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FORTIFICATION AND RETENTION OF VITAMIN D_3 IN CHEDDAR CHEESE, YOGURT AND ICE CREAM

by

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ABSTRACT

Fortification and Retention of Vitamin D_3 in Cheddar Cheese, Yogurt and Ice Cream.

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Investigations were conducted into the fortification and retention of vitamin D_3 in Cheddar cheese, yogurt and ice cream. Two different formulations of vitamin D_3 were used to fortify these products: a water-dispersible emulsion of vitamin D_3 (Vitex D) and crystalline vitamin D_3 dissolved in ethanol. A newly-developed extraction method followed by reverse phase High Pressure Liquid Chromatography analysis was used to assess vitamin D_3 retention in these products. Stability studies of vitamin D_3 in Cheddar cheese aged for three months, in yogurt aged for one month, and in ice cream aged for one month were made. There were no significant differences in the recovery of vitamin D_3 between the two formulations of vitamin D_3 used in any of the products ($P>0.05$). The recovery of vitamin D_3 in cheese, whey, yogurt and ice cream immediately after processing was 87-90%, 7-9%, 97-99% and 99-100%, respectively. However, retention of vitamin D_3 in cheese, yogurt and ice cream over the storage periods

was 93-100%, 100-102% and 98-99%, respectively. In conclusion, cheese, yogurt and ice cream were good vehicles for vitamin D_3 fortification, using either form.

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1. LITERATURE REVIEW

1.1 Vitamin D

Vitamin D, also known as calciferol, is a fat-soluble vitamin. It is found in some fortified foods, but also can be made in the human body via exposure to ultra violet rays from the sun. In the body, the liver and kidney help to convert vitamin D to its active hormone form (Feldman *et al.*, 1997).

The prime function of vitamin D is to maintain normal blood levels of calcium and phosphorus. Vitamin D aids in the absorption of calcium, helping to form and maintain strong bones. Deficiency of this vitamin may cause rickets in children and osteoporosis in adults, which are skeletal diseases that result in defects that weaken bones (www.vitamind.ucr.edu/milk.html). An excess of vitamin D may cause hypercalcemia, which is due to the excess absorption of calcium by the intestine and enhanced bone resorption (Collins and Norman, 1991).

There are two forms of vitamin D, namely vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). The natural form of vitamin D in humans and animals is vitamin D_3, which is produced in the body from cholesterol and 7-dehydrocholesterol. An alternative, vitamin D_2, is derived from the yeast sterol ergosterol by chemical synthesis (www.vitamind.ucr.edu/milk.html)

Foods are fortified with vitamin D to protect the population, which may be insufficiently exposed to sunlight (Thompson and Plouffe, 1993; Reichel *et al.*, 1989). In the diet, fluid milk is considered to be the major source of vitamin D. Nutritional recommendations specify 200 IU (5 μg) of vitamin D per day for infants and for adults under age 50. For those between 51-70 years age, the recommended intake is 400 IU (10 μg) of vitamin D per day; and for adults over 70 years, 600 IU (15 μg) per day is recommended (Vieth and Chem, 2001). Fluid milk is supplemented in US and Canada with vitamin D to meet these requirements.

Variations in vitamin D concentration have been reported for fortified whole and partially skimmed milk products. In 1991 and 1993, 70% of milk samples taken from Canada and the US contained ≤ 80% of the amount of vitamin D claimed, and half had less than 50% (Chen *et al.*, 1993 and Holick *et al.*, 1992). Also, 14% of the skim milk samples contained no detectable vitamin D. Tanner *et al.* (1988) reported that only 15.8% of the US samples of fluid milk had concentrations within 81 to 120% of the label claims. Handerson and Wickrowski (1978) found wide variation between the amounts found in the milk and the amount printed on the label. This discrepancy of vitamin D between claimed values and measured values in skim milk may have been due to

chemical degradation as concluded by Renken and Warthesen (1993) or to other errors during manufacturing (Faulkner *et al.*, 2000).

