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DEVELOPMENT OF ICE CREAM FORTIFIED WITH CALCIUM AND VITAMIN D_3 EMULSION

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Thesis Title	DEVELOPMENT OF ICE CREAM FORTIFIED WITH CALCIUM AND VITAMIN D_3 EMULSION
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นาถอุมา ทิพย์ชูวงศ์ : การพัฒนาไอศกรีมเสริมแคลเซียมและอิมัลชันวิตามินดี 3 (DEVELOPMENT OF ICE CREAM FORTIFIED WITH CALCIUM AND VITAMIN D_3 EMULSION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ภญ. ดร.รสริน ตันสวัสดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.พนิตา งามเชื้อชิต, 84 หน้า.

การศึกษานี้มีจุดประสงค์เพื่อพัฒนาไอศกรีมเสริมแคลเซียมและอิมัลชันวิตามินดีที่ใช้โปรตีนนมเป็นสารก่ออิมัลชัน โดยจะใช้โปรตีนนมทั้งหมด 3 ชนิด ได้แก่ นมผงขาดมันเนย (nonfat dry milk, NFDM), โซเดียมเคซีเนต (sodium casienate, NaCN) และเวย์โปรตีนไอโซเลท (whey protein isolate, WPI) ในการเตรียมอิมัลชันวิตามินดี การศึกษาคุณสมบัติทางเคมีกายภาพของอิมัลชันวิตามินดี พบว่าอิมัลชันวิตามินดีที่ใช้ NaCN เป็นสารก่ออิมัลชันจะมีขนาดของหยดน้ำมันในอิมัลชันเล็กที่สุดและมีดัชนีการเกิดครีมน้อยที่สุดตลอดระยะเวลาการเก็บรักษา ($P < 0.05$) ดังนั้นอิมัลชันวิตามินดีที่ใช้ NaCN เป็นสารก่ออิมัลชันจึงถูกเลือกมาเสริมลงในไอศกรีม 3 ชนิด ได้แก่ ไอศกรีมชนิดปกติ (RF, ไขมันมากกว่าร้อยละ 10) ชนิดไลท์ (LF, ไขมันต่ำกว่าร้อยละ 5) และชนิดปราศจากไขมัน (FF, ไขมันต่ำกว่าร้อยละ 0.625) โดยปริมาณวิตามินดีที่เติมในไอศกรีมเท่ากับ 250 ยูนิตต่อหนึ่งหน่วยบริโภค การวิเคราะห์ปริมาณของอิมัลชันวิตามินดีในไอศกรีมที่เก็บที่อุณหภูมิ -20 องศาเซลเซียส ณ วันที่ 0, 7, 14, 28 และ 56 เมื่อเปรียบเทียบกับกลุ่มควบคุมซึ่งเป็นรูปผงแห้ง พบว่ารูปอิมัลชันสามารถทำให้ความคงตัวของวิตามินดีดีขึ้นในไอศกรีมทั้ง 3 ชนิดที่มีปริมาณไขมันในตำรับแตกต่างกัน นอกจากนี้ยังมีการเสริมแคลเซียม 200 มิลลิกรัมร่วมกับอิมัลชันวิตามินดีลงในไอศกรีม RF, LF และ FF ผลการศึกษาพบว่าทั้งแคลเซียมและวิตามินดีในไอศกรีมมีความคงตัวดีตลอดระยะเวลา 28 วันของการเก็บรักษา การศึกษาคุณสมบัติทางกายภาพและจุลชีววิทยาของไอศกรีมเสริมแคลเซียมและอิมัลชันวิตามินดี พบว่าร้อยละของการขึ้นฟูและความหนืดของไอศกรีมทุกสูตรตำรับไม่แตกต่างกัน ความแข็งและเวลาที่ใช้ในการละลายไอศกรีมจะเพิ่มขึ้นเมื่อปริมาณของไขมันในตำรับลดลง แอโรบิคแบคทีเรียในทุกตำรับน้อยกว่า 1,000 โคโลนีต่อมิลลิลิตร และไม่พบเชื้ออีโคไลและโคลิฟอร์มตลอดการเก็บรักษานาน 28 วัน โดยการศึกษานี้อาจเป็นประโยชน์สำหรับอุตสาหกรรมการผลิตไอศกรีมที่ต้องการเสริมแคลเซียมและวิตามินดีลงในผลิตภัณฑ์

ภาควิชา	อาหารและเภสัชเคมี	ลายมือชื่อนิสิต .....
สาขาวิชา	อาหารเคมีและโภชนศาสตร์ทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
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NARDAUMA TIPCHUWONG: DEVELOPMENT OF ICE CREAM FORTIFIED WITH CALCIUM AND VITAMIN D_3 EMULSION. ADVISOR: ROSSARIN TANSAWAT, Ph.D., CO-ADVISOR: PANITA NGAMCHUACHIT, Ph.D., 84 pp.

This study aimed to develop ice cream fortified with calcium and emulsified vitamin D_3 using milk proteins as an emulsifier. Physicochemical stability of vitamin D_3 emulsion using different milk protein emulsifiers including nonfat dry milk (NFDM), sodium caseinate (NaCN), and whey protein isolate (WPI) was investigated. Emulsion using NaCN had the smallest oil droplet size and the lowest creaming index throughout the storage time ($P < 0.05$). Then, vitamin D_3 emulsified by NaCN was selected to fortify in regular fat (RF, > 10% fat), light fat (LF, < 5% fat) and fat free (FF, < 0.625% fat) ice creams at 250 IU per serving. Retention of vitamin D_3 in each ice cream formulation was determined compared to control (non-emulsified vitamin D_3) at day 0, 7, 14, 28 and 56 of storage at -20 °C. The results indicated that emulsified form of vitamin D_3 remarkably improved vitamin D_3 stability in all ice cream formulations. The ice creams were further fortified with 200 mg elemental calcium. Calcium content and vitamin D retention were investigated through 28-day storage. The results showed that both nutrients were preserved in the ice creams throughout the storage time. Physical characteristics and microbial properties of the ice creams were also determined. Overrun and viscosity of all ice cream formulations were not significantly different. Hardness and melting behavior increased with decreasing fat content. Aerobic bacteria were < 1,000 CFU/mL and no *Escherichia coli* and coliform were detected through 28 days. Our findings could be useful for the ice cream industries that seek to add vitamin D and calcium to their products.

Department:	Food and Pharmaceutical Chemistry	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Food Chemistry and Medical Nutrition	Co-Advisor's Signature	.....
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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CFU	colony-forming unit
CI	creaming index
E. coli	*Escherichia coli*
et al.	and others
FF	fat free ice cream (< 0.625%, w/w, fat)
g	gram
GRAS	generally recognized as safe
h	hours
ID	inner diameter
IU	International Units
kcal	kilocalorie
LF	light fat ice cream (< 5%, w/w, fat)
M	molar
mg	milligram
min	minutes
mL	milliliters
mm/s	millimeters per second
mV	millivolts

Na_2HPO_4	disodium phosphate
NaCN	sodium caseinate
ND	not detected
NFDM	nonfat dry milk
NIH	National institutes of health
nM	nanomolar
O/W	oil-in-water
oz	ounces
RDAs	recommended dietary allowances
RDI	recommended dietary intake
RF	regular fat ice cream (> 10%, w/w, fat)
rpm	rounds per minute
UV	ultraviolet radiation
VDR	vitamin D receptor
v/v	volume by volume
WHO/FAO	World Health Organization, Food and Agricultural Organization of the United Nations
WPC	whey protein concentrate
WPH	whey protein hydrolysate
WPI	whey protein isolate
W/O	water-in-oil

w/v	weight by volume
w/w	weight by weight
°C	degree Celsius
µm	micron

CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Vitamin D plays an important role in calcium homeostasis. To maintain level of calcium serum, parathyroid hormone is secreted when calcium serum level is low. This hormone promotes the conversion of vitamin D to active form (1, 25 dihydroxyvitamin D_3) to stimulate calcium absorption from intestine and calcium resorption from bone. Both vitamin D and calcium are essential for bone development. The recommendation of the amounts of daily vitamin D and calcium required to maintain healthy bone depends on aged, sex, and some condition such as pregnant and lactation. For healthy adult, vitamin D_3 and calcium requirements are 400 IU and 1,000-1,200 mg per day, respectively (Holick and Chen, 2008; Institute of Medicine, 2010; National Institutes of Health, 2016a, 2016b). Vitamin D and calcium deficiencies increase risk of rickets in children and osteoporosis in adults. Lack of both nutrients is associated with some chronic diseases such as multiple sclerosis, diabetes, hypertension, autoimmune disease, and common cancer (Holick, 2006; Heaney, 2008; National Institutes of Health, 2016a).

A number of studies have shown that vitamin D deficiency (serum calcidiol < 50 nM) and insufficiency (serum calcidiol < 80 nM) are the global burden occurred in many regions such as Europe (Kaganov et al., 2015), North America (Mangano et al.,

2011), and Asia (Mithal and Kaur, 2012). Accordingly, taking vitamin D fortified foods could be one of the strategies to solve the problem. There are two types of vitamin D; vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Generally, vitamin D is unstable and easily degraded by heat, light, oxidation, humidity, and inappropriate pH levels (Remington, 1975; O'Neil M. et al., 2001). In addition, fat content, and its stability play an important role in shelf life of vitamin D fortified products. Therefore, vitamin D emulsion is introduced to enhance the stability (Kazmi, Vieth, and Rousseau, 2007; Wagner et al., 2008; Tippetts et al., 2012). Emulsion is a mixture of two or more immiscible liquid phases, a dispersed phase, and a continuous phase, which is stabilized by emulsifiers. Many previous studies developed emulsified vitamin D by using synthetic emulsifying agents such as Tween and sodium dodecyl sulfate (Ziani, Fang, and McClements, 2012; Guttoff, Saberi, and McClements, 2015). However, the information about vitamin D emulsion with the use of natural substances is still limited.

Milk proteins can be used as natural emulsifiers due to the amphiphilic nature in its molecule that contain both hydrophilic and hydrophobic regions. Proteins coat oil droplet surfaces, lower the interfacial tension, resulting in separation of the individual droplets for long periods of time. In addition, milk proteins are generally recognized as safe (GRAS) and are a high-quality source of dietary protein.

Vitamin D fortification of milk and dairy products has received considerable attention because of their ability to provide rich sources of calcium. Even though milk is typically fortified with vitamin D, common dairy products such as cheese, yogurt,

butter, and ice cream are usually not vitamin D fortified (Calvo, Whiting, and Barton, 2004; Chansathirapanich, Ngamchuachit, and Tansawat, 2016). Some previous studies documented that emulsified form of vitamin D improved vitamin D stability as well as prevented vitamin D degradation by the manufacturing process in cheese (Kazmi et al., 2007; Wagner et al., 2008; Tippetts et al., 2012) and yogurt (Kazmi et al., 2007). Yet, little is known about fortification of ice cream with vitamin D. Although Kazmi et al. (2007) have examined the retention of crystalline vs. emulsified vitamin D_3 added to lab-scale ice cream before; their level of vitamin D_3 was 8,000 IU per serving, which was over the tolerable upper intake values (1,000 - 4,000 IU per day, depending on age) (National Institutes of Health, 2016a). Recently, Chansathirapanich et al. (2016) investigated the effect of fat content on characteristics of ice creams fortified with calcium and 200 IU dry vitamin D_3 per serving. However, due to the poor dispersion of vitamin D_3 in ice cream mix, the inconsistent of vitamin D_3 content during 28-day storage was reported. Therefore, the retention of vitamin D_3 in ice cream at suitable consumption level is still required.

Many studies suggested that supplementation of both calcium and vitamin D was better for maintaining healthy bone than supplementation with either calcium or vitamin D alone (Boonen et al., 2007; Reid, 2014). Therefore, the purpose of this study was to develop calcium and vitamin D_3 fortified ice cream, using vitamin D_3 emulsified with milk protein emulsifier.

1.2 Objectives

1) To examine vitamin D_3 retention in ice creams fortified with vitamin D_3 emulsion using milk protein including nonfat dry milk (NFDM), sodium caseinate (NaCN), and whey protein isolate (WPI) as emulsifiers.
2) To develop regular fat (RF; > 10%, w/w, fat), light fat (LF; < 5%, w/w, fat), and fat-free (FF; < 0.625%, w/w, fat) ice creams fortified with calcium and vitamin D_3 emulsion.

1.3 Benefits of the Study

This study provides helpful information for ice cream manufacturers that seek to add vitamin D and calcium to their products. This innovation can be used as a functional food for consumers or patients who have high risk of calcium and vitamin D insufficiency.

CHAPTER II

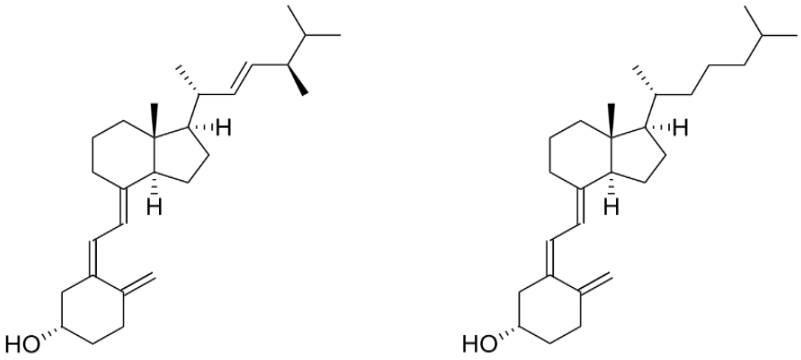
LITERATURE REVIEW

2.1 Vitamin D

2.1.1 General Introduction of Vitamin D

Vitamin D is a fat-soluble vitamin. There are two major forms of vitamin D; vitamin D_2 and vitamin D_3 (Figure 1). Vitamin D_2, also known as ergocalciferol, is usually found in mushroom. Vitamin D_3 (cholecalciferol) can be synthesized by human skin when exposed to sunlight. Other sources of vitamin D_3 are egg yolk, liver, and fatty fish such as salmon and sardine (Lamberg-Allardt, 2006; Institute of Medicine, 2010). Typically, the amount of vitamin D from natural sources are varied (Borradale and Kimlin, 2009). In fact, there is very little or no vitamin D naturally found in milk and dairy products (Thomson and Cressey, 2011). The United States and Canada routinely fortified fluid milk with vitamin D (Calvo et al., 2004). New Zealand has specific foods for vitamin D fortification such as baby foods, cereal, milk, cheese, dairy desserts, margarine, and soy milk (Thomson and Cressey, 2011).

The National Institutes of Health (2016a) suggests the recommended dietary allowances (RDAs) of vitamin D for healthy people, depended on age and specific conditions, for example, pregnancy and lactation. The RDA for babies under 12 months is 400 international units (IU), for person aged 1-70, pregnancy or lactation is 600 IU, and for elder aged over 70 years is 800 IU.



(A) vitamin D_2 (ergocalciferol) (B) vitamin D_3 (cholecalciferol)

Figure 1 Chemical structures of vitamin D_2 (A) and vitamin D_3 (B)

Vitamin D metabolism is shown in Figure 2. When serum concentration of calcium or phosphate is low, parathyroid gland is induced to secrete parathyroid hormone. Parathyroid hormone stimulates the enzymatic hydroxylation of calcidiol in kidney to generate calcitriol (1,25-dihydroxyvitamin D_3), the active form of vitamin D. Calcitriol that bound to vitamin D binding protein circulates in the blood circulation to the target tissues such as bone, kidney, immune cell, intestine and muscle (Institute of Medicine, 2010; Holick, 2013). For calcium homeostasis, calcitriol binds with vitamin D receptor (VDR) in bone (increase bone resorption), kidney (increase calcium and phosphate reabsorbtion) and intestine (decrease calcium excretion) to maintain blood calcium level (Holick and Chen, 2008; National Institutes of Health, 2016a). Nowadays, VDR is found nearly every tissue in the body. Thus, vitamin D may be act on the several organs (Bikle, 2014) . Both calcidiol and calcitriol are catabolized by CYP24A1 and these catabolites are most excreted through feces (Jones, Strugnell, and DeLuca, 1998).

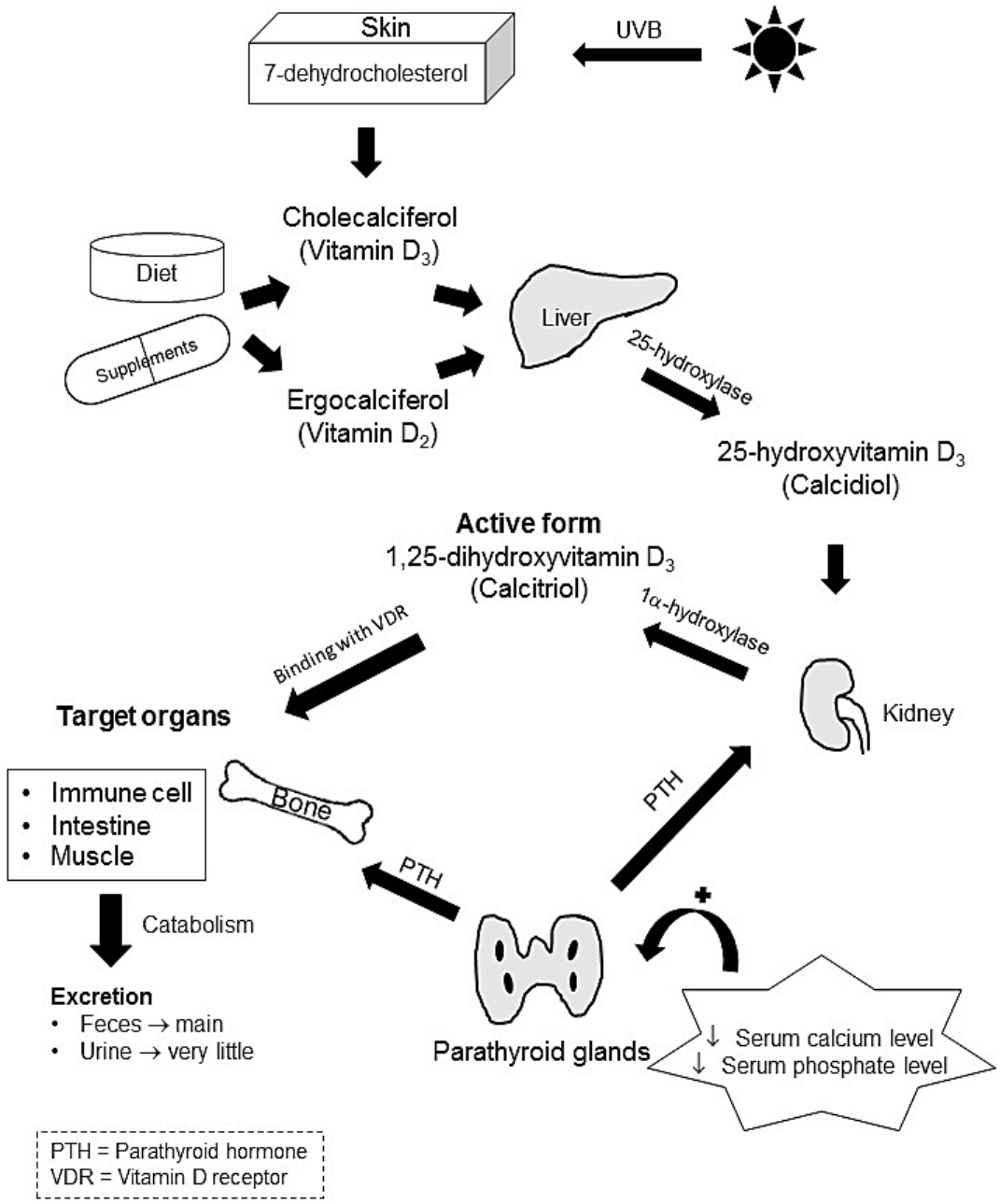


Figure 2 Vitamin D metabolism

2.1.2 Health Benefits of Vitamin D

The principal role of vitamin D is to maintain the normal concentrations of calcium and phosphate in blood. Vitamin D induces calcium absorption from intestine, so it can prevent bone diseases such as rickets, osteoporosis and osteomalacia (Holick and Chen, 2008; Borradale and Kimlin, 2009). Since there are several locations of VDR, vitamin D is associated with many human diseases. Many studies showed that VDR were found in immune system, especially T cell-mediated immunity (DeLuca, 2004). Thus, vitamin D intake may suppress and prevent the autoimmune disease such as multiple sclerosis (Cantorna, Hayes, and DeLuca, 1996), type 1 diabetes mellitus (Zella and DeLuca, 2003), and inflammatory bowel disease (Cantorna et al., 2000). Many studies also found that the strong relationship between vitamin D deficiency and increased risk of colorectal, breast and prostate cancers (Bischoff-Ferrari et al., 2006; Holick 2007; Borradale and Kimlin, 2009). Moreover, vitamin D can promote muscle function as a result of muscle weakness from vitamin D deficiency (Bischoff-Ferrari et al., 2006).

2.1.3 Vitamin D Deficiency

As described above, calcidiol concentration is considered the best biomarker for definition vitamin D status. Normally, healthy people should have serum calcidiol greater than or equal to 50 nM (≥ 20 ng/mL). The National Institutes of Health (2016a) concluded that vitamin D deficiency occurs when serum calcidiol less than 30 nM (<12 ng/mL). Serum calcidiol ranged between 30–50 nM (12–20 ng/mL) is considered

inadequacy vitamin D intake. In contrast, adverse effects of vitamin D may occur at serum calcidiol level greater than 125 nM (>50 ng/mL).

Risk groups of vitamin D deficiency are children, elders, pregnant women, and persons living in the urban area (Lips, 2010; Chailurkit, Aekplakorn, and Ongphiphadhanakul, 2011). Major cause of vitamin D deficiency results from low formation of vitamin D in the skin (50-90% of vitamin D in the body is produced by this pathway) (Lips, 2010). Moreover, low vitamin D intake, dark skin tone, activities such as working indoor and living in the countries far from the equator also lead to poor vitamin D status (Mithal et al., 2009; Lips, 2010). The study by Brock et al. (2013) demonstrated that using sun protection was associated with vitamin D deficiency. However, some studies inversely concluded that sun protection behavior did not influence vitamin D status (Jayaratne, Russell, and van der Pols, 2012; Linos et al., 2012; Hansen et al., 2016).

Insufficiency and deficiency of vitamin D are currently worldwide problem. Vitamin D deficiency problem was reported in many regions such as Europe (Kaganov et al., 2015), North America (Mangano et al., 2011), Middle-East Asia (Lips, 2010), Australia and New Zealand (Lips, 2010), and South and Southeast Asia (Mithal et al., 2009). Some recent studies showed that vitamin D insufficiency and deficiency are current problem in Thailand although Thailand locates in the tropics. Soontrapa et al. (2006) observed the prevalence of vitamin D deficiency among postmenopausal women. The results showed that prevalence of vitamin D deficiency was 60.2%.

Chailurkit et al. (2011) investigated vitamin D status of Thai healthy adults in various geographical regions. The calcidiol level of subjects living in Bangkok were lower than the other regions in Thailand. The prevalence of vitamin D insufficiency was 64.6%, 46.7%, and 33.5% in Bangkok, municipal areas except Bangkok, and outside municipal area in other parts of the country, respectively. This study concluded that the vitamin D insufficiency is common and varies across geographical regions of Thailand. Kruavit et al. (2012) showed that elderly participants living in nursing homes had low vitamin D status with prevalence rate of 38.7%.

2.1.4 Vitamin D Fortified Foods

Vitamin D is unstable and easily degraded by heat, light, oxidation, humidity, and inappropriate pH levels (Remington, 1975; O'Neil M. et al., 2001). Banville, Vuillemard, and Lacroix (2000) showed that fortified vitamin D in cheddar cheese was lost during ripening at 4 $^{\circ}$C for 7 months. Ganesan, Brothersen, and McMahon (2011) demonstrated that vitamin D was degraded by homogenized process. Moreover, vitamin D was unstable in translucent container during the shelf life (Jafari et al., 2016). Vitamin D was also degraded by heat and pasteurized process (Upreti, Mistry, and Warthesen, 2002). Many research suggested that emulsified form improved stability of vitamin D in foods such as cheese and yogurt. (Kazmi et al., 2007; Wagner et al., 2008; Tippetts et al., 2012).

In general, both vitamin D_2 and D_3 can be fortified in foods. However, numerous studies showed that vitamin D_3 was more effective than vitamin D_2. The study by Trang

et al. (1998) found that intake vitamin D_3 increased serum vitamin D as 1.7 times compared with intake vitamin D_2 when subjects took 4,000 IU of each vitamin D for 14 days. Heaney et al. (2011) compared potencies of vitamin D_2 and D_3. Healthy participants took 50,000 IU of each vitamin D once a week for 12 weeks. Vitamin D content in serum and in subcutaneous fat were evaluated. They reported that vitamin D_3 was greater potent in increasing serum vitamin D than vitamin D_2. Moreover, vitamin D_3 was more accumulated in subcutaneous fat than vitamin D_2. According to the meta-analysis study of Tripkovic et al. (2012), the authors revealed that vitamin D_3 supplementation was better than vitamin D_2 because vitamin D_3 was more effective to raise serum vitamin D than vitamin D_2. Therefore, vitamin D_3 is widely used to fortify in foods.

2.2 Calcium

2.2.1 General Introduction of Calcium

Calcium is the most abundant mineral found in human body. Ninety percent of the body's calcium is the composition of bones and teeth. The remaining of total body calcium plays a role in cellular functions. The rich natural sources of calcium are milk, dairy products, fish with edible bones and some leafy green vegetables such as kale and broccoli. Nowadays, some calcium fortified foods such as fruit juices, soy products and cereals are available in the market (Titchenal and Dobbs, 2007; National Institutes of Health, 2016b).

There are many types of calcium salts that can be used for food fortification and supplementation. The amounts of elemental calcium from each calcium salt are shown in Table 1. Calcium carbonate is suitable to fortify in milk and dairy products because it has the highest elemental calcium content with colorless and odorless characteristics (World Health Organization Food and Agricultural Organization of the United Nations, 2006).

The National Institutes of Health (2016b) recommends the amounts of calcium for maintaining healthy bone. Adequate intake of calcium for infant age 0-6 months and 7-12 months are 200 mg and 260 mg, respectively. The RDA of calcium for children aged 1-3 and 4-8 years are 700 mg and 1,000 mg, respectively. The RDA becomes 1,300 mg for children aged 9-18 years, pregnant and lactating women. The RDA 1,000 mg and 1,000-1,200 mg are suggested for people aged 19-50 years and > 50 years, respectively.

2.2.2 Calcium Metabolism and Homeostasis

Dietary calcium is absorbed through the intestines. Two important movements of calcium across the intestinal wall are active transport and passive diffusion. When person consumes low to moderate calcium, calcium is absorbed by active transport. On the other hand, high calcium intake promotes calcium absorption by passive diffusion. The level of serum calcium is directly controlled by hormonal system and vitamin D metabolism. The maintenance of serum calcium level, also known as calcium homeostasis, is concluded in Figure 3. When serum calcium level is low, parathyroid glands increase parathyroid hormone secretion to stimulate calcitriol

Table 1 The elemental calcium from each calcium salt

Calcium salt	Elemental calcium (%)
Calcium carbonate	40
Calcium chloride	36
Calcium sulfate	29
Calcium phosphate	
monobasic	17
dibasic	30
tribasic	38
Calcium pyrophosphate	31
Calcium glycerophosphate	19
Calcium acetate	25
Calcium lactate	13
Calcium citrate	24
Calcium citrate malate	23
Calcium gluconate	9
Calcium hydroxide	54
Calcium oxide	71

Source: World Health Organization Food and Agricultural Organization of the United Nations, 2006

formation in kidney. Calcitriol promotes calcium absorption from intestine, calcium reabsorption from kidney and bone resorption to raise serum calcium level. Conversely, when serum calcium level is high, negative feedback works to maintain its level. Thyroid gland is stimulated to secrete calcitonin to inhibit bone resorption. Moreover, high serum calcium concentration also suppresses parathyroid hormone secretion. Calcium is excreted through both urine and feces. For urinary excretion, kidney simultaneously excretes and reabsorbs calcium to balance its level. Unabsorbed calcium is excreted through feces (Weaver and Heaney, 2007; Institute of Medicine, 2010).

2.2.3 Health Benefits of Calcium

The important role of calcium is formation of calcium hydroxyapatite to be a composition of bone and teeth. Calcium hydroxyapatite increases bone mineral density to provide skeletal strength (Aspray, 2017). Calcium deficiency leads to risks of bone diseases; for example, rickets, osteomalacia and osteoporosis, just like vitamin D deficiency (Gershon-Cohen and Jowsey, 1964; Birge et al., 1967; Davidovits et al., 1993). Other major functions of calcium in human body include supporting nerve and heart muscle functions, being a cofactor for blood clotting and involving in membrane functions through calcium-dependent channels (Aspray, 2017). Many studies reported the relationship between calcium deficiency and various diseases such as breast cancer (Cui and Rohan, 2006) and cardiovascular disease (Rautiainen et al., 2013).

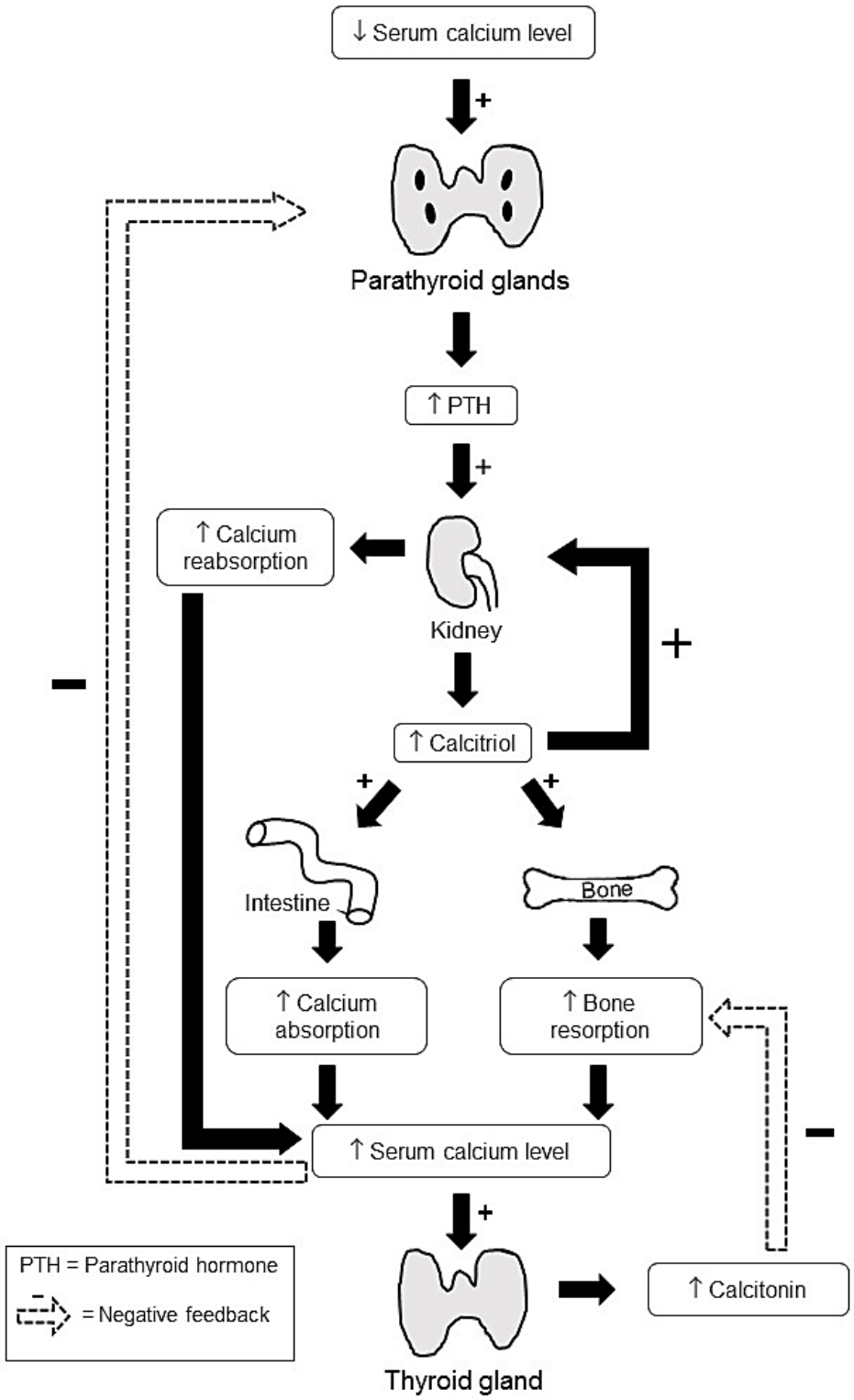


Figure 3 Calcium homeostasis

Vitamin D directly promotes calcium absorption. Vieth, Fraser, and Kooh (1987) studied the effect of dietary factors on the level of serum calcidiol in rat. The results showed that calcidiol decreased when rat obtained low dietary calcium. Similarly, Berlin and Bjorkhem (1988) showed that serum calcidiol levels of healthy men significantly enhanced when they consumed calcium enriched diet. Moreover, additional calcium supplementation in persons who were supplemented with vitamin D reduced risk of hip fracture more than single supplement (Boonen et al., 2007; Reid, 2014).

2.2.4 Calcium Deficiency

Calcium blood test is not suitable for estimation of inadequate calcium status. The Agriculture Organization of the United Nations (2016) suggests that evaluation of dietary calcium intake is the best method for the study of prevalence of calcium deficiency.

People who are at risk of calcium deficiency are postmenopausal women, amenorrhea, person with lactose intolerance or cow's milk allergy and vegetarians (National Institutes of Health, 2016b). Bailey et al. (2010) investigated total calcium intake of the United States citizens aged $\geq$ 1 year by using National Health and Nutrition Examination Survey (NHANES) 2003–2006 data. The results indicated that low calcium intake was found in every ages and gender groups. Children aged 1-3 year had the lowest prevalence of calcium insufficiency. Women aged over 3 years tended to

have higher calcium insufficiency than men. Mangano et al. (2011) revealed that low calcium intake was found in American adults and calcium supplement use was not sufficient to meet the RDA.

Nowadays, calcium deficiency becomes a worldwide problem especially in developing countries. The main reason of calcium deficiency is inadequate dietary calcium intake. The global study in 2001 showed that 3.5 billion people were at risk of calcium deficiency, where 90% of these people lived in Africa and Asia. Moreover, this study demonstrated that a lack of calcium was also found in America, Europe and Oceania (Kumssa et al., 2015). Calcium deficiency is also a widespread problem in Thailand, which mainly caused by low milk and dairy products intake. The data using 3-day food records and interviewer-administered quantitative food-frequency questionnaire demonstrated that healthy adults aged 20-85 years (both men and women) consumed average dietary calcium 378.6 and 265.6 mg/day, respectively. Moreover, the participants who consumed dietary calcium less than 400 mg/day were 67% of those men and 87% of those women (Pongchaiyakul et al., 2008). In addition, Thai postmenopausal women who lived in Khon Kaen Province consumed very low dietary calcium with the average 236 ± 188 g/day (Pongchaiyakul et al., 2004). The part of the South East Asian Nutrition Survey (SEANUTS) by Yamborisut et al. (2015) reported that boys and girls aged 6-12 years, living in various regions of Thailand, had insufficiency dietary calcium intake. The averages of total calcium intakes were 42% and 39% of the RDA for boys and girls, respectively. Similarly, the study by

Rojroongwasinkul et al. (2013) reported that children aged 3-12 years consumed calcium less than the RDA.

2.2.5 Calcium Fortified Foods

In general, milk and dairy products are major sources of calcium. Although calcium in milk is simply absorbed by the intestines and its bioavailability is higher than other sources, cow's milk contains only 300 mg of calcium per serving (250 mL) (Guéguen and Pointillart, 2000). This amount of calcium is only 30% of RDA for healthy adults. Hence, milk manufacturers often fortify their products with calcium. Calcium is also added in other food products such as juices, bread, beverages, yoghurt, cheese and soy beverages (World Health Organization Food and Agricultural Organization of the United Nations, 2006).

The relationship between calcium and vitamin D influences the development of food products fortified with both nutrients together. For example, the development of milk fortified with both calcium and vitamin D increased their bioavailability compared with fortification of either calcium or vitamin D alone (Kaushik et al., 2014). In Canada and the United State, soy beverages are recommended to fortify with both calcium and vitamin D for improving a nutrient profile to become like the nutrients of cow's milk (Calvo et al., 2004).

2.3 Emulsion

Emulsion is a mixture of at least two immiscible liquids, one is dispersed in the other. Different types of emulsions are showed in Figure 4. In general, there are two

types of primary emulsions; oil-in-water (O/W) emulsion and water-in-oil (W/O) emulsion. The O/W emulsion contains oil droplets dispersed in aqueous phase. On the other hand, W/O emulsion contains water droplets dispersed in the oil phase. Multiple emulsions consist of primary emulsion that dispersed in another liquid phase again (Chiralt, 2005; McClements, 2010). Emulsions are produced via various methods such as homogenization, membrane emulsification, microchannel emulsification and spontaneous emulsification (Leal-Calderon, Schmitt, and Bibette, 2007). However, high-speed mixer, one of the uncomplicated homogenization devices, is the most widely used for preparing emulsion in the food industry (McClements, 2004; Mao and Miao, 2015).

The O/W emulsion has been commonly used as a delivery system to improve hydrophobic nutrients bioavailability (Prichapan and Klinkesorn, 2014; McClements et al., 2016). Moreover, the other benefits of emulsion-based delivery system for bioactive compounds comprise of physical stability enhancement against heat, pH and oxidation, shelf life improvement, and controlling nutrients release in the intestinal systems (Schlyter and Piene, 2002; Hategekimana et al., 2015; Mao and Miao, 2015). Examples of hydrophobic bioactive nutrients that were designed in emulsified form include β-carotene (Liu et al., 2012), Coenzyme Q10 (Estevez et al., 2012), vitamin D (Tippetts et al., 2012), and vitamin E (Hategekimana et al., 2015).

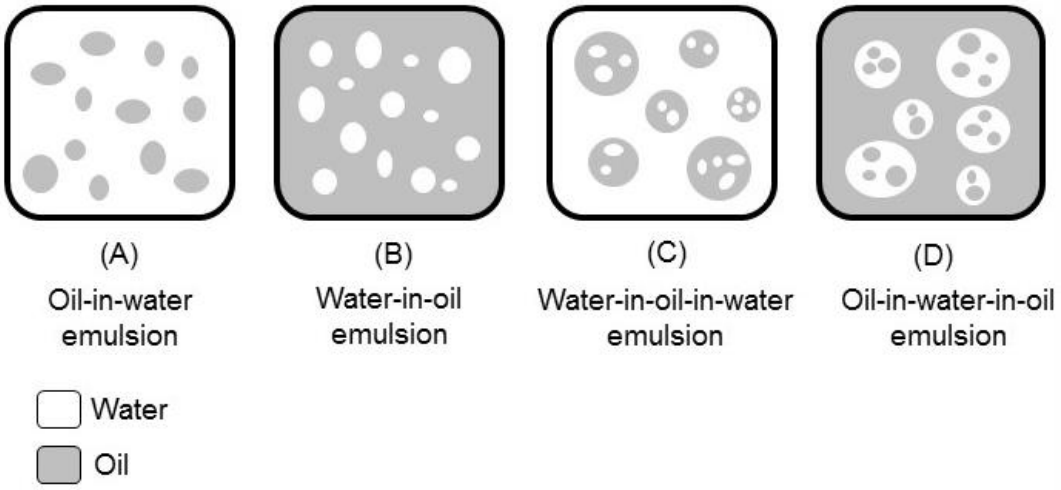


Figure 4 Types of emulsion

Normally, emulsions are thermodynamically unstable due to the incompatibility between the immiscible phases. The dispersed droplets tend to aggregate over time because of the large interfacial area. The breakdown processes consist of flocculation, coalescence, Ostwald ripening, creaming, sedimentation, phase inversion and separation (Figure 5) (McClements, 2004; Tadros, 2013). Mostly, two or more types of these processes coexist. Increasing in droplets size is caused by flocculation and coalescence. Flocculation occurs when one droplet is aggregated with the others, but they do not merge to each other. This situation can be re-dispersed. In contrast, coalescence is an irreversible process. Coalescence consists of merging of two or more droplets to form a bigger droplet.

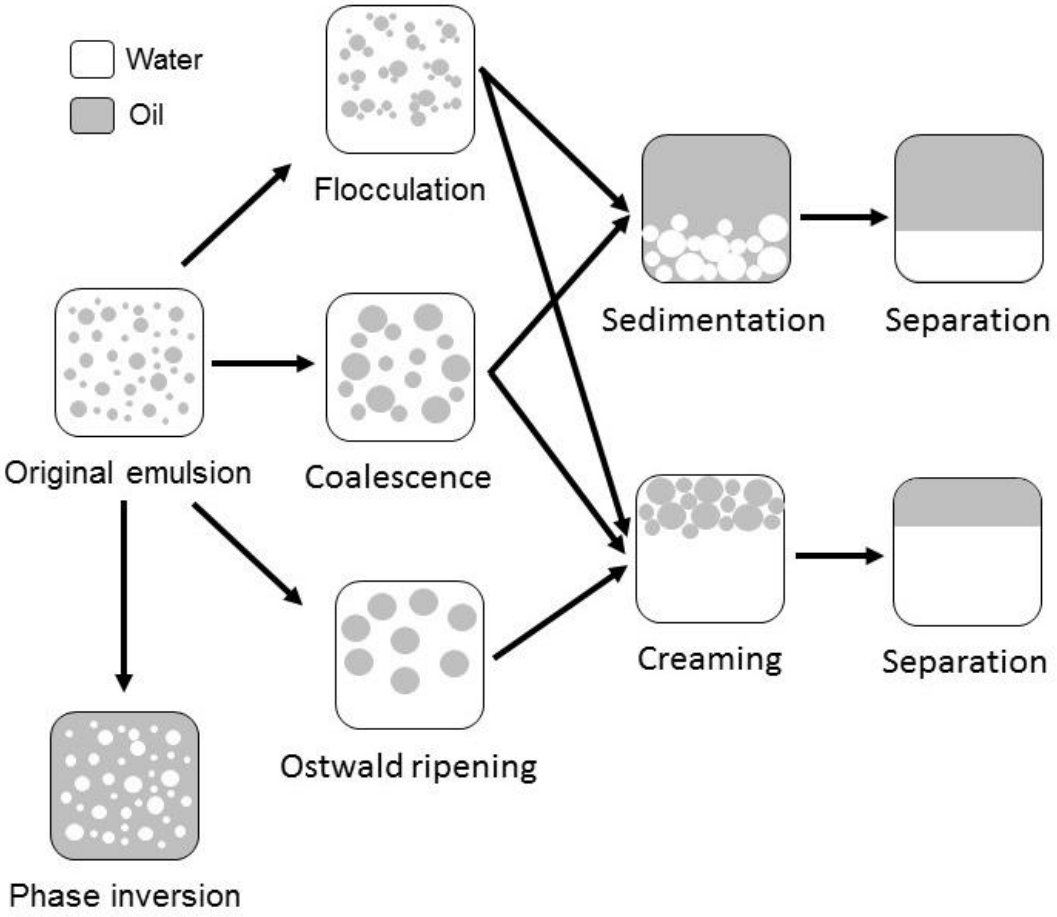


Figure 5 Schematic diagram of the breakdown processes of emulsion

Average droplets size and size distribution are used to monitor the flocculation and coalescence. Light scattering method is commonly used to detect the size and distribution of the droplets; however, it cannot directly identify the flocculation. Flocculation can be detected by electron microscopy. Zeta potential, which is the value of electrostatic field around the droplets, can be used to predict the aggregation of the droplets. Both flocculation and coalescence lead to creaming or sedimentation. Creaming is an unstable process of O/W emulsion whereas sedimentation happens in W/O emulsion. Both are driven by the gravitational force or centrifugal force. The lower

density phase moves to the top of emulsion after the immiscible phases are separated. Creaming is directly evaluated by creaming index of the emulsion. In the case of O/W emulsion, smaller oil droplets diffuse through the aqueous phase and merge into the other bigger droplets to form bigger one, this process is called Oswald ripening. Phase inversion generally occurs when the properties of emulsifier is changed when the O/W emulsion is converted to W/O emulsion or W/O emulsion is converted to O/W emulsion (Robins, 2000; Friberg, Larsson, and Sjoblom, 2003; McClements, 2004; Tadros, 2013; Mao and Miao, 2015)

2.4 Emulsifiers

Due to thermodynamic instability of emulsion, emulsifier is added to improve the stability. Emulsifier is an amphiphilic compound which contains both hydrophilic and hydrophobic portions. Emulsifiers connects the interfaces between the immiscible phases. Not only hydrophilic heads interact with aqueous phase, but hydrophobic tails also interact with the oil phase to prevent droplets aggregation (AACC International, 1996).

There are four general classes of emulsifiers: surfactant, polysaccharide, phospholipids and protein (McClements, 2004). Proteins are macromolecules emulsifier that are widely used in the food industries because they are cheap, nontoxic, natural, and easily available (Wilde et al., 2004; Vanessa, Roberto, and Amelia, 2008). Furthermore, some proteins, i.e., milk proteins, have high nutritive value.

The structure of protein is long polymeric chain of amino acids with amphiphilic property (Boom, 2008). The structures of proteins are complex. Proteins can unfold and rearrange their structure before they absorbed around the droplet interfaces. Hydrophobic region reacts with the oil phase and hydrophilic region reacts with aqueous phase. Consequently, proteins can protect droplet aggregation by decreasing interfacial tension and steric effects (Wilde et al., 2004; Boom, 2008; Vanessa et al., 2008).

2.5 Milk Proteins

Cow's milk is one of the most popular consumed milk that consisted of approximately 3 to 3.5 % protein and the other substances such as fat, lactose and various minerals and vitamins. Protein is a main nutrient in milk. Milk proteins have an emulsifying property because of its amphiphilic characteristic. The major types of milk proteins produced by mammary gland are casein and whey. Both proteins have high nutritive value. They are excellent sources of all essential amino acids and some bioactive peptides (Bylund, 1995; McSweeney and Fox, 2013).

2.5.1 Nonfat Dry Milk (NFDM)

Nonfat dry milk (NFDM) is one of the most popular food ingredients containing high milk proteins, which is used to prepare the recombined milk and used as emulsifiers of salad dressing and ice cream. NFDM is produced by removing fat and water to prolong its shelf life. This dried milk consists of lactose particles, fat globule, casein micelle and powder of serum proteins. NFDM has nutritive value similar to a

skim milk fluid. Protein content of NFDM are 80% of casein and 20% of whey. The industrial methods used to produce NFDM include roller drying and spray drying. During the drying process, the moisture is removed from a skim milk by heating; therefore, some nutrition such as vitamin A can be degraded. Moreover, partial whey in the NFDM can be denatured and deformed depending on a degree of temperature used to dry the skim milk. The denatured whey can decrease emulsifying properties of NFDM. However, the effect of temperature is less impact on casein, which has higher thermal stability than whey. (Schwartz, 1987; Bylund, 1995; Sharma, Jana, and Chavan, 2012).

2.5.2 Sodium Caseinate (NaCN)

Eighty percent of total milk proteins are casein. The major subtypes of casein are α_{s1}-, α_{s2}-, β- and κ- caseins. The difference among these subtypes depends on amino acid compositions, which are varied by genetic variants. The α_s- and β- caseins are calcium-sensitive that rapidly precipitated by binding with calcium, while κ- casein is insensitive and easily soluble. In general, caseins in milk are found the form of micelles, which are the complex of casein molecules, calcium, and inorganic phosphate (Figure 6). These micelles are soluble in milk like a colloid dispersion (Bylund, 1995; Sharma et al., 2012). κ- caseins surround its surface to cover the hydrophobic core including other subtypes of caseins and calcium phosphate. Casein can be precipitated by enzyme and acid. Caseins precipitated by enzyme such as chymosin is called casein rennet. In acid condition, casein will precipitate from the

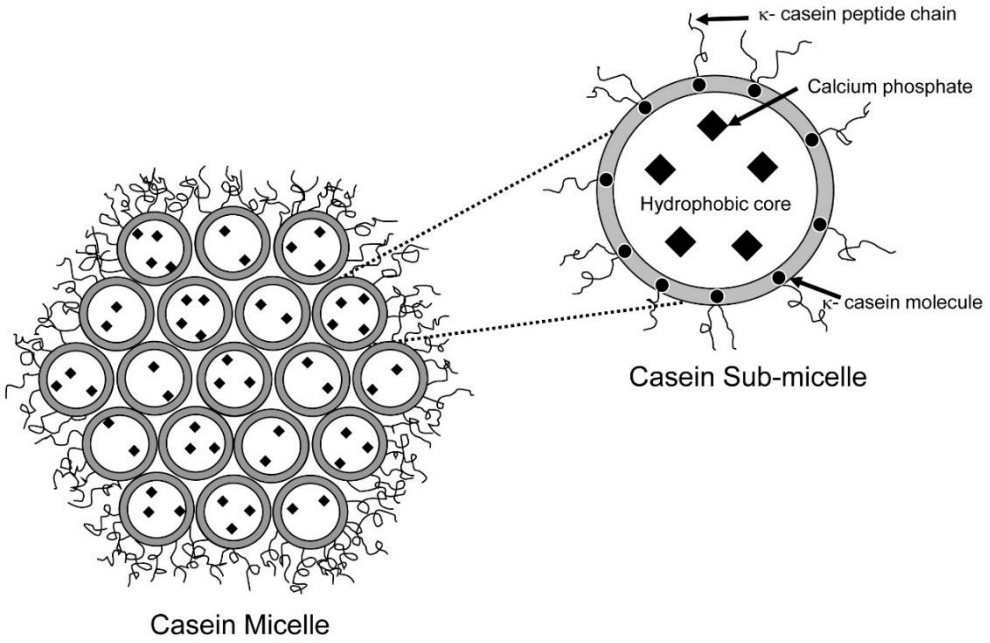


Figure 6 Casein micelle structure

skim milk when the pH is closed to its isoelectric point (approximately 4.6). This precipitated casein is called acid casein. Although both acid casein and casein rennet are not soluble in water, the interaction between these caseins and alkalis can improve their solubility. The final product is named from the types of alkali. For example, sodium caseinate is the product from the reaction between casein and sodium hydroxide (Srinivasan, Singh, and Munro, 1996; Sindayikengera and Xia, 2006; Southward, 2008).

Sodium caseinate strongly adsorb at the oil-water interface of the emulsion. When the concentration of sodium caseinate below 2%, β-casein is the main subtype of casein that coats the oil droplets. The emulsion is stable because the cover of

β-casein at the interface has high electrostatic and steric effects especially at pH of 7. However, an increasing of sodium caseinate concentration to above 4% offers the multilayers of protein at the interface and increases aggregation of caseinate in the emulsion (Srinivasan et al., 1996; Dickinson, 2001). Nowadays, sodium caseinate is widely used as an emulsifier in the food industry due to its good emulsifying property and good solubility. For example, it is an emulsifier for meat products and coffee creamers, a texture improvers and stabilizer for ice cream, soup and cheese products (Southward, 2008). Wei and Gao (2016) showed that the β-carotene coated by sodium caseinate to form a monolayer emulsion had higher heat stability than the beta-carotene coated by α-lactalbumin. Sabouri, Geng, and Corredig (2015) indicated that sodium caseinate emulsion could be a good carrier for tea polyphenols and the physicochemical properties of the sodium caseinate emulsion were not affected by tea polyphenols. Penalva et al. (2015) reported that entrapped folic acid in casein nanoparticles improved its oral bioavailability and folic acid in nanoparticles was released from the particles only in the intestine. In 2011, (Zimet, Rosenberg, and Livney, 2011) demonstrated that docosahexaenoic acid (DHA) in casein micelle and casein nanoparticles had a good physical stability and these systems also preserved the bioactive effects of DHA because the casein micelle and nanoparticles can protect DHA against oxidation reaction and thermal treatment. Apart from the above, caseins can be used as a delivery system for vitamin D in forms of micelle, nano-capsule or

emulsion to improve vitamin D stability and protect vitamin D from light and heat (Forrest, Yada, and Rousseau, 2005; Semo et al., 2007; Tippetts et al., 2012).

2.5.3 Whey Protein Isolate (WPI)

Twenty percent of the total milk proteins are soluble whey, also known as serum proteins. Two major types of whey consist of α-lactalbumin and β-lactoglobulin. The other proteins include lactoferrin, transferrin, blood serum albumin and immunoglobulin. Whey has high nutritive values and provides all the essential amino acids. Alpha-lactalbumin is found in mammalian milk and plays an important role in lactose biosynthesis. Beta-lactoglobulin is considerably found in ungulates such as cow milk (Bylund, 1995; Oluk and Karaca, 2016). Beta-lactoglobulin can rapidly unfold at temperature above 60 °C. At this temperature, the hydrophobic residues in its structure can bind with small hydrophobic molecules. This interaction has been used as a method to develop the delivery system of some nutrients such as β-lactoglobulin-epigallocatechin gallate nanoparticles (Shpigelman, Cohen, and Livney, 2012), β-lactoglobulin-retinoic acid complex (Lynen et al., 2003) and nanoparticles of vitamin D (Abbasi et al., 2014; Diarrassouba et al., 2015). β-lactoglobulin also provides an antioxidant activity because of their free thiol groups (Oluk and Karaca, 2016).

Whey is a by-product from cheese manufacture. There are three types of the developed whey products; whey protein concentrate (WPC), whey protein isolate (WPI)

and whey protein hydrolysate (WPH). Ultrafiltration is used to produce WPC and WPI to remove fat, minerals, and lactose. WPC contains 20-89% protein whereas WPI contains at least 90% protein. WPH is partially hydrolyzed to simply digest. (Lee, 1992; Onwulata and Huth, 2009).

WPI has been used widespread in food researches due to its high protein content and low price. For instance, Hu, McClements, and Decker (2003) showed that the oxidation of corn oil was inhibited by using casein, WPI and soy protein isolate as emulsifiers. Wang et al. (2016) demonstrated the higher WPI concentration in vitamin E emulsion increased the prevention of vitamin E degradation and addition of resveratrol or ascorbic acid enhanced the vitamin E stability. WPI has also been used to prepare vitamin D in form of oil-in-water emulsion, nanoparticles and coagulum.

2.6 Ice Cream

Ice cream is defined as a frozen dairy dessert that contains air cell homogeneously dispersed in an aqueous matrix. The U.S. Food and Drug Administration (2016) defines the standard composition for the ice cream. Standard or regular ice cream must contain at least 10% (w/w) milk fat and 10% (w/w) milk solids non-fat. Ice creams that contain lower fat content should label the percentages of fat; for example, reduced fat (< 7.5%, w/w, fat), light (< 5%, w/w, fat), low fat (< 3.75%, w/w, fat) and fat free (< 0.625%, w/w, fat) (Marshall, 2003; The U.S. Food and Drug Administration, 2016). Ice creams can be divided into four categories according to the ingredients; 1) ice creams that made from milk products, 2) ice creams that contain

vegetable fat, 3) sherbet ice cream that contain fruit juice mixed with milk fat and milk solids non-fat, and 4) water ice that made from water, sugar and fruit concentrate (Bylund, 1995). The standard of Thailand also divides ice cream according to the ingredients used (The Ministry of Public Health Thailand (Regulation no.222), 2002).

The The Ministry of Public Health Thailand (Regulation no.222) (2002) suggests that the preservative must not add into the ice creams. Moreover, one gram ice cream must contain less than 600,000 CFU of total bacteria. There must be no *Escherichia coli* (*E. coli*) in 0.01 g of ice cream. For the U.S. standard, one gram ice cream must contain less than 20,000 CFU of aerobic bacteria, less than 10 CFU of coliform, and no *E. coli* (Marshall, 2003; Goff and Hartel, 2013).

Ice cream has been popular milk product for a long time all over the world. The highest per capita consumers of ice cream in the world is in New Zealand (Statistics New Zealand, 2016). In the U.S., people consume ice cream average 22 pounds of ice cream per year and vanilla is the most popular flavor (International Dairy Foods Association, 2016). In 2014, Nestlé company showed that sale of ice cream has continuously grown up in Americas, Asia, Oceania, and Africa (Nestlé, 2014). Kasikorn Research Center (2011) presented that Thais consume ice cream average 1.7 liters per capita per year. In addition, the turnover of ice cream market may reach THB 15 billion in 2011 and grow up to about 15 percent per year.

2.6.1 Ice Cream Composition Mix Ingredients

The main compositions of ice cream consist of milk solids-not-fat (MSNF), milk fat or nondairy fats, stabilizers, emulsifiers, sweeteners, and flavors. The proportion of the compositions depends on categories of ice cream. For example, standard ice cream is specified the content of 10% fat, 10-11% milk solids non-fat, 15% sweeteners, and 0.3% stabilizers/emulsifiers (Arbuckle, 1986). The ingredients are mixed, pasteurized, and homogenized. After that, the mixture is aged to let the ingredients entirely absorb water and to let fats completely crystallize. Finally, the mixture is whipped and frozen to incorporate air into the mix to enhance smoothness, softness and overrun before hardening (Marshall, 2003; Goff and Hartel, 2013).

2.6.1.1 Fat

The fat component of ice cream provides a smooth texture and enhances flavor quality (Goff and Hartel, 2013). The amounts of fat content also affect physical properties of the ice cream including hardness, melting behavior, overrun and viscosity (Babcock, 1931; Roland, Phillips, and Boor, 1999; Aime et al., 2001; Warren and Hartel, 2014). The best source of fat in standard ice cream is fresh cream (Arbuckle, 1986). Plants or vegetables oil such as palm oil, coconut oil, sunflower oil, canola oil and soybean oil can be used as an alternative source of fat (Bylund, 1995; Goff and Hartel, 2013). Moreover, fat replacers; i.e., Simplesse, Olestra, maltodextrins and inulin, have been used to manufacture reduced fat or fat free ice creams (Goff and Hartel, 2013; Mahdian and Karazhian, 2013). Many recent studies recommended using inulin

in reduced fat ice cream due to its benefits for health and good improvement of physical properties and sensory quality of the ice cream (Wood, 2011; Mahdian and Karazhian, 2013; Tiwari et al., 2015).

2.6.1.2 Milk Solids-Non-Fat

Milk solids-non-fat refers to the dried skim milk that contains protein, lactose and minerals (Bylund, 1995). Its milky flavor can increase palatability of the ice cream. Not only it has high nutritive values, but also improves the texture of the ice cream, for example, enhancing smoothness, viscosity and melting time (Arbuckle, 1986; Goff and Hartel, 2013).

2.6.1.3 Sweetener

Sugar is used to give sweet taste of ice cream. In addition, it reduces freezing point and the amount of ice crystal in the ice cream mix (Marshall, Goff, and Hartel, 2003; Clarke, 2004). Commonly, the best choice of sweetener for ice cream manufacturing is sucrose. However, corn sugar can replace approximately 45% of sucrose to reduce cost (Arbuckle, 1986). The other sweeteners that can be used as ice cream ingredients are contains maple sugar, brown sugar, honey, sugar alcohol, and nonnutritive sweeteners such as aspartame, saccharin, and sucralose (Goff and Hartel, 2013).

2.6.1.4 Stabilizer and Emulsifier

Stabilizer provides a high viscosity of ice cream mix by binding with water molecules to form the network (Bylund, 1995). Furthermore, it can enhance

physical stability of the ice cream by preventing shrinkage of the ice cream volume and reducing ice and lactose crystal growth during storage. Stabilizer also increases the melting resistance and smoothness of the ice cream (Clarke, 2004). The recommended stabilizers include alginates, modified cellulose compounds, guar gum, locust bean gum, carrageenan, gelatin, and pectin (Arbuckle, 1986; Goff and Hartel, 2013).

Emulsifier can be used to prevent the instability of ice cream emulsion system. Besides, emulsifier is used to improve smoothness of the ice cream (Arbuckle, 1986; Bylund, 1995). Emulsifiers that commonly used in the ice cream mix include mono- and diglycerides and sorbitan esters (Bylund, 1995; Goff and Hartel, 2013). Currently, the commercial mix of stabilizer and emulsifier is widely used in the ice cream industry (Marshall et al., 2003). For instance, Palsgaard® ExtruIce consist of stabilizers (guar gum, cellulose, and carrageenan), emulsifiers (mono- and di-glycerides of fatty acids) and antioxidant (Palsgaard, 2014).

2.6.2 Ice Cream Fortification

Nowadays, ice cream is the popular dairy product to be fortified with some nutrients and bioactive compounds. It can be consumed as a functional food to increase health and nutritional benefits. For instance, Friedeck, Aragul-Yuceer, and Drake (2003) fortified low fat dairy-based ice cream with soy protein and the fortification did not influence consumer sensory evaluation. Consumers were interested in health benefits of soy protein and low fat food so researchers concluded that the fortified ice cream may possibly be marketed. Salem and Mowafy (2001)

established the ice cream fortified with antioxidants containing lycopene and β-carotene by adding tomato paste and carrot concentrate. The other substances such as soluble soybean polysaccharide (Chen et al., 2010), probiotic (Mohammadi et al., 2011), fish protein (Shaviklo et al., 2011), orange fiber (Crizel et al., 2014), etc., were also developed to fortify in the ice cream. Moreover, the recent study showed that calcium fortified ice cream had bioavailability as high as fortified milk (Van der Hee et al., 2009) and it was no loss during storage (Chansathirapanich et al., 2016). Thus, ice cream can be a good carrier for fortified calcium. Kazmi et al. (2007) fortified ice cream with high levels of vitamin D (8,000 IU/serving). Two forms of fortified vitamin D included dry and commercial emulsified forms. The results showed that both forms were stable throughout 28-day storage. In contrast, the study by Chansathirapanich et al. (2016) demonstrated that fortification of dry vitamin D in ice cream was inconsistent.

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of Vitamin D_3 Emulsion

Two hundred milliliters of vitamin D_3 emulsion containing 90:10 w/w of aqueous phase to oil phase was prepared, using milk proteins including nonfat dry milk (NFDM), sodium caseinate (NaCN), and whey protein isolate (WPI) as an emulsifier. Each treatment was made in three separate batches. NFDM, obtained from Dusit Dairy Product, Bangkok, Thailand, contains 25.0% (w/w) protein, 3% (w/w) sugar, and 8% (w/w) fat. NaCN (Erie Foods International, IL, USA) and WPI (Glanbia, WA, USA) consist of 95.4% and 90.0% (w/w) protein, respectively. The amount of emulsifier used in this study was equivalent to 2 g of protein, calculated from protein content of each milk protein emulsifier. An aqueous phase was prepared by dissolving milk protein emulsifier into 178 g of 0.01 M Na_2HPO_4 (pH 7.0). Sodium benzoate (0.2%, w/v) was added as a preservative. The mixture was continuously stirred at room temperature for 2 h to ensure adequate protein hydration. An oil phase was a mixture of 0.5 g dry vitamin D_3 (500,000 IU) (DSM Nutritional Products, Heerlen, Netherlands) and 20 g soy bean oil. The oil-in-water emulsions were emulsified using a rotor-stator homogenizer (Ultra Turrax T25, IKA Instruments, Staufen, Germany) at 20,500 rpm for 5 min at room temperature. The control was prepared using the same method without adding milk

protein emulsifiers. All homogenized emulsions were transferred into test tubes and kept at 4 ºC.

3.2 Oil Droplet Diameter and Zeta Potential Measurements of Vitamin D_3 Emulsion

Oil droplet diameter and zeta potential of vitamin D_3 emulsions were determined from day 0-14 of storage at 4 ºC in triplicate. Oil droplets diameter and zeta potential were measured using a particle size analyzer (Zetasizer Nano-ZS, Malvern Instruments, Malvern, UK). The analytical emulsions were re-dispersed by shaking and diluted with ultrapure water (1:1,000, v/v) to prevent multiple scattering effects prior to the analysis.

3.3 Creaming Index of Vitamin D_3 Emulsion

Thirty-five milliliters of each vitamin D_3 emulsion sample was poured into a flat bottom tube, sealed with the Parafilm M®, and stored at 4 ºC. Creaming index (CI) of vitamin D_3 emulsion was investigated every day from day 0-14. The height of total emulsion (H_E) and the height of the serum were recorded. Creaming index was calculated using the following equation (1):

$$CI = 100 \times (H_S/H_E) \quad (1)$$

3.4 Preparation of Vitamin D_3 Fortified Ice Creams

Two forms of vitamin D_3; emulsified vitamin D_3 and dry vitamin D_3 (control), were used in this study. Emulsified vitamin D_3 was prepared by choosing the better candidate milk protein emulsifier. Types of ice cream included regular fat (RF), light fat

(LF), and fat free (FF). The ice cream samples were made in three separate batches per formulation. Compositions of the ice cream samples are shown in Table 2. Firstly, skim milk, nonfat dry milk, whipping cream, sugar, and stabilizer (Extrulce 278, Palsgaard, Juelsminde, Denmark) were mixed and pasteurized at 82 ºC for 8 min. Inulin (Cosucra, Hainaut, Belgium) was added as a fat replacer in the FF formulation. After that, the mixes were quickly cooled down to 15 ºC in ice-water bath, followed by the addition of vanilla flavor (Durkee, OR, USA) and 250 IU vitamin D_3 per serving (80 g). Next, the ice cream mixes were homogenized by T25 Ultra Turrax ® homogenizer (IKA Instruments, Staufen, Germany) at 20,500 rpm for 15 min and aged at 4 ºC for 4 h. Subsequently, the mixes were blended by the ice cream maker (Cuizimate RBSICECREAM, China) at -35 ºC, 25 rpm for 45 min. The ice cream samples were kept at -20 ºC until the analysis at day 0, 7, 14, 28, and 56.

3.5 Preparation of Calcium and Vitamin D_3 Fortified Ice Creams

Three types of ice creams; RF, LF and FF, were fortified with 500 mg of calcium carbonate containing 200 mg elemental calcium (VWR, Fontenay-sous-Bois, France) and 250 IU vitamin D_3 per serving (80 g). Each ice cream formulation was made in three separate batches. Vitamin D_3 was an emulsion form, using NaCN as emulsifier. The main compositions of the ice creams were calculated according to Table 2. Skim milk, nonfat dry milk, whipping cream, sugar, stabilizer, and calcium carbonate were mixed before pasteurization at 82 ºC for 8 min. For FF formulation, inulin was mixed in this step.

Table 2 Compositions of the ice cream samples

Compositions (%, w/w)	Types of ice cream samples		
	Regular fat	Light fat	Fat free
Milk solids-non-fat[a]	11.0	11.0	11.0
Milk fat	10.0	5.0	< 0.625
Sugar	12.0	12.0	8.0
Stabilizer	0.5	0.5	0.6
Vanilla liquid	2.0	2.0	2.0
Inulin	0.0	0.0	5.0

[a] Milk solids-non-fat was calculated from total solids of skim milk, nonfat dry milk, and whipping cream. See Appendix A for more information.

Vanilla flavor and vitamin D_3 emulsion were added after the ice cream mixes were cooled down to 15 ºC. Then, the ice cream mixes were homogenized, aged, and blended according to the same condition in section 3.4. The ice cream samples were kept at -20 ºC until the analysis of vitamin D_3 retention, calcium content and microbiological properties at day 0, 7, 14, and 28. Physical properties (overrun, hardness, viscosity, and melting behavior) of the ice creams were determined on day 0.

3.6 Vitamin D_3 Retention of Ice Creams

Vitamin D_3 content of the ice cream samples fortified with emulsified vitamin D_3 was determined on day 0, 7, 14, 28, and 56 of the storage. Vitamin D_3 content of calcium and vitamin D_3 fortified ice creams was determined on day 0, 7, 14, and 28.

Vitamin D_3 was extracted from the ice creams according to the official method 2002.05 (AOAC, 2012a), with slight modification. Ten grams of ice cream sample were saponified with 25 mL of 50% (w/v) KOH. A 50 mL aliquot of 0.5% (w/v) ethanolic pyrogallol was added to prevent the oxidation of vitamin D. The mixture was heated at 95 ºC for 30 min and immediately cooled in the ice bath. The saponified sample was transferred into the separatory funnel and extracted with 35 mL of petroleum ether:diethyl ether (1:1, v/v). The aqueous layer was collected and transferred to another separatory funnel to extract again. Ether extract was collected and washed by deionized water until reaching a neutral pH. After that, the ether extract was evaporated and clearly dried under nitrogen gas. The dried extract was reconstituted in 2 mL acetonitrile (VWR, Fontenay-sous-Bois, France) and filtered through a 0.45 µm syringe filter (Whatman, Maidstone, UK) before the analysis.

The amount of vitamin D_3 in the ice creams was evaluated by high performance liquid chromatography (Shimadzu, Kyoto, Japan). The operating conditions as described by Upreti et al. (2002) were applied. The analytical column was C_{18} (5 µm particles, 4.6 mm ID, 150 mm length) (GL Sciences, Tokyo, Japan). Vitamin D chromatogram was recorded by UV detector at 254 nm at the retention time of approximately 9 min.

Concentrations of vitamin D_3 were calculated by the standard plot of the peak area against the concentrations of standard vitamin D_3.

3.7 Calcium Content of Ice Creams

Calcium analysis of the ice cream was carried out at the Food Research and Testing Laboratory (FRTL), Chulalongkorn University. Calcium content of the ice cream samples was measured by inductively coupled plasma atomic emission spectrometry (JobinYvon, Horiba, France) according to AOAC Official Method 984.27 (AOAC, 2012b). The test solutions were prepared by dry ash method in AOAC Official Method 975.03 (AOAC, 2012c). See Appendix B for more information.

3.8 Proximate Analysis

Total solid, fat, ash, and protein contents of ice creams were determined by the standard method of AOAC 941.08, 952.06, 945.46 and 930.33, respectively (AOAC, 2012d; 2012e 2012f, 2012g). Modified mojonnier method with ether extraction was performed to extract fat from the ice creams. Ash was determined by gravimetric method. Kjeldahl method was used to determine protein content. Total carbohydrate and total caloric content of the ice creams were calculated by formula as described by Food and Agricultural Organization of the United Nations (2003). See Appendix B for more information.

3.9 Physical Characteristics of Calcium and Vitamin D_3 Fortified Ice Creams

3.9.1 Overrun

The air incorporated in the ice creams was determined by the following equation (2):

$$\text{Overrun (\%)} = [(W1-W2)/W2] \times 100 \quad (2)$$

where W1 is the weight of ice cream mix and W2 is the weight of ice cream in the same volume.

3.9.2 Hardness

Hardness of ice cream samples was determined by method of Whelan et al. (2008). Hardness was immediately evaluated by Texture analyzer (TA-XT2i, Stable Micro Systems, Godalming, UK) with acrylic conical probe (Perspex cone probe P/45C) after the ice creams were taken out of -20 °C on day 0 of the storage. Probe was set to penetrate into the ice creams with depth as 20 mm at speed 2 mm/s. The compressed force was recorded for hardness of the ice cream.

3.9.3 Viscosity

Viscosity of the ice creams was analyzed according to Aime et al. (2001). Ice cream samples were placed at 4 °C for 4 h before the analysis. Bohlin CVOR 105 software version 6.32.2.0 viscometer (Worcestershire, UK) with cup and bob was used for viscosity measurements at 30 °C. Eight milliliters of the sample were filled into a cup. Bob was set to penetrate into the samples for 2.5 mm gap width. Viscosity was recorded at shear rate 29.3/s.

3.9.4 Melting Behavior

Melting behaviors of the ice cream samples were determined according to the method of Biasutti et al. (2013). One hundred and thirty grams of ice creams were immediately determined after they were removed from -20 ºC on day 0. The ice creams were placed on a 2-mm wire mesh at 25 ± 2 ºC. Weight of melted ice creams were recorded every 2.5 min for 30 min. Percentage of melted ice creams at each time point was calculated. Melting rate is presented as the time required to melt 5% and 10% of the ice creams.

3.10 Microbial Properties of Calcium and Vitamin D_3 Fortified Ice Creams

To estimate microbiological stability of the ice cream samples, 3M™ Petrifilm (St. Paul, MN, USA) was used to quantify the colony of *Escherichia coli* (*E. coli*)/ coliform and aerobic bacteria according to AOAC Official Method 989.10 (AOAC, 2012h). See Appendix B for more information.

3.11 Statistical Analysis

Experimental samples were performed in replications of three. Data were reported as mean ± standard deviation. Statistical Analysis Software version 9.0 (SAS Institute, NC, USA) was used to identify differences among the treatments at 95% confidence level ($P < 0.05$). Repeated measures design, using PROC MIXED function with Tukey adjustment, was applied for statistical analysis of creaming index, oil droplet size, vitamin D_3 retention, calcium content, and microbial counts of the ice cream. The comparison of variables at each time point, as well as proximate analysis

and physical characteristics of the ice creams, was performed by means of ANOVA and Kruskal-Wallis analyses, as appropriate.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Physicochemical Stability of Vitamin D_3 Emulsions

In the first part of this study, we evaluated the role of milk protein emulsifiers in the physicochemical stability of vitamin D_3 emulsion. Milk proteins including NFDM, NaCN, and WPI were investigated. Mean diameter of oil droplets, zeta potential and creaming index were determined as indicators of emulsion stability. The results indicated that stability of vitamin D_3 emulsion was significantly different when the three different milk protein emulsifiers were applied. Mean droplet diameters of vitamin D_3 emulsions are shown in Table 3. Sodium caseinate exhibited the smallest oil droplet size throughout the study from day 0 to day 14. Oil droplet diameter of the control group (no emulsifier added) could not be detected at day 11 or longer, because the oil and aqueous phases were completely separated. Table 4 displays the effects of the types of milk protein emulsifiers and storage time on zeta potential values of vitamin D_3 emulsions. Zeta potential of control, NFDM, NaCN, and WPI treatments were in a range of -44 to -51 mV, -32 to -37 mV, -37 to -44 mV, and -39 to -45 mV, respectively. Generally, high negative (-30 mV) or positive (+30 mV) zeta potential values could stabilize the emulsion by preventing oil droplet coalescence and increasing electrostatic repulsion between oil droplet surface and the external phase (Moore and Cerasoli, 2010). At pH 7, oil droplets are surrounded by the negative

charges of the milk proteins that could protect against oil droplet aggregation by electrostatic repulsion (Surh, Decker, and McClements, 2006; Taherian et al., 2011).

The effects of types of milk protein emulsifiers on the creaming behavior of vitamin D_3 emulsions are illustrated in Figure 7. Statistical analysis showed that CI values were influenced by type of milk protein emulsifier ($P < 0.0001$), storage time ($P < 0.0001$) and the interaction between type of emulsifier*storage time ($P < 0.0001$). Sodium caseinate emulsion presented the lowest CI throughout 14-day storage. Creaming indices of the control, NFDM and WPI treatments were not affected by the duration of storage ($P > 0.05$). Moreover, CI of vitamin D_3 emulsion using NaCN was gradually increased as compared to the other treatments. (See vitamin D_3 content in vitamin D_3 emulsion throughout 14-day storage in Appendix C)

Creaming index is related to the extent of droplet flocculation in the emulsion. The increase in oil droplet diameter results from coalescence and destabilization of the emulsion, and leads to the formation of the cream layer. The more the flocculation is, the larger the particles and the faster the creaming rate (Robins, 2000; Chiralt, 2009). In this study, vitamin D_3 emulsion using NaCN as emulsifier showed the smallest oil droplet sizes throughout the storage time, which was correlated with its low CI values. This result is in agreement with a number of previous studies. Katoh et al. (1995) demonstrated that emulsion containing NaCN exhibited the smallest oil droplet size as compared to the other emulsifiers including sodium dodecyl sulfate, sucrose esters and polyglycerol esters. Similarly, Hung and Zayas (1991) studied emulsifying

properties of NaCN, whey protein concentrate and NFDM as well as corn germ protein flour. The results showed that NaCN had the highest emulsifying capacity and emulsion stability. Sourdet et al. (2002) reported that fat droplets at high total protein concentration and high proportion of casein emulsion were less aggregated than casein-free emulsion.

Casein is an excellent candidate to generate oil-in-water emulsions because of its high physical stability, its ability to produce a thick layer around the oil droplet interface along with its chelating properties (Lethuaut, Métro, and Genot, 2002; Hu et al., 2003). β-casein is the main subtype of casein protein that coats the oil droplets. At pH 7, the covering of β-casein at the interface provides high electrostatic and steric effects (Srinivasan et al., 1996; Dickinson, 2001). The amphiphilic property of NaCN lowers interfacial tension between the oil and the aqueous phases. In addition, NaCN is highly flexible and easily unfolds at the interface of the emulsion, and eventually the emulsion is more stable (Zayas, 1997). On the other hand, whey was found to have not as much of emulsifying effect due to the absence of balance between hydrophobic and hydrophilic groups (Yamauchi, Shimizu, and Kamiya, 1980). Sourdet et al. (2002) pointed out that using WPI as emulsifier resulted in large dispersed particles and low adsorbed proteins at the surface of the oil droplets. Tippetts et al. (2012) compared stability of the emulsion using nonfat dry milk, whey protein concentrate, sodium caseinate and calcium caseinate as emulsifier. Their results illustrated that whey

protein concentrate had significantly higher phase separation than emulsions formulated with NFDM or caseins ($P < 0.05$).

Casein is also reported to be more heat tolerant than whey protein (Srinivasan, Singh, and Munro, 2002), which is suitable for food processing. The conformation of NaCN does not change by heat. Therefore, the emulsifying property is not decreased (Srinivasan et al., 2002). Semo et al. (2007) discovered that vitamin D was protected from UV degradation by the entrapment of casein. Haham et al. (2012) revealed that casein micelles positively protected vitamin D against thermal degradation and cold storage compared to un-encapsulated or Tween-80 emulsified vitamin D. Besides, the phosphoseryl group of casein gives anionic properties to its structure, which functioned as metal ion chelation that prevents the oil phase from oxidation reactions (Hu et al., 2003).

Table 3 Z-average sizes of the oil droplets in vitamin D_3 emulsions as affected by types of milk protein emulsifiers and storage time

Sample groups	Z-average size of oil droplets (nm)				
	Day 0	Day 3	Day 7	Day 11	Day 14
Control	922.64 (168.62)[a]	757.71 (71.53)[a,b]	768.07 (71.21)[a,b]	ND	ND
NFDM	991.18 (243.52)[a]	1064.53 (229.34)[a]	955.54 (202.03)[a]	1392.44 (259.79)[a]	2355.78 (448.86)[a]
NaCN	434.07 (53.67)[b]	498.34 (2.19)[b]	433.03 (93.67)[b]	706.29 (162.30)[b]	1036.31 (207.39)[b]
WPI	777.79 (171.06)[a,b]	876.67 (138.39)[a]	783.03 (173.38)[a,b]	1178.01 (132.04)[a,b]	1348.72 (463.42)[b]

The data were shown as mean (standard deviation) of the three replications.

Control = no emulsifier added.

NFDM = nonfat dry milk, NaCN = sodium caseinate, WPI = whey protein isolate.

ND = Not detected because of a complete separation of the oil phase.

[a,b] The data within the same day with different superscripts are significantly different ($P < 0.05$).

Table 4 Zeta potential of vitamin D_3 emulsions as affected by types of milk protein emulsifiers and storage time

Sample groups	Zeta potential (mV)				
	Day 0	Day 3	Day 7	Day 11	Day 14
Control	-51.07	-49.23	-44.18	ND	ND
	(1.71)	(6.27)	(7.47)		
NFDM	-34.66	-37.17	-34.24	-36.98	-32.22
	(3.01)	(1.18)	(4.55)	(2.30)	(0.69)
NaCN	-37.40	-44.73	-44.38	-43.97	-43.80
	(7.79)	(2.21)	(4.85)	(4.04)	(0.62)
WPI	-44.01	-43.63	-39.58	-45.57	-45.21
	(2.77)	(1.91)	(2.91)	(1.56)	(3.64)

The data were shown as mean (standard deviation) of the three replications.
NFDM = non-fat dry milk, NaCN = sodium caseinate, WPI = whey protein isolate.
ND = Not detected because of a complete separation of the oil phase.

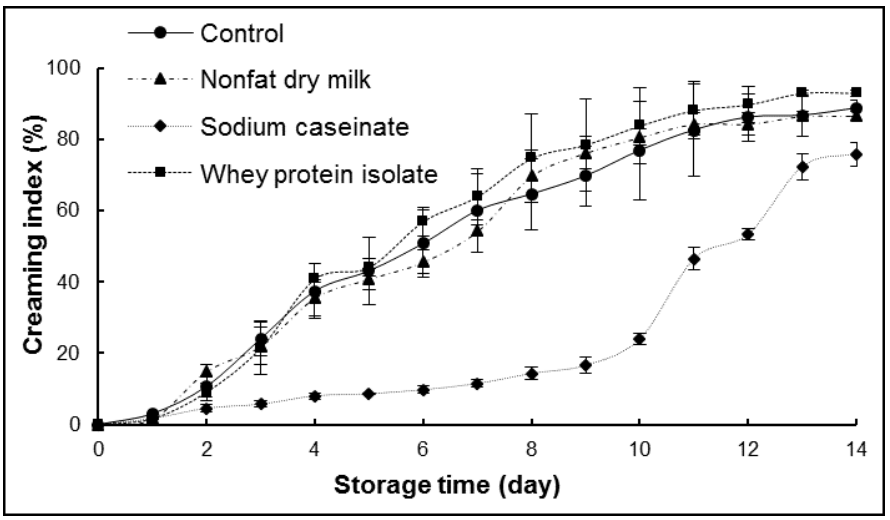


Figure 7 Creaming indices of vitamin D_3 emulsions as affected by types of milk protein emulsifiers and storage time. Error bars = standard deviation.

4.2 Vitamin D_3 Retention in Fortified Ice Creams

According to the results of physicochemical stability of vitamin D_3 emulsion, it can be concluded that NaCN was an outstanding milk protein to prepare stable vitamin D_3 emulsion. Thus, for the second part of the study, vitamin D_3 that was emulsified with NaCN was selected to incorporate in the experimental ice creams. Regular fat (RF), light fat (LF), and fat free (FF) ice creams fortified with vitamin D_3 were formulated as shown in Table 2 (See standard curve of vitamin D_3 in Appendix D). The control groups were RF, LF, and FF ice creams that were fortified with the dry form of vitamin D_3.

The stability of emulsified vitamin D_3, using NaCN as an emulsifier, in the ice creams with three different levels of fat content was excellent up to 2 months at -20 °C. Retention of vitamin D_3 was investigated at day 0, 7, 14, 28, and 56 of the storage. The amounts of vitamin D_3 in the ice creams were significantly affected by

type of ice cream, form of vitamin D_3, and storage time ($P < 0.0001$). Vitamin D_3 levels of the ice creams fortified with emulsified vitamin D_3 compared to the control were not significantly different until day 7, 14, and 28 for FF, LF and RF ice creams, respectively (Figure 8). For RF formulation, dry vitamin D_3 decreased approximately 90% at day 28 (Figure 8A) and approximately 80% for the LF at day 14 (Figure 8B). Vitamin D_3 content in an emulsion form in FF ice cream was not significantly changed throughout the storage time whereas the non-emulsified form was rapidly depleted at day 7 (Figure 8C). Typically, vitamin D is a fat-soluble vitamin so that the amount of fat in the foods could influence vitamin D levels. In this study, vitamin D_3 in the control groups demonstrated the longest shelf-life in RF ice creams, followed by LF and FF. Nevertheless, this study clearly showed for the first time that the emulsified form of vitamin D_3, using NaCN as emulsifier, improved the stability of vitamin D_3 not only in the ice creams with regular fat content, but also in the low fat and fat-free formulations. The amounts of detected vitamin D_3 were higher than 250 IU/serving in all ice cream samples. In general, very low levels of vitamin D were fortified in food products. Due to the low ratio between vitamin D and food mass, other compounds in the products might disturb vitamin D extraction and analysis (Parrish, 1979; Upreti et al., 2002; Kazmi et al., 2007).

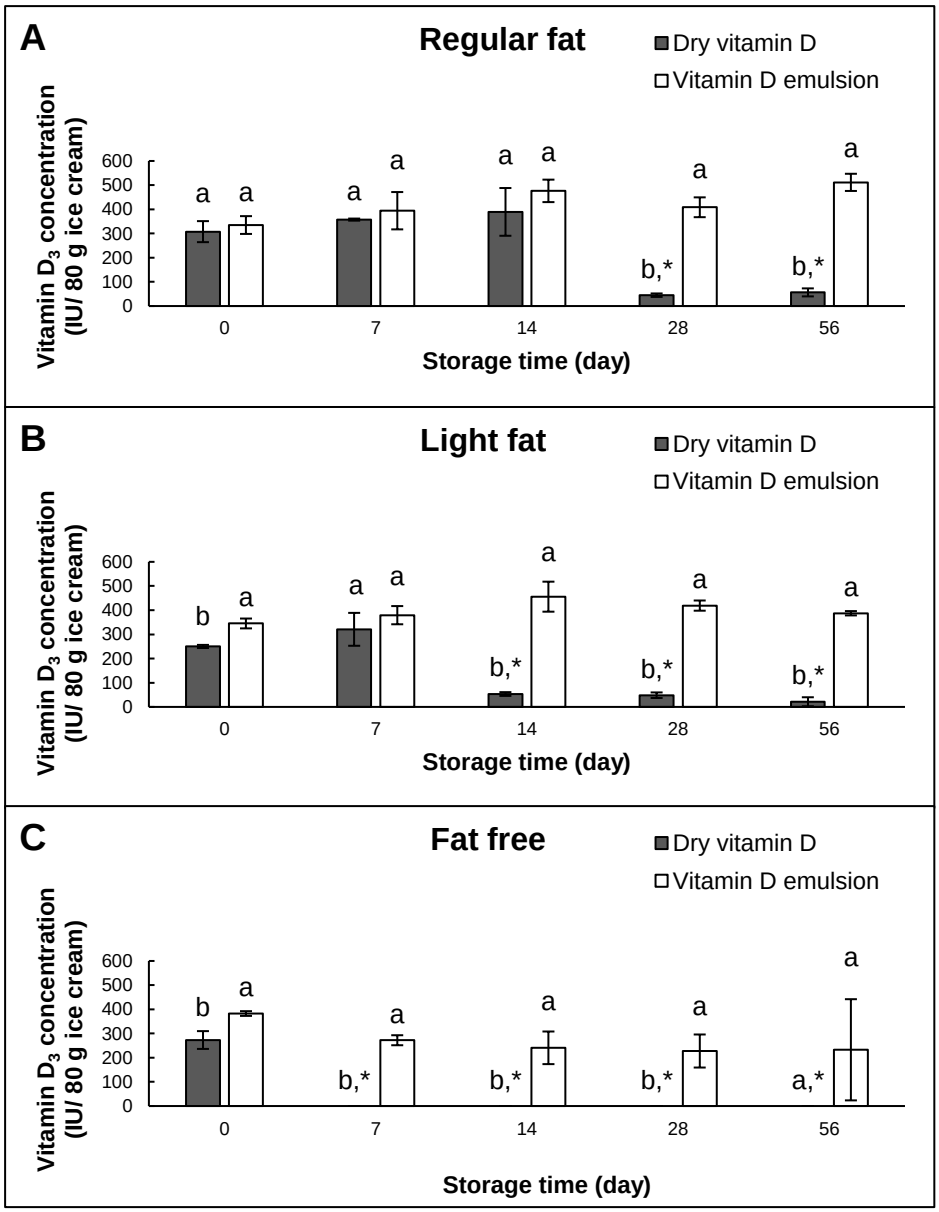


Figure 8 Effect of the different forms of vitamin D_3 on the retention of vitamin D_3 in regular fat (A), light fat (B) and fat free (C) ice creams during storage at -20 ºC for 56 days. Sodium caseinate was used as emulsifier for vitamin D_3 emulsion. Error bars = standard deviation. Letters indicate significant differences ($P < 0.05$) between data points within the same day. * The amount of vitamin D is significantly different from day 0 for the same treatment ($P < 0.05$).

Kazmi et al. (2007) formerly explored the retention of vitamin D_3 added to the fortified ice cream at the level of 8,000 IU per serving. These authors reported that vitamin D_3 content of both crystalline and emulsified forms were stable and did not degrade by the storage time. However, little details on the type of emulsifier used in their study were given and the levels of vitamin D_3 were exceeded the tolerable upper intake levels. Therefore, their findings may not represent vitamin D retention when used on more practical levels.

4.3 Characteristic of Ice Creams Fortified with Calcium and Vitamin D_3 Emulsion

A 200 mg of elemental calcium per serving (80 g) was added into the ice cream samples. Proximate analyses of RF, LF and FF ice creams fortified with calcium and vitamin D_3 emulsion, using NaCN as an emulsifier, were performed and energy per serving was calculated as shown in Table 5. Calcium content in the fortified ice cream was determined and illustrated in Figure 9. The control group was ice cream recipe that was not fortified with calcium. Calcium contents of RF, LF and FF ice creams were significantly higher than the control throughout 28-day storage ($P < 0.05$) (Figure 9A-C). National Institutes of Health (2014) indicates that general vanilla ice cream contains only 84 mg calcium per 4 oz or approx. 74 mg per 80 g serving. The control ice creams of this study had approx. 100 mg calcium per serving. Still, this calcium amount is insufficient for calcium requirement (1,000-1,200 mg per day for healthy adult). Thus, food fortified with calcium is an alternative source of dietary calcium for consumers to

Table 5 Compositions and energy value of the ice creams fortified with calcium and vitamin D_3 emulsion, using sodium caseinate as emulsifier

Compositions (%, w/w)	Types of ice cream		
	Regular fat	Light fat	Fat free
Total solids	34.87 ± 0.51	30.11 ± 0.33	25.78 ± 0.08
Moisture	65.13 ± 0.51	69.89 ± 0.33	74.22 ± 0.08
Protein	0.74 ± 0.04	0.81 ± 0.08	0.88 ± 0.01
Fat	10.11 ± 0.44	5.70 ± 0.23	0.62 ± 0.03
Ash	1.49 ± 0.24	2.45 ± 0.10	2.81 ± 0.38
Carbohydrate	22.52 ± 0.35	21.15 ± 0.61	21.47 ± 0.47
Energy (kcal/serving)	147.22 ± 3.59	111.33 ± 0.58	76.00 ± 1.37

The data were shown as mean ± standard deviation of the three replications.
One serving of ice cream = 80 g.

achieve the RDI levels. Previous studies by Chansathirapanich et al. (2016) also demonstrated that fortified calcium in ice cream was not loss during storage. Van der Hee et al. (2009) reported that calcium from calcium-fortified ice cream had bioavailability as high as fortified milk. Hence, our finding confirmed that fortified calcium was stable in different fat levels of ice creams.

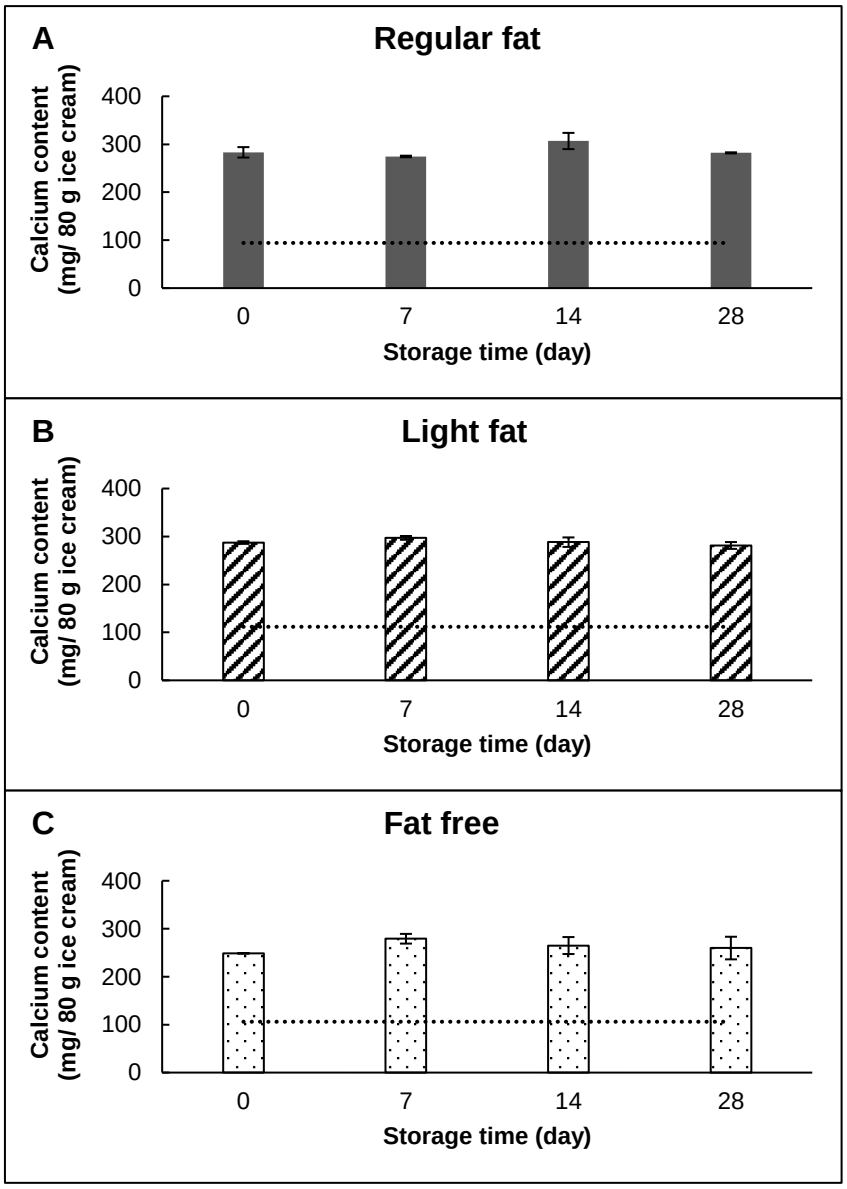


Figure 9 Calcium content of regular fat (A), light fat (B), and fat free (C) ice creams fortified with calcium and vitamin D_3 emulsion, using sodium caseinate as emulsifier, during storage at -20 ºC for 28 days. Baseline represents a calcium content of each ice cream treatment that was not fortified with calcium. Error bars = standard deviation. Means with the same letter are not significantly different ($P > 0.05$).

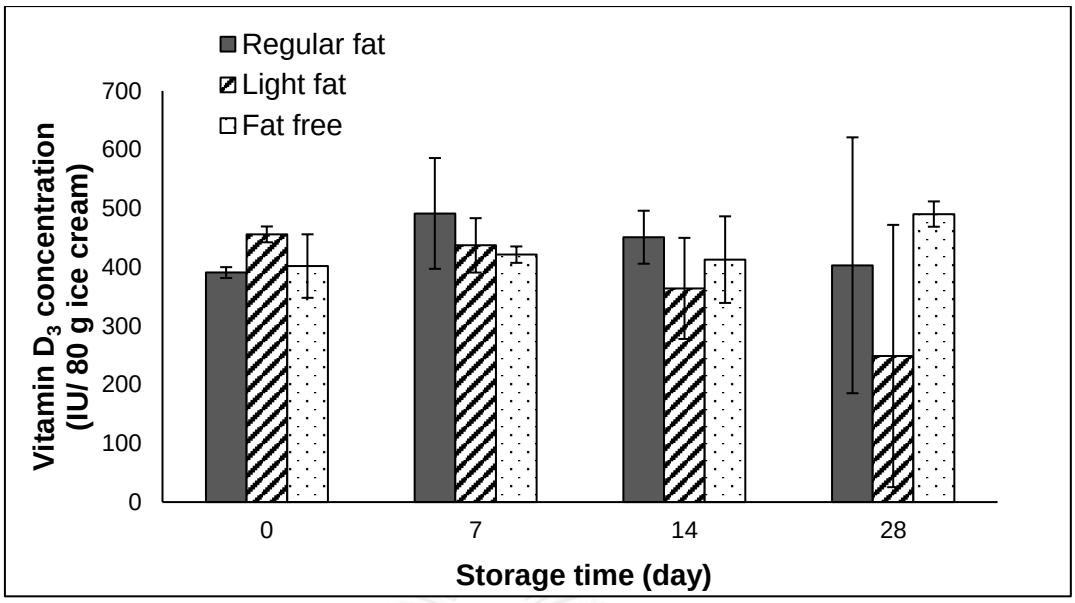


Figure 10 Retention of vitamin D_3 in regular fat, light fat, and fat free ice creams fortified with calcium and vitamin D_3 emulsion, using sodium caseinate as emulsifier, during storage at -20 ºC for 28 days. Error bars = standard deviation.

Vitamin D_3 content in the ice cream samples was examined again after the addition of calcium. The results indicated that vitamin D_3 emulsion, using NaCN as emulsifier, had excellent stability. Retentions of vitamin D_3 in all ice cream samples were not different during 28-day storage ($P > 0.05$) (Figure 10). Statistical analysis also demonstrated that vitamin D_3 retentions was not affected by levels of fat content in the ice cream. The amounts of vitamin D_3 in all ice cream samples were ranged approximately 250 to 400 IU per serving. Dickinson and Golding (1998) and Radford, Dickinson, and Golding (2004) pointed out that calcium ion could inhibit the depletion flocculation of emulsion by promoting calcium-casein binding that produced thick

layer of adsorbed casein at oil droplets surface to increase steric effects. Thus, adding calcium to the ice cream may be advantageous to stabilize emulsified vitamin D_3.

Physical characteristics and microbial properties of the ice creams were investigated for a potential industrial transfer of the present method. Overrun, hardness, and viscosity are shown in Table 6. Overrun of RF, LF, and FF ice creams fortified with calcium and vitamin D_3 emulsion were about 40% and they were not significantly different between the groups ($P > 0.05$). Similarly, the studies by Chang and Hartel (2002) and Biasutti et al. (2013) revealed that overrun was not affected by fat content of the ice cream. In this study, FF ice cream has the highest hardness, followed by LF and RF ($P < 0.05$). It could be pointed out that hardness was affected by fat content of the ice creams. Viscosity was evaluated at shear rate 29.3/s, which is the approximate shear rate of oral cavity when eating ice cream (Aime et al., 2001). The results showed that viscosity values were not significantly different among type of ice cream ($P > 0.05$). Melting behavior of the ice creams was illustrated in Figure 11. FF had significantly longer melting time than RF and LF ($P < 0.05$). Mahdian and Karazhian (2013) reported that using inulin as a fat replacer in ice cream could increase melting resistance of the ice cream.

Table 6 Overrun, hardness and viscosity of regular fat (RF, 10% fat), light fat (LF, 5% fat) and fat free (FF, < 0.625% fat) ice creams fortified with calcium and vitamin D_3 emulsion using sodium caseinate as emulsifier

Physical properties	Types of ice cream		
	Regular fat	Light fat	Fat free
Overrun (%)	40.21 ± 2.17[a]	41.23 ± 0.52[a]	39.68 ± 2.04[a]
Hardness (kg)	17.24 ± 0.36[a]	23.33 ± 1.45[b]	41.56 ± 2.30[c]
Viscosity (Pa/s)[*]	6.04 ± 2.97[a]	1.76 ± 0.29[a]	1.64 ± 0.34[a]

[*] All data were reported at shear rate 29.3/s.
The data were shown as mean ± standard deviation of the three replications.
Means with different superscript letters in the same row are significantly different ($P < 0.05$).

For microbial tests, the results demonstrated that no *E. coli* and coliform detected in 0.01 g of all ice creams in this study throughout 28 days. The numbers of aerobic bacteria in the ice cream samples are indicated in Figure 12. All ice cream samples had less than 1,000 CFU/g of aerobic bacteria throughout 28 days and there were no significant changes by storage time ($P > 0.05$). Therefore, according to The Ministry of Public Health Thailand (Regulation no.222) (2002), it can be concluded that ice cream samples in our study were appropriate for consumption because the microbial counts were not exceeded the legal limit (Total bacteria must not exceed 600,000 CFU per 1 g of ice cream; *E. coli* must not find in 0.01 g of ice cream).

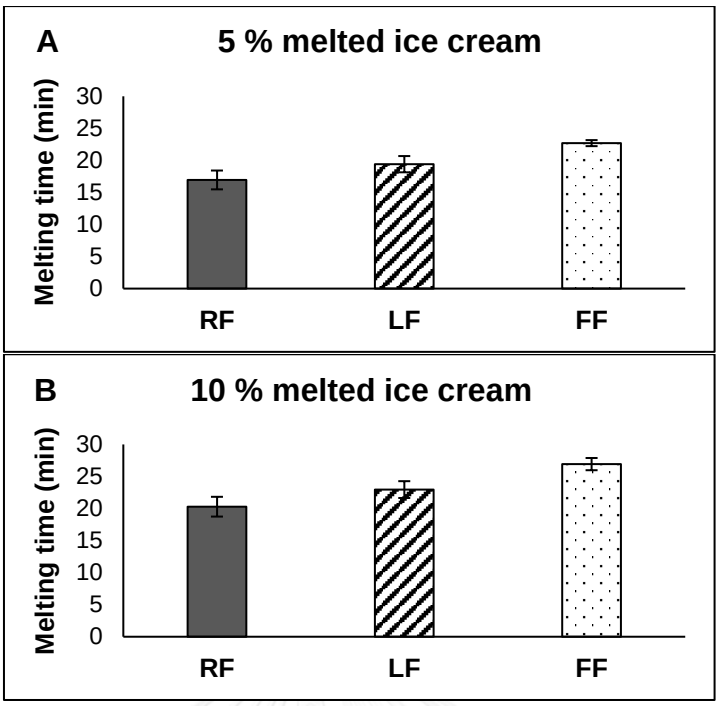


Figure 11 Melting time for 5% (A) and 10% (B) of regular fat (RF), light fat (LF), and fat free (FF) ice creams fortified with calcium and vitamin D_3 emulsion, using sodium caseinate as emulsifier. Error bars = standard deviation. Means with difference letter are significantly different ($P < 0.05$).

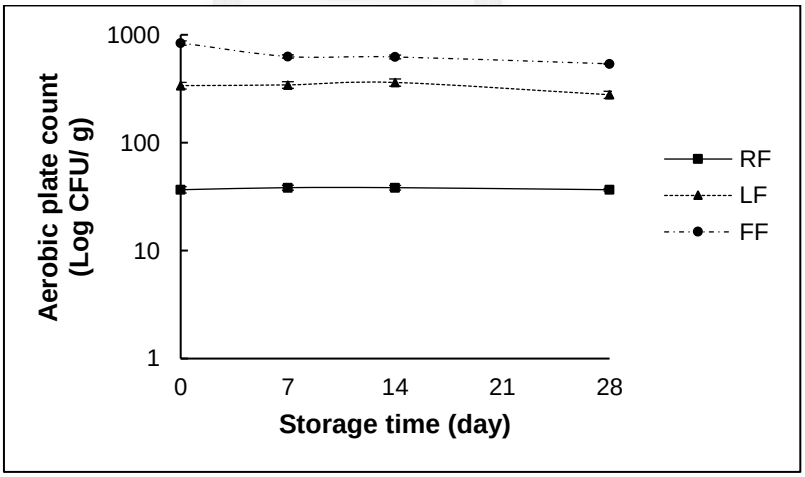


Figure 12 Aerobic plate counts in regular fat (RF), light fat (LF) and fat free (FF) ice cream fortified with calcium and vitamin D_3 emulsion, using sodium caseinate as emulsifier, during storage at -20 ºC for 28 days. Error bars = standard deviation.

CHAPTER V

CONCLUSION

Ice creams, with different fat levels ranged from < 0.625 - 10%, fortified with vitamin D_3 and calcium were successfully developed in this study. Our finding demonstrates that the lab-scale vitamin D_3 fortified ice cream at a nutritionally acceptable consumption level had a remarkable improvement of vitamin D_3 stability, especially in the fat free formulation. Vitamin D_3 retention in the ice cream is enhanced by incorporating vitamin D_3 as an emulsion using milk protein as an emulsifier. Sodium caseinate is an excellent milk protein emulsifying agent that can be used for vitamin D_3 emulsion preparation. Sodium caseinate not only increases emulsion stability, but also easily combines with the dairy products. Calcium content is preserved in the ice creams throughout the storage time. Our finding provides helpful information for ice cream manufacturers that seek to add vitamin D and calcium to their products. Calcium and vitamin D_3 fortified ice cream can be used as a functional food for consumers or patients who have risk of calcium and vitamin D insufficiency. However, further study regarding sensory evaluation of the ice cream fortified with calcium and vitamin D_3 emulsion may be needed to additionally explain consumers' acceptance of this product.

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APPENDICES

APPENDIX A

FAT, TOTAL SOLIDS, AND MILK SOLIDS-NON-FAT

OF MILK INGREDIENTS OF ICE CREAM

Table A-1 Fat, total solids, and milk solids-non-fat of milk ingredients of ice cream

Milk ingredients	Fat (%)	Total solids (%)	Milk solids-non-fat (%)
Milk	0.20 ± 0.00	10.30 ± 0.01	10.1 ± 0.01
Whipping cream	34.50 ± 0.02	41.59 ± 0.01	7.09 ± 0.03
Nonfat dry milk	6.07 ± 0.08	96.13 ± 0.03	90.06 ± 0.11

The data were shown as mean ± standard deviation of the three replications.

APPENDIX B

THE STANDARD METHODS OF AOAC FOR CALCIUM CONTENT ANALYSIS, PROXIMATE ANALYSIS, AND MICROBIOLOGICAL STABILITY TEST OF ICE CREAM

1. Calcium Content of Ice Cream (AOAC official method 975.03 and 984.27, 2012)

To prepare the test solutions of the ice cream samples, approximately 1 g of ice cream sample was weighed in porcelain crucible. Then, the sample was heated by hot plate until smoke was depleted. After that, sample was ignited in furnace at 500 ºC for 2 h until the remaining residue became white ash. To dissolve cooled white ash, 10 mL of 50% (v/v) hydrochloric acid was added to the sample and heated on hot plate for about 2-3 min until it was clear solution. The cooled solution was analyzed by inductively coupled plasma atomic emission spectrometry.

2. **Total Solid Content of Ice Cream** (AOAC official method 941.08, 2012)

The ice cream samples were weighed approximately 2 g into round, flat-bottom dish. The accurate weight was recorded. Samples were then heated on water bath for 30 min and dried in forced air oven at 100 ºC for 3.5 h to completely remove all liquid. The dried samples were rapidly weighed after they were cooled in a desiccator. The total solid of the ice cream samples was calculated by the following equation:

$$\% \text{ Total solid (w/w)} = \frac{\text{Weight of total solid}}{\text{Weight of sample}} \times 100$$

3. Fat Content of Ice Cream (AOAC official method 952.06, 2012)

Fat content of the ice cream samples was determined using modified Mojonnier method. Samples were extracted with ether. Firstly, the ice cream samples were weighed 4-5 g into the Mojonnier flask and diluted with deionized water to about 10 mL. Two milliliters of ammonium hydroxide were added and phenolphthalein was used as an indicator to observe the interface between aqueous and ether phase. Then, samples were heated in water bath at 60 ºC for 20 min and periodically shaken. After that, samples were cooled down and 10 mL of 95% ethanol were added. Twenty-five milliliters of diethyl ether and petroleum ether were used for extracting fat in ice creams. Each sample was extracted in triplicate. The ether layers from each of extraction were kept and evaporated in force air oven at 100 ºC for 30 min. The percentage of fat of the ice cream samples was calculated as follows:

$$\% \text{ Fat (w/w)} = \frac{\text{Weight of Fat}}{\text{Weight of sample}} \times 100$$

4. Ash Content of Ice Cream (AOAC official method 945.46, 2012)

Five grams of the ice cream samples in porcelain crucible were dried on steam bath followed by ignited in furnace at 550 ºC until ash became white. Ash was cool in desiccator and weighed. The percentage of ash of the ice cream samples was calculated as follows:

$$\% \text{ Ash (w/w)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

5. Protein Content of Ice Cream (AOAC official method 930.33, 2012)

Kjeldahl method was used to determine protein in ice cream. The ice cream samples were weighed 4-5 g into Kjeldahl flask and digested with 25 mL concentrated sulfuric acid, using 2 tablets of Kjeltabs (containing 3.5 g K_2SO_4 and 0.4 g $CuSO_4$, Foss analytical A/S, USA) as catalyst until the digested solution was clear. The cooled digested solution was automatically distilled by the distillation unit (Buchi, Switzerland). For distillation, 100 mL of 40% (w/v) sodium hydroxide was added to convert ammonium sulfate in the solutions to ammonia gas. The gas was collected by 150 mL of 4% (w/v) boric acid in order to convert ammonia to ammonium borate. Modified methyl red was used as an indicator. The ammonium borate solution was titrated with 0.1 N sulfuric acid. The volume of the acid at the end point was recorded to calculate protein content by the following equation:

$$\% \text{ Protein (w/w)} = \frac{0.014 \times N \times V \times \text{Empirical factor}}{\text{Weight of sample}} \times 100$$

N = Normality of sulfuric acid solution

V = mL of sulfuric acid titrant used

Empirical factor = 6.38 (an empirical factor of milk and milk products)

6. Total Carbohydrate of Ice Cream (Food and Agriculture Organization of the United Nations, 2013)

Total carbohydrate of ice cream was calculated by the following formula:

% Total carbohydrate (w/w) = 100 - [(% Moisture + % Fat + % Ash + % Protein) of sample]

7. Total Caloric Content of Ice Cream (Food and Agriculture Organization of the United Nations, 2013)

Total caloric content of ice cream was calculated from sources of energy including fat, protein and carbohydrate. The values of energy per one gram of these sources are 9 kcal for fat, 4 kcal for protein and 4 kcal for carbohydrate. Total caloric content was calculated by the following formula:

Total caloric content (kcal/100 g) = [(9 × % Fat) + (4 × % Protein) + (4 × % Carbohydrate)]

8. Microbiological Stability of Ice Cream (AOAC Official Method 989.10, 2012)

The 3M™ Petrifilm (Minnesota, USA) was used to quantify the colony of *Escherichia coli* (*E. coli*)/coliform and aerobic bacteria. The ice cream samples were diluted with Butterfield's phosphate buffer (pH = 7.2) as a serial dilution. Then, 1 mL of the diluted solutions were placed onto the bottom film by automatic pipette and the film was immediately and carefully closed. For *E. coli*/coliform count plates, the films were incubated at 35 ± 1 ºC for 24 ± 2 h. The red colonies were counts as *E. coli*. All red and blue colonies with gas were counted as a colony of total coliform. For aerobic plate counts, the films were incubated at 32 ± 1 ºC for 48 ± 3 h. All colonies were counted and calculated as colony forming unit bacteria.

APPENDIX C

RETENTION OF VITAMIN D EMULSION

USING SODIUM CASEINATE AS AN EMULSIFIER

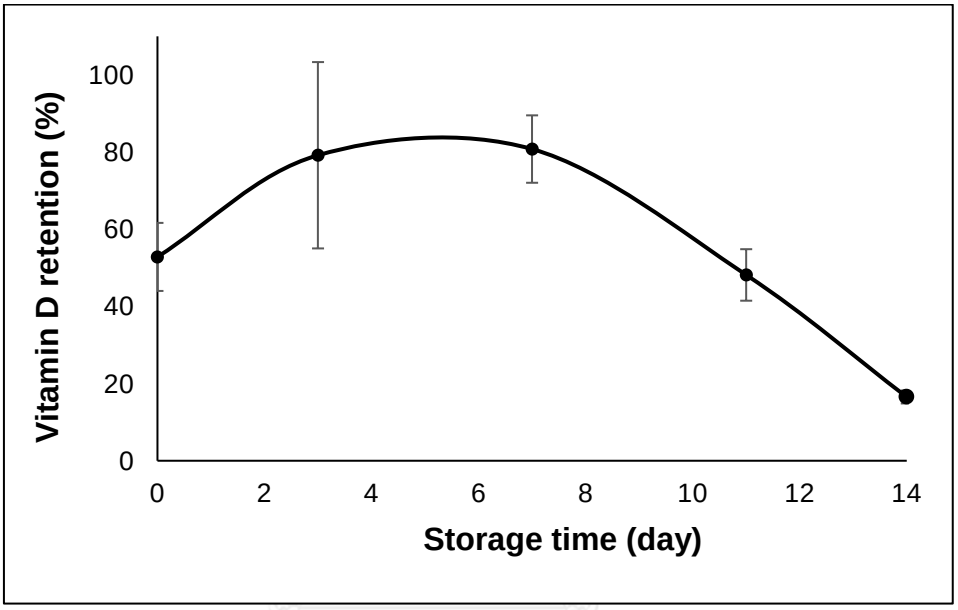
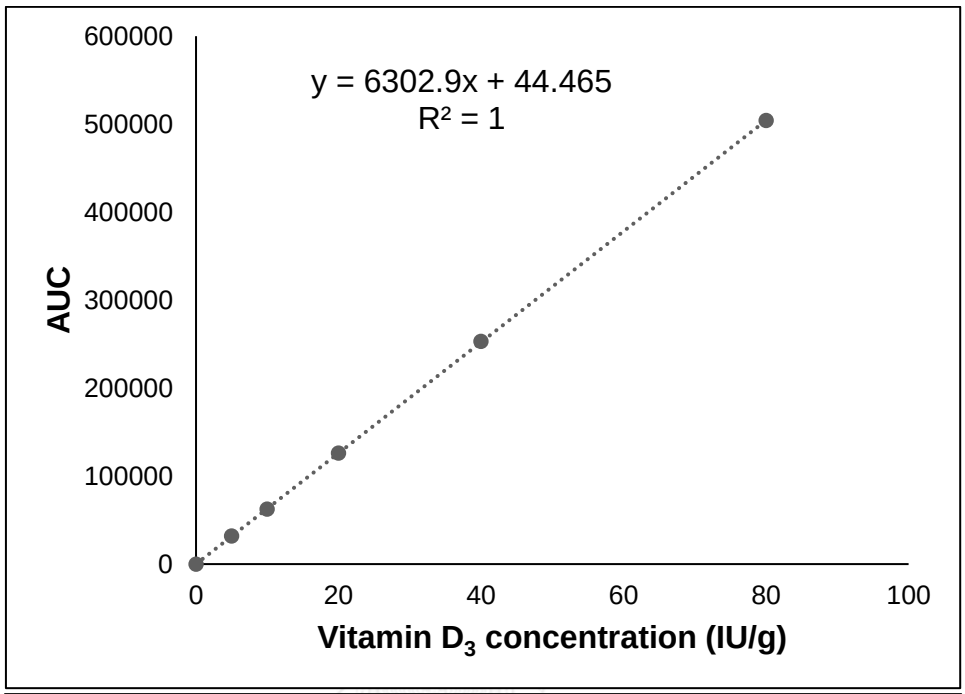


Figure A-1 Retention of vitamin D_3 emulsion using sodium caseinate as an emulsifier. Error bars = standard deviation.

APPENDIX D

STANDARD CURVE OF VITAMIN D_3



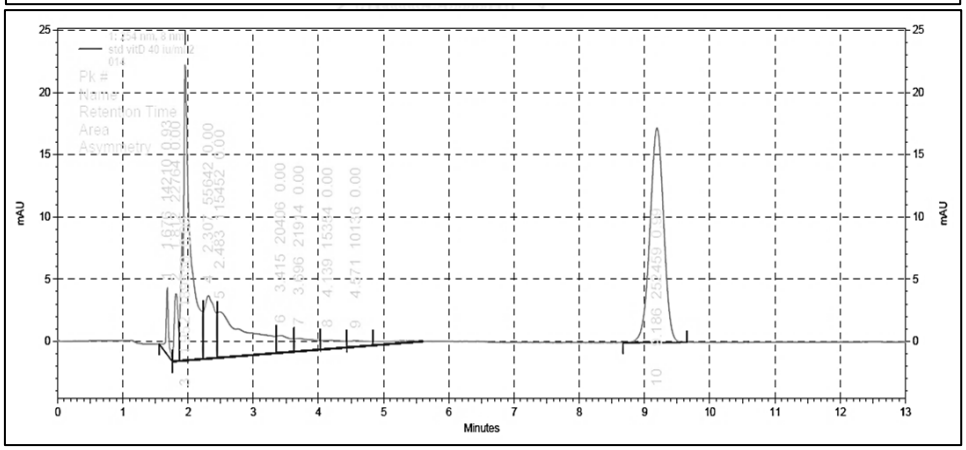


Figure A-2 Standard curve of vitamin D_3.

VITA

Miss Nardauma Tipchuwong was born on February 12, 1989 in Bangkok, Thailand. In 2012, she received her Bachelor of Sciences in Pharmacy from the Faculty of Pharmacy, Srinakharinwirot University. After graduation, she had been worked as a community pharmacist of Boots Retail (Thailand) Ltd. for 1 year. Her responsibilities included prescription, advising the information to maintain and improve people's health, training the drug store's staff, managing the stock of medicines, and sale planning.