730					
C.V. %	0.32					

Table 76 Analysis of variance of %total solids of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.27	2	0.14	55.51	< 0.0001	significant
Linear Mixture	0.27	2	0.14	55.51	< 0.0001	
Residual	0.020	8	2.457E-003			
Lack of Fit	0.019	6	3.186E-003	11.78	0.0803	not significant
Pure Error	5.407E-004	2	2.704E-004			
Cor Total	0.29	10				
The model to Fit						
R-Squared	0.9328					
Adj R-Squared	0.9160					
C.V. %	0.34					

Table 77 Analysis of variance of pH of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.038	5	7.501E-003	4.42	0.0644	not significant
Linear Mixture	7.563E-003	2	3.781E-003	2.23	0.2034	
AB	1.250E-003	1	1.250E-003	0.74	0.4300	
AC	1.218E-003	1	1.218E-003	0.72	0.4356	
BC	0.012	1	0.012	7.02	0.0454	
Residual	8.489E-003	5	1.698E-003			
Lack of Fit	7.815E-003	3	2.605E-003	7.73	0.1167	not significant
Pure Error	6.741E-004	2	3.370E-004			
Cor Total	0.046	10				
The model to Fit						
R-Squared	0.8154					
Adj R-Squared	0.6309					
C.V. %	0.63					

Table 78 Analysis of variance of total plate count of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	675.93	2	337.96	1.68	0.2466	not significant
Linear Mixture	675.93	2	337.96	1.68	0.2466	
Residual	1612.96	8	201.62			
Lack of Fit	1427.78	6	237.96	2.57	0.3064	not significant
Pure Error	185.19	2	92.59			
Cor Total	2288.89	10				
The model to Fit						
R-Squared	0.2953					
Adj R-Squared	0.1191					
C.V. %	32.77					

Table 79 Analysis of variance of temperature of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	1.44	5	0.29	2.19	0.2055	not significant
Linear Mixture	0.55	2	0.28	2.09	0.2189	
AB	0.18	1	0.18	1.38	0.2923	
AC	0.53	1	0.53	4.01	0.1016	
BC	0.050	1	0.050	0.38	0.5654	
Residual	0.66	5	0.13			
The model to Fit						
R-Squared	0.6861					
Adj R-Squared	0.3723					
C.V. %	7.02					

Table 80 Analysis of variance of L* of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	14.67	2	7.33	25.62	0.0003	significant
Linear Mixture	14.67	2	7.33	25.62	0.0003	
Residual	2.29	8	0.29			
Lack of Fit	1.48	6	0.25	0.61	0.7299	not significant
Pure Error	0.81	2	0.40			
Cor Total	16.96	10				
The model to Fit						
R-Squared	0.8650					
Adj R-Squared	0.8312					
C.V. %	0.82					

Table 81 Analysis of variance of a* of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	5.83	5	1.17	10.32	0.0114	significant
Linear Mixture	2.03	2	1.01	8.97	0.0222	
AB	0.42	1	0.42	3.75	0.1105	
AC	3.08	1	3.08	27.24	0.0034	
BC	1.36	1	1.36	12.04	0.0179	
Residual	0.56	5	0.11			
Lack of Fit	0.36	3	0.12	1.21	0.4823	not significant
Pure Error	0.20	2	0.10			
Cor Total	6.39	10				
The model to Fit						
R-Squared	0.9116					
Adj R-Squared	0.8233					
C.V. %	3.15					

Table 82 Analysis of variance of b* of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	3.63	5	0.73	1.58	0.3151	not significant
Linear Mixture	0.95	2	0.48	1.03	0.4218	
AB	1.36	1	1.36	2.94	0.1469	
AC	2.35	1	2.35	5.09	0.0737	
BC	0.086	1	0.086	0.19	0.6837	
Residual	2.30	5	0.46			
Lack of Fit	1.97	3	0.66	3.88	0.2118	not significant
Pure Error	0.34	2	0.17			
Cor Total	5.94	10				
The model to Fit						
R-Squared	0.6117					
Adj R-Squared	0.2234					
C.V. %	3.44					

Table 83 Analysis of variance of TPC of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	34.45	2	17.23	19.96	0.0008	significant
Linear Mixture	34.45	2	17.23	19.96	0.0008	
Residual	6.90	8	0.86			
Lack of Fit	6.30	6	1.05	3.51	0.2385	not significant
Pure Error	0.60	2	0.30			
Cor Total	41.35	10				
The model to Fit						
R-Squared	0.8331					
Adj R-Squared	0.7913					
C.V. %	1.33					

