INVESTIGATION OF VITAMIN D_3 CONTENT IN FORTIFIED FLUID MILK AND THE STABILITY OF VITAMIN D_3 IN MILK TO LIGHT EXPOSURE

by

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Abstract

Fortified milk is the main dietary source of vitamin D, an important nutrient for skeletal health and reducing the risk of some chronic diseases. However, non-compliance of vitamin D fortification of fluid milk has been reported as a recurring problem. The purpose of this research is to analyze vitamin D contents of fortified fluid milk sold in Vancouver, the stability of commercial vitamin D formulations in milk upon light exposure during refrigerated storage, and the thermal stability of vitamin D_2 and D_3.

Vitamin D content was determined for 104 fluid milk products purchased from the Vancouver retail market from October 2011 to September 2012. Fortification non-compliance was defined as vitamin D levels outside the range of 35.2-46.9 IU/100 mL, corresponding to regulatory requirements stated by the Canadian Food Inspection Agency. The results showed 54% under-fortification and 4% over-fortification with significant variation in vitamin D content among milk from different brands, with different fat content and sampled at different times. In particular, higher incidence of under-fortification was observed in skim milk samples.

To investigate the stability of vitamin D_3 to light exposure, skim milk was fortified with one of four vitamin D_3 formulations along with vitamin A: crystalline vitamin D_3, two water dispersible formulations and an emulsified vitamin A/D premix. Vitamin D loss after 22 days of storage at 4 ℃ with exposure to light of 2000 lux intensity ranged from 37% to 71% depending on the different formulation, and was accompanied by vitamin A loss.

To investigate the effects of pH and heat on stability, vitamin D_2 and D_3 in citrate-phosphate buffer at pH 3.5 and 6.6 were heated at 72 ℃ for 15 seconds. Less than 8%

vitamin D loss was observed. No significant difference was found in thermal stability between vitamin D_2 and D_3 or between the pH conditions during heating.

These results indicate the possible approach to improve vitamin D fortification compliance in fluid milk by choosing a vitamin D formulation with improved dispersibility and greater stability against light exposure. Further research is needed to investigate other potential factors affecting the vitamin D content in fortified milk.

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List of Abbreviations

1, 25$(OH)_2$D: 1α,25-Dihydroxyvitamin D

25(OH)D: 25-Hydroxyvitamin D

AD premix: Emulsified mixture of vitamin D_3 and vitamin A palmitate from Kingsway Chocolate

AOAC: Association of Official Analytical Chemists

BHT: Butylated Hydroxytoluene

CFIA: Canadian Food Inspection Agency

Crystalline D_3: Food grade crystalline vitamin D_3 from DSM nutrition

CWS D_3: Dry vitamin D_3 100 CWS/AM from DSM nutrition

FTIR: Fourier-Transform Infrared

HPLC: High Performance Liquid Chromatography

HTST: High Temperature Short Time

IOM: Institute of Medicine

IU: International Unit

RDA: Recommended Dietary Allowance

SDS D_3: Dry vitamin D_3 100 SD/S from DSM nutrition

SPE: Solid Phase Extraction

UHT: Ultra High Temperature

UVB: Ultraviolet B

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Dedication

This thesis is dedicated to my parents and my husband

for their unconditional love and support

As well as my dear friend Isabelle Lacroix for her precious friendship

Chapter 1: Introduction and literature review

1.1 Background

The importance of vitamin D has increased in the past few decades as emerging studies show the sunshine vitamin provides many other health benefits besides the well-known contribution to skeletal health. The deficiency of vitamin D may raise the risks of some chronic diseases such as type II diabetes, autoimmune disorders, cardiovascular disease, and certain types of cancer (Holick, 2004; Vatanparast et al., 2010; IOM, 2011).

Vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) are two natural forms of vitamin D. Vitamin D_2 is obtained from irradiation of plants or yeasts. Vitamin D_3 is naturally occurring in some foods. In the human body, vitamin D_3 can be synthesized in skin via exposure to sunlight. Skin pigment, latitude, seasons, clothing, amount of skin exposure and the use of sunscreen cosmetics are main factors affecting the endogenous vitamin D level (Calvo & Whiting, 2003; IOM, 2011). The concern of skin cancer is a reason to avoid long exposure to sunlight.

The prevalence of vitamin D insufficiency and deficiency has long been a worldwide concern, especially in high risk groups such as young children, pregnant women and elderly people. These problems have been observed even in sun drenched areas, such as southern Europe, the Middle East, India, Africa and Australia (Mithal *et al.*, 2009; Lips, 2010).

The prevalence of vitamin D insufficiency in Canada, especially in dark skinned people living in northern latitudes has been reported (Cole *et al.*, 2001; Rucker *et al.*, 2002). In North America, food fortification and dietary supplements are the main approaches to ensure adequate vitamin D intake (Calvo *et al.*, 2004). However, the study

of Whiting *et al.* (2007) showed that despite the fortification, the vitamin D intakes in Canada are not adequate to prevent vitamin D insufficiency. Non-compliance of vitamin D fortification that has been reported in several studies (e.g., Holick *et al.*, 1992; Blank *et al.*, 1995; Murphy *et al.*, 2001) might be one of the reasons behind the vitamin D insufficiency, since consumers might access less or more vitamin D than what is being claimed on the labels of fortified foods. These previous studies did not indicate what caused the fortification non-compliance and whether or not the vitamin stability played a role in these cases. To understand the possible reasons of vitamin D non-compliance, it is important to investigate the content as well as stability of vitamin D in fluid milk.

1.2 Literature review

1.2.1 General introduction of vitamin D

Vitamin D refers to a group of secosteroid compounds with antirachitic activity. Cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) are two natural forms of vitamin D. In the human body, upon UVB irradiation, the 7-dehydrocholesterol in skin is first converted to previtamin D_3 in which the vitamin precursor B-ring is open, then subsequently isomerized to vitamin D_3. Similarly, vitamin D_2 is formed via the conversion of ergosterol to previtamin D_2 by UVB irradiation; this process mainly occurs in certain fungi or yeasts. The molecular structures of vitamins D_2 and D_3 are shown in Figure 1.1.

The research of vitamin D has a long history. According to Norman & Henry (2007), Hirsch and Palm first associated the disease rickets with the lack of sunlight near the end of 19^{th} century. In 1922, McCollum discovered a compound in cod-liver oil with antirachitic activity and named it vitamin D (Norman & Henry, 2007).

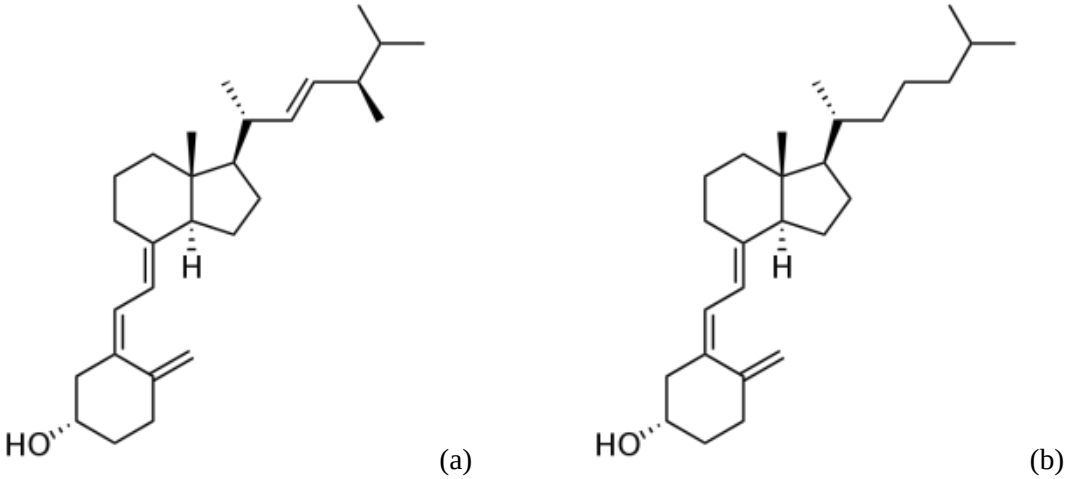


Figure 1.1 Molecular structures of vitamin D_2 (a) and vitamin D_3 (b).

The figure for vitamin D_3 is adapted from Cholecalciferol in Wikipedia (retrieved March 19, 2011, from http://en.wikipedia.org/wiki/Cholecalciferol, Copyright Public Domain). The figure for vitamin D_2 is adapted from Ergocalciferol in Wikipedia (retrieved March 19, 2011, from http://en.wikipedia.org/wiki/Ergocalciferol, Copyright Public Domain).

Vitamin D modulates calcium and phosphorus absorption, which is necessary for the health of bones and teeth. Vitamin D had been considered as the active form for healthy bone development until the discovery of its metabolites, 25-hydroxyvitamin D (25(OH)D) and 1, 25-dihydroxyvitamin D (1, $25(OH)_2D$). After synthesis in skin or intake from diet, vitamin D is converted into 25(OH)D in the liver, and then further transformation to 1, $25(OH)_2D$ is completed in the kidney. The 25(OH)D is the main circulating metabolite while 1, $25(OH)_2D$ is the active metabolite of vitamin D (Norman & Henry, 2007).

At the early stage, scientists mainly focused on the function of vitamin D to develop healthy bones. Recent studies showed that the benefits of vitamin D are not limited to bone health. As a hormone, 1, $25(OH)_2D$ was also found in different tissues such as intestine, kidney, liver, lung, brain, breast, cancer cells, muscle, and ovary (Norman & Henry, 2007). It is believed to modulate the immune system, associate with neuropsychological development of infants and reduce the risk of diabetes, cardiovascular diseases, and certain cancers (Holick, 2004; Vatanparast *et al.*, 2010; IOM, 2011). However, excessive intake of vitamin D is dangerous. High dose of vitamin D may lead to weight loss, polyuria, heart arrhythmias, raised blood levels of calcium and result in vascular and tissue calcification, damage to the heart, blood vessels and kidneys (IOM, 2011).

Table 1.1 shows the recommended dietary allowance and upper tolerance of vitamin D, as revised by Health Canada in 2011. The vitamin D level in selected foods can be found in Table 1.2.

Table 1.1 Dietary reference intakes of vitamin D by Health Canada.

Age group	**Recommended dietary allowance per day (RDA)**	**Tolerable upper intake level per day**
Infants 0-6 months	400 IU (10 μg)	1000 IU (25 μg)
Infants 7-12 months	400 IU (10 μg)	1500 IU (38 μg)
Children 1-3 years	600 IU (15 μg)	2500 IU (63 μg)
Children 4-8 years	600 IU (15 μg)	3000 IU (75 μg)
Children and adults 9-70 years	600 IU (15 μg)	4000 IU (100 μg)
Adults > 70 years	800 IU (20 μg)	4000 IU (100 μg)
Pregnancy & lactation	600 IU (15 μg)	4000 IU (100 μg)

Source: Health Canada, 2011[a]

Table 1.2 Vitamin D content in selected foods.

	IU per serving	% RDA [a]
Natural foods [b]		
Cod liver oil, 1 tablespoon	1,360	227
Salmon (sockeye), cooked, 3 ounces	794	132
Mackerel, cooked, 3 ounces	388	65
Tuna fish, canned in water, drained, 3 ounces	154	26
Liver, beef, cooked, 3.5 ounces	46	8
Egg, 1 whole (vitamin D in yolk)	25	4
Fortified foods [c]		
Milk, vitamin D-fortified, 1 cup	90	15
Orange juice fortified with vitamin D, 1 cup	100	17
Yeast-leavened bakery products, 100 g	90	15
Margarine, fortified, 1 tablespoon	72	12
Infant formula, 100 kcal	100	25
Formulated liquid diets, 1,000 kcal (2,500 kcal/day)	800	133

[a] The RDA of 600 IU for people from 1 to 70 years old was used for the calculation of % RDA in all food items except infant formula, for which 400 IU was used.

[b] U.S. Department of Agriculture, Agricultural Research Service (2012).

[c] Food and Drug Regulations (2012) and Health Canada (2004, 2006 and 2011[b])

In Canada, vitamin D fortification is mandatory for fluid milk and milk powder, fluid goat milk and powder, margarine and other butter substitutes, infant formula and formulated liquid diet, foods used in very low energy diet, whole egg products and yolk products (Food and Drug Regulations, 2012). Health Canada (2006 & 2011b) also issued interim marketing authorizations for vitamin D addition to orange juice and yeast-leavened bakery products.

Both vitamin D_2 and D_3 can be used for food fortification. Published studies have not shown consistent conclusions on the potency of these two forms of vitamin D. Vitamin D_2 was initially recognized as being equivalent to vitamin D_3 although research revealed vitamin D_2 was significantly less potent than vitamin D_3 in some animals (Norman & Henry, 2007). The development of methods using serum 25(OH)D as a vitamin D biomarker provided an alternative to animal bioassays for the study of vitamin D potency. Trang *et al.* (1998) compared the ability of vitamin D_2 and D_3 to elevate serum 25(OH)D and reported that vitamin D_3 increased serum 25(OH)D 1.7 times as much as vitamin D_2 did at an intake level of 4,000 IU/day for two weeks. Armas *et al.* (2004) studied the effect of a single dose of 50,000 IU vitamin D_2 or vitamin D_3 on serum 25(OH)D over four weeks. They reported that the two forms maintained the serum 25(OH)D at a similar level during the first three days. However, the serum 25(OH)D dropped rapidly in the vitamin D_2 group and was close to the baseline at day 14 while the serum 25(OH)D content in the vitamin D_3 group reached a peak at that time. The vitamin D_2 group showed a serum 25(OH)D content even lower than the control group at the end of the trial. As a response to the conclusion of Trang *et al.* (1998) and Armas *et al.* (2004), Holick *et al.* (2008) claimed, based on their study, that there was no significant

difference between vitamin D_2 and D_3 to increase the circulating 25(OH)D. They divided the subjects into three groups to receive 1000 IU/day of either vitamin D_2 or vitamin D_3 or a combination for 11 weeks. By the end of the experiment, all of the subjects in the three groups showed an increase in serum 25(OH)D of 8-10 ng/mL. On the other hand, Heaney *et al.* (2011) provided more evidence in favour of vitamin D_3 being more potent by comparing circulating 25(OH)D and fat storage of the subjects who were given the two vitamin D forms.

Different methods and dosages were applied in these studies, which makes the comparison between studies difficult. The potency of vitamin D_2 and D_3 is still a controversial topic that remains unsettled, despite more evidence showing that vitamin D_3 might be more effective than vitamin D_2. More research needs to be conducted to establish the potency of vitamin D_2 versus D_3.

1.2.2 Vitamin D status of Canadians

The importance of vitamin D has increased since more and more research indicates that vitamin D provides multiple health benefits. In contrast to the increased awareness of the nutritional functions, low vitamin D status has long been a worldwide problem and needs to be addressed.

Currently, the level of serum 25(OH)D is widely accepted for use as a biomarker to evaluate the status of vitamin D in the human body since it represents both skin synthesis and dietary intake of vitamin D (Calvo & Whiting , 2003; Langlois *et al.*, 2010). However, various cut-offs of serum 25(OH)D level are being applied to define different vitamin D status in published studies. For example, serum 25(OH)D ranging from 27.5 nmol/L to 37.5 nmol/L have been used to define vitamin D deficiency based on

different health outcomes (Mithal *et al.*, 2009; Langlois *et al.*, 2010). The reported optimal level of serum 25(OH)D ranges from 40 nmol/L to 125 nmol/L in various studies (Rucker *et al.*, 2002; Whiting *et al.*, 2007; Lips, 2010; IOM, 2011). Recently, more studies agree that serum 25(OH)D level above 75 nmol/L is desirable for overall health (Mithal *et al.*, 2009; Lips, 2010).

There is no central organization that is responsible to establish the clinical standard of vitamin D deficiency, insufficiency and adequacy. In Canada, IOM (2011) suggests that adults with 25(OH)D levels below 30 nmol/L are considered at risk of poor bone health and that a concentration above 50 nmol/L is sufficient for most persons.

A national survey was conducted between 2007 to 2009 to obtain health information of Canadians (Langlois *et al.*, 2010). The cut-offs of 27.5 nmol/L and 37.5 nmol/L for vitamin D deficiency and insufficiency respectively, were applied in this survey. Considering these concentrations were lower than those suggested in the IOM 2011 report, the prevalence of vitamin D deficiency and insufficiency might be underestimated. According to the results of this survey, 4.1% of the population were deficient in vitamin D and the percentage insufficient was only 10%. However, if using 75 nmol/mL as the optimal vitamin D level cut-point, then only one-third of the population had sufficient 25(OH)D. The authors did not report the percentage of population having serum 25(OH)D less than the IOM insufficiency cut-off of 50 nmol/L.

Rucker *et al.* (2002) reported that more than one third of western Canadians have 25(OH)D less than 40 nmol/L in winter, spring and fall. Cole *et al.* (2001) indicated that more than 20% of young Canadian women have low vitamin D status (less than 40 nmol/L). Vitamin D status might be affected by many factors such as skin colour,

latitude, season, dietary and supplementary intake. In North America, fortified foods and supplements are the main sources of vitamin D during seasons with limited sunlight (Calvo *et al.*, 2004).

Based on the 2011 report of IOM, Health Canada (2011a) increased the recommended intake of vitamin D from 200 IU to 600 IU for people aged 1 to 70. Daily intake of 400 IU is considered adequate for infants while people over 70 need to consume 800 IU.

1.2.3 Vitamin D fortification in milk

1.2.3.1 Milk consumption and serum 25(OH)D

In Canada, fortification of milk with vitamin D is mandatory. As a result, milk is the main dietary vehicle of vitamin D for Canadians, followed in descending order by "meat and meat alternatives, other foods, grain products, vegetable and fruit" (Vatanparast *et al.*, 2010). Vitamin D intake via milk consumption was reported to contribute 49% of the total dietary vitamin D (Vatanparast *et al.*, 2010).

The 2007 to 2009 Canadian Health Measures survey (Langlois, 2010) indicated that consumption of milk was a significant factor contributing to the increase of serum 25(OH)D concentration (Figure 1.2). The mean serum 25(OH)D of people who consumed milk more than once per day was 75.0 nmol/L while those who drank milk less than once per day had mean serum level of 62.7 nmol/L.

The average serum 25(OH)D of different age groups are plotted against the percentage of that population group drinking milk more than once per day in Figure 1.3. The proportion of high frequency of milk consumption declined with increasing age to

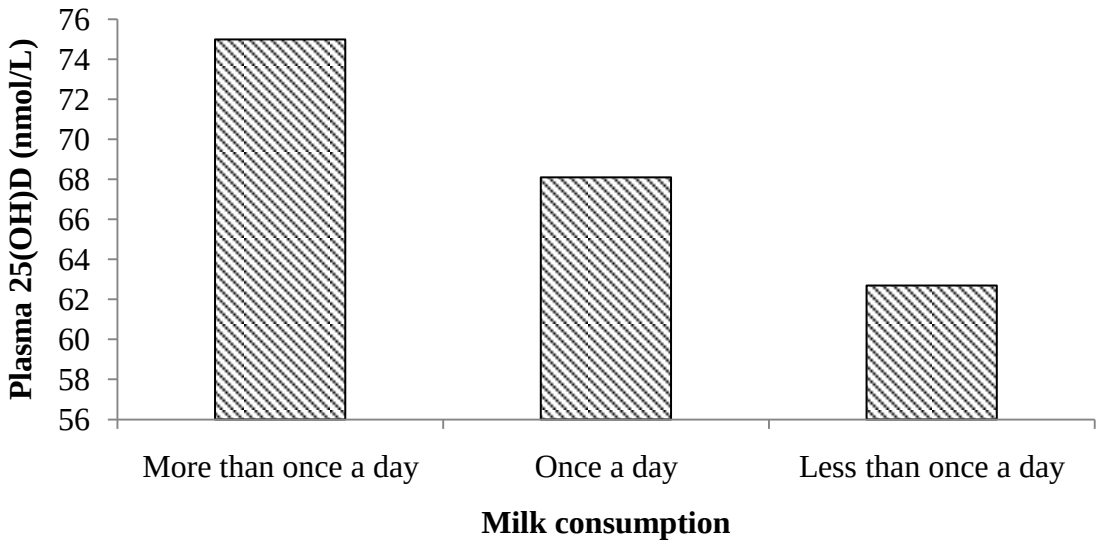


Figure 1.2 The impact of milk consumption on serum 25(OH)D of Canadians.

Based on data of national survey conducted between 2007 and 2009 (Langlois *et al.*, 2010).

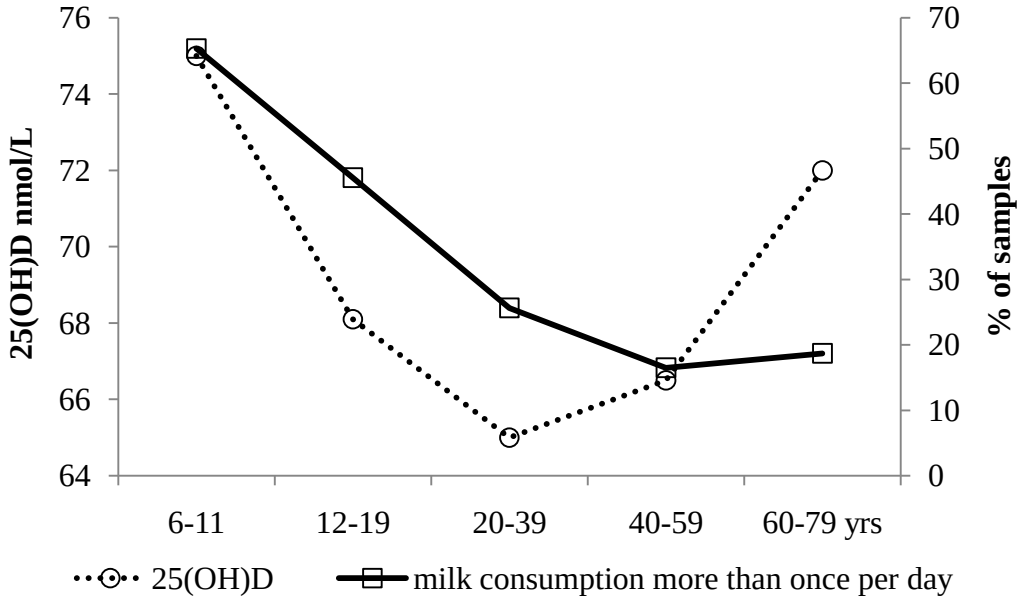


Figure 1.3 Average serum 25(OH)D concentration versus the percentage of different age groups in Canada with high milk consumption.

(Based on the data from the 2007-2009 Canada Health Measures Survey, as reported by Langlois *et al.*, 2010).

40-59 years and then increased slightly in the 60-79 year old group. The serum 25(OH)D was distributed in a U shape from the young group to senior group. Although the distribution of serum 25(OH)D did not follow the exact pattern of high frequency milk drinkers, the trends were similar for the groups between 6-39 years of age. For different age groups, the serum 25(OH)D levels of those drinking milk more than once per day were all above 70 nmol/L despite the age difference (Langlois *et al.*, 2010).

1.2.3.2 Non-compliance of vitamin D fortification

Vitamin D is classified as risk category B nutrient by Health Canada, which causes serious adverse effects at high intake level, but with low risk of excessive intake at the proposed level of addition for discretionary fortification (Health Canada, 2005).

In 1991, over-fortification of vitamin D in home-delivery milk in Boston resulted in 41 patients being hospitalized and 2 deaths (Blank *et al.*, 1995). Jacobus *et al.* (1992) reported the analysis of milk products being associated with the 1991 hypervitaminosis D cases in Boston. The result showed that none of the products contained vitamin D at the required level of 400 IU/quart. In this over-fortification incident, the vitamin D concentration ranged from less than 40 IU/quart to more than 200,000 IU/quart in fortified products. Half of the samples were fortified with a concentration at 73-580 times that of the required level. Furthermore, vitamin D levels varied greatly in milk sampled in April and June 1991. For example, homogenized whole milk from a Boston local dairy contained over 200,000 IU vitamin D per quart in April, while in the follow up test in June, vitamin D was found at a level of less than 40 IU/quart in this product. The investigation also found the concentrate used for milk fortification was labelled vitamin D_2 while analysis revealed it contained vitamin D_3.

The fortification non-compliance of vitamin D has been a recurring problem in milk products and not limited to North America. Table 1.3 summarizes the findings of several surveys on vitamin D content in fluid milk in the past 25 years.

