OPTIMIZING THE LOADING OF VITAMIN A AND VITAMIN D INTO RE-ASSEMBLED CASEIN MICELLES AND INVESTIGATING THE EFFECT OF MICELLAR COMPLEXATION ON VITAMIN D STABILITY

by

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Abstract

Food products may be fortified with vitamins A and D as public health intervention strategies. However, vitamin instability is a problem during storage before and after addition to foods. Re-assembled casein micelles (rCM) are reported to be effective carriers of hydrophobic compounds providing enhanced stability. The aim of this research was to form rCM with high loading of vitamin A palmitate (VA) or vitamin D_3 (VD). The protection given to VD by rCM was compared to that given by the controls composed of the re-assembly components not in micellar form, and to commercial vitamin products (CWS D_3, AD premix). Stability was investigated during storage of dry powders and of fortified skim milk exposed to light.

Response surface methodology was employed to ascertain the influence of phosphate, citrate and calcium on vitamin loading during CM re-assembly, and to identify optimal vitamin loading conditions. Average optimal VD loading of 14.0 or 15.1 mg VD/g casein was found at 4.9 or 10.5 mM phosphate, 4.0 mM citrate and 26.1 mM calcium, respectively. VA loading was optimal at 9.7 mM phosphate, 5.5 mM citrate and 30.0 mM calcium, resulting in 14.7 mg VA/g casein.

Significantly more vitamin was retained in VD-rCM powders than control powders during storage at 37°C and 75% relative humidity for 48 and 72 hours. Additionally, loss of VD was not significantly different in VD-rCM powders compared to CWS D_3 during storage at 37°C and 75% relative humidity. Significantly more VD was retained in VD-rCM powders than control powders when stored at ambient temperature and humidity for up to 42 days. Retention of VD in skim milk fortified using different formulations and exposed to light for 21 days conformed to the following order: CWS D_3 > VD-rCM L, CD > AD premix.

In conclusion, response surface methodology was an effective tool to optimize vitamin loading of rCM. Stability of VD was improved by incorporation into rCM for some storage conditions. Protection may depend on the physical state of the VD-rCM and on the applied stress.

Preface

I, Anisa Loewen, conducted the original work of this thesis under the supervision of Dr. Eunice Li-Chan. My committee members, Dr. Christine Scaman, Dr. Xiaonan Lu and Dr. Urs Hafeli, provided additional advice and support in developing this research. I had the responsibility to design this project, conduct the experiments, collect data, analyze and interpret the data, and write this thesis, with guidance from Dr. Li-Chan. This thesis has been reviewed by Dr. Li-Chan, Dr. Christine Scaman, Dr. Xiaonan Lu, Dr. Urs Hafeli and Dr. Tim Green.

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List of Abbreviations

25(OH)D = 25-hydroxyvitamin D

AD premix = An emulsified vitamin A palmitate and vitamin D_3 product from Kingsway Chocolate

BHT = Butylated hydroxytoluene

CCD = Central composite design

CD milk = Milk fortified with the same level of casein, vitamin D_3, phosphate, citrate and calcium used in the fortification of VD-rCM L milk

CFIA = Canadian Food Inspection Agency

CH = Control powder, high phosphate

CL = Control powder, low phosphate

CWS A = Dry vitamin A palmitate type 250 CWS/F from DSM nutrition

CWS D3 = Dry vitamin D3 100 CWS/AM from DSM nutrition

DRI = Dietary Reference Intake

EAR = Estimated Average Requirement

HPLC = High performance liquid chromatography

HTST = High temperature short time

IU = International unit

ND = Not determined

rCM = Re-assembled casein micelles

RDA = Recommended Dietary Allowance

RSM = Response surface methodology

UV = ultraviolet

VA = Vitamin A palmitate

VA-rCM = Vitamin A palmitate – re-assembled casein micelles

VD = Vitamin D_3

VD-rCM H = Vitamin D_3 – re-assembled casein micelles, high phosphate

VD-rCM L = Vitamin D_3 – re-assembled casein micelles, low phosphate

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There are many people who helped me throughout the completion of this thesis, all of whom I would not have been able to do this project without. I would like to give special thanks to the following groups and individuals.

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Lastly I'd like to thank my family, friends and lab mates for providing support and inspiration during the long days of work on this project.

Dedication

I dedicate this thesis to my mother, Marjorie Jantzen.

Chapter 1: Introduction

1.1 Background

Vitamins D and A are fat soluble vitamins essential for human health (Dusso, Brown, & Slatopolsky, 2005; Takahashi, 2010). In Canada, fortification of skim milk with vitamins D and A is mandatory (FDR, 2013). Unfortunately under- and over-fortification of vitamins D and A have been reported for fluid milk in Canada, though under-fortification seems to be more prevalent (Faulkner, Hussein, Foran, & Szijarto, 2000; Liu, 2013; Murphy, Whited, Rosenberry, Hammond, Bandler, & Boor, 2001).

There are many possible causes for low vitamin levels in milk. Vitamin D powders may be unstable to oxygen, temperature and humidity (Grady & Thakker, 1980). Similarly, vitamin A is sensitive to oxygen, trace metals, ultraviolet (UV) light, and acid pH (DeRitter, 1982). As such, vitamin fortification products may degrade during storage resulting in less active vitamin being added to milk than believed. Vitamin D is quite stable during processing and storage of food products (Kazmi, Vieth, & Rousseau, 2007; Wagner, Rousseau, Sidhom, Pouliot, Audet, & Vieth, 2008). However, light exposure can result in significant loss of vitamins D and A in low-fat UHT milk (Saffert, Pieper, & Jetten, 2009).

It would be valuable to improve vitamin stability during storage before and after addition to fluid milk, as this may result in more consistent vitamin levels in milk. Consumers would benefit by gaining a healthier product in regards to vitamin content. In this research the focus was on re-assembled casein micelles as carriers for vitamins D and A to be used for fortification of fluid milk. Improved vitamin stability was the main concern, as well as feasibility and practicality of the product within industry.

Re-assembled casein micelles (rCM) can improve oxidative stability of hydrophobic compounds during cold storage, thermal treatment, and exposure to UV light (Haham et al., 2012; Sáiz-Abajo, Gonzalez-Ferrero, Moreno-Ruiz, Romo-Hualde, & Gonzalez-Navarro, 2013; Semo, Kesselman, Danino, & Livney, 2007; Zimet, Rosenberg, & Livney, 2011). Interestingly enough, altering the composition of rCM, has an influence on micelle structure and stability (Schmidt, Both, & Koops, 1979) and may also influence the efficiency of loading hydrophobic compounds into the rCM.

Also of importance is the form of the vitamin fortification product, for example liquid versus dry. Vitamin fortification products for milk are often sold and purchased as dry powders. Drying is advantageous because, as a result of the lower volume and weight, a lower product transportation cost accompanies dry products as opposed to liquids. Greater stability against microbial growth can also be associated with dry products when compared to liquids.

The purpose of the current study was to form rCM with high loading of vitamin A palmitate or vitamin D_3 and investigate the effect of rCM as vitamin carriers on vitamin D stability. Vitamin D_3 stability was assessed during storage of dry powders at different temperatures, and during light exposure throughout refrigerated storage of fortified fluid skim milk.

1.2 Vitamins and health

1.2.1 Health aspects of vitamin D

Vitamin D is a fat-soluble vitamin that is essential for maintaining bone and teeth health in the human body. It is involved in calcium and phosphate absorption and bone mineralization

(Dusso et al., 2005). As such, chronic insufficient levels of vitamin D can lead to rickets in children and osteomalacia or osteoporosis in adults. Vitamin D deficiency may increase risk for certain chronic diseases, for example type II diabetes, cardiovascular disease, multiple sclerosis, and some types of cancers (Holick, 2004; Holick, 2010). The exact effect and required amount of vitamin D for such effects on chronic disease risk are of debate (Holick, 2010). On the other side of the coin, excessive consumption of vitamin D is potentially toxic and may result in weight loss, heart arrhythmias, vascular and tissue calcification, and damage to the heart, blood vessels and kidneys (IOM, 2011).

There are two natural forms of vitamin D: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) (IOM, 2011). Vitamin D_2 is formed in certain yeasts and fungi by conversion of ergosterol to previtamin D_2 via ultraviolet B (UVB = 290-315 nm) light exposure (Calvo & Whiting, 2003). Theoretically, a major source of vitamin D_3 for humans is synthesis in the skin (Holick, 2010). When skin is exposed to UVB light the body converts 7-dehydrocholesterol to previtamin D_3, which is quickly isomerized to vitamin D_3. The amount of vitamin D obtained through this pathway is highly variable because of the variety of factors that can alter production. Factors that affect the skin's production of vitamin D_3 include skin pigmentation, latitude, season, clothing, time of day, aging, and use of sunscreen. Canada's location on the earth's latitudinal axis dictates that for a large portion of the year very limited vitamin D_3 can be formed in the skin of the inhabitants. This goes the same for other countries far from the equator.

Vitamin D or vitamin D precursors can be found naturally in oily fish such as salmon and mackerel, liver and other organ meat, mushrooms, and egg yolk (Holick, 2010). Liver and other organ meats are often avoided in the diet because they contain appreciable amounts of cholesterol. Mushrooms and egg yolk typically contain low and variable levels of vitamin D

(Calvo, Whiting, & Barton, 2004). Cow's milk may also contain vitamin D. A wide range of vitamin D contents for whole bovine milk is reported in the literature, for example 0.27-1.08 IU/100 mL (Kurmann & Indyk, 1994), 34.1-62.9 IU/100 mL (Bulgari, Caroli, Chessa, Rizzi, & Gigliotti, 2013) and 100-800 IU/ 100 mL (Hollis, Roos, Draper, & Lambert, 1981). However, it is generally assumed that limited vitamin D is found naturally in bovine milk. As natural sources of vitamin D are sparse in the diet for many individuals, fortified foods and supplements are common sources of vitamin D (Calvo & Whiting, 2003).

The Institute of Medicine (IOM) has established Dietary Reference Intakes (DRIs) for vitamin D based solely on requirements for skeletal health (IOM, 2011). DRIs are reference values for the recommended nutrients intakes designed for different populations. The DRIs for vitamin D have been established considering vitamin D intake from food, supplements, and synthesis in the skin. Specifically, the Estimated Average Requirement (EAR) and Recommended Daily Allowance (RDA) for individuals ≥ 1 year old is 400 IU/day and 600-800 IU/day, respectively. To avoid symptoms associated with vitamin D toxicity, the Tolerable Upper Intake Levels (ULs) have been set from 1,200 to 4,000 IU/day for children 1-8 years of age and 4,000 IU/day for all individuals ≥ 9 years old.

1.2.2 Health aspects of vitamin A

Vitamin A is a fat soluble vitamin that has many important functions in the human body. Its role as a chromophore involved in normal vision function may be vitamin A's most well-known function (Takahashi, 2010). Vitamin A also takes part in cell proliferation and differentiation, as well as gene expression and immune regulation. Epidemiological evidence suggests that increased consumption of certain forms of vitamin A may reduce the risk of several

chronic diseases. Xerophthalmia, which is characterized by night blindness, conjunctival xerosis, Bitot's spots, corneal xerosis, corneal ulceration, and scarring is a major clinical effect of vitamin A deficiency (IOM, 2001).

In addition, vitamin A deficiency is linked to increased risk of morbidity and mortality in humans. Diminished immune function, along with increased risk for respiratory infections and diarrhea may occur with vitamin A deficiency. It is important to ensure adequate intake of vitamin A to prevent deficiency; however, problems may also occur if intake is excessive. In general, toxic levels of vitamin A can result in liver damage, headaches, vomiting, alopecia, abnormalities in bones and pain in joints (WHO, 2004). To this extent, vitamin A toxicity has been associated with osteoporosis and spontaneous fractures, specifically hip fractures (Penniston & Tanumihardjo, 2006). Fortunately, these circumstances of toxicity typically only occur with ingestion of high doses of preformed vitamin A exceeding around 3000 μg retinol activity equivalents (RAE) for adults (Penniston & Tanumihardjo, 2006; WHO, 2004).

Vitamin A compounds can be classified into two major groups: preformed vitamin A and compounds with provitamin A activity. Preformed vitamin A occurs as retinoids, such as retinol and retinyl esters. Retinoids can be found in some animal products. Fortified foods and supplements may also be sources of retinoids in the diet (Penniston & Tanumihardjo, 2006). Some carotenoids have provitamin A activity, including β-carotene, α-carotene, and β-cryptoxanthin (IOM, 2001). These compounds serve as precursors to retinol and their RAE have been set at 12, 24 and 24 μg, respectively. Carotenoids can be found in colored fruits and vegetables.

Another source of preformed vitamin A is cow's milk, which also contain carotenoids (Agabriel et al., 2007; Marino et al., 2012; Plozza, Trenerry, & Caridi, 2012). In contrast to the

amount of preformed vitamin A in cow's milk, the level of carotenoids widely varies depending on numerous factors including cow breed and physiological state, and the carotenoid level in the feed. Of all factors that have been identified, feed is thought to be the most influential in determining milk carotenoid content.

DRIs for vitamin A have been established by the IOM as guidelines for consumption (IOM, 2001). The IOM has set the EARs and RDAs for males and females over the age of 18 at 625 and 500 μg RAE/day, and 900 and 700 μg RAE/day, respectively. For males and females ages 14 to 18, the UL has been set at 2800 μg RAE/day preformed vitamin A. The UL for all adults has been set at 3,000 μg/day of preformed vitamin A.

1.3 Vitamin intake and status of Canadians

1.3.1 Vitamin D intake and status of Canadians

Vitamin D status is typically reported using serum 25-hydroxyvitamin D or 25(OH)D (Calvo & Whiting, 2003; Langois, Greene-Finestone, Little, Hidiroglou, & Whiting, 2010). This biomarker represents synthesis of vitamin D in the skin, as well as dietary intake. Reference levels for 25(OH)D based on bone health have been established by the IOM (IOM, 2011). Levels below 30 nmol/L are linked with risk of deficiency, around 40 nmol/L are correlated with the EAR and 50 nmol/L are harmonious with the RDA for those aged ≥ 1 year. Additionally, researchers have cited 75 nmol/L as indicative of positive health effects and disease prevention (Langois et al., 2010).

Data from the 2004 Canadian Community Health Survey Cycle 2.2 was used to investigate the vitamin D intake of Canadians (Vatanparast, Calvo, Green, & Whiting, 2010).

The mean intake of vitamin D for the 34,789 participants was 5.8 µg/day. In general, males had higher intakes than females. The highest intake levels were seen for males 9-19 years of age and the lowest intake levels were seen for females 51-70 years years of age. Sources of dietary vitamin D in descending order were milk products (49%), meat and meat alternatives (31.1%), others foods (11.8%), grain products (5.5%) and vegetables and fruits (2.4%). The overall contribution of milk products to dietary vitamin D decreased by age for individuals over 9 years of age with a concurrent increase in the proportion vitamin D obtained from meat and meat alternatives.

Dietary data and plasma 25(OH)D levels from 5,306 Canadians aged 6 to 79 years was collected in the Canadian Health Measures Survey and assessed according to the IOM reference levels (Whiting, Langois, Vatanparast, Greene-Finestone, 2011). Of all the participants, 5.4 percent, 12.7 percent and 25.7 percent had 25(OH)D levels below 30, 40 and 50 nmol/L, respectively. Plasma concentrations for children and seniors were the highest, whereas the lowest concentrations were seen for individuals 20 to 39 years of age. The association between milk consumption was found to be positive, with a mean concentration of 75 nmol/L for those who consumed milk more than once per day and 62.7 nmol/L for those who consumed milk less than once per day. Additionally, significantly higher 25(OH)D concentrations were found for supplement users than nonusers.

1.3.2 Vitamin A intake and status of Canadians

Vitamin A intake of Canadians was reported from the Canadian Community Health Survey 2.2 (Health Canada & Statistics Canada, 2009). Excluding the territories, 11.6-49 percent of males and females resident of all Canadian regions aged 9 to > 70 had an intake of vitamin A

less than their respective EAR. Results from the literature available on the vitamin A intake for those living in northern Canada indicate that this population may be at risk for inadequate intake. A culturally-appropriate food frequency questionnaire was administered to 106 women in Nunavut, Canada (Schaefer, Erber, Trzaskos, Roache, Osborne, & Sharma, 2011). Sixteen percent of women 19-44 years of age reported intake below the EAR. A food frequency questionnaire was also used to asses the vitamin A intake of 92 randomly selected women aged 19-44 in three communities in the Northwest Territories (Kolahdooz, Spearing, Corriveau, & Sharma, 2013). Participants were classified as drinkers or nondrinkers, where drinkers had a mean consumption of 20.1 g alcohol per day. The percent of participants with an intake of below the EAR was 24.5 and 31.0 for drinkers and nondrinkers, respectively. Higher levels of vitamin A inadequacy were found for adult Inuvialuit in the Northwest Territories using a different method of dietary analysis (Erber, Hopping, Beck, Sheehy, De Roose, & Sharma, 2010). Analysis of a 24 hour recall found that 93 percent of males ≥ 19 years, 90 percent of females 19-50 years and 84 percent of females > 50 years had an intake of vitamin A below the EAR for their age group.

There is limited literature reporting the serum vitamin A levels in Canadians (Hoffer, Ruedy, & Verdier, 1981; Plante, Blanchet, Rochette, & O'Brien, 2011). The nutritional status, including vitamin A status, of individuals from native populations in Quebec was investigated during the summer of 1978 (Hoffer et al., 1981). Of the total participants, 10.2 percent had serum vitamin A concentrations ranging from 10 to 30 µg/100 mL, which was stated to be associated with moderate risk for deficiency. A more recent study of 466 Inuit women in Nunavik aged 18-74 found that over 99 percent of the participants had normal serum vitamin A levels (Plante et

al., 2011). These reports seem to suggest that the percentage of the population at risk for vitamin A deficiency is relatively low.

1.4 Fortification

1.4.1 Food fortification

Food fortification refers to the addition of vitamins and/or minerals to foods. Typically the practice is intended to reduce the prevalence of nutrient deficiencies in individuals, and at the same time reduce the burden on the health care system caused by these nutrient deficiencies (WHO, 2006). For many years, vitamins A and D, iodine, iron, and several B vitamins have been added to foods to reduce nutrient deficiencies. For example, it is common to find salt that is fortified with iodine in many countries. The advantage to mass fortification of appropriate foods is that a large segment of the population can be reached, but there are also some problems that may be encountered with fortification of foods. These problems can generally be divided into the following areas: appropriate levels of fortification, stability of fortificants, interactions between nutrients in the product, and how fortification affects physical properties of the product. Additionally, effective quality control measures need to be in place to protect against over- or under-fortification (WHO, 2006).

1.4.2 Vitamin D fortification

Fortification of food with vitamin D in Canada began as a method of reducing the prevalence of rickets in children (Holick, Shao, Liu, & Chen, 1992). Fortification of all fluid milk, milk powder, evaporated milk, margarine, butter substitutes, infant formula, formulated

liquid diet, foods used in very low energy diets, meal replacements and nutritional supplements, whole egg products and yolk products with vitamin D is mandatory in Canada (FDR, 2013). These include whole, skim and partially skimmed milk. Fluid milk "shall contain added vitamin D in such an amount that a reasonable daily intake of the milk contains not less than 300 International Units and not more than 400 International Units of vitamin D" (FDR, 2013). The Canadian population largely relies on these fortified foods, as well as dietary supplement pills, for dietary vitamin D (Calvo et al., 2004).

1.4.3 Vitamin A fortification

Vitamin A fortification is mandatory in Canada for skim and partially skimmed milk and evaporated milk, margarine, infant formulas and formulated liquid diets, low energy diet foods, breakfast replacement foods, simulated whole egg products, whole egg products and yolk products (FDR, 2013). Skim and partially skimmed milk is required to "contain added vitamin A in such an amount that a reasonable daily intake of the milk contains not less than 1,200 International Units and not more than 2,500 International Units of vitamin A" (FDR, 2013). As vitamin A is a fat soluble vitamin, much of it is removed from milk with the lipid phase during the skimming process. Hence, the addition of vitamin A takes place for skim and partially skimmed milks but not for whole milk. Preformed vitamin A, as retinyl acetate or retinyl palmitate, may be used to fortify fluid milk (WHO, 2006).

1.4.4 Non-compliance of milk fortification

A number of studies have been conducted on the vitamin D and A contents of fluid milk. Within these studies a trend of non-compliance has been found (Chen, Shao, Heath, & Holick,

1993; Faulkner et al., 2000; Holick et al., 1992; Liu, 2013; Murphy et al., 2001; Patterson et al., 2010). In 1992, vitamin D analysis from 42 samples of milk taken from five states in the eastern United States was published (Holick et al., 1992). Only 29 percent of milk samples contained between 80 to 120 percent of the vitamin D claimed on the label. Most notably, 62 percent of the samples had less than 80 percent of the amount of vitamin D stated on the label. Later in 1993, additional results were reported on these same states and 2 additional states (Chen et al., 1993). Similar results were found for non-compliance in this second study, such that 80 percent of the 79 samples were either under- or over-fortified with vitamin D. In the same study, Chen and colleagues (1993) published data on 15 milk samples from British Columbia, finding that 73 percent of the samples did not contain between 80 to 120 percent of the vitamin D claimed on the label.

Investigation of vitamin D and A levels in Ontario's retail milk identified non-compliance as well (Faulkner et al., 2000). Of the 45 milk samples, only 20 percent of skim milk, 40 percent of 2% fat milk, and 20 percent of whole milk were within the acceptable range for vitamin D content. Non-compliance was also seen for vitamin A, where only 77 percent of 2% fat milk and 46 percent of skim milk were within the limits of vitamin A fortification. All samples not in compliance for vitamin A fortification were under-fortified. Hence, over-fortification of vitamin A was not observed in this study. Another survey of fluid milk fortification for vitamin D and A levels was conducted in New York State in 2001 (Murphy et al., 2001). Non-compliance for vitamin D fortification was observed for 52.3 percent of the 648 samples, of which 46.3 percent contained less than the acceptable levels of vitamin D as defined by the Food and Drug Administration. In addition, 55.5 percent of the samples did not fall within the acceptable range for vitamin A fortification. Overall, under-fortification occurred

in 51.4 percent of the milks sampled. In 2010, Patterson and colleagues published data from a nationwide survey in the United States on vitamin D levels in fluid milk. Large variability for vitamin D levels in fluid milk was observed, though only 16 percent of the 120 milk samples were found to be under-fortified (Patterson et al., 2010). More recently, non-compliance for vitamin D fortification was observed in 58 percent of the milks sampled from Vancouver (Liu, 2013).

There are a number of possible reasons for the variation in levels of vitamins found in milk. Inconsistent procedures used to fortify milks may contribute to variability (Hicks, Hansen, & Rushing, 1996). The stability of vitamin fortification products may vary during storage which may contribute to under-fortification. Additionally, dairy processors may over-fortify milk to account for vitamin instability during processing. Over-estimation of vitamin instability can result in vitamin levels remaining too high after processing and during storage. Also, fortification of low fat milk products with fat soluble vitamins can be difficult. Solubility or dispersibility of the vitamin fortification product is essential for proper fortification of low fat milk products. Ultimately, stability and dispersibility of vitamins in fortified milk is important for the maintenance of proper vitamin levels until consumption.

1.5 Vitamin stability

Degradation of vitamins D and A may be induced by oxidation (Byrn, 1976; Huber & Barlow, 1943). Double bonds contained within their structures may be altered as a result of exposure to light, air or high temperatures causing deactivation of the vitamin (DeRitter, 1982; Stewart, Midland, & Byrn, 1984). The structures of vitamin D_2, vitamin D_3, and vitamin A

palmitate can be found in Figure 1.1. Environmental stress causing vitamin deactivation may occur at any point during processing, storage, and food product processing and storage.

1.5.1 Vitamin D stability

All forms of vitamin D may be prone to oxidation, even more so in the presence of air. Pure crystalline vitamin D_2 remained relatively stable for up to nine months when sealed in evacuated amber vials at refrigerated temperature; however, vitamin D_2 degraded significantly after one to two months when stored in air-filled vials at refrigerated temperature (Huber & Barlow, 1943). When air-filled vials were stored at room temperature pure crystalline vitamin D_2 began to degrade after two to three days. Negligible deterioration was reported for pure crystalline vitamin D_3 stored in evacuated amber vials at refrigerated temperatures. In comparison, pure crystalline vitamin D_2 and vitamin D_3 in propylene glycol were stable for up to 30 months when stored in air-filled amber bottles at 38 to 40°C (Huber & Barlow, 1943). Vitamin D_2 (crystalline) completely decomposed after six months of storage at room temperature in air (Byrn, 1976). Byrn (1976) also reported that in the absence of oxygen, crystalline vitamin D_2 remained stable for one week during heat treatment at 80°C.

Stability of vitamin D_2 and D_3 powders was compared by Grady and Thakker (1980) at 25 and 40°C with varying levels of relative humidity. Vitamin D_3 powder remained relatively stable when stored in a desiccator at 25°C for 56 days. Vitamin loss was greater (11-14 percent) when relative humidity was increased to 85%. In contrast, the stability of vitamin D_2 improved with an increase in relative humidity along with storage at room temperature. At 56 days of room temperature storage, 95-98 percent loss was observed in samples stored in a desiccator, compared to 21-23 percent loss of vitamin D_2 when stored at 85% relative humidity. Storage at

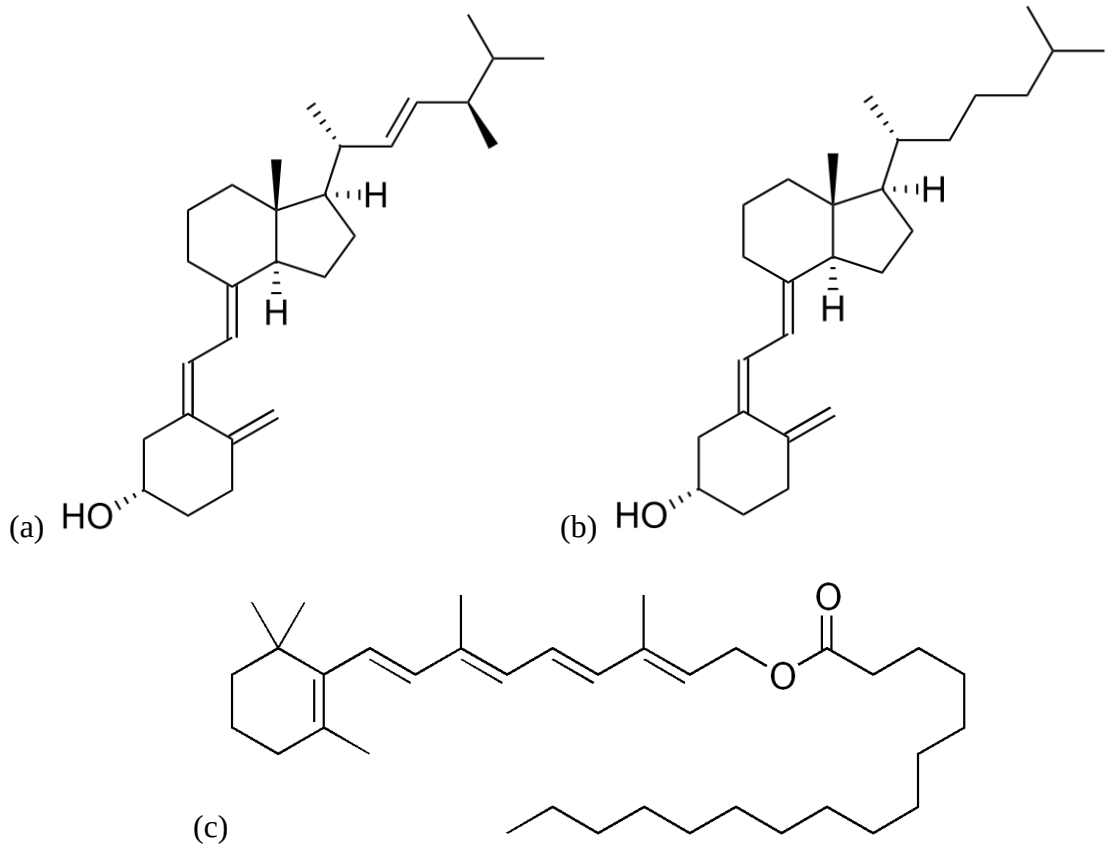


Figure 1.1 Molecular structures of vitamin D_2 (a), vitamin D_3 (b) and vitamin A palmitate (c) The figures for vitamin D_2 and vitamin D_3 are adapted from Vitamin D in Wikipedia (retrieved July 9, 2014, from http://en.wikipedia.org/wiki/Vitamin_D, Copyright Public Domain). The figure for vitamin A palmitate is adapted from http://en.wikipedia.org/wiki/Retinyl_palmitate, Copyright Public Domain).

40°C intensified the rate of degradation for both forms of vitamin D when at 45% relative humidity. At 40°C and relative humidity of 45 and 85%, 96-97 percent loss of vitamin D_3 was observed after 21 days of storage. No vitamin D_2 was measureable after 10 days of storage at 40°C and 45% relative humidity. Stability of vitamin D_2 improved when relative humidity was increased at 40ºC. Between 69 and 65 percent of vitamin D_2 remained after 21 days of storage at 40°C and 85% relative humidity. Stewart and colleagues (1984) reported the occurrence of vitamin D_2 oxidative degradation with light exposure when held at room temperature and average humidity for six months, though the extent of degradation and light intensity were not given.

It is important to note that despite the knowledge that vitamin D is important to human health, there is limited literature available examining the stability of vitamin D used in fortification of milk and other food products. What's more, much of the existing literature is dated and conclusions may not hold true in the current time because of changes to vitamin production technology and improved vitamin purity over time.

1.5.2 Vitamin A stability

Isolated vitamin A is sensitive to oxygen, trace metals, UV light, and acid pH (DeRitter, 1982). Vitamin A palmitate was the most stable of the esters when in the presence of moisture and more stable than retinol to heat in aqueous dispersions lower than pH 5.5. Retinoids in caprylic/capric triglyceride, an oily solvent, degraded significantly after 15 hours of UVA or UVB light exposure at room temperature with accelerated deterioration in the presence of oxygen (Failloux, Bonnet, Perrier, & Baron, 2004). However, photodegradation of retinoids decreased in the presence of butylated hydroxyl toluene (BHT). Along with BHT, several

compounds have been shown to stabilize vitamin A in vitamin preparations including aluminum salts of fatty acids such as stearic acid, and a combination of gelatin and dextrin (Byrn, 1976).

1.5.3 Stability of vitamins in food products

A variety of factors may affect the final vitamin content of a food product such as fluid milk. Processes and procedures employed during preparation and storage of the food product may cause vitamin degradation, resulting in lower than expected vitamin levels by the time the food product is consumed. Other factors include reactions between multiple components of the products that result in degradation reactions.