Table 84 Analysis of variance of melatonin of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.68	2	0.34	37.91	< 0.0001	significant
Linear Mixture	0.68	2	0.34	37.91	< 0.0001	
Residual	0.071	8	8.923E-003			
Lack of Fit	0.069	6	0.012	11.32	0.0834	not significant
Pure Error	2.042E-003	2	1.021E-003			
Cor Total	0.75	10				
The model to Fit						
R-Squared	0.9046					
Adj R-Squared	0.8807					
C.V. %	6.70					

Table 85 Analysis of variance of free tryptophan of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	8.929E-003	5	1.786E-003	5.36	0.0446	significant
Linear Mixture	5.194E-003	2	2.597E-003	7.79	0.0291	
AB	1.781E-003	1	1.781E-003	5.34	0.0688	
AC	1.325E-004	1	1.325E-004	0.40	0.5561	
BC	3.387E-003	1	3.387E-003	10.16	0.0243	
Residual	1.667E-003	5	3.334E-004			
Lack of Fit	1.359E-003	3	4.529E-004	2.94	0.2641	not significant
Pure Error	3.082E-004	2	1.541E-004			
Cor Total	8.929E-003	5	1.786E-003	5.36	0.0446	significant
The model to Fit						
R-Squared	0.8427					
Adj R-Squared	0.6854					
C.V. %	6.86					

Table 86 Analysis of variance of DPPH-Trolox of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	3.395E-003	2	1.697E-003	49.69	< 0.0001	significant
Linear Mixture	3.395E-003	2	1.697E-003	49.69	< 0.0001	
Residual	2.732E-004	8	3.416E-005			
Lack of Fit	2.036E-004	6	3.394E-005	0.98	0.5860	not significant
Pure Error	6.960E-005	2	3.480E-005			
Cor Total	3.668E-003	10				
The model to Fit						
R-Squared	0.9255					
Adj R-Squared	0.9069					
C.V. %	1.15					

Table 87 Analysis of variance of DPPH-Trolox of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	3.395E-003	2	1.697E-003	49.69	< 0.0001	significant
Linear Mixture	3.395E-003	2	1.697E-003	49.69	< 0.0001	
Residual	2.732E-004	8	3.416E-005			
Lack of Fit	2.036E-004	6	3.394E-005	0.98	0.5860	not significant
Pure Error	6.960E-005	2	3.480E-005			
Cor Total	3.668E-003	10				
The model to Fit						
R-Squared	0.9255					
Adj R-Squared	0.9069					
C.V. %	1.15					

Table 88 Analysis of variance of ABTS-Trolox of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.032	2	0.016	103.57	< 0.0001	significant
Linear Mixture	0.032	2	0.016	103.57	< 0.0001	significant
Residual	0.032	2	0.016	103.57	< 0.0001	
Lack of Fit	1.219E-003	8	1.524E-004			
Pure Error	1.127E-003	6	1.878E-004	4.08	0.2099	not significant
Cor Total	9.209E-005	2	4.604E-005			
The model to Fit						
R-Squared	0.9628					
Adj R-Squared	0.9535					
C.V. %	1.69					

Table 89 Analysis of variance of FRAP-Trolox of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	0.011	2	5.438E-003	65.37	< 0.0001	significant
Linear Mixture	0.011	2	5.438E-003	65.37	< 0.0001	
Residual	6.656E-004	8	8.320E-005			
Lack of Fit	5.626E-004	6	9.376E-005	1.82	0.3961	not significant
Pure Error	1.030E-004	2	5.150E-005			
Cor Total	0.012	10				
The model to Fit						
R-Squared	0.9423					
Adj R-Squared	0.9279					
C.V. %	1.86					

Table 90 Analysis of variance of DPPH-Melatonin of pasteurized milk treatments using mixture design