Tanner *et al.* (1988) summarized the results of two surveys on vitamin D content of fluid milk conducted in Oregon and Ohio states, US. In the Oregon survey, 449 milks and milk products were analyzed during 1979-1983. In the Ohio survey, 23 milk products were determined without indicating analysis dates. A general trend was found in these two surveys, which was lower vitamin D content in milk of lower fat content. For example, in the Oregon survey, the percentage of milk containing vitamin D at less than 50% of label value was 28% for the whole milk and 47% for the skim milk. In the Ohio survey, all whole milk samples contained vitamin D at a level more than 50% of the claimed amount while 37.5% of the skim milk contained less than half of the label amount. Tanner *et al.* (1988) indicated vitamin addition before fat separation might be the reason for lower fat soluble vitamin in milk with lower fat content.

Holick *et al.* (1992) examined vitamin D content in 42 milk samples of 13 brands and 10 infant formula samples of 5 brands from five Eastern states in United States. The results showed that more than 60% of the milk samples were under-fortified and only 29% of the milk samples contained 80 -120 % of the declared label amount. More than 30% of the 42 samples contained vitamin D less than 50% of the label value. Three out of 14 skim milk samples contained vitamin D lower than the detection limit of 0.5 IU/100 mL. One sample contained vitamin D_3 despite the fact that vitamin D_2 was stated on the label. All 10 infant formula samples were over-fortified with vitamin D and 70% of the

Table 1.3 Summary of surveys conducted for vitamin D content in fluid milk.

Reference	Location	Sample size	Under-fortification	Over-fortification	Standard of compliance
Tanner *et al.*, 1988	US	539	53%	23%	33.8 - 50.7 IU/100 mL
Holick *et al.*, 1992	US	42	62%	10%	33.8 - 50.7 IU/100 mL
Chen *et al.*, 1993	US	79	51%	29%	33.8 - 50.7 IU/100 mL
	BC, Canada	15	27%	47%	28.8 - 43.2 IU/100 mL
Faulkner *et al.*, 2000	ON, Canada	45	36%	38%	31.7 - 51.6 IU/100 mL
Murphy *et al.*, 2001	US	648	28%	25%	33.8 - 50.7 IU/100 mL
Thomson, 2006*	New Zealand	18	28%	39%	Various label claim
Laleye *et al.*, 2009	United Arab Emirates	54	31%	30%	32 - 48 IU/100 mL
Patterson *et al.*, 2010	US	120	19%	41%	42.3 - 52.9 IU/100 mL

* The percentages for the study by Thomson (2006) were based on all fortified foods investigated including fluid milk.

samples contained more than 200% of the labelled amount and the highest was over-fortified with more than four times the declared vitamin D content.

In a follow-up study (Chen *et al.*, 1993), the research group of Holick, Chen and colleagues analyzed 79 milk samples from United States and 15 samples from British Columbia (BC), Canada. The fortification situation was not improved in the United States with 51% of the samples containing less than 80% of the claimed vitamin D amount and 29% samples being fortified with over 120% of the claimed amount. The samples from BC also showed fortification non-compliance, with 47% over-fortified samples and 27% under-fortified samples.

Faulkner *et al.* (2000) reported an investigation on fortified fluid milk in Ontario. Whole milk, 2% and skim milk from 15 brands were analyzed in this research. More than 70% of the 45 samples were either under or over-fortified. Vitamin D was not detected in four products including one 2%, one skim and two whole milk products. Whole milk and skim milk showed similar patterns, with almost 50% over-fortification and 30% under-fortification. The fortification compliance in 2% milk was more acceptable, with close to 50% of the samples satisfying the standard of 31.7 - 51.6 IU/100 mL. The criteria of acceptance applied in this research was wider than the CFIA (2012) requirement of 35.2 - 46.9 IU/100mL, thus the non-compliance might be even worse if applying the more strict CFIA range.

Murphy *et al.* (2001) evaluated the fortification of fluid milk in New York states. Whole milk, 1%, 2% and skim milk were sampled over four years for vitamin D determination. More than half of the 648 samples showed fortification non-compliance.

The Institute of Environmental Science and Research Limited conducted a survey on vitamin A, D and calcium contents in the fortified foods of New Zealand (Thomson, 2006). Eighteen samples including milk, margarine, food drink, and baby food were analyzed for vitamin D content. Overages of 25-70% were observed in 39% of the samples and 28% of the samples were under-fortified with vitamin D levels 47-68% less than the label claim. Laleye *et al.* (2009) sampled whole milk, low fat milk and skim milk in United Arabs Emirates and found 31% of the 54 samples were under-fortified and 30% were over-fortified.

In a recent investigation, Patterson *et al.* (2010) analyzed 120 milk products sampled across the US. Wide variation was observed with vitamin D content ranging from undetectable to almost 200% of the label value.

1.2.3.3 Vitamin D fortification practices

The conditions of vitamin storage and addition might affect the final fortification level. In a survey conducted in North Carolina (Hicks *et al.*, 1996), 54% of the surveyed dairy manufacturers stored vitamin D at ambient temperatures despite the suppliers' recommendation for refrigerated storage. The 13 manufacturers added the vitamin at 9 different points during processing and most of them added vitamin preparation before pasteurization. This survey divided the dairy processors into two groups based on the method of vitamin addition. For those who used injection meter, seven processors added vitamin D at five different points after standardization and before homogenization. Two processors injected the vitamin D after homogenization and before the pasteurization. When using batch addition method, three processors added vitamin D before milk fat separation, and four processors added vitamin D after standardization and before

homogenization. This survey indicated possible losses of vitamin D during the storage and processing. The researchers also suggested that similar fortification practices might be used nationwide in the United States.

No information of the actual vitamin D addition practices in Canada was found, although CFIA does provide detailed guidance on the specific procedures and protocols for vitamin addition to dairy products (CFIA, 2012). In the Dairy Products Inspection Manual, CFIA (2012) provides equations to calculate the amount of vitamin D for batch addition and injection of continuous processing. CFIA (2012) also suggests that vitamin D can be added at different points in the processing system, from batch pasteurizer or HTST pasteurizer balance tank to finished powder prior to final packaging.

1.2.4 Vitamin D stability

1.2.4.1 Stability of solid vitamin D

Huber and Barlow (1943) indicated that all forms of vitamin D were susceptible to oxidation, and more rapidly oxidized under dry condition than in emulsions. Byrn (1976) reported in his study of solid-state reactions of drugs that crystalline vitamin D_2 was sensitive to light, air, and heat and yielded several decomposed products whose structures were not determined. The crystalline vitamin D_2 was observed to have completely turned to yellow after six months at ambient temperature with free access to air, and the elemental composition was changed from $C_{28}H_{44}O$ to $C_{28}H_{44}O_8$. The crystalline vitamin D_2 was fully degraded when heated at 80 ℃ no matter whether light was present or not but was stable at this high temperature for one week if there was no contact with oxygen.

In their accelerated degradation study, Kozlov *et al.* (1977) observed changes in the infrared spectrum in terms of the shape and intensity of bands around 890 cm^{-1} and 970 cm^{-1}, which they attributed to the breakdown of the conjugated system caused by oxidation. Ketones were also generated during the oxidation and showed bands at 1660 cm^{-1} and 1715 cm^{-1}.

Grady and Thakker (1980) compared the stability of vitamin D_2 and D_3 powders under four different storage conditions (Figure 1.4). When stored in a desiccator and in the presence of oxygen, less than 5% vitamin D_2 was left while 99% of vitamin D_3 was maintained after 56 days. Vitamin D_3 became more sensitive to the temperature when humidity was increased. The stability of vitamin D_2 was greatly improved at 85% relative humidity and room temperature. When the environment temperature increased from 25 ℃ to 40 ℃, the degradation of both forms was accelerated. At room temperature both forms were stable in nitrogen during 56 days storage. The authors also indicated that the decomposed products of vitamin D_2 possessed higher polarity.

DeRitter (1982) indicated that vitamin D_3 was also sensitive to trace metals. Stewart *et al.* (1984) reported that under room temperature in the presence of light, the crystalline vitamin D_2 would be gradually degraded to an orange colour powder in 6 months, which confirmed the observation of Byrn (1976). The oxidation resulted in the destruction of the triene structure.

1.2.4.2 Stability of vitamin D in foods

It is reported that vitamin D is generally stable during food processing and storage (Kazmi *et al.*, 2007; Hanson & Metzger, 2010). However, vitamin D loss was reported in some other research. Table 1.4 summarizes the studies of vitamin D stability in foods.

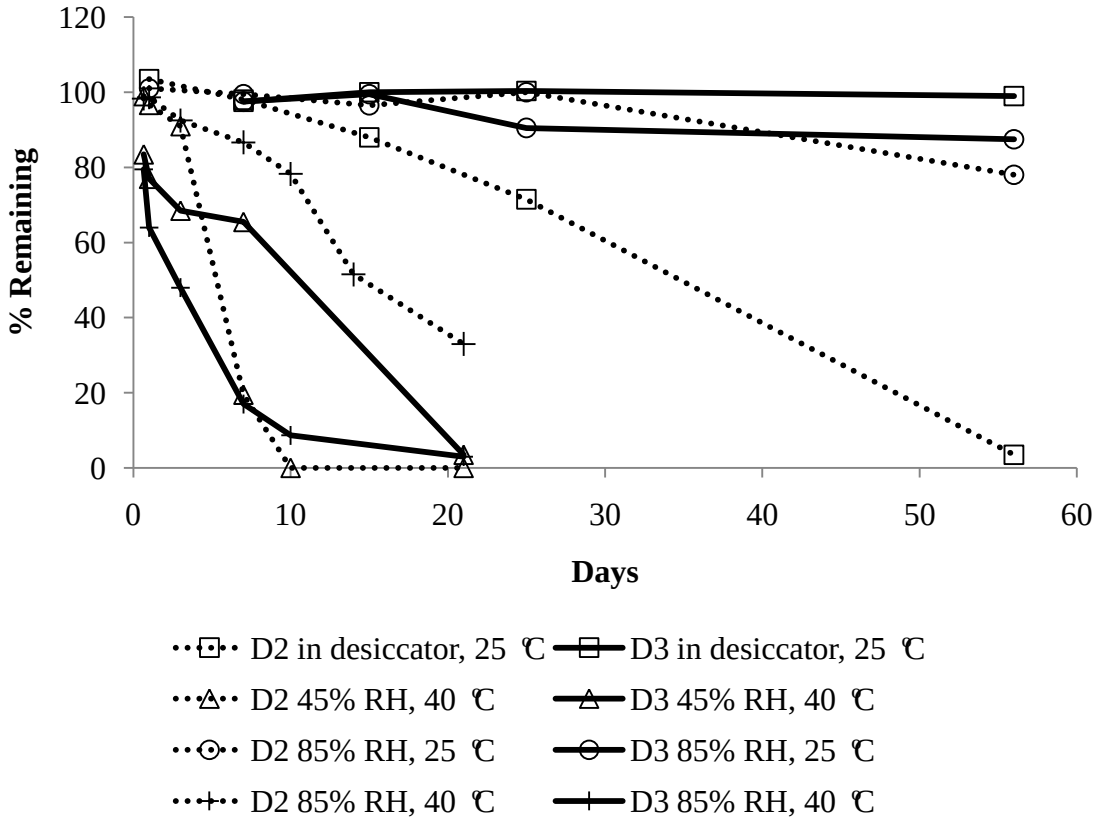


Figure 1.4 Degradation of solid vitamin D under different storage conditions.

(Based on data of Grady and Thakker, 1980).

Table 1.4 Summary of studies on vitamin D stability in model system or foods.

Analyte	Result	Reference
Vitamin D_3 in acetonitrile	60% loss by light exposure and storage at 21 °C after 10 days	Renken & Warthesen, 1993
Vitamin D_3 in skim milk	25% loss by light exposure in 1.8 L plastic container after 10 days	
Vitamin D_3 in spray-dried milk powder	No loss during processing	Indyk *et al.*, 1996
Vitamin D_2 in model system (12% H_2O+88% acetone)	Vitamin D oxidation caused by riboflavin + light	King & Min, 1998
Vitamin D_3 in fish, eggs, & vitamin D_2 in mushrooms	Less than 10 % loss during household cooking	Mattila *et al.*, 1999
Vitamin D_3 in orange juice	Stable for 30 days storage	Tangpricha *et al.*, 2003
Vitamin D_3 in cheese, yogurt, & ice cream	Stable during processing and cold storage	Kazmi *et al.*, 2007
Vitamin D_3 in UHT whole milk	20 -57% loss caused by light during 12 weeks storage	Saffert *et al.*, 2008
Vitamin D_3 in UHT low fat milk	35-65% loss induced by light during 12 weeks storage	Saffert *et al.*, 2009

Renken and Warthesen (1993) studied the impact of light, air, storage temperature on the stability of vitamin D_3 in an acetonitrile model system and skim milk. They exposed the acetonitrile solution of vitamin D_3 to fluorescent light of 2,260 lux (210 foot-candle) and air was bubbled into samples by a pump. Two storage temperatures, 4 °C and 21 °C, were applied. In the acetonitrile model system, the impact of light exposure was not significant on vitamin D_3 stability. Air contributed the greatest loss of vitamin D_3, while the combination of air and high storage temperature also caused significant loss of vitamin D. In skim milk, the light exposure alone contributed the greatest vitamin D_3 loss, followed by air with light and air alone. In a 1.8 L plastic container, the vitamin D_3 level in skim milk was decreased by 25% after being exposed to 3229 lux (300 foot-candle) for 10 days. The difference between acetonitrile model system and skim milk might be caused by different ways of aeration. Air was flushed into the headspace of skim milk while being bubbled through the acetonitrile solution. Renken and Warthesen (1993) assumed that the photo-degradation of vitamin D was caused by the singlet oxygen. This hypothesis was confirmed by later research (King & Min, 1998 & 2002; Li & Min, 1998). Singlet oxygen is produced by light exposure and has higher energy than regular oxygen. It may react with susceptible bonds in vitamin D and cause degradation. Renken and Warthesen (1993) also noticed the stratification of vitamin D_3 in milk with more vitamin D_3 in the top layer.

In Renken and Warthesen's research, light was a significant factor for vitamin D loss in skim milk while not in acetonitrile model system. This might be caused by the presence of riboflavin in milk. As a photo-sensitizer, riboflavin can trigger the formation of singlet oxygen, which may cause degradation of vitamin D. King and Min (1998)

tested the stability of vitamin D_2 with or without riboflavin in a model system of 12% distilled water and 88% acetone. Instead of vitamin D_2 measurement, oxygen loss in the headspace of sealed sample container was analyzed to confirm the oxidation of vitamin D_2. It was based on the following reaction: singlet oxygen was produced by a sensitizer (in this research, riboflavin), then reacted with a compound to be depleted from the headspace. Similar to the result of Renken and Warthesen (1993), no oxidation was found in sole vitamin D_2 solution, even though it was exposed to light of 4,000 lux. In the absence of riboflavin, no singlet oxygen was produced and triplet oxygen could not react with vitamin D_2. Oxygen loss was also not found in the sample with riboflavin but stored in the dark since riboflavin was not excited. King and Min (2002) further explored the mechanism using multiple instruments. Their result of high performance liquid chromatography (HPLC) showed that two extra peaks appeared in the chromatogram of the vitamin D solution containing riboflavin on light exposure, that were not found in other samples. UV scan confirmed the reaction occurred on the triene ring of vitamin D_2. The mass spectrum showed that the mass of vitamin D_2 was increased by one oxygen atom. Hydroxyl group was presented in oxidized vitamin D_2 while no carbonyl group was found by IR spectrum. Based on the results of different instrumental analysis, King and Min (2002) concluded that oxygen reacted with the triene ring of vitamin D_2 and a 5, 6-epoxide was formed.

Indyk *et al.* (1996) studied stability of vitamin D_3 in milk during spray-drying. Fortified milk went through low pressure preheating, direct steam injection at 95 °C, evaporation and spray-drying at 149 °C and fluid bed finish at 107 °C. No significant change of vitamin D content was found after the processing. Thus the common practice

of 30% overage addition of vitamin D to ensure the final fortification level was not necessary.

Mattila *et al.* (1999) studied the stability of vitamin D_3 found naturally in egg, fish and vitamin D_2 in wild mushroom and claimed that vitamin D was generally stable. The losses during the storage and thermal processing of these foods were less than 10%. Tangpricha *et al.* (2003) reported vitamin D_3 in fortified orange juice was stable during 30 days storage at 4 ℃.

Kazmi *et al.* (2007) studied the stability of vitamin D_3 in crystalline and emulsion forms during processing and storage of yogurt, ice cream and cheese. The acidification of yogurt and aeration of ice cream showed little impact on the vitamin D_3 content. No significant difference was found in the loss of the two forms of vitamin D_3. The vitamin D_3 losses subsequent to processing were 3% and 1% in yogurt and ice cream respectively. Pike and Brown (1984) indicated that vitamin D was not stable in acidic media and the instability increased with increased temperature. Whether the low temperature contributes to the low vitamin D loss during production and storage of yogurt was still unknown. Vitamin D_3 loss of 3-5% was observed during cheese making. Of the vitamin D_3 recovered, about 7-9% was found in whey. The crystalline form showed slightly higher loss than the emulsified one, with more vitamin D_3 found in whey for the product fortified with crystalline form.

Saffert *et al.* (2008) observed vitamin D_3 loss in ultrahigh temperature (UHT) pasteurized whole milk stored in clear or pigmented bottles at 23 ℃. More than half of the vitamin D_3 content in milk was lost after 12 weeks storage in clear bottles. Most samples lost at least 20% of vitamin D_3 content despite the protection provided by

pigmented bottles. In low fat UHT milk the vitamin D_3 was more sensitive, with a loss up to 65% of the original content (Saffert *et al.*, 2009).

1.2.5 Encapsulation of fat soluble vitamins

Encapsulation technology provides many benefits to the food fortification. Sensitive ingredients can be protected by encapsulation matrix from oxygen and light. Encapsulation may change the physical properties of food ingredients to improve the quality of fortified products, for example, reducing the size of particles or droplets to achieve a more homogeneous dispersion of nutrients in final products. Liquid agents can be converted into solid particles and fat soluble ingredients might be added into an aqueous system after encapsulation.

Fat soluble substances are often dissolved in vegetable oil. An emulsion is then formed by dispersing the oil droplets into an aqueous protein or polysaccharide solution. The last step is either spray drying or freeze drying to produce a free-flowing powder (Palzer, 2009).

Milk proteins can be used as encapsulating materials for fat soluble nutrients. Forrest *et al.* (2005) investigated the interactions between β-lactoglobulin A, β-casein and vitamin D_3. Both proteins showed strong ability to bind vitamin D_3. β-Lactoglobulin is able to bind a variety of lipophilic compounds, thus is considered as a suitable natural vehicle for fat soluble nutrient delivery. Forrest *et al.* (2005) suggested that β-lactoglobulin A might provide protection to vitamin D_3 during fermentation since the binding between the two compounds was stable from pH 8.0 to 2.5. Ron *et al.* (2010) reported nano-encapsulation of vitamin D_2 for the fortification of clear beverages. Lowest turbidity was achieved by adjusting pectin concentration in a β-lactoglobulin-vitamin D_2

solution at pH 4.25. Semo *et al.* (2007) encapsulated vitamin D_2 within casein micelles and suggested that this nano-vehicle could provide partial protection against the UV light induced degradation of vitamin D_2.

Modified starch, corn starch, vegetable oil, acacia gum, and sucrose might be used in commercial formulas of vitamin D (DSM, Netherlands). Disaccharides can be included in the matrix to improve the retention of characteristics of the substance of interest(Sosa *et al.*, 2011). Sucrose has been used to form coating matrix with other compounds for various oil products because of its good solubility in water, low cost and long shelf-life (Kaushik & Roos, 2006). Encapsulation of fish oil also involves sucrose in coating matrix since sucrose can develop cross-linking by Maillard reactions and thus improve the protection from oxygen (Beindorff & Zuidam, 2010). Silicon oxides, aluminum oxides or tripolyphosphate can be used alone or combined during encapsulation of active compounds. These chemicals are believed to improve the barrier ability of coatings, thus protect active compounds from oxygen, water vapor, and flavour permeation (Amberg-Schwab *et al.*, 2006; Desai & Park, 2006).

1.2.6 Analysis of vitamin D content in food samples

The analysis of the vitamin D content in foods is important to ensure that amounts claimed on labels are met. Different types of methods have been developed and improved for vitamin D determination.

1.2.6.1 Biological assays

The earliest official methods for vitamin D determination were bioassays, in which the bioactivity of vitamin D was determined by its ability to prevent or improve deficiency symptoms *in vivo* (Otles & Karaibrahimoglu, 2005). Rat line test was the first

official method to measure vitamin D content in foods or pharmaceutical products (Norman & Henry, 2007). According to AOAC method 936.14 (Cunniff, 1995), rachitic rats are fed with foods containing different amounts of vitamin D for the establishment of standards. After seven days, the bone of rats are processed with a silver nitrate solution. The newly deposited calcium in bone stains dark. Standards are set up by associating the dark regions on bone to the vitamin D amount in diet. Vitamin D content in sample is determined by comparing the dark region with standards. This method does not distinguish vitamin D_2 from vitamin D_3. To overcome this disadvantage, chick assay was introduced. Vitamin D_3 shows 10 times greater potency than vitamin D_2 in the chick, thus can be distinguished from vitamin D_2 (Norman & Henry, 2007). It involves setting up standards by feeding newborn chicks with fortified feed containing different levels of vitamin D. The percentage of bone ash is determined after three weeks on the diet.

Other bioassays include *in vivo* and *in vitro* intestinal calcium absorption, which investigate the ability of the test compound to stimulate the absorption of calcium in the small intestine. In these assays, $^{45}Ca^{2+}$ is used to indirectly quantify vitamin D content (Norman & Henry, 2007).

Bioassays can detect vitamin D at very low concentration. For example, the rat line test can detect as low as 12 ng of vitamin D. However, bioassays require long preparation times, which make them less convenient as a routine analysis method compared to other methods. The sacrifice of animals is also against the increasing awareness of animal welfare.

1.2.6.2 Spectrophotometric methods

IR spectroscopy has been used for qualitative analysis of vitamin D. In solid forms, vitamin D_2 can be distinguished from vitamin D_3 using the vitamin D_2 peak at 907 cm^{-1} (Westermark, 1991). Typical FTIR spectra of crystalline vitamin D_2 and D_3 (Liu, Y., unpublished data) are shown in Figure 1.5.

There is no published methodology on quantification of vitamin D using vibrational spectroscopy. The reason might be the inadequate detection limit and accuracy of the method since the concentration of vitamin D is normally low in foods.

Vitamin D can be quantified by measuring its maximum UV absorption at 264 nm. The extinction coefficients for vitamin D_2 and D_3 are 19,400 and 18,300 M^{-1} cm^{-1} respectively. The limitation of this method is that other UV absorbing compounds in solution will interfere with the quantitation of vitamin D (Norman & Henry, 2007).

1.2.6.3 HPLC methods

High-performance liquid chromatography (HPLC) methods have been widely used for the determination of fat soluble vitamins since the mid-1970s and have been adopted as official methods due to good selectivity and detection ability (Ball, 2006). Table 1.5 lists the details of AOAC official methods for analysis of vitamin D in dairy products. The analytical procedure normally involves saponification, liquid extraction by organic solvents, concentration by evaporation, and purification by separating the vitamin from other interfering compounds and HPLC quantification. Reverse phase columns are dominant in the HPLC determination for vitamin D. After extensive sample preparation steps, the vitamin D extracts are injected into a reverse phase column and eluted by one or more solvents with or without gradient. Light exposure is avoided during the whole procedure.