1.5.3.1 Stability of vitamin D in food products

Vitamin D can be quite stable during processing and storage of food products (Hanson & Metzger, 2010; Indyk, Littlejohn, & Woollard, 1996; Kazmi et al., 2007; Upreti, Mistry, & Warthesen, 2002; Wagner et al., 2008). Kazmi et al. (2007) found emulsified and crystalline forms of vitamin D_3 to be stable during yogurt fermentation and during storage of fortified yogurt at 5°C and ice cream at -25°C for four weeks each. Stability was greater for emulsified vitamin D_3 than crystalline vitamin D_3 during storage of a fortified lab scale Cheddar cheese-like matrix for three months at 4°C. Upreti et al. (2002) looked at the stability of vitamin D_3 in process cheese. No loss of vitamin D_3 was found during cheese manufacturing including during pasteurization. As well, no loss was observed over a nine month storage period at 21 to 29°C or 4 to 6°C. Loss of between 25 to 35 percent was found when cheese was heated at 232°C for five minutes. Wagner et al. (2008) reached similar conclusions on the stability of vitamin D during storage and some types of processing. Vitamin D_3 in Cheddar and low-fat cheeses was stable

during HTST pasteurization and over one year of ripening at 3 to 8°C. Similar to the findings of Upreti et al. (2002), here no loss of vitamin D was found after treatment for 5 minutes at 232°C. Hanson & Metzger (2010) studied the stability of a commercial water dispersible vitamin D product in HTST pasteurized 2% fat milk, UHT-processed 2% fat chocolate milk, and low-fat strawberry yogurt stored at 4°C for 21, 60, and 42 days, respectively. No significant loss in vitamin D was found during processing or over the shelf life of the products at 4°C. Findings published by Indyk et al. (1996) are in agreement that vitamin D_3 is stable throughout processing conditions. These authors reported vitamin D_3 to be stable to spray-drying, which included steps of pasteurization, high pressure evaporation and drying.

Other reports have indicated vitamin D instability in certain conditions and environments. Renken & Warthesen (1993) looked at the impact of light, air, and storage temperature on vitamin D_3 stability in skim milk. Skim milk stored in plastic containers at 4°C and exposed to light of around 3200 lux for 10 days resulted in 25 percent loss of vitamin D_3. Instability to light was stated to be very small or insignificant in terms of potential loss in real retail settings because of the extreme light intensity used in this study. The authors posited that vitamin D_3 loss is a result of photosensitized oxidation where singlet oxygen is produced when milk is exposed to light. Later research explored this hypothesis using model systems containing riboflavin (King & Min, 1998; King & Min, 2002). Riboflavin can act as a photosensitizer, triggering the production of singlet oxygen. Singlet oxygen may then react with vitamin D resulting in degradation.

UHT milk is typically not exposed to light as intense as pasteurized chilled milk; however, the longer shelf life of UHT milk leaves potential for longer exposure to light (Saffert, Pieper, & Jetten, 2008). Whole UHT milk fortified with vitamins was stored for 12 weeks at

23°C with light of different intensity (Saffert et al., 2008). Vitamin D_3 was stable when milk was stored in the dark, but loss between 20 to 57 percent was observed when light was introduced. Similar observations were made for low-fat UHT milk stored for 12 weeks at 23°C (Saffert et al., 2009). A reduction of 35 to 65 percent vitamin D_3 was found for low-fat UHT milk dependent on milk packaging.

1.5.3.2 Stability of vitamin A in food products

The degradation of vitamin A appears to be greatly dependent on exposure to light. Changing the carrier of vitamin A has some effect on stability to light. Low-fat and skim milks were fortified with the *trans* form of retinyl palmitate using butter, coconut oil, corn or peanut oil as carriers (Zahar, Smith, & Warthesen, 1986). Fortified milks were pasteurized, homogenized, and exposed to light at 1614 lux for 48 hours at 4°C. Vitamin A loss was greater for milk fortified using corn and peanut oil as vitamin carriers, such that ~ 55 percent of vitamin A remained in these samples at 48 hours compared to ~70 percent in milk fortified using coconut oil and butter as carriers. Bartholomew & Ogden (1990) looked at the influence of carriers on vitamin A light stability in a recombined milk system. All treatment groups had between 30 to 40 percent vitamin A remaining at 32 hours of storage at 4°C and light intensity of 4300 lux, with no significant differences between carriers.

Other components of the food system may affect the stability of vitamin A. Addition of 3% non-fat dry milk to fortified low-fat milk resulted in greater retention of vitamin A palmitate when stored at 4°C for 48 hours and exposed to light at 1614 lux (Gaylord, Warthesen, & Smith, 1986). The effect of β-carotene on the stability of vitamin A to light was investigated by fortifying skim milk with retinyl palmitate in corn oil and adding β-carotene to some samples

(Zahar, Smith, & Warthesen, 1992). Milk was stored for 96 hours at 4°C and exposed to light at 1614 lux. Less degradation of vitamin A was seen in milk samples containing added β-carotene than those without added β-carotene. As discussed previously, levels of carotenoids in milk vary greatly depending on many factors (Agabriel et al., 2007). This could account for some of the variability in vitamin A levels found for fluid milk.

Packaging may also influence the stability of vitamin A in fortified fluid milk when exposed to light. Many results have been published for vitamin A stability in milk (Saffert, Pieper, & Jetten, 2006; Saffert et al., 2008; Saffert et al., 2009; Zygoura, Moyssiadi, Badeka, Kondyli, Sawaidis, & Kontominas, 2004). Vitamin A seems to be moderately stable in pasteurized whole milk exposed to light; however, this is greatly dependent on packaging material such that vitamin A will significantly degrade when milk is packed in clear packaging (Saffert et al., 2006; Zygoura et al., 2004). When compared to clear packaging, loss of vitamin A was reduced in UHT milk packaged in all types of non-clear packaging that were exposed to light. After 12 weeks of storage at 23°C, vitamin A loss in UHT whole milk exposed to light was between 66 to 88 percent and in UHT low-fat milk was between 70 to 93 percent. When milk was stored in the dark, only 5 percent loss and 16 percent loss of vitamin A was seen for UHT whole milk and UHT low-fat milk, respectively (Saffert et al., 2008; Saffert et al., 2009). By comparing the loss of vitamin A in UHT whole milk and low-fat milk it is apparent that fat content may play a role in the extent of vitamin degradation.

1.6 Carrier systems for hydrophobic compounds

Since vitamins D and A are fat soluble and have limited solubility in low fat products, it is advantageous to formulate these vitamins as dispersible or water soluble forms for use in

low- or non-fat food products. As well, both these vitamins are vulnerable to degradation when exposed to certain stresses depending on their environment. Encapsulation or complexation of bioactives with biopolymers has demonstrated advantages such as increased solubility, bioavailability, and stability (Esmaili et al., 2011; Haham et al., 2012; Zimet et al., 2011).

1.6.1 Processing and physicochemical methods

Systems developed to entrap, protect, and subsequently release these compounds need to use food-grade materials and more preferably natural materials. These systems are typically formed using two main methods, physicochemical and processing, and a combination of both is often applied (Matalanis, Jones, & McClements, 2011). Physicochemical methods involve using physical forces, including hydrogen bonding or electrostatic interactions, to form particles that contain the desired ingredient. Processing uses specific processing methods to achieve the formation of particles containing the lipophilic compound. Typical methods include spray drying, freeze drying and homogenization. The methods have been reviewed in detail elsewhere (Matalanis et al., 2011) but a few specific examples will be discussed briefly here.

Physicochemical methods have been increasingly popular ways to provide protection to lipophilic compounds. Methods of preparation rely on changing the physicochemical properties of the system such as pH, concentration, holding time, holding temperature, and ionic strength Matalanis et al., 2011). Using different combinations of these, solutions can be manipulated so particles are formed between lipophilic compounds of interest and another compound, or multiple other compounds. Food grade proteins and polysaccharides are used in the formation of these particles. A variety of different whey proteins, casein, starch, cellulose and hydrocolloids are manipulated into complexes with lipophilic compounds. Essential to this method of particle

formation is knowledge of molecular characteristics and behavior. Conformation, structure and charge can all be important when developing a method to form particles through physicochemical methods. When choosing a particular protein or polysaccharide to use, it is important that the polymer characteristics cater to the function and final application of the particle. For example, a particle that is designed to deliver its load to the mouth to impart flavor must break down in the mouth, as opposed to delivering a compound to the intestine for absorption where the particle must then release its cargo. The current discussion will focus on methods that have been employed for the encapsulation of vitamin D and vitamin A in various forms.

Beta-lactoglobulin is a globular protein found in the whey fraction of mammalian milk. It has one main ligand binding site within the conical β-barrel in the protein structure (Ron, Zimet, Bargarum, & Livney, 2010). Two other binding sites may also exist where lipophilic compounds can associate. These binding sites are accessible to solvents and therefore, may provide only partial protection to the ligand. Complexes formed using β-lactoglobulin may provide protection to the ligand that has been trapped in the protein structure (Hattori, Watabe, & Takahashi, 1995; Shimoyamada, Yoshimura, Tomida, & Watanabe, 1996). Hattori and colleagues (1995) formed β-lactoglobulin/retinol complexes by adding ethanolic ligand solution to β-lactoglobulin dissolved in phosphate buffer. The solution was shaken to assist with complex formation. Retinol was more stable when bound to β-lactoglobulin than unbound retinol during heat treatment up to 73°C, exposure to oxygen and exposure to fluorescent light (Hattori et al., 1995). Shimoyamada and colleagues (1996) used a similar method of complex preparation for β-lactoglobulin/retinol and β-lactoglobulin/retinoic acid complexes. A protective effect of these complexes on retinol

and retinoic acid during exposure to UV light was found when compared to free retinol and retinoic acid.

Some methods of physicochemical encapsulation use the electrostatic interactions between two biopolymers to entrap a ligand. The attraction that occurs between positively charged proteins at a pH less than their isoelectric point and anionic polysaccharides, or negatively charged proteins at a pH greater than their isoelectric point and cationic polysaccharides can be exploited to capture a ligand (Ron et al., 2010). More specifically, both soluble and insoluble complexes can be formed. Soluble complexes result when the charges carried by the two compounds forming the complex around the ligand are not equal. This leads to a net charge allowing solubilization of the complex. Conversely, when charges are neutralized the complex becomes insoluble and precipitates.

Vitamin D_2 can be entrapped in an electrostatic complex formed using β-lactoglobulin and low methoxyl pectin that is soluble at low pH (Ron et al., 2010). By varying the concentration of low methoxyl pectin, net charge or charge neutralization can be obtained. Vitamin D_2 was found to be more stable in soluble β-lactoglobulin – pectin complexes than when the vitamin was bound to only β-lactoglobulin as well as when vitamin D_2 was alone (Ron et al., 2010). The optimal complexes were stable at pH 4.25 and formed a transparent solution with no precipitate.

Of the processing techniques used to encapsulate compounds, spray drying is the most common and has been used since the 1930's (Matalanis et al., 2011). First, an emulsion is formed with the lipophilic compound and a suitable emulsifier. Certain systems also require additional fats or oils to accompany the lipophilic compound of interest into the emulsion. The emulsifier material is commonly a polysaccharide or protein, or mixture thereof, and forms the

wall of the resulting particles (Jafari, Assadpoor, & Bhandari, 2008). Often a mixture of materials is utilized to obtain a variety of desirable properties within the wall material (Sosa, Zamora, Chirife, & Schebor, 2011). Acacia gum is a standard wall material for encapsulating flavors and oils that is often mixed with other compounds to lower the cost of use (Jafari et al., 2008). Effective encapsulation of fatty acids, and soy oil have been achieved using a mixture of gum acacia and maltodextrin. Carbohydrates such as alginates, sucrose and flour, and modified cellulose have also been explored as wall materials. Gelatin is a common protein used to form the wall of spray dried particles. Other proteins include soy protein and milk proteins.

The emulsion containing the lipophilic compound and desired wall materials is forced through a small nozzle or past a centrifugal wheel, sending the liquid as a mist into a hot chamber. The liquid phase quickly evaporates and a powder remains containing particles usually 10-100 μm in size (Matalanis et al., 2011). Even with a chamber temperature greater than 150°C, the core of the particles does not reach higher than 100°C because of the latent heat associated with liquid evaporation. The time the particle is exposed to heat lasts only a few seconds. To obtain a suitable particle that provides sufficient protection to the lipophilic compound it is important to consider four main factors: the attributes of the wall material, characteristics of the lipophilic compound, properties of the emulsion that is formed and the conditions used in the spray drying process (Jafari et al., 2008).

Spray drying is an advantageous technique because it is low cost. It has been used for many years so the technology is widely available and well-studied. However, there are a few drawbacks to using spray drying as a primary encapsulation method. It may be undesirable to use this technique for highly unstable lipophilic compounds as exposure to heat, even for a short time, may have a significant effect on the stability of the compound and cause oxidation.

Additionally, the particles that are formed provide protection to the encapsulated material as a dry powder. Nonetheless, protection does not automatically remain once the dry particles have been rehydrated.

A combination of physicochemical and processing techniques may be used to encapsulate vitamins that are used for the fortification of food and beverages. Two of the vitamin fortification products listed in Appendix C may have been formed using physicochemical methods, followed by spray drying. Dry vitamin D3 100 CWS/AM contains cholecalciferol, medium chain triglycerides, corn starch, acacia gum, sucrose and *dl*-α-tocopherol. Additionally, silicon dioxide is used as a processing agent. The purpose of acacia gum and corn starch may be to provide structure to the wall surrounding cholecalciferol and medium chain triglycerides (Matalanis et al., 2011). Inclusion of sucrose in spray dried formulations is intended to improve retention of the encapsulated compound (Sosa et al., 2011; Galmarini, Zamora, Baby, Chirife, & Mesina, 2008), which may be attributed to its role in fabricating an oxygen barrier (Desobry, Netto, & Labuza, 1998). As an antioxidant, the role of *dl*-α-tocopherol is to minimize oxidation and extend the shelf life of the product. Finally, silicon dioxide is used to enhance the flowing properties of a spray dried powder (Beindorff & Zuidam, 2010). Dry vitamin A palmitate, Type 250 CWS/F contains vitamin A palmitate, cornstarch, gelatin, sucrose and *dl*-α-tocopherol. It has a similar composition to dry vitamin D3 100 CWS/AM, however gelatin has been substituted for acacia gum.

1.7 Casein micelles as vitamin carriers

A variety of different milk proteins have been discussed as natural vehicles to carry bioactive compounds (El-Salam & El-Shibiny, 2012; Livney, 2010; Tavares, Croguennec,

Carvalho, & Bouhallab, 2014). Recent research has focused on casein micelles as natural carriers for hydrophobic compounds (Zimet et al., 2011; Haham et al., 2012; Sáiz-Abajo et al., 2013).

1.7.1 Casein micelles

1.7.1.1 Features

Bovine caseins are milk phosphoproteins that account for approximately 80% of bovine milk protein. They can be divided into four types based on their primary structure, and type and extent of post-translational modification. These types are α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, which are present in bovine milk at ratios of approximately 4:1:3.5:1.5, respectively (Dalgleish & Corredig, 2012). Caseins are essential for the transfer of calcium, phosphate and protein to the neonate through milk (McMahon & Oommen, 2013).

Most caseins present in bovine milk associate together into colloidal particles. These colloidal particles are termed casein micelles (McMahon & Oommen, 2013). Natural casein micelles are made up of 93.3 percent casein and 6.6 percent inorganic constituents (Aoki, Uehara, Yonemasu, & El-Din, 1996). The inorganic material is mainly represented by calcium and phosphate, often termed colloidal calcium phosphate (CCP), and is accompanied by small quantities of citrate, magnesium, and even smaller amounts of other species (O'Mahony & Fox, 2013). Casein micelles contain high levels of water. Interestingly, only 15 percent of the water is bound to protein, while the rest is merely held within the particle. Micelles in bovine milk exist as spherical particles ranging from 50 to 700 nm in diameter depending on the method used to measure particle size (McMahon & Oommen, 2013) with an average diameter of 150 to 200 nm (Dalgleish & Corredig, 2012) .

1.7.1.2 Structure

The structure of casein micelles has been the subject of much debate for over half a century. Over this time a variety of models have been proposed for how the components within the micelle are structured (McMahon & Oommen, 2013). Interpretations of the structure have been based on physicochemical evidence available at the time. Hydrophobic interactions, ionic and electrostatic interactions, hydrogen bonds, disulphide bonds, and steric stabilization may all be involved in maintaining the structure of micelles. Recently emphasis has been placed on bridging between phosphoserine groups of caseins by calcium and phosphate, as well as hydrophobic interactions between caseins resulting in association. Calcium and phosphate are found as self-assembled calcium phosphate nanoclusters within micelles. Of the caseins found in bovine milk, α_{s1}-casein, α_{s2}-casein, and β-casein are calcium sensitive, whereas κ-casein is not. Calcium sensitive caseins contain clusters of phosphoserine residues. Caseins may associate through phosphoserine groups interacting with calcium, calcium bridging, hydrophobic or hydrophilic region interactions, hydrogen bonding, or electrostatic interactions. Calcium insensitive caseins, κ-caseins, are found at increased levels on the outer edge of the micelles. The lack of phosphoserine residues excludes κ-caseins from interacting with calcium phosphate nanoclusters throughout the micelle. Nevertheless, κ-caseins may appear within the micelle by means of other stabilization forces. In addition, there is a glycomacropeptide within the protein sequence of κ-casein. The κ-caseins located on the exterior of the micelle prevent aggregation and growth of micelles by extension of the hydrophilic glycomacropeptide away from the micellar surface (McMahon & Oommen, 2013).

1.7.2 Re-assembled casein micelles

1.7.2.1 Composition and properties

Casein micelles can be reconstructed by mixing a source of casein together with phosphate, calcium and citrate (Knoop, Knoop, & Wiechen, 1979). Sodium caseinate is a readily available source of casein that has been used for this purpose (Haham et al., 2012; Sáiz-Abajo et al., 2013; Semo et al., 2007; Zimet et al., 2011). Production of sodium caseinate begins by lowering the pH of milk to 4.6, causing caseins to aggregate. Then the liquid (whey) is removed from the aggregated casein, and followed by addition of alkali such as sodium hydroxide to the aggregated casein (O'Kennedy, 2011). The resulting product is readily dispersable in water. Re-assembled micelles are significantly affected by changes in pH, temperature, and concentrations of the system components during the process of formation (Aoki, 1989; Aoki et al., 1987; Aoki et al., 1996; Dalgleish & Law, 1989; Gatti, Alvarez, & Sal, 1999). Additionally, altering the composition of re-assembled casein micelles has an influence on micelle properties. Modifying the amount of α_{s1}-casein, β-casein, κ-casein, colloidal phosphate, or citrate present in micelles induces changes in size and stability to dialysis pressure and heat stability, as well as the pH of maximum heat stability (Schmidt, 1979; Schmidt et al., 1979; Schmidt, Koops, & Westerbeek, 1977).

1.7.2.2 Re-assembled casein micelles as carriers

Re-assembled casein micelles (rCM) have been proposed to be used for transportation of functional compounds into the body. Applications of casein micelles as carrier structures have been in the medical and nutritional fields using a variety of preparation methods. Bioavailability,

safety and stability of the encapsulated compounds have been investigated depending on the proposed use. Safety is particularly important for medicinal uses because the encapsulated compounds can often be harmful, especially if highly bioavailable or in large doses. Stability of encapsulated compounds within casein micelles has been investigated most often for nutritional uses. The functional nutrients being carried typically are prone to oxidation and casein micelles are used to provide protection to the enclosed nutrient.

Self-assembling β-casein micelles have been employed as carriers for medically relevant molecules including celecoxib, mitoxantrone, and curcumin (Bachar et al., 2012; Esmaili et al., 2011; Shapira, Markan, Assaraf, & Livney, 2010). Beta-casein has been used to facilitate oral delivery (Bachar et al., 2012; Shapira et al., 2010), and to improve solubility and effectiveness of the encapsulated compound (Esmaili et al., 2011). The physical and chemical stability of the celecoxib powder over long-term storage was enhanced by the use of β-casein micelles. The need for a cryoprotectant to be added before lyophilisation was also eliminated (Bachar et al., 2012). Micelles similar to native casein micelles have been explored as carriers for curcumin (Sahu, Kasoju, & Bora, 2008; Yazdi & Corredig, 2012). Remarkably, curcumin was found to be more potent to cancer cells and significantly more soluble when carried by casein micelles than alone (Sahu et al., 2008). Cross-linking of casein nanoparticles by genipin has been used to enhance stability of the casein micelles as drug carriers (Elzoghby, Helmy, Samy, & Elgindy, 2013). Genipin locates between two free amino groups of lysine resides on casein chains and links them together. Through this method, solubility of nanoparticles was enhanced after spray drying.

Casein micelles can also function to carry nutritional factors such as vitamins and minerals. Reverse acidification of reconstituted skim milk resulted in association of iron with

casein micelles (Raouche, Dobenesque, Bot, Lagaude, & Marchesseau, 2009). Semo et al. (2007) used rCM for encapsulation of vitamin D_2 and reported an increase in UV light stability for the vitamin. A patent by Livney and Dalgleish (2009) resulted from the work of Semo et al. (2007) and outlines the use of rCM for encapsulation of hydrophobic compounds for nutraceutical purposes. Various publications have used this method for encapsulation of compounds by rCM patented by Livney & Dalgleish (2009) and similar methods have also been developed and explored (Zimet et al., 2011; Haham et al., 2012; Sáiz-Abajo et al., 2013). Oxidative stability of docosahexanoic acid during cold storage (4°C, 16 days) and heat treatment (74°C, 20 seconds) was improved by encapsulation in rCM (Zimet et al., 2011). Similarly, rCM provided increased protection to vitamin D_3 against oxidative degradation during thermal treatment of 80°C for one minute and cold storage for 28 days (Haham et al., 2012). Haham and colleagues (2012) also reported that bioavailability of vitamin D_3 in a clinical trial was preserved with the use of rCM. More recently, β-carotene in rCM displayed greater stability to sterilization and pasteurization than β-carotene alone (Sáiz-Abajo et al., 2013). Also, β-carotene in rCM was more stable to baking at 180°C for 20 minutes than free β-carotene in a cookie matrix.

1.7.3 Casein micelle stability

1.7.3.1 Stability to freezing

Specific alterations to the casein micelle structure are seen with slow freezing and storage between -10 and -20°C (O'Mahony & Fox, 2013). Calcium concentration in the unfrozen phase rises and a decrease in pH is observed due to precipitation of calcium phosphate. When

cryo-destabilized casein is dispersed in water, particles have micelle-like properties; however, not much information is available on the structure and properties of these particles.

1.7.3.2 Stability to heating

Heat treatment of milk can cause significant changes to casein micelles contained in the system. Anema and Klostermeyer (1997) looked at the effect of heat and pH on dissociation of casein micelles. Dissociation of κ-casein from micelles increased in a linear fashion as temperature was increased from 60 to 90°C when pH was held above 6.5. Anema and Li (2003) investigated the effect of heat on casein micelles in skim milk. The size of casein micelles in heated milk increased as much as 35 nm when whey proteins were present. When milk was heated without whey proteins the size of casein micelles changed very little. Thus, instability of casein micelles to heat treatment was explained to be a result of interactions with whey proteins. When milk is heated to above 70°C at its natural pH, whey proteins denature and aggregate. Complexes form mainly through disulphide reactions between whey proteins consisting of mainly α-lactalbumin and β-lactoglobulin, together with κ-caseins and to a lesser degree with α_{s2}-caseins causing changes to the micelle structure (Donato & Guyomarc'h, 2009). Hydrophobic and ionic interactions are also involved in the interaction of caseins with whey proteins to a lesser extent. These interactions during heat treatment are affected by pH, salt system and ionic strength (McMahon & Oommen, 2013).

Surface changes on casein micelles induced by heat mediated whey protein interactions were hypothesized by Yazdi and Corredig (2012) to alter the binding of curcumin to casein micelles in skim milk. Heating milk at 80°C for 10 minutes resulted in an increase of curcumin

incorporation into micelles, likely due to the presence of whey proteins on the surface. There was also a smaller but significant increase in binding when whey proteins were not present.

1.7.3.3 Stability to cooling

Although casein micelles are quite stable to many processes typically used in the manufacture of milk, alterations to the casein micelle structure are seen with cooling to between 0 and 5°C (O'Mahony & Fox, 2013). Cooling results in the dissociation some β-casein and smaller amounts of κ-casein from the micellar structure in greater proportions than α_{s1}-casein and α_{s2}-casein. This suggests that the former two types of caseins are held in the structure by hydrophobic interactions, which decrease as the temperature is lowered (McMahon & Oommen, 2013). Dissociation of β-caseins can occur by up to 60 percent when micelles are cooled (Downey, 1973). Using labelled ^{14}C, Creamer et al. (1977) demonstrated that this process is reversible.

Yazdi (2012) explored the potential effects of cooling on binding of cucumin and resveratrol to casein micelles in milk. A series of centrifugation and dilution steps were employed to deplete milk of β-caseins, after which curcumin or resveratrol was added. Compared to unaltered milk, increased levels of curcumin and resveratrol were bound to micelles in β-casein depleted milk. There was no difference in micellar integrity as viewed by cryo-tranmission electron microscopy and no shift in the level of colloidal calcium phosphate present. Yazdi concluded that β-casein release causes changes to the internal structure of the micelle, without affecting the surface structure or colloidal calcium phosphate.

Chapter 2: Hypotheses and experimental overview

2.1 Rationale

As indicated in Section 1.4.4, there is a history of non-compliance for fortification of milk with vitamins D and A. This non-compliance may be partly due to instability of the vitamins, as well as solubility problems in low-fat milk products. Re-assembled casein micelles (rCM) have been proposed as carriers for vitamins used in fortification since they may combat both of these hurdles.

When forming rCM, changing the concentrations of different constituents will alter the structure and stability of the resulting micelles (Aoki, 1989; Aoki et al., 1987; Aoki et al., 1996; Gatti et al., 1999; Schmidt, 1979; Schmidt et al., 1979; Schmidt & Koops, 1977), but no report has been found on whether altering the concentration of casein micelle components during formation will affect loading of a bioactive compound into the rCM. Changes within the rCM construct might allow more vitamins to bind within the structure.

Additionally, it is financially advantageous and more convenient to transport dry product as compared to a more bulky and weighty fluid product (Augustin & Hemar, 2008). Thus, it is valuable to investigate dried storage of the rCM vitamin product, more specifically in reference to the stability of the vitamins. Furthermore, the effect of milk processing and storage conditions on the stability of vitamins loaded into rCM has yet to be determined.

The purpose of the present research was to optimize loading of rCM with vitamins D and A, and to examine the efficacy of rCM as carriers for vitamin D in terms of improving vitamin stability. The protection that rCM can provide to vitamin D throughout storage of a dry powder and refrigerated storage of fortified fluid skim milk exposed to light was explored.

2.2 Hypotheses

Hypothesis 1: Vitamin – re-assembled casein micelles prepared using different concentrations of phosphate, citrate and calcium have different levels of vitamin loaded into the re-assembled casein micelles.

Hypothesis 2: Vitamins in dry powders of vitamin – re-assembled casein micelles are more stable during storage than vitamins stored with the same dry powdered materials that are not in micellar form.

Hypothesis 3: Vitamins that have been loaded into re-assembled casein micelles are more stable in milk exposed to light than vitamins added to milk along with the same materials used to form the re-assembled casein micelles.

2.3 Objectives

Objective 1: To investigate the levels of phosphate, citrate and calcium resulting in optimal loading of vitamin D_3 or vitamin A palmitate within re-assembled casein micelles.

Objective 2: To compare the protection provided to vitamin D_3 by re-assembled casein micelles to that provided by the same dry powdered materials not in micellar form during accelerated storage at 37˚C with 75% relative humidity and at ambient temperature and relative humidity.

Objective 3: To investigate the light stability of vitamin D_3 during cold storage of fluid skim milk fortified with vitamin D_3 – re-assembled casein micelles and fluid skim milk fortified with the same materials not in micellar form.

2.4 Experimental overview

An overview of the experimental approach used in this study is shown in Figure 2.1. The sections of experimental design that match each objective are shown in the figure. First, different concentrations of phosphate, citrate and calcium were used in the process of forming re-assembled casein micelles loaded with vitamin D_3 or vitamin A palmitate. Levels of phosphate, citrate and calcium resulting in the highest levels of vitamin loading were selected as optimal loading conditions. These re-assembled casein micelles loaded with optimal levels of vitamins were characterized.

The next two parts of the study were conducted using re-assembled casein micelles formed with optimal loading conditions for vitamin D_3. Vitamin D_3– re-assembled casein micelle suspensions formed using optimal loading conditions were freeze-dried and stored in the following conditions: accelerated storage (37˚C with 75% relative humidity) for a total of 96 hours, and ambient temperature and relative humidity for a total of 42 days. The same powdered ingredients used in the formation of optimally loaded vitamin – re-assembled casein micelles but not in micellar form ("control" powders), as well as a commercially available vitamin D_3 product (CWS D_3) were stored under the same conditions. Vitamin content of powders was tested after drying and throughout the storage period. See Figure 2.1 for time points when vitamin D was analyzed.

