การพัฒนาไอศกรีมเสริมแคลเซียมและคอเลแคลซิเฟอรอล

นางสาววีรยา จันสถิรพานิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ภาควิชาอาหารและเภสัชเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

DEVELOPMENT OF CALCIUM AND CHOLECALCIFEROL FORTIFIED ICE CREAM

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Food Chemistry and Medical Nutrition

Department of Food and Pharmaceutical Chemistry

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2015

Thesis Title	DEVELOPMENT OF CALCIUM AND CHOLECALCIFEROL FORTIFIED ICE CREAM
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วีรยา จันสถิรพานิช : การพัฒนาไอศกรีมเสริมแคลเซียมและคอเลแคลซิเฟอรอล (DEVELOPMENT OF CALCIUM AND CHOLECALCIFEROL FORTIFIED ICE CREAM) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ภญ. ดร.รสริน ตันสวัสดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.พนิตา งามเชื้อชิต{, 63 หน้า.

แคลเซียมและวิตามินดีมีความสำคัญต่อการควบคุมสมดุลของการสร้างและสลายเซลล์กระดูก งานวิจัยหลายชิ้นรายงานว่าประชากรหลายกลุ่มได้รับแคลเซียมและวิตามินดีไม่เพียงพอ ดังนั้นการเสริมแคลเซียมและวิตามินดีในอาหารจึงเป็นวิธีหนึ่งในการแก้ไขปัญหาดังกล่าว การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของการเสริมแคลเซียมและวิตามินดี3 ลงในไอศกรีมที่มีปริมาณไขมันแตกต่างกันจำนวน 5 ชนิด ได้แก่ ไอศกรีมสูตร regular fat (RF; 10%), reduced fat (RDF; 7.5%), light (L; 5%), low fat (LF; 2.5%) และ fat free (FF; < 0.625%) มีการใช้อินนูลินเพื่อเป็นสารทดแทนไขมันในตำรับ LF และ FF ไอศกรีมทุกตำรับเสริมด้วยแคลเซียม 200 มิลลิกรัม และวิตามินดี3 200 ยูนิต และเก็บไว้ในอุณหภูมิ -20 องศาเซลเซียส เพื่อวิเคราะห์คุณสมบัติทางกายภาพและจุลชีววิทยาในวันที่ 0, 7, 14 และ 28 ผลการศึกษาพบว่าร้อยละการขึ้นฟูของไอศกรีมชนิด L และ FF สูงกว่าชนิด RF, RDF และ FF ($P < 0.05$) ความแข็งของไอศกรีมมีแนวโน้มมากขึ้นเมื่อปริมาณไขมันลดน้อยลงและเมื่อเก็บรักษายาวนานขึ้น ไอศกรีมที่มีปริมาณไขมันน้อยมีแนวโน้มของอัตราการละลายช้ากว่าไอศกรีมชนิดที่มีปริมาณไขมันมากกว่าในวันที่ 0 ในขณะที่อัตราการละลายของไอศกรีมแต่ละชนิดในวันที่ 14 และ 28 ไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ไอศกรีมที่มีอินนูลินเป็นส่วนประกอบมีความหนืดต่ำกว่าไอศกรีมชนิดที่ไม่มีอินนูลินทุกระยะการเก็บรักษา ($P < 0.05$) ผลการทดสอบทางจุลชีววิทยาพบเชื้อแบคทีเรียน้อยกว่า 100 โคโลนีต่อมิลลิลิตร และไม่พบเชื้ออีโคไลและโคลิฟอร์มในไอศกรีมที่เจือจางในอัตราส่วน 1 ต่อ 100 ตลอดระยะเวลาในการศึกษา ทั้งนี้พบว่าแคลเซียมไม่สูญเสียไประหว่างการเก็บรักษา แต่อาจจำเป็นต้องมีการใช้เทคนิคอื่นๆในการเสริมวิตามินดีในไอศกรีมเพื่อเพิ่มความคงตัว

ภาควิชา	อาหารและเภสัชเคมี	ลายมือชื่อนิสิต	.....
สาขาวิชา	อาหารเคมีและโภชนศาสตร์ทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....
ปีการศึกษา	2558		

5576225333 : MAJOR FOOD CHEMISTRY AND MEDICAL NUTRITION

KEYWORDS: ICE CREAM / CALCIUM / VITAMIN D

WEERAYA CHANSATHIRAPANICH: DEVELOPMENT OF CALCIUM AND CHOLECALCIFEROL FORTIFIED ICE CREAM. ADVISOR: ROSSARIN TANSAWAT, Ph.D., CO-ADVISOR: PANITA NGAMCHUACHIT, Ph.D.{, 63 pp.

Calcium and vitamin D play important roles in bone homeostasis. Several studies reported inadequate intakes of these two nutrients in many population groups. Food fortification is one way to solve the problem. The objective of this study was to determine the effects of fortification of ice cream with calcium and vitamin D3. Five ice cream formulations contained different amounts of fat including regular fat (RF), reduced fat (RDF), light (L), low fat (LF) and fat free (FF) (10%, 7.5%, 5%, 2.5% and < 0.625% fat content, respectively). Inulin was used as a fat replacement in LF and FF formulas. Two hundreds mg of elemental calcium and 200 IU of vitamin D3 per serving were fortified in each formula. Ice creams were stored at -20 ˚C. Physical and microbiological properties were evaluated on day 0, 7, 14, and 28. Overrun of L and FF were higher than RF, RDF and FF ($P < 0.05$). Hardness tended to increase as the lower of fat content of an ice cream and as the longer the products stored. Melting rate inclined with further reduction of fat on day 0, but no significant difference was found among the formulations on day 14 and 28. The ice creams formulated with inulin had lower viscosity ($P < 0.05$) compared to the non-containing inulin formulas at all the time points. Aerobic plate counts were less than 100 CFU/mL, and no *E. coli* and coliform found at 1:100 dilutions throughout the study. Calcium was preserved; however, alternative techniques of vitamin D fortification may be needed to improve the stability.

Department:	Food and Pharmaceutical Chemistry	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Food Chemistry and Medical Nutrition	Co-Advisor's Signature	.....
Academic Year:	2015		

ACKNOWLEDGEMENTS

I honestly thank to the Graduate School Thesis Grant, Chulalongkorn University, Bangkok, Thailand for the financial support. I would like to thank the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences and Department of Food Technology, Faculty of Science, Chulalongkorn University, for laboratory equipment.

I would like to express my sincere gratitude to my advisor Dr. Rossarin Tansawat for giving knowledge and supporting everything for my research study. Besides my advisor, I would like to express my thankfulness to my co-advisor, Dr. Panita Ngamchauchit for the laboratory work. I would also like to extend my deepest gratitude for the rest of my thesis committee including Assistant Professor Dr. Suyanee Pongthananikorn, Dr. Tippawan Siritientong and Associate Professor Thitirat Panmaung for their comments and encouragements. I am grateful to Assistant Professor Dr. Suyanee Pongthananikorn in particular for enlightening me the first glance of this research. I am very grateful to all my instructors at the Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. My sincere thanks goes to Miss Phornphan Rohitrat, who gave me a lot of help from the first day here until the last day of my research. I would like to also thank Miss Roongnapa Ornchoo, Mr. Chawanphat Muangnoi and Miss Sirirat Kopandung for helping me in the equipment set. Above of all, I would like to express my gratitude to my parents, my sister, my brother and my friends for always supporting me spiritually.

CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AIs	Adequate Intakes
APC	Aerobic plate count
CFU	colony-forming unit
cm	centimeter
DBP	vitamin D-binding protein
ES	the US Endocrine Society
E/S	ice cream emulsifier and stabiliser
et al.	*et alii* (and others)
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FF	fat free ice cream (0.625% fat)
IOM	the Institute of Medicine Committee's
IU	international unit
L	liter
L	light ice cream (5% fat)
LF	low fat ice cream (2.5% fat)
mL	milliliter
MSNF	milk solids nonfat

N	normality
RDF	reduced fat ice cream (7.5% fat)
RF	regular ice cream (10% fat)
TRAP5b	tartrate-resistant acid phosphatase isoform 5b
nmol	nanomole
ng	nanogram
µg	microgram
US	The United States of America
UV	ultraviolet radiation
˚C	degree Celsius
$1,25(OH)_2D$	1,25-dihydroxy vitamin D (calcitriol)
25(OH)D	25-hydroxy vitamin D (calcidiol)

CHAPTER I

INTRODUCTION

1.1 Background

Calcium and vitamin D play important roles in bone homeostasis. Calcium is a major constituent of bones and a second messenger in cell signalling pathways. Obtaining sufficient calcium is important to decrease risk of osteoporosis, hypertension, colorectal cancer, gastric cancer, nephrolithiasis, neurogenerative disease and degenerative joint disease (Beto, 2015; Fujita, 2000; Keum *et al*., 2014). The recommended daily allowance of calcium varies for different age groups, and ranges from 700 – 1,300 mg/day (Institute of Medicine, 2012). Several studies have reported instances of inadequate calcium intake throughout the world including in Europe (Kaganov *et al*., 2015), United States (Mangano *et al*., 2011), and Asia (Mithal and Kaur, 2012; Wang *et al*., 2010). Vitamin D is also essential for bone health. The Institute of Medicine (2012) recommended that vitamin D requirements are 400 IU/day in infants (0 – 1 year), 400 IU/day in children and adults (1 – 70 years), and 800 IU/day in elderly (800 IU/day). Long-term vitamin D deficiency (< 30 ng/ml or < 75 nmol/L) (National Institutes of Health, 2014) can result in osteoporosis in adults and rickets in children. Many other health problems such as cardiovascular disease, type 2 diabetes, several

cancers and autoimmune disorders are also related to low vitamin D levels (Calvo, Whiting and Barton, 2005; Pearce and Cheetham, 2010).