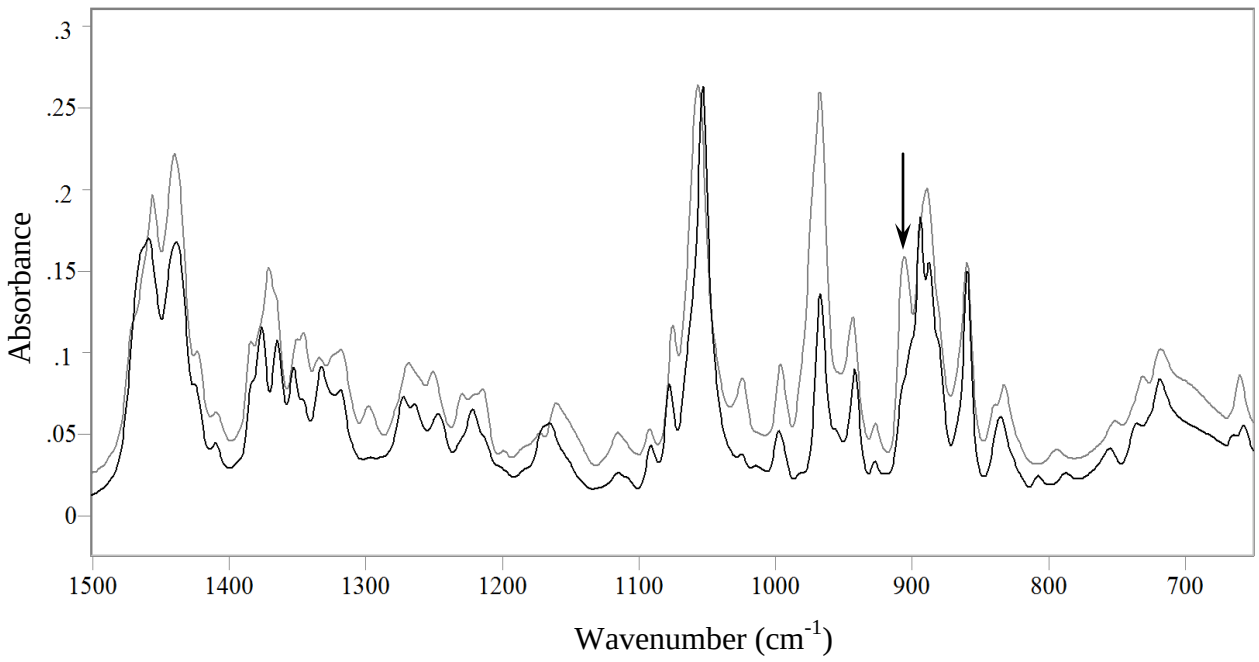


Figure 1.5 FTIR spectra of crystalline vitamin D_2 (grey line) and vitamin D_3 (black line). The differentiating peak of 907 cm^{-1} is marked by the arrow (Liu, Y., unpublished data).

Table 1.5 Summary of AOAC HPLC methods for vitamin D in dairy products.

Matrix	Saponification	Solvent Extraction	Purification	Column / Mobile phase	Detection	Reference
Fortified milk and milk powder	Aqueous KOH, 60 ℃, 30 min	Ethyl ether, pentane	Sil60D-10CN (250×4.6 mm)/ Amyl alcohol (0.35%) in hexane	Stainless Partisil (250×4.6 mm)/ Amyl alcohol (0.35%) in hexane	UV 265 nm	AOAC, 981.17 (Devries & Borsje, 1982)
Ready to feed milk based infant formula	KOH pellets, 75 ℃, 30 min	Ethyl ether, petroleum ether	Silica (250×4.6 mm)/ hexane-amyl alcohol (99.2+0.8)	Silica (150×4.6 mm)/ hexane-amyl alcohol (99.85/0.15)	UV 254 nm	AOAC, 992.26 (Tanner *et al.*, 1993)
Infant formulas and enteral products	Ethanolic KOH, 60 ℃, 30 min	Hexane	Silica SPE cartridge	C18 (250×4.6 mm)/ Gradient mixture of acetonitrile, methanol and ethyl acetate	UV 265 nm	AOAC, 995.05 (Sliva & Sanders, 1996)
Fortified milk, infant formula, margarine, milk powder	Aqueous KOH, 95 ℃, 30 min	n-Heptane	Silica (250×4.6 mm)/ 0.5% isopropanol-2% Methy tertiary butyl ether-cyclohexane–*n*-heptane	C18 (250×4.6 mm)/ methanol-acetonitrile (20/80)	UV 265 nm	AOAC, 2002.05 (Staffas & Nyman, 2003)

Various temperature and time combinations have been reported for the saponification of food samples, ranging from room temperature, overnight to 95 °C, 30 minutes (Devries & Borsje, 1982; Tanner *et al.*, 1993; Faulkner *et al.*, 2000; Staffas & Nyman, 2003). Johnsson and Hessel (1987) reported that the ambient saponification was insufficient and might give uncontrollable emulsions. Official methods normally use the short time high temperature saponification to saponify fat content thoroughly. After the saponification, different solvents can be applied to extract vitamin D from aqueous phase of the samples. For example, hexane is normally used alone as the extracting solvent, but ethanol is sometimes added into hexane to prevent formation of an emulsion (Faulkner *et al.*, 2000). Ethyl ether is often combined with petroleum ethyl or other solvents to extract vitamin D (Devries & Borsje, 1982; Tanner *et al.*, 1993).

During the saponification, vitamin D partially isomerizes into its precursor previtamin D (Johnsson and Hessel, 1987). In the four AOAC official methods for dairy products, various methods are used to correct the conversion from vitamin D to previtamin D. The AOAC 981.17 applies a correction factor of 1.25 on the measured vitamin D (Devries & Borsje, 1982) and AOAC 992.26 assumes 14% of the vitamin D converted into previtamin D during the saponification (Tanner *et al.*, 1993). An internal standard of vitamin D_2 is used in AOAC 995.05 and AOAC 2002.05 since the conversion rates of vitamin D_2 and D_3 to the respective previtamins are similar (Sliva & Kanders, 1996; Staffas & Nyman, 2003).

Before being applied to the analytical column, an extensive purification of the crude extract is needed. As shown in previous Table 1.5, most AOAC official methods use a normal phase column to separate vitamin D from interfering compounds. Samples need to be individually purified using a silica column and then collected for the determination on an

analytical column. In comparison, AOAC 995.05 uses a silica solid phase extraction (SPE) cartridge to clean up the crude extract, which allows multiple samples to be processed using a vacuum manifold.

SPE is also applied in some other methods to replace the normal phase column for purification (Mattila *et al.*, 1992; Renken & Warthesen, 1993; Hagar *et al.*, 1994; Faulkner *et al.*, 2000). Direct extraction using a SPE cartridge for vitamin D in non-fortified sample was also reported (Blanco *et al.*, 2000; Iwase, 2000; Heudi *et al.*, 2004; Bartolucci *et al.*, 2011). Table 1.6 summarizes various SPE protocols involved in vitamin D determination.

Antioxidants are often added to protect vitamin D before HPLC analysis. Pyrogallol, ascorbic acid or sodium ascorbate are used during saponification since they are water soluble (Faulkner *et al.*, 2000; Trenerry *et al.*, 2011). Butylated hydroxytoluene (BHT) is often used to protect vitamin D in organic solvent (Devries & Borsje, 1982; Grace & Bernhard, 1984; Staffas & Nyman, 2003).

With high reproducibility and relatively short protocol, the HPLC method has become dominant for routine analysis of vitamin D.

Table 1.6 Solid phase extraction techniques for vitamin D determination.

SPE cartridges	Purpose	Analytes	Conditioning/ Extraction	Wash	Elute	Reference
Mega Bond Elut Silica SPE column	Extract purification	D in egg yolk	Hexane	Hexane Hexane/isopropanol (99.5/0.5)	Hexane/isopropanol (99.5/0.5)	Mattila *et al.*, 1992
Bond Elute SPE silica cartridge	Extract purification	D in milk	Hexane	Hexane	Hexane/chloroform (21.5/78.5)	Renken & Warthesen, 1993
Sep-Pak Florisil	Extract purification	Vitamin D in milk	Methanol (3ml) Isopropanol (4ml) Hexane (7ml)	Hexane	Isopropanol	Hagar *et al.*, 1994
Mega Bond Elut C18	Direct extraction	Fat-soluble vitamins	Methanol (25ml) and MIlli-Q water	Methanol/water (10/90)	Methanol	Blanco *et al.*, 2000
Extract-clean Si	Extract purification	Vitamin D	Hexane 1-2 volumes	Hexane/chloroform (22/78)	Methanol	Faulkner *et al.*, 2000
Bond Elute C18	Direct extraction	Vitamin D_2 as emulsified nutritional supplements	0.2 M K_2HPO_4 aqueous solution with 1 mM EDTA $\cdot 2Na \cdot 2H_2O$	Deionized water then 10% aqueous methanol	Methanol	Iwase, 2000
Chromabond XTR®	Direct extraction	Vitamin A, D_3 & E	-	-	Hexane	Heudi *et al.*, 2004
Supelclean LC-NH2	Direct extraction	Vitamin D_3 in cod liver supplement	Hexane	Hexane	Ethyl acetate	Bartolucci *et al.*, 2011

Chapter 2: Hypotheses and research outline

2.1 Hypotheses and objectives

As introduced in the literature review, the non-compliance of vitamin D fortification of milk has been a recurring problem and little information is available regarding the current situation of milk fortification in British Columbia since the last report in 1993. The reasons behind the fortification problem are also not clear despite frequently reported non-compliance. In this situation, information about the stability of vitamin D during processing and storage may provide some hints for processors to fortify foods with targeted concentration.

The purpose of the present research is to investigate whether the non-compliance of vitamin D fortification still exists in fluid milk sold in Vancouver and whether the instability of vitamin D might be a reason for the variation of vitamin D content in milk.

Hypothesis 1: Non-compliance of vitamin D fortification exists in fluid milk sold in Vancouver.

To examine this hypothesis, vitamin D_3 content of commercial fluid milk from seven brands in Vancouver grocery stores was analyzed.

Hypothesis 2: Different commercial formulations of vitamin D_3 differ in their stability to light exposure during storage.

To examine this hypothesis, four commercial formulations of vitamin D were added into skimmed milk and exposed to light at 2000 lux intensity for 22 days. Vitamin D retention of each formulation in milk was calculated and compared.

Hypothesis 3: Vitamin D_2 and D_3 have different stabilities at different pH values during thermal treatment.

To examine this hypothesis, crystalline vitamin D_2 and D_3 content in pH 3.5 and 6.6 buffers were analyzed before and after thermal treatment of 72°C for 15 seconds.

Chapter 3: Vitamin D of fortified fluid milk in Vancouver

Milk fortification with vitamin D is mandatory in Canada. However, wide variation has previously been noticed in vitamin D content of fortified milk (Chen *et al.*, 1993; Faulkner *et al.*, 2000). Moreover, the non-compliance of vitamin D fortification has been reported from time to time. Some recent studies indicated that the non-compliance of milk fortification is still a problem in the US (Murphy *et al.*, 2001; Patterson *et al.*, 2010) while the last available information about vitamin D in BC fluid milk was published in 1993 (Chen *et al.*, 1993).

This investigation was conducted to acquire the information on vitamin D of fluid milk in Vancouver. In total 104 cartons of fluid milk from seven brands were purchased from Vancouver grocery stores from November 2011 to September 2012. The vitamin D content in these products was analyzed on their "Best Before Date" or previous day and compared with the CFIA requirement (Figure 3.1).

3.1 Materials

3.1.1 Chemicals and apparatus

Potassium hydroxide, ascorbic acid, butylated hydroxytoluene, sodium sulphate, hexane, and absolute ethanol were purchased from Fisher Scientific Canada (Ottawa, ON) and were of reagent grade or higher. Dichloromethane, methanol, isopropanol and ethyl acetate from Fisher Scientific were HPLC grade. Vitamin D_2 (ergocalciferol, E5750), and vitamin D_3 (cholecalciferol, C9756), were purchased from Sigma Aldrich Canada (Oakville, ON).

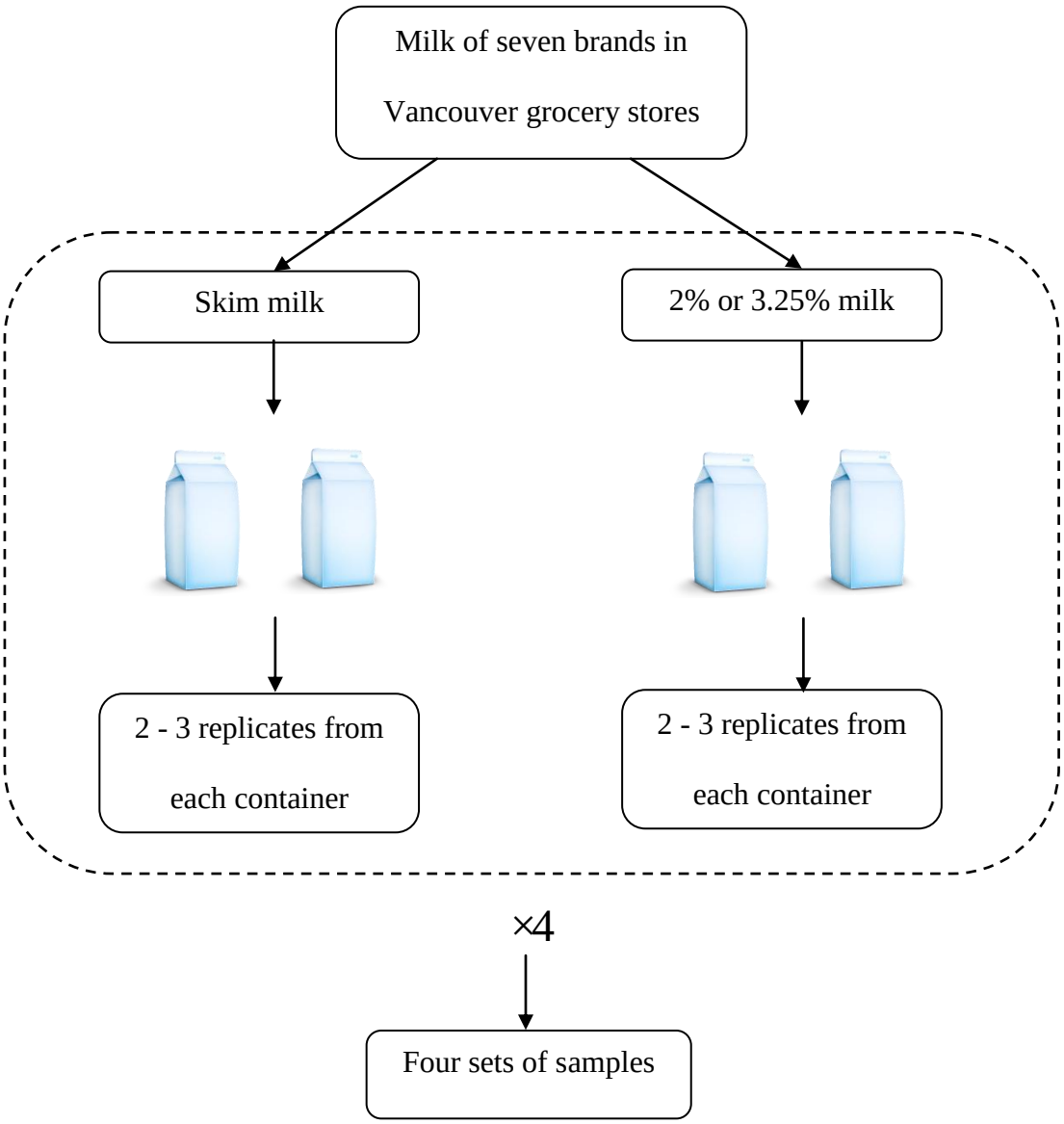


Figure 3.1 Overview of sampling of commercial milk products for vitamin D measurement.

Agilent 1100 Series LC (Mississauga, ON) with quaternary pump system and diode array detector was used for vitamin D determination. A Grace Vydac 201TP54 C18 column (Deerfield, IL) of 4.6 mm internal diameter and 250 mm length was installed on the HPLC system as the analytical column. A Vydac 201TP C18 guard column (Deerfield, IL) was installed to protect the analytical column.

3.1.2 Vitamin standards

Vitamin D_3 standard solutions were prepared in methanol at concentrations ranging from 0.200 to 4.000 µg/mL (8-160 IU/100 mL). Vitamin D_2 internal standard solutions were prepared in methanol at concentrations of 50.0 µg/mL and 5.00 µg/mL for calibration samples and milk samples respectively. The concentration of vitamin D_3 standards and vitamin D_2 internal standard were verified by UV absorption at 264 nm. All vitamin D_3 standard solutions and vitamin D_2 internal standards were stored in amber vials and kept at -80 ℃ when not in use. Solutions were discarded after two weeks and new standards were made. Calibration samples were prepared daily by adding 20 µL internal standard to 1 mL of vitamin D_3 standard solution.

3.1.3 Sampling of commercially fortified fluid milks

Fluid milk from seven brands was collected from retail stores on four separate occasions (referred to as four "sets") from November 2011 to September 2012. The exception was brand 2, which was only collected in two sets as it was withdrawn from the Vancouver market and was no longer available after the second set was sampled. Each set was purchased after the procurement of the previous set was completed. In a few instances, sampling had to be repeated for a particular brand in a set due to analytical

problems, and thus occasionally there was an overlap in the time periods for some samples across the sets.

For each set, two containers of each milk product (i.e. with the same lot number) were purchased from the same location. To evaluate the potential effect of milk fat content on fortification, skim milk and 3.25% milk were selected for the analysis, except for brand 6, for which 2% milk was used since 3.25% milk was not found. Table 3.1 shows an overview of the milk products. Samples of brand 1 were in 1 litre clear glass bottles, samples of brand 2 were packed in 1 litre opaque plastic containers, and the milk of the other five brands were in 1 litre laminated paperboard cartons. Brand 5 and Brand 7 were produced by the same dairy processor. The processor of brand 2 was unknown since this brand was withdrawn from the market after the second set, thus the information of processor was not available anymore. In other words, the seven brands represented fluid milks from at least 5 different dairy processors as identified by the CFIA Registered Dairy Establishment 4 digit codes. All milks were immediately stored in a cold room at 4 °C after purchase. Since the production date for each sample was not available on milk package, the vitamin D was measured either on the "Best Before Date" or the day before this date. Details of the sampling dates and locations as well as analysis dates for each of the commercially fortified fluid milks can be found in Appendix A.

3.2 Vitamin D determination

Among different types of methods for vitamin D determination, the HPLC methods have been dominant and have been accepted as AOAC official methods for measuring vitamin D in various food samples. Based on the equipment availability, AOAC method 995.05 was adapted for the vitamin D measurement in this research.

Table 3.1 Overview of milk samples of seven brands.

Brand	Processor	Milk type	Package	Volume
1	A	Skim & 3.25%	Clear glass bottle	1 litre
2	Unknown	Skim & 3.25%	Opaque plastic container	1 litre
3	B	Skim & 3.25%	Laminated paperboard cartons	1 litre
4	C	Skim & 3.25%	Laminated paperboard cartons	1 litre
5	D	Skim & 3.25%	Laminated paperboard cartons	1 litre
6	E	Skim & 2%	Laminated paperboard cartons	1 litre
7	D	Skim & 3.25%	Laminated paperboard cartons	1 litre

3.2.1 Saponification

Samples were prepared according to AOAC method 995.05 (Sliva & Kanders, 1996) with slight modification. The milk container was inverted 20 times in case there was any stratification of vitamin D, then an aliquot of about 15 mL (15.20 g weighed accurately) of milk was added into a 125 mL Erlenmeyer flask. To the flask was added 20 µL vitamin D_2 internal standard, 1.5 mL of ascorbic acid solution in water (19.4% wt/vol), and 19 mL potassium hydroxide solution (30.7% wt/vol in 20:163 ethanol:water vol/vol). The flask was stoppered and heated in a shaking water bath (Blue M, Blue Island, Illionois) at 60 ℃ for 30 min. Aluminum foil was used to cover the water bath to avoid light exposure.

3.2.2 Extraction

The sample mixture was transferred to a 250 mL clear separatory funnel along with 15 mL water and 60 mL hexane was used to rinse the flask. The contents were shaken for 90 s and the layers were allowed to separate. The aqueous layer was drained and the organic phase washed with 15 mL water. The aqueous layer was drained to waste and the sample mixture washed again with 15 mL of water after being adjusted to neutrality by drop wise addition of 10% acetic acid (vol/vol) using phenolphthalein as indicator. The aqueous layer was drained. The organic phase was passed through sodium sulfate into a 100 mL round bottom flask and to it was added 1 mL of BHT dissolved in hexane (1 mg/mL). The solvent was removed under vacuum using a rotary evaporator (Buchi, Flawil, Switzerland) with a water bath temperature of 40 ℃.

3.2.3 Sample purification

The crude extract was purified on a vacuum manifold (Honeywell Burdick Jackson, Morristown, New Jersey, USA) using Sampliq silica SPE cartridges (3 mL capacity, 500 mg packing, Agilent Canada) that had been conditioned with 4 mL dichloromethane-isopropanol solution (80:20 vol/vol) and then 5 mL dichloromethane-isopropanol solution (99.8:0.2 vol/vol). The crude sample extract was dissolved in 2 mL dichloromethane-isopropanol (99.8:0.2 vol/vol) and transferred to the cartridge along with a 1 mL rinse of dichloromethane-isopropanol (99.8:0.2 vol/vol). The cartridge was then washed with 2 mL dichloromethane-isopropanol (99.8:0.2 vol/vol). Finally, the sample was eluted with 7 mL dichloromethane-isopropanol (99.8:0.2 vol/vol) into a clean test tube. The purified sample was filtered through Millex 0.45 μm 4 mm PTFE syringe filter (Millipore, Billerica, Massachusetts) and then evaporated in a Rapidvap Vertex Evaporator (Labconco, Kansas City, Missouri). The dried sample was transferred to an HPLC vial and then dried under a stream of nitrogen. The residue was reconstituted in 100 μL methanol/dichloromethane (80:20 vol/vol) for analysis by HPLC.

3.2.4 HPLC analysis of vitamin D

The injection volume was 40 μl out of the 100 μl concentrated sample. Vitamin D_2 and D_3 were eluted by isocratic mobile phase of 98% acetonitrile and 2% methanol. Then the column was washed using 98% ethyl acetate and 2% methanol. By the end of the run, the mobile phase was switched back to acetonitrile and methanol. The total running time was 26 minutes. The mobile phase change is shown in Table 3.2.

Vitamin D_3 and the internal vitamin D_2 standard were monitored at 264 nm. A six-point calibration curve was constructed before the sample analysis on a daily basis.

Table 3.2 Flow rates and concentrations of mobile phase components for determination of vitamin D in fluid milk by HPLC.

Time, min	Flow rate, mL/min	Acetonitrile, %	Methanol, %	Ethyl acetate, %
0.0	1.2	98	2	0
15.5	1.5	98	2	0
17.0	1.5	0	2	98
20.0	1.2	0	2	98
21.5	1.2	98	2	0

The area ratio of the vitamin D_3 standard peak and the vitamin D_2 internal standard peak were plotted against vitamin D_3 standard concentration. Vitamin D concentration in the sample expressed as IU/100 mL was quantified by applying the vitamin D_3:vitamin D_2 ratio of the sample on the equation derived from the six-point standard calibration curve.

3.3 Statistical analysis

Three-way ANOVA and Tukey's test (Minitab 16, State College, PA) was conducted to determine the factors contributing to significant variation of vitamin D ($p<0.05$). Brand, milk fat content and sampling sets were defined as potential factors for vitamin D variation.

3.4 Results and discussion

3.4.1 Establishment of the adapted AOAC official method

Currently there are four AOAC official methods for vitamin D measurement in dairy products. The AOAC 995.05 method was selected and adapted for this research because it uses SPE cartridge to purify the vitamin D extract. Comparing with LC column purification, SPE speeds up the preparation for each sample while removing most interfering compounds. Furthermore, multiple samples can be purified and concentrated simultaneously.

In many AOAC official methods measuring vitamin D, ascorbic acid or sodium ascorbic acid were used in saponification and BHT during evaporation on water bath (Devries & Borsje, 1982; Tanner *et al.*, 1993; Staffas & Nyman, 2003). In the original 995.05 method, none of these anti-oxidants are applied. To protect vitamin D_3 and internal standard of vitamin D_2 in the present study, ascorbic acid was added together

with the internal standard before saponification in water bath and BHT solution was added before rotary evaporation at 60 ℃.

In the original AOAC 995.05 method, the HPLC running time is 35 minutes with gradient mobile phase. The flow rate was increased to speed up the HPLC analysis in this research.

System suitability is to verify that the whole system of instrument, reagent, column and samples are suitable for the intended method. Vitamin D_2 and vitamin D_3 solutions were stored at room temperature for four days to cause degradation of vitamin D to previtamin D. The system suitability was tested using the stressed vitamin D_2 and vitamin D_3 solutions. The resolution of vitamin D peaks and the reproducibility were examined. Figure 3.2 shows that the peaks of previtamin D and vitamin D were well separated.

In the original method, the ratio of vitamin D_2 : vitamin D_3 is calculated using peak height. In this research, the peak area ratio was applied instead of peak height to improve the precision of quantification. The test of replicate injections showed that relative standard deviation (RSD) of vitamin D_3 calculation from 5 injections ranged from 0.7% to 5.5% for concentrations ranging from 9 to 0.3 μg/ mL. Recovery was calculated by spiking fortified milk in three replicates. Vitamin D was added to the skim and 3.25% milk at 3.6 and 36 IU/100 mL. The low concentration spiking showed a recovery of 96% and 102% for skim and 3.25% milk respectively. The high concentration spiking was recovered at 100% and 101% for skim and 3.25% milk respectively. Baseline drift was observed during method verification, thus a reference wavelength of 345 nm was used to correct for any baseline drift.