1.1.1 Chemistry of Vitamin D

1.1.1.1 Structure

The structures of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) and their provitamins are presented in Figure 1 and 2. Vitamin D is a generic term and indicates a molecule of the general structure shown for rings A, B, C, and D with differing side chain structures. The A, B, C, and D ring structure is derived from the cyclopentanoperhydrophenanthrene ring structure for steroids. Technically, vitamin D is classified as a seco-steroid. Seco-steroids are those in which one of the rings has been broken; in vitamin D, the 9,10 carbon-carbon bond of ring B is broken, and it is indicated by the inclusion of "9,10-seco" in the official nomenclature (www.vitamind.ucr.edu/milk.html).

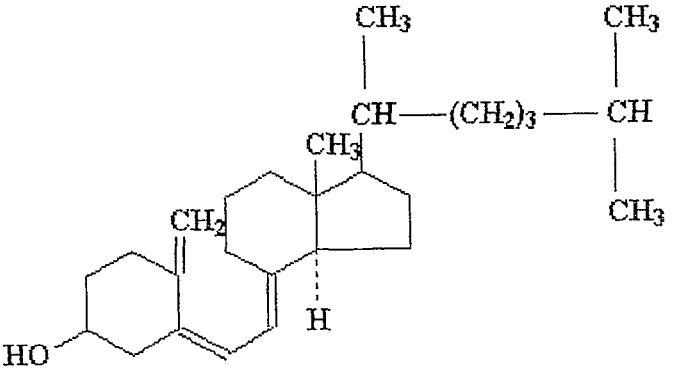

FIGURE 1: Chemical structure of vitamin D_3 (www.medicinalfoodnews.com)