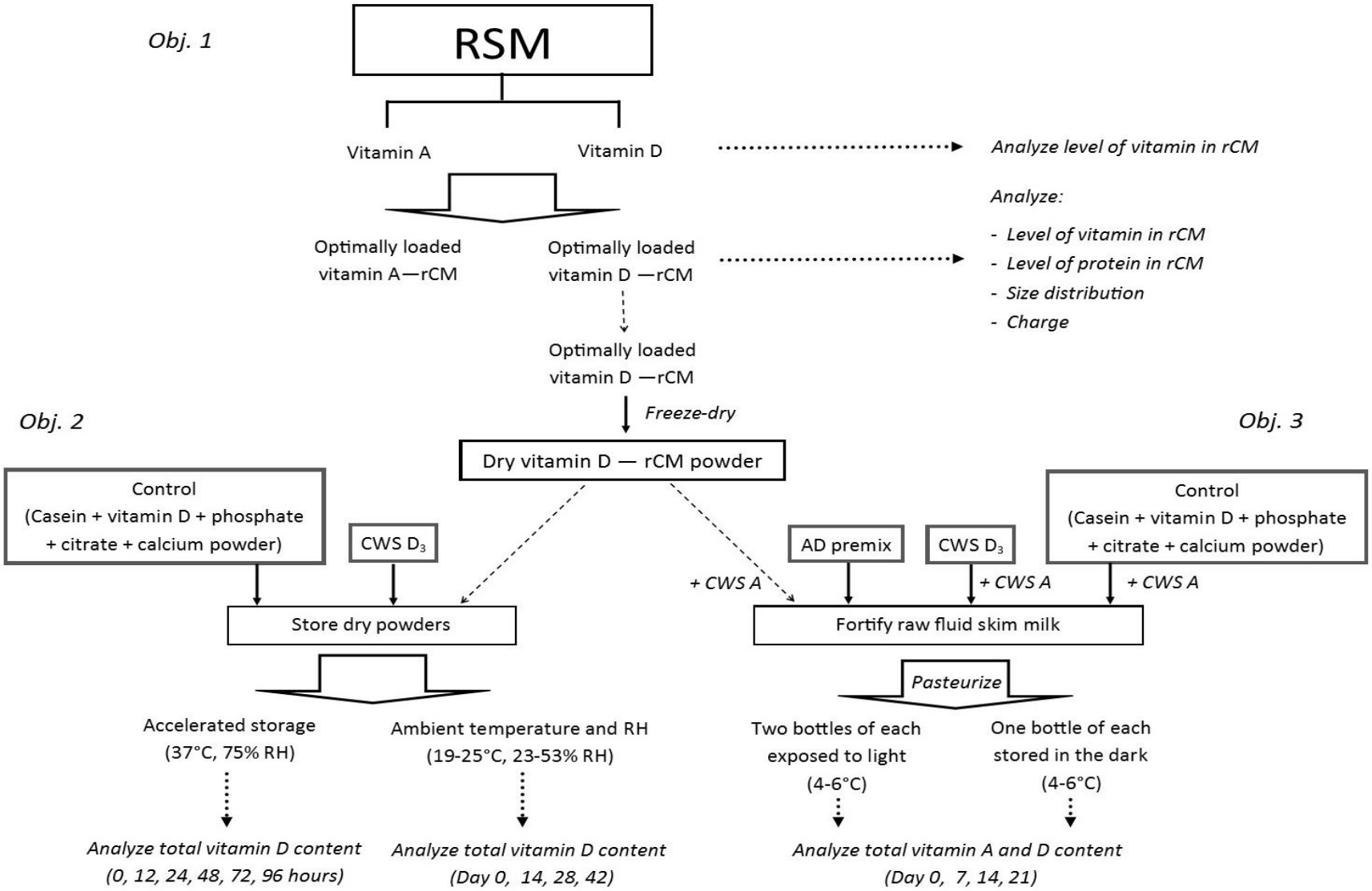


Figure 2.1 Overview of this thesis research

*RSM = response surface methodology; rCM = re-assembled casein micelle

The third objective was met by carrying out the following study. Fluid skim milk was fortified with optimally loaded vitamin D_3 – re-assembled casein micelles that were freeze-dried. Fluid skim milk was also fortified using the same materials used in the process of forming optimally loaded vitamin D_3 – re-assembled casein micelles ("control"), and with two commercially available products, CWS D_3 and vitamin AD premix. The fortified milk was pasteurized and exposed to light at 2000 lux intensity for 21 days at 4°C.

Chapter 3: Optimizing the incorporation of vitamins into re-assembled casein micelles

3.1 Introduction

Numerous studies in the past few decades have detailed the existence of non-compliance for milk fortification with vitamins D and A (Chen, Shao, Heath, & Holick, 1993; Faulkner et al., 2000; Holick et al., 1992; Liu, 2013; Murphy et al., 2001; Patterson et al., 2010). In each study, a significant number of milk samples tested were not in compliance with the label amount. As well, under-fortification of both vitamins seemed to be a substantial contributor to the non-compliance that was reported.

There are many potential contributors to high or low levels of vitamins in milk. These can be generalized into those stemming from the vitamin itself such as stability and solubility, and from external errors such as inconsistent vitamin preparation or addition procedures. Vitamin D and vitamin A are inherently unstable compounds because the double bonds contained within their structures can be altered by many stresses, including exposure to light, air and high temperatures (DeRitter, 1982; Stewart et al., 1984). Therefore, the stability of vitamins in fortification products may be affected even prior to addition to milk.

Furthermore, vitamins D and A may be unstable once added into dairy products. In some instances vitamin D is quite stable during heat treatment of dairy products such as process cheese, milk and yogurt (Hanson & Metzger, 2010; Indyk et al., 1996; Upreti et al., 2002; Wagner et al., 2008). Degradation is also limited during cold storage of cheese (Upreti et al., 2002; Wagner et al., 2008). Of note, Kazmi et al. (2007) observed that stability of emulsified

vitamin D_3 was greater than crystalline vitamin D_3 during storage of cheese for three months at 4°C.

Looking at how vitamin D fares in dairy products when exposed to light, there is some disparity within the literature as to the extent of vitamin D degradation (Liu, 2013; Renken & Warthesen, 1993). Renken & Warthesen (1993) stored skim milk in plastic containers at 4°C and exposed them to light of around 3200 lux for 10 days. Loss of 25 percent vitamin D_3 was found at the end of the storage time. Liu (2013) reported a significant variation in the degradation of different vitamin D formulations in fluid skim milk exposed to light at 2000 lux during storage at 4°C for three weeks. Between 29 and 63 percent of vitamin D_3 was retained in fortified skim milk for four different formulations, compared to the same formulations stored in the dark.

When compared to vitamin D, the influence of light exposure on vitamin A degradation is clearer. Vitamin A levels significantly decrease in skim milk, low-fat milk, UHT low fat and UHT whole milk when exposed to light in clear packaging (Saffert et al., 2006; Saffert et al., 2008; Saffert et al., 2009; Zahar et al., 1986). The carrier of vitamin A also may have a significant effect on the sensitivity of vitamin A to light. Zahar et al. (1986) demonstrated that coconut oil and butter provide greater protection to vitamin A palmitate than corn and peanut oil when used as vitamin carriers in low-fat and skim milk stored at 4°C for 96 hours and exposed to light at 1614 lux.

Carriers of vitamins D and A may play a role in improving vitamin stability to different stresses. One approach to a vitamin carrier is to look at encapsulation or complexation of these vitamins with biopolymers. Recent work has focused on rCM as carriers for hydrophobic compounds providing improved stability to stresses such as thermal treatment and UV light (Zimet et al., 2011; Haham et al., 2012; Sáiz-Abajo et al., 2013; Semo et al., 2007).

Because the ions used to make rCM each play a role in determining the size and composition of the micelles (Schmidt, 1979; Schmidt et al., 1979; Schmidt & Koops, 1977), they also may influence the vitamin loading and size of rCM complexes. Optimization of the conditions for forming rCM with high loading of a hydrophobic compound has not been previously investigated. Therefore, response-surface methodology (RSM) was employed to determine the optimum levels of three rCM components to achieve optimal vitamin loading in the rCM.

The objective of this study was to investigate the effect of phosphate, citrate, and calcium concentrations on vitamin D and vitamin A loading of rCM. Through this investigation, the levels of these three factors required for optimal vitamin loading of rCM were determined.

3.2 Materials

Vitamin D_2 (ergocalciferol), vitamin D_3 (cholecalciferol), sodium caseinate, phenolphthalein, retinyl palmitate, sodium azide, and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich Canada (Oakville, ON). Hydrochloric acid (HCl), potassium hydroxide (KOH), sodium hydroxide (NaOH), potassium phosphate dibasic anhydrous (K_2HPO_4), tripotassium citrate (K_3citrate), calcium chloride dehydrate ($CaCl_2$), ascorbic acid, acetic acid, sodium sulphate, ethanol (absolute), acetone, methanol (HPLC grade), hexane, dichloromethane (HPLC grade), ethyl acetate (HPLC grade), isopropanol (HPLC grade), and acetonitrile (HPLC grade) were purchased from Fisher Scientific Canada (Ottawa, ON). Ethylenediamine tetraacetic acid (EDTA) was obtained from BDH Chemicals, VWR (Radnor, PA). Retinyl propionate was purchased from Santa Cruz Biotech (Santa Cruz, CA). Protein assay dye reagent concentrate was obtained from Bio-Rad (Hercules, CA).

3.3 Methods

3.3.1 Experimental design

RSM using a central composite rotatable design (CCRD) was chosen for optimization of vitamin – re-assembled casein micelle preparations. RSM is a statistical technique commonly used to improve and optimize processes (Nakai, Li-Chan, & Dou, 2006). In this technique, input or independent variables are set at several levels in order to study how changes in these variables affect output or response variables. More specifically, the CCRD is most often used since the equations generated from this design remain quite accurate through to the boundaries of the tested factor limits.

Experiments with vitamin D and vitamin A used the same basic design. The experiment was a star design with axial points at ± α, cube points at ± 1, and a center point at zero. The values for α can be represented by the following equation: $\alpha = 2^{k/4}$ where k is the number of factors. In the present design $\alpha = 1.682$ which gives the design its rotatable characteristic. The number of experiments within this type of design is $2^k + 2k + 1$. The center point is typically replicated as a representation of error for the experiment (Nakai et al., 2006). These coded values can be converted to decoded values to be used in experiments. In the present case, decoded values refer to final concentrations. Coded experimental design points for forming rCM loaded with vitamin D or vitamin A are listed in Table 3.1.

Three factors: phosphate, citrate and calcium concentrations, were used within the current experiment design as these are factors reported to influence casein micelle formation and micellar size (Schmidt, 1979). The lower limits for phosphate, citrate and calcium in the RSM for vitamin D_3 were set at 1, 1, and 5 mM final concentration in the rCM preparation,

Table 3.1 Coded units for final concentrations of factors in central composite rotatable design for forming re-assembled casein micelles with vitamin D_3 and vitamin A palmitate

Standard Order	***Phosphate***	***Citrate***	***Calcium***
1	- 1	- 1	- 1
2	+ 1	- 1	- 1
3	- 1	+ 1	- 1
4	+ 1	+ 1	- 1
5	- 1	- 1	+ 1
6	+ 1	- 1	+ 1
7	- 1	+ 1	+ 1
8	+ 1	+ 1	+ 1
9	- α	0	0
10	+ α	0	0
11	0	- α	0
12	0	+ α	0
13	0	0	- α
14	0	0	+ α
15 [a]	0	0	0

[a] 15-20 = replication of the center point

respectively. The upper limits for phosphate, citrate and calcium in the RSM for vitamin D_3 were set at 20, 15 and 30 mM final concentrations, respectively. These concentrations were chosen to encompass conditions previously employed for the formation of rCM (Aoki, 1989; Aoki et al., 1996; Dalgleish & Law, 1989; Gatti et al., 1999).

After conducting the RSM with vitamin D_3 using these concentration limits, it was decided that it was not necessary to test such high concentrations of citrate and low concentrations of calcium. The lower limits for phosphate, citrate and calcium in the RSM for vitamin A palmitate were set at 1, 1, and 10 mM final concentration, respectively. The upper limits for the final concentrations of phosphate, citrate and calcium in the RSM for vitamin A palmitate were set at 20, 10 and 30 mM, respectively.

Levels of the three factors for rCM loaded with vitamin D_3 or vitamin A palmitate have been converted to decoded units, or final concentrations, and shown together with the standard order and run order for each CCRD in Table 3.2. For all of these experiments, a final casein concentration of 1% (w/v) was used (Haham et al., 2012). Casein concentration was kept constant as preliminary experiments showed that casein concentration did not influence vitamin D_3 loading. The amount of vitamin contained in the rCM was measured in duplicate samples from each experiment. Once optimal conditions for loading of rCM with vitamin D_3 and vitamin A palmitate were determined using surface and contour plots of the regression models, suspensions containing rCM loaded with vitamins were formed. Optimal suspensions were prepared twice, resulting in two productions runs (P1 & P2) for each optimal rCM suspension. Vitamin loading of rCM contained in these optimally loaded rCM suspensions was measured on duplicate samples from each production run. Also, the size and charge of particles present in the

Table 3.2 Final concentrations of factors (decoded units) in central composite rotatable design for forming re-assembled casein micelles with vitamin D_3 and vitamin A palmitate

		Vitamin D_3			***Vitamin A palmitate***			
Standard Order	*Run Order*	*Phosphate (mM)*	*Citrate (mM)*	*Calcium (mM)*	*Run Order*	*Phosphate (mM)*	*Citrate (mM)*	*Calcium (mM)*
1	*3*	4.9	3.8	10.1	*11*	4.9	2.8	14.1
2	*6*	16.1	3.8	10.1	*14*	16.1	2.8	14.1
3	*14*	4.9	12.2	10.1	*18*	4.9	8.2	14.1
4	*9*	16.1	12.2	10.1	*15*	16.1	8.2	14.1
5	*11*	4.9	3.8	24.9	*19*	4.9	2.8	25.9
6	*4*	16.1	3.8	24.9	*9*	16.1	2.8	25.9
7	*7*	4.9	12.2	24.9	*10*	4.9	8.2	25.9
8	*5*	16.1	12.2	24.9	*20*	16.1	8.2	25.9
9	*8*	1.0	8.0	17.5	*1*	1.0	5.5	20.0
10	*17*	20.0	8.0	17.5	*2*	20.0	5.5	20.0
11	*15*	10.5	1.0	17.5	*17*	10.5	1.0	20.0
12	*2*	10.5	15.0	17.5	*6*	10.5	10.0	20.0
13	*12*	10.5	8.0	5.0	*12*	10.5	5.5	10.0
14	*1*	10.5	8.0	30.0	*3*	10.5	5.5	30.0
15	*13*	10.5	8.0	17.5	*13*	10.5	5.5	20.0
16	*20*	10.5	8.0	17.5	*4*	10.5	5.5	20.0
17	*18*	10.5	8.0	17.5	*5*	10.5	5.5	20.0
18	*10*	10.5	8.0	17.5	*16*	10.5	5.5	20.0
19	*16*	10.5	8.0	17.5	*8*	10.5	5.5	20.0
20	*19*	10.5	8.0	17.5	*7*	10.5	5.5	20.0

optimal suspensions were determined. Finally, the amount of protein held within the rCM in the optimal suspensions was also measured.

3.3.2 Vitamin binding and re-assembly of casein micelles

The procedure for rCM preparation in the present study was based on a method described by Haham et al. (2012) and took place at ambient temperature:

A 60 mL volume of 2% sodium caseinate solution in distilled water was prepared. Sodium azide was added to 0.05 % (w/v) of the final volume as a preservative. This solution was stirred while adding 1.48 mL of ethanolic vitamin D_3 (13 mg/mL) or vitamin A palmitate in acetone (14 mg/mL). Stirring continued for 30 minutes. Solutions of K_2HPO_4, K_3citrate, and $CaCl_2$ were prepared in distilled water as per calculations from final concentrations dictated by the CCRD using Minitab software (Table 3.2). Slowly, 13.2 mL K_2HPO_4 solution and then 1.2 mL K_3citrate solution were added. For vitamin D_3 RSM experiments, 44 mL $CaCl_2$ solution was added drop by drop from a 50 mL burette while stirring. For vitamin A palmitate RSM experiments, 44 mL $CaCl_2$ solution was added drop by drop from a 50 mL syringe, using a programmable syringe pump (Braintree Scientific, BS-8000; Braintree, MA) set at 1.2 mL per minute, while stirring. The total volume was 120 mL. The final suspension was stirred moderately for 1 hour.

Prior to settling on using 14 mg/mL vitamin A palmitate solubilized in acetone, higher concentrations of vitamin A palmitate were used in trial experiments for forming re-assembled casein micelles loaded with vitamin A palmitate. At concentrations higher than 14 mg/mL, vitamin A palmitate was found to be insoluble after being dropped into the sodium caseinate solution at the beginning of the re-assembly procedure. This presented as yellow clumps attached

to the sides of the beaker used to contain the suspension and also attached to the magnetic stir bar used for agitation. Testing was conducted on the use of ethanol to solubilize vitamin A palmitate. The concentration of vitamin A palmitate used thereafter (14 mg/mL) was also determined to be too high to solubilize properly in ethanol.

3.3.3 Re-assembled casein micelle separation and vitamin release from micelles

Samples of 10 mL were taken from each rCM preparation and centrifuged at 25,000 *g* for 1 hour at 20°C (Semo et al., 2007). The supernatant was separated from the pellet since rCM were assumed to be located in the pellet.

Dissociation of rCM to release vitamins was conducted in a similar manner as Semo et al. (2007). The pellet was suspended in 10 mL 0.1 M EDTA (prepared in water and adjusted to pH 7.2 with 1 N NaOH) within 15 minutes of centrifugation. This pellet + EDTA solution was sonicated using an Elmasonic S 30/(H) at 37 KHz (Fisher Scientific Canada; Ottawa, ON) for 15 minutes, and then stirred at 4°C for 14 – 18 hours to allow for dissociation of micelles. The pellet solution was diluted to suitable approximate concentrations for the extraction and HPLC analysis methods employed for vitamin D_3 or vitamin A palmitate.

3.3.4 Vitamin extraction and analysis

3.3.4.1 Vitamin D

Vitamin D_3 extraction and HPLC analysis for each sample was conducted in duplicate using a procedure modified from the AOAC method 995.05 (Sliva & Sanders, 1996). The employed method has been used previously for extraction and analysis of vitamin D_3 from skim

milk in our lab (Liu, 2013) and is explained in detail in Appendix A.1. A description of the flow rate and concentrations of mobile phase components for HPLC analysis is listed in Appendix A.3. The same procedure was followed for all vitamin D_3 analyses.

3.3.4.2 Vitamin A

A method was developed in our lab previously to extract vitamin A palmitate from skim milk (Liu, 2013). This procedure is modified from the AOAC method 2002.06 (Hite, 2003); it requires small amounts of sample and uses reverse phase HPLC instead of normal phase (Liu, 2013). The extraction and analysis procedures are described fully in Appendix A.2. The flow rate and concentrations for mobile phase components used for HPLC analysis are listed in Appendix A.3. The same method of extraction was employed for all samples. Vitamin A analysis for each sample was conducted in duplicate.

3.3.5 Protein determination

Protein content of the rCM was assumed to be equal to protein in the pellet, which was calculated as the difference between total protein concentration and protein concentration determined in the supernatant. The supernatant was diluted to a total of 25 or 50 mL with distilled water and frozen at -20°C for analysis at a later date. These supernatant samples required further dilution to fit within the determination range of the protein assay.

Protein concentration of the supernatant was determined using the Bio-Rad Protein assay (Bio-Rad, 2013). This assay is based on the Bradford method. The manufacturer's directions for the standard procedure with a 96 well microplate were followed. The dye reagent was prepared by diluting 1 part dye reagent concentrate with 4 parts distilled, deionized water. Volumes of 10

μL for each sample and standard were put into wells of a microplate. To each sample and standard, 200 μL of the diluted reagent was added. The plate was incubated at ambient temperature for 15 minutes and samples were read at 595 nm using a microplate reader (Infinite M200 PRO, TECAN; Maennedorf, Switzerland) at ambient temperature.

Standard protein solutions containing 0, 50, 125, 250 and 500 μg/mL sodium caseinate in water were used to make a standard curve to determine the protein concentration in each sample. Protein concentration was determined from triplicate samples of supernatant and each supernatant sample was assayed in triplicate.

3.3.6 Charge determination

Charge is reported as zeta potential (in mV) measured on a Malvern Zetasizer Nano ZS using Zetasizer 7.0 software. Samples of 1 mL were diluted with water at a ratio of 1:10. Zeta potential was measured on two preparations for each type of V-rCM suspension. Three samples from each preparation were analyzed. Three measurements were taken for each sample and a mean value was given by the Zetasizer software. These three mean values were averaged to give one value per preparation, and the two preparations of each V-rCM suspension were averaged resulting in one value representing the zeta potential of that preparation type.

3.3.7 Size determination

3.3.7.1 Light scattering

Particle size was measured by light scattering on a Malvern Mastersizer with a Hydro2000SM small volume wet dispersion accessory. This instrument uses laser diffraction to

determine particle size. The principle behind laser diffraction is that particles passing through the laser beam will scatter light at angles directly related to their size. Measurements were conducted on the whole suspension formed from optimal loading conditions. The small volume wet dispersion accessory was filled with tap water and flushed through the system at least three times for cleaning. Following this, distilled water was used to flush the system. Samples were pipetted into the small volume wet dispersion accessory, which was filled with distilled water, to reach a minimum obscuration of 5 percent. Duplicate experiments (Production Run 1 and Production Run 2) were prepared for size measurements. For each experiment, measurements were conducted on triplicate samples of optimally loaded rCM suspensions. Three measurements were taken per sample and averaged by the software. Size is reported as volume weighted particle size, calculated as the average of the three samples. For each run a particle size distribution curve was recorded in volume (%).

3.3.7.2 Light microscopy

Suspensions of VD-rCM were also viewed by light microscopy at the Bioimaging Facility (The University of British Columbia). For each suspension, 3 glass slides were prepared by pipetting 5 µL into the center of the slide. A slide cover was placed over the suspension. Slides were viewed immediately after preparation using a Zeiss Axioplan Fluorescent Microscope (Carl Zeiss Microscopy, Thornwood, NY) with a QImaging 12 bit cooled CCD camera and QCapture software. For each slide, contrast enhancement was done using a phase contrast objective and differential interference contrast components.

3.4 Statistical analysis

The CCRD RSM was analyzed using Minitab 16 software (State College, PA). Regression analysis and analysis of variance (ANOVA) at a 95 percent confidence level was conducted to determine the significance of each factor and fit the data to a model. Validity of the model was determined through coefficient of determination (R^2 values), lack of fit tests, and observation of residual plots. Optimum conditions were determined through critical evaluation of surface and contour plots. Values were considered significantly different if $p < 0.05$. Minitab 16 software (State College, PA) was also used to analyze size distributions, including skew, kurtosis and goodness of fit for the type of distribution.

3.5 Results and discussion

3.5.1 Vitamin loading of rCM

Results for vitamin D_3 loading of rCM in the vitamin D_3 CCRD experiments are listed according to standard order in Table 3.3. The expected total vitamin D_3 in each sample taken for analysis was 1605 μg. Preliminary results showed that total recovery of vitamin D_3 was around 95 percent or higher. Therefore, percent of vitamin D found in pellet calculated from total expected vitamin D_3 is interpreted as being loading of rCM. The remaining vitamin D_3 is assumed to have partitioned into the supernatant. Vitamin loading seen within the replicated experiments (standard order 15-20) ranged between 26 and 34 percent (mean ± standard deviation = 30 ± 3). These experiments provide a measure of repeatability or level of error in terms of vitamin D_3 loading of rCM formed under the center point conditions. There was a wide range in vitamin loading for all experiments in the CCRD, from 3 (standard order 3) to 91

Table 3.3 Vitamin D_3 (mean, n = 2) found in pellet of 10 mL samples of vitamin D_3 – re-assembled casein micelle suspension from central composite rotatable design

Standard Order	***Mean vitamin D_3 in pellet (µg)***	***Mean vitamin D_3 in pellet (%)[a]***
1	745	46
2	245	15
3	46	3
4	163	10
5	1445	90
6	1459	91
7	881	55
8	693	43
9	1213	76
10	355	22
11	1455	91
12	153	10
13	67	4
14	1375	86
15	503	31
16	433	27
17	480	30
18	553	34
19	510	32
20	414	26

[a] Calculated as the percent of vitamin D_3 recovered in the pellet out of the expected total vitamin in the 10 mL sample (1605 µg)

percent (standard order 6 and 11).

Table 3.4 lists the results for vitamin A palmitate loading of rCM in the CCRD experiments according to standard order. Assuming no loss of vitamin A palmitate, the expected total vitamin A palmitate in each sample taken for analysis was 1727 μg. Vitamin loading for replicated experiments (standard order 15 to 20) varied from 29 to 42 percent (mean ± standard deviation = 39 ± 4). Similar to results of the CCRD experiments with vitamin D_3, there was a wide range of vitamin loading between the experiments, from 10 (standard order 3) to 72 percent (standard order 14). The smaller range in loading for vitamin A palmitate experiments than vitamin D_3 experiments might be credited to the narrower ranges in factor levels used for the experimental design.

3.5.2 Establishing a model for the effect of calcium, phosphate and citrate on vitamin loading of re-assembled casein micelles

It is evident from the range of vitamin loading seen in experiments within the CCRD for vitamin D_3 and for vitamin A palmitate that one or more of the tested factors have an effect on vitamin loading. To determine the level of significance and direction of effects, multiple regression analysis was conducted. Appendix B contains the regression coefficients and ANOVA analysis for vitamin D_3 loading of rCM that was conducted using coded units of the factors. It was determined that a linear and squares model best fit the data for vitamin D_3 loading with a coefficient of determination (R_{adj}^2) of 0.97. This means that 97 percent of the variability within the range of values can be explained using this model. Lack of fit from ANOVA testing was insignificant (p = 0.072), and there were no unusual observations on residual plots, which are further indications for the validity of the model. In this model, citrate, calcium, citrate^2, and

Table 3.4 Vitamin A palmitate (mean, n = 2) found in pellet of 10 mL samples of vitamin A palmitate – re-assembled casein micelle suspension from central composite rotatable design

Standard Order	***Mean vitamin A palmitate in pellet (µg)***	***Mean vitamin A palmitate in pellet (%)[a]***
1	680	39
2	329	19
3	178	10
4	283	16
5	894	52
6	854	49
7	789	46
8	728	42
9	387	22
10	257	15
11	784	45
12	827	48
13	311	18
14	1243	72
15	493	29
16	694	40
17	602	35
18	736	43
19	603	35
20	719	42

[a] Calculated as the percent of vitamin A palmitate recovered in the pellet out of the expected total vitamin in the 10 mL sample (1727 µg)

calcium^2 all had highly significant effects ($p \leq 0.001$) on vitamin D_3 loading. The regression coefficient for citrate was negative and the regression coefficient for the squared term of citrate was positive. This means that as citrate was increased from the lower concentration limit,vitamin loading decreased. Then at a certain level of citrate past the center of the concentration limits, further increases in the citrate resulted in greater vitamin loading. Positive regression coefficients were found for calcium and the squared term of calcium. This indicates that as calcium increased from the lower concentration limit, vitamin loading increased. At a point before the calcium concentration reached the mid-point of the concentration limits, further increases in calcium resulted in higher vitamin loading. Throughout the upper half of the calcium concentrations used, increases in calcium resulted in greater vitamin loading.

Multiple regression analysis was also used to fit a model to data from the CCRD for vitamin A loading of rCM. Regression coefficients and ANOVA analysis of vitamin A palmitate loading of rCM are shown in Appendix B. As with vitamin D_3, a model including linear and squared terms was the best fit with a coefficient of determination (R_{adj}^2) of 0.82. There were no concerning observations from residual plots, and lack of fit testing ($p = 0.270$) also indicated that the model selected was valid. Significant factors for vitamin A palmitate loading were calcium ($p = 0.000$) and phosphate^2 ($p = 0.001$). The regression coefficient for calcium was positive, indicating that loading of vitamin A palmitate increased with greater concentrations of calcium. The squared term of phosphate was significant with a negative regression coefficient. Loading of vitamin A palmitate was highest around the center of the range of phosphate concentrations that were tested, and loading decreased as the concentration of phosphate was moved towards the higher and lower edges of this range.