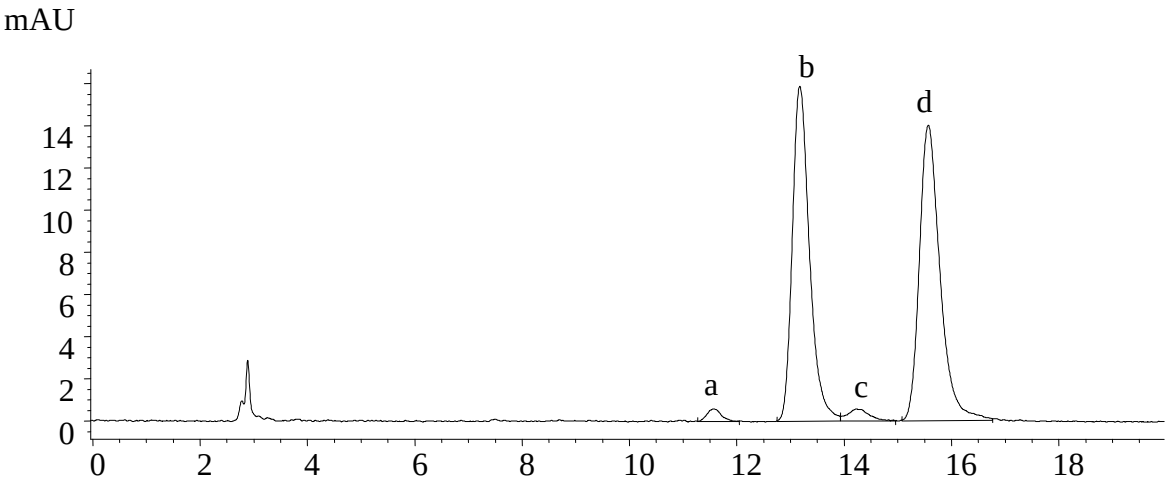


Figure 3.2 HPLC chromatogram of vitamin D_2 and vitamin D_3 standards.

Peak identification: a) previtamin D_2; b) vitamin D_2; c) previtamin D_3; d) vitamin D_3.

The criteria of acceptable HPLC performance were as follows. Shift of retention times should not be more than ± 0.3 min. Peaks should be nearly symmetrical with only minimal tailing at high concentrations. System pressure should not exceed 100 bar. Standard curves should have a minimum of five points and correlation factor r^2 no less than 0.997. Figure 3.3 is an example of the standard curve.

The column was cleaned thoroughly when contamination was indicated by peak shift. The column was reversed in order to flush out contaminants accumulated at the front part of the column. Four solvents were used in this procedure at a flow rate of 1 mL/min. The change of mobile phase is shown in Figure 3.4. Each solvent was run at 100% for 15 min before switching to the next solvent. After the wash from acetonitrile to ethyl acetate, the solvent was switched back from 100% ethyl acetate back to 100% acetonitrile. Each solvent was run about 5 min in this step. Guard column replacement was also conducted to improve the performance of analytical column after thorough cleaning.

3.4.2 Vitamin D in commercially fortified fluid milk

Two containers of the same product were purchased in each of the four sets in case there was container to container variation in vitamin D content. The measured vitamin D content for each container ("A" and "B") of the same product in each set and the results of paired comparison of vitamin D in the two containers are shown in Appendix B. Student t-test analysis showed that there was no significant difference ($p>0.05$) between container A and B with the exception of the two containers of 3.25% milk for brand 5 in set 2 ($p=0.0493$). Therefore, in this research the total four or six replicate data measured from both containers of the same product in each set were considered as one data for subsequent data analysis.

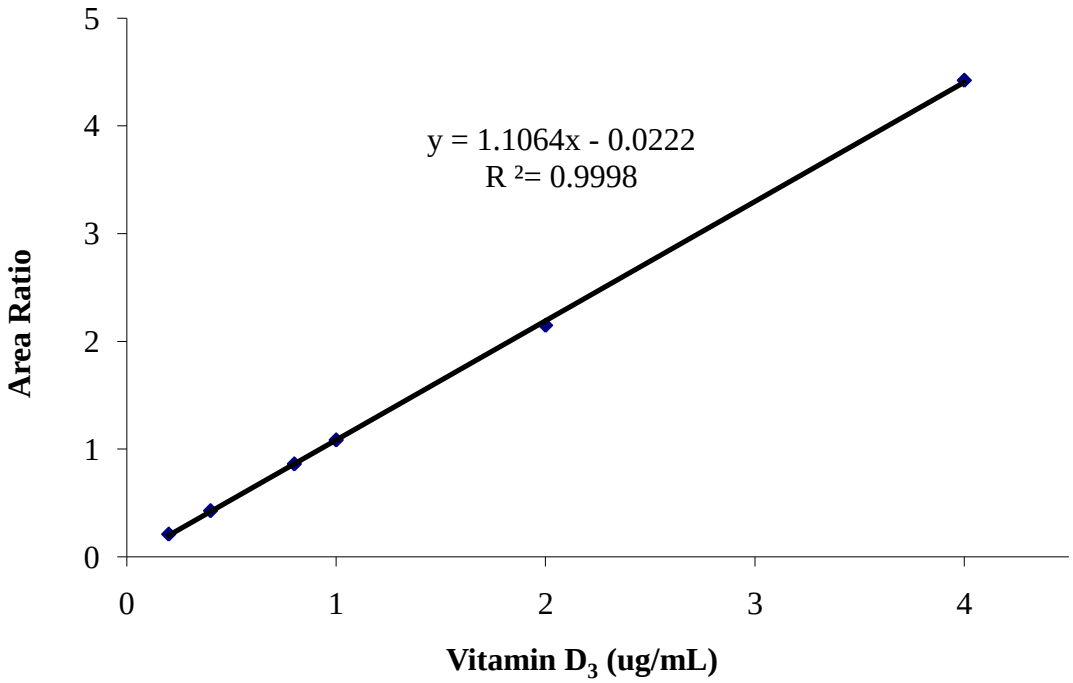


Figure 3.3 Standard curve of vitamin D_3 determination using HPLC (vitamin D_2 peak area ratio versus vitamin D_3 concentration).

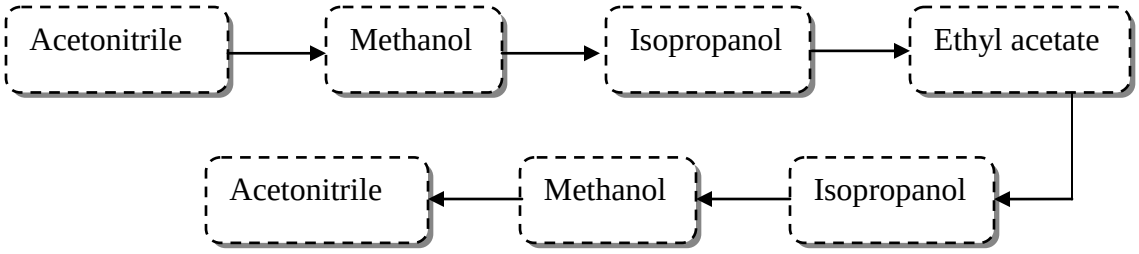


Figure 3.4 Sequence of solvents used for washing and re-conditioning the analytical HPLC column.

The mean vitamin D content and standard deviation of each product in each set are shown in Table 3.3. All milk samples were labelled as containing 45% of the daily value of vitamin D_3 per 250 mL, which represents 36 IU/100 mL based on 200 IU as the recommended daily allowance according to the Food and Drug Regulations (2012), since the new recommended daily value of 600 IU has not yet been adopted in the regulations. Generally, fortification of 3.25% or 2% milk was more compliant than that of skim milk. CFIA (2012) requires that the vitamin content in fortified milk should be within the range of 35.2 to 46.9 IU/100 mL and the processors should target the final concentration of 41 IU/100 mL. Only 42% of the 104 cartons of sampled milks in this study were compliant with this requirement. The compliance of milk samples with higher fat content was better than that of skim milk, with 57% of 2 or 3.25% milk and 27% of skim milk satisfying the CFIA standard respectively. Under-fortification was the major problem found in milk samples in this research, especially skim milk. In total 73% of skim milk samples and 35% of 2 or 3.25% milk samples were found to be under-fortified. None of the skim milk samples contained vitamin D greater than 46.9 IU/100 mL, while 8% of milk with higher fat content were over-fortified.

As demonstrated in Figure 3.5, in general higher vitamin D_3 content was found in 2 or 3.25% fat content milk than skim milk. Three-way ANOVA confirmed that significant difference existed among different brands, sampling sets, and products with different fat content. In addition, the interaction between brand and milk fat content was also significant. The results are summarized in Table 3.4.

The vitamin D content in skim milk of brands 5 and 7 were consistently low, with the mean value of four sets ranging from 8.53 to 15.1 IU/100 mL (Table 3.3), which were

Table 3.3 Vitamin D_3 content (mean ± standard deviation, n = 4 or 6) of seven brands of fluid milks collected in four sampling sets.

	Milk fat	Vitamin D_3 content (IU/100mL)			
		Set 1	**Set 2**	**Set 3**	**Set 4**
Brand 1	Skim	32.2 ± 1.3	36.5 ± 1.0	1.3 ± 0.1	35.0 ± 0.6
	3.25%	29.5 ± 1.4	29.4 ± 0.3	25.8 ± 0.7	26.8 ± 0.3
Brand 2	Skim	23.0 ± 0.7	31.0 ± 0.6	N/A[a] ± N/A	N/A ± N/A
	3.25%	39.4 ± 1.1	35.9 ± 0.9	N/A ± N/A	N/A ± N/A
Brand 3	Skim	45.6 ± 0.6	33.3 ± 1.1	36.8 ± 0.8	31.3 ± 0.8
	3.25%	43.2 ± 0.9	42.9 ± 0.9	27.5 ± 2.0	30.3 ± 1.1
Brand 4	Skim	34.5 ± 0.4	31.2 ± 1.1	40.8 ± 0.5	44.5 ± 0.9
	3.25%	40.9 ± 0.9	41.8 ± 0.7	35.5 ± 1.6	57.3 ± 0.4
Brand 5	Skim	15.1 ± 0.7	14.1 ± 0.5	9.9 ± 0.4	13.7 ± 1.6
	3.25%	32.2 ± 0.9	47.3 ± 0.8	36.6 ± 1.5	41.3 ± 0.4
Brand 6	Skim	32.8 ± 1.1	38.8 ± 0.8	34.3 ± 0.8	39.3 ± 1.0
	2%	38.7 ± 1.6	33.9 ± 0.6	35.6 ± 0.2	40.5 ± 1.1
Brand 7	Skim	12.0 ± 0.7	11.7 ± 0.3	8.5 ± 0.3	14.3 ± 0.2
	3.25%	45.1 ± 0.8	13.7 ± 0.9	35.2 ± 1.1	38.0 ± 0.9

[a] N/A = data not available

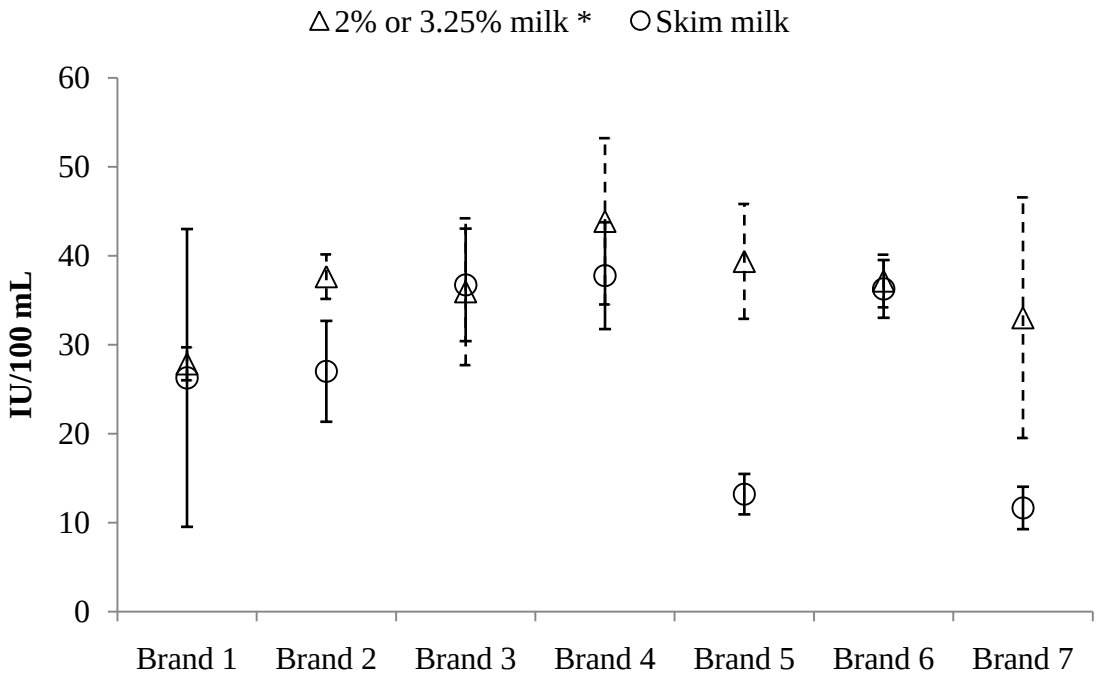


Figure 3.5 Comparison of vitamin D_3 content in skim milk versus milk with 2 or 3.25% fat. Values shown are mean and standard deviation of four sets ($n \geq 8$).

(* 2% fat milk was analyzed for brand 6; 3.25% fat milk was analyzed for other brands)

Table 3.4 Results of three way ANOVA for vitamin D in commercial fluid milk.

Factors	DF	Seq SS	Adj SS	Adj MS	F value	Probability
Brand	6	9325	9364	1561	38.8	0.000
Milk fat content	1	4614	4745	4745	117.9	0.000
Sampling set	3	1467	1371	457	11.4	0.000
Brand × Milk fat content	6	5742	5742	957	23.8	0.000
Set × Milk fat content	3	171	164	55	1.4	0.258
Error	218	8776	8776	40		
Total	237	237	30096			

less than 50% of the claimed amount. Brands 5 and 7 are produced by the same milk processor according to the registration number marked on the package. Compared to skim milk, milk with 3.25% fat of these two brands showed better fortification compliance with vitamin D content close to the claimed value. Wide variation was also observed in the vitamin content in milk products with same fat content but from different brands or sampling sets. For example, only trace amount of vitamin D_3 content was found in set 3 skim milk of brand 1, while the first two sets and the fourth set contained vitamin D_3 content close to each other (Table 3.3). A dramatically lower vitamin D content was also observed in 3.25% milk of set 2 of brand 7. The inconsistency in vitamin D_3 content in the same products from different sets can be visually shown by the standard deviation of vitamin D content of the same product in four sets, which was demonstrated as error bars in Figure 3.5. The product with longer error bars means big variation was found among the samples in different sets. Except for brand 6, fluctuating vitamin D contents were observed in the other brands, either in skim milk or 3.25% milk, or both products.

Brands 1 and 2 were packed in glass bottles and plastic jugs respectively, while paperboard cartons were used in the other five brands. As a result, vitamin D loss might be greater in brands 1 and 2 since vitamin D is light sensitive. As expected, the mean vitamin D content in 3.25% milk of brand 1 was the lowest among the seven brands. However, its skim milk had a moderate vitamin D concentration comparing with other brands. Both skim milk and 3.25% milk of brand 2 contained vitamin D at a moderate level among the seven brands. The impact of different container on vitamin D content was not very clear.

3.5 Conclusions

Wide variation among brands or sets in vitamin D content was found in milk samples purchased in Vancouver. The fortification non-compliance was observed in 58% of the products sampled. There are several possible reasons contributing to the variation and non-compliance of vitamin D fortification in milk products. Commercial vitamin D_3 products used for fortification may have different stability during storage. Different fortification protocol might be applied by milk producers. The fortification of skim milk is more difficult than 2% or 3.25% milk since vitamin D is fat soluble. All of these factors may cause variation in vitamin D content in final products. More information about the fortification practices of milk producers indicating the type and stability of vitamin D formulations used will be helpful to improve the compliance of vitamin D fortification in milk.

Chapter 4: Vitamin D in laboratory fortified milk exposed to light

Vitamin D is sensitive to light. Published studies confirmed that light is a significant factor contributing to vitamin D loss in fortified milk (Renken & Warthesen, 1993). Riboflavin, as a photo-sensitizer, produces singlet oxygen on light exposure and thus trigger the oxidation of vitamin D in milk (King & Min, 1998). Since riboflavin is naturally present in milk, significant loss of vitamin D may occur on light exposure when milk is packed in clear plastic bags or glass bottles. Milk in plastic jugs with various light transmittance might be impacted as well (Saffert *et al.*, 2008 & 2009). According to the Food and Drug Regulations (2012), vitamin A and D fortification is mandatory for skim milk while in other fluid milk products, only vitamin D addition is required. Vitamin A is sensitive to light exposure and the added vitamin A might be even more sensitive than indigenous vitamin A in milk (Gaylord et al., 1986; Bartholomew & Ogden, 1990). The impact of vitamin A degradation on vitamin D is still unknown.

Commercial vitamin D formulations may differ in the stability to light exposure because of the different encapsulating materials or emulsifiers. In this research, four commercial vitamin D formulations were used to fortify milk and the vitamin D retention on light exposure was measured during three weeks of storage at 4 °C.

In-house fortified skimmed milk samples were used to investigate the impact of light exposure on four different commercial formulations of vitamin D_3 during cold storage (Figure 4.1). The vitamin D content during the storage of light-exposed samples was compared with that of the corresponding control samples stored in the dark.

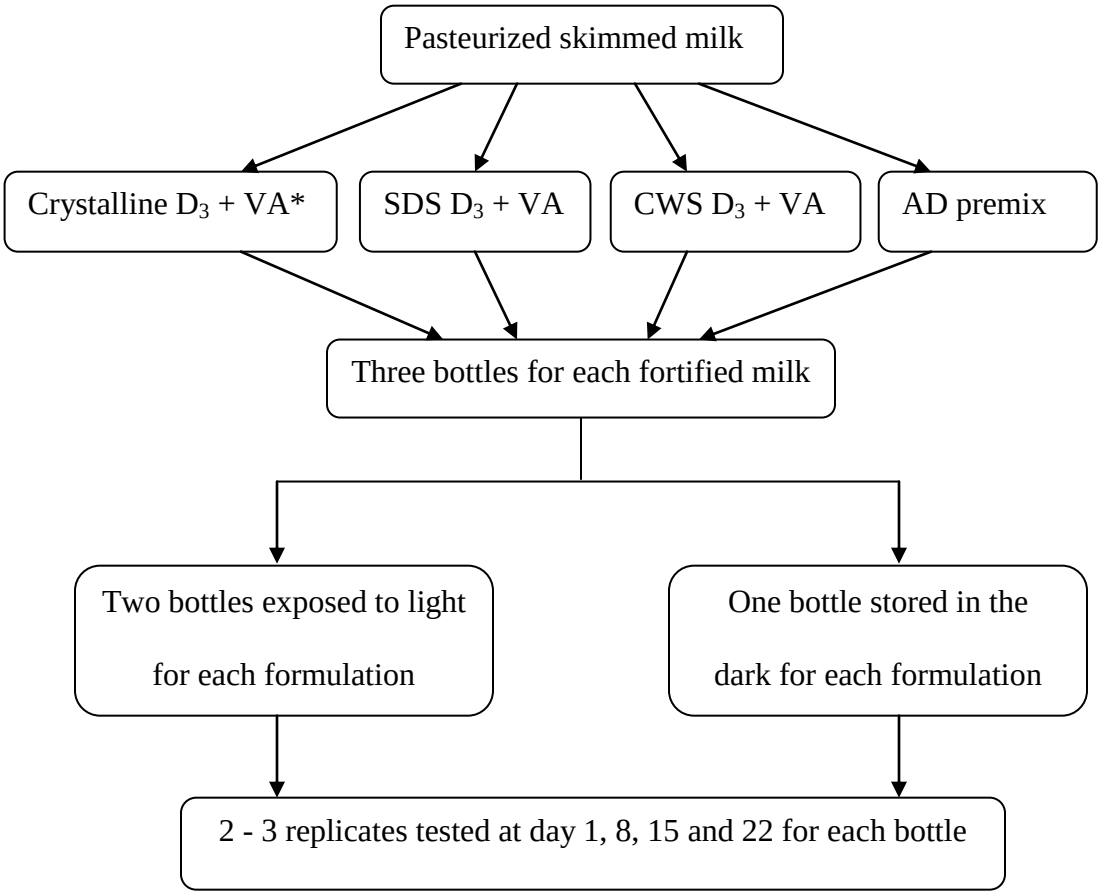


Figure 4.1 Overview of study design to test stability of different vitamin D formulations in milk during storage with light exposure.

* VA = vitamin A

4.1 Materials

4.1.1 Commercial Vitamin D Formulations

Vitamin D formulations were donated by DSM Nutritional Products (Belvidere, New Jersey, USA) and Kingsway Chocolate (Mississauga, Ontario, Canada). Vitamin A, in the form of a water dispersible powder of retinyl palmitate, was donated by DSM Nutritional Products. Two of the vitamin D formulations from DSM Nutritional Products came in the form of water dispersible encapsulated powders, dry vitamin D_3 100 CWS/AM (CWS D_3) and dry vitamin D_3 100 SD/S (SDS D_3), while the third vitamin D used in this research was food grade crystalline vitamin D_3 (crystalline D_3). The formulation from Kingsway Chocolate came in the form of an emulsified mixture of vitamin D_3 and vitamin A palmitate (AD premix). The ingredients of the vitamin formulations are summarized in Table 4.1. The detailed product information of each formulation is attached as Appendix C. Erythromycin (E0774) was purchased from Sigma-Aldrich (Oakville, ON).

4.1.2 Preparation of fortified milks for the study of stability of vitamin D to light exposure

Raw whole milk donated by the UBC Dairy Education and Research Centre (Agassiz, BC) was skimmed by centrifugation at 10,000 *g* at ambient temperature for 20 minutes on a Sorvall RC 5B Plus centrifuge (Thermo Fisher Scientific, Ottawa, ON). The skimmed milk was stored immediately at -30 ℃ before thermal treatment. The skimmed milk was thawed under cold running water, then pasteurized at 72 ℃ for 15 seconds using a FT74X HTST/UHT Heat Exchanger Processing Unit at the Food Technology

Table 4.1 Ingredients of vitamin formulations used in the present research.

Vitamin formulations	Ingredients
Crystalline D_3	Vitamin D_3
SDS D_3	Vitamin D_3, medium chain triglycerides, modified food starch, sucrose, *dl*-α-tocopherol, sodium ascorbate and silicon dioxide
CWS D_3	Vitamin D_3, medium chain triglycerides, corn starch, acacia gum and sucrose, *dl*-α-tocopherol, and silicon dioxide
AD Premix *	Vitamin D_3, vitamin A palmitate, sunflower oil, polysorbate 80, polyglycerol monooleate
Water dispersible vitamin A	Vitamin A palmitate, corn starch, gelatin, sucrose and *dl*-α-tocopherol.

* The vitamin supplier suggested that the AD premix containing 10 mg of mixed tocopherol and 5 mg of BHT per 1.7 million IU of vitamin A palmitate although such information was not included in Appendix C.

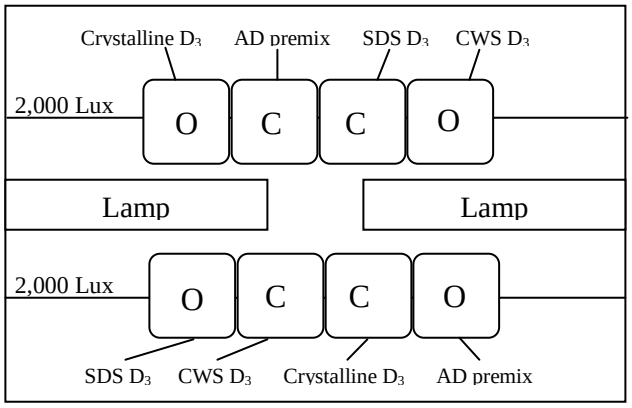
pilot plant of British Columbia Institute of Technology (BCIT) (Burnaby, BC). Pasteurized milk was stored at -30 ℃ before fortification.

Stock solutions of CWS D_3 and SDS D_3 were prepared by dissolving in water. Ethanol was used for preparing the crystalline D_3 stock solution, while vitamin A and D premix was dissolved in 75% ethanol and 25% dichloromethane. All vitamin D_3 stock solutions were made to target concentration of 320 IU/mL. For the purpose of comparison to the emulsified vitamin AD premix from Kingsway Chocolate, vitamin A dissolved in water to 6663.8 IU/mL was used as the stock solution for addition to the three milk samples fortified with CWS D_3, SDS D_3 and crystalline D_3.

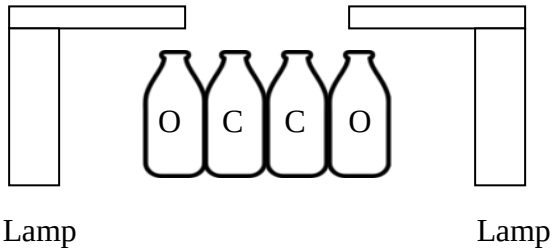
Milk samples were fortified with the prepared vitamin stock solutions to a final vitamin D concentration of 40 IU/100 mL and to a final vitamin A concentration of 218 IU/100 mL by adding a 2 mL aliquot of vitamin stock solution into 1.6 L of milk. Erythromycin was added at 100 mg/L at the same time of fortification to minimize the growth of microorganisms during the three weeks of storage. Milk was stirred for 10 minutes after the addition of vitamins and erythromycin. Each fortified milk sample was portioned into three 500 mL glass bottles (Avalon Dairy, Burnaby, BC), with two bottles exposed to light as described in Figure 4.2, and one control bottle which was wrapped in aluminum foil to prevent light exposure.

4.1.3 Chemicals and apparatus for vitamin D and vitamin A determination

The chemicals and apparatus for vitamin D were as described in section 3.1.1. Vitamin A in the form of retinyl palmitate (R3375) was purchased from Sigma-Aldrich (Oakville, ON). Retinyl propionate (sc-236667) was purchased from Santa Cruz



(a)



(b)

(c)

Figure 4.2 Set up for the exposure of fortified skimmed milk samples to light.

(a) Schematic of top view: skimmed milk fortified with four vitamin D formulations were aligned along the line of 2,000 lux, which was typical light intensity of showcase in local grocery. C - center position; O - outside position; (b) Schematic of front view; (c) photograph of light exposed samples.

Biotechnology (Santa Cruz, CA) and served as the internal standard for vitamin A determination. Ethanol, methanol, hexane, isopropanol, acetonitrile, and ethyl acetate were purchased from Fisher Scientific Canada (Ottawa, ON) in HPLC grade.

Vitamin A stock solutions ranging from 0.20 to 3.96 μg/mL and internal standard of 10 μg/mL were prepared in isopropanol and then in methanol by serial dilution. The vitamin A standards were prepared by mixing 100 μL internal standard, 400 μL vitamin A stock solution, and 500 μL dichloromethane in a HPLC vial.

4.2 Experimental set-up for the light exposure study

Milk samples were exposed to 2000 lux light intensity over the course of 22 days storage at 4 ℃. Two fluorescent lamps (Blue Planet 052-5109-0 fluorescent tubes with colour temperature of 4,100 K; Canadian Tire, Vancouver, BC) were used as the source of light. As shown in Figure 4.2, positions on both side of the lamps providing 2000 lux intensity were determined by measuring light intensity using a LuxMaster light meter (Harry's Pro Shop, Toronto, ON). For each vitamin D formulation, the control milk was stored in the dark at 4 ℃, the other two bottles were placed along the 2000 lux lines, at center and outside positions respectively.

4.3 Analysis of vitamin D and vitamin A

Milk was analyzed for vitamin D content in triplicate from light exposed bottles on day 1, day 8, day 15, and day 22 of the fortification. The control bottle was analyzed in duplicate at the same time. Vitamin D was determined using the HPLC method described in section 3.2. The retention of vitamin D in each fortified milk was calculated relative to the vitamin D content of the corresponding control milk stored in the absence of light.

Milk samples were extracted for vitamin A, retinyl palmitate based on the method of Hite (2003). In a 16×100 test tube, 0.25 mL skim milk, 0.25 mL water and 1.25 mL ethanol were added with 10 μL internal standard, then mixed on vortex for 30 seconds. The mixture was allowed to stand still for 5 min before adding 1.25 mL hexane, then vortexed again for 30 seconds. After 2 min, the addition of hexane and vortex was repeated two more times, then 0.75 mL water was added and the sample was mixed on vortex for 5 seconds. The mixture was centrifuged at 633 g for 10 min. The top hexane layer was filtered through a 0.45 μm syringe filter, then evaporated under nitrogen flush and reconstituted in 100 μL 50:50 CH_2Cl_2/MeOH for HPLC analysis.

Vitamin A was analyzed at 325 nm on an HPLC system using the same columns described in section 3.1.1 at the column temperature of 24 °C. A gradient mobile phase of 95:5 acetonitrile/water to 100% methanol was applied, followed by ethyl acetate wash (Table 4.2). The total running time was 34 min.

4.4 Microbial total plate count

3M Petrifilm Aerobic Count Plates (3M Canada, London, ON) were used to evaluate the total microbial counts. At the end of the storage of the milk samples, samples were diluted aseptically from 10^{-1} to 10^{-6} using peptone water (0.1% wt/vol) and incubated at 32 ℃ for 48 hours in an Innova®40 incubator (New Brunswick Scientific, Enfield, CT, USA) before conducting the microbial counts. Microbial analysis was conducted in duplicate.

Table 4.2 Flow rates and concentrations of mobile phase components for determination of vitamin A in fluid milk by HPLC.

Time, min	Flow rate, mL/min	Acetonitrile, %	Methanol, %	Ethyl acetate, %	Water, %
0.0	1.5	95	0	0	5
3.5	1.5	95	0	0	5
8.0	1.5	0	100	0	0
19.0	1.5	0	100	0	0
22.0	1.5	0	5	95	0
27.0	1.5	0	5	95	0
30.0	1.5	95	0	0	5

4.5 Statistical analysis

Statistical analysis was performed using Minitab 16 (State College, PA). Three-way ANOVA was conducted using general linear model analysis for repeated measures. Tukey's test was applied to investigate the significant variation in vitamin D content among the different formulations and changes with time. Regression analysis was conducted to fit the degradation of vitamin D over time.

4.6 Results and discussion

Vitamin D contents measured at days 1, 8, 15 and 22 for milk samples fortified with different vitamin formulations are shown in Table 4.3. Vitamin D_3 content dropped dramatically in all milk samples after being exposed to 2000 lux light for 22 days, while the control group retained vitamin D_3 at a similar level to that measured at day 1.

The aerobic microbial count in milk samples ranged from 0 to 10^8 CFU/mL, but no correlation was found between the vitamin D retention and microbial counts ($r = 0.147$, $n=2$).

The results of three-way ANOVA indicated that the retention of vitamin D content was significantly different between milk samples fortified with the four vitamin D_3 formulations (Table 4.4). Generally, vitamin D formulation, and exposure time were significant factors contributing to the variation of vitamin retention while no significant difference was observed between milk being stored in outside bottle versus center bottle. The interaction between sample position and vitamin D formulation was significant, indicating that vitamin D content of samples at different positions did not follow the same pattern in all formulations. The interaction of vitamin D formulation and exposure time

Table 4.3 Vitamin D_3 content (mean and standard deviation or range*) during 22 days of storage at 4 ℃ of milks fortified with four different vitamin formulations.

		Vitamin D_3 content (IU/100 mL)			
	Day	**Crystalline D_3**	**SDS D_3**	**CWS D_3**	**AD premix**
Outside	1	40.4 ± 0.6	37.4 ± 1.0	37.2 ± 0.7	40.5 ± 2.2
bottle	8	24.8 ± 0.9	26.4 ± 0.9	34.6 ± 0.2	25.4 ± 0.3
	15	19.0 ± 0.6	18.3 ± 0.5	29.0 ± 0.5	15.5 ± 0.1
	22	14.5 ± 0.4	13.8 ± 0.7	24.4 ± 0.9	10.6 ± 0.2
Center	1	40.4 ± 0.6	37.4 ± 1.0	37.2 ± 0.7	40.5 ± 2.2
bottle	8	21.9 ± 0.1	29.4 ± 0.3	34.1 ± 0.5	25.0 ± 1.1
	15	17.6 ± 0.4	18.8 ± 0.0	28.6 ± 0.2	15.2 ± 0.1
	22	14.7 ± 0.4	14.2 ± 0.7	23.8 ± 0.6	11.4 ± 0.5
Control	1	40.4 ± 0.6	37.4 ± 1.0	37.2 ± 0.7	40.5 ± 2.2
	8	37.9 ± 1.5	42.3 ± 2.5	38.1 ± 1.5	38.5 ± 0.4
	15	37.6 ± 0.4	41.1 ± 0.9	38.2 ± 0.9	38.2 ± 1.1
	22	37.7 ± 0.8	36.4 ± 0.9	38.0 ± 1.7	37.6 ± 0.5

* Standard deviation was calculated from the triplicate values of the outside and center bottles exposed to light, while range was calculated from the duplicate values of the control bottle held in the dark.

Table 4.4 Results of three-way ANOVA for retention of vitamin D_3 in fortified skimmed milk exposed to light.

Factors	DF	Seq SS	Adj SS	Adj MS	F value	Probability
Vitamin D formulation	3	0.824	0.824	0.275	1830	0.000
Exposure time	3	4.611	4.611	1.537	10230	0.000
Sample position	1	0.000	0.000	0.000	1.74	0.191
Sample position × vitamin D formulation	3	0.008	0.008	0.003	17.7	0.000
Vitamin D formulation × exposure time	9	0.316	0.316	0.035	233	0.000
Sample position × exposure time	3	0.001	0.001	0.000	1.96	0.129
Three-way interaction	9	0.011	0.011	0.001	8.41	0.000
Error	64	0.010	0.010	0.000		
Total	95	5.780				

was also significant, indicating that the vitamin D in different formulations degraded at different rates during the storage. The three way interaction was also significant. Each significant factor or interaction is further discussed in the following paragraphs.

The trend of vitamin D retention in the four formulations can be seen in Figure 4.3. CWS D_3 was obviously more stable than the other three vitamin D formulations. Since the mechanism of vitamin D degradation is not established, regression analysis using different models was tried to fit the degradation trend. The polynomial models gave the highest R^2 values. Based on the four time points of data, the decrease in retention of SDS D_3 followed a second order polynomial trend while the retention of CWS D_3, crystalline D_3 and AD premix was best fit using third order polynomial equations.

The result of Tukey's test for mean vitamin retention at different storage time is shown in Table 4.5. It confirmed that the retention of vitamin D in CWS D_3 samples was significantly greater than that in the other three groups after exposure to light. At day 8, SDS D_3, AD premix and crystalline D_3 had a similar retention of vitamin D on light exposure, while at day 15, retention of vitamin D in all formulations were significantly different from one another. In particular, the AD premix started to show greater vitamin D loss compared with the other three. At day 22, AD premix showed lowest retention of vitamin D while vitamin D in crystalline D_3 fortified milk remained at a similar level to that of SDS D_3 fortified milk.

The crystalline D_3 was expected to be labile to light since it was reported that vitamin D is sensitive to light especially when riboflavin is present (King & Min, 1998). The AD premix was an emulsified form and showed a lower stability than the crystalline

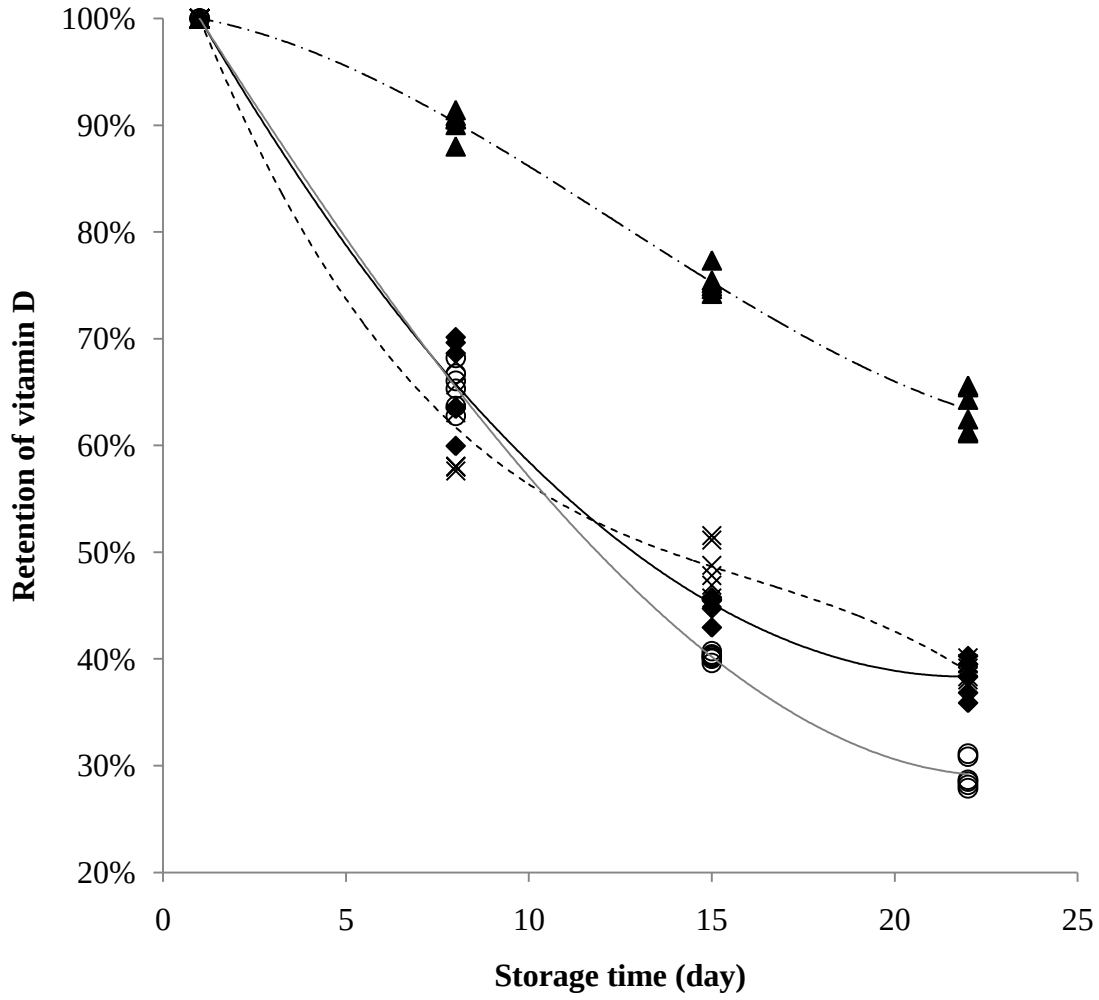


Figure 4.3 Trends of percent retention of vitamin D_3 content in skimmed milk fortified with four different formulations during 22-day storage at 4 °C with light exposure.

Crystalline D_3 SDS D_3 CWS D_3 AD premix

Crystalline D_3	$y = -0.0001x^3 + 0.0051x^2 - 0.0932x + 1.0882$	$R^2 = 0.990$
SDS D_3	$y = 0.0014x^2 - 0.0616x + 1.0607$	$R^2 = 0.992$
CWS D_3	$y = 4E\text{-}05x^3 - 0.0015x^2 - 0.0034x + 1.0049$	$R^2 = 0.993$
AD premix	$y = 2E\text{-}05x^3 + 0.0004x^2 - 0.0544x + 1.054$	$R^2 = 0.998$

Table 4.5 Tukey's test of vitamin D retention of four formulations with different light exposure times.

Exposure time, days	Vitamin D retention, %			
	Crystalline D_3	**SDS D_3**	**CWS D_3**	**AD premix**
1	100 AW*	100 AW	100 AW	100 AW
8	62 BX	66 BX	90 AX	65 BX
15	49 BY	45 CY	75 AY	40 DY
22	39 BZ	38 BZ	63 AZ	29 CZ

* A, B, C, & D indicate significant difference between formulations (within row) and W, X, Y, & Z indicate significant differences between days of storage (within column).

D_3. The significant difference between the retention of AD premix and crystalline D_3 may be due to the instability of other compounds in the premix. The two encapsulated vitamin D_3 forms showed different vitamin D retention. The CWS D_3 was formulated by dissolving vitamin D_3 in droplets of medium chain triglycerides, then encapsulated in the matrix of acacia gum and sucrose with a coating of corn starch. In SDS formulation, vitamin D_3 was finely dispersed in a matrix of modified starch, sucrose and medium chain triglycerides. The properties and structure of encapsulation matrix may cause the variation of vitamin D stability to light.

Despite having more surface area exposed to light, vitamin D in outside bottles was not always higher than center bottles (Figure 4.4). Furthermore, vitamin D content was not significantly different in milk placed at center and outside positions according to ANOVA analysis.

A significant decline in vitamin D content was observed at each test day during the storage. This indicated the degradation of vitamin D in all formulations was ongoing during the 22-day storage.

The interaction of sample position and vitamin formulation was significant, which means higher vitamin D retention was not always found in one position in all formulations. For example, as shown in Figure 4.4, crystalline D_3 fortified milk in outside bottles retained more vitamin D than milk in center bottles at the 8th day. Milk fortified with SDS D_3 showed a reversed pattern, in which more vitamin D was found in center bottles. Interaction of formulation and exposure time was also significant, which indicated that vitamin D in different formulations degraded at different rate, and this rate was changing during the storage, as shown by the trend lines in Figure 4.3.

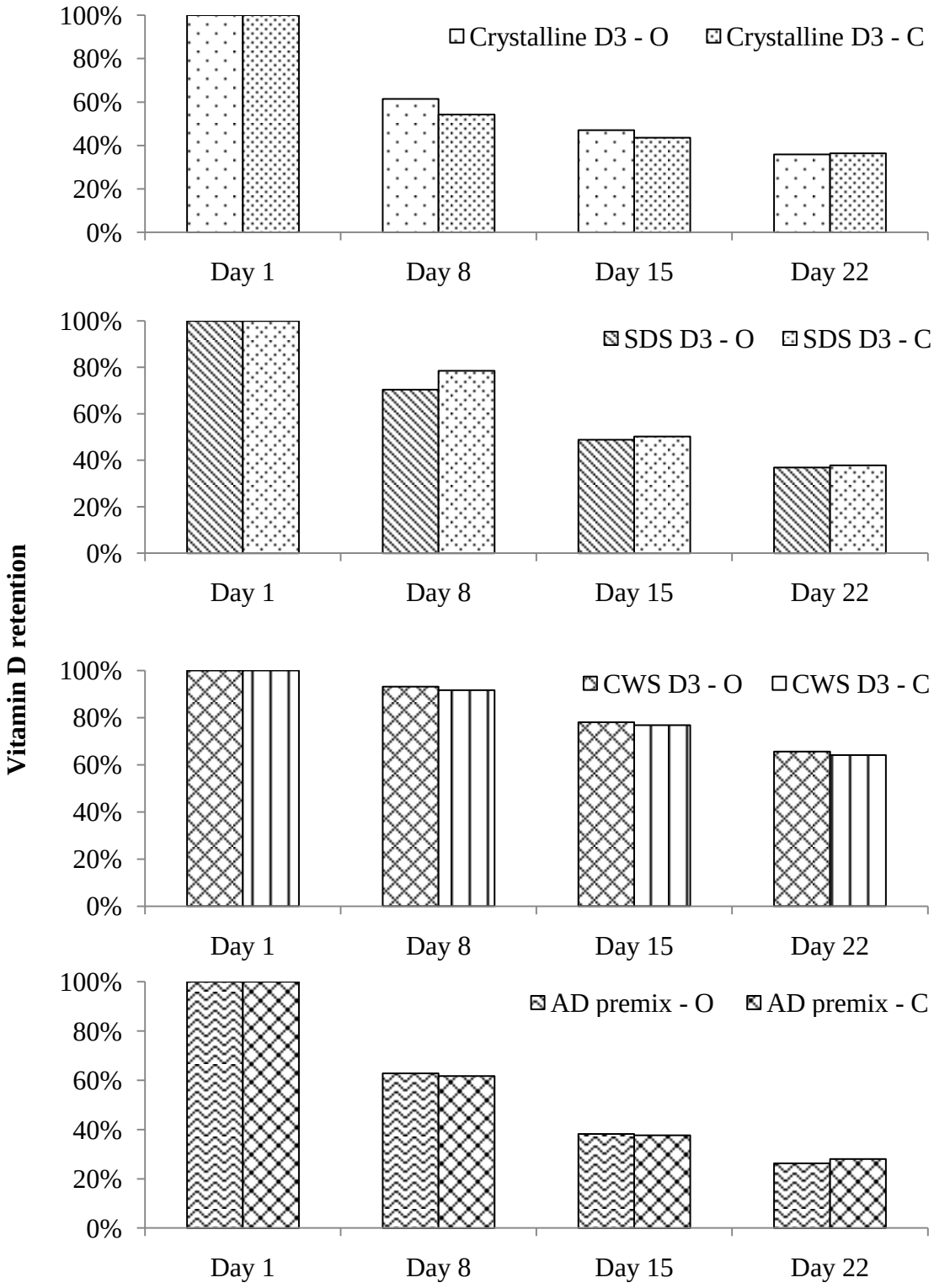


Figure 4.4 Vitamin D % retention in outside bottle (- O) and center bottle (- C) of skimmed milk fortified with four vitamin D_3 formulations (n=3).

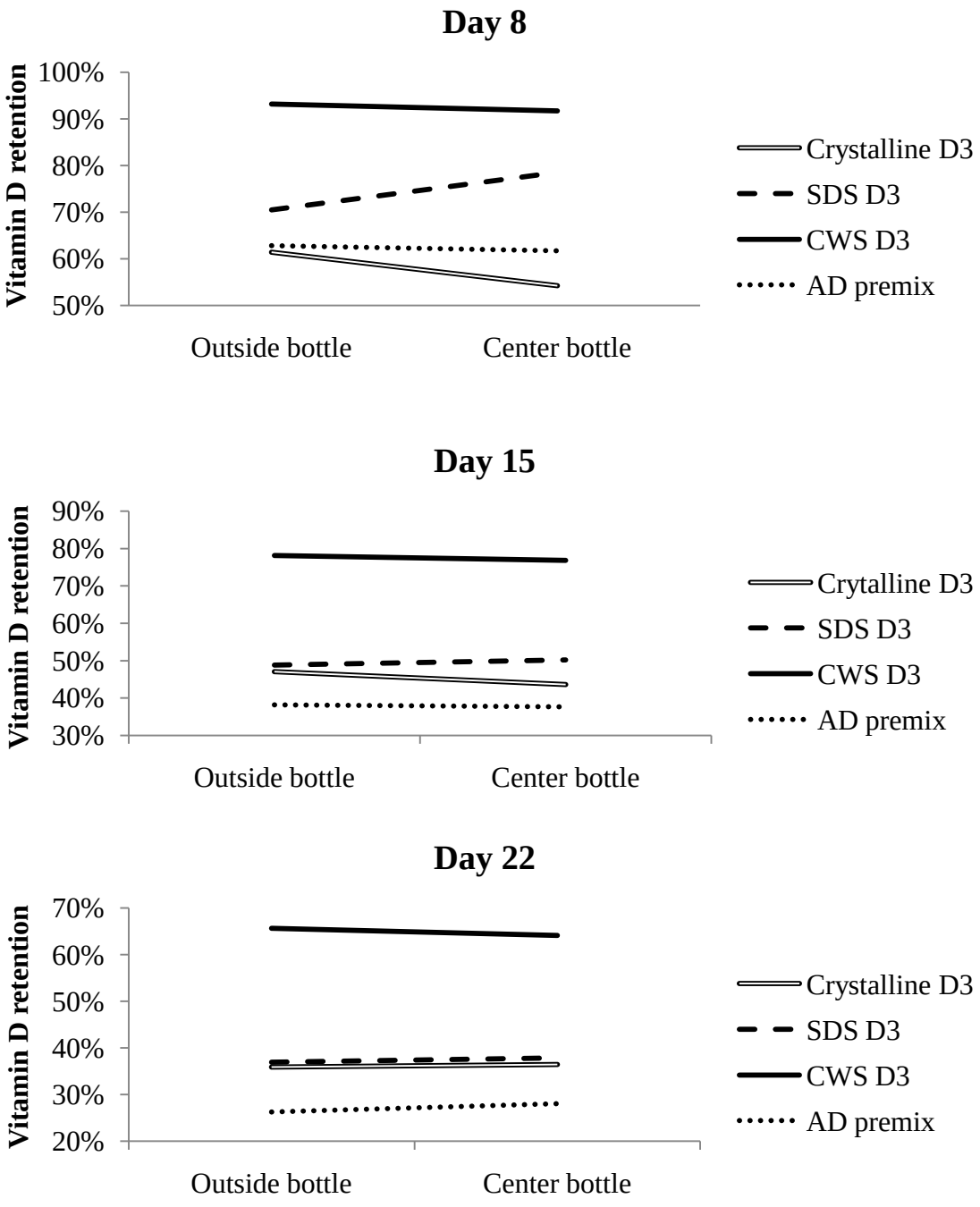
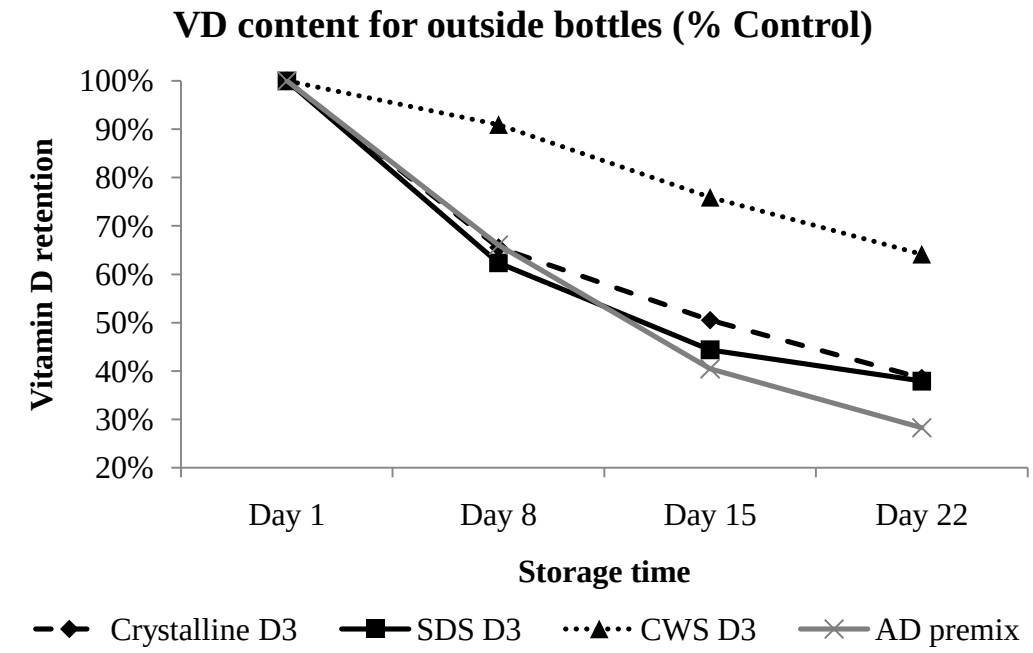


Figure 4.5 Vitamin D retention as a function of the interaction of formulation and position at different exposure times.

The two way interaction between vitamin D formulation and sample position or exposure time were both significant. The significant three way interaction indicated the interaction between two factors changes when introducing the third factor. For example, the interaction of formulation and position was changed by the exposure time. The interaction of formulation, position and exposure time was plotted in Figure 4.5. The interaction between the formulation and time exposure also followed a different pattern in the outside bottle and center bottle, as shown in Figure 4.6.

Since vitamin A and D fortification is mandatory for skim milk (Food and Drug regulations, 2012), industry usually uses a premix of vitamin A and D for skim milk fortification. For the purpose of comparison with vitamin AD premix, water dispersible vitamin A was added into the milk samples fortified with the other three vitamin D formulations, and the contents of vitamin A were analyzed for the samples collected at day 8 and 22 of storage at 4 °C. The results are summarized in Table 4.6. Vitamin A in milk samples were almost degraded completely on light exposure by the end of the storage. However, in the control bottles kept in the dark, more than 90% of vitamin A still remained in milk fortified with AD premix while little water dispersible vitamin A remained in control milk fortified with the other three vitamin D formulations. A follow-up experiment was conducted, in which skimmed milk was fortified with only the water dispersible vitamin A or together with either CWS D_3 or crystalline D_3, and stored in the dark at 4 °C. The results showed that the degradation of water dispersible vitamin A started within one week even in the dark (Table 4.7). The vitamin D loss in this research was higher than those reported by Renken and Warthesen (1993) and Saffert et al. (2008



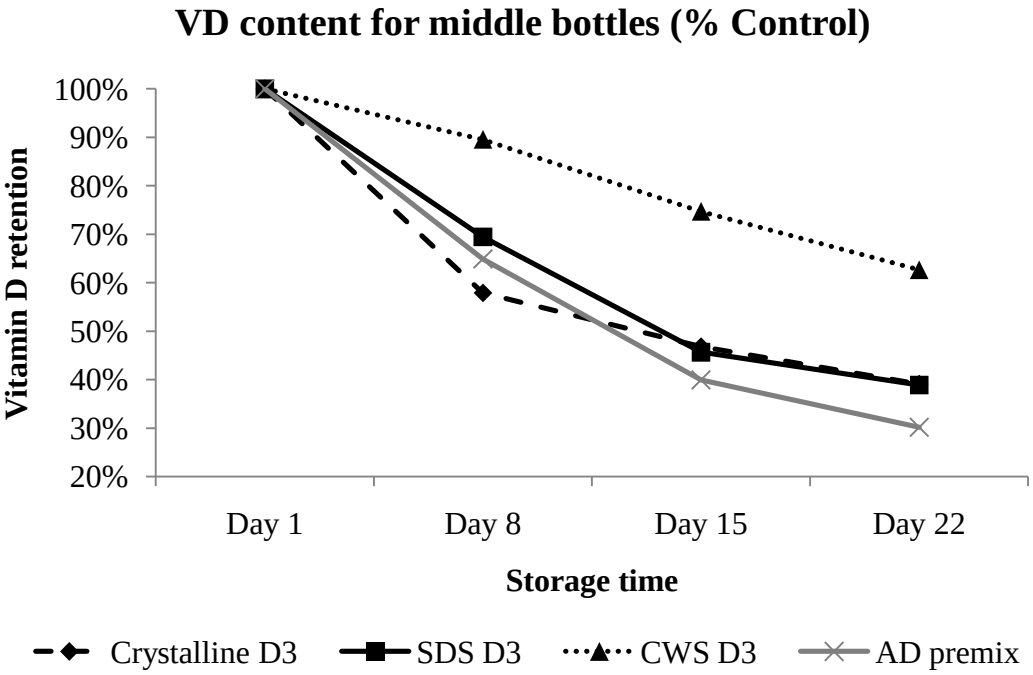


Figure 4.6 Vitamin D retention as a function of the interaction of formulation and exposure time at different positions.

Table 4.6 Vitamin A content (IU/100 mL) in skimmed milk fortified with vitamin A and D exposed to light of 2000 lux or stored in the dark (n=2).

Vitamin D formulation	**Day 8**		**Day 22**	
	Light exposed	**In the dark**	**Light exposed**	**In the dark**
Crystalline D_3	0.0	9.8	0.0	11.4
SDS D_3	0.0	16.8	0.0	9.9
CWS D_3	0.0	20.3	0.0	7.3
AD premix	12.0	206.3	0.0	185.2

Table 4.7 Vitamin A content (IU/100 mL) in skimmed milk fortified with vitamin A or vitamin A and D, and stored in the dark (n=2).

Milk fortification	**Initial concentration**	**After 1 week**	**After 3 weeks**
Vitamin A only	220.6	49.3	6.5
CWS D_3 and Vitamin A	206.3	44.3	7.3
Crystalline D_3 and vitamin A	217.7	54.0	9.3

and 2009). Whether the rapid degradation of vitamin A accelerated the degradation of vitamin D remains to be confirmed.

Bartholomew and Ogden (1990) suggested that added vitamin A might be more sensitive to light than the indigenous vitamin A but commercial emulsifiers might improve the stability of fortified vitamin A since it could provide as good protection as the milk fat. In the present research, the AD premix was an emulsified form containing polysorbate 80 and polyglycerol monooleate as emulsifiers. This may enhance the stability of vitamin A in AD premix compared to the water dispersible form when being stored in the dark. However, both vitamin A forms were not stable upon light exposure.

4.7 Conclusion

Commercial vitamin D_3 formulations did show different stability to the light exposure in skimmed milk. Significant vitamin D losses were observed for all milk samples being exposed to light. Vitamin D retention was not associated with the position of the sample container although the center container had less area being exposed to light than outside container.

The added water dispersible vitamin A and the vitamin A in AD premix degraded completely on light exposure after 22 days storage. However, the water dispersible vitamin A degraded significantly even when being stored in the dark, while the vitamin A content in AD premix was retained at more than 90% of the original concentration in the dark. More research needs to be done to determine whether the degradation of vitamin A may have an impact on vitamin D stability.

Chapter 5: Thermal stability of vitamin D_2 and vitamin D_3 solutions at pH 3.5 and 6.6

Both vitamin D_2 and D_3 can be used for food fortification despite the debate on potency of these two forms. The Food and Drug Regulations (2012) does not specify which form of vitamin D should be used for food fortification. Although usually vitamin D_3 is often added to milk products, milk fortified with vitamin D_2 has also been reported. Vitamin D_2 might also be used for fortification for some milk substitutes or other beverages, such as soy milk, almond milk, and orange juice.

Studies in the pharmaceutical field indicated that in solid form, vitamin D_2 and D_3 demonstrated different stability under various conditions (Byrn, 1976; Grady & Thakker, 1980). However there are few studies published for the stability comparison of the two vitamin D forms in liquid solution or foods. In this research, a model system at typical pH of orange juice and milk was set up to compare the thermal stability of vitamin D_2 and D_3. Crystalline vitamin D_2 and D_3 content in buffer of pH 3.5 and 6.6 were analyzed before and after thermal treatment of 72°C for 15 seconds since this condition is typical for milk pasteurization (CFIA, 2010). The experiment design is shown in Figure 5.1.

5.1 Materials

Buffer solutions with pH 3.5 and pH 6.6 were used to test the thermal stability of vitamin D_2 and D_3. Sodium phosphate dibasic (0.2 M) and citric acid (0.1 M) were used to prepare the buffer. The buffer pH was adjusted to the specific level using a Corning Pinnacle M530 pH meter (Gold River, CA). Crystalline vitamin D_2 (E5750) and D_3 (C9756) from Sigma-Aldrich (Oakville, ON) were used to make methanol stock solution

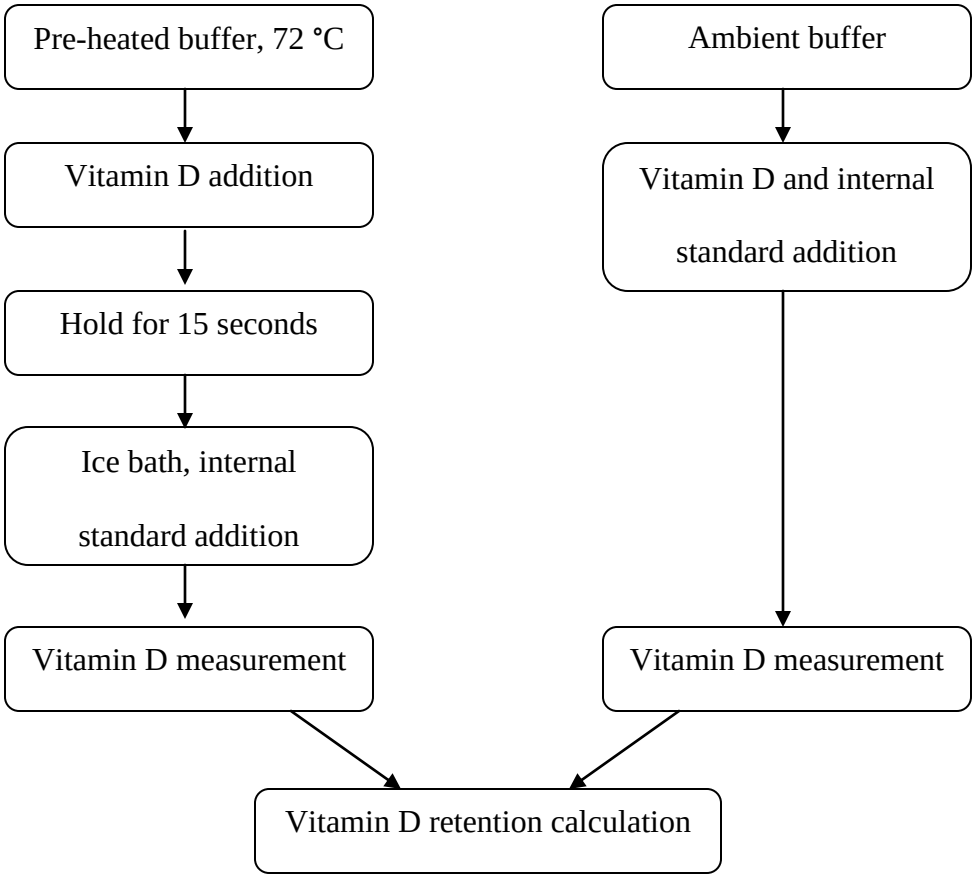


Figure 5.1 Overview of study design for stability of vitamin D_2 and D_3 to thermal treatment in a model system.

of 200 IU/mL and 160 IU/mL respectively. For the purpose of quantification, vitamin D_2 was used as internal standard for vitamin D_3 and vitamin D_3 was internal standard for vitamin D_2 determination.

5.2 Thermal processing

Three glass tubes each containing 4 mL of pH 3.5 or pH 6.6 buffer were heated up to 72 ℃ using water bath (Blue M, Blue Island, IL). A thermometer was placed in a fourth tube to monitor the temperature of the buffer. An 80 µl aliquot of vitamin D_2 or 100 µl of vitamin D_3 (ca. 16 IU) was added into the pre-heated buffer solution without taking the tubes from the water bath. This amount of vitamin D gives a similar final concentration as vitamin levels in fortified milk. After holding the temperature for 15 seconds, the tube was removed and placed into an ice bath immediately. Then 80 µl of vitamin D_2 or 100 µl of vitamin D_3 (ca. 16 IU) was added as an internal standard into the tube in ice bath. The thermal treatment was conducted in three replicates. Another set was held at ambient temperature as controls.

5.3 Vitamin D analysis

The vitamin D was extracted and analyzed on HPLC using a similar protocol described in section 3.2 except that saponification and SPE purification steps were not necessary. The vitamin D retention was calculated by comparing the vitamin D content in the thermal treated milk with that in the unheated milk. The retentions were processed using two-way ANOVA.

5.4 Statistical analysis

Two-way ANOVA was conducted using Minitab 16 (State College, PA) to investigate

the difference in the vitamin D retention between vitamin D_2 and D_3 in pH 3.5 and 6.6 buffers.

5.5 Results and discussion

Over 90% retention of vitamin D was observed after thermal treatment for both forms of vitamin D (Table 5.1). Only the treatment at pH 6.6 of vitamin D_3 showed a significant difference ($P < 0.05$) when comparing with the corresponding unheated sample using Tukey's test. However, the difference was less than 8%.

The results of ANOVA are shown in Table 5.2. Two way ANOVA indicated that there was no significant difference between vitamin D_2 and D_3 samples in vitamin D retention after thermal treatment. The thermal stability of vitamin D in pH 3.5 versus pH 6.6 buffer was also not significantly different . These results are similar to what Mattila *et al.* (1999) found in house cooking process of egg, fish and mushroom.

5.6 Conclusion

Vitamin D_2 and D_3 showed a similar thermal stability at pH 3.5 and 6.6 to High Temperature Short Time (HTST) treatment of 72 ℃ for 15 seconds. The total vitamin D loss was less than 8%. For the purpose of fortification, both vitamin D_2 and D_3 can be added to liquid food at pH range of 3.5 to 6.6 since the two forms of vitamin D showed similar stability. Both forms can be added before the HTST pasteurization since this process showed little impact on vitamin D content.

Table 5.1 The impact of thermal treatment (72 °C for 15 seconds) on vitamin D content in pH 3.5 and pH 6.6 buffers. Values are mean values ± standard deviation (SD, n=3) or range (n=2).

Vitamin D form and pH of buffer	Vitamin D content (IU)						Vitamin D retention %
	Unheated (n=2)			72 ℃ 15"(n=3)			
	Mean	±	Range	Mean	±	SD	
Vitamin D_3, pH 3.5	15.39	±	0.19	15.01	±	0.46	97.54%
Vitamin D_2, pH 3.5	16.70	±	0.62	15.74	±	0.46	94.23%
Vitamin D_3, pH 6.6	16.79	±	0.38	15.51	±	0.20	92.32%
Vitamin D_2, pH 6.6	16.05	±	0.52	15.28	±	0.72	95.15%

Table 5.2 The result of two-way ANOVA for vitamin D_2 and D_3 retention after thermal treatment (72 ℃ for 15 seconds) at pH 3.5 or pH 6.6.

Factors	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F value**	**Probability**
pH	1	0.001	0.001	0.001	1.46	0.262
Vitamin D form	1	0.000	0.000	0.000	0.02	0.896
pH * vitamin D form	1	0.003	0.003	0.003	2.96	0.123
Error	8	0.008	0.008	0.001		
Total	11	0.012				

Chapter 6: General discussion and conclusions

The present research investigated the vitamin D content in 104 cartons of fluid milk samples sold in Vancouver between November 2011 and September 2012. Fortification non-compliance was identified in 58% of the sampled milk using 35.2 to 46.9 IU/100 mL as the criteria for compliance.

The fortification compliance of vitamin D in fluid milk in the present research was compared with the investigation on BC milk reported in 1993 by Chen *et al.* For the purpose of comparison, 20% margin of tolerance relative to the claimed fortification level was applied to evaluate the compliance (Figure 6.1). After 19 years, there was an obvious improvement in vitamin D fortification of milk. The percentage of samples containing 80-120% of the claimed vitamin D amount increased from 27% to 65%. However, 27% of the sampled milks sampled in 2012 are under-fortified, which is the same percentage of under-fortification reported for the 15 milks sampled from British Columbia as reported by Chen *et al.* in 1993.

Wide variation was found among different brands and between skim versus 2% or 3.25% milk. Vitamin D content was also found to fluctuate between milk sampled at different times. All three factors (brand, fat content and sampling set) were significant contributors to the vitamin D variation. Some possible reasons are summarized in Table 6.1. More research needs to be done to understand the causes of the recurring under-fortification. Of seven brands sampled in the present research, vitamin D in skim milk from brand 5 and 7 were consistently low. These two brands were produced by the same dairy processor. Consistently low vitamin D content may indicate a systematic

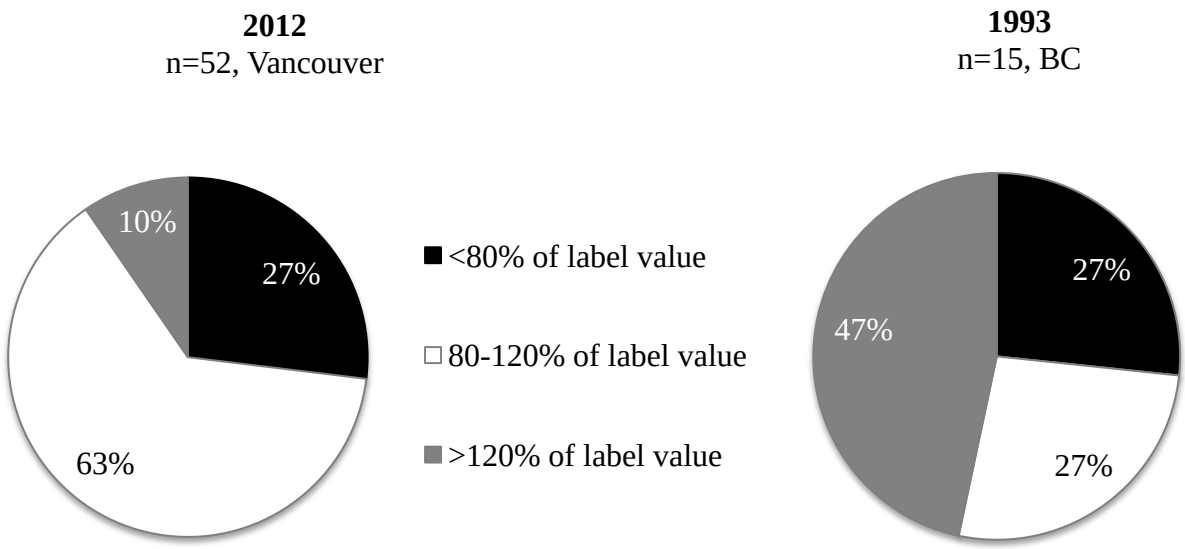


Figure 6.1 The percentages of milk samples categorized as under-fortified, over-fortified or satisfying the fortification target for vitamin D (based on a 20% margin of tolerance) in the present study (2012) compared to the study by Chen *et al.* (1993).

Table 6.1 Possible reasons contributing to the variation in vitamin D content in sampled milk.

Significant Factor	Possible reasons for the variation in vitamin D content
Brand	Different vitamin formulation
	Different vitamin addition method (injection or batch addition)
	Different processing procedure for vitamin addition
	Different storage condition for both dry vitamin and working solution
	Different packaging
Milk fat content	Possible protection provided by milk fat
	Different vitamin formulation used for milk with different fat content considering the solubility of vitamin D
Sampling set	Poor quality management
	Degradation of vitamin working solution during storage
	Any changes in the production (formulation or fortification procedure) may change the final concentration of vitamin D

mistake in the vitamin D addition protocol from this processor. For example, the calculation of vitamin D addition might be incorrect because of inaccurate concentration information from vitamin supplier or simply using wrong calculation. Since vitamin A needs to be added into skim milk as well, the vitamin D formulation used for skim milk is usually different from that used for the 3.25% milk. Low vitamin D content might also be caused by the low solubility of the fat soluble vitamin formulations in skim milk.

The investigation in North Carolina (Hicks *et al.*, 1996) showed vitamin D was added at different processing steps in different plants and different storage conditions were applied on vitamins. The impact of milk processing on vitamin D is not well studied. However, Bartholomew and Ogden (1990) suggested that the homogenization step improved the blending of fat soluble vitamin with milk solid and milk fat, which acted as natural emulsifiers and provided protection. Bartholomew and Ogden (1990) also reported that blending vitamin A into milk fat after separation was more effective than adding vitamin A as a highly concentrated solution. Since vitamin D is also a fat soluble vitamin, different addition points and solution preparation might introduce variation into the final concentration in milk.

Besides the processing procedure, vitamin D formulations also have different stability, as indicated in Chapter 4. If different vitamin formulations are applied in milk fortification by milk processors, the vitamin D concentration of final products may be variable as well. The storage conditions also affect the stability of both dry and emulsified vitamin D. If processing and formulation information could be collected from processors, it would be helpful to understand why wide variation of vitamin D content existed in milk products.

In the present research, only fluid milk of Vancouver was included. To understand the national trend, milk with different fat content of different areas should be analyzed.

The stability to light exposure of four commercial vitamin D formulations was found to be different. A well encapsulated formulation may reduce the vitamin D loss in milk during storage. The impact of light exposure on vitamin D in fluid milk has been reported previously (Renken & Warthesen, 1993; Saffert et al., 2008 and 2009). Although different milk samples and storage conditions were applied in these studies, significant vitamin D losses were observed in milk when being exposed to light. The results of previous studies and the present study are summarized in Table 6.2.

In the study of Saffert et al. (2008), unfortified milk samples were studied and thus only indigenous vitamin D was present. In the other three studies, milk samples were fortified with vitamin D at concentration ranging from 40 to 48 IU/100 mL. The vitamin D loss in the study reported by Saffert *et al.* (2009) at the end of week 4 was much lower than that at the end of week 3 in the present research, despite higher storage temperature. By the end of week 12, the total exposure reached 1092 hours, much longer than 504 hours in the present research. However the vitamin D loss is still lower. The lower light intensity of 700 lux might be the reason. In the study of Saffert *et al.* (2008), after 1092 hours exposure at 3000 lux, the vitamin D loss also remained lower than the present research. For both studies of Saffert *et al.* (2008 & 2009), the details of vitamin D formulation and fortification protocol were not given. A different vitamin D formulation might have been used and affected the vitamin D loss.

Table 6.2 Comparison of vitamin D loss in milk exposed to light during storage.