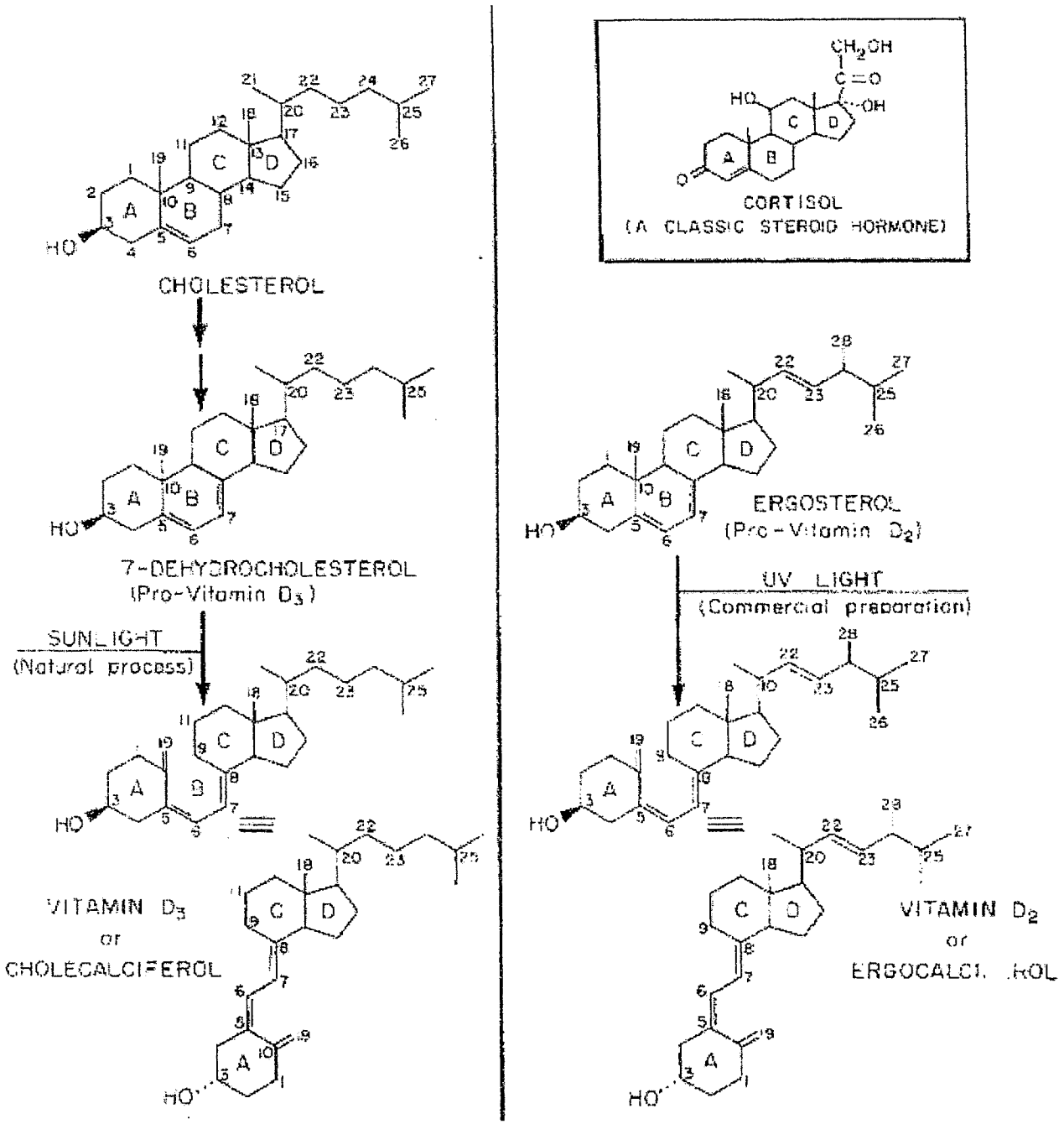

FIGURE 2: Structure of vitamin D_3 and vitamin D_2

(www.vitamind.ucr.edu/milk.html)

1.1.1.2 Nomenclature

Since vitamin D is derived from a steroid nucleus, its official nomenclature is based on the guidelines of the Revised Tentative Rules for Nomenclature of steroids prepared by the IUPAC-IUB Commission on Biochemical Nomenclature. Also, given the steroid derivation, the structure retains its numbering from the parent compound, cholesterol. Asymmetric centers are designated by using the R, S notation; the configuration of the double bonds are notated E for "entgegen" or *trans*, and Z for "zuzammen" or *cis*. Thus the official name of vitamin D_3 is 9,10-seco (5Z, 7E)-5,7,10(19) cholestatriene-3β-ol, and the official name of vitamin D_2 is 9,10-seco (5Z, 7E)-5,7,10(19), 22-ergostatetraene-3β-ol (www.vitamind.ucr.edu/milk.html).

1.1.1.3 Chemical Properties

1. Vitamin D_3 ($C_{27}H_{44}O$)
    - Three double bonds
    - Melting point, 84-85°C
    - UV absorption maximum at 264-265 nm with a molar extinction coefficient of 18,300 in alcohol or hexane, $\alpha_D 20$ + 84.8°C.
    - Molecular weight, 384.65
    - Insoluble in H_2O.

- Soluble in benzene, chloroform, ethanol, and acetone.
- Stable to heat, acid, and alkali.
- Unstable in light.
- Will undergo oxidation if exposed to air at 24°C for 72 hrs.
- Best stored at 0°C.

2. Vitamin D_2 ($C_{28}H_{44}O$)
    - Four double bonds.
    - Melting point, 121°C.
    - UV absorption maximum at 265 nm, $\alpha_D 20 + 106°C$.
    - Same solubility and stability properties as D_3.

1.1.2 Health benefits of vitamin D

Vitamin D helps in the absorption and utilization of calcium and phosphorus. Vitamin D is necessary for normal growth in children. In adults, it helps with any function that utilizes calcium or phosphorus, such as transmission of nerves, the beating of the heart, blood clotting, and many others (Feldman *et al.*, 1997)

In children, vitamin D will insure that the body forms healthy teeth and bones. Postmenopausal women are already advised to take vitamin D and calcium to combat bone-thinning osteoporosis (Vieth and Chem, 2001). Other benefits of vitamin D supplementation include: prevention of certain cancers,

osteoarthritis progression, multiple sclerosis and hypertension. Animal studies show that Vitamin D is important for immune function. There is also evidence that vitamin D may not just help prevent cancer but may help in tumor regression. Additionally, vitamin D deficiency has been associated with an increased risk for type 1 diabetes. The converse is also true - adequate vitamin D lowers diabetes.