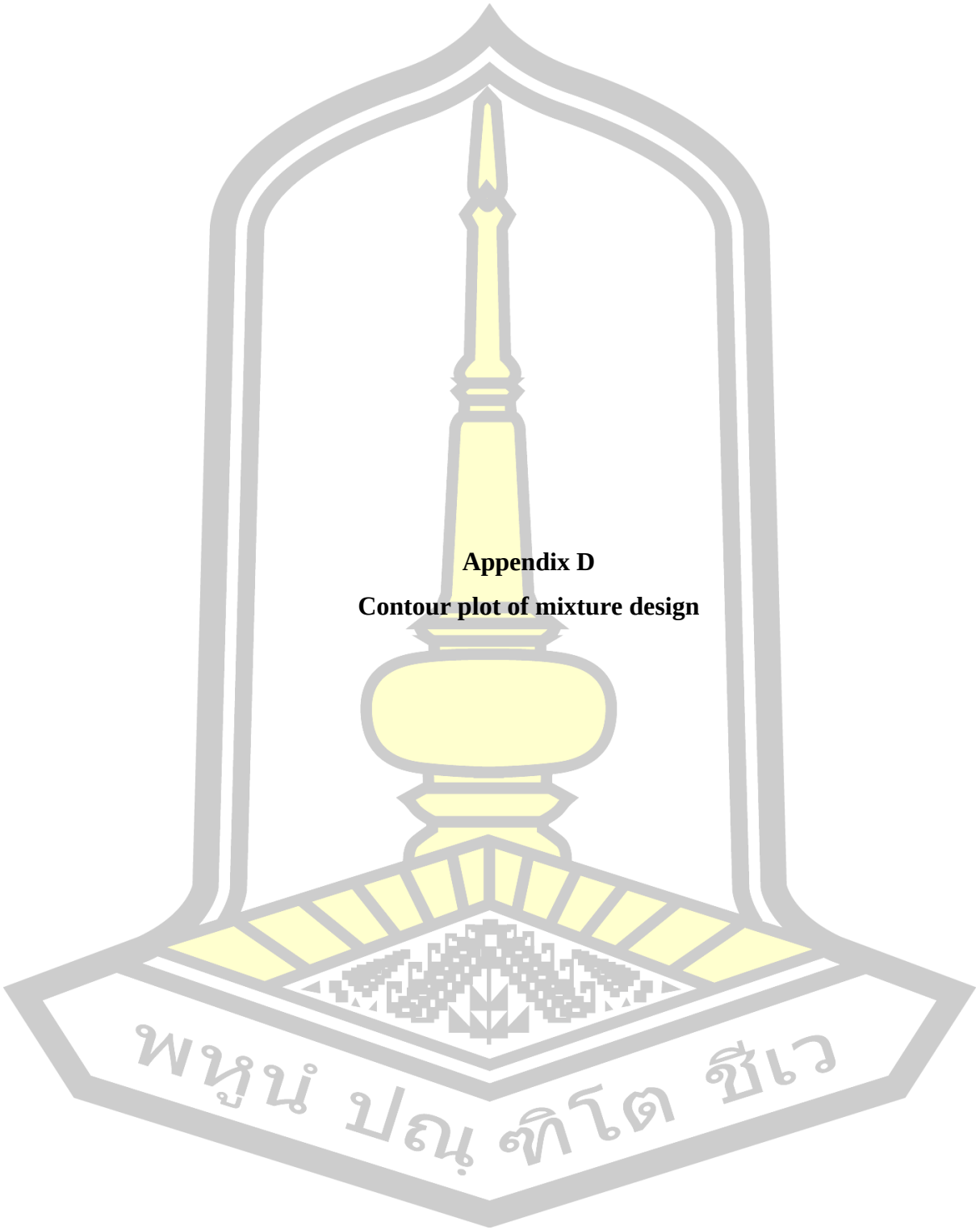
Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	199.20	2	99.60	47.27	< 0.0001	significant
Linear Mixture	199.20	2	99.60	47.27	< 0.0001	
Residual	16.86	8	2.11			
Lack of Fit	12.81	6	2.13	1.05	0.5615	not significant
Pure Error	4.05	2	2.03			
Cor Total	216.06	10				
The model to Fit						
R-Squared	0.9220					
Adj R-Squared	0.9025					
C.V. %	3.11					

Table 91 Analysis of variance of ABTS-Melatonin of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	4.61	2	2.31	103.03	< 0.0001	significant
Linear Mixture	4.61	2	2.31	103.03	< 0.0001	
Residual	0.18	8	0.022			
Lack of Fit	0.17	6	0.028	4.05	0.2110	not significant
Pure Error	0.014	2	6.802E-003			
Cor Total	4.79	10				
The model to Fit						
R-Squared	0.9626					
Adj R-Squared	0.9533					
C.V. %	2.52					

Table 92 Analysis of variance of FRAP-Melatonin of pasteurized milk treatments using mixture design