Hypovitaminosis D is a worldwide problem (Chapuy *et al*., 1997; Holick and Chen, 2008; Mithal *et al*., 2009), including tropical countries such as Thailand (Chailurkit, Kruavit and Rajatanavin, 2011; Dawson-Hughes *et al*., 2010; Lips *et al*., 2006; Rojroongwasinkul *et al*., 2013). Lim *et al.* (2008) and Dawson-Hughes *et al.* (2010) reported that approximately 50% of postmenopausal women in Thailand and Malaysia had vitamin D insufficiency. Chailurkit *et al.* (2011) stated that vitamin D insufficiency is common and varies across geographical regions in Thailand, including 64.6% of people in the capital city, Bangkok. Rojroongwasinkul *et al.* (2013) reported the prevalence of vitamin D insufficiency in Thailand ranged from 27.7% to 45.6% among children aged 0.5 - 12.9 years.

Risk factors of vitamin D deficiency include lack of exposure to sunlight, wearing sunscreen or concealing clothes, dietary inadequacy, mal-absorption, or use of anticonvulsants (Holick *et al*., 2011; Teagarden, Meador and Loring, 2014). Obtaining vitamin D either from food or supplements could be one of the effective ways to improve vitamin D status. However, supplements may depend on individual conditions such as financial status and may not be easily applied to an entire population (Lamberg-Allardt, 2006). Very few foods naturally contain vitamin D (Calvo, Whiting and Barton, 2004; Cribb *et al*., 2014; Holick *et al*., 2011; National Institutes of Health, 2014) and the main

sources are fatty fish and liver, which are not typically consumed every day. Thus, fortifying foods that are commonly consumed by a whole population are important. (Black *et al*., 2012) showed that vitamin D food fortification is a potentially effective public health strategy that can increase circulating 25(OH)D concentration in community-dwelling adults. Milk is one of the food products commonly fortified with vitamin D. However, other dairy products such as cheese, butter, cream, yogurt, as well as ice cream are usually not fortified with vitamin D (Calvo *et al*., 2004; National Institutes of Health, 2014), which leads to public misconceptions that all milk products are a rich source of vitamin D (Calvo, 2000; Calvo *et al*., 2004). Trang *et al.* (1998) and Tripkovic *et al.* (2012) also indicated that supplementation with vitamin D3 could raise more efficiently serum calcidiol levels as compared to the effect of vitamin D2.

The use of both calcium and vitamin D supplements were more effective than using calcium alone without vitamin D (Beto, 2015; Kaushik *et al*., 2014). Dairy products fortified with vitamin D and calcium increased serum calcidiol level in children aged 9 – 12 years old (Neyestani *et al*., 2014), elderly women (Bonjour *et al*., 2013), and in pre- and postmenopausal women (Kruger *et al*., 2015), as compared to unfortified milk products. A study by (Bonjour *et al*., 2012) also revealed that vitamin D and calcium-fortified soft white cheese lowered bone resorption biomarker TRAP 5b in postmenopausal women. Calcium-fortified ice cream was an effective system to deliver

calcium to the human body since the absorption of calcium from ice cream was reported to be as high as from milk (van der Hee *et al.*, 2009).

1.2 Objectives

Due to the fact that ice cream is one of the milk products currently consumed worldwide, the objectives of this study were to determine the effect of calcium and vitamin D fortification and to develop ice cream as a functional food for resolving inadequate intakes in many regions of the world at every age.

1.3 Scope

Five ice cream formulations including regular fat (10%), reduced fat (7.5%), light fat (5%), low fat (2.5%), and fat free (< 0.625%) were prepared with addition of 500 mg calcium carbonate (equivalent to 200 mg ionic calcium) and 200 IU vitamin D3 per 80 g serving. Physical, microbiological, and vitamin D3 quantification were determined through 28-day storage at -20 $^{\circ}$C.

CHAPTER II

LITERATURE REVIEW

2.1 Calcium and Vitamin D

2.1.1 Calcium

Major role of calcium in human body is bone homeostasis. Other functions are involved in intracellular fluid (mitochondria, nucleus, endoplasmic reticulum and vesicle) and extracellular fluid (blood, lymph and body fluids) such as blood clotting mechanism, nerve conduction, muscle contraction, enzyme regulation and membrane permeability (Gropper, Smith and Groff, 2005). Sources of calcium include milk, dairy products (e.g. cheese, yogurt), seafood (e.g. salmon, sardine with bone, oyster), legumes and dried fruits. Vegetables such as kale, celery, collard, Chinese cabbage, soybean sprouts, ivy gourd, and winged bean young pod are also high in calcium (Charoenkiatkul *et al*., 2008; Kamchan *et al*., 2004). In addition, calcium can be obtained from calcium-fortified foods and supplements. There are many calcium supplement products available in the market. Each product contains different salts that affect levels of calcium element as shown in Table 1.

Table 1 Percentage of ionic calcium from different calcium salts

Calcium salt	%Ionic calcium
Calcium glubionate	6.5
Calcium gluconate	9
Calcium lactate	12
Calcium citrate	21
Calcium acetate	25
Calcium carbonate	40

Source: De-Regil LM *et al*., 2013

2.1.2 Vitamin D

Vitamin D is an oil-soluble vitamin that functions in human bone mineralization. Other functions of vitamin D include cell differentiation, cell proliferation, cell growth and activating calcium and phosphorus absorption and gene expression. Vitamin D in a form of calcitriol can act as a hormone in many tissues (Gropper *et al*., 2005). There are two major forms of vitamin D; ergocalciferol (vitamin D2) and cholecaciferol (vitamin D3) (Figure 1). Ergocalciferol is produced in plants while cholecalciferol is found in animal products such as liver, beef, egg, some oily fish (herring, salmon, tuna, and sardine) (Romagnoli *et al*., 2013). In some countries such as United States, some foods are

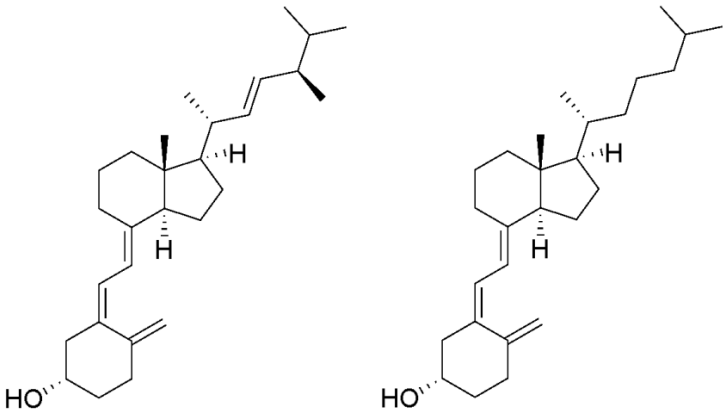

Figure 1 Structure of vitamin D2 and vitamin D3.

fortified with vitamin D, e.g., milk, margarine, bread, cereals. Cholecalciferol can also form in the skin when exposed to sunlight. Generally, both vitamin D3 and vitamin D2 are not stable in air, heat, and light. Moisture can cause oxidation of vitamin D (The Merck Index, 2006).

2.1.3 Calcium and vitamin D metabolism

Vitamin D and calcium play important roles in human bone homeostasis. Bone mineralization occurs when vitamin D and calcium levels in the body are sufficient. Vitamin D induces calcium binding proteins, enhancing calcium and phosphate absorption in small intestine. Long term vitamin D deficiency will lead to osteoporosis in adult and osteopenia in childhood (Lips and van Schoor, 2011).

In human skin, 7-dehydrocholesterol changes to previtamin D3 when exposed to ultraviolet radiation of 290 – 315 nm (Lips *et al*., 2006). Then, previtamin D3 will be thermally isomerised to cholecalciferol within 2 - 3 days (Gropper *et al*., 2005). Pathway of vitamin D metabolism is shown in Figure 2. Vitamin D transports in association with α-2 globulin vitamin D-binding protein (DBP) or transcalciferin. About 60% of plasma cholecalciferol binds to DBP and transports to liver and other organs such as muscle and adipose tissue. Vitamin D from food is absorbed via passive diffusion into the intestinal cells, which is the most rapid absorption occuring in duodenum. Vitamin D is then incorporated with chylomicron and transported to lymphatic and blood system. Chylomicron remnant binds to cholecalciferol and transports to the liver. In the liver, vitamin D is hydroxylated by hydroxylase enzyme to 25-hydroxy vitamin D (calcidiol). Another hydroxylation occurs in the kidney where calcidiol is converted by 1-hydroxylase enzyme to 1,25-dihydroxy vitamin D (calcitriol), the active form of vitamin D. Calcitriol will bind to DBP and deliver to the target tissues such as intestinal, brain, kidney, bone, cardiac, muscles, pancreas, skin, hematopoietic and immune system via the bloodstream (Gropper *et al*., 2005).

Calcitriol induces calcium and phosphate absorption in the intestine. This hormone stimulates nuclear receptors that important for luminal calcium channels gene expression. Calcitriol binds with enterocyte receptor, then transports to the nucleus and interact with genes involved in calcium transporting system leading to DNA

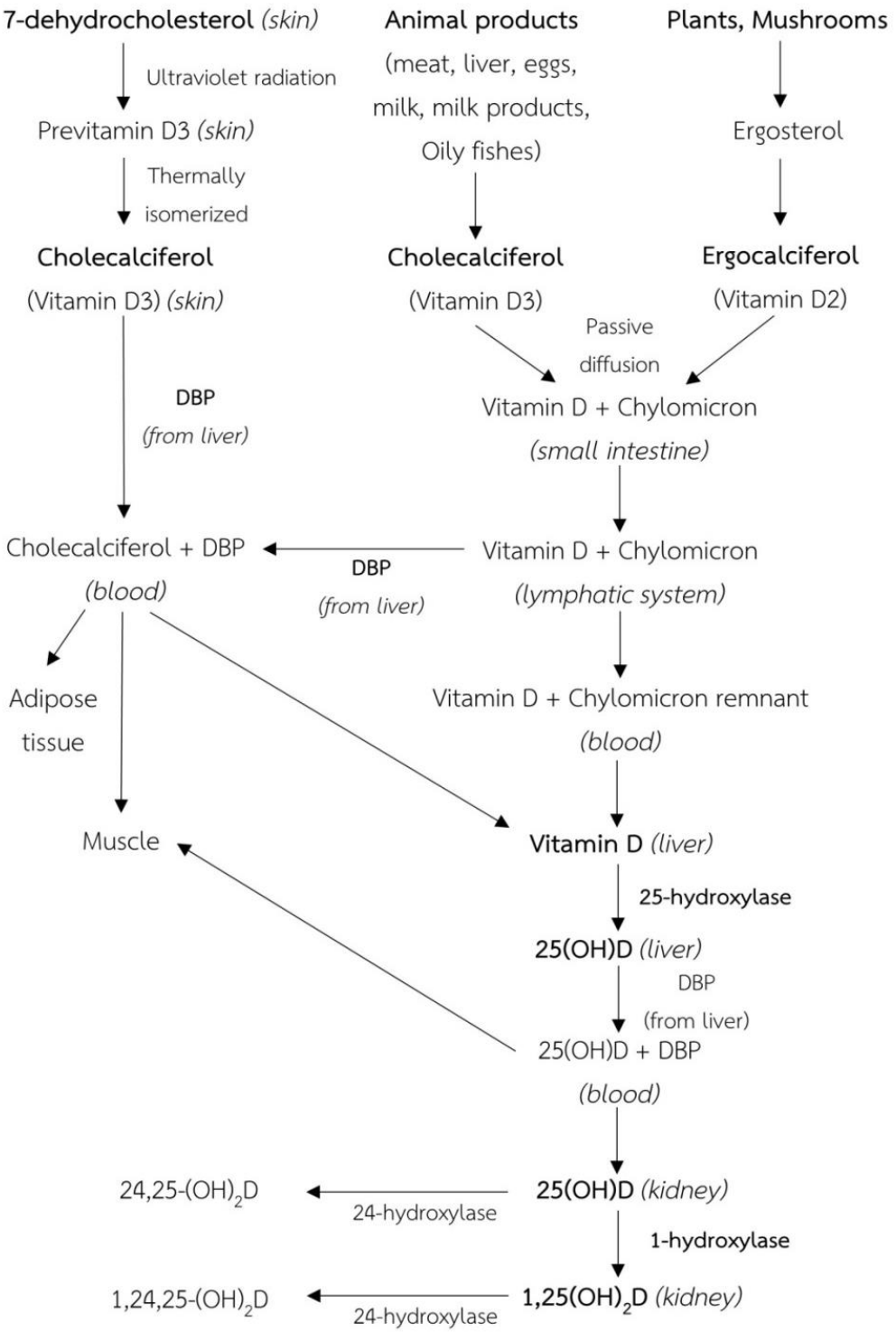

Figure 2 Vitamin D metabolism.

transcription of calcium absorption proteins. Low plasma calcium level raises parathyroid hormone (PTH) secretion, which stimulates bone desorption to maintain normal calcium level. PTH also activates 1-hydroxylase in the kidney to convert 25-dihydroxyvitamin D to 1,25-dihydroxyvitamin D. In case of sufficient calcitriol, 1-hydroxylase activity will be minimized, and 24-hydroxylase (enzyme from kidney, cartilage and intestine) activity will be increased in order to convert 25-hydroxy vitamin D to 24,25-dihydroxy vitamin D. Figure 3 illustrated roles of vitamin D in calcium homeostasis.

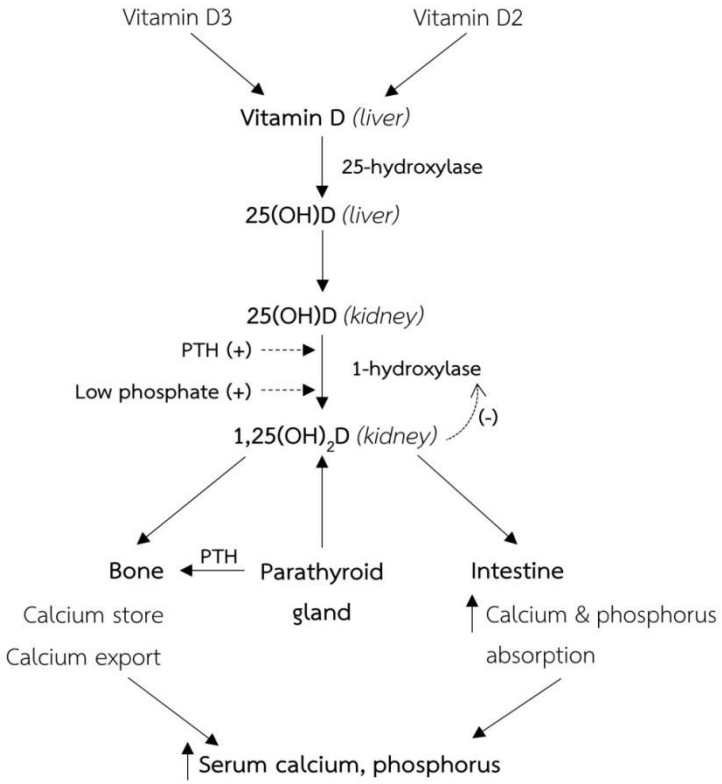

Figure 3 Vitamin D metabolism and regulation for calcium homeostasis.

2.1.4 Calcium and vitamin D requirements

The dietary requirements of calcium and vitamin D slightly vary around the world. Recommended daily intake (RDI) of calcium for children 1 - 3 and 9 - 18 years old are 700 mg/day and 1,300 mg/day, respectively (Institute of Medicine, 2012). RDI of calcium for children age 4 - 8 years and adults 19 - 50 years, pregnant and lactating women and over 70 years old men are 1,000 mg/day. Ross *et al.* (2011) suggested that women age between 50 and 70 years old should take at least 1,200 mg of ionic calcium per day, which is the same amount as adult age over 70 years (both men and women). In addition, (Beto, 2015) revealed that getting enough calcium from foods is more effective than from the supplements.

Microgram of vitamin D can convert to international unit (IU) by multiply with 40. The Institute of Medicine (IOM) committee's 2011 recommends that vitamin D requirements in children age 0 - 1 year are 10 µg/day (400 IU/day), children age over 1 year to adults age 70 years are 15 µg/day (600 IU/day), and adults age > 70 years old are 20 µg/day (800 IU/day). The US Endocrine Society suggests that children age 1 - 17 years old need 10 - 25 µg/day (400 - 1,000 IU/day), and adults age over 18 years need 37.5 – 50 µg/day (1,500 – 2,000 IU/day) of Vitamin D (Romagnoli *et al*., 2013). Gropper *et al.* (2005) also stated that an adequate intake (AI) of vitamin D was 5 µg/day or 200 IU/day for infant age over 6 months, children, adolescents, adults 19 - 50 years

old, pregnant and lactating women. Adequate intakes of elderly age 50 - 70 and above 70 years old are 10 µg/day (400 IU/day) and 15 µg/day (600 IU/day), respectively.

Benefits of calcium supplementation without vitamin D are questionable. Supplementation of calcium along with vitamin D was reported to be more effective than using calcium alone (Barice and Hennekens, 2015; Beto, 2015; Kaushik *et al*., 2014). However, excessive vitamin D intake for long term may cause hypervitaminosis D, which could lead to hypercalcemia and renal damage (Hanley *et al*., 2010).

2.1.5 Prevalence of calcium deficiency

Calcium deficiency is an important global nutrition problem, which can cause osteoporosis in adults. Calcium imbalance also causes calcium paradox disease. When intracellular calcium loads occurs in the vascular cell, it may lead to cardiovascular disease, neurodegenerative disease, colon cancer, degenerative joint disease and nephrolithiasis (Fujita, 2000). Kumssa *et al.* (2015) reported that prevalence of calcium deficiency around the world was 76% in 1992 and decreased to 51% in 2011. These numbers were associated with the average calcium intakes in 1992 that were 547 ± 230 mg/day, and went up to 684 ± 211 mg/day in 2011. Risk of calcium deficiency in Africa, Asia, America, Europe and Oceania were reported 80%, 57%, 29%, 11% and 11%, respectively (Kumssa *et al.*, 2015).

People who are at risk of calcium deficiency include postmenopausal women, female athlete triad, amenorrhea women, people who are milk allergy or lactose intolerance, and low dietary consumption especially in adolescent and elderly (Beto, 2015). The study of the National Health and Nutrition Examination Survey in 2003 - 2006 in the United States reported that calcium intakes of more than one-third of people age 31 - 50 years and two-third of people age 51 years and over were less than the AIs level (Mangano *et al.*, 2011). Children living in Europe (Belgium, Denmark, France, Germany, Netherlands, Poland, Spain, and United Kingdom) age 10 - 17 years have an average calcium intake lower than the reference standard (Kaganov *et al.*, 2015).

In 2011, the South East Asian Nutrition Survey (SEANUTS) investigated the amount of calcium intake per day of children living in urban and rural areas of Thailand. Both children groups were reported lower calcium intakes than the reference standard. Children age 0.5 - 2.9, 3.0 - 5.9, 6.0 - 12.9 years in urban area consumed 593 ± 8.6, 602 ± 8.5 and 602 ± 8.5 mg calcium per day while kids in the rural obtained 541 ± 8.7, 527 ± 26.5 and 352 ± 20.9 mg/day, respectively. These data indicated that calcium intake of children age 3 - 12.9 years is not sufficient (Rojroongwasinkul *et al.*, 2013). Sornsuvit *et al.* (2011) revealed that University students in Khon Kaen, Thailand, had an average calcium intake 477.4 ± 261.9 mg/day (calculated from 3-day dietary record). Pongchaiyakul *et al.* (2004) reported that women age 60 - 97 years who lived in Bangkok and Khon Kaen had average calcium intake of 309.5 ± 147.2 mg/day and

235.7 ± 188.0 mg/day, respectively. Pongchaiyakul *et al.* (2008) also pointed out that calcium intakes of women age 20 - 85 years in Khon Kaen were 265 mg/day, which only 2.8% met the recommended level.

2.1.6 Prevalence of vitamin D deficiency

Serum level of calcidiol is an indicator for vitamin D deficiency due to the fact that blood stream is the largest storage site of 25-hydroxy vitamin D. Half-life of calcidiol is between 10 days to 3 weeks. Vitamin D deficiency in infants and children may lead to rickets; the mineralization of the bone before epiphyseal closure. Epiphyseal cartilage still grows without bone matrix and mineral replacement. The deformities of wrists, knees, ankles, pelvic and thoracic can be seen such as bow legs, knock knees and rachitic rosary (Gropper *et al*., 2005). Osteoporosis is a result of vitamin D deficiency in adults.

Prevalence of vitamin D deficiency were reported in many countries. The study of Lips *et al.* (2006) revealed that postmenopausal women with osteoporosis in Europe, middle East, Asia, Latin America and Australia had an average serum calcidiol 26.8 ± 13.2 ng/mL. The prevalence of vitamin D deficiency (serum calcidiol < 30 ng/ml or 75 nmol/L) in each continent was 57.7%, 81.8%, 71.4%, 53.4% and 60.3%, respectively. Lim *et al.* (2008) stated that 71% of postmenopausal women in Eastern Asia, 92% in South Korea, 90% in Japan, 47% in Malaysia and 47% in Thailand were at vitamin D

inadequacy state. The study pointed out that consumption of low-vitamin D foods was one of the main factors that caused vitamin D insufficiency in Taiwan, Hong Kong, Japan, South Korea and China. The researchers also indicated that no enough sunlight exposure perhaps caused vitamin D insufficiency in Taiwan, Hong Kong, Japan, South Korea, and Thailand, especially in elderly. The study by the National Health and Nutrition Examination Survey (NHANES) in 2003 - 2006 (Bailey *et al.*, 2010) reported that only 24% of males and 22% of females in the United States had adequate vitamin D consumption. Moreover, elderly was mentioned to have more risk of vitamin D insufficiency than people in lower ages.

In Thailand, (Soontrapa, Soontrapa and Chailurkit, 2002) revealed that 66.3% of women in Khon Kaen were at vitamin D insufficiency state (serum calcidiol equal or lesser than 35 ng/mL or 87.5 nmol/L) with an average serum calcidiol 33.24 ng/ml (83.10 nmol/L). Chailurkit *et al.* (2011) pointed out that the prevalence of vitamin D insufficiency (serum calcidiol equal or lesser than 28 ng/mL or 70 nmol/L) of women in Bangkok was 31.8% with the average serum calcidiol 27.04 ng/mL (67.6 nmol/L), which was greater in elderly women who lived in the nursing home. In addition, Kruavit *et al.* (2012) also indicated that there were 61.3% of women with vitamin D insufficiency at the nursing home in Bangkok (serum calcidiol equal or lesser than 28 ng/mL or 70 nmol/L; data were collected in winter season). Rojroongwasinkul *et al.* (2013) reported that the

incidence of vitamin D deficiency (serum calcidiol < 20 ng/ml or 50 nmol/L) in children (< 13 years old) was 45.6% in urban area and 27.7% in rural area of Thailand.

Low exposure to sunlight, low intake of vitamin D-containing foods and poor skin activity against UV radiation could be major causes of vitamin D deficiency. Low exposure to sunlight could be influenced by using sunscreen, less outside activities, living in less sunlight area or living in long winter season area, wearing clothes over vast skin area (Lim *et al.*, 2008).

Nowadays, several criteria are used to identify vitamin D deficiency. Chailurkit *et al.* (2011) stated that serum levels of calcidiol were correlated with parathyroid hormone levels. Optimum level of serum calcidiol suggested by Chailurkit *et al.* (2011) was 70 nmol/L. However, the IOM (2011) advise that serum level of calcidiol above 20 ng/ml (50 nmol/L) is an optimum level for bone health. The US Endocrine Society (ES) announced that less than 20 ng/mL (50 nmol/L) of serum calcidiol is a cutoff point of vitamin D deficiency, 21 – 29 ng/mL (52.5 – 72.5 nmol/L) is vitamin insufficiency, and > 30 ng/ml (> 75 nmol/L) is an optimal level for vitamin D in human body (Romagnoli *et al.*, 2013).

2.2 Calcium- and Vitamin D- Fortified Foods

Food fortified with calcium and vitamin D can increase consumption of these two nutrients. There are many types of calcium-fortified foods available in the market such

as juice, cereal, cooking flour and soy milk. Milk is generally considered as calcium-rich food, but calcium content is lower in other dairy products such as frozen yogurt and ice cream (National Osteoporosis Foundation, 2015). Calcium-fortified foods are popular in European countries where (Rizzoli *et al.*, 2013) reported that foods fortified with calcium increased 35 – 40% of calcium intake compared to unfortified diets. Johnson-Down *et al.* (2003) pointed out that consumption of fortified foods contained 55 mg and 165 - 275 mg elemental calcium/serving raised 54 mg and 280 - 1,019 mg/day, respectively, of calcium intake in women. The study of Zhao *et al.* (2005) revealed that bioavailability of calcium from calcium carbonate fortified in soymilk that available in the United States market was as high as calcium bioavailability from cow milk, and higher than bioavailability of calcium from tricalcium phosphate fortification. Heaney *et al.* (2000) stated that calcium absorption from soymilk fortified with tricalcium phosphate was 75% compared to calcium absorption from milk.

Milk is typically fortified with vitamin D in the United States, Canada and European countries to increase calcium bioavailability. Other food products commonly fortified with vitamin D include infant foods, cereal, cake, biscuits and fat spreads. Infant formulas are required to add vitamin D in the U.S. and Canada. Non-fat and whole fat dry milk, evaporated milk, fluid milk and margarine in Canada also have to fortify with vitamin D margarine (Calvo and Whiting, 2013). Margarine, infant formulas, energy-restricted diets are allowed to fortify with vitamin D in the United Kingdom (Spiro

and Buttriss, 2014). In Australia, 1 µg/100 mL and 3.5 µg/100 g of vitamin D is typically added in milk and breakfast cereals, respectively. Jayaratne *et al.* (2013) reported that about 50% of vitamin D intake in Australian adults came from food intake whereas 28% of vitamin D consumption in the women came from vitamin D-fortified margarine. Nevertheless, there is still little available information about food fortified with both calcium and vitamin D together.

2.3 Ice Cream

The U.S. Food and Drug Administration (US21CFR135.110, 2015) described that "Ice cream is a food produced by freezing, while stirring, a pasteurized mix consisting of one or more of the optional dairy ingredients" (Department of health and human services, 2013). Ice cream may contain casein or hydrolyzed milk protein or other suitable non-milk derived ingredients. Only natural fat from ice cream ingredients are allowed. Total solids in the ice cream must not less than pounds per gallon (191.7 g/L). Either milk fat or milk solids nonfat must not less than 10%. However, proportion of milk fat and milk solids nonfat could be changed if the ice cream contains > 10% fat content (Department of health and human services, 2013).

Marshall (2003) classified ice creams by fat content into five types as followed; regular fat or standard ice cream (> 10% fat), reduced-fat ice cream (< 7.5% fat), light ice cream (< 5% fat), low-fat ice cream (< 3.75% fat) and nonfat ice cream (< 0.625% fat).

According to the regulation of ice cream by ice cream by The Ministry of Public Health, Thailand 2002 (Regulation no.222), ice cream is categorized into five different types including milk ice cream, modified ice cream, mixed ice cream, liquid or dry/powdered ice cream and water ice (ice cream made from water and sugar). Milk ice cream must contain at least 5% of milk fat and 7.5% of MSNF by weight. Amounts of each ingredient have to display on the label in percentage. Ice cream must contain no preservative. In Thailand, total bacterial counts should not exceed 600,000 CFU/g and no *Escherichia coli* allowed in 0.01 g of ice cream, whereas standards for aerobic plate counts and coliform count are 20,000 CFU/g and 10 CFU/g, respectively in the United States (Marshall, 2003).

In 2012, the value of ice cream manufacturer in Thailand increased up to 85,552 tons, which was 2-fold increase compared with 2002 (Office of Industrial Economics, 2013). The average consumption rate of ice cream in Thailand was about 1.7 liter per capita per year (Kasikorn Research Center, 2013) while 12.9 pounds per person was reported in the United States (United States Department of Agriculture, 2014).

2.3.1 Ice cream ingredients

2.3.1.1 Fat

Fats provide body and affect creaminess and melting behavior of the ice cream (Dairy processing handbook, 1995). Either milk fat such as cream and butter oil or

vegetable oil, e.g. palm kernel oil and flax seed oil, can be used. Vegetable fat may alter color and odor of the ice cream and is not allowed to use in some countries (นรินทร์ ทองศิริ, 2528).

2.3.1.2 Milk solids nonfat (MSNF)

Milk solid nonfat (MSNF) is solid matter left in the milk products after milk fat is separated. MSNF consists of casein micelles, whey protein, lactose, minerals and vitamins, leading to high nutrition quality. Proteins in MSNF function as emulsification, whipping and water-holding capacity (Goff, 2011). MSNF improves ice cream texture by binding to water molecules and acts as emulsifier. Other function is to disperse air bubbles in ice cream, making good texture and creaminess (Dairy processing handbook, 1995). Ice cream mix with 10 – 12% milk fat contains 11% of MSNF.

2.3.1.3 Sugar

Sugar is a sweetener and it is used to adjust the total solid amounts of the ice cream. Sugar decreases the freezing point and controls temperature-hardness relationship of the ice cream mix. Sugar content of ice cream ranges from 10 – 18%. Sugar used in the ice cream industries includes cane sugar, glucose, glucose syrup, lactose and invert sugar (นรินทร์ ทองศิริ, 2528). Glucose syrup or starch hydrolysate syrup were reported to increase smoothness, improve meltdown characteristic as well as lower incidence of heat shock of ice cream (Goff, 2011). Artificial sweeteners such

as sorbitol, aspartame, glycerol, mannitol may be used. Bulking agent, i.e. maltodextrin, is typically added together with the non-caloric sweetener (Dairy processing handbook, 1995).

2.3.1.4 Emulsifier

Emulsifier makes bubbles incorporates in ice cream better, resulting in the smoother texture. Emulsifier also makes ice cream melt-resistant. Mechanism of action of emulsifier in the ice cream is to decrease surface tension of liquid, thus reducing size making partial coalescence of fat globules (Dairy processing handbook, 1995; Goff, 2011). Two types of emulsifiers are wildly used in the ice cream industries; 1) mono- and di-glycerides and 2) sorbitan esters (e.g. polysorbate 80). Other emulsifiers such as egg yolks, glycerine ester and sugar ester may be used. In general, amounts of emulsifier used in the ice cream is 0.3 - 0.5% by weight (นรินทร์ ทองศิริ, 2528).

2.3.1.5 Stabiliser

Stabiliser binds with water in ice cream mix and making the mix more viscous. Stabiliser also increases smoothness of ice cream body by controlling ice crystal and lactose crystal growth during storage, reducing heat shock and controlling the melting time of ice cream (Dairy processing handbook, 1995; Goff, 2011). Most stabilisers are polysaccharides such as guar gum, locust bean gum, carboxymethyl cellulose, xanthan gum (Goff, 2011).

Emulsifier and stabiliser may be integrated together, e.g. Palsgaard® ExtruIce (Denmark), which composts of mono- and di-glycerides of fatty acids, guar gum, cellulose, carrageenan and antioxidant (See Palsgaard® ExtruIce 278 data sheet in Appendix A).

2.3.1.6 Flavour and colour

Flavouring and colouring agents are associated with customer preference. Addition of flavour and colour may be done after pasteurisation (นรินทร์ ทองศิริ, 2528)

2.3.2 Production of ice cream

Production of the ice cream begins with preparation of the ingredients. The manufacturing process can be divided into two stages; 1) mixing stage and 2) freezing stage. The mixing stage starts with blending ice cream ingredients, followed by pasteurization, homogenisation and ageing. The freezing stage includes whipping, hardening and storage (Goff, 2011).

2.3.2.1 Preparation of ice cream ingredients

Dry and liquid ingredients need to be mixed separately. Dry ingredients are prepared by weighing in the bag. Wet ingredients are prepared in suitable container and storage condition, i.e. keeping fresh milk at < 5 ^{o}C. Pre-heat is required for some

ingredients such as sweet condense milk, vegetable oil and butter, for better condition in blending step (Dairy processing handbook, 1995; Goff, 2011).

2.3.2.2 Blending

Liquid ingredients, e.g. milk, cream, syrup and condense milk, are mixed at temperature about 50 – 60 °C to increase solubility (Dairy processing handbook, 1995). Dry ingredients are thoroughly mixed, e.g. MSNF, egg yolk powder, sugar and stabiliser, and add in liquid components before temperature reach 50 °C. Flavouring and colouring agents should be added last step (วรรณา ตั้งเจริญชัย และวิบูลย์ศักดิ์ กาวิละ, 2531).

2.3.2.3 Pasteurisation

Pasteurisation is heat treatment to destroy pathogenic bacteria. Pasteurisation also increases solubility of some ice cream ingredients, for example, proteins and stabilisers. There are two pasteurising methods as described by Goff (2011) that are batch method (using temperature at 68.5 °C for at least 30 minutes) and continuous method (using temperature at 80 °C for at least 25 seconds).

The regulation no.222 (2002) by the Ministry of Public Health, Thailand, stated that all ice creams (except ice cream powder) have to be pasteurized by heating the ice cream to at least 68.5 °C for 30 min or 80 °C for minimum 25 sec, and cooled down immediately to 4 °C.

2.3.2.4 Homogenisation

Homogenisation is performed by passing ice cream mix through small holes. This process reduces the size of fat globules to 1 - 2 μm, thus increasing fat globules surface area and making ice cream mix more uniformity (Goff, 2011). Homogenisation also protects ice cream from separation, helps making better ice cream texture (วรรณา ตั้งเจริญชัย และวิบูลย์ศักดิ์ กาวิละ, 2531), as well as enhances whipping ability of the mix (Dairy processing handbook, 1995). Pressure used in homogenising process may be set at two steps; the first step at 15.5 - 18.9 MPa then lowered to 3.4 MPa in the second step. The purpose of using second step homogenisation is to increase more time for surface adsorption (Goff, 2011).

2.3.2.5 Ageing

Ice cream is cooled down immediately after homogenisation, then age at 2 – 5 ^{o}C for at least 4 hours. Continuous stirring is needed in ageing process. Ageing is a process to let milk protein and stabiliser get trap with water. Fat globule will crystallise and membrane rearrangement will occur. Benefits of this step are the smoothness of the ice cream and high whippability of the mix (Goff, 2011).

2.3.2.6 Whipping

Whipping is a process to incorporate air into the mix and stir until ice cream becomes frozen. Temperature used is between -3 to -6 ^{o}C depends on type of the ice

cream. Thai regulation no.222 (2002) indicated that whipping process should perform at the temperature less than -2.2 °C. Whipping period affects ice cream quality. The less time the better texture because size of ice crystals will become smaller (Dairy processing handbook, 1995). Generally, whipping process is finished when ice cream texture is thick enough. Air cell, ice crystal, fat globule, milk protein, lactose crystal, stabiliser and sucrose will disperse in the liquid phase and the mix will become 3-phase system that consists of liquid, air and solid phases (Dairy processing handbook, 1995). Other ingredients such as dried fruits, nuts, candies and cookies should be added at the last step before ice cream is frozen. Ice cream products should be kept in suitable container before freezing (วรรณา ตั้งเจริญชัย และวิบูลย์ศักดิ์ กาวิละ, 2531).

2.3.2.7 Hardening

After whipping step, ice cream will become soft in texture. Hardening is the process that makes ice cream firm by lowers the temperature to -20 °C (วรรณา ตั้งเจริญชัย และวิบูลย์ศักดิ์ กาวิละ, 2531). This step is needed to be done instantly to prevent sandiness (caused by lactose crystallisation) during ice cream storage (นรินทร์ ทองศิริ, 2528).

2.3.2.8 Storage

Thailand regulation no. 222 (2002) suggested that the suitable storage temperature for the ice cream is 20 °C or lower. วรรณา ตั้งเจริญชัย และวิบูลศักดิ์ กาวิละ (2531) recommended that the freezer should be set at between -23 to -18 °C. Shelf-life depends on type of the ice cream. In general, shelf life of ice cream is about 0 – 9 months (Dairy processing handbook, 1995).

CHAPTER III

MATERIALS AND METHODS

3.1 Study Design

Five ice cream formulations including regular fat (10 %; RF), reduced fat (7.5%; RDF), light (5%; L), low fat (2.5%; LF), and fat free (< 0.625%; FF) ice creams were designed (*n* = 3/treatment). A 200 mg elemental calcium and 200 IU vitamin D3 per serving were fortified in each treatment. Ice creams were stored at -20 ^{o}C throughout the study. Proximate analyses of each sample were evaluated. Overrun was analyzed on day 0. Other physical parameters consisted of hardness, melting rates and viscosity were measured on day 0, 7, 14, and 28. Microbiological properties (aerobic plate counts and coliform counts) as well as vitamin D3 retention were also determined on 0, 7, 14, and 28 days of storage.

3.2 Ice Cream Production

Compositions and energy of each ice cream formulation were shown in Table 3. All samples consisted of non-fat milk, skim milk powder, fresh whipping cream, sugar, imitation vanilla flavor (Durkee®, Iowa, USA) and a mixture of emulsifier and stabiliser (Palsgaard® extruIce 278, Denmark). Inulin (Cosucra®, Belgium) was used as a fat replacement in low fat and fat free treatments. Ice creams were fortified with 500 mg

calcium carbonate (VWR international, Belgium) and 5 µg cholecalciferol (100,000 IU Vitamin D3/g; DSM Nutritional Products Inc., Singapore) per serving (serving size = 80 g).

For ice cream production, all ingredients (except cholecalciferol) were mixed together and pasteurized at 68.5 ^{o}C for 30 min. After cooling to 15 ^{o}C, cholecalciferol was added into the mix. Next, homogenisation was performed by Ultra-turrax T25 homogenizer (Janke & Kunkel IKA Labortechnik, Germany) at 20,500 min^{-1} for 15 min. Ice cream mixes were aged at 4 ^{o}C for 4 h before processing by a tabletop ice cream maker (Grace KA-608, China). Ice creams were then hardened at -20 ^{o}C for at least 12 h before the analyses.

3.3 Proximate Analysis

Proximate compositions of ice creams including crude protein, crude fat and ash were analyzed in this study according to AOAC official methods 2012 (The Association of Official Analytical Chemists, 2012). Protein content was determined using Kjeldahl method (Method 930.33). Fat content was measured by Rose-Gottlieb method (Method 952.06). Ash content was determined by official method 945.46. Moisture content was calculated from moisture loss at oven drying step during total solids examination by gravimetric analysis according to AOAC method 941.08 for ice cream and frozen dessert. Carbohydrate was calculated as described by Food and Agricultural

Organization (FAO, 2003). Calories of each ice cream formulation were also calculated using factors of 4, 4 and 9 calories per gram protein, carbohydrate and fat, respectively. See Appendix B for more details.

3.4 Physical Measurements

3.4.1 Overrun

Overrun is a measure of air incorporation of ice creams. Overrun of the ice creams were calculated using the following equation (Whelan *et al.*, 2008).

$$\text{Overrun} = \frac{\text{Weight of mix} - \text{Weight of ice cream}}{\text{Weight of ice cream}} \times 100$$

where weight of mix and weight ice cream are in the same volume.

3.4.2 Hardness

Hardness of ice cream samples was determined according to Whelan *et al.* (2008), using a TA-XT2i texture analyzer (Stable Micro Systems Ltd., UK) with P/30C cone Perspex probe. The highest force was recorded as the probe penetrated into the ice cream to a depth of 20 mm at a speed of 2 mm/s.

3.4.3 Melting rate

Melting rates of the ice creams were examined according to Whelan *et al.* (2008), with slightly modification. One cup (80 g) of ice cream was placed on a mesh at

room temperature (21 ± 1 °C). Weight of the drip was recorded every 10 min until ice cream entirely melted down. The highest slope from a plot between time and weight of the drip was used to determine the melting rate of the ice cream.

3.4.4 Viscosity

Ice cream viscosity was analyzed according to (Aime *et al.*, 2001). Samples were brought to 4 °C for 4 h before measurements. Eight millilitres of melted ice cream were transferred to a cup to measure the viscosity using a Bohlin C-VOR 105 viscometer (Worcestershire, UK) at shear rate 0.01 – 150 sec^{-1}. The viscosity was read at a shear rate of 11.1 sec^{-1} (T = 25 °C).

3.5 Vitamin D3 Retention

Retention of cholecalciferol was evaluated by method as described by (Kazmi, Vieth and Rousseau, 2007). Saponification with 60% KOH was performed before extraction by high-performance liquid chromatography (Shimadzu LC-10A, Shimadzu, Kyoto, Japan) using C18 column (Water Spherisorb ODS2 10 µm 4.6 x 250 mm, Ireland). Methanol: acetonitrile: water (47.5: 47.5: 5) was used as a mobile phase. The sample injection volume was 50 µl. A flow rate of 1.0 mL/min was maintained throughout the test period (5 min, T = 26 °C). The elution of vitamin D3 was detected at 254 and 228 nm on an ultraviolet detector at 2.7 min.

3.6 Microbiological Analysis

Ice cream samples were quantified for aerobic plate counts (APC) and *E. coli*/coliform counts using 3M™ Petrifilm (Minnesota, USA) according to AOAC Official Method 989.10 (The Association of Official Analytical Chemists, 2012). See Appendix B for more details.

3.7 Statistical Analysis

Statistical Analysis Software version 9.0 (SAS Institute Inc., NC, USA) was used for analysis of variance (ANOVA) to identify differences among ice cream treatments at the 95% confidence level ($P < 0.05$). Completely randomized design with *proc glm* function was used for proximate analysis and overrun experiments. Repeated measures design with *proc mixed* function, using Tukey adjustment to obtain differences of least means squares, was used for other physical analyses, microbial measurements and vitamin D3 retention of the samples. Mean values and standard error of mean from triplicate analysis were reported.

CHAPTER IV

RESULTS

4.1 Proximate composition

Five ice cream formulations were analysed for the amounts of fat, ash, protein, carbohydrate and moisture as shown in Table 2. Ash contents were not significantly different among the groups, but were higher than non-calcium fortified ice cream ($P < 0.05$; average 1.21% for calcium fortified ice creams versus 0.74% for control) (Figure 4). There was a higher protein content in L, LF and FF than RF and RDF ice creams ($P < 0.05$). Calories of each ice cream formulation were also calculated using factors of 4, 4 and 9 calories per gram protein, carbohydrate and fat, respectively. The energy value of 80 g ice cream varied with the ingredients of the mix, and ranged from 82 – 145 kcal/serving as shown in Table 3.

Table 2 The proximal composition of ice creams

	RF	RDF	L	LF	FF
%Protein	2.79 ± 0.04[a]	2.84 ± 0.02[a]	2.98 ± 0.06[b]	2.92 ± 0.03[b]	2.90 ± 0.05[b]
%Fat	9.78 ± 0.48[e]	7.08 ± 0.28[d]	4.72 ± 0.17[c]	2.44 ± 0.10[b]	0.61 ± 0.03[a]
%Ash	1.20 ± 0.14[b]	1.21 ± 0.04[b]	1.20 ± 0.12[b]	1.22 ± 0.08[b]	1.25 ± 0.07[b]
%Moisture	65.53 ± 0.28[a]	67.77 ± 0.34[b]	70.27 ± 0.10[c]	71.78 ± 0.36[d]	73.73 ± 0.19[e]

RF = Regular fat, RDF = Reduced fat, L = Light, LF = Low fat, FF = Fat free

Table 3 Ice cream mix formulations and energy content

Ingredients	Ice cream formulations				
	RF	RDF	L	LF	FF
Fat (%)	10.02	7.50	5.00	2.50	0.61
MSNF (%)	11.00	11.00	11.00	11.00	11.00
Sugar (%)	12.00	12.00	12.00	8.00	8.00
Inulin (%)	0.00	0.00	0.00	5.00	5.00
Emulsifying/stabilizing agent (%)	0.50	0.50	0.60	0.60	0.60
Vanilla flavour (%)	2.00	2.00	2.00	2.00	2.00
Ionic calcium (mg/serving)	200.00	200.00	200.00	200.00	200.00
Vitamin D3 (IU/serving)	200.00	200.00	200.00	200.00	200.00
Energy (kcal/serving)	145.00	128.00	110.00	96.00	82.00

RF = Regular fat, RDF = Reduced fat, L = Light, LF = Low fat, FF = Fat free;

MSNF = Milk solids non fat

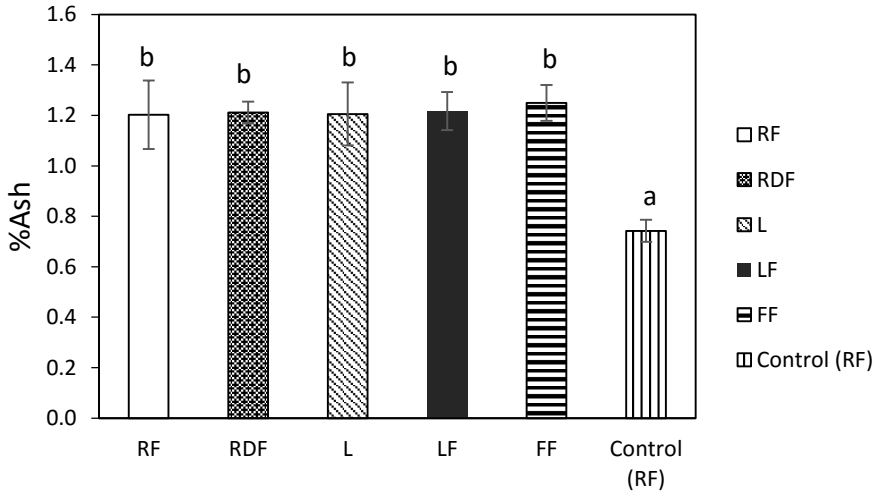

Figure 4 Ash contents in ice cream.

4.2 Properties of ice cream

Overrun of L and FF ice creams was greater than other formulations ($P < 0.05$; Table 4). Hardness, melting rate and viscosity of the samples were illustrated in Figure 5A-C. There was no significant difference in hardness between the groups on day 0. However, FF and LF ice creams had the highest hardness on day 7 and day 28, respectively (Figure 5A; $P < 0.05$). Statistics showed that only the hardness of LF treatment changed over time. Hardness of LF ice cream on day 28 was significantly higher than day 0 and 7. Melting rate of RF was faster than FF and L samples on day 0 and 7, respectively (Figure 5B; $P < 0.05$). No significant difference was found between the treatments on day 14 and 28, and the melting rates of all ice creams were not changed by storage time ($P > 0.05$). Viscosity of each ice cream treatment was not

changed over time. Nevertheless, the results showed that LF and FF ice cream tended to have lower viscosity than other groups through 28-day storage (Figure 5C). All ice cream formulas exhibited pseudoplastic behavior as shown in (Figure 6).

Microbial examination of the ice creams is shown in Figure 7. APC values were expected to be low because the ice cream mix was pasteurized before proceeding with ice cream processing. APCs in this study were lower than 40 CFU/g throughout 28 days for every ice cream treatments. There was no *E. coli* or coliform found in a 0.01 g of ice cream in every samples.

Table 4 Overrun (measure of air incorporation) of ice creams (%)

RF	RDF	Light	LF	FF
32.70 ± 1.62^{a}	30.49 ± 2.93^{a}	55.73 ± 1.02^{b}	23.61 ± 3.28^{a}	49.18 ± 1.87^{b}

RF = Regular fat (10%), RDF = Reduced fat (7.5%), L = Light (5%), LF = Low fat (2.5%), FF = Fat free (< 0.625%). Data is shown as mean ± SEM. Means not sharing a common superscript letter within the same row (group) are significantly different ($P < 0.05$).

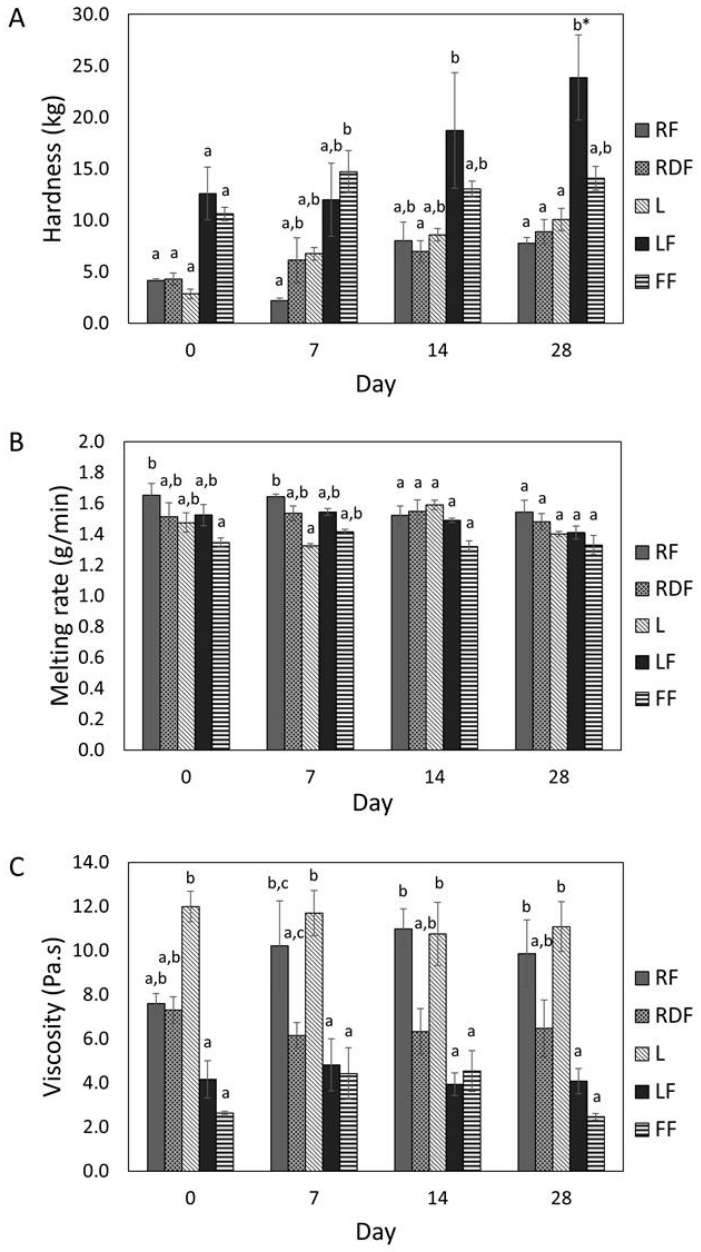

Figure 5 Hardness (A), melting rate (B), viscosity (C) of ice creams on 0, 7, 14 and 28 days of storage at -20 °C. RF = Regular fat (10%), RDF = Reduced fat (7.5%), L = Light (5%), LF = Low fat (2.5%), FF = Fat free (< 0.625%). Data is shown as mean ± SEM. Means not sharing a common superscript letter within the *same* day are significantly different ($P < 0.05$). An asterisk (*) indicates a significant difference from day 0.

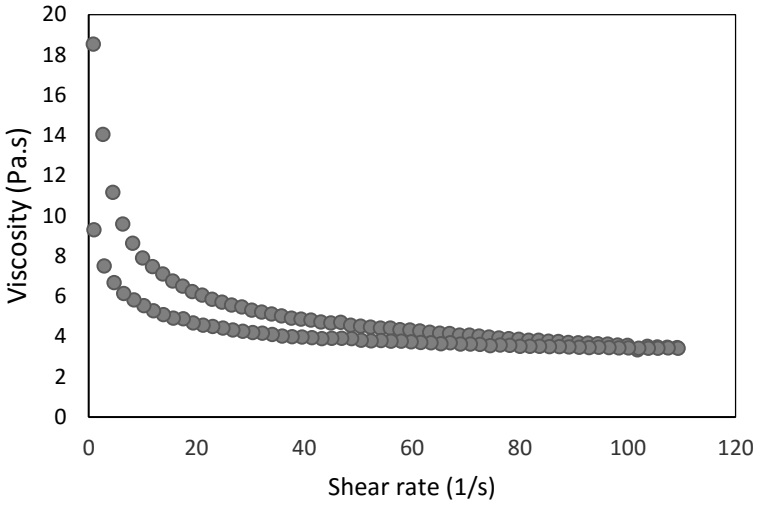

Figure 6 Effect of the shear rate on the viscosity of regular fat formula on day 0.

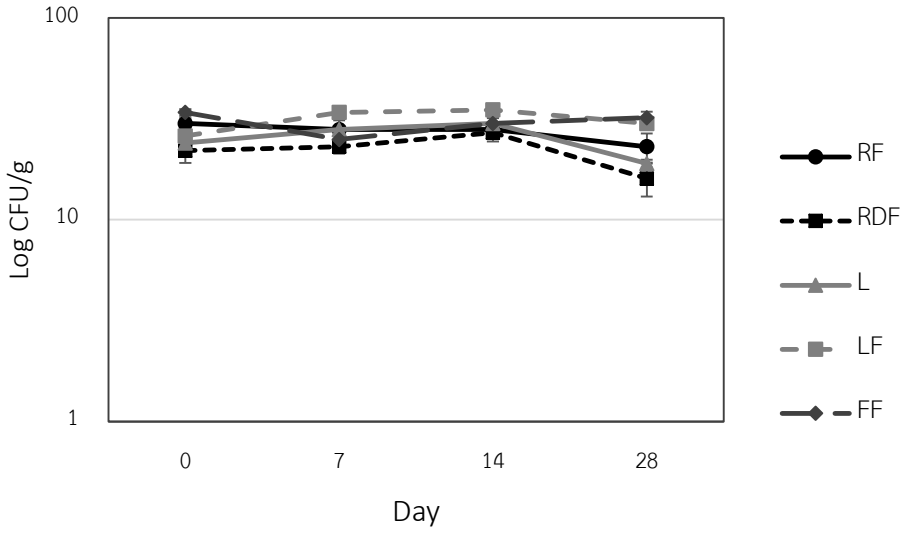

Figure 7 Aerobic plate counts of ice creams on 0, 7, 14 and 28 days of storage at -20 °C. RF = Regular fat (10%), RDF = Reduced fat (7.5%), L = Light (5%), LF = Low fat (2.5%), FF = Fat free (< 0.625%). Error bars represent standard error of the mean.

4.3 Cholecalciferol retention

Retention of cholecalciferol in the ice creams is presented in Figure 8. Vitamin D3 in RF and LF samples was found to be lower on day 7, compared to day 0 ($P < 0.05$). However, it was elevated again on day 14. Vitamin D3 content in RDF and FF ice creams tended to go down during storage, but the change was not statistically significant. L sample showed the highest cholecalciferol level on day 7.

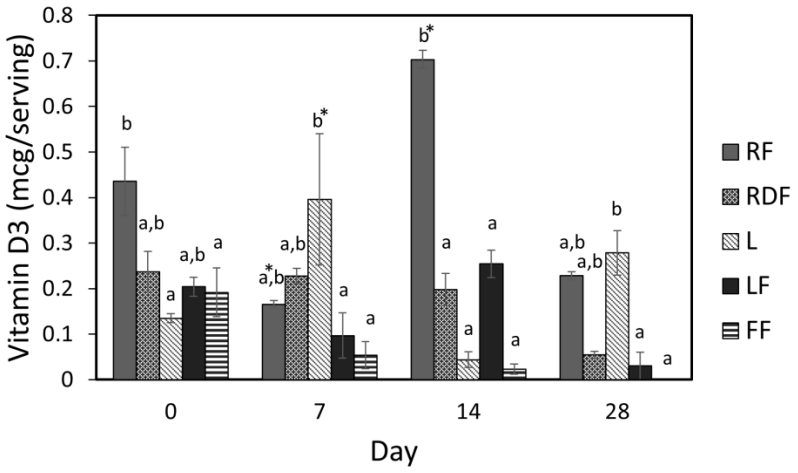

Figure 8 Vitamin D3 retention in ice creams on 0, 7, 14 and 28 days of storage at -20 °C. RF = Regular fat (10%), RDF = Reduced fat (7.5%), L = Light (5%), LF = Low fat (2.5%), FF = Fat free (< 0.625%). Data is shown as mean ± SEM. Means not sharing a common superscript letter within the same day are significantly different ($P < 0.05$). An asterisk (*) indicates a significant difference from day 0.

CHAPTER V

DISCUSSION

Ice cream can be characterized as a multiphase food product consisting of air bubbles and ice crystals surrounded with the aqueous phase of the mix (sugar, protein, salts, polysaccharides and water), intermixed with a network of fat in a partial coalescence state (Biasutti *et al*., 2013; Goff and Hartel, 2013). The study of Biasutti *et al*. (2013) indicated that factors affecting the overrun (air incorporation) of ice cream were ingredients of the mix and the manufacturing process including extrusion and homogenisation steps. Conventional homogenisation was found to make high overrun ice cream while high pressure homogenisation produced the lower overrun (Biasutti *et al*., 2013). Results from this study showed that overrun of FF and L ice creams were higher than RF, RDF and LF groups, which could be explained by the higher ratio of non-fat dry milk / milk fat in RF and RDF formulas. There was the same level of milk solids nonfat (MSNF) in all treatments, which was 11% in all formulations. It seems that higher fat levels decreased air entrapment. Air bubbles and fat droplets will both be coated and emulsified with milk proteins after mixing. If there is high fat, then some protein will emulsify the fat, and there will be less protein to stabilize the air bubbles. Thus, there will be fewer stable air bubbles in high fat samples.

All ice cream formulations presented pseudoplastic behaviours, which were similar to the previous study on rheological properties of ice creams by Aime *et al*. (2001). Their study revealed that ice cream viscosity decreased as shear rate increased. Viscosity of ice cream can be influenced by the mixing process, homogenisation pressure, aging time and ice cream composition (Goff and Hartel, 2013). Higher levels of stabiliser, protein, corn syrup solid, fat and total solid can increase ice cream viscosity as well (Goff and Hartel, 2013). The results from this study showed that RF and RDF treatments, which tended to have higher viscosity than LF and FF, had significantly lower overrun than the FF ice cream. Inulin is a carbohydrate-based fat replacer that functions as a bulking agent. It is resistant to hydrolysis in the stomach and intestine, and can be used as a prebiotic (Aykan, Sezgin and Guzel-Seydim, 2008). Inulin makes ice cream chewy and protects ice cream against heat shock (Schaller-Povolny and Smith, 1999). Inulin interacts with the aqueous phase of the ice cream and thus lowers the concentration of free water, therefore, thickening the mix (El-Nagar *et al.,* 2002) and slowing the melting rate of ice cream (Akalın and Eri**Ş**ir, 2008). High viscosity ice cream can incorporate less air than samples with less viscosity (Biasutti *et al*., 2013), resulting in low overrun (Goff and Hartel, 2013).

Several studies reported that ice cream with high viscosity had slow melting rate (Biasutti *et al.,* 2013; El-Nagar *et al*., 2002; Goff and Hartel, 2013). Granger *et al*. (2005) found that melting time is related to ice cream ingredients, especially type of emulsifier.

Other factors influencing ice cream melting behaviour are the interactions among the ingredients such as fat, air, protein and the polymer network (Granger *et al., 2005)*. Meltdown characteristics are also associated with fat and non-fat milk solids content (El-Nagar *et al.,* 2002). Nevertheless, the melting rate and viscosity of all ice cream formulations in this study were stable through 28 days storage at -20 ^{o}C.

All of the ice cream formulations in this study did not show significant differences in hardness, except the LF formula. Hardness of LF treatment on day 28 was significantly higher than day 0 and 7. Moreover, hardness of LF and FF tended to be higher than RF and RDF ice creams on day 0. This finding is in agreement with the report by El-Nagar *et al*. (2002) that reviewed the inverse relationship between hardness and freezing point, as well as sugar content and total solids in the mix, overrun, and the type and quantity of stabiliser added into the ice cream.

Amounts of $CaCO_3$ and cholecalciferol in the ice cream were 500 mg and 5 µg per 80 g serving, respectively, which equals 200 mg ionic calcium and 200 IU vitamin D3 per serving (80 g). Proximate analysis was performed to confirm the retention of calcium in the products after 28-day storage. Ash, including added calcium, was greater in all fortified ice cream treatments than in the control (unfortified ice cream).

Cholecalciferol is a fat-soluble vitamin that is not stable in light, heat and air. In this study, cholecalciferol was added into the mix after pasteurization but before homogenisation, when temperature was 15 ^{o}C, to minimize heat degradation. However,

the amount of vitamin D3 in ice cream on day 0, 7, 14 and 28 were inconsistent and did not show any predictive trend. In contrast to the study by Kazmi *et al*. (2007), which reported that cholecalciferol in butter oil was stable through 28-day storage, vitamin D3 in FF ice cream was not detected on day 28 of this study. It appears that cholecalciferol was lost in the low fat system in this study. The results could be influenced by the crystallisation of milk fat, which is a vehicle for fat-soluble vitamins that could affect the solubility of cholecalciferol (Breitschuh and Windhab, 1998). Melting temperature of milk fat is in the range between -40 to 40 ^{o}C. (Wiking *et al*., 2009) found that anhydrous milk fat could crystallise at temperatures below 20 ^{o}C. Another factor that probably affected the solubility of cholecalciferol was non-uniform dispersion of vitamin D3 in the cold mix. Thus, fortification method may also impact on the retention of vitamin D3 in ice cream.

Every ice cream product in this research met the standard in microbiological testing, which indicated that the manufacturing process was clean and no contamination occurred during processing and storage. No *E.coli* were detected, and APCs were less than 40 CFU/g throughout the study. Aerobic plate count is an indicator of the bacterial level in the sample and processing environment, while coliform count indicates the contamination after pasteurization or efficacy of the pasteurization process. Goff and Hartel (2013) suggested that APCs and coliform counts should not be greater than 20,000 and 10 CFU/g, respectively. According to the regulation for ice cream by the

ministry of public health, Thailand (regulation no. 222, 2002), APC must not be greater than 600,000 CFU/g and a 0.01 g ice cream sample must not contain any *E.coli.*

CHAPTER VI

CONCLUSIONS

The development of calcium vitamin D3 fortified ice cream could provide an alternative consumer choice for additional dietary calcium and vitamin D, especially for those who are at high risk of calcium and vitamin D deficiency. Ice cream physical properties including overrun, viscosity, hardness and melting rate were slightly different among the treatments, but microbiological counts were not affected by fat content or by calcium and vitamin D3 fortification during 28-day storage at -20 $^{\circ}$C. Ice cream of all fat levels (< 0.625 – 10% fat) could be fortified with calcium, without calcium loss during 28 day frozen storage. However, unconventional methods of vitamin D fortification such as encapsulation pre-treatment may be needed to improve vitamin D stability in ice cream, particularly in low-fat and fat-free formulas.

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APPENDIX

APPENDIX A

PALSGAARD® EXTRUICE 278 DATA SHEET

Palsgaard®
Heart working people

Palsgaard® Extrulce 278 Product Profile

Product Type: Palsgaard® Extrulce 278 is an integrated mixture of emulsifier and stabilizers.

Application Areas: Palsgaard® Extrulce 278 is developed for use in extruded ice cream and soft ice, but may also be used for standard ice cream and milk ice.

Functional Properties: Palsgaard® Extrulce 278 should be added to the mix while stirring continuously.

Addition may take place at any temperature, but complete dissolution of the product is only obtained when heated to 65°C.

Palsgaard® Extrulce 278 provides the following advantages:

* Facilitates the incorporation of air into the mix giving a high and stable overrun.
* Prevents the formation of coarse ice crystals giving a creamy and uniform texture.
* Provides a creamy and refreshing texture to the ice cream
* Provides dryness on extrusion and excellent stand-up and meltdown properties
* Protects the ice cream against heat shock damages when exposed to fluctuating temperatures during distribution and storage.

Dosage: The dosage of Palsgaard® Extrulce 278 depends on the composition of the mix and the viscosity required. Generally we recommend the following levels:

Fat content in the mix/recommended dosage:

4%	6%	8%	10%	12%
0.65%	0.60%	0.55%	0.50%	0.45%

Palsgaard®
Heart working people

Palsgaard® Extrulce 278 Data sheet

Identification
005978

Product type
Integrated mixture of emulsifier and stabilizers.

Declaration
Mono- and diglycerides of fatty acids E 471
Guar gum E 412
Cellulose gum E 466
Carrageenan E 407 (standardised with dextrose or sucrose)

Antioxidant, containing:
Tocopherol-rich extract E 306, max. 100 ppm
Ascorbyl palmitate E 304, max. 20 ppm

Physical/chemical data

Appearance	Off-white beads
Fat, approx. %	68
Lipid source	Vegetable fat

Microbiological data

Total plate count, max. cfu/g	5000
Yeasts, max. cfu/g	500
Moulds, max. cfu/g	500
Enterobacteriaceae, cfu/g	<100
Staphylococcus aureus, cfu/g	<1
Salmonella, in 25 g	Absent
E. coli, cfu/g	<1

Heavy metals

Arsenic (As), max. ppm	3
Lead (Pb), max. ppm	5
Mercury (Hg), max. ppm	1
Cadmium (Cd), max. ppm	2

Nutritional values

Nutritional values per 100 g (calculated values)	
Energy, kJ/kcal	2700/650
Protein, g	1.5
Carbohydrate, g	<1
Dietary fibre, g	24
Fat, g	68
Sodium, g	0.9

Packaging
20 kg net in multiply paper bag with an inner polyethylene bag.

Batch coding
Seven digits = DDMMY##

Palsgaard®
Heart working people

Palsgaard® Extrulce 278 Data sheet

Shelf-life and storage

Minimum 24 months from date of production when stored cool and dry in unopened packing.

GMO status

According to regulations Nos. 1829/2003/EC and 1830/2003/EC this product does not contain or consist of GMOs and is not produced from or contain ingredients produced from GMOs.

This non-GMO statement is based on information from raw material suppliers.

Allergens

According to directive 2000/13/EC including all amendments the below table indicates the presence (as added component) of the following allergens and products thereof:

No	Cereals containing gluten
No	Crustaceans
No	Eggs
No	Fish
No	Peanuts
No	Soybeans
No	Milk
No	Nuts
No	Celery
No	Mustard
No	Sesame seeds
No	Sulphur dioxide and sulphites (>10mg/kg)
No	Lupin
No	Molluscs

Identity and purity

The product is made from carefully selected raw materials. The food additives are in full conformity with the requirements for purity and identity laid down in EU Council Directives and the "FAO Food and Nutrition Papers".

Legal status

Due to the fact that legislation on application of this product in foodstuffs may vary from country to country, the local food law should always be examined.

Country of origin

EU

APPENDIX B

AOAC OFFICIAL METHODS

FOR PROXIMATE COMPOSITION AND MICROBIAL ANALYSES

Protein content of ice creams (AOAC 2012, Method 930.33)

Protein content of the ice cream was determined using Kjeldahl method. A 4.0 mL of ice cream sample was pipetted into beaker and weighed. Samples were digested in 25 mL concentrated sulfuric acid with Kjeltabs® catalyst (Cu/3.5, Foss analytical A/S, USA) at 370 – 410 °C until the solution became clear. Forty mL of NaOH and 50 mL of 2% H_3BO_3 solutions were added to the digested. Samples were then distilled in a close system. After that, samples were titrated with sulfuric acid (0.1 N) using modified methyl red as an indicator. A conversion factor of 6.38 was used for the calculation.

Fat content of ice creams (AOAC 2012, Method 952.06)

Crude fat content was measured using Rose-Gottlieb method. Five mL of ice cream sample was accurately weighed and transferred to a Rohlig tube. Sample was diluted with H_2O (1:10 dilution) and 2 mL of NH_4OH was added. The solution was thoroughly mixed and heated in a water baht at 60 °C for 20 min. Ten mL of 95% ethyl alcohol was added to a cool mix. Fat was extracted with diethyl ether and petroleum

ether. After the extraction, ether layer was evaporated. The residue was heated in the oven at 100 °C for at least 30 min, cooled in a desiccator and weighed.

Ash content of ice creams (AOAC 2012, Method 945.46)

Five grams of ice cream sample was accurately weighed in a porcelain crucible. Sample was heated on hot plate until well charred and stopped smoking. After that, sample was ashed in a muffle furnace at 550 °C until ash became white (C-free). Ash was cooled in desiccator and weighed. %Ash was calculated.

Moisture content of ice creams (AOAC 2012, Method 941.08)

Moisture content was determined from moisture loss at oven drying step during total solids examination. A 2 mL of ice cream sample was accurately weighed. Sample was heated at 100 °C for 3.5 h. Sample was then cooled in a desiccator and quickly weighed for total solids. %Moisture was calculated as the following equation.

$$\%\text{Moisture} = \frac{\text{Weight of sample} - \text{Weight of total solid}}{\text{Weight of sample}} \times 100$$

Aerobic plate counts and *E. coli*/coliform counts of ice cream (AOAC 2012, Method 989.10)

Ice cream samples were quantified for aerobic plate counts (APC) and *E. coli*/coliform counts using 3M™ Petrifilm (Minnesota, USA). Serial dilutions were prepared in Butterfield's phosphate buffer. For aerobic plate counts, 1 mL was inoculated onto the plate and incubated at 32 ± 1 ^{o}C for 48 ± 3 h. Colonies were counted in countable range of 25 – 250 colony/plate. For *E. coli*/coliform counts, the incubation condition was 35 ± 1 ^{o}C for 24 ± 2 h with a countable range of 15 – 150 colonies.

VITA

Miss Weeraya Chansathirapanich was born in March 19, 1984 in Songkhla Province, Thailand. She received a Bachelor of Pharmacy (with honors) from the Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Thailand in 2006. After graduation, she has been working as a pharmacist at Naradhiwasrajanagarindra Hospital, Naradhiwas, Thailand. Her responsibilities include drug dispensing, drug information services and chemotherapy preparation.