1.1.3 Canadian legislation

The Canadian Food and Drug Act mandates that all fluid milk sold should contain vitamin D_3 in such an amount that a reasonable daily intake of milk contains not less than 300 and not more than 400 IU of the vitamin. The Canadian Food Inspection Agency (CFIA) has simplified the regulation by requiring all fortified milk to contain between 31.7 to 51.6 IU of vitamin D per 100 ml. This requirement, which has been in place for decades, has resulted in a dramatic reduction of disorders caused by deficiency of this vitamin (Faulkner *et al.*, 2000).

Currently, milk-processing plants rely on the manufacturer's information and recommendations for implementing their fortification processes with no adequate monitoring of the final products. The Health Protection Branch of Health and Welfare Canada has undertaken periodic sampling and testing for vitamin D nationally. However, there is presently no systematic monitoring of

this vitamin by CFIA in the Ontario retail milk supply. Dairy processors voluntarily have some samples tested for vitamin A and then use the result of vitamin A to extrapolate vitamin D content in skimmed and partially skimmed milk. This is theoretically possible because the premix used for the fortification of skimmed and partially skimmed milk contains vitamin A and D, but without actual analysis, it is difficult to monitor vitamin losses during processing.

1.2 Background information on dairy products

Dairy products are traditionally classified into two broad product groups: i) *fluid or market milk products* and *ii) manufactured milk products.*

Some of the more common foods which are normally considered fluid milk products include whole milk, 2% milk, low-fat milk, skim milk, chocolate milk, egg nog, half and half cream, and cultured butter milk. These products may be fortified with vitamins or minerals. Processing conditions may be changed to produce pasteurized, homogenized, or other types of special products (Harper and Hall, 1976).

Manufactured milk products include most of the non-beverage products, such as cheese, yogurt, ice cream and frozen desserts, as well as condensed, evaporated, and/or dried milks.

1.2.1 Processing of dairy products

The general methods of processing cheese, yogurt and ice cream will be discussed here, with some details on fortification with vitamin D.

1.2.1.1 Cheese

Cheese is the most common processed dairy product. Milk from various animals, particularly cattle, buffalo, goat, and sheep is used to make cheese (www.cip.ukcentre.com/cheese4html). Cheese is the generic name for a group of fermented milk-based food products produced throughout the world in a great diversity of flavors, textures, and forms. Despite the limited range of raw materials (bovine, ovine, caprine, or buffalo milk), there are ~500 varieties of cheese recognized by the International Dairy Federation (Bukhalter, 1981).

1.2.1.1.1 Cheese production

The production of all varieties of cheese involves a generally similar protocol as depicted in Figure 3.