		Initial content (IU/100 mL)			Vitamin D loss			
Reference	**Sample and package**	**Vitamin D**	**Vitamin A**	**Storage conditions**	**Week 2**	**Week 3**	**Week 4**	**Week 12**
The present study	Pasteurized skimmed milk 500 ml in glass bottle	40	200	2000 lux, 24 hrs/day, 4 ℃	25-60%	37-71%	NA *	NA
Saffert *et al.*,. 2009	UHT 1.5% fat milk 1 litre in clear plastic jug	46 - 48	675-739	700 lux, 13 hrs/day, 23 ℃	10%	NA	33%	65%
Saffert *et al.*, 2008	Unfortified UHT whole milk 1 litre in clear plastic jug	0.49	110	a - 700 lux b - 1700 lux c - 3000 lux 13 hrs/day, 23 ℃	NA	NA	a - 14% b - 12% c - 24%	a - 39% b - 49% c - 57%
Renken & Warthesen, 1993	Pasteurized skim milk a - 1.8 litre in plastic jug b - 30 ml in 20×150 mm glass tube	42	Unknown	3229 lux, 24 hrs/day, 4 ℃	a - 25% b - 50%	NA	NA	NA

*NA = not applicable

The storage condition of the present study is close to that of Renken and Warthesen's research. Light exposure was applied in a 24 hours/day manner and skim milk was fortified at a similar concentration. Plastic containers and glass tubes were used in the research of Renken and Warthesen. The plastic container might provide better protection than the clear glass containers used in the present research. The vitamin D loss in glass tubes was expected to be higher than that in the 500 mL glass bottle of the present research since higher light intensity was applied. However, the vitamin D loss was slightly lower than that of the present research at the end of week 2 although higher light intensity was applied. Again, different vitamin D formulation might have been used.

In the present research, the vitamin D stability was not different between the center bottle and outside bottle despite more surface area being exposed to light in the outside bottles. Saffert *et al.* (2008) found that the vitamin D loss in unfortified milk held in clear packages was independent of the light intensity. However, the initial vitamin D content was so low in unfortified milk, the conclusion of Saffert *et al.* (2008) may not be applicable to the degradation of vitamin D in fortified milk.

The instability of vitamin D to light is caused by the singlet oxygen produced by a photo-sensitizer (Renken and Warthesen, 1993; King & Min, 1998 & 2002). As a photo-sensitizer, riboflavin can produce singlet oxygen on the exposure of light (King & Min, 1998 & 2002). According to U.S. Department of Agriculture, Agricultural Research Service (2012), the riboflavin content in 100 g skim milk is about 0.182 mg. A clear package, either plastic or glass, with high transmittance of light, may provide enough energy for riboflavin to produce adequate singlet oxygen and start the oxidation of

vitamin D, and thus no significant difference of vitamin D loss was found between bottles despite the different light intensity being applied and different exposure area.

As mentioned in section 4.6, vitamin A in fortified skimmed milk was completely degraded after storage with light exposure. Crank and Pardijanto (1995) suggested that the photosensitized oxidation of vitamin A is initiated by singlet oxygen. Since riboflavin produces singlet oxygen on light exposure, its presence in the milk may accelerate the degradation of vitamin A. However, whether the degradation of vitamin A affects the stability of vitamin D or not still remains unknown. If the degradation of vitamin A also has an impact on vitamin D stability, then the different amount of vitamin A in the four studies of Table 6.2 may also have caused the different vitamin D loss. To confirm this, more research needs to be done.

As a conclusion, non-compliance, especially under-fortification, is still a problem of vitamin D fortification of milk in Vancouver. Both vitamin D_2 and D_3 are stable during HTST treatment in liquid system at pH 3.5 or 6.6. Different vitamin D formulations differ in their stability to light. Encapsulated vitamin D with good protection may reduce the vitamin D loss on light exposure.

The present research only analyzed skim and 2% or 3.25% milk. To better understand the vitamin D variation in commercial milk, analysis of vitamin D needs to be conducted on milk products with different fat content from different brands across the country. Considering the fluctuating vitamin D content between different lots, information about the fortification protocols provided by milk processors would be helpful to screen the possible sources of lot-to-lot variation. However, such information

might be very difficult to obtain from the industry since many processors consider processing information confidential.

The light stability of different vitamin D formulations were only tested in skimmed milk. Whether the stability might be affected by fat content is still unclear despite higher vitamin D content was found in 3.25% commercial milk than in skim milk. The potential protection provided by milk fat still needs to be confirmed by further research.

The possible impact of vitamin A degradation on vitamin D also needs to be investigated. Based on the results obtained in the present research, using formulation with higher stability or avoiding light exposure during storage would improve the retention of vitamin D content in milk product and thus may help to improve the compliance of fortification. Vitamin D_2 showed similar thermal stability as vitamin D_3 at pH 3.5 and 6.6. Both vitamin D forms are suitable for the fortification of food system at these pH values from the point of stability under pasteurization.

References

Amberg-Schwab, S., Weber, U., Burger, A., Nique, S., & Xalter, R. (2006). Development of passive and active barrier coatings on the basis of inorganic–organic polymers. *Monatshefte für Chemie - Chemical Monthly, 137,* 657-666.

Armas, L. A. G., Hollis, B. W., & Heaney, R. P. (2004). Vitamin D_2 is much less effective than vitamin D_3 in humans. *Journal of Clinical Endocrinology & Metabolism, 89,* 5387-5391.

Ball, G. F. M. (2006). *Vitamins in foods: Analysis, bioavailability, and stability*. Boca Raton, FL: CRC Press.

Bartholomew, B. P., & Ogden, L. V. (1990). Effect of emulsifiers and fortification methods on light stability of vitamin A in milk. *Journal of Dairy Science, 73,* 1485-1488.

Bartolucci, G., Giocaliere, E., Boscaro, F., Vannacci, A., Gallo, E., Pieraccini, G., & Moneti, G. (2011). Vitamin D_3 quantification in a cod liver oil-based supplement. *Journal of Pharmaceutical and Biomedical Analysis, 55,* 64-70

Beindorff, C.M. & Zuidam, N.J. (2010) Microencapsulation of fish oil. In N.J. Zuidam & V.A. Nedovic (Ed.), *Encapsulation technologies for active food ingredients and food processing* (pp. 161-185). New York, NY: Springer.

Blake, C. J. (2007). Status of methodology for the determination of fat-soluble vitamins in foods, dietary supplements, and vitamin premixes. *Journal of the Association of Official Analytical Chemists*, 90, 897-910.

Blanco, D., Fernandez, M. P., & Gutierrez, M. D. (2000). Simultaneous determination of fat soluble vitamins and provitamins in dairy products by liquid chromatography with a narrow-bore column. *Analyst, 125,* 427-431.

Blank, S., Scanlon, K. S., Sinks, T. H., Lett, S., & Falk, H. (1995). An outbreak of hypervitaminosis D associated with the overfortification of milk from a home-delivery dairy. *American Journal of Public Health, 85,* 656-659.

Byrn, S. R. (1976). Mechanisms of solid-state reactions of drugs. *Journal of Pharmaceutical Sciences, 65,* 1-22.

Calvo, M. S., & Whiting, S. J. (2003). Prevalence of vitamin D insufficiency in Canada and the United States: Importance to health status and efficacy of current food fortification and dietary supplement use. *Nutrition Reviews, 61,* 107-113.

Calvo, M. S., Whiting, S. J., & Barton, C. N. (2004). Vitamin D fortification in the United States and Canada: Current status and data needs. *American Journal of Clinical Nutrition, 80,* 1710S-1716S.

CFIA. (2010). Dairy Establishment Inspection Manual: Chapter 11 HTST pasteurization Tasks. Retrived from http://www.inspection.gc.ca/english/fssa/dailai/man/estman/ch11e.shtml

CFIA. (2012). Dairy products inspection manual: Chapter 9 vitamin addition. Retrieved from http://www.inspection.gc.ca/english/fssa/dailai/man/proman/ch9e.shtmlChen, T. C., Shao, Q., Heath, H., & Holick, M. F. (1993). An update on the vitamin-D content of fortified milk from the United States and Canada. *New England Journal of Medicine, 329,* 1507-1507.

Cole, D. E., Hawker, G. A., Rubin, L. A., Trang, H. M., & Vieth, R. (2001). Wintertime vitamin D insufficiency is common in young Canadian women, and their vitamin D intake does not prevent it. *European Journal of Clinical Nutrition, 55,* 1091-1097.

Crank, G., & Pardijanto, M.S. (1995). Photooxidations and photosensitized oxidations of vitamin A and its palmitate ester. *Journal of Photochemistry and Photobiology A: Chemistry*, *85,* 93-100.

Cunniff, P. (Ed.). (1995). *Official methods of analysis of AOAC International.* Arlington, VA: AOAC International.

DeRitter, E. (1982). Vitamins in pharmaceutical formulations. *Journal of Pharmaceutical Sciences, 71,* 1073-1096.

Desai K. G. H., & Park H. J. (2006). Effect of manufacturing parameters on the characteristics of Vitamin C encapsulated tripolyphosphate-chitosan microspheres prepared by spray-drying. *Journal of Microencapsulation, 23,* 91-103.

Devries, E. J., & Borsje, B. (1982). High performance liquid chromatographic and gas-liquid chromatographic determination of vitamin D in fortified milk and milk powder - collaborative study. *Journal of the Association of Official Analytical Chemists, 65,* 1228-1234.

Faulkner, H., Hussein, A., Foran, M., & Szijarto, L. (2000). A survey of vitamin A and D contents of fortified fluid milk in Ontario. *Journal of Dairy Science, 83,* 1210-1216.

Food and Drug Regulations. (2012). Retrieved from the Department of Justice Canada website: http://laws.justice.gc.ca/eng/regulations/C.R.C.,_c._870/FullText.html

Forrest, S. A., Yada, R. Y., & Rousseau, D. (2005). Interactions of vitamin D_3 with bovine β-lactoglobulin A and β-casein. *Journal of Agricultural and Food Chemistry, 53,* 8003-8009.

Gaylord, A. M., Warthesen, J. J., & Smith, D. E. (1986). Influence of milk fat, milk solids, and light intensity on light stability of vitamin A and riboflavin in lowfat milk. *Journal of Dairy Science, 69,* 2779-2784.

Gosta B. (Eds.). (1995). *Handbook of dairy processing*. Lund, Sweden: Tetra Pak Processing Systems.

Grace, M. L., & Bernhard, R. A. (1984). Measuring vitamins A and D in milk. *Journal of Dairy Science, 67,* 1646-1654.

Grady, L. T., & Thakker, K. D. (1980). Stability of solid drugs: Degradation of ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) at high humidities and elevated temperatures. *Journal of Pharmaceutical Sciences, 69,* 1099-1102.

Hagar, A.F., Madsen, L., Wales, L., & Bradford, H. B. (1994). Reversed phase liquid chromatographic determination of vitamin-D in milk. *Journal of the Association of Official Analytical Chemists, 77,* 1047-1051.

Hanson, A. L., & Metzger, L. E. (2010). Evaluation of increased vitamin D fortification in high temperature, short time processed 2% milk, UHT processed 2% fat chocolate milk, and low fat strawberry yogurt. *Journal of Dairy Science, 93,* 801-807.

Health Canada. (2004). Interim marketing authorization. Canada Gazette. 138. Retrieved from http://gazette.gc.ca/archives/p1/2004/2004-11-06/html/notice-avis-eng.html

Health Canada. (2005). Addition of vitamins and minerals to foods, 2005: Health Canada's proposed policy and implementation plans. Retrieved from http://www.hc-sc.gc.ca/fn-an/nutrition/vitamin/fortification_final_doc_1-eng.php

Health Canada. (2006). Interim marketing authorization. *Canada Gazette, 140*(28). Retrieved from http://gazette.gc.ca/archives/p1/2006/2006-07-15/html/notice-avis-eng.html

Health Canada. (2006). Interim marketing authorization. *Canada Gazette, 140*(39). Retrieved from http://gazette.gc.ca/archives/p1/2006/2006-09-30/html/notice-avis-eng.html

Health Canada. (2011a). *Reference values for vitamins.* Retrieved from http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/ref_vitam_tbl-eng.php

Health Canada. (2011b). Interim marketing authorization. *Canada Gazette, 145*(8). Retrieved from http://www.gazette.gc.ca/rp-pr/p1/2011/2011-02-19/html/notice-avis-eng.html

Heaney, R. P., Recker, R. R., Grote, J., Horst, R. L., & Armas, A. G. (2011). Vitamin D_3 is more potent than vitamin D_2 in humans. *Journal of Clinical Endocrinology & Metabolism, 96,* E447-E452.

Heudi, O., Trisconi, M. J., & Blake, C. J. (2004). Simultaneous quantification of Vitamins A, D_3 and E in fortified infant formulae by liquid chromatography-mass spectrometry. *Journal of Chromatography A, 1022,* 115-123.

Hicks, T., Hansen, A. P., & Rushing, J. E. (1996). Procedures used by North Carolina dairies for vitamins A and D fortification of milk. *Journal of Dairy Science, 79,* 329-333.

Hite, D. A. (2003). Determination of retinyl palmitate (vitamin A) in fortified fluid milk by liquid chromatography: collaborative study. *Journal of AOAC International, 86,* 375-385.

Holick, M. F., Shao, Q., Liu, W. W., & Chen, T. C. (1992). The vitamin-D content of fortified milk and infant formula. *New England Journal of Medicine, 326,* 1178-1181.

Holick, M. (2004). Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. *American Journal of Clinical Nutrition, 80,* 1678S-1688S.

Holick, M. F., Biancuzzo, R. M., Chen, T. C., Klein, E. K., Young, A., Bibuld, D., Reitz, R., Salameh, W., Ameri, A., & Tannerbaum, A. D. (2008). Vitamin D_2 is as effective as vitamin D_3 in maintaining circulating concentrations of 25-hydroxyvitamin D. *Journal of Clinical Endocrinology & Metabolism, 93,* 677-681.

Huber W. & Barlow O. W. (1943). Chemical and biological stability of crystalline vitamins D_2 and D_3 and their derivatives. *The Journal of Biological Chemistry,* 149, 125-137.

Indyk H., Littlejohn, V., & Woollard, D.C. (1996). Stability of vitamin D_3 during spray-drying of milk. *Food Chemistry, 57,* 283-286.

IOM. (2011). *Dietary reference intakes for calcium and vitamin D*. Washington, DC: The National Academies Press.
Retrieved from http://www.nap.edu/catalog.php?record_id=13050

Iwase, H. (2000). Determination of vitamin D_2 in emulsified nutritional supplements by solid-phase extraction and column-switching high-performance liquid chromatography with UV detection. *Journal of Chromatography A, 881,* 189-196.

Jacobus, C.H., Holick, M.F., Shao, Q., Chen, T.C., Holm, I.A., Kolodny, J.M., Fuleihan, G.E.H., & Seely, E.W. (1992). Hypervitaminosis D Associated with Drinking Milk, *The New England Journal of Medicine, 326,* 1173–1177.

Johnsson, H., & Hessel, H. (1987). High performance liquid chromatographic determination of cholecalciferol (vitamin D_3) in food - a comparison with a bioassay method. *International Journal for Vitamin and Nutrition Research, 57,* 357-365.

Kaushik, V., & Roos, Y. H. (2007). Limonene encapsulation in freeze-drying of gum Arabic–sucrose–gelatin systems. *LWT-Food Science and Technology, 40,* 1381-1391.

Kazmi, S.A., Vieth, R., & Rousseau, D. (2007). Vitamin D_3 fortification and quantification in processed dairy products. *International Dairy Journal, 17,* 753-759.

King, J. M., & Min, D. B. (1998). Riboflavin photosensitized singlet oxygen oxidation of vitamin D. *Journal of Food Science, 63,* 31-34.

King, J. M., & Min, D. B. (2002). Riboflavin-photosensitized singlet oxygen oxidation product of vitamin D_2. *Journal of the American Oil Chemists' Society, 79,* 983-987.

Kozlov, E. I., Ivanova, R. A., Finkelshtein E. I., Lopatnikova, E., & Aleskeev, E. V. (1977). Model systems for studying the stability of fat-soluble vitamins. *Pharmaceutical Chemistry Journal, 11,* 973-978.

Laleye, L. C., Wasesa, A. A. H., & Rao, M. V. (2009). A study on vitamin D and vitamin A in milk and edible oils available in the United Arab Emirates. *International Journal of Food Sciences and Nutrition, 60*(S5), 1-9.

Langlois K., Greene-Finestone, L., Little, J., Hidiroglou, N., & Whiting, S. (2010). Vitamin D status of Canadians as measured in the 2007 to 2009 Canadian Health Measures Survey (Report No. 82-003-XPE). Retrieved from: http://www.statcan.gc.ca/pub/82-003-x/2010001/article/11131-eng.pdf

Li, T., & Min, D. (1998). Stability and photochemistry of vitamin D_2 in model system. *Journal of Food Science, 63,* 413-417.

Lips, P. (2010). Worldwide status of vitamin D nutrition. *Journal of Steroid and Molecular Biology, 121,* 297-300.

Mattila, P., Piironen, V., Backman, C., Asunmaa, A., Uusi-Rauva, E., & Koivistoinen, P. (1992). Determination of vitamin D-3 in egg yolk by high-performance liquid chromatography with diode array detection. *Journal of Food Composition and Analysis, 5,* 281-290.

Mattila, P., Ronkainen R., Lehikoinen K., & Piironen V. (1999). Effect of household cooking on the vitamin D content in fish, eggs, and wild mushrooms. *Journal of Food Composition and Analysis, 12,* 153-160.

Mithal A., Wahl, D. A., Bonjour, J., Burckhardt, P., Dawson-Hughes., B., Eisman, J. A., Fuleihan, G. E., Josse, R. G., Lips, P., Morales-Torres, J. (2009). Global vitamin D status and determinants of hypovitaminosis D. *Osteoporosis International, 20,* 1807-1820.

Murphy, S. C., Whited, L. J., Rosenberry, L. C., Hammond, B. H., Bandler, D. K., & Boor, K. J. (2001). Fluid milk vitamin fortification compliance in new york state. *Journal of Dairy Science, 84,* 2813-2820.

Norman, A. W., & Henry, H. L. (2007). Viamin D. In J. Zempleni, R. B. Rucker, D. B. McCormick, & J. W. Suttie (Eds.), *Handbook of vitamins* (pp. 42-88). Boca Raton, FL: CRC Press.

Otles, S., & Karaibrahimoglu, Y. (2005). Analysis of vitamins for the health, pharmaceutical, and food science. In S. Otles (Ed.), *Methods of analysis of food components and additives* (pp. 211-230). Boca Raton, FL: Taylor & Francis Group.

Palzer S. (2009). Food Technology. In Bullinger H. (Ed.), *Technology guide: principles - applications - trends* (pp.230-235). New York, NY: Springer.

Patterson, K.Y., Philips, K. M., Horst, R. L., Byrdwell, W. C., Exler, J., Lemar, L. E., & Holden, J. M. (2010). Vitamin D content and variability in fluid milks from a US Department of Agriculture nationwide sampling to update values in the National Nutrient Database for Standard Reference. *Journal of Dairy Science, 93,* 5082-5090.

Perales, S., Alegria, A., Barbera, R., & Farre, R. (2005). Review: Determination of vitamin D in dairy products by high performance liquid chromatography. *Food Science and Technology International, 11,* 451-462.

Pike, R. L., & Brown, M. L. (1984). Nutrition, an Integrated Approach. New York, NY, USA: Wiley.

Renken, S. A., & Warthesen, J. J. (1993). Vitamin-D stability in milk. *Journal of Food Science, 58,* 552-556.

Ron, N., Zimet, P., Bargarum, J., & Livney, Y. D. (2010). Beta-lactogloblulin-polysaccharide complexes as nanovehicles for hydrophobic nutraceuticals in non-fat foods and clear beverages. *International Dairy Journal, 20,* 686-693.

Rucker, D., Allan, J. A., Fick, G. H., & Hanely, D. A. (2002). Vitamin D insufficiency in a population of healthy western Canadians. *Canadian Medical Association Journal, 166,* 1517-1524.

Saftert, A., Pieper, G., & Jetten, J. (2008). Effect of package light transmittance on vitamin content of milk, part 2: UHT whole milk. *Packaging Technology and Science, 21,* 47-55.

Saffert, A., Pieper, G., & Jetten, J. (2009). Effect of package light transmittance on the vitamin content of milk part 3: Fortified UHT low-fat milk. *Packaging Technology and Science, 22,* 31-37.

Semo, E., Kesselman, E., Danino, D., & Livney, Y. D. (2007). Casein micelle as a natural namo-capsular vehicle for nutraceuticals. *Food Hydrocolloids, 21,* 936-942.

Sliva, M. G., & Sanders, J. K. (1996). Vitamin D in infant formula and enteral products by liquid chromatography: Collaborative study. *Journal of AOAC International, 79*, 73-80.

Sosa, N., Zamora, M.C., Chirife, J., & Schebor, C. (2011). Spray-drying encapsulation of citral in sucrose or trehalose matrices: physicochemical and sensory characteristics. *International Journal of Food Science & Technology, 46,* 2096-2102.

Staffas, A., & Nyman, A. (2003). Determination of cholecalciferol (vitamin D-3) in selected foods by liquid chromatography: NMKL collaborative study. *Journal of AOAC International, 86,* 400-406.

Stewart, B. A., Midland, S. L., & Byrn, S. R. (1984). Degradation of crystalline ergocalciferol. *Journal of Pharmaceutical Sciences, 73,* 1322-1323.

Tangpricha, V., Koutkia, P., Rieke, S.M., Chen, T.C, Perez, A.A., & Holick, M.F. (2003). Fortification of orange juice with vitamin D: A novel approach for enhancing vitamin D nutritional health. *American Journal of Clinical Nutrition, 77,* 1478-1483.

Tanner, J. T., Smith, J., Defibaugh, P., Angyal, G., Villalobos, M., Bueno, M. P., & McGarrahan, E. T. (1988). Survey of vitamin content of fortified milk. *Journal of AOAC International, 71,* 607-610.

Tanner, J. T., Barnett, S. A., & Mountford, M. K. (1993). Analysis of milk-based infant formula - phase-IV - iodide, linoleic-acid, and vitamin D and vitamin K - collaborative study. *Journal of AOAC International, 76,* 1042-1056.

Thomson, B. (2006). *Fortification overages of the food supply: vitamin A, vitamin D and calcium* (Report No. FW0637). Retrieved from http://www.foodsafety.govt.nz/elibrary/industry/fortification_overages-measuring_actual.pdf

Trang, H. M., Cole, D. E. C., Rubin, L. A., Pierratos, A., Siu, S., & Vieth, R. (1998). Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. *American Journal of Clinical Nutrition, 68,* 854-858.

Trenerry, V. C., Plozza, T., Caridi, D., & Murphy, S. (2011). The determination of vitamin D_3 in bovine milk by liquid chromatography mass spectrometry. *Food Chemistry, 125,* 1314-1319.

U.S. Department of Agriculture, Agricultural Research Service. (2012). USDA National Nutrient Database for Standard Reference, Release 25. Retrieved from http://www.ars.usda.gov/ba/bhnrc/ndl

Vatanparast, H., Calvo, M. S., Green, T. J., & Whiting, S. J. (2010). Despite mandatory fortification of staple foods, vitamin D intakes of Canadian children and adults are inadequate. *Journal of Steroid Biochemistry and Molecular Biology, 121,* 301-303.

Westermark, M. (1991). WHO Collaborating Centre for Chemical Reference Substances: Report on the Work in 1990 (Report No. WHO/PHARM/91.555). Retrieved from World Health Organization website: http://whqlibdoc.who.int/hq/1991/WHO_PHARM_91.555.pdf

Whiting, S. J., Green, T. J., & Calvo, M. S. (2007). Vitamin D intakes in North America and Asia-pacific countries are not sufficient to prevent vitamin D insufficiency. *Journal of Steroid Biochemistry and Molecular Biology, 103,* 626-630.

Appendices

Appendix A Time and places for commercial milk sampling and analysis

Brand & Type	Set #	Date of purchase	Date of analysis	Period of storage (day)	Place of procurement
Brand 1 skim	Set 1	23-Nov-11	28-Nov-11	5	Safeway at Sasamat St & 10th Ave
(Processor A)	Set 2	10-Jan-12	17-Jan-12	7	Safeway at Sasamat St & 10th Ave
	Set 3	28-Feb-12	7-Mar-12	8	Safeway at Mcdonald St & Broadway
	Set 4	9-Aug-12	22-Aug-12	13	Safeway at Sasamat St & 10th Ave
Brand 1 3.25%	Set 1	28-Dec-11	13-Jan-12	16	T&T at China Town
(Processor A)	Set 2	23-Jan-12	8-Feb-12	16	Donald's Market at East Hasting
	Set 3	29-Feb-12	7-Mar-12	7	Save on Food at UBC
	Set 4	28-Aug-12	12-Sep-12	15	Save on Food at UBC
Brand 2 skim	Set 1	25-Nov-11	1-Dec-11	6	Safeway at Commercial Drive & Broadway
(Processor unknown)	Set 2	10-Jan-12	19-Jan-12	9	Safeway at Commercial Drive & Broadway
	Set 3	N/A	N/A	N/A	N/A
	Set 4	N/A	N/A	N/A	N/A
Brand 2 3.25%	Set 1	25-Nov-11	8-Dec-11	13	Safeway at Mcdonald St & Broadway
(Processor unknown)	Set 2	24-Jan-12	2-Feb-12	9	Safeway at Mcdonald St & Broadway
	Set 3	N/A	N/A	N/A	N/A
	Set 4	N/A	N/A	N/A	N/A

Appendix A Time and places for commercial milk sampling and analysis (continued)

Brand & Type	Set #	Date of purchase	Date of analysis	Period of storage (day)	Place of procurement
Brand 3 skim	Set 1	24-Nov-11	5-Dec-11	11	Shoppers Drug Mart at UBC
(Processor B)	Set 2	18-Jan-12	30-Jan-12	12	Shoppers Drug Mart at UBC
	Set 3	14-Mar-12	26-Mar-12	12	Shoppers Drug Mart at UBC
	Set 4	16-Aug-12	27-Aug-12	11	Shoppers Drug Mart at UBC
Brand 3 3.25%	Set 1	25-Nov-11	9-Dec-11	14	Shoppers Drug Mart at UBC
(Processor B)	Set 2	11-Jan-12	23-Jan-12	12	Shoppers Drug Mart at Broadway & Commercial Drive
	Set 3	21-Feb-12	27-Feb-12	6	Shoppers Drug Mart at UBC
	Set 4	16-Aug-12	30-Aug-12	14	Shoppers Drug Mart at UBC
Brand 4 skim	Set 1	5-Dec-11	14-Dec-11	9	Donald's Market at East Hastings
(Processor C)	Set 2	23-Jan-12	1-Feb-12	9	Donald's Market at East Hastings
	Set 3	24-Feb-12	8-Mar-12	13	Donald's Market at East Hastings
	Set 4	9-Aug-12	23-Aug-12	14	Donald’s Market at East Hastings
Brand 4 3.25%	Set 1	30-Nov-11	13-Dec-11	13	Donald's Market at East Hastings
(Processor C)	Set 2	27-Jan-12	8-Feb-12	12	Donald's Market at East Hastings
	Set 3	24-Feb-12	8-Mar-12	13	Donald's Market at East Hastings
	Set 4	9-Aug-12	23-Aug-12	14	Donald’s Market at East Hastings

Appendix A Time and places for commercial milk sampling and analysis (continued)

Brand & Type	Set #	Date of purchase	Date of analysis	Period of storage (day)	Place of procurement
Brand 5 skim	Set 1	29-Nov-11	12-Dec-11	13	Safeway at Sasamat St & 10th Ave
(Processor D)	Set 2	7-Feb-12	20-Feb-12	13	Safeway at Mcdonald St & Broadway
	Set 3	21-Feb-12	28-Feb-12	7	Safeway at Commercial Drive & Broadway
	Set 4	9-Aug-12	20-Aug-12	11	Safeway at Sasamat St & 10th Ave
Brand 5 3.25%	Set 1	30-Nov-11	13-Dec-11	13	Safeway at Sasamat St & 10th Ave
(Processor D)	Set 2	21-Jan-12	3-Feb-12	13	Safeway at Commercial Drive & Broadway
	Set 3	7-Mar-12	20-Mar-12	13	Safeway at Commercial Drive & Broadway
	Set 4	31-Aug-12	13-Sep-12	13	Safeway at Commercial Drive & Broadway
Brand 6 skim	Set 1 *	15-Dec-11	22-Dec-11	7	Safeway at Commercial Drive & Broadway
(Processor E)	Set 2	30-Jan-12	10-Feb-12	11	Safeway at Mcdonald St & Broadway
	Set 3	25-Feb-12	9-Mar-12	13	Safeway at Commercial Drive & Broadway
	Set 4	16-Aug-12	7-Sep-12	22	Safeway at Commercial Drive & Broadway
Brand 6 2%	Set 1	21-Dec-11	16-Jan-12	26	Safeway at 4th Ave near Kitsilano
(Processor E)	Set 2	5-Feb-12	27-Feb-12	22	Superstore at Grandview Highway
	Set 3	2-Mar-12	19-Mar-12	17	Safeway at Commercial Drive & Broadway
	Set 4	16-Aug-12	10-Sep-12	25	Safeway at Commercial Drive & Broadway

Appendix A Time and places for commercial milk sampling and analysis (continued)

Brand & Type	Set #	Date of purchase	Date of analysis	Period of storage (day)	Place of procurement
Brand 7 skim	Set 1	29-Jan-12	7-Feb-12	9	Superstore at Grandview Highway
(Processor D)	Set 2	3-Feb-12	14-Feb-12	11	No Frill at Broadway
	Set 3	17-Mar-12	27-Mar-12	10	Superstore at Grandview Highway
	Set 4	16-Aug-12	27-Aug-12	11	Superstore at Grandview Highway
Brand 7 3.25%	Set 1	29-Jan-12	10-Feb-12	12	Superstore at Grandview Highway
(Processor D)	Set 2	3-Feb-12	14-Feb-12	11	No Frill at Broadway
	Set 3	24-Feb-12	9-Mar-12	14	Superstore at Grandview Highway
	Set 4	24-Aug-12	4-Sep-12	11	Superstore at Grandview Highway

* 2-L package was sampled instead of 1 L.

Appendix B Vitamin D content of commercial fortified fluid milks and t-test for two containers of same product in each set.

		Set 1	Set 2	Set 3	Set 4	Set 1	Set 2	Set 3	Set 4
Carton	**Rep.**		**Brand 1 - Skim**				**Brand 1 - 3.25%**		
A	1	31.98	36.93	1.37	35.16	28.16	29.48	26.44	26.75
	2	30.98	37.12	1.48	35.77	30.29	29.39	25.98	27.04
	3	31.32	36.76			28.58			
B	1	34.61	35.56	1.18	34.72	31.24	28.98	25.98	26.46
	2	31.66	35.14	1.20	34.51	30.49	29.63	24.77	26.90
	3	32.63	37.62			28.04			
T-test		NS*	NS	NS	NS	NS	NS	NS	
			Brand 2 - skim				**Brand 2 - 3.25%**		
A	1	22.00	31.27			39.20	36.13		
	2	22.51	31.78			40.75	35.42		
	3	23.46	31.22			38.27			
B	1	23.67	31.13			38.20	35.02		
	2	23.70	30.14			40.37	36.95		
	3	22.66	30.57			39.73			
T-test		NS	NS			NS	NS		
			Brand 3 - skim				**Brand 3 - 3.25%**		
A	1	45.34	34.54	36.28	31.76	42.15	42.14	29.30	31.64
	2	46.53	33.91	38.01	31.72	43.24	43.81	28.71	30.19
	3	45.86				42.97	43.74		
B	1	45.04	32.12	36.59	30.10	43.08	42.58	24.96	28.94
	2	44.84	32.52	36.45	31.42	44.83	41.69	26.93	30.36
	3	45.74				42.84	43.32		
T-test		NS	NS	NS	NS	NS	NS	NS	NS

Appendix B Vitamin D content of commercial fortified fluid milks and t-test for two containers of same product in each set (continued).

		Set 1	Set 2	Set 3	Set 4	Set 1	Set 2	Set 3	Set 4
			Brand 4 - skim				Brand 4 - 3.25%		
A	1	34.91	32.68	40.41	44.70	39.55	42.81	35.82	57.77
	2	33.99	31.25	41.60	43.37	41.31	41.40	36.33	57.18
	3	34.64				41.36			
B	1	34.73	31.08	40.65	45.41	39.98	41.77	36.75	57.21
	2	34.20	29.92	40.59	44.57	41.90	41.14	33.12	56.85
	3	34.51				41.54			
T-test		NS	NS	NS	NS	NS	NS	NS	NS
			Brand 5 - skim				Brand 5 - 3.25%		
A	1	15.80	13.81	9.59	14.70	30.49	46.38	36.04	41.08
	2	15.84	14.93	9.57	11.24	32.88	46.94	35.44	40.90
	3	15.46				32.25			
B	1	14.00	13.98	10.13	14.32	32.08	47.59	38.89	41.73
	2	14.90	13.80	10.35	14.45	32.63	48.34	36.15	41.36
	3	14.58				33.14			
T-test		NS	NS	NS	NS	NS	p=0.05	NS	NS
Carton									
	Rep.		Brand 6 - skim				Brand 6 - 2%		
A	1	32.06	38.86	33.17	38.02	38.70	34.44	35.63	41.79
	2	34.01	38.30	34.16	40.32	41.82	33.97	35.83	39.16
	3	32.27				38.11			
B	1	31.67	38.08	34.75	39.23	37.94	33.00	35.64	40.26
	2	32.24	39.89	34.99	39.52	38.24	34.18	35.37	40.71
	3	34.34				37.11			
T-test		NS	NS	NS	NS	NS	NS	NS	NS

Appendix B Vitamin D content of commercial fortified fluid milks and t-test for two containers of same product in each set (continued).

		Set 1	Set 2	Set 3	Set 4	Set 1	Set 2	Set 3	Set 4
			Brand 7 - skim				Brand 7 - 3.25%		
A	1	11.96	11.83	8.27	14.15	44.29	14.49	36.12	39.22
	2	13.01	11.90	8.60	14.18	45.57	12.73	36.14	37.09
B	1	11.30	11.25	8.92	14.56	44.61	14.54	34.88	38.26
	2	11.86	11.89	8.34	14.45	46.08	13.20	33.83	37.53
T-test		NS	NS	NS	NS	NS	NS	NS	NS

Appendix C Vitamin formulations used in light exposure study.

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

Product Information
Product Data Sheet

Vitamin D3 Crystalline

Description

Vitamin D3 Crystalline is a crystalline powder containing 40 000 000 IU vitamin D_3 per gram (1 000 000 µg cholecalciferol per gram).

Product identification

Product code: 50 0927 8

Chemical name: (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3β-ol

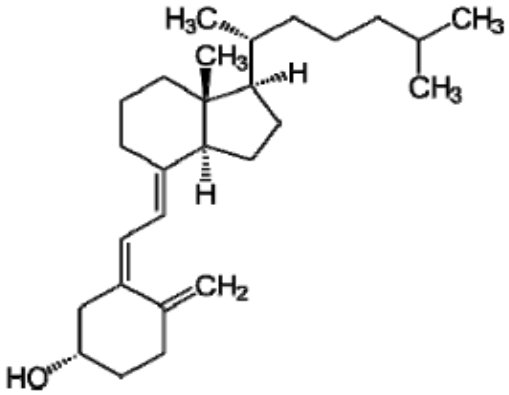
Synonyms: cholecalciferol; calciol; vitamin D_3

CAS No.: 67-97-0

EINECS No.: 200-673-2

Empirical formula: $C_{27}H_{44}O$

Molecular mass: 384.6 g/mol



Specifications

Appearance:	crystalline powder
Colour:	white to almost white
Identity:	corresponds
Specific rotation, 589 nm (c= 0.8 in ethanol 96%):	+105° to +112°
Maximum UV absorption in ethanol (A 1%, 1 cm at 263-267 nm):	460 - 500
Loss on drying:	max. 0.1%
Related substances (Ph.Eur.)	
• 5,6-trans Vitamin D3 (impurity A)	max. 0.1%
• Unspecified impurities (each)	max. 0.10%
• Total	max. 1.0%
Assay:	97.0-102.0%

Appendix C Vitamin formulations used in light exposure study (continued).

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

Product Information
Product Data Sheet

Vitamin D3 Crystalline

Residual solvents:

• Methanol	max. 3000 ppm
• Pyridine	max. 200 ppm
• Hexane	max. 290 ppm
• Acetone	max. 1000 ppm
• Methyl formate	max. 1000 ppm

Solubility

Vitamin D3 Crystalline is insoluble in water, freely soluble in acetone, alcohol, chloroform and ether, and soluble in oils.

Stability and storage

Vitamin D3 Crystalline is sensitive to air, heat, humidity and light. The product may be stored for 60 months from the date of manufacture in the unopened original container (which is sealed under inert gas) and at 2°- 8°C. The 'best use before' date is printed on the label. Keep container tightly closed. Once opened, use contents quickly.

Uses

For pharmaceutical and food preparations as a source of vitamin D_3.

This product is not intended for use in the manufacture of sterile drug products. The purchaser assumes all responsibility for additional processing, testing and labelling required for such use.

Compendial compliance

Vitamin D3 Crystalline meets all requirements of the USP, FCC and Ph. Eur. when tested according to these compendia.

Safety

This product is safe for the intended use. Avoid ingestion, inhalation of dust or direct contact by applying suitable protective measures and personal hygiene.

For full safety information and necessary precautions, please refer to the respective DSM Material Safety Data Sheet.

Appendix C Vitamin formulations used in light exposure study (continued).

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Product Information
Product Data Sheet

Dry Vitamin D_3 100 SD/S

Description

Dry Vitamin D_3 100 SD/S consists of free-flowing particles containing 100,000 IU Vitamin D_3 (cholecalciferol) per gram finely dispersed in a matrix of Modified Food Starch, Sucrose and Medium Chain Triglycerides. *dl*-α-Tocopherol and Sodium Ascorbate are added as antioxidants. Silicon Dioxide is added as a processing aid.

Product identification

Product code: 50 1095 0

Chemical name: (5*Z*,7*E*)-(3*S*)-9,10-secocholesta-5,7,10(19)-trien-3-ol; (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3β-ol

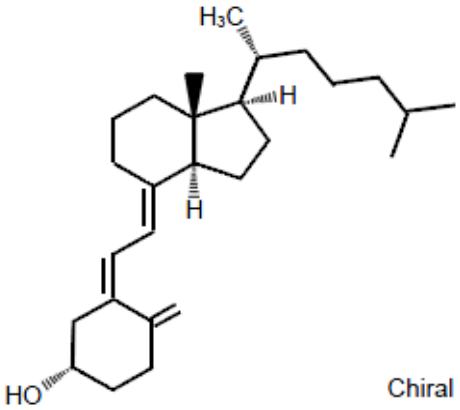
Synonyms: cholecalciferol; calciol; vitamin D_3

CAS No.: 67-97-0

EINECS No.: 200-673-2

Empirical formula: $C_{27}H_{44}O$

Molecular mass: 384.65 g/mol



Chiral

Specifications

Appearance:	free flowing particles
Colour:	off-white to yellowish
Fineness (US standard sieves):	
• through sieve No. 80	min. 95%
Dispersibility in water:	satisfactory
Loss on drying:	max. 6%
Identity for Vitamin D_3:	corresponds
Identity for tocopherol:	corresponds
Vitamin D_3 content (Ph. Eur.):	100'000 - 110'000 IU/g.

Appendix C Vitamin formulations used in light exposure study (continued).

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Product Information

Product Data Sheet

Dry Vitamin D_3 100 SD/S

Microbiological purity:

• Total aerobic microbial count	max. 10^3 CFU/g
• Total combined yeast/moulds count	max. 10^2 CFU/g
• Enterobacteria	< 10 CFU/g
• Escherichia coli	negative in 10 g
• Salmonella spp.	negative in 25 g
• Staphylococcus aureus	negative in 10 g
• Pseudomonas aeruginosa	negative in 10 g

Dispersibility

Dry Vitamin D_3 100 SD/S disperses quickly and completely in cold water, fruit juices, milk and other liquids. High concentrations may give cloudy dispersions which, however, remain uniform for relatively long periods of time.

Stability and storage

Dry Vitamin D_3 100 SD/S is sensitive to air, light and humidity. The product may be stored for 24 months from the date of manufacture in the unopened original container and at a temperature below 15 °C. The 'best use before' date is printed on the label. Keep container tightly closed. Once opened, use contents quickly.

Uses

For food preparations.

For direct-compression tableting of chewable or coated tablets

Compendial compliance

The Vitamin D_3 Crystalline, contained in this product, meets all requirements of the USP, FCC and Ph. Eur. when tested according to these compendia.

Safety

This product is safe for the intended use. Avoid ingestion, inhalation of dust or direct contact by applying suitable protective measures and personal hygiene.

For full safety information and necessary precautions, please refer to the respective DSM Material Safety Data Sheet.

Appendix C Vitamin formulations used in light exposure study (continued).

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Product Information
Product Data Sheet

Dry Vitamin D3 100 CWS/AM

Description

Dry Vitamin D3 100 CWS/AM consists of off-white to yellowish, free-flowing particles (beadlets). They contain Vitamin D_3 (cholecalciferol) in Medium Chain Triglycerides finely dispersed in a Corn Starch coated matrix of Acacia Gum and Sucrose. *dl*-α-Tocopherol is added as an antioxidant. Silicon Dioxide is used as a processing aid.

Product identification

Product code: 50 1147 7

Chemical name: (5*Z*,7*E*)-(3*S*)-9,10-secocholesta-5,7,10(19)-trien-3-ol; (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3β-ol

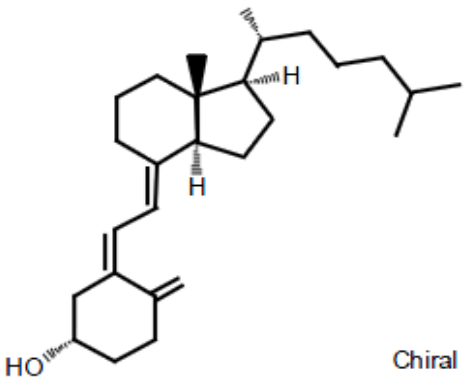
Synonyms: cholecalciferol; calciol; vitamin D_3

CAS No.: 67-97-0

EINECS No.: 200-673-2

Empirical formula: $C_{27}H_{44}O$

Molecular mass: 384.65 g/mol



Chiral

Specifications

Appearance:	free-flowing particles
Colour:	off-white to yellowish
Fineness (US standard sieves):	
100% through sieve No. 20 min. 90% through sieve No. 40 max. 15% through sieve No. 100	
Dispersibility in water:	satisfactory
Loss on drying:	max. 8%
Identity for vitamin D_3:	corresponds
Identity for tocopherol:	corresponds
Vitamin D_3 content (Ph. Eur.):	90'000-110'000 IU/g

Appendix C Vitamin formulations used in light exposure study (continued).

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Product Information
Product Data Sheet

Dry Vitamin D3 100 CWS/AM

Microbiological purity:

• Total aerobic microbial count	max. 10^3 CFU/g
• Total combined yeast/moulds count	max. 10^2 CFU/g
• Enterobacteria	< 10 CFU/g
• Escherichia coli	negative in 10 g
• Salmonella spp.	negative in 25 g
• Staphylococcus aureus	negative in 10 g
• Pseudomonas aeruginosa	negative in 10 g

Dispersibility

Dry Vitamin D3 100 CWS/AM disperses quickly and completely in cold water, fruit juices, milk and other liquids. High concentrations may give cloudy dispersions which, however, remain uniform for relatively long periods.

Stability and storage

Dry Vitamin D3 100 CWS/AM is sensitive to air, light and humidity. The product may be stored for 27 months from the date of manufacture in the unopened original container and at a temperature below 15 °C. The 'best use before' date is printed on the label. Keep container tightly closed. Once opened, use contents quickly.

Uses

For food preparations which are reconstituted with liquids.

Compendial compliance

The Vitamin D_3 crystalline, contained in this form, meets all requirements of the USP, Ph.Eur., and FCC when tested according to these compendia.

Safety

This product is safe for the intended use. Avoid ingestion, inhalation of dust or direct contact by applying suitable protective measures and personal hygiene.

For full safety information and necessary precautions, please refer to the respective DSM Material Safety Data Sheet.

Appendix C Vitamin formulations used in light exposure study (continued).

Kingsway
Established in 1949

KINGSWAY VITAMIN AD 50-10 GMO FREE PREMIX
FOR THE VITAMIN FORTIFICATION OF SKIMMED MILK

DESCRIPTION	A blend of Vitamin A Palmitate and Vitamin D_3 Cholecalciferol emulsified in a refined food grade GMO free sunflower oil base. Vitamin Premixes are subject to stringent quality control and are guaranteed for vitamin content. Vitamin AD 50-10 is manufactured and distributed in Canada by Kingsway Chocolate Company Limited.	
APPEARANCE	Amber liquid	
PHYSICAL CHEMICAL	Vitamin A content	50,000 I.U./ml
	Vitamin D_3 content	10,000 I.U./ml.
	Specific Gravity	0.96 to 0.98 g/ml
MICROBIOLOGICAL	Standard Plate Count	5,000 per gram maximum
	Yeast & Mould	250 per gram maximum
	Coliform/E.Coli	10 per gram maximum
	Salmonella	Negative per 375 grams
	Listeria monocytogenes	Negative per 50 grams
INGREDIENTS	Sunflower Oil, Polysorbate 80, Polyglycerol Monooleate, Vitamin A Palmitate, Vitamin D_3 (Cholecalciferol).	
PACKAGING	2 Litre high density polyethylene jugs, 4 per case. 4 Litre high density polyethylene jugs, 4 per case.	
STORAGE	Store at ambient temperature 18°C to 23°C or store refrigerated. DO NOT FREEZE. Store in original carton to avoid direct light contact. Solidification of triglycerides may occur at lower refrigeration temperatures.	
SHELF LIFE	Six months maximum under ambient storage conditions. Ten months maximum under refrigerated storage conditions.	
HANDLING	Pre-mix should be used at room temperature in order to minimize fluctuations in addition rates due to viscosity changes with varying temperatures. Do not shake the vitamin pre-mix container. This will incorporate air, which can cause oxidation, vitamin dissipation and may affect the flow rate accuracy of the metering pump. DO NOT add the vitamin pre-mix prior to the separator. Vitamin AD 50-10 should be added directly into the skim or partly skimmed milk flow prior to homogenization and pasteurization.	

Appendix C Vitamin formulations used in light exposure study (continued).

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Product Information
Product Data Sheet

Dry Vitamin A Palmitate, Type 250 CWS/F

Description

Dry Vitamin A Palmitate, Type 250 CWS/F consists of light yellow, free-flowing particles (beadlets). They contain vitamin A palmitate finely dispersed in a cornstarch-coated matrix of gelatin* and sucrose. *dl*-α-Tocopherol is added as an antioxidant.

*Gelatin obtained from fish skins of food fish processed for human consumption

Product identification

Product code: 04 2187 1

Chemical name: all-*trans*-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexene-1-yl)-2,4,6,8-nonatetraene-1-yl palmitate

Synonyms: retinyl palmitate; all-*trans*-vitamin A palmitate; vitamin A palmitate

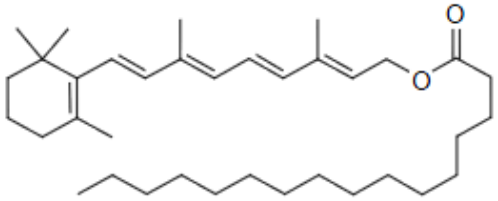
CAS No.: 79-81-2

EINECS No.: 201-228-5

INCI name: retinyl palmitate

Empirical formula: $C_{36}H_{60}O_2$

Molecular mass: 524.87g/mol



Specifications

Appearance:	light yellow, free-flowing particles
Fineness (US standard sieves): 100% through sieve No. 20 min. 90% through sieve No. 40 max. 15% through sieve No. 100	
Dispersibility in water:	satisfactory
Loss on drying:	max. 8%
Identity for vitamin A palmitate:	corresponds
Identity for tocopherol:	corresponds
Vitamin A content:	min. 250 000 IU/g
Microbiological purity:	corresponds

Appendix C Vitamin formulations used in light exposure study (continued).

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Product Information
Product Data Sheet

Dry Vitamin A Palmitate, Type 250 CWS/F

Dispersibility

Dry Vitamin A Palmitate, Type 250 CWS/F disperses quickly and completely in cold water, fruit juices, milk and other liquids. High concentrations give cloudy dispersions which, however, remain uniform for relatively long periods.

Stability and storage

Dry Vitamin A Palmitate, Type 250 CWS/F is sensitive to air, heat, light and humidity. The product may be stored for 24 months from the date of manufacture in the unopened original container and at a temperature below 15 °C. The 'best use before' date is printed on the label. Keep container tightly closed. Once opened, use contents quickly.

Uses

For dry pharmaceutical and food preparations which are reconstituted with liquids, especially for effervescent tablets.

Compendial compliance

Dry Vitamin A Palmitate, Type 250 CWS/F meets all requirements of the relevant monographs of the USP, the FCC and the Ph. Eur. when tested according to these compendia.

Safety

This product is safe for the intended use. Avoid ingestion, inhalation of dust or direct contact by applying suitable protective measures and personal hygiene.

For full safety information and necessary precautions, please refer to the respective DSM Material Safety Data Sheet.