The influence of all factors in a CCRD including linear and squared terms in the model can be expressed using the following regression equation:

$$\mathbf{Y = b_0 + \sum b_n X_n + \sum b_{nn} X^2_n}$$

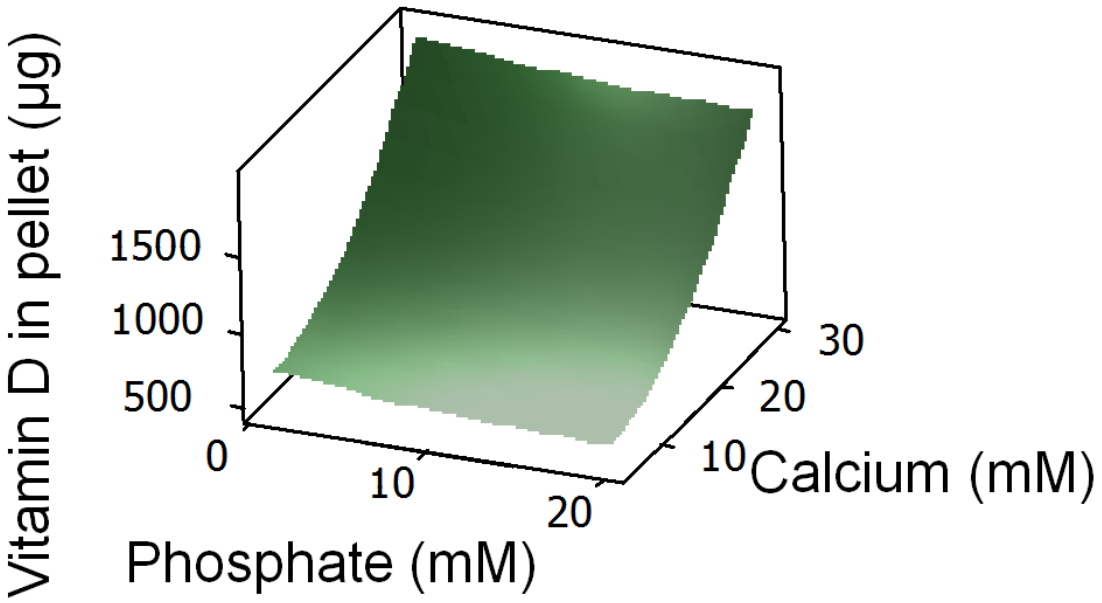
In this equation Y is the predicted response (vitamin loading), b_0 represents the value for the fixed response at the central point of the experiment (constant), b_n are linear coefficients, b_{nn} are quadratic coefficients, and X values are independent variables (X_1 = phosphate, X_2 = citrate, X_3 = calcium). Using this prescribed format, the following regression equations can be obtained that represent the effect of the three factors on vitamin D_3 loading of rCM and on vitamin A palmitate loading of rCM.

$$\mathbf{VD = 479 - 42\ X_1 - 348\ X_2 + 374\ X_3 + 13\ X_1^2 + 131\ X_2^2 + 101\ X_3^2}$$

$$\mathbf{VA = 643 - 41\ X_1 - 52\ X_2 + 246\ X_3 - 122\ X_1 + 49\ X_2^2 + 39\ X_3^2}$$

The 3D response surfaces and 2D contour plots of vitamin loading can be formed using these equations when one factor is fixed at a specific level and the other two are allowed to vary along the axes. These plots allow visual examination of the effect of different factors on a certain response. Figure 3.1 shows surface and contour plots for the effect of phosphate and calcium on vitamin D_3 loading of rCM. The effect of citrate and calcium on vitamin D_3 loading of rCM is shown in surface and contour plots in Figure 3.2. Also, the effect of phosphate and citrate on vitamin D_3 loading of rCM is depicted in Figure 3.3. Additionally, surface and contour plots showing the effect of calcium and phosphate on vitamin A palmitate loading of rCM are found in Figure 3.4.

a)



b)

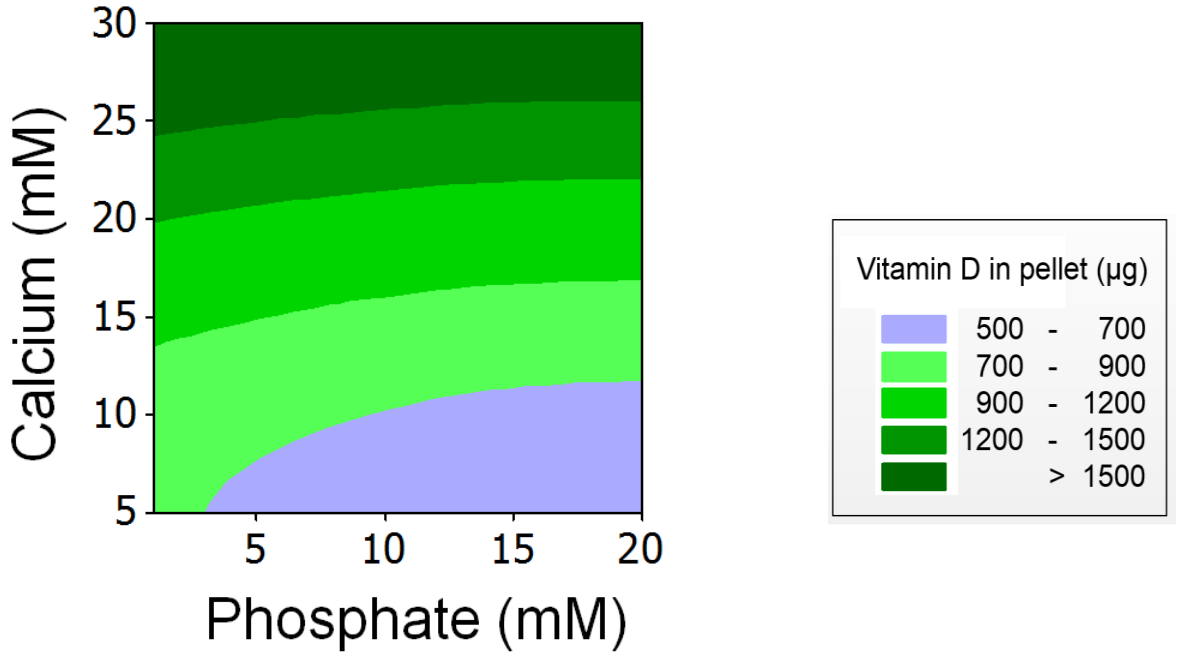
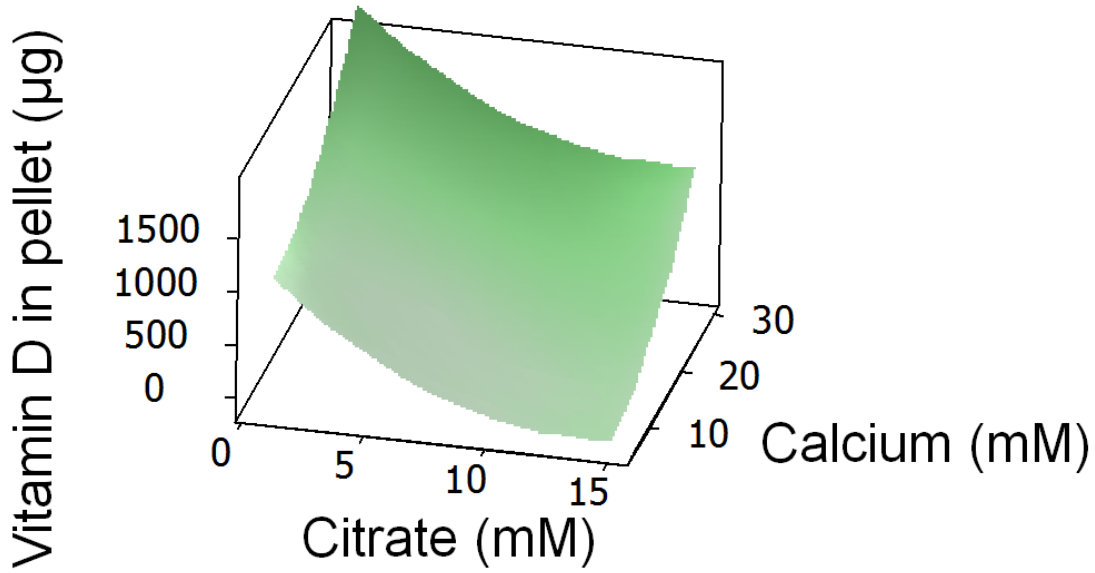


Figure 3.1 Surface (a) and contour (b) plots of vitamin D recovered in the pellet with phosphate vs calcium, and citrate held at a final constant concentration of 3.8 mM

a)



b)

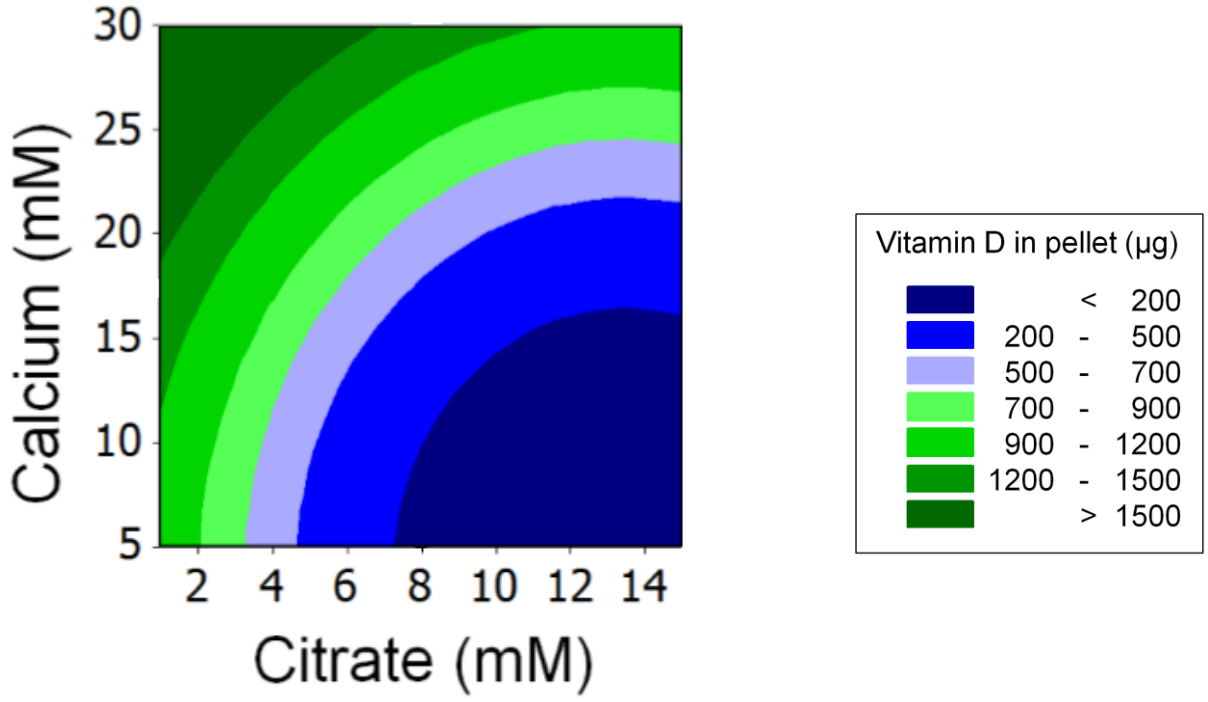
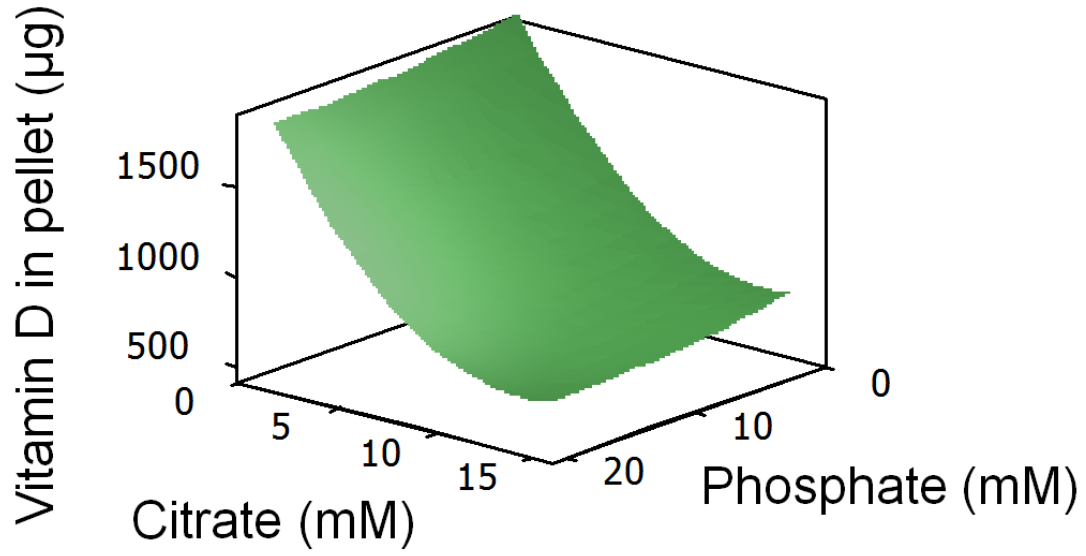


Figure 3.2 Surface (a) and contour (b) plots of vitamin D recovered in pellet with calcium vs citrate, and phosphate held at a final concentration of 10.5 mM

a)



b)

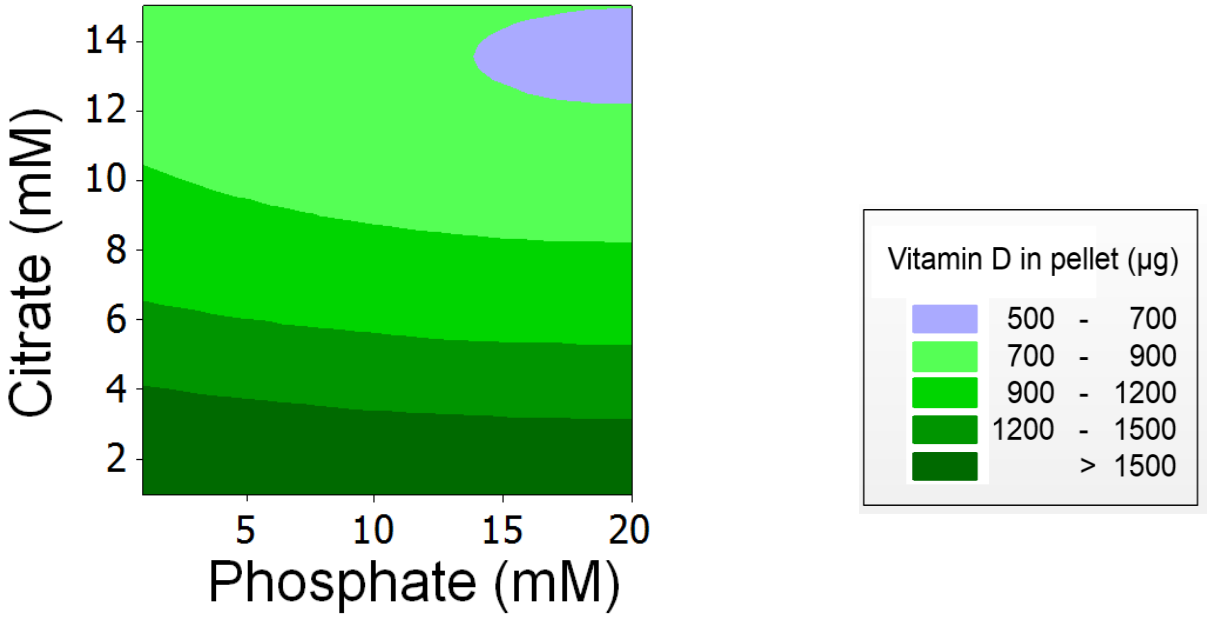
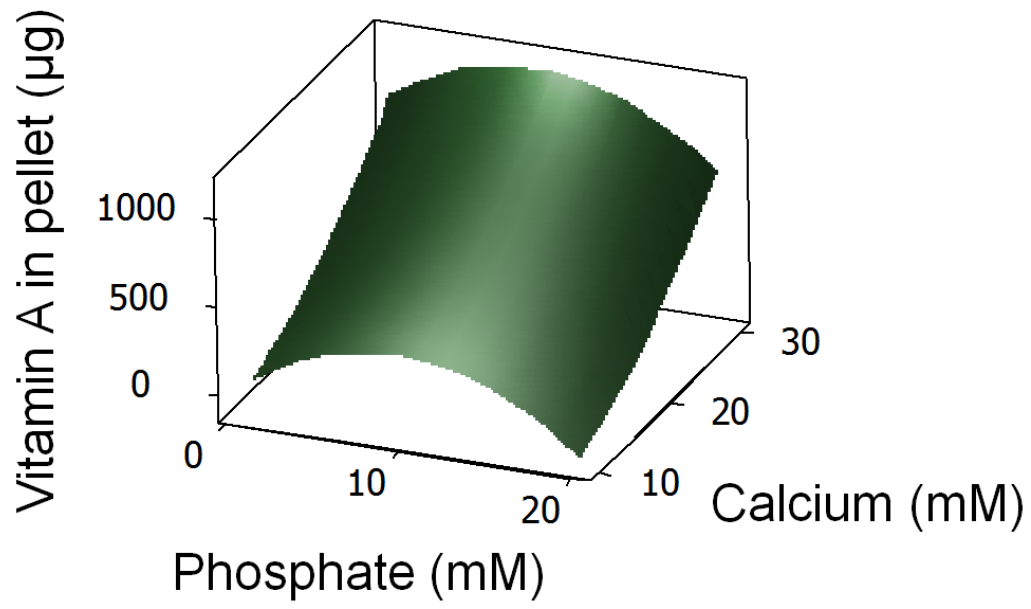


Figure 3.3 Surface (a) and contour (b) plots of vitamin D recovered in pellet with citrate vs phosphate, and calcium held at a final concentration of 24.9 mM

a)



b)

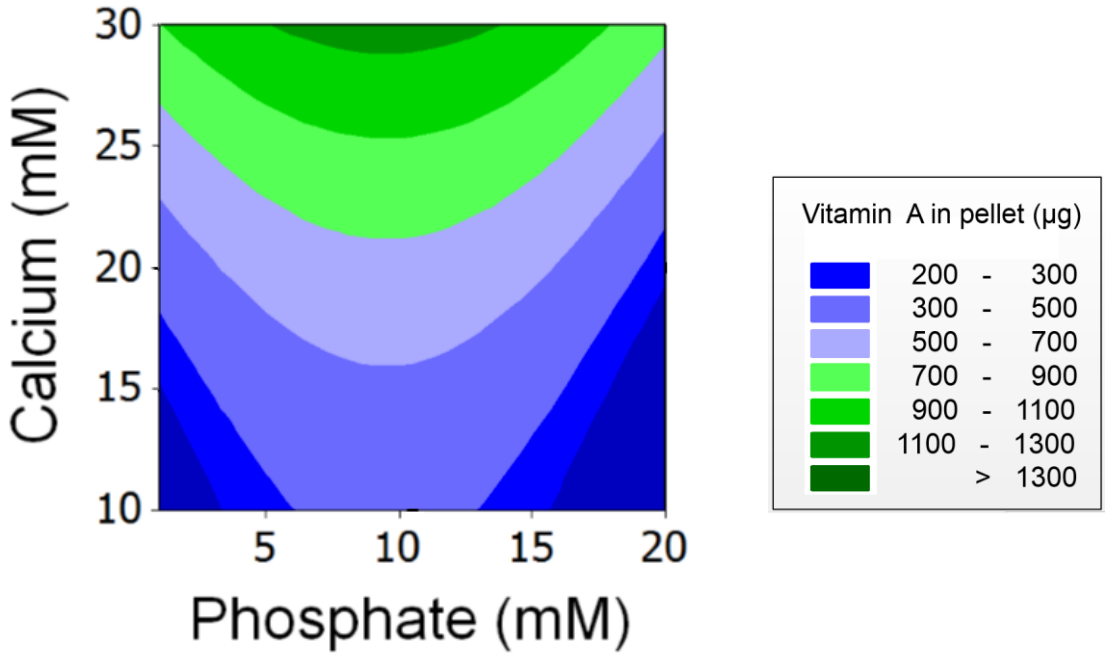


Figure 3.4 Surface (a) and contour (b) plots of vitamin A recovered in pellet with calcium vs phosphate, and citrate held at a final concentration of 5.5 mM

A common factor found to increase loading of both vitamins when the factor was increased was calcium. Calcium has a significant impact on the level of casein cross-linking which results in the formation of casein micelles (Aoki et al., 1996). Lipophilic ligands bind to caseins, in particular β-casein which is the most hydrophobic of the casein proteins (Forrest, Yada, & Rousseau, 2005). This would suggest that since higher levels of calcium cause more caseins to cross-link and become part of the rCM, increases in calcium would cause vitamin loading to heighten because the vitamin is expected to be associated with casein. The results in the current work are consistent with this assumption. Aoki et al. (1996) also reported that phosphate has a significant positive effect on casein cross-linking, though to a lesser extent than calcium. With tested ranges of 30-40 and 0-30 mM calcium and phosphate, respectively, Aoki and colleagues (1996) concluded this effect was more obvious at lower levels of calcium.

The ranges of calcium and phosphate tested in the present work were 5-30 and 1-20 mM for the vitamin D_3 CCRD and 10-30 and 1-20 mM for the vitamin A palmitate CCRD, respectively. At a glance, it might be thought that the range for phosphate concentration used in the current study was not sufficiently large to provide meaningful information on the effect of phosphate; however, for vitamin A palmitate loading of rCM, the squared term of phosphate was a significant factor. Vitamin loading was the highest at close to 10 mM and decreased when phosphate was increased or decreased from this value. Schmidt (1979) stated that increasing phosphate in the system results in an increase in micellar size. Thus, we may infer that vitamin loading, particularly for vitamin A palmitate, does not correlate directly to an increase in micellar size; however, further research is required to substantiate this claim.

Citrate and citrate^2 were significant terms in the model developed to describe factor effects on loading of rCM with vitamin D_3. As citrate was increased from the lower limit,

vitamin loading decreased, and then at a certain level of citrate past the center of the concentration limits, further increases in the concentration of citrate resulted in higher vitamin loading. Previous reports have stated that an increase in citrate results in a decrease in size and causes casein micelles to disperse (Schmidt, 1979). Gatti and colleagues (1999) reported that this is not an entirely linear relationship. When calcium and phosphate concentrations were 25 and 20 mM, respectively, and citrate ranged between 5 and 15 mM, the largest casein micelles were formed when the citrate concentration was between 7 and 10 mM. The methods used to prepare casein micelles in these two studies were slightly different, mainly in the order and timing that compounds were mixed together. Furthermore, Schmidt (1979) reported citrate concentration as the total measured citrate after casein micelle preparation, whereas Gatti et al. (1999) reported citrate as the total amount added. Since size was not a measured response in the current work, it is not known whether changes in vitamin loading were related to changes in the size of rCM due to altering the concentration of citrate.

Caseins are cross-linked by calcium phosphate nanoclusters (McMahon & Oommen, 2013). The quantity of nanoclusters present in reformed casein micelles increases when less citrate is present (Schmidt et al., 1979), thus enabling more casein to cross-link and increase the mass of the colloidal particle. Moreover, β-casein is postulated to be more strongly bound to calcium phosphate nanoclusters than α_{s1}-casein (Schmidt, 1979). It is known that vitamin D binds to β-casein and the interaction is highly hydrophobic in nature (Forrest et al., 2005); however, it is not known whether this binding is dominant when other caseins are present. When citrate was increased in the current system, the number of calcium phosphate nanoclusters in the rCM may have decreased resulting in the dispersion of β-casein and vitamin D_3 out of the rCM.

Binding behaviour of retinol and retinoic acid towards α- and β-caseins can be both hydrophilic and hydrophobic, though hydrophobic interaction dominates (Bourassa, N'soukpoé-Kossi, & Tajmir-Riahi, 2013). Complexes formed with α-caseins are more stable than those formed with β-caseins for retinol and retinoic acid. Differences in vitamin binding to α- and β-caseins may point to an explanation as to why citrate has such an influence on loading of rCM with vitamin D_3 but not vitamin A palmitate in the current study. In addition, an increase in phosphate within the rCM is associated with a decrease in α_{s1}-casein (Schmidt, 1979). This may provide an explanation for the apparent decrease in vitamin A palmitate loading with an increase in phosphate past the mid-level of phosphate concentrations used in the present research. Further investigation is required to elucidate the relationship between vitamin A palmitate loaded into rCM and the concentrations of different caseins, as related to the concentrations of inorganic compounds.

There are other factors that may affect vitamin loading of rCM that were beyond the scope of the current work. Small changes in protein conformation are seen with retinol and retinoic acid binding to α- and β-caseins (Bourassa et al., 2013) which may influence the inner arrangement and loading of this vitamin into the rCM. No literature has been found on changes in protein conformation as a result of vitamin D binding to caseins. The small conformational changes in protein due to binding of vitamin A may contribute to differences in the predicted levels of loading when compared to vitamin D_3 loaded rCM prepared using the same conditions.

3.5.3 Testing the model and establishing optimal conditions for vitamin loading

By viewing the surface and contour plots for vitamin D_3 and vitamin A palmitate loading of rCM in Section 3.5.2, areas can be identified where high vitamin loading is predicted. For

vitamin D_3 loading of rCM, a lower limit of 90 percent vitamin loading was chosen as criteria for optimal loading conditions. A lower limit (80 percent) was chosen for optimal vitamin A palmitate loading conditions than vitamin D_3 loading conditions to account for greater loss of vitamin A palmitate during rCM preparation and vitamin analysis preparation.

It was previously discussed in Section 3.5.2 that calcium and citrate were significant factors that affected the loading of rCM with vitamin D_3. In Figure 3.2b a contour line is shown at 1500 µg vitamin D_3, or 93 percent of total vitamin D_3. To obtain vitamin loading of 93 percent or greater, calcium may range from approximately 19 to 30 mM if there is an accompanying change in citrate concentration from about 1 to 6 mM, while keeping phosphate constant at 10.5 mM. A point in this area with 26.1 mM calcium and 4.0 mM citrate was chosen to produce rCM with optimal vitamin loading. It may be desirable to limit the level of phosphate used in the procedure because of cost savings if scaled up. Also, phosphate may have an effect on rCM size and stability (Schmidt, 1979). To investigate the effect this might have on the properties of the rCM, two levels of phosphate were chosen to test with the aforementioned levels of calcium and citrate: 4.9 mM and 10.5 mM phosphate. These conditions selected for optimal vitamin D_3 loading of rCM are referred to as vitamin D_3 – re-assembled casein micelle, low phosphate (VD-rCM L) and vitamin D_3 – re-assembled casein micelle, high phosphate (VD-rCM H) and are displayed in Table 3.5.

Significant factors that affected vitamin A palmitate loading of rCM were calcium and the squared term of phosphate. Examination of these factor effects can lead to the determination of optimal vitamin loading conditions for forming VA-rCM. Evaluation of Figure 3.4b where citrate is held at a constant concentration of 5.5 mM shows a small area of high vitamin loading. The contour at the highest level of vitamin loading shows an area with greater than 75 percent

Table 3.5 Optimal loading conditions for vitamin A – and vitamin D – re-assembled casein micelle suspensions, and predicted and experimental values for vitamin loading in the pellet of 10 mL samples of re-assembled casein micelle suspensions

	Phosphate (mM)	***Citrate (mM)***	***Calcium (mM)***	***Prediction interval (μg)***	***Experimental value (μg)***	***Experimental value (IU)[d]***
VD-rCM L[a]	4.9	4.0	26.1	1331 - 1773	1375 - 1462	55000 - 58500
VD-rCM H[b]	10.5	4.0	26.1	1290 - 1710	1490 - 1530	59600 - 61200
VA-rCM[c]	9.7	5.5	30.0	860 - 1480	1460 - 1480	4870 - 4930

[a] Vitamin D_3 – re-assembled casein micelles, low phosphate

[b] Vitamin D_3 – re-assembled casein micelles, high phosphate

[c] Vitamin A palmitate – re-assembled casein micelles

[d] Range of n = 4

vitamin A palmitate loading. Within this area, phosphate ranges from approximately 6 to 12 mM. To keep vitamin loading above 75 percent within this range of phosphate levels, calcium must begin at 30 mM, may decrease to a minimum of around 29 mM if phosphate is increased to approximately 9 mM, and then increase again to 30 mM with any further increase in phosphate levels. As the lower values within this range result in close to 75 percent vitamin loading, they do not match the criteria for greater than 80 percent loading of vitamin A palmitate. The point at 9.7 mM phosphate and 30.0 mM calcium, with citrate held at 5.5 mM (Table 3.5), was chosen in order to result in maximum loading of rCM with vitamin A palmitate.

To validate the models that were developed to describe the effect of phosphate, citrate and calcium on vitamin D_3 and vitamin A palmitate loading, conditions that were selected from each model as optimal vitamin loading were produced experimentally. Two production runs were generated for each set of optimal loading conditions, Production Run 1 (P1) and Production Run 2 (P2). Vitamin loading was assessed and compared to prediction intervals given by the models. Since the models were developed using 95 percent confidence levels, prediction intervals were defined using the same confidence level. As shown in Table 3.5, the range of experimental values for vitamin loading of all optimal vitamin loading conditions were within prediction limits of the models.

3.5.4 Characterization of re-assembled casein micelles loaded with optimal levels of vitamin D_3 and vitamin A palmitate

3.5.4.1 Protein

For all optimal vitamin loading conditions, protein analysis showed that close to 100 percent of protein in the suspension was incorporated into rCM. These values can be found in Table 3.6. No differences were found between P1 and P2 for VD-rCM L, VD-rCM H or VA-rCM. The amount of protein found in rCM was also not significantly different between VD-rCM L and VD-rCM H.

3.5.4.2 Vitamin loading in relation to protein

Loading of rCM with vitamin D_3 or vitamin A palmitate can also be expressed in terms of the amount of vitamin in relation to the amount of protein. Since protein analysis showed that close to 100 percent of protein was incorporated into rCM for all production runs, it can be assumed that 100 mg of protein is in each 10 mL sample. Therefore, it is estimated that 13.3-14.6 mg vitamin D_3/g casein, 14.9-15.3 mg vitamin D_3/g casein, and 14.6-14.8 mg vitamin A palmitate/g casein are incorporated into rCM found in VD-rCM L, VD-rCM H, and VA-rCM suspensions, respectively. Alternatively vitamin loading can be reported in terms of IU/g casein. For VD-rCM L, VD-rCM H, and VA-rCM suspensions 550-585 IU vitamin D_3/g casein, 596-612 IU vitamin D_3/g casein, and 48.7-49.3 IU vitamin A palmitate/g casein are incorporated into rCM, respectively.

Table 3.6 Protein (mean ± standard deviation, n = 3) found in pellet of 10 mL samples of optimal vitamin A and D – re-assembled casein micelle suspensions

	Production Run 1 ***Protein (%)***	***Production Run 2*** ***Protein (%)***
VD-rCM L[a]	104 ± 5	101 ± 2
VD-rCM H[b]	98 ± 5	95 ± 4
VA-rCM[c]	103 ± 6	102 ± 5

[a] Vitamin D_3 – re-assembled casein micelles, low phosphate

[b] Vitamin D_3 – re-assembled casein micelles, high phosphate

[c] Vitamin A palmitate – re-assembled casein micelles

3.5.4.3 Charge

The charge of particles present in a solution is often given as an indicator of stability against aggregation. Particles with a zeta potential of greater than 30 mV or less than -30 mV are thought to be stable (Clogston & Patri, 2011). If variability between production runs of V-rCM is accounted for, there are no significant differences ($p > 0.05$) between the zeta potential for VD-rCM L, VD-rCM H and VA-rCM (Table 3.7). The recorded zeta potentials were -17 to -20 mV, suggesting that particles may not be stable; however, zeta potential of natural casein micelles range from -13 to -20 mV depending on pH and nature of the dispersant (Anema & Klostermeyer, 1996). The pH of all suspensions prepared in optimal conditions for the current study ranged from pH 6 to 6.5, which is close to the natural pH of milk. Anema and Klostermeyer (1996) measured the zeta potential for casein micelles within this pH range to be -15 to -17 mV. The zeta potential of rCM carrying β-carotene was much more negative, reported at -34 mV (Sáiz-Abajo et al., 2013). Also, Raouche et al. (2009) reported zeta potential of -26 to -28 mV for control casein micelles and -23 to -28 mV for casein micelles exposed to ferrous and ferric chloride. Zeta potential measurements ranged between -19.1 ± 0.75 and -31.2 ± 1.45 mV for casein micelles when spray dried with flutamide which varied between different ratios of flutamide to casein (Elzoghby et al., 2013).

The conditions used to form VD-rCM L were also prepared without vitamin D_3 to investigate the effect vitamin D_3 has on zeta potential of rCM. No difference in zeta potential was observed when VD-rCM L conditions were prepared without vitamin D_3 (P1 = -17.1 ± 0.3 mV, P2 = -18.5 ± 0.1 mV) compared to VD-rCM L (Table 3.7). Consequently it can be concluded that the addition of vitamin D_3 does not significantly change the zeta potential of particles in VD-rCM suspensions. On the other hand, the zeta potential of particles is

Table 3.7 Zeta potential (mean ± standard deviation, n = 3) of particles in optimal vitamin A – and vitamin D – re-assembled casein micelle suspensions

	Production Run 1 ***Zeta potential (mV)***	***Production Run 2*** ***Zeta potential (mV)***
VD-rCM L[a]	- 17.1 ± 0.1	-18.1 ± 0.2
VD-rCM H[b]	-19.7 ± 0.4	-18.4 ± 1.0
VA-rCM[c]	- 17.3 ± 0.2	-17.8 ± 0.2

[a] Vitamin D_3 – re-assembled casein micelles, low phosphate

[b] Vitamin D_3 – re-assembled casein micelles, high phosphate

[c] Vitamin A palmitate – re-assembled casein micelles

affected by salt concentrations. When compared to Sáiz-Abajo and colleagues (2013), high levels of calcium chloride were used in the formation of all optimal formulations of V-rCM in the current study. Excess calcium in the solution surrounding rCM would have resulted in association of calcium with rCM, which would neutralize the negative zeta potential.

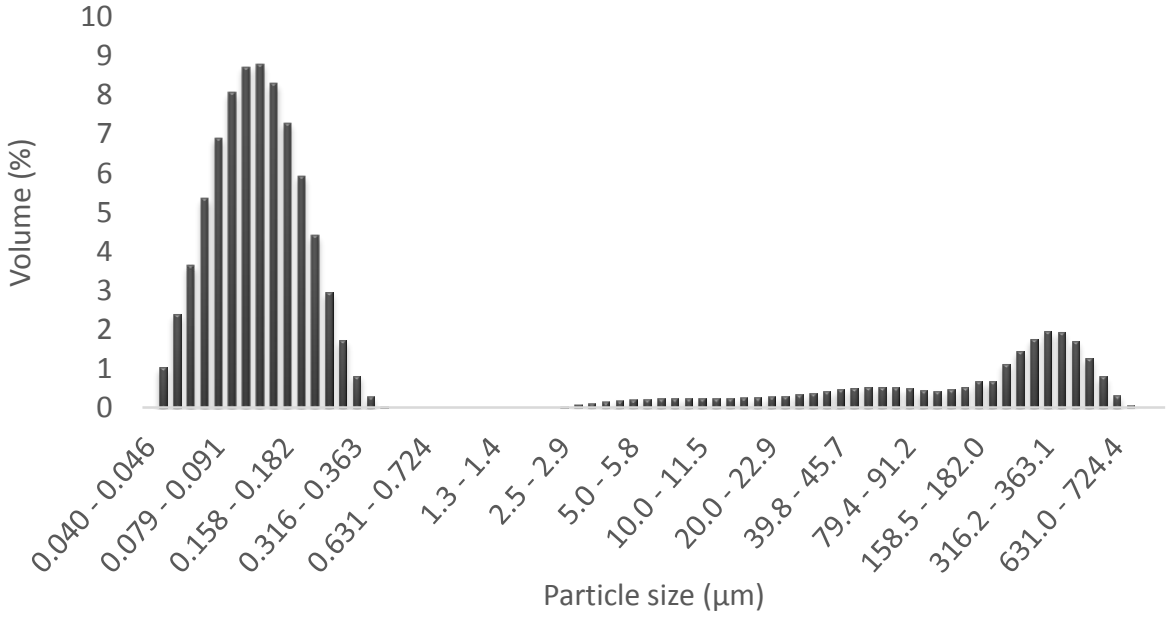
3.5.4.4 Size

Laser diffraction was used to characterize the size of particles present in suspensions prepared using optimal loading conditions for vitamin D_3 and vitamin A palmitate . This technique uses the intensity and angle that light is scattered as a laser beam is passed through a sample. Light is scattered at smaller angles and higher intensities by larger particles. If particles are small, scattering angles are large and light is scattered with lower intensity. Important to this technique is the assumption that particles are spherical in shape as size is reported as hydrodynamic diameter.

Preliminary examination of rCM size using a ZetasizerNano determined that size distributions in suspensions formed using optimal vitamin loading conditions for vitamin D_3 contained particles larger than the capability of this instrument. Thus, size of particles in rCM suspensions loaded with optimal levels of vitamins was measured using a Mastersizer, which is capable of measuring nano- to micro-sized particles.

The range of particles present in suspensions prepared using VD-rCM L conditions is shown in Figure 3.5a as volume weighted size distribution. These figures show the average of P1 (n = 3) and P2 (n = 3). Particles in these suspensions were present in bimodal size distributions, where the majority of the particle volume occurred as particles approximately 0.04 to 0.63 µm and a smaller population of large particles stretched between 182.0 to 724.4 µm.

a)



b)

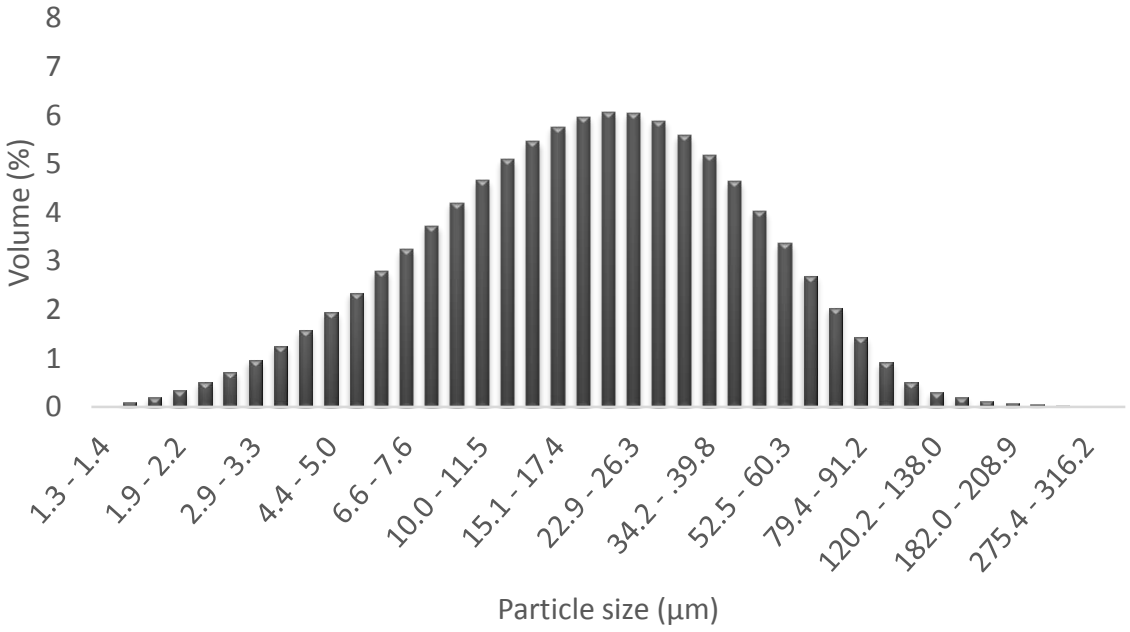
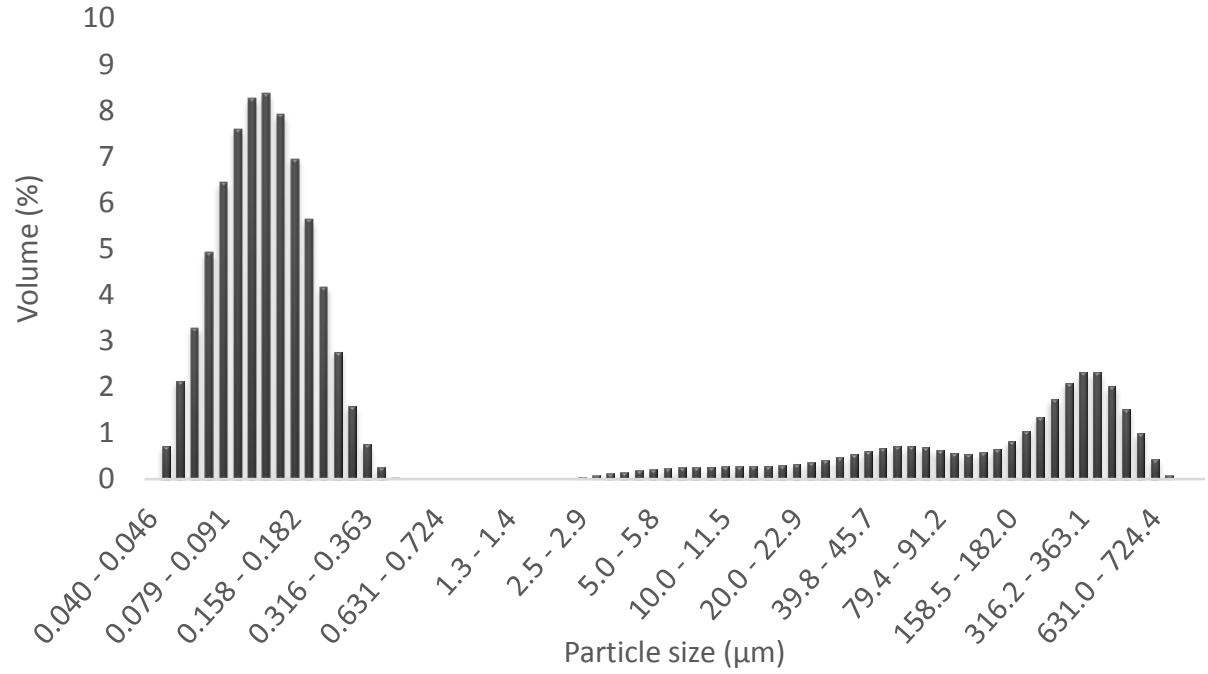


Figure 3.5 Average volume weighted particle size distribution of particles formed with optimum conditions for vitamin D_3 – re-assembled casein micelles from Production Run 1 and Production Run 2 (n = 2), low phosphate (a) and high phosphate (b) as measured using a Mastersizer

Average volume weighted particle size is a value often used to described particle size when measured using this technique. Using this statistic in the present case would provide a flawed interpretation of the data because the distribution has two distinct populations. A small number of particles with large volume can skew the average volume weighted particle size by bringing up the average significantly. To illustrate this point, the individual volume weighted size distributions for P1 and P2 of VD-rCM L suspensions are shown in Figure 3.6. These distributions are very similar. Differences are seen in the volume (%) of particles present in each population. The area of the peak for small particles in P1 is smaller than the same peak in P2. When looking at the total volume (%) of larger particles present, P1 has a greater volume of large particles than P2. Even though the appearance of the trends for size distribution are very similar, the average volume weighted particle size for P1 and P2 was 0.83 ± 1.10 and 49.13 ± 31.77 µm, respectively (Table 3.8). This can be attributed to differences in the total volume of the two size populations of particles present in P1 and P2. One way to better describe this data would be to use the D(0.5) value. Half of the total particles present are below this size and half of the particles are above this size. The D(0.5) values for P1 and P2 of VD-rCM L were 0.13 ± 0.03 and 0.16 ± 0.02 µm, respectively (Table 3.8).

Figure 3.5b shows the volume weighted particle size distribution present in suspensions prepared using VD-rCM H conditions. Minitab analysis showed that the distribution was skewed slightly to the right. Particles in these suspensions ranged from approximately 1.3 to 316.2 µm. P1 and P2 of VD-rCM H suspensions had average volume weighted particle sizes of 19.04 ± 2.34 µm and 16.10 ± 0.24 µm, respectively (Table 3.8). For both P1 and P2, slightly more particles were present below the average volume weighted particle size than above. This is shown by D(0.5) values of 13.59 ± 1.53 µm and 12.26 ± 0.13 µm for P1 and P2, respectively.

a)



b)

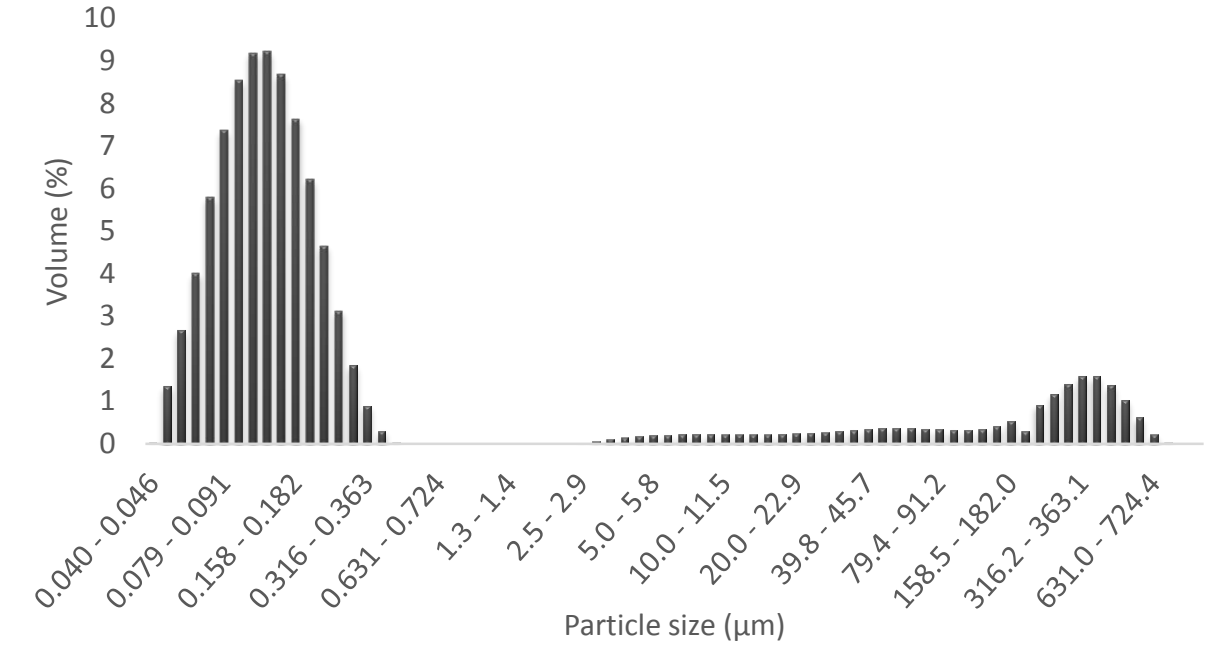


Figure 3.6 Average volume weighted particle size distribution (n = 3) of particles formed with optimum conditions for vitamin D_3 – re-assembled casein micelles, low phosphate for Production Run 1 (a) and Production Run 2 (b) as measured using a Mastersizer

Table 3.8 Size (mean ± standard deviation, n = 3) of particles in optimal vitamin A – and vitamin D – re-assembled casein micelle suspensions

	Production Run 1		***Production Run 2***	
	Average volume weighted particle size (µm)	**D(0.5)* (µm)**	**Average volume weighted particle size (µm)**	**D(0.5)[d] (µm)**
VD-rCM L[a]	0.83 ± 1.10	0.13 ± 0.03	49.13 ± 31.77	0.16 ± 0.02
VD-rCM H[b]	19.04 ± 2.34	13.59 ± 1.53	16.10 ± 0.24	12.26 ± 0.13
VA-rCM[c]	26.09 ±2.52	18.52 ± 4.27	27.21 ± 1.98	18.68 ± 2.65

[a] Vitamin D_3 – re-assembled casein micelles, low phosphate

[b] Vitamin D_3 – re-assembled casein micelles, high phosphate

[c] Vitamin A palmitate – re-assembled casein micelles

[d] D(0.5) is the diameter where 50% of the particle size distribution is above and 50% is below.

The volume weighted particle size distribution present in suspensions of VA-rCM was similar to that of VD-rCM H, appearing as single populations for both P1 and P2 (Figure 3.7). A slight difference can be observed between the shapes of size distributions of VA-rCM and VD-rCM H. In addition, the span of the distribution for VA-rCM was smaller than for VD-rCM H, ranging from approximately 1.3 to 120.0 µm. These observations can be described numerically using the skew and kurtosis for each distribution. Both of these values should be zero or close to zero for a normal distribution, with positive skew values indicating skew to the right and negative kurtosis signifying flatness of the distribution. Skew and kurtosis were 0.23 and -0.74 for VA-rCM compared to 0.67 and -0.85 for VD-rCM H. Using goodness of fit, it was shown that the distribution for VA-rCM was normal ($p = 0.331$) as opposed to that for VD-rCM H ($p < 0.005$). The average volume weighted particle size for VA-rCM was 26.09 ± 2.52 µm for P1 and 27.21 ± 1.98 µm for P2 (Table 3.8). When looking at particle size alone, more particles were present that were smaller than the average volume weighted particle size than larger than the average volume weighted particle size in VA-rCM suspensions. This is shown by the D(0.5) values, at 18.52 ± 4.27 µm and 18.68 ± 2.65 µm for P1 and P2, respectively (Table 3.8).

As discussed previously, laser diffraction gives particle size as hydrodynamic diameter and assumes that particles are spherical in shape. Previous observation of rCM using cryo-electron transmission microscopy (Semo et al., 2007) and environmental scanning electron microscopy (Sáiz-Abajo et al., 2013) showed nano-sized spherical rCM, similar in shape to natural casein micelles. While some particles were shown to be nano-sized in the present research (VD-rCM L, Figure 3.5a and Figure 3.6), others were measured to be micro-sized. To investigate this further, VD-rCM L and VD-rCM H were observed using light microscopy.

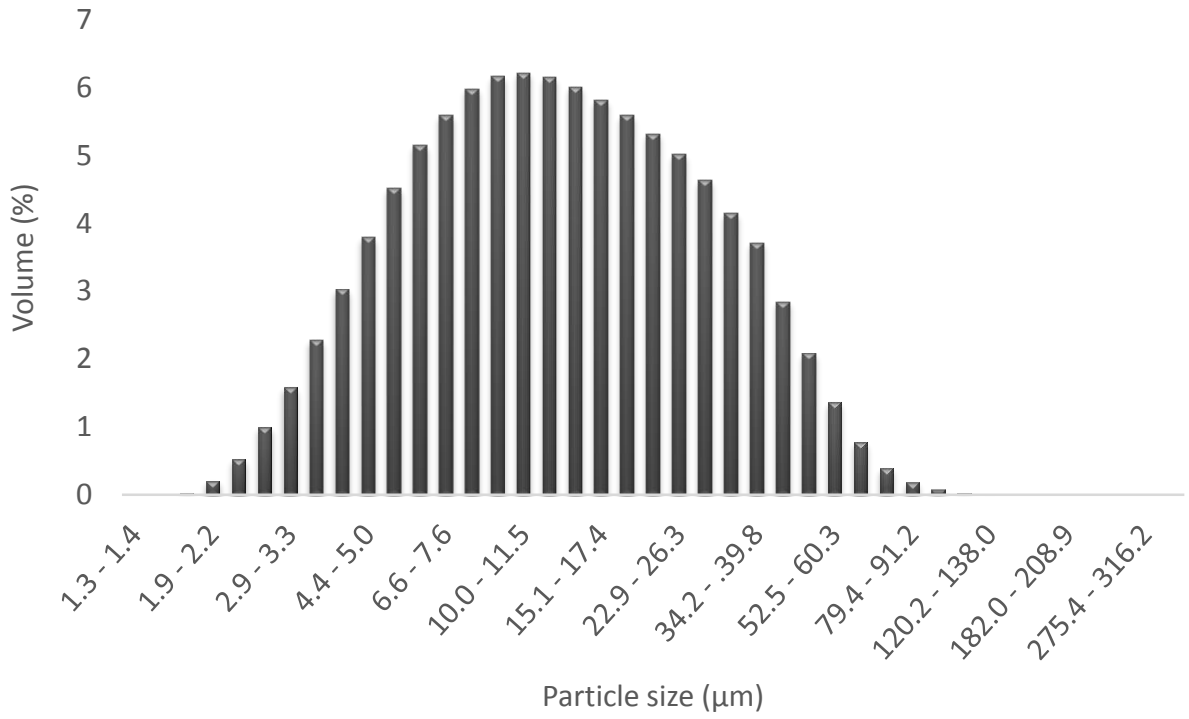


Figure 3.7 Average volume weighted particle size distribution of particles formed with optimum conditions for vitamin A palmitate – re-assembled casein micelles from Production Run 1 and Production Run 2 (n = 2) as measured using a Mastersizer

Figure 3.8 and Figure 3.9 show suspensions of VD-rCM L viewed by phase contrast and differential interference contrast (DIC) microscopy. These images partially support the results from laser diffraction measurements for VD-rCM L suspensions, showing a large portion of particles of small size. It is clear that Figure 3.8 shows the presence of a few larger particles. It is unknown whether these particles represent the population of micro-sized particles seen in Figure 3.5a and Figure 3.6 or if they are artifacts in the image. Examination of Figure 3.9 uncovers the possibility that the small particles viewed in the image are spherical in shape, which would be in agreement with previous conclusions concerning rCM shape (Sáiz-Abajo et al., 2013).
Similar to the results of laser diffraction measurements performed on VD-rCM H suspensions, images taken using phase contrast and DIC microscopy show a wide variation in the size of particle present (Figure 3.10 and Figure 3.11).

Using microscopy allows us to view the shape of particles and using different techniques can provide a variety of information. Using phase contrast microscopy (Figure 3.10) particles in a VD-rCM H suspension appear as numerous branched structures of varying sizes. Small branched structures spotted in the image appear to be similar to the larger particles present in Figure 3.8 for VD-rCM L. Given this observation, the theory that the larger particles seen in Figure 3.8 are due to particles in the VD-rCM L suspension, and not artifacts on the slide, may be substantiated. DIC microscopy provides an interesting view of the VD-rCM H suspension (Figure 3.11). Similar to Figure 3.9, small particles are viewed that appear to be spherical in shape. In addition to the small particles, large aggregates are seen that look to be made up of smaller spherical particles. It is postulated that the particles begin as small and spherical, and then these particles aggregate together forming interconnected branches of small particles.

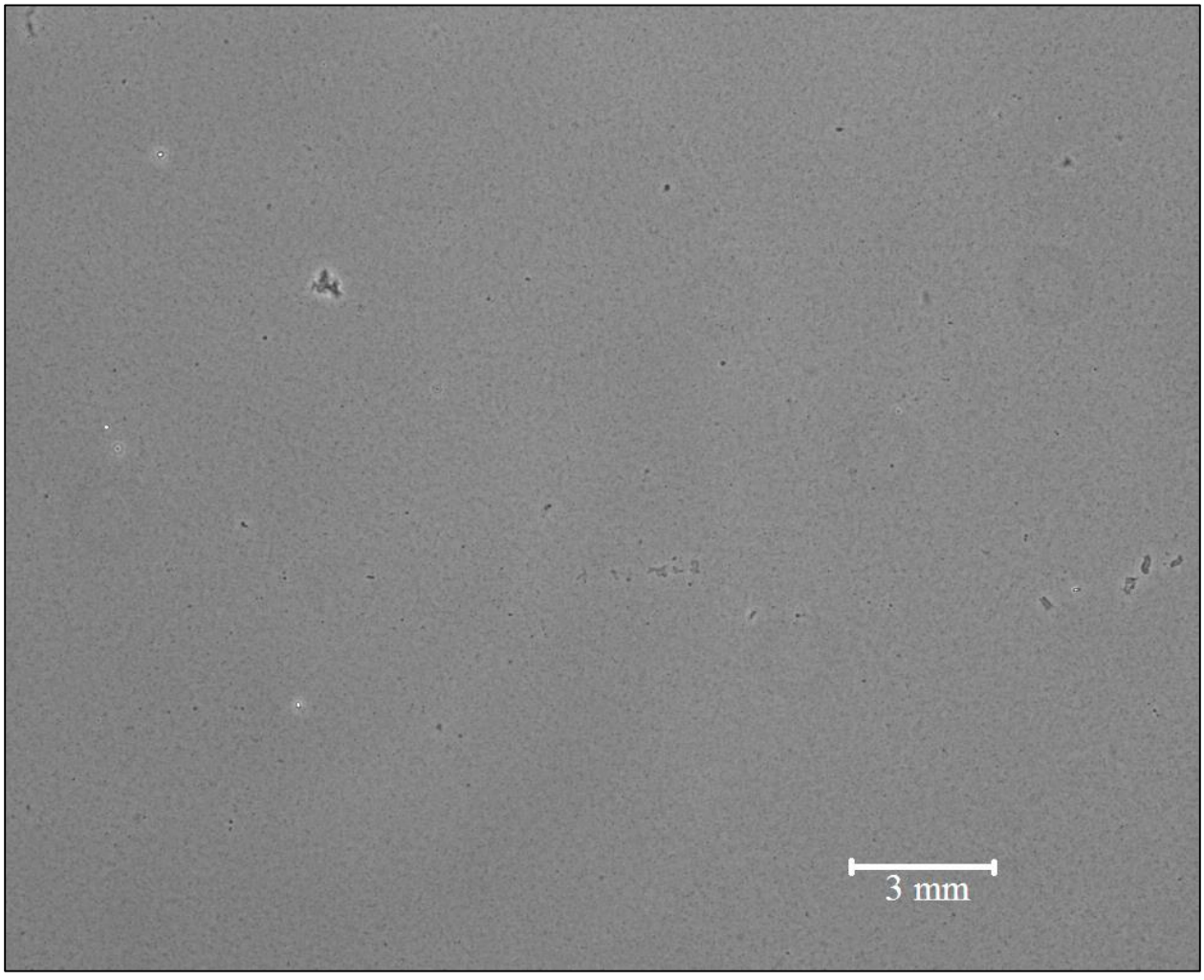


Figure 3.8 Phase contrast microscopy of re-assembled casein micelle suspension prepared using conditions defined for optimal loading of vitamin D_3, with low phosphate

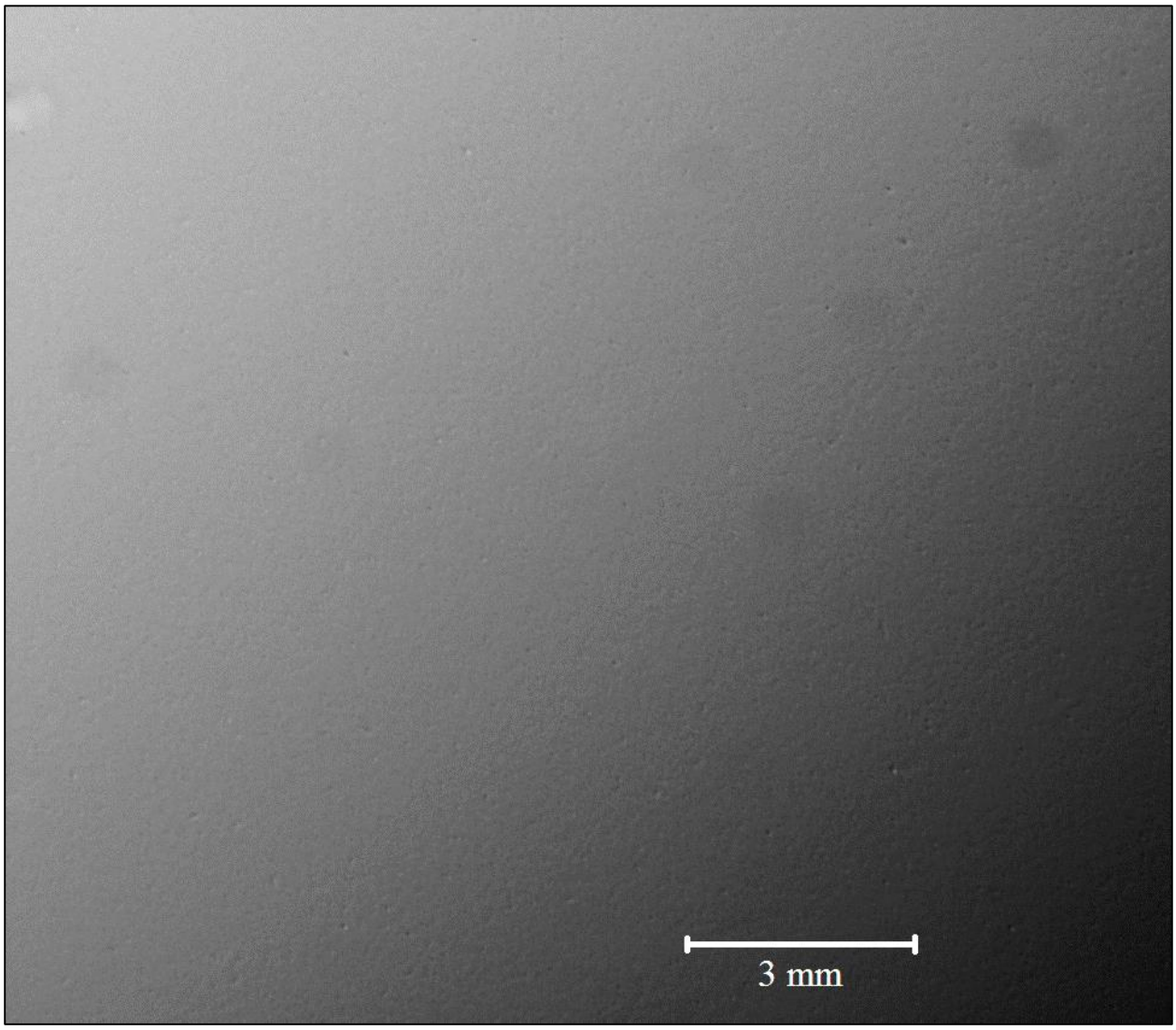


Figure 3.9 Differential interference contrast microscopy of re-assembled casein micelle suspension prepared using conditions defined for optimal loading of vitamin D_3, with low phosphate

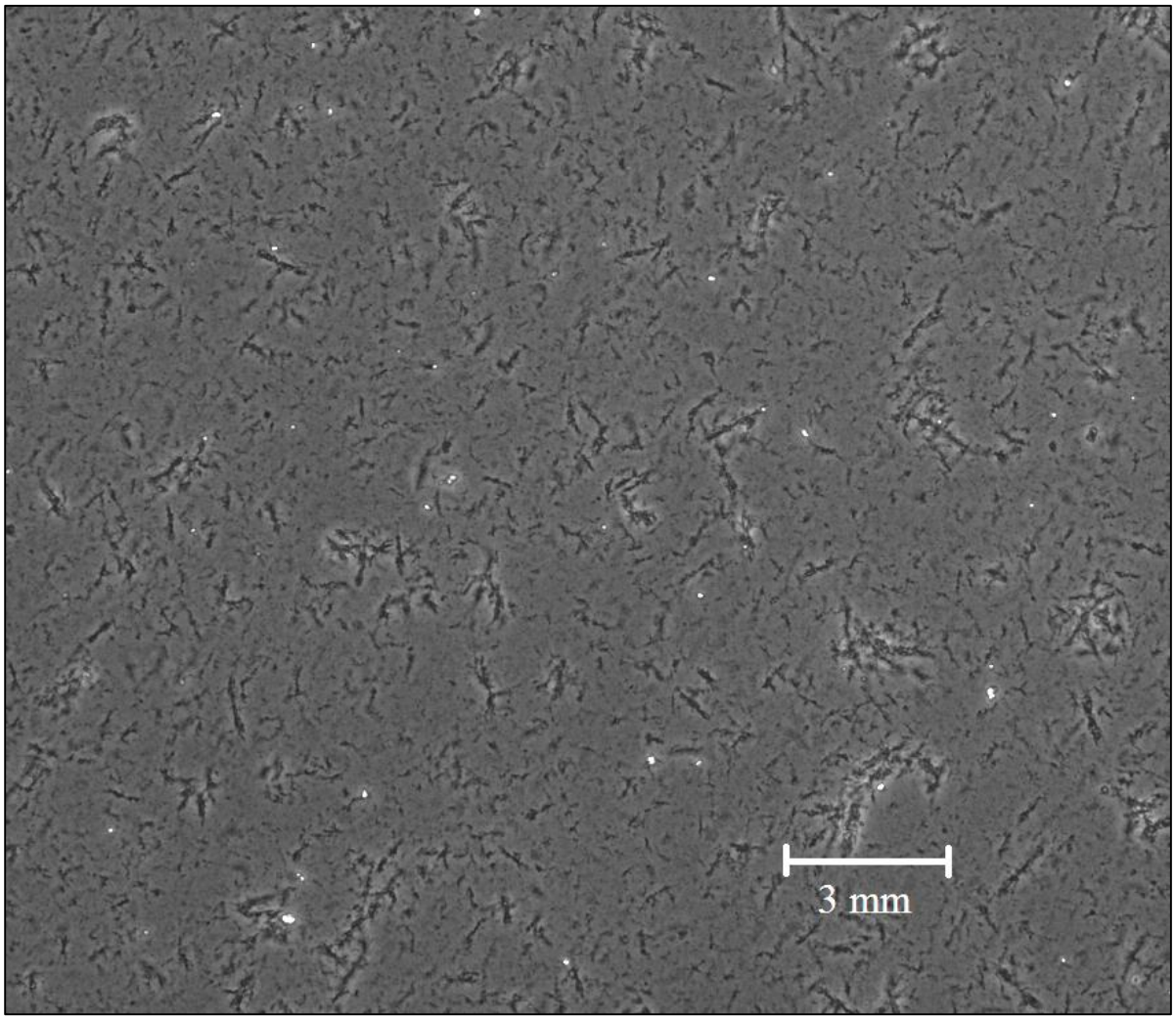


Figure 3.10 Phase contrast microscopy of re-assembled casein micelle suspension prepared using conditions defined for optimal loading of vitamin D_3, with high phosphate

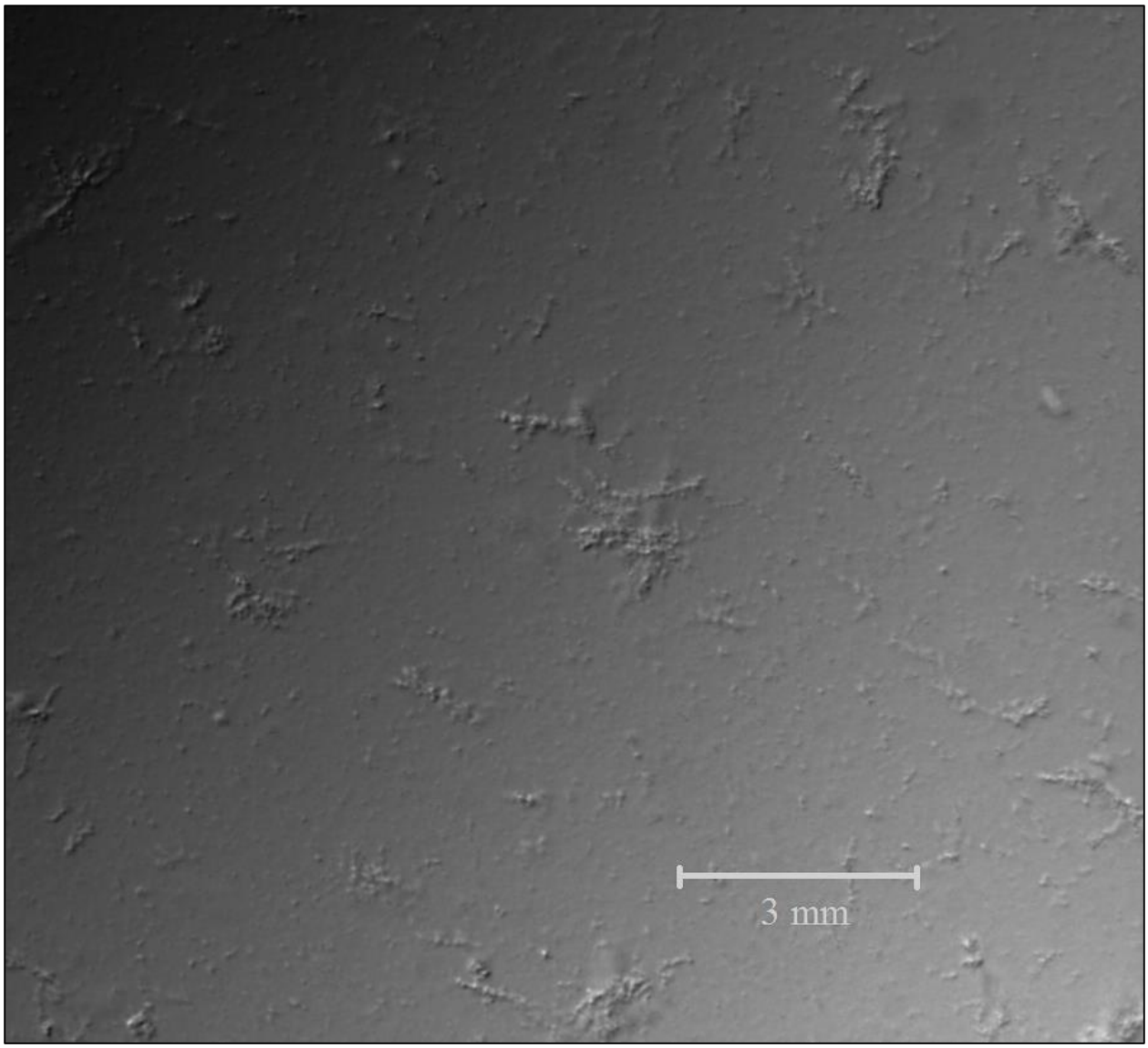


Figure 3.11 Differential interference contrast microscopy of re-assembled casein micelle suspension prepared using conditions defined for optimal loading of vitamin D_3, with high phosphate

3.5.5 Estimating the levels of vitamin – re-assembled casein micelles for milk fortification

A dry powder of VA-rCM and VD-rCM was initially proposed to be used as a milk fortification product. Given that a dry fortification product would be hydrated before use, it is valuable to look at the mass of solids that would be added to milk, as well as volume required to meet fortification targets. As discussed in Section 0, the amount of vitamin A palmitate used in the procedure to load rCM was at the upper limit of solubility. If the numbers for the concentration of vitamin in suspension are translated into the mass of solids containing the level of vitamin necessary to reach fortification targets of 217 IU/100 mL for vitamin A and 41 IU/100 mL for vitamin D (CFIA, 2013), approximately 0.9 mg of VD-rCM and 0.14 g VA-rCM would be added to 1 L of milk. Phrasing this in terms of volume of the initial rCM suspensions, approximately 63 µL of VD-rCM and 10 mL of VA-rCM would be required to reach the target concentrations. While a total of 10 mL water may not be necessary to order to hydrate a dry VA-rCM powder properly, a significant volume of water would be compulsory for powder rehydration. Adding this volume of water into milk would alter the composition of milk, which is undesirable and unethical. Another approach might be to rehydrate VA-rCM powder in milk. Still, VA-rCM were not carried through into the next phase of this research.

3.6 Conclusion

Loading of vitamin D_3 and vitamin A palmitate into rCM was affected by the concentrations of phosphate, citrate and calcium used to prepare them. Increasing levels of vitamin D_3 were loaded into rCM when calcium was increased through the calcium concentration range. A different effect was seen for citrate, where vitamin loading increased with increases of citrate in the lower end of the concentration range, and further increases in citrate

resulted in the lowering of vitamin D_3 loading. More vitamin A palmitate was loaded into rCM when calcium was increased. In addition, the squared term of phosphate was also significant for vitamin A palmitate loading. Vitamin loading was the highest in the center of the phosphate concentration range, then decreased as phosphate concentration strayed from this value.

Two sets of conditions were chosen to produce VD-rCM with optimal levels of vitamin loading: VD-rCM L with 4.9 mM phosphate, 4.0 mM citrate and 26.1 mM calcium, and VD-rCM H with 10.5 mM phosphate, 4.0 mM citrate and 26.1 mM calcium. Vitamin loading of rCM in VD-rCM L and VD-rCM H suspensions was estimated to be 13.3-14.6 mg vitamin D_3/g casein and 14.9-15.3 mg vitamin D_3/g casein, respectively. Factor levels defined to produce optimally loaded VA-rCM were 9.7 mM phosphate, 5.5 mM citrate and 30.0 mM calcium, and resulted in approximately 14.6-14.8 mg vitamin A palmitate/g casein being incorporated into rCM.

The particle size distributions in VD-rCM L and VD-rCM H suspensions were bimodal and unimodal, respectively, when measured using dynamic light scattering. When viewed using light microscopy, particles in VD-rCM L suspensions were small and round, compared to large and highly branched structures that were seen in VD-rCM H suspensions. Additionally, the size distribution of VA-rCM was similar to VD-rCM H when measured using dynamic light scattering. No differences were found between the zeta potential measurements for any of the three optimal experimental conditions. Similarly, the level of protein bound within V-rCM was not significantly different between VD-rCM L, VD-rCM H and VA-rCM.

RSM was an effective tool to optimize loading of vitamin D_3 and vitamin A palmitate into rCM. The rCM loaded with high levels of vitamin D and vitamin A prepared in this research are valuable since the loading efficiency in reference to the total vitamin added is higher than

previously reported (Haham et al., 2012; Semo et al., 2007). Though the volume of these complexes required to meet the target vitamin A fortification level in skim milk is unrealistic for addition to milk, VA-rCM may be useful in other food products where a lower concentration is desired. More importantly, VD-rCM complexes have potential to be used for the fortification of foods and beverages, such as milk.

Chapter 4: Storage of vitamin D – re-assembled casein micelle powders

4.1 Introduction

The aim of using rCM to carry vitamins is to improve stability of these vitamins. Vitamin D_2 and D_3 are sensitive to light, oxygen, humidity and heat when alone in powder form (Grady & Thakker, 1980). Semo et al. (2007) examined the stability of vitamin D_2 within rCM in suspension to UV light stability. Vitamin D_2-rCM suspensions were prepared to a final concentration of 63.5 µM vitamin D_2. About 85 percent of this was recoverable in the pellet and supernatant after analysis. Of this, 27 percent was found to be incorporated into the rCM and the remaining 73 percent was recovered in the supernatant. Re-assembled casein micelles carrying vitamin D_2 provided more protection to the vitamin when compared to the remaining casein and vitamin in the supernatant (rCM removed). Semo et al. (2007) suggested that vitamin D_2 in the rCM was 5.5 times more concentrated than that in the supernatant, indicating that these two samples were not comparable in concentration of vitamin. No other vitamin D solution was included in this experiment to compare this level of protection to vitamin D alone or to another water dispersible vitamin D product.

In a more recent study, the protection conferred to vitamin D_3 by rCM was investigated by subjecting rCM carrying vitamin D_3, vitamin D_3 emulsified in water using Tween-80 and vitamin D_3 in water and 0.1% ethanol (v/v) to heat treatment and cold storage (Haham et al., 2012). Final concentrations of vitamin D_3 in these were 162.5 µg/mL, 12.5 µg/mL and 12.5 µg/mL, respectively. Vitamin D_3 levels in Tween-80 and water were significantly lower after heating at 80°C for 1 minute, while no significant reduction in vitamin D_3 was found for rCM suspensions. Corroborating protective effects of rCM were found after 28 days of cold storage

where vitamin D_3 levels in vitamin D_3 – rCM suspensions decreased significantly less than both controls.

Dry vitamin products are financially advantageous because of the lower cost associated with transport due to lower mass and volume compared to liquid products (Augustin & Hemar, 2008). Limited literature is available on the stability of vitamin D in dry form, and no information has been found on the effect that rCM might have on vitamin stability after they have been dried.

The objective of this study was to determine if rCM as carriers for vitamin D_3 provide more protection to the vitamin than the materials used in the re-assembly process but not in micellar form. Stability of vitamin D_3 in freeze-dried VD-rCM suspensions formed using optimal loading conditions was compared with powders containing the same concentration of materials used to form the VD-rCM suspensions. This study looked at the stability of vitamin D_3 in these powders during accelerated storage conditions (37°C with 75% relative humidity) and storage at ambient temperature and relative humidity, and compared vitamin stability to that measured in a commercial vitamin D fortification product.

4.2 Materials

Vitamin D_2 (ergocalciferol), vitamin D_3 (cholecalciferol), sodium caseinate, phenolphthalein, sodium azide, and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich Canada (Oakville, ON). Hydrochloric acid (HCl), potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium chloride (NaCl), potassium phosphate dibasic anhydrous (K_2HPO_4), tripotassium citrate (K_3citrate), calcium chloride dehydrate ($CaCl_2$), ascorbic acid, acetic acid, sodium sulphate, ethanol (absolute), methanol (HPLC grade), hexane, dichloromethane (HPLC

grade), ethyl acetate (HPLC grade), and acetonitrile (HPLC grade) were purchased from Fisher Scientific Canada (Ottawa, ON). Ethylenediamine tetraacetic acid (EDTA) was obtained from BDH Chemicals, VWR (Radnor, PA). Retinyl propionate was purchased from Santa Cruz Biotech (Santa Cruz, CA). A water dispersible vitamin D powder, dry vitamin $D_3$100 CWS/AM (CWS D_3) was obtained from DSM Nutritional products (Belvidere, New Jersey, USA).

4.3 Sample preparation

Four different vitamin D powders were prepared: two VD-rCM powders and two powders to serve as controls.

4.3.1 Preparation of vitamin D – re-assembled casein micelle powders

Two VD-rCM suspensions were prepared following the micelle preparation procedure detailed in Section 3.3.2. The concentrations of salts used for these two VD-rCM suspensions were those from the low and high phosphate conditions determined for optimal vitamin D loading. These concentrations are listed in Table 3.5. For this procedure, the $CaCl_2$ solution was added drop by drop from a 50 mL syringe, using a syringe pump set at 1.2 mL per minute. The procedure was conducted twice each for low and high phosphate conditions. The two production runs were mixed together and stirred for 1 minute, then poured into a flat metal pan covered in foil and transferred to -80°C until frozen thoroughly (1.75 hours) to avoid alterations to the rCM structure that may accompany slow freezing (O'Mahony & Fox, 2013).The frozen samples were freeze-dried in a bulk tray drier accessory using a FreeZone console freeze dry system (Labconco, Kansas City, MO) for 5 days. Once dry, the powder was crushed and sifted through a 16 mesh (No. 18 USA) sieve.

4.3.2 Preparation of control powders

Two control powders were prepared using the following method: Two solutions were prepared by mixing the same concentrations of vitamin D_3 (in ethanol), sodium caseinate, and sodium azide as in the procedure for VD-rCM. Each of these mixtures were poured into a flat metal pan, covered in foil and transferred to -80°C until frozen thoroughly (1.75 hours). The frozen samples were freeze-dried for 5 days in a bulk tray drier accessory using a FreeZone console freeze dry system (Labconco, Kansas City, MO). Following freeze-drying, the same concentrations of dry salts used in forming VD-rCM L were added to one pan of dry powder and the amount of dry salts used for VD-rCM H were added to the other pan of dry powder. The powder in each pan was mixed and sifted through a 16 mesh (No. 18 USA) sieve separately. The low phosphate and high phosphate control powders are referred to as CL and CH, respectively.

4.4 Experimental set-up and sampling

4.4.1 Accelerated storage

VD-rCM powders and control powders (1.5 g) were placed in three covered glass petri dishes (70 mm diameter) each. Commercially available dry vitamin D Type CWS/AM (1.5 g) was also placed in three covered glass petri dishes (Corning, Fisher Scientific Canada, Ottawa, ON). All powders were spread to an even thickness.

A saturated solution of NaCl was positioned in the bottom of a desiccator covered in foil to create an environment of 75% relative humidity. The desiccator was then placed in a 37°C incubator to equilibrate for 24 hours. Petri dishes were then placed in the desiccator.

Total vitamin analysis was conducted for 0, 12, 24, 48, 72, and 96 hours. Time 0 samples (three samples each, 0.1 g) were taken from powders prior to placing in the experimental set-up. One sample of 0.1 g was taken at 12, 24, 48, 72 and 96 hours from each petri dish by removing the powder from the petri dish, mixing thoroughly in a foil covered beaker to avoid light exposure, and then weighing out a sample. Preliminary analysis showed no difference between vitamin D content of powder samples that were diluted and stored at -80°C for 4 weeks, samples stored at -80°C for 8 weeks then diluted, and samples diluted followed by same-day analysis. Therefore, samples from two of the petri dishes were diluted for vitamin D_3 analysis and frozen at -80°C for up to 4 weeks. The remaining sample was directly frozen at -80°C and diluted within 8 weeks for vitamin D_3 analysis. The samples of control and VD-rCM powders were diluted with 10 mL 0.1 M EDTA in a covered volumetric flask, stirred, and left for 3 hours at 4°C in the dark to allow powder to dissolve, then was made up to volume with distilled water. Samples of commercial vitamin D powder, CWS D_3, were diluted with distilled water in covered volumetric flasks.

4.4.2 Ambient storage

Vitamin formulations were stored at ambient temperature and humidity. One 50 mL clear glass bottle (2.5 cm diameter x 10 cm height) was filled with 8 g of powder for each formulation. Bottles were placed in a box within a closed cupboard. Temperature and humidity within the box was monitored on a daily basis using a digital hygrometer/thermometer (Model 4088, VWR, Radnor, PA). Initially, CL and CH powders were prepared by mixing all materials together in dry form. It was found that using this method resulted in unreliable results for vitamin analysis. A new method was developed for forming CL and CH powders that included adding vitamin D_3

(in ethanol) to sodium caseinate in water, followed by freeze-drying and adding all other dry ingredients. It is because of this that CL and CH powders stored at ambient temperature and relative humidity were prepared at a later date than VD-rCM L and VD-rCM H powders. and therefore the recorded temperature and humidity during storage of CL and CH powders was different than for VD-rCM L, VD-rCM H and CWS D_3. On Day 0, 14, 28, and 42 duplicate samples of 0.1 g were taken from each bottle and diluted for vitamin analysis using the same procedure for VD-rCM L, VD-rCM H, CL, CH, and CWS D_3 as described in Section 4.4.1. Prior to sampling, bottles were shaken for 15 seconds, and then stirred with a metal spatula for 15 seconds to allow for proper mixing. Single vitamin analysis was conducted for each of the duplicate samples.

4.5 Vitamin D and vitamin A analysis

The method for determining vitamin D, modified from the AOAC method 995.05 (Sliva & Sanders, 1996), is described fully in Appendix A.1. This procedure has been used previously for extraction and analysis of vitamin D_3 from skim milk in our lab (Liu, 2013). The method used for vitamin A determination is found in Appendix A.2. The procedure is modified from the AOAC method 2002.06 (Hite, 2003). Additionally, the flow rate and concentrations of mobile phase components for HPLC analysis of both vitamin D_3 and vitamin A palmitate analysis can be found in Appendix A.3.

4.6 Statistical analysis

Remaining vitamin D_3 was calculated as the percent of vitamin remaining compared to initial vitamin content (Day 0). Minitab 16 (State College, PA) was used to conduct statistical

analysis. Data was analyzed using a one-way ANOVA in a general linear model with repeated measures on one factor at a 95 percent confidence level. Tukey's test was then used to investigate significant differences ($p \leq 0.05$) between vitamin levels measured in different formulations at different time points. Additionally, reaction kinetics for degradation of vitamin D_3 in each formulation was determined using Microsoft Excel 2014 (Redmond, WA).

4.7 Results and discussion

4.7.1 Accelerated storage of vitamin D_3 formulations

Using a one-way ANOVA with repeated measures on one factor (vitamin D_3 formulation) (Table 4.1), vitamin D_3 formulation with exposure time as a repeated measure was determined to be a significant factor for loss of vitamin. This means that there were significant differences between at least some formulations. Additionally, exposure time had a significant effect on the loss of vitamin D_3.

Tukey's test was applied for pairwise comparison of vitamin levels in different formulations over time. The results of this test are shown in Table 4.2. There was no significant difference in vitamin D_3 content between any powders at 12 hours. At 24 hours, the percent of vitamin remaining in CL powder was significantly lower than that for both VD-rCM powders as well as the commercial powder; however, no significant difference was found between the percent of vitamin D_3 remaining in the CH powder compared to both VD-rCM powders and CWS D_3. By 48 hours of storage time, the percent of vitamin remaining in both control powders was significantly lower than in VD-rCM L, VD-rCM H and CWS D_3 powders, and remained as such at 72 hours of storage time. At 96 hours of storage time, no significant difference was found

Table 4.1 Results of a one-way ANOVA, with repeated measures, on the degradation of vitamin D_3 in dry vitamin formulations held in accelerated storage conditions (37°C and 75% relative humidity)

Factors[a]	**DF**[b]	**Adj SS**[c]	**Adj MS**[d]	***F*-value**	***p*-value**[e]
Exposure time	5	94100	18800	323	**0.000**
Vitamin D_3 formulation (Exposure time)	24	17400	723	12.4	**0.000**
Error	90	5250	58		
Total	119	117000			

[a] Vitamin D formulation and exposure time (the repeated factor) are fixed factors.

[b] Degrees of freedom

[c] Adjusted sum of squares

[d] Adjusted mean squares

[e] Significant effects ($p \leq 0.05$) are shown in bold font.

Table 4.2 Vitamin D_3 remaining in dry powders of five formulations at different storage times while held in accelerated storage conditions (37°C and 75% relative humidity)

Exposure time (hours)	**Vitamin D_3 (% of initial) [a]**				
	VD-rCM L[b]	**VD-rCM H[c]**	**CL[d]**	**CH[e]**	**CWS D_3[f]**
0	100 a	100 a	100 a	100 a	100 a
12	75 b	65 bcd	71 bc	65 bcd	76 b
24	61 bcde	59 bcde	28 fghi	38 efgh	61 bcde
48	60 bcde	54 bcde	23 ghi	18 hi	58 bcde
72	48 cdef	46 defg	19 hi	13 i	61 bcde
96	39 efgh	37 efgh	17 hi	10 i	57 bcde

[a] Means (n = 3) that do not share common lowercase letters are significantly different ($p \leq 0.05$).

[b] Vitamin D_3 – re-assembled casein micelles, low phosphate

[c] Vitamin D_3 – re-assembled casein micelles, high phosphate

[d] Control powder, low phosphate

[e] Control powder, high phosphate

[f] Dry vitamin D3 100 CWS/AM from DSM nutrition

between the percent of vitamin D_3 remaining in the CL powder compared to both VD-rCM powders. The percent of vitamin remaining in the CH powder at 96 hours of storage was significantly lower than VD-rCM L and VD-rCM H powders. At any given time point during storage, no significant difference was found between amount of vitamin remaining in VD-rCM L, VD-rCM H and CWS D_3 powders.

No reports have been found in the literature on reaction kinetics for vitamin D_3 degradation over time; therefore, an attempt was made to fit vitamin loss to different orders of reaction. Individual data points from the triplicate samples (one from each petri dish) were used to generate the regression equations. Poor R^2 values were found when vitamin D degradation was fit to zero, first and second order reaction kinetics for all formulations (Appendix D Poor fit may, in part, be due to variability within the data points from the three petri dishes, as illustrated by the relative standard deviations for each time point within every formulation (Appendix E). Additionally, the degradation reaction may be too complex to be described using these methods.

4.7.2 Ambient storage of vitamin D_3 formulations

The results of a one-way ANOVA with repeated measures for the significance of vitamin powder formulation with exposure time as the repeated measure, and time exposed to storage conditions on vitamin D_3 loss in powders stored at ambient temperature and humidity are shown in Table 4.3. Parallel to analysis of vitamin loss in powders exposed to accelerated storage conditions, vitamin D formulation with exposure time as a repeated measure was significant. This shows that there were at least some differences between formulations in regards to loss of vitamin D_3. Exposure time was also significant, meaning that the amount of vitamin D_3 remaining in the powder changed over time for one or more formulations.

Table 4.3 Results of a one-way ANOVA, with repeated measures, on the degradation of vitamin D_3 in dry vitamin formulations stored at ambient temperature and humidity

Factors[a]	**DF**[b]	**Adj SS**[c]	**Adj MS**[d]	***F*-value**	***p*-value**[e]
Exposure time	3	24700	8230	380	**0.000**
Vitamin D_3 formulation (Exposure time)	16	5060	316	14.6	**0.000**
Error	20	433	22		
Total	39	30200			

[a] Vitamin D formulation and exposure time (the repeated factor) are fixed factors.

[b] Degrees of freedom

[c] Adjusted sum of squares

[d] Adjusted mean squares

[e] Significant effects ($p \leq 0.05$) are shown in bold font.

The amount of vitamin D remaining in formulations held at ambient temperature and humidity was compared between formulations at each exposure time and within each formulation over time using Tukey's test (Table 4.4). At 14 days of exposure to these storage conditions, the level of vitamin remaining in both VD-rCM powders (VD-rCM L and VD-rCM H) was significantly greater than both control formulations (CL and CH). The amount of vitamin D_3 remaining in VD-rCM H powder was significantly lower than CWS D_3 at 14 days of storage. The remaining percentage of vitamin D_3 was not significantly different between VD-rCM L, VD-rCM H, and CWS D_3 powders at 28 and 42 days of storage time. For days 28 and 42, the percent of vitamin remaining for both control powders was significantly less than both VD-rCM powders.

Ambient temperature and relative humidity were recorded during the storage of all samples and shown in Figure 4.1. It is important to note that VD-rCM L, VD-rCM H and CWS D_3 powders were stored and sampled during the same time period, while CL and CH were prepared at a later date and stored for the same amount of time. A short description of the reason for this difference can be found in Section 4.4.2. Thus, temperature and relative humidity were different between these two sets of formulations. The storage temperature for VD-rCM L, VD-rCM H and CWS D_3 was higher during the first 8 days of storage (Figure 4.1a). Shown in Figure 4.1b, relative humidity during storage of CL and CH samples was higher on many days than relative humidity during storage of VD-rCM L, VD-rCM H and CWS D_3 powders. Grady and Thakker (1980) reported that storage at 40°C and relative humidity of 45 and 85%, 96-97 percent loss of vitamin D_3 was observed after 21 days of storage; however, vitamin loss was 11-14 percent when stored at 25°C and 85% relative humidity for 56 days. This might suggest that the difference between loss of vitamin D_3 in CL and CH powders

Table 4.4 Vitamin D remaining in five formulations at different storage times held at ambient temperature and humidity

Exposure time (days)	**Vitamin D_3 (% of initial)[a]**				
	VD-rCM L[b]	**VD-rCM H[c]**	**CL[d]**	**CH[e]**	**CWS D_3[f]**
0	100 a	100 a	100 a	100 a	100 a
14	60 bc	67 b	35 efg	30 fg	46 cdef
28	54 bcd	56 bcd	31 fg	25 g	44 cdef
42	52 bcde	55 bcd	27 fg	24 g	39 defg

[a] Means (n = 2) that do not share common lowercase letters are significantly different ($p \leq 0.05$).

[b] Vitamin D_3 – re-assembled casein micelles, low phosphate

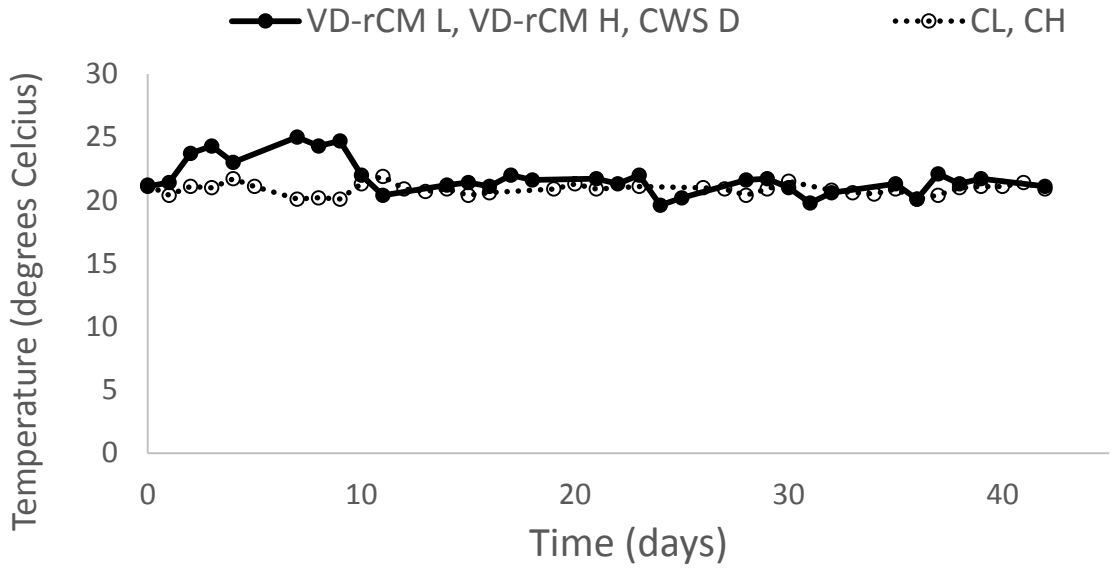
[c] Vitamin D_3 – re-assembled casein micelles, high phosphate

[d] Control powder, low phosphate

[e] Control powder, high phosphate

[f] Dry vitamin D3 100 CWS/AM from DSM nutrition

a)



b)

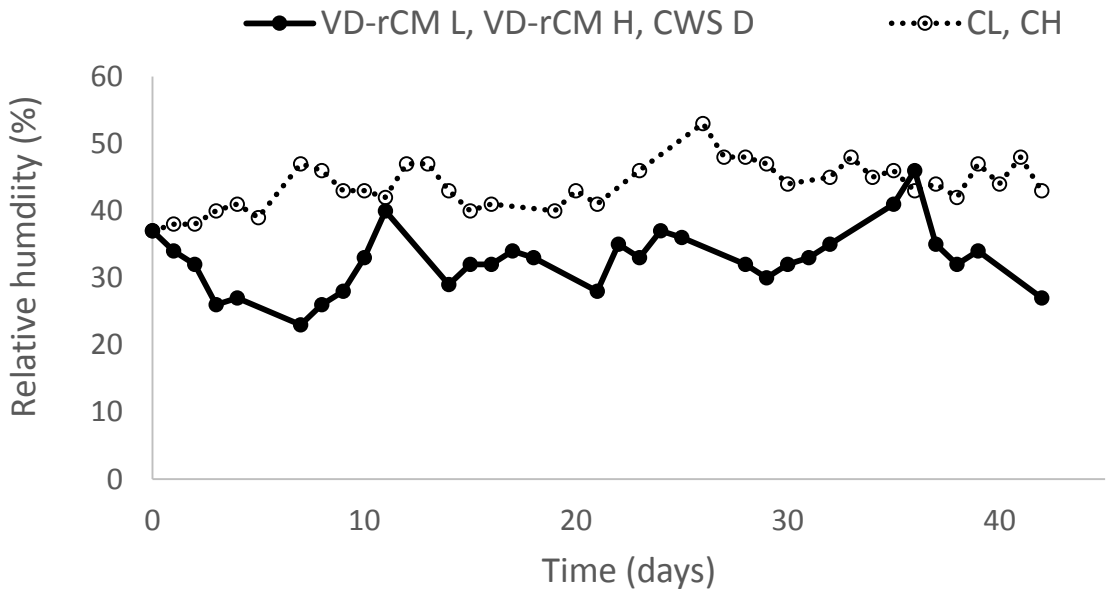


Figure 4.1 Recorded temperature (a) and relative humidity (b) during storage of five formulations held at ambient temperature and relative humidity

compared to the other three formulations may be partially attributed to the difference in storage conditions, in particular the difference in storage temperature.

Vitamin D_3 loaded into VD-rCM was more stable than vitamin D_3 stored with the same materials not in micellar form during storage at ambient temperature and relative humidity. Vitamin D in CL and CH powders was first mixed with sodium caseinate, then dried and mixed with phosphate, citrate and calcium. The stability provided to vitamin D_3 by the micellar structure as opposed to casein binding may be due to a few different effects.

Firstly, vitamin D is known to bind to β-casein (Forrest et al., 2005). If vitamin D binds to β-casein within the micelle structure, the exterior of the rCM may provide a physical barrier to moisture since β-caseins are theorized to mainly reside in the interior of micelles (Dalgleish & Corredig, 2012). Additionally, binding of vitamin D_3 by casein will limit the mobility of vitamin D_3 and thereby decrease its availability for chemical reactions. Haham et al. (2012) proposed that an antioxidative mechanism plays a role in protecting vitamin D_3 from degradation within rCM. This is because caseins have antioxidative properties that may be attributed partly to free thiol groups on κ-casein.

As lipid oxidation is autocatalytic, once oxidation is initiated it continues to propagate. Interestingly, the majority of rCM in VD-rCM L were smaller than those in VD-rCM H (Table 3.8). In addition, vitamin loading of rCM in VD-rCM L and VD-rCM H suspensions was estimated to be 13.3-14.6 mg vitamin D_3/g casein and 14.9-15.3 mg vitamin D_3/g casein, respectively. This suggests that, by mass, there is more vitamin D_3 per casein for VD-rCM H than VD-rCM L. Since oxidation reactions would continue to propagate as more vitamin D_3 is available, greater degradation might be assumed to occur for VD-rCM H where more vitamin D_3 per casein were found compared to VD-rCM L; however, no significant difference was found

between the degradation of vitamin D_3 in VD-rCM L and VD-rCM H powders (Table 4.2 and Table 4.4). One possible explanation for this finding is the actual structure of rCM and location of vitamin D_3. Observation of Figure 3.10 and Figure 3.11 reveals structures than are highly branched, made up of spherical subunits. It is proposed that vitamin D_3 is located mainly in the interior of these subunits and separated by a barrier of casein at each linkage.

No significant difference was found for degradation of vitamin D_3 in CWS D_3 powder compared to VD-rCM L or VD-rCM H powders during storage, except for Day 14 of ambient temperature and relative humidity storage where VD-rCM H had significantly less degradation than CWS D_3 (Table 4.4). This is a significant finding that the protection given to vitamin D_3 by rCM is at least comparable to if not better than that provided by a commercial formula, especially when considering that CWS D_3 contains *dl*-α-tocopherol, which acts as an antioxidant and may contribute to the protection this formula provides against degradation of vitamin D_3. Visual observation of VD-rCM L and VD-rCM H powders compared to CWS D_3 powder indicated that rCM powders were more porous and less compact than CWS D_3. Freeze drying creates a porous and less stable structure than other methods of drying such as spray drying (Augustin & Hemar, 2008). Thus, changing the method of drying as well as adding antioxidants to VD-rCM are potential opportunities to enhance the protection provided to vitamin D_3 by rCM.

Degradation of vitamin D_3 occurred much more quickly in all formulations when stored in accelerated storage conditions as opposed to ambient temperature and humidity. Grady and Thakker (1980) reported that crystalline vitamin D_3 degraded to 86-89 percent of initial values when stored at 25°C for 56 days with 85% relative humidity. At 40°C and relative humidity of 45 and 85%, 96-97 percent loss of vitamin D_3 was observed after 21 days of storage. The results of

the present study are in agreement that an increase in temperature and humidity negatively affect the stability of vitamin D_3.

It is difficult to extrapolate the potential vitamin degradation in each formulation from the present research to a lower temperature such as 4°C. The Arrhenius equation can be used to predict degradation at different temperatures by determining the dependence of reaction rate on temperature; however, utilization of this method requires rate constants to be determined for multiple storage temperatures (Burdurlu, Koca, & Karadeniz, 2006). Only four data points were recorded for loss of vitamin D_3 when stored at ambient temperature and humidity, which may be insufficient to determine statistically valid rate constants. In addition, poor R^2 values were found when vitamin D_3 degradation during accelerated storage conditions was fit to zero, first and second order reaction kinetics for all formulations (Appendix D).

The degradation of vitamin D_3 in VD-rCM L, VD-rCM H and CWS D_3 powders was measured after seven weeks of storage at 4°C. Powders were stored in tightly sealed vials and were not exposed to light during the entire storage period. Vials were of the same type as those used during ambient temperature and relative humidity storage. Powders were sampled in triplicate. For VD-rCM L, VD-rCM H, and CWS D_3 powders 100 ± 6, 98 ± 3, and 74 ± 9 percent (mean ± standard deviation, n = 3) of initial vitamin D_3 remained after seven weeks of storage. Vitamin D_3 loaded into rCM may degrade at a much slower rate when temperature is decreased; however, further replicates and data points are required for confirm this finding.

4.8 Conclusion

When stored under accelerated storage conditions, degradation of vitamin D_3 in CL and CH powders was greater than in VD-rCM L and VD-rCM H powders at 48 and 72 hours of

storage. Additionally, loss of vitamin D_3 in VD-rCM L and VD-rCM H powders was less than in CL and CH powders when stored at ambient temperature and humidity. This difference was already noticeable at 14 days of storage. These findings suggest that the rCM structure may provide protection to vitamin D_3 from heat and humidity. Comparing the loss of vitamin D_3 in rCM powders to that measured in CWS D_3 powder, it is seen that there is little difference between these formulations in the protection provided against heat and humidity. Thus, VD-rCM may be suitable for use as a vitamin product where storage as a dry powder is desired.

Chapter 5: Exposure of fortified fluid skim milk to light

5.1 Introduction

Vitamin D_3 is typically stable during processing and storage when in a food matrix (Hanson & Metzger, 2010; Kazmi et al., 2007; Upreti et al., 2002). Pasteurization did not affect vitamin D_3 content of process cheese, nor did storage at 21 to 29°C or 4 to 6°C (Upreti et al., 2002). Processing of HTST pasteurized 2% fat milk, UHT-processed 2% fat chocolate milk, and low-fat strawberry yogurt did not affect retention of vitamin D from a commercial water dispersible vitamin D product (Hanson & Metzger, 2010). Stability was found to be greater for emulsified vitamin D_3 than crystalline vitamin D_3 in a fortified lab scale Cheddar cheese-like matrix during storage for three months at 4°C (Kazmi et al., 2007).

Instability of vitamin D is observed when food products are exposed to light. Renken & Warthesen (1993) concluded that the effect of light exposure of degradation of vitamin D_3 was small when skim milk was exposed to light at 3200 lux for 10 days. However, Saffert et al. (2009) observed a reduction of 35 to 65 percent vitamin D_3 in low-fat UHT milk exposed to light for 12 weeks at 23°C, where the extent of degradation was dependent on milk packaging. Additionally, Liu (2013) reported high levels of vitamin D_3 degradation when comparing the retention of vitamin D in skim milk fortified with four commercial formulations exposed to light at 2000 lux over 22 days of storage. By the end of storage 29, 38, 39 and 63 percent of vitamin D_3 was retained in fortified skim milk for AD premix, SDS D_3, crystalline D_3 and CWS D_3, respectively, compared to the same formulations stored in the dark.

Protection conferred to vitamins by rCM has been investigated following preparation of V-rCM suspensions. UV light stability of vitamin D_2 was improved by using rCM as vitamin carriers (Semo et al., 2007). More protection was provided to vitamin D_2 in suspensions of VD-rCM compared to a solution where rCM had been removed (supernatant containing casein and vitamin D_2). Stability of vitamins carried by rCM to light exposure compared to other commercial vitamin formulations has not been reported in the literature.

The objective of this study was to investigate the protection provided to vitamin D_3 by rCM in skim milk during exposure to light and compare this to commercially available vitamin products. To accomplish this, fluid skim milk was fortified with four different vitamin formulations: VD-rCM that had been freeze-dried, the same materials used in the process of forming VD-rCM not in micelle form, a commercial vitamin D product, and a commercial vitamin D and A product. Since fortification of fluid skim milk with vitamins A and D is mandatory in Canada (FDR, 2013), the first three milks were fortified with a commercial vitamin A product to allow comparisons with commercial milk, as well as the milk fortified using the commercial vitamin D and A product. After fortification, the milk was pasteurized and exposed to light for 21 days.

5.2 Materials

Vitamin D_2 (ergocalciferol), vitamin D_3 (cholecalciferol), sodium caseinate, phenolphthalein, retinyl palmitate, sodium azide, and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich Canada (Oakville, ON). Hydrochloric acid (HCl), potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium chloride (NaCl), potassium phosphate dibasic anhydrous (K_2HPO_4), tripotassium citrate (K_3citrate), calcium chloride dehydrate

($CaCl_2$), ascorbic acid, acetic acid, sodium sulphate, ethanol (absolute), methanol (HPLC grade), hexane, dichloromethane (HPLC grade), ethyl acetate (HPLC grade), and acetonitrile (HPLC grade) were purchased from Fisher Scientific Canada (Ottawa, ON). Erythromycin was purchased from Sigma-Aldrich Canada (Oakville, ON). Retinyl propionate was purchased from Santa Cruz Biotech (Santa Cruz, CA).

5.2.1 Vitamin formulations

Commercial vitamin formulations were donated by DSM Nutritional products (Belvidere, New Jersey, USA) and Kingsway Chocolate (Mississauga, Ontario, Canada). Water dispersible vitamin powders, namely dry vitamin $D_3$100 CWS/AM ("CWS D_3") and vitamin A palmitate type 250 CWS/F ("CWS A"), were obtained from DSM Nutritional products. An emulsified vitamin A palmitate and vitamin D_3 product ("AD premix") came from Kingsway Chocolate. Specific product information for each vitamin formulation can be found in Appendix C.

VD-rCM L was chosen over VD-rCM H to fortify milk in this study for a few reasons. Although no difference was observed between VD-rCM L and VD-rCM H in regard to percent loss of vitamin during storage as dry powders (Chapter 4), slightly less phosphate is required to prepare VD-rCM L. Though slight, this reduction in necessary material provides opportunity for cost savings if VD-rCM were prepared as a fortification product. Moreover, particles in the suspensions of VD-rCM L are smaller than those in VD-rCM H. Particles in the range of 2 to 3 mm were observed in suspensions of VD-rCM H when viewed by light microscopy (Figure 3.10 and Figure 3.11). When hydrating a dry powder for use, small particle size is desirable for uniform dispersion in the resulting suspension, as well as in the fortified food or beverage.

Difficulty was also experienced when attempting to hydrate VD-rCM H powder, with high levels of sedimentation.

5.2.2 Preparation of fluid skim milk

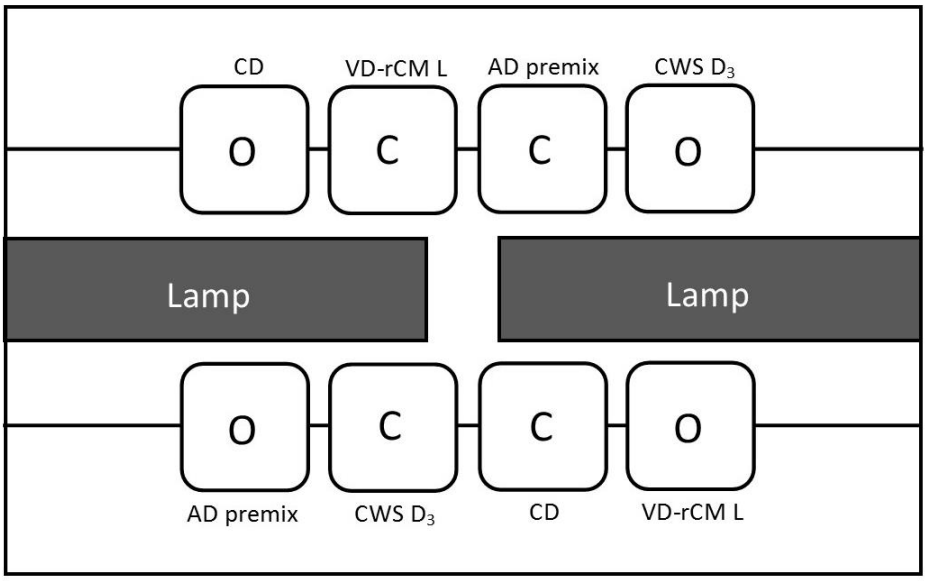
Raw whole milk was obtained from the UBC Dairy Education and Research Centre (Agassiz, BC) and skimmed by centrifugation at 10,000 *g* for 20 minutes at ambient temperature using a Sorvall RC 6+ centrifuge. Four different milk fortifications were prepared from skimmed milk: VD-rCM L, a control for VD-rCM L (CD), CWS D_3, and AD premix. Milk was fortified to target concentrations of 43 IU vitamin D/100 mL and 218 IU vitamin A/100 mL (CFIA, 2012). VD-rCM L milk was prepared by fortifying 2 L of skimmed milk with 1 mL of 2.1 mg/mL VD-rCM L suspension (powder hydrated in distilled water and stirred for 30 minutes prior to use). CD milk was prepared by adding 1 mL of vitamin D_3 in ethanol (21.6 µg/mL) to 2 L of milk; at the same time, the following compounds were added: 0.5 mg $CaCl_2$, 0.2 mg K_3citrate, 0.1 mg K_2HPO_4, and 45.3 mg sodium caseinate. CWS D_3 milk was fortified by adding 1 mL of 6.4 mg/mL of CWS D_3 solution prepared in distilled water to 2 L of milk. VD-rCM L, CD, and CWS D_3 milks were also fortified with vitamin A by adding to each 2 L batch of milk, 1 mL of 3.75 mg/mL CWS A solution (prepared in distilled water). Lastly, 2 L of milk was fortified with AD premix by adding 2 mL of AD premix stock solution, which had been prepared by diluting 4.15 mL AD premix to 100 mL using 75:25 ethanol:dichloromethane. Directly after fortification, to each 2 L batch of milk 100 mg/L erythromycin was added to slow milk quality deterioration over the period of storage. Milk was stirred for 10 minutes to ensure even distribution of vitamins and erythromycin.

The fortified milk was batch pasteurized at 63°C for 30 minutes with constant agitation. For each fortification type, milk was split into two 1 L batches that were pasteurized simultaneously. Each batch was poured into a 1.5 L stainless steel canister. The canister was suspended in a 63°C water bath and milk was stirred continuously at 600 rpm using an overhead stirrer. The come up time for each batch was 28 minutes. After pasteurization, the two batches per fortification type were combined in a sterilized 2 L glass bottle, which was inverted 8 times to ensure thorough blending. Then, three sterilized 500 mL glass bottles (Avalon Dairy, Burnaby, BC) were filled with fortified milk. Bottles were sealed using molded plastic caps (Avalon Dairy, Burnaby, BC) that were cleaned and rinsed with ethanol.

5.3 Experimental set-up for light exposure

The two glass bottles of fortified milk were exposed to light at 2000 lux for 21 days during storage at 4°C. Previously light intensity of 3200 lux was reported to be unrealistic compared to retail settings (Renken & Warthesen, 1993); therefore, lower intensity light was used here. The experimental set-up in this study was used previously in our lab to investigate the stability of vitamins in fluid milk to light exposure (Liu, 2013). Milk bottles were placed under two fluorescent lamps (Blue Planet 052-5109-0 fluorescent tubes with color temperature of 4,100 K; Canadian Tire, Vancouver, BC) in a straight line providing 2000 lux light intensity at the bottom of the bottles. Light intensity was measured using a LuxMaster light meter (Harry's Pro Shop, Toronto, ON). For each fortification, two bottles were placed in the light set-up with one in the center and one in an outside position, while one bottle was covered in foil and stored in the dark at 4°C. An overview of the set-up is shown in Figure 5.1.

a)



b)

Figure 5.1 Top view (a) and side view (b) of the experimental set-up for the exposure of fortified fluid skim milk samples to light. C – center position; O – outside position

AD premix = An emulsified vitamin A palmitate and vitamin D_3 product from Kingsway Chocolate; CWS D_3 = Dry vitamin D_3 100 CWS/AM from DSM nutrition; VD-rCM L = Vitamin D_3 – re-assembled casein micelles, low phosphate; CD = Milk fortified with the same level of casein, vitamin D_3, phosphate, citrate and calcium used in the fortification of VD-rCM L milk

5.4 Sampling and analysis of vitamin D and vitamin A

Vitamin D_3 content of fortified milk was analyzed in duplicate before pasteurization, after pasteurization (Day 0) and on Day 7, Day 14, and Day 21 of storage in the experimental set-up. The method for determining vitamin D_3 is described fully in Appendix A.1. This method is modified from the AOAC method 995.05 (Sliva & Sanders, 1996) and has previously been used for the extraction and analysis of vitamin D_3 from skim milk (Liu, 2013). Milk was also analyzed for vitamin A palmitate content before pasteurization, after pasteurization (Day 0) and on Day 1, Day 2, Day 3, Day 5, Day 7, Day 10, Day 14, and Day 21 of storage in the experimental set-up. Analysis was conducted in duplicate. The method used for vitamin A determination is found in Appendix A.2., and is modified from the AOAC method 2002.06 (Hite, 2003). The flow rate and concentrations of mobile phase components used for HPLC analysis of vitamin D and vitamin A are described in Appendix A.3.

5.5 Microbial plate count

Directly after pasteurization and at the end of storage milk samples were evaluated for total aerobic microbial counts. Samples of 1 mL were placed on 3M Petrifilm Aerobic Count Plates (3M Canada, London, ON) and incubated at 32°C in an Innova 40 Incubator (New Brunswick Scientific, Enfield, CT). Microbial counts were conducted after 48 hours of incubation. Samples were diluted aseptically from 10^{-1} to 10^{-6} using peptone water (0.1% wt/vol). Two plates were prepared for each sample type and dilution.

5.6 Statistical analysis

The change in vitamin concentration due to pasteurization was analyzed using a one-way ANOVA in a general linear model with repeated measures on one factor at a 95 percent confidence level. Tukey's test was then used to investigate significant differences ($p \leq 0.05$) between vitamin levels measured in different formulations before and after pasteurization. Statistical analysis was conducted using Minitab 16 (State College, PA).

Vitamin D_3 levels in fortified milk were converted to retention of vitamin D_3 (%). Retention was calculated as the percent of vitamin remaining in bottles exposed to light compared to control bottles at the same time point in the dark. Statistical analysis of vitamin retention was conducted using Minitab 16. Data was analyzed using a two-way ANOVA in a general linear model with repeated measures on one factor. Tukey's test was then used to explore significant variations in vitamin levels between formulations and changes over time ($p \leq 0.05$).

5.7 Results and discussion

5.7.1 Vitamin D_3 content of fluid skim milk

The amount of vitamin D_3 measured in skim milks fortified with AD premix, CWS D_3, VD-rCM L, and CD is shown in Table 5.1. Though milks were fortified to the same target concentration of vitamin D, differences are seen between fortified milks. This may be due to experimental error during milk preparation or also because actual values were not exactly the contents listed on the vitamin product certificates of analysis. The concentration of vitamin D_3 measured in milk after pasteurization was not significantly different than before pasteurization for any formulation type ($p > 0.05$). The amount of vitamin D remaining on Day 21 for all

Table 5.1 Vitamin D_3 content (mean ± range, n = 2) of milks fortified with four different vitamin formulations and stored for 21 days at 4°C

		Vitamin D_3 content (IU/100 mL)			
	Day	**AD premix[a]**	**CWS D_3[b]**	**VD-rCM L[c]**	**CD[d]**
	Prior to pasteurization	48.8 ± 0.9	38.1 ± 1.1	43.6 ± 3.1	45.1 ± 0.6
	0	46.4 ± 2.1	34.6 ± 1.3	40.4 ± 0.4	45.2 ± 2.3
Center	7	17.0 ± 0.3	19.1 ± 4.8	16.9 ± 0.0	20.4 ± 6.6
	14	10.2 ± 0.8	16.4 ± 0.9	11.8 ± 1.3	14.2 ± 0.4
	21	8.5 ± 0.3	15.7 ± 1.9	11.7 ± 1.4	15.4 ± 0.2
Outside	7	27.0 ± 0.2	25.4 ± 0.1	22.1 ± 0.1	30.0 ± 0.1
	14	15.3 ± 0.1	20.5 ± 0.5	16.2 ± 0.0	16.7 ± 0.7
	21	10.5 ± 1.1	20.1 ± 0.2	12.4 ± 0.5	15.9 ± 5.1
Control	7	45.1 ± 0.3	34.4 ± 0.3	39.6 ± 1.3	47.0 ± 2.7
	14	43.2 ± 0.7	32.1 ± 0.9	36.4 ± 1.7	41.0 ± 0.5
	21	35.7 ± 4.2[e]	28.7 ± 2.3	35.7 ± 2.2	38.5 ± 2.0

[a] An emulsified vitamin A palmitate and vitamin D_3 product from Kingsway Chocolate

[b] Dry vitamin D_3 100 CWS/AM from DSM nutrition

[c] Vitamin D_3 – re-assembled casein micelles, low phosphate

[d] Milk fortified with the same level of casein, vitamin D_3, phosphate, citrate and calcium used in the fortification of VD-rCM L milk

[e] For this value: mean ± standard deviation, n = 3

bottles exposed to light was lower than on Day 0. Additionally, both bottles for each type of vitamin formulation had lower vitamin D_3 on Day 7, 14, and 21 than control bottles that were stored in the dark. From this it is apparent that light exposure resulted in a decrease in vitamin D content.

A two-way ANOVA with repeated measures on one factor (bottle position) was used to investigate the effect of vitamin formulation, exposure time, and bottle position on vitamin D_3 retention in bottles exposed to light as compared to control bottles kept in the dark (Table 5.2). All factors were fixed factors. Vitamin formulation and exposure time both significantly affected the retention of vitamin D_3. Bottle position with exposure time as a repeated measure, the interaction between vitamin D_3 formulation and exposure time, as well as the interaction between vitamin formulation and bottle position (with exposure time as a repeated measure) were all significant factors to retention of vitamin D_3. Within each formulation there were two bottles, located in two positions: outside and center. Bottle position was significant, meaning that bottle position was a significant factor for vitamin retention even within the same vitamin formulation. Furthermore, a significant interaction also existed between vitamin formulation and time exposed to light. This is an indication that rates of vitamin D_3 degradation in different formulations were not the same.

Tukey's test was used to examine variation between vitamin retention of formulations at different exposure times more closely. Results are shown in Table 5.3. No significant difference in vitamin retention was seen between CWS D_3 and CD fortified milks at Day 7. In addition, the retention of vitamin D_3 was not significantly different between AD premix and VD-rCM L fortified milks at Day 7. Vitamin retention at Day 7 was significantly lower in milk fortified using AD premix and VD-rCM L than in milk fortified using either CWS D_3 or CD powders. At

Table 5.2 Results of a two-factor ANOVA, with repeated measures on one factor, on retention of vitamin D_3 in fortified skim milk exposed to light

Factors[a]	**DF**[b]	**Adj SS**[c]	**Adj MS**[d]	***F*-value**	***p*-value**[e]
Vitamin D_3 formulation	3	2150	716	21.8	**0.000**
Bottle position (Exposure time)	4	2580	644	50.7	**0.000**
Exposure time	3	39100	13000	1020	**0.000**
Vitamin D_3 formulation × Exposure time	9	1190	132	10.4	**0.000**
Vitamin D_3 formulation x Bottle position (Exposure time)	12	824	69	5.40	**0.000**
Error	32	407	13		
Total	63	46200			

[a] Vitamin D formulation and bottle position are fixed factors, and exposure time is the repeated measure conducted for each bottle position.

[b] Degrees of freedom

[c] Adjusted sum of squares

[d] Adjusted mean squares

[e] Significant effects ($p \leq 0.05$) are shown in bold font.

Table 5.3 Vitamin D_3 retention of four formulations at different light exposure times in skim milk

Exposure time (days)	**Vitamin D_3 retention (%)[a]**			
	AD Premix[b]	**CWS D_3[c]**	**VD-rCM L[d]**	**CD[e]**
0	100 a	100 a	100 a	100 a
7	49 cd	60 b	50 cd	60 b
14	30 fg	52 bc	38 ef	39 ef
21	27 g	57 bc	36 efg	41 de

[a] Means (n = 2) that do not share common lowercase letters are significantly different ($p \leq 0.05$).

[b] An emulsified vitamin A palmitate and vitamin D_3 product from Kingsway Chocolate

[c] Dry vitamin D3 100 CWS/AM from DSM nutrition

[d] Vitamin D_3 – re-assembled casein micelles, low phosphate

[e] Milk fortified with the same level of casein, vitamin D_3, phosphate, citrate and calcium used in the fortification of VD-rCM L milk

Day 14 and Day 21, vitamin retention was significantly higher in milk fortified with CWS D_3 than with AD premix, VD-rCM L and CD formulations. Additionally, on Day 21 vitamin retention for AD premix milk was significantly lower than vitamin retention for CWS D_3 and CD fortified milks, though not significantly different than retention for VD-rCM L fortified milk. No significant difference in vitamin retention was found on Day 14 or 21 between milks fortified with VD-rCM L and CD. CD milk was fortified using crystalline vitamin D_3. Unprotected vitamin D such as crystalline vitamin D_3 is expected to be sensitive to degradation in milk when exposed to light, because riboflavin can act as a photosensitizer triggering singlet oxygen production which then reacts with vitamin D (King & Min, 1998; King & Min, 2002). In spite of this, retention of vitamin D_3 was not significantly greater in milk fortified using VD-rCM L when compared to CD fortified milk.

AD premix is a commercial formula intended for the fortification of milk. It is an emulsion containing vitamin D_3, vitamin A palmitate, sunflower oil, polysorbate 80, and polyglycerol monooleate. The supplier indicated that the formulation also included tocopherol and BHT though these ingredients are not listed in Appendix C. Even with the inclusion of antioxidants in the AD premix formulation, vitamin D_3 retention was not better in milk fortified using AD premix than milk fortified with VD-rCM L. On Day 14 and 21, vitamin D_3 retention was the greatest for milk fortified using CWS D_3. CWS D_3 contains cholecalciferol, medium chain triglycerides, corn starch, acacia gum, sucrose and *dl*-α-tocopherol. The materials used in this formulation may provide enhanced protection to vitamin D_3 even after reconstitution and addition to milk.

The retention of vitamin D_3 in milk fortified in the present work with CWS D_3 was lower than that reported by Liu (2013) for CWS D_3 fortified milk at all storage times. Additionally,

retention of vitamin D_3 in milk fortified using AD premix was lower than that reported by Liu (2013) for the first 14 days of storage. Liu (2013) fortified milk after pasteurization, whereas in the present study milk was fortified prior to pasteurization. Heat treatment may have played a significant role in the initiation of degradation reactions in the current work. Renken and Warthesen (1993) reported much lower loss of vitamin D_3, in the range of 25%, after exposure to light at 3200 lux for 10 days. As well, Saffert et al. (2009) reported loss of between 35 to 65 percent vitamin D_3 in low-fat UHT milk during exposure to light; however, the conditions were even more severe than the present study as Saffert et al. (2009) measured vitamin retention in over 12 weeks of storage at 23°C.

Changes to the surface of casein micelles with heat treatment have been suggested to alter the binding of hydrophobic compounds, such as curcumin (Yazdi & Corredig, 2012). It is undetermined whether heat treatment had any effect on the retention of vitamin D_3 in milks exposed to light in the current study. Since whey proteins denature at temperatures above 70°C (Donato & Guyomarc'h, 2009) and milk in the current research was pasteurized at 63°C, we can presume that heat mediated whey protein interactions with casein micelles did not influence the retention of vitamin D_3.

Alterations to the casein micelle structure are seen with cooling to between 0 and 5°C (O'Mahony & Fox, 2013). Some β-casein and smaller amounts of κ-casein dissociate from the micellar structure in greater proportions than α_{s1}-casein and α_{s2}-casein when casein micelles are cooled (McMahon & Oommen, 2013). As much as 60 percent of β-caseins can dissociate from casein micelles when they are cooled (Downey, 1973) and this release changes the internal structure of the casein micelles (Yazdi, 2012). Vitamin D_3 binds to β-casein (Forrest et al., 2005) though it is unknown whether binding to β-casein is prevalent when other caseins are present

such as in the case of rCM. Release of β-casein from the casein micelle structure upon cooling may affect the protection that is provided to vitamin D_3 from light when in fluid skim milk stored at 4 to 6°C if this release results in the concurrent release of vitamin D_3 from rCM. It is not currently known where vitamin D_3 is located in relation to different caseins within rCM nor where the vitamin is located in milk once VD-rCM powder is added to fluid skim milk.

5.7.2 Vitamin A palmitate content of fluid skim milk

The concentrations (IU/100 mL) of vitamin A palmitate measured for bottles of milk fortified with vitamin A and D stored for 21 days at 4°C are shown in Table 5.4. Though values given on the certificate of analysis of the AD premix product for vitamin content were used for the preparation of stock solution, there is a discrepancy between predicted values (218 IU/100 mL) and measured values for both vitamin A palmitate and vitamin D_3 prior to pasteurization in the milk fortified with AD premix. The remaining milks (VD-rCM L, CWS D_3 and CD) were fortified with CWS A. Manufacturer's directions and vitamin content given on the certificate of analysis were used to prepare the vitamin solution (CWS A). All values for vitamin A palmitate in fluid skim milk fortified with CWS A exceeded the target concentration of 218 IU/100 mL prior to pasteurization, as well as on Day 0 (after pasteurization).

The concentration of vitamin A palmitate measured in milk after pasteurization was not significantly different than before pasteurization for any formulation type ($p > 0.05$). By Day 7 no vitamin A palmitate was detectable in all bottles exposed to light. More vitamin A was detected on Day 1, 2 and 3 in the center and outside position bottles of milk fortified with AD premix than CWS D_3, and VD-rCM and CD milks. The level of vitamin measured in milk fortified with AD premix stored in the dark remained largely constant during the entire storage

Table 5.4 Vitamin A palmitate content (mean ± range, n = 2) of milks fortified with four different vitamin formulations and stored for 21 days at 4°C

		Vitamin A palmitate content (IU/100 mL)			
	Day	**AD premix[a]**	**CWS D_3[b]**	**VD-rCM L[c]**	**CD[d]**
	Prior to pasteurization	288 ± 3	334 ± 9	310 ± 16	324 ± 18
	0	272 ± 2	306 ± 16	293 ± 8	320 ± 13
Center	1	139 ± 20	16 ± 3	14 ± 3	25 ± 5
	2	87 ± 1	ND	ND	ND
	3	48 ± 4	-	-	-
	5	10 ± 14	-	-	-
	7	ND	-	-	-
	14, 21	-	-	-	-
Outside	1	156 ± 7	23 ± 4	23 ± 0	31 ± 0
	2	114 ± 5	ND	ND	ND
	3	79 ± 0	-	-	-
	5	ND	-	-	-
	7, 14, 21	-	-	-	-
Control	1	274 ± 2	324 ± 13	279 ± 9	311 ± 2
	2	262 ± 5	318 ± 9	273 ± 8	293 ± 20
	3	261 ± 3	297 ± 16	270 ± 3	288 ± 8
	5	253 ± 10	287 ± 4	270 ± 18	285 ± 16
	7	252 ± 4	242 ± 2	265 ± 4	271 ± 4
	14	247 ± 16	74 ± 7	111 ± 1	110 ± 17
	21	245 ± 5	47 ± 1	57 ± 7	71 ± 8

ND – None detected

[a] An emulsified vitamin A palmitate and vitamin D_3 product from Kingsway Chocolate

[b] Dry vitamin D3 100 CWS/AM from DSM nutrition

[c] Vitamin D_3 – re-assembled casein micelles, low phosphate

[d] Milk fortified with the same level of casein, vitamin D_3, phosphate, citrate and calcium used in the fortification of VD-rCM L milk

time. It is not clear if the rapid degradation of vitamin A palmitate had a significant influence on vitamin D_3 degradation.

5.7.3 Microbial analysis

At Day 0, the aerobic microbial count in all individual bottles was below the limit of detection for the enumeration method. This is a good indication of sufficient pasteurization and avoidance of contamination. By Day 21, the aerobic microbial count of each bottle ranged from 0 to 7.5 x 10^8 CFU/mL. No correlation was found between aerobic microbial counts and vitamin D_3 retention ($R^2 = 0.065$).

5.8 Conclusion

Vitamin formulation played a significant role in vitamin D_3 retention during exposure to light in fortified skim milk. After 7 days of storage, vitamin retention was significantly greater in CWS D_3 and CD fortified milks than AD premix and VD-rCM L fortified milks. No difference was found at Day 7, 14 or 21 between vitamin retention in milk fortified using VD-rCM L or AD premix. In addition, no difference was found at Day 7 between vitamin retention in CWS D_3 or CD fortified milks. Vitamin retention was significantly higher in CWS D_3 fortified milk than AD premix, VD-rCM L and CD fortified milks at Day 14 and Day 21. Most importantly, retention of vitamin D_3 in VD-rCM L fortified milk was not significantly greater than retention in CD fortified milk during 21 days of storage.

Chapter 6: Conclusions

6.1 Main findings

The process of re-assembling casein micelles was developed many years ago in an attempt to understand the structure and function of natural casein micelles more fully. Information from these endeavors has been available for many years on the effect that different levels of casein micelle constituents play on casein micelle size and stability (Schmidt, 1979; Schmidt et al., 1979; Schmidt et al., 1977). More recently, rCM have been used as carriers for a variety of different hydrophobic compounds. Studies report different levels of loading, or encapsulation efficiency, for different compounds loaded into casein micelles. However, until now no attempt to find conditions that result in optimal levels of loading of a hydrophobic compound into rCM has been described. Chapter 3 explored the effect of phosphate, citrate and calcium concentration on vitamin loading of rCM. RSM was successfully employed to describe these effects and establish levels of phosphate, citrate and calcium that result in optimal levels of vitamin loading into rCM. This procedure was followed for vitamin D_3 and vitamin A palmitate. The following hypothesis was made prior to executing the study described in Chapter 3:

Hypothesis 1: Vitamin – re-assembled casein micelle suspensions with different concentrations of phosphate, citrate and calcium have different levels of vitamin loaded into the re-assembled casein micelles.

An increasing amount of vitamin D_3 was loaded into rCM when calcium was increased. A different effect was seen for citrate, where vitamin loading increased with increases of citrate in

the lower end of the concentration range, and further increases in citrate resulted in the lowering of vitamin D_3 loading. Loading of vitamin A palmitate increased with increases in calcium concentration. The phosphate^2 term was also significant for vitamin A palmitate loading. Vitamin loading was the highest in the center of the tested phosphate concentration range, then decreased as phosphate concentration strayed from this value. Since the loading of vitamins into rCM was affected by one of more of the three factors, hypothesis 1 can be accepted.

Application of VD-rCM as a fortification product for milk has previously been discussed in the literature (Haham et al., 2012). Haham and colleagues (2012) fortified 1% fat milk with freshly prepared liquid suspension of VD-rCM to examine the effect of rCM on vitamin D_3 bioavailability once they were consumed. The practicality of fortifying milk with freshly prepared liquid VD-rCM as compared to dry powder is limited for a variety of reasons, including shorter shelf-life and higher transportation cost. Considering that fortification products are often sold as dry powders, the current study sought to explore the use of VD-rCM further by investigating vitamin stability in a dry powder during storage. In Chapter 4 the storage stability of vitamin D_3 in VD-rCM (VD-rCM L, VD-rCM H) was compared to the stability in controls composed of the materials used to form VD-rCM (CL, CH), all in dry powder form. To obtain a well-rounded picture of stability at different temperatures and humidity, two different storage conditions were chosen to investigate the stability of vitamin D_3 during storage. Before beginning this study a hypothesis was made regarding the protective effect of rCM towards vitamins:

Hypothesis 2: Vitamins that have been loaded into re-assembled casein micelles are more stable during storage in dry powdered form than vitamins stored with the same dry powdered materials used to form the re-assembled casein micelles.

When powders were stored at 37°C and 75% relative humidity, significantly more vitamin was retained in VD-rCM L and VD-rCM H powders than CL and CH powders at 48 and 72 hours of storage (Table 4.2). At no point during accelerated storage was the loss of vitamin D_3 greater in VD-rCM L and VD-rCM H powders than CL and CH powders. Furthermore, the level of vitamin remaining was significantly greater in VD-rCM L and VD-rCM H powders than CL and CH powders for all storage times when powders were stored at ambient temperature and humidity (Table 4.4). The results from storage at ambient temperature and relative humidity support the acceptance of hypothesis 2; however, findings from storage at 37°C and 75% relative humidity are not in full agreement. Therefore, hypothesis 2 can be accepted for storage of powders at ambient temperature and humidity, but must be rejected for storage of powders at 37°C and 75% relative humidity.

Re-assembled casein micelles have been proposed as carriers for vitamin D_3 to provide stability to the vitamin. VD-rCM have also been suggested for use as vitamin carriers in fluid milk. Though Haham and colleagues (2012) fortified 1% fluid skim milk with VD-rCM, no report was given on the protection rCM provide to vitamin D_3 once deposited into the milk. The stability that rCM (VD-rCM L) and the materials used to form rCM (CD) provide to vitamin D_3 in fluid skim milk during light exposure was investigated in Chapter 5. Dry VD-rCM L was

hydrated and used for fortification to mimic procedures that would be in place for milk fortification in the industry. Hypothesis 3 was formulated and tested in this study.

Hypothesis 3: Vitamins that have been loaded into re-assembled casein micelles are more stable in milk exposed to light than vitamins added to milk along with the same materials used to form the re-assembled casein micelles.

Retention of vitamin D_3 in fluid skim milk fortified with VD-rCM L was not significantly greater than retention of vitamin D_3 in CD milk (Table 5.3). The results of this study warrant the rejection of hypothesis 3.

In summary, conditions resulting in high loading of vitamin D_3 into rCM were found while loading of vitamin A palmitate was insufficient to warrant further investigation in regards to milk fortification. As dry powder, VD-rCM L and VD-rCM H were able to provide protection to vitamin D_3 during storage at ambient temperature and relative humidity when compared to control samples. Additionally, no difference was found between vitamin degradation in VD-rCM L and VD-rCM H powders compared to the CWS D_3 commercial formulation when stored at 37°C and 75% relative humidity. When stored at ambient temperature and relative humidity for 14 days, the level of vitamin D_3 remaining in VD-rCM L and VD-rCM H powders was significantly greater than in CWS D_3 powder. Differences in vitamin D retention were seen for milk fortified with different vitamin D formulations exposed to light, compared to control milk samples fortified with the same formulations and stored in the dark. Retention was significantly higher in CWS D_3 fortified milk than VD-rCM L and CD milks at 14 and 21 days of

storage. No significant difference in vitamin retention was found between AD premix and VD-rCM L milks at any time during storage. This is a significant finding because, though not listed in Appendix C, the supplier indicated that AD premix contains 10 mg of mixed tocopherol and 5 mg of BHT per 1.7 million IU of vitamin A palmitate, whereas VD-rCM L powder contains no supplementary preservatives. No protective effect of rCM on vitamin degradation in fluid milk was found when compared to a milk fortified with the same materials, though not in micellar form.

6.2 Future direction

The loading of vitamin D_3 and vitamin A palmitate into rCM was optimized in the present research. To do this, the concentrations of phosphate, citrate and calcium in the system were altered to investigate their influence on vitamin loading. Factors that affected the loading of vitamin D_3 were different than those that affected the loading of vitamin A palmitate. It is proposed that the concentration of different caseins present in source of casein used in the re-assembly process influences the loading of these vitamins in different ways. It may be important to consider the composition of casein utilized in the formation of rCM, which could be another factor employed to further optimize vitamin loading. Further investigation is needed to investigate the concentration of different caseins incorporated into rCM loaded with different vitamins and whether this can be altered by altering the composition of the casein source, as well as the influence this has on the level of vitamin incorporated into the rCM.

While protection provided to the dry powder form of vitamin D_3 by incorporation into rCM was investigated during accelerated storage conditions and at ambient temperature and humidity, it is essential that stability be investigated during actual storage conditions. The

manufacturer's directions for storage of CWS D_3 are at a temperature below 15°C; therefore, refrigerated temperatures might be more suitable for storage of vitamin D powders. As part of the present study, samples of VD-rCM L, VD-rCM H, and CWS D_3 were stored for seven weeks at 4°C. Loss of vitamin D_3 was greater in CWS D_3 than both VD-rCM L and VD-rCM H. Further investigation with additional data points and replicates is required to make any substantial conclusions on the comparative vitamin degradation for each formulation. Additionally, CL and CH powders were not stored at 4°C, so it is unknown whether there is a difference between vitamin degradation at 4°C in VD-rCM L and VD-rCM H powders compared to CL and CH powders.

It is critical to understand the behavior of a powder once it is rehydrated. Consequently, there is opportunity to explore the characteristics of rehydrated V-rCM. It is unknown whether the structure of V-rCM in rehydrated suspension is similar to when V-rCM are initially prepared. Modification of V-rCM structure during the drying or rehydration processes could influence the protection that is provided to vitamins after rehydration and addition to foods.

VD-rCM L, VD-rCM H, CL and CH powders were freeze dried to form powders that were used in Chapter 4 and Chapter 5. In addition to being more costly and less accessible to industry, freeze drying produces a more porous structure in the dry material compared to spray drying (Augustin & Hemar, 2008). It is unknown whether spray drying would increase the protection provided to vitamins by rCM compared to freeze drying. As spray drying is more industrially relevant, future research should focus on this drying method to prepare dry V-rCM powder.

6.3 Conclusion

To conclude, rCM can be prepared with high levels of vitamin D_3 loading; however, loading of vitamin A palmitate was not high enough to warrant further investigation for the purpose of milk fortification. Other factors may affect loading of vitamins into rCM such as the levels of different caseins in the casein source. Further investigation is needed to determine the influence of casein concentrations on vitamin loading. Additionally, vitamin D_3 degradation was significantly lower in VD-rCM L and VD-rCM H powders than in CL and CH powders at 48 and 72 hours of storage at 37°C and 75% relative humidity, and at all times when vitamin content of powders was measured during storage at ambient temperature and humidity. Though the micellar structure may provide protection to vitamin D_3 during storage in a dry powder, vitamin loss was not significantly different from loss in the commercial powder (CWS D_3). Incorporation of antioxidants into the VD-rCM powder may be one avenue to explore to preserve vitamin activity. Finally, no additional protection was provided to vitamin D_3 by the micellar structure during exposure to light in fortified skim milk. Knowledge of the effect drying and rehydration has on rCM may be fundamental to understanding how to properly dry, rehydrate and utilize rCM as vitamin carriers in food products to provide enhanced vitamin protection.

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Appendices

Appendix A

A.1 Vitamin D extraction and analysis

Saponification

The following solutions were added to a 125 mL Erlenmeyer flask: 15 mL of sample, 20 µL of internal standard (5 µg/mL vitamin D_2), 1.5 mL of 19.4% wt/vol ascorbic acid in water (made fresh), 19 mL of 30.7% wt/vol of potassium hydroxide in water/ethanol (42 g KOH in 15:121.8 water/ethanol, made fresh). The flask were stoppered tightly and heated in a covered, shaking water bath at 60 °C for 30 min.

Extraction

The sample was cooled to room temperature and added to a 250 mL separatory funnel. The flask was washed with 15 mL distilled water and then 60 mL hexane and added to the funnel. The sample was shaken in the separatory funnel for 90 s and allowed to separate for approximately 10 minutes. The lower aqueous layer was drained to waste. The upper hexane layer was washed with 15 mL distilled water. The sample was shaken for 90 seconds and allowed to separate. The aqueous layer was drained to waste. The hexane layer was washed with 15 mL distilled water along with two drops of phenolphthalein indicator (0.5% in ethanol/water). If a pink color was observed, 10% vol/vol acetic acid was added drop wise until the aqueous layer was colorless (2 – 4 drops).

A funnel with sodium sulphate was set up on top of a 100 mL round bottom flask. The funnel was rinsed with ~30 mL hexane into the 100 mL round bottom flask. The hexane was swirled in the flask to rinse. The hexane was discarded to waste. The aqueous layer in the separatory funnel was drained to waste. Then, the hexane layer was passed through sodium sulphate into the round bottom flask containing 1 mL 0.1% wt/vol butylated hydroxytoluene in hexane. The separatory funnel was rinsed with 10 mL hexane and the rinse was passed through sodium sulphate into the 100 mL round bottom flask. The sodium sulphate was rinsed with 10 mL hexane before discarding. The sample was dried under vacuum using a rotary evaporator with the water bath at 35 – 40 °C and the residue was dissolved in 2 mL dichloromethane-isopropanol solution (99.8:0.2 vol/vol).

Sample purification

A 3 mL silica solid phase extraction cartridge (Agilent Technologies, Santa Clara, CA) and 13x100 test tube was set up on the vacuum manifold. The cartridge was conditioned with 4 mL dichloromethane-isopropanol solution (80:20 vol/vol) and drained to waste. Then the cartridge was conditioned with 5 mL dichloromethane-isopropanol solution (99.8:0.2 vol/vol) and drained to waste. The sample was then transferred to the cartridge along with 1 mL of dichloromethane-isopropanol solution (99.8:0.2 vol/vol) rinse and drained to waste. The cartridge was washed with 2 mL dichloromethane-isopropanol solution (99.8:0.2 vol/vol) and drained to waste. The vitamin D was eluted with 7 mL dichloromethane-isopropanol solution (99.8:0.2 vol/vol) into a new 13x100 test tube. Using a glass syringe, a 4 mm polytetrafluoroethylene filter was pre-rinsed with 2 mL dichloromethane-isopropanol solution (99.8:0.2 vol/vol) to remove any extractable compounds. The sample was passed through the

filter into a new 16x100 tube and dried under nitrogen. The sides of the tube were rinsed with 1 mL dichloromethane-isopropanol solution (99.8:0.2 vol/vol) to concentrate the sample at the bottom of the tube and dried under nitrogen. The sample was dissolved in 600 µL dichloromethane-isopropanol solution (99.8:0.2 vol/vol) wash and transferred to a micro-V HPLC vial, where it was dried under nitrogen. The residue was dissolved in 100 µL methanol/dichloromethane (80:20 vol/vol) for HPLC analysis at 264 nm.

HPLC analysis of vitamin D

Vitamin analysis was conducted on an HPLC system using Agilent 1100 series LC (Mississauga, ON) with a quaternary pump system and diode array detector. The column on the HPLC system was a Grace Vydac 201TP54 C18 column (Deerfield, IL) with 4.6 mm internal diameter and 250 mm length. Protection was provided to the column by installation of a Vydac 201TP C 18 guard column (Deerfield, IL). Column temperature was 24˚C.

An aliquot (40 µL) of the purified sample was injected into the HPLC for analysis, with detection of the vitamin D peak at 264 nm. The mobile phase was 98% acetonitrile and 2% methanol. Washing of the column used 98% ethyl acetate and 2% methanol, followed by a switch back to acetonitrile and methanol at the end of the run. The change in mobile phase components is shown in Appendix A.3. The total running time was 28 minutes.

Vitamin D_3 standard solutions were prepared in methanol at concentrations ranging from 0.20 to 4.00 µg/mL, using at least six concentrations. Internal standard solutions of vitamin D_2 were prepared in methanol at concentrations of 50.0 µg/mL for calibration samples and 5.00 µg/mL for experimental samples. An aliquot (40 µL) of the standards was injected into the HPLC for analysis, with detection of the vitamin D peak at 264 nm. All standard solutions were

stored at -80 °C when not in use for a maximum of 4 weeks. The area ratio of the vitamin D_2 standard peak and the vitamin D_2 internal standard peak were plotted against the vitamin D_3 standard concentration. Vitamin D_3 concentration in the sample was determined from the ratio of the sample on the equation derived from the standard curve.

A.2 Vitamin A extraction and analysis

The following items were added to a test tube: 10 µL internal standard (10 µg/mL retinyl propionate), 0.25 mL sample, 0.25 mL double-distilled water, and 1.25 mL ethanol. The test tube was vortexed for 30 seconds. After waiting 5 minutes, 1.25 mL hexane was added. The test tube was vortexed for 30 seconds and let rest for 2 minutes. Vortexing for 30 seconds and waiting 2 minutes was repeated for a total of 3 cycles. After the 3 cycles were complete, 0.75 mL water was added to the tube. The tube was vortexed for 5 seconds, the centrifuged at 633 *g* (~1800 RPM) for 10 minutes. The top hexane layer was transferred into a 1 mL plastic syringe that was fitted with a 0.45 µm syringe filter. The hexane layer was passed through the syringe filter into a micro-V HPLC vial. The sample in the vial was dried under nitrogen. The residue in the vial was dissolved in 100 µL 50:50 dichloromethane/methanol for HPLC analysis and 40 µL of the sample was injected into the HPLC for analysis.

Vitamin A was analyzed on the Agilent 1100 series LC HPLC system described above in the procedure for vitamin D analysis, with detection of the vitamin A peak at 325 nm. The gradient mobile phase was 95% acetonitrile and 5% water, then 100% methanol, and followed by ethyl acetate wash. The change in mobile phase components is shown in Appendix A.3. The total running time was 34 minutes.

Vitamin A standards contained 100 μL internal standard, 400 μL vitamin A palmitate stock solution and 500 μL dichloromethane. Vitamin A palmitate and internal standard (10 μg/mL retinyl propionate) stock solutions were prepared in isopropanol and then methanol through serial dilutions. Standards of at least five concentrations were used to make a standard curve. An aliquot (40 μL) was injected into the HPLC for analysis at 325 nm. The vitamin A palmitate in the sample was calculated from this standard curve.

A.3 Flow rates and concentrations of mobile phase components for determination of vitamin D and vitamin A

Table A.1 Flow rates and concentrations of mobile phase components for determination of vitamin D by HPLC

Time (min)	Flow rate (mL/min)	Acetonitrile (%)	Methanol (%)	Ethyl acetate (%)
0	1.2	98	2	0
16.5	1.2	98	2	0
18	1.5	0	2	98
21	1.5	0	2	98
22.5	1.2	98	2	0

Table A.2 Flow rates and concentrations of mobile phase components for determination of vitamin A by HPLC

Time (min)	Flow rate (mL/min)	Acetonitrile (%)	Methanol (%)	Ethyl acetate (%)	Water (%)
0	1.5	95	0	0	5
3.5	1.5	95	0	0	5
8	1.5	0	100	0	0
19	1.5	0	100	0	0
22	1.5	0	5	95	0
27	1.5	0	5	95	0
30	1.5	95	0	0	5

Appendix B Statistical analysis of data obtained in the central composite rotatable designs for vitamin D_3 and vitamin A palmitate loading of re-assembled casein micelles

Table B.1 Estimated regression coefficients for factors influencing vitamin D_3 loading of re-assembled casein micelles

Term	Regression Coefficient	Standard error of regression coefficient	t-value	*p*-value[a]
Constant	479	34	13.9	**0.000**
Phosphate	-42	30	-1.4	0.181
Citrate	-348	24	-14.5	**0.000**
Calcium	374	24	15.6	**0.000**
Phosphate x Phosphate	13	29	0.4	0.667
Citrate x Citrate	131	23	5.7	**0.000**
Calcium x Calcium	101	23	4.4	**0.001**

$R^2 = 0.981$; R^2 (pred) = 0.904; R^2 (adj) = 0.970

Analysis was done using coded units

[a] Significant effects ($p \leq 0.05$) are shown in bold font.

Table B.2 Analysis of variance for vitamin D_3 loading of re-assembled casein micelles

Source	**DF**[a]	**Seq SS**[b]	**Adj SS**[c]	**Adj MS**[d]	**F-value**	***p*-value**[e]
Regression	6	3950000	3950000	658000	92.7	**0.000**
Linear	3	3620000	3580000	1190000	168	**0.000**
Phosphate	1	48300	14500	14500	2.0	0.181
Citrate	1	1750000	1500000	150000	211	**0.000**
Calcium	1	1820000	1730000	1730000	244	**0.000**
Square	3	325000	325000	108000	15.3	**0.000**
Phosphate x Phosphate	1	7110	1380	1380	0.2	0.667
Citrate x Citrate	1	181000	229000	229000	32.3	**0.000**
Calcium x Calcium	1	138000	138000	138000	19.4	**0.001**
Residual error	11	78100	78100	7100		
Lack-of-Fit	6	64800	64800	10800	4.1	0.072
Pure error	5	13200	13200	2650		
Total	17	4020000				

Analysis was done using coded units

[a] Degrees of freedom

[b] Sequential sum of squares

[c] Adjusted sum of squares

[d] Adjusted mean squares

[e] Significant effects ($p \leq 0.05$) are shown in bold font.

Table B.3 Estimated regression coefficients for factors influencing vitamin A palmitate loading of re-assembled casein micelles

Factors	**Regression Coefficient**	**Standard error of regression coefficient**	**T-value**	***p*-value[a]**
Constant	643	46	14.0	**0.000**
Phosphate	-41	31	-1.4	0.198
Citrate	-52	31	-1.7	0.114
Calcium	246	31	8.1	**0.000**
Phosphate x Phosphate	-122	30	-4.1	**0.001**
Citrate x Citrate	49	30	1.7	0.122
Calcium x Calcium	39	30	1.3	0.212

$R^2 = 0.878$; R^2 (pred) = 0.665; R^2 (adj) = 0.821

Analysis was done using coded units

[a] Significant effects ($p \leq 0.05$) are shown in bold font.

Table B.4 Analysis of variance for vitamin A palmitate loading of re-assembled casein micelles

Source	**DF[a]**	**Seq SS[b]**	**Adj SS[c]**	**Adj MS[d]**	**F-value**	***p*-value[e]**
Regression	6	1190000	1190000	198000	15.6	**0.000**
Linear	3	888000	888000	296000	23.3	**0.000**
Phosphate	1	23400	23400	23400	1.8	0.198
Citrate	1	36600	36600	36600	2.9	0.114
Calcium	1	828000	828000	828000	65.1	**0.000**
Square	3	299000	299000	99700	7.8	**0.000**
Phosphate x Phosphate	1	247000	214000	214000	16.8	**0.001**
Citrate x Citrate	1	29800	34800	34800	2.7	0.122
Calcium x Calcium	1	22000	22000	22000	1.7	0.212
Residual error	13	165000	165000	12700		
Lack-of-Fit	8	123000	123000	15300	1.8	0.270
Pure error	5	42800	42800	8560		
Total	19	1350000				

Analysis was done using coded units

[a] Degrees of freedom

[b] Sequential sum of squares

[c] Adjusted sum of squares

[d] Adjusted mean squares

[e] Significant effects ($p \leq 0.05$) are shown in bold font.

Appendix C Commercial vitamin formulations in dry powder storage study and light exposure study

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

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

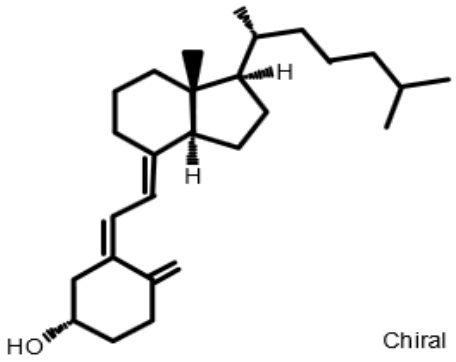
Synonyms: cholecalciferol; calciol; vitamin D_3

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

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.

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

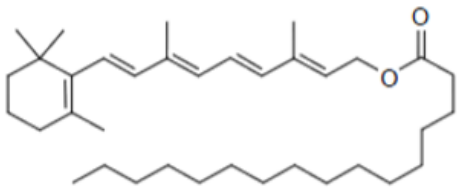
CAS No.: 79-81-2

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

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.

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 D Reaction kinetics for vitamin D_3 content of vitamin D_3 powders

Table D.1 Reaction kinetics for vitamin D_3 content of vitamin D_3 powders subjected to storage at 37°C and 75% relative humidity

	Zero order		**First order**		**Second order**	
Formulation	Regression equation	R^2	Regression equation	R^2	Regression equation	R^2
VD-rCM L[a]	y = -2.549x + 413.43	0.782	y = -0.009x + 6.035	0.851	y = 3E-05x + 0.0023	0.866
VD-rCM H[b]	y = -2.810x + 450.15	0.713	y = -0.008x + 6.108	0.813	y = 3E-05x + 0.0022	0.858
CL[c]	y = -3.178x + 314.38	0.631	y = -0.018x + 5.687	0.716	y = 0.0001x + 0.0033	0.674
CH[d]	y = -4.160x + 375.1	0.7522	y = -0.024x + 5.963	0.893	y = 0.0002x + 0.0013	0.844
CWS D_3[e]	y = -0.618x + 150.12	0.4567	y = -0.005x + 4.986	0.482	y = 3E-05x + 0.007	0.494

[a] Vitamin D_3 – re-assembled casein micelles, low phosphate

[b] Vitamin D_3 – re-assembled casein micelles, high phosphate

[c] Control powder, low phosphate

[d] Control powder, high phosphate

[e] Dry vitamin D3 100 CWS/AM from DSM nutrition

Appendix E Vitamin D_3 content of powders subjected to accelerated storage conditions as a percentage of initial vitamin D_3 concentration

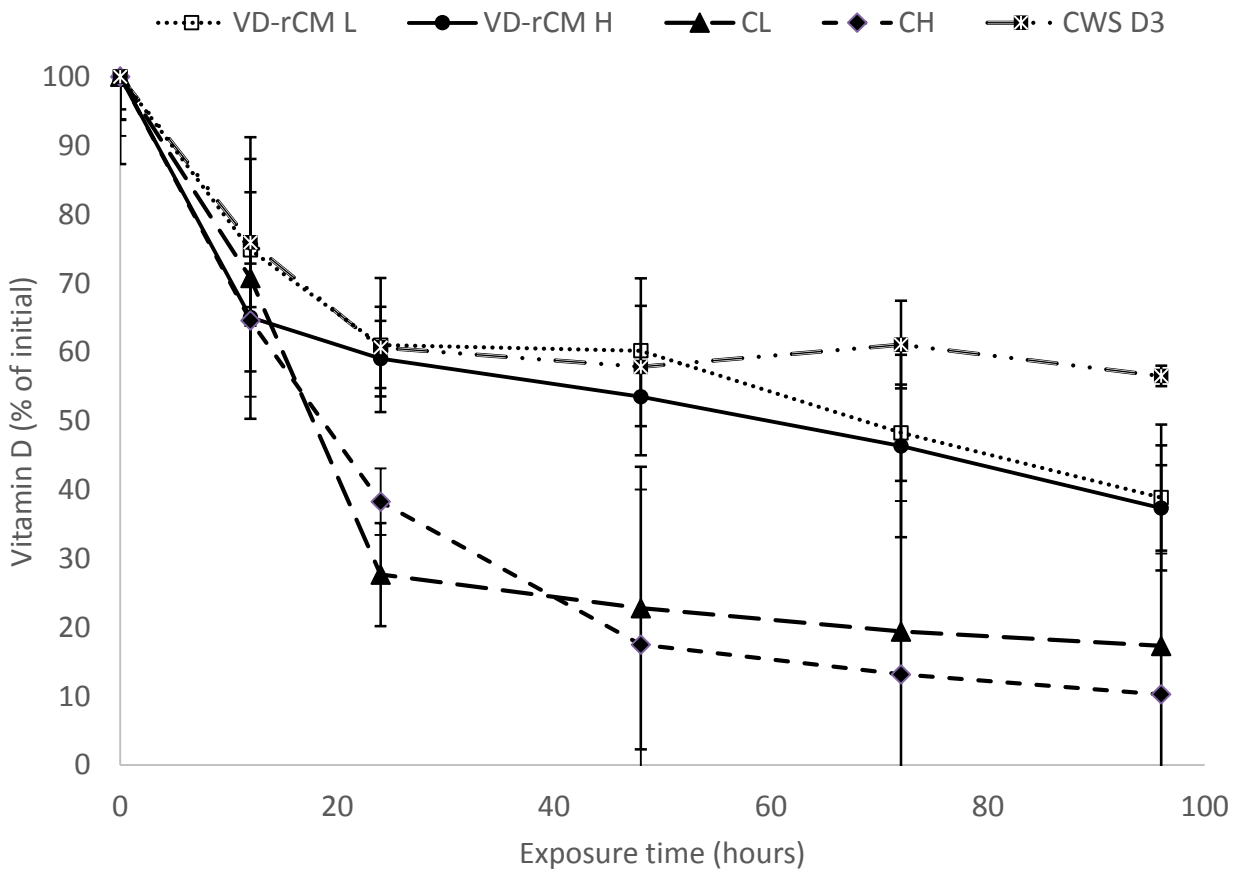


Figure E.1 Vitamin D_3 content of vitamin D_3 – re-assembled casein micelles with low phosphate (VD-rCM L), vitamin D_3 – re-assembled casein micelles with high phosphate (VD-rCM H), control with low phosphate (CL), control with high phosphate (CH), and commercial vitamin D_3 (CWS D3) powders subjected to storage at 37°C and 75% relative humidity as a percentage of initial vitamin D_3 concentration

Error bars show the relative standard deviation (n = 3).