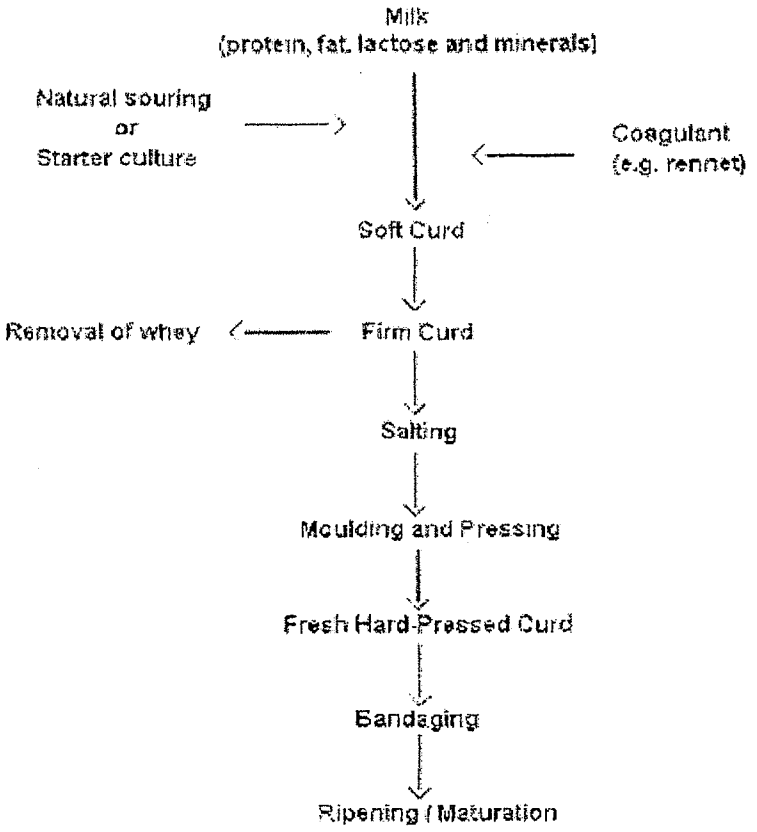

FIGURE 3: General protocol for cheese manufacturing

(www.efr.hw.ac.uk/SDA/cheese2.html)

Cheese is made by coagulating milk, cream, skim milk, buttermilk, or a combination of these and then draining part or most of the whey. Processing into cheese serves to concentrate the protein and fat while reducing the carbohydrate content. Pasteurized milk is used in most, but not all, cases. Use of milk concentrated by ultrafiltration is also used (Sharma *et al.*, 1989). Ultrafiltration is a high-pressure filtration process that results in the removal of water and low-molecular weight solutes. The resulting retentate coagulates

more rapidly than regular milk and the formed curd is also firmer than with regular milk (Kosikowski, 1982).

1) Coagulation

Milk used for cheese production may be clotted with rennin or acid or both. A bacterial culture is added for acid production. Formerly, the clotting of milk was achieved by using rennin (the calf gastric enzyme chymosin). Rennin usually is used as a crude extract, rennet. Rennin acts most effectively at pH 6.7 (Bingham, 1971) and a temperature of about 40°C. Enzymatic coagulation of milk involves two phases. Rennin is involved in the first phase, in which a bond of κ-casein is cleaved to form insoluble para-κ-casein and a soluble peptide (Bingham, 1971). In the second phase, a clot is formed by the para-κ-casein and calcium (Cheryan *et al.*, 1976). Coagulation time decreases as the pH of the second phase is decreased from 6.7 to 5.6 and as the temperature increases (Cheryan *et al.*, 1976b). Heating milk above 65°C and then cooling it prior to treatment with rennin reduces clotting rate and curd firmness. A heat-induced interaction between κ-casein and β-lactoglobulin may delay the action of rennin on κ-casein (Sawyer, 1969).

Rennet is used from three animal sources (veal calf, adult bovine, and porcine pepsin) and three microbial sources (*Mucor miehei, Mucor pusillus*, and *Endothia parasitica*). Use of enzymes from different sources has necessitated

alterations in processing techniques and in some cases resulted in cheese that is pasty and bitter. More encouraging results may be possible for microbial enzymes because they contain nonspecific proteases, which enhance opportunity for formation of a better flavor (Peczczola, 1988).

2) Curd treatment

Following coagulation, the curd is cut to allow drainage of the whey. Cutting the curd into various-sized pieces controls this process. The curd is heated at low temperatures to hasten the loss of whey and produce a more compact texture. Salts, acids and bacterial cultures are added at various steps in the process. The curd then is pressed to make the cheese firmer and more compact. At that stage, variations in treatments lead to diversity in the kinds of cheese available.

Cheese may be classified on the basis of their moisture content. For example, hard cheeses, such as Cheddar, contain 30-40% moisture whereas semi soft cheeses, such as Muenster, contain 50-75% moisture (Fox *et al.*, 2000)

3) Curing

Cheese may be cured for varying length of time and at varying temperature and humidity in order to allow the product to ripen (Fox *et al.*, 2000). The chemical and physical changes associated with ripening result in a

change from a bland, tough, rubbery mass to a full-flavored, soft, mellow product. Salt level is related to control of ripening. Omission of salt speeds up the ripening process, resulting in a pasty texture and the development of an unnatural, bitter, fruity, or flat flavor in Cheddar cheese. These effects are attributed to increased bacterial activity in the unsalted cheese. During ripening, carbohydrate, fat, and protein contents are altered. Lactose is rapidly converted to lactic acid, which helps to inhibit the growth of undesirable microorganisms (Fox *et al.*, 2000). Lipolytic enzymes liberate fatty acids, which contribute to the flavor. The typical flavor of Cheddar cheese is not developed when skim milk is used, suggesting an important role for fat in flavor (Ohren and Tuckey, 1969). Proteolytic enzymes such as rennin are responsible for the formation of nitrogenous products of intermediate size, such as peptones and peptides. Enzymes act on these and other substances to form amino acids, amines, fatty acids, esters, aldehydes, alcohols, and ketones, in part responsible for the characteristic sensory attributes of cheese (Creamer and Olson, 1982).

1.2.1.1.2 Processing of cheese on industrial scale

The manufacture of cheese has become a major activity in many dairy-processing plants. It has been one of the last branches of the dairy industry to become well mechanized. In fact, even today, there is no real continuous process used commercially for the various types of cheese, although there are several

new and interesting developments, which are employing automation to a certain extent.

1.2.1.1.2.1 Cheese vat

A vat consists of a metal outer box with an inner metal tank so placed that the bottom of the inner tank is supported on a second tank, allowing a space for water between the two tanks. Note that an H-shaped steam pipe lies in the bottom of the outer tank to distribute heat evenly to the inner tank. The inner tank, or vat pan, is usually made of stainless steel with welded-type construction. Cheese vat are normally made in sizes with a 400 to 6000 liters capacity.

1.2.1.1.2.2 Cheese curd mill

The curd mill is used for cutting the curd to the proper size for finishing in the manufacture of Cheddar and some other types of cheese. This machine is made to fit on the top of the cheese vat, is mounted on a special dolly so that it can be readily moved so that the cheese can be cut to different degrees of fineness. The standard mill cut is a 1.5 cm x 1.5 cm.

1.2.1.1.2.3 Agitator

A metal cheese vat with an automatic, forking type agitator is used commercially to cool the curd after cutting. It is motor-driven and travels on a track above the vat so that it passes back and forth from one end of the vat to the other, or between the limits of adjustable stops.

1.2.1.1.2.4 Cheese press

There are different types of cheese press used commercially, but the most common is hydraulic cheese press. The cheese is put in hoops that are placed in a press to which pressure is applied. Hoops are usually from 30 to 40 cm in diameter.

1.2.1.2 Yogurt

Yogurt is an acidified, coagulated product obtained from milk by fermentation with lactic acid-producing bacteria. It is a perishable cultured dairy product that normally has a shelf life of up to 6 weeks at 4°C. The storage life of yogurt depends on the sanitary conditions maintained during processing and packaging.

Flavour, aroma and texture of yogurt vary from country to country, and these depend on its origin as well as other factors including raw material formulation and manufacture process. In some areas, yogurt is produced in the form of a highly viscous liquid, whereas in other countries it takes the form of a softer gel. Yogurt is also produced in drinking form and can be frozen or blended with other ingredients (e.g., mousse type products, sorbets, yogurt ice cream) (Early, 1998). The popularity of yogurt has increased significantly in recent years, especially in the Western world.

1.2.1.2.1 Manufacturing of yogurt

Whole milk, skimmed milk, partially skimmed milk may be used. In order to ensure the development of the yogurt culture, the following standards for the raw milk must be considered:

- Low bacterial count
- Free from antibiotics, sanitizing chemicals, mastitis milk, colostrums, and rancid milk.
- No contamination by bacteriophages.

Other yogurt ingredients may include concentrated skim milk, non-fat dry milk, whey, lactose (to increase the non-fat solids), sweeteners, stabilizers, flavors and colors (www.foodsci.uoguelph.ca/dairyedu).