Source of variable	Sum of Squares	df	Mean Square	F	Sig. (*p*-value)	
Model	24.11	2	12.06	66.39	< 0.0001	significant
Linear Mixture	24.11	2	12.06	66.39	< 0.0001	
Residual	1.45	8	0.18			
Lack of Fit	1.22	6	0.20	1.78	0.4023	not significant
Pure Error	0.23	2	0.11			
Cor Total	25.57	10				
The model to Fit						
R-Squared	0.9432					
Adj R-Squared	0.9290					
C.V. %	1.95					



Appendix D

Contour plot of mixture design

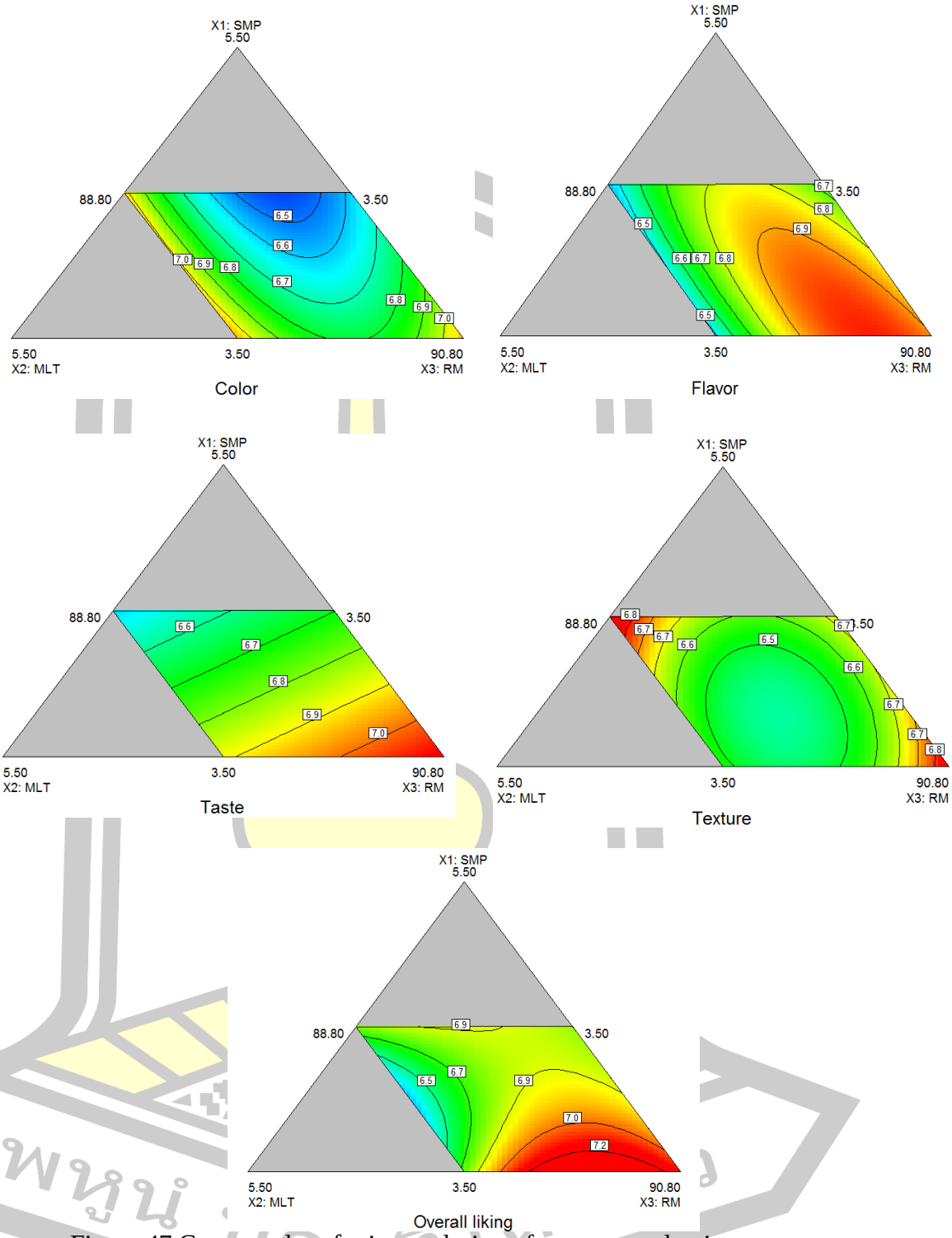


Figure 47 Contour plot of mixture design of sensory evaluation

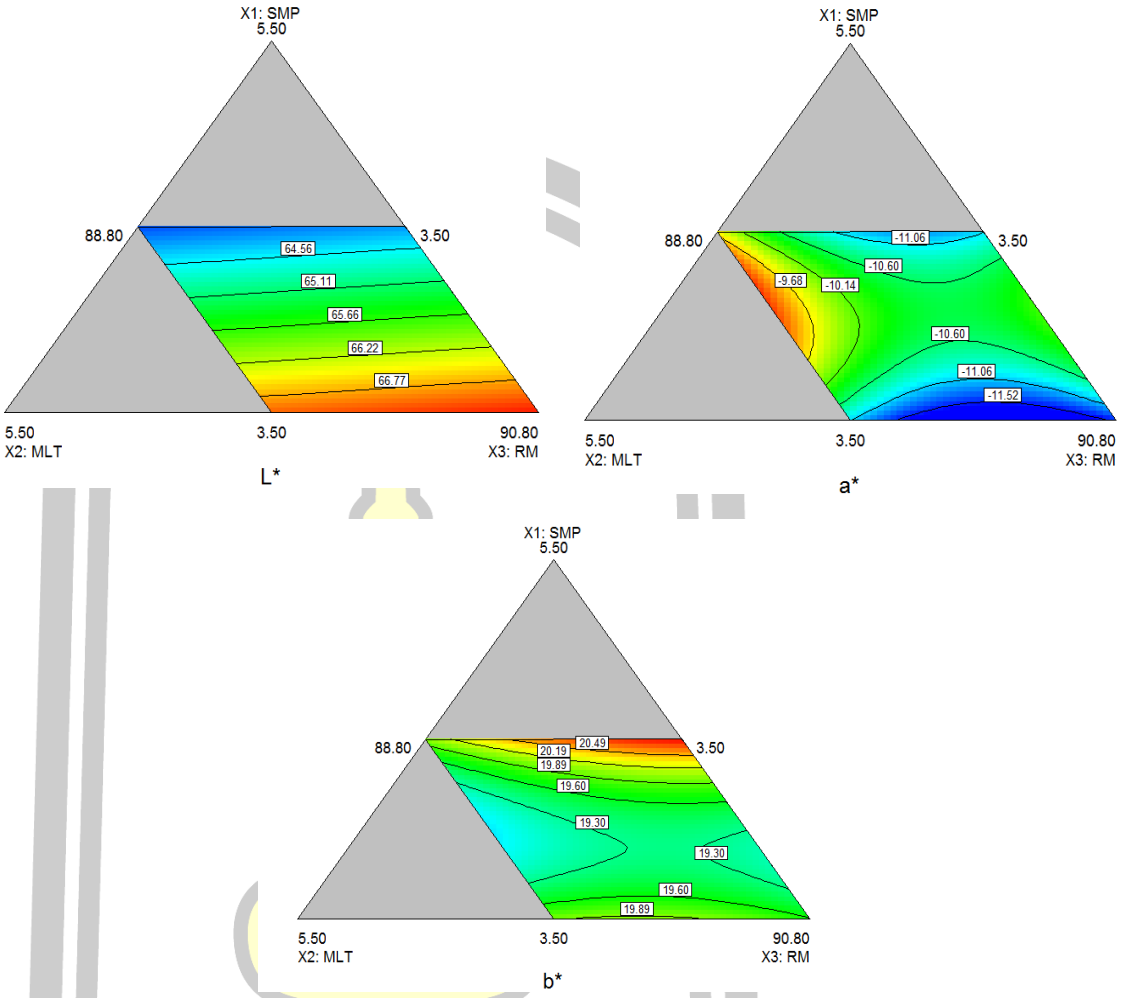


Figure 48 Contour plot of mixture design of color parameter

BIOGRAPHY

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PLACE OF WORK	-
EDUCATION	2006 Bachelor degree, Bs.C. Major of Food Technology and Nutrition, Mahasarakham University 2009 Master degree, Ms.C. Major of Food Technology, Mahasarakham University Mahasarakham University 2020 Doctor of Philosophy, Ph.D. Major of Food Technology, Mahasarakham University
Research grants & awards	- Research and Researchers for Industries (RRi) of The Thailand Research Fund (TRF) or Thailand Science Research and Innovation (TSRI) - Postgraduate Scholarship of faculty of technology, Mahasarakham University - Postgraduate Scholarship of Mahasarakham University
Research output	- Dietary sources of melatonin and benefits from production of high melatonin pasteurized milk - Optimization of pasteurized milk with soymilk powder and mulberry leaf tea based on melatonin, bioactive compounds and antioxidant activity using response surface methodology