The starter culture for most yogurts in North America is a symbiotic blend of *Streptococcus thermophilus* (ST) and *Lactobacillus bulgaricus* (LB). These cultures are used together in order to hasten the rate of acid production. ST grows faster and produces acid and carbon dioxide, which stimulates LB growth. On the other hand, LB produces peptides and amino acid for use by ST. These microorganisms are ultimately responsible for the formation of flavor and texture. The yogurt mixture coagulates during fermentation due to the reduction in pH. The ST is responsible for the initial pH drop of the yogurt mixture to ~5.0; further decrease in the pH to 4.3 is due to the LB (Tamime and Robinson, 1999).

The essential steps in the manufacture of fresh stirred and set yogurt are outlined in Figure 4.

During yogurt manufacture, milk is clarified and separated into cream and skim milk, then standardized to achieve the desired fat content. The various ingredients are then blended together in a mix tank equipped with a powder funnel and an agitation system. The mixture is then pasteurized, typically with a continuous plate heat exchanger for 30 minutes at 85°C. The heat treatment, which is much more severe than fluid milk pasteurization, is necessary to achieve the following:

- Produce a relatively sterile and conducive environment for the starter culture.
- Denature and coagulate whey proteins to enhance the viscosity and texture and prevent rapid wheying off.

The milk is then homogenized. Besides thoroughly mixing the stabilizers and other ingredients, homogenization also helps to prevent creaming and wheying off during incubation and storage. Stability, consistency and body are enhanced by homogenization. Once the homogenized mix has cooled to an optimum growth temperature, the yogurt starter culture is added (www.foodsci.uoguelph.ca/dairyedu).

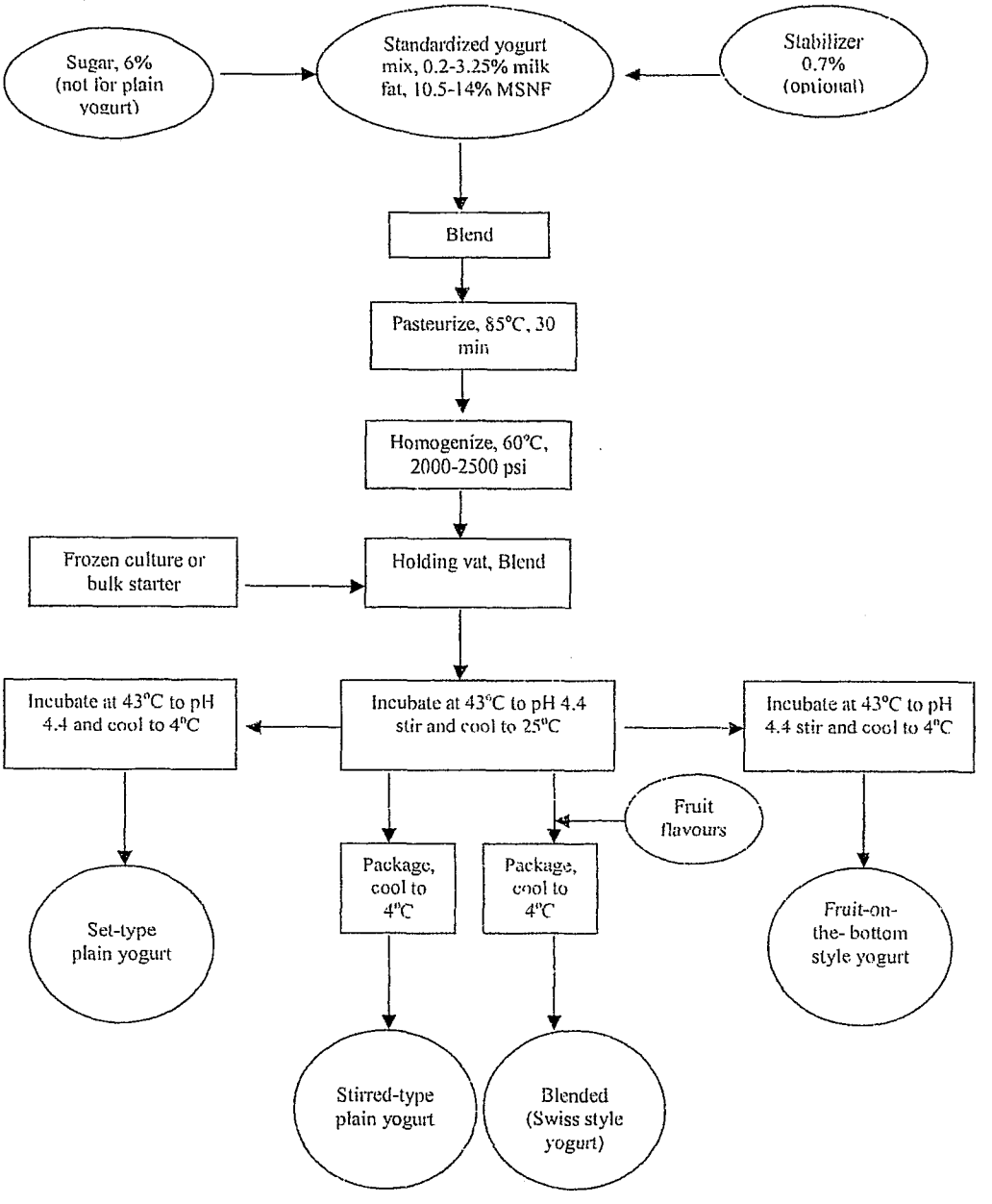

FIGURE 4: Steps in yogurt manufacturing (Chandan, 1997)

MSNF= milk solids-non-fat

Afterwards, the inoculate is added at a 1:1 ratio of ST to LB to a jacketed fermentation tank. A temperature of 43°C is maintained for 4-6 hours under quiescent conditions. This temperature is a compromise between the optimal growth temperatures of the two bacteria (ST @ 39°C vs LB @ 45°C). The titrable acidity is closely monitored until it reaches to 0.85 to 0.90%. At this stage, the hot water jacket is replaced with cool water and agitation begins, both of which stops the fermentation. The coagulated product is cooled to 4-25°C, depending on the type of product. Fruit and flavors may be added at this time, and then packaged. The product is now cooled and stored at refrigerated temperature (4°C) to slow down physical, chemical and microbiological degradation (www.foodsci.uoguelph.ca/dairyedu).

The above description is the manufacturing procedure for stirred type yogurt. In set style, the yogurt is packaged immediately after inoculation with the starter and is incubated inside the container.

1.2.1.2.2 Yogurt manufacture on industrial scale

On an industrial scale, the equipment employed for the manufacture of yogurt is specially designed to handle thousands of liters per day and a highly sophisticated technology has evolved offering a dairy plant both improved mechanization and automation.

There are several approaches that can be employed for the production of yogurt and, each yogurt manufacturer has his own specific requirements.

1.2.1.2.2.1 Hand Operated Vat

In some parts of the world, equipment manufacturers may produce specially designed processing vessels where the agitation of the milk base during heating and cooling is done manually. The different steps involved during the production of yogurt can be summarized as follows:

- Pour the milk into the vat, add the required amount of dry ingredients and mix with the aid of stainless steel wire whisk.
- Start the heating cycle using an electric element to heat the insulated water jacket.
- After reaching the pasteurization temperature, the heating element is switched off and the milk is held for 10-30 minutes, prior to cooling.
- During cooling, the water in the jacket is replaced by circulating mains water. At 40-45°C the milk is inoculated with starter culture and left undisturbed during the fermentation period.
- After a few hours, or at the desired acidity, mains water is circulated through the jacket to cool the coagulum, a process that may be assisted by gentle agitation.

- At 15-20°C, a known volume of yogurt is drained out and mixed with fruit/flavoring additives and packaged into plastic cups.

1.2.1.2.2.2 Multipurpose vat

This type of vat is really a batch pasteurizer, which is slightly modified to meet the requirements of yogurt manufacture, and it is widely used for the production of viscous yogurt. These vats are usually made of stainless steel and insulated with a water jacket. When this type of vat is used, it usually follows two alternative patterns. In the first approach, the vat is utilized for all the necessary steps required for the production of yogurt. However, in the second approach the vat is merely used for the preparation of milk, that is, mixing of all the dried ingredients with milk, heat treatment, fermentation of milk with lactic acid producing bacteria and cooling to incubation temperature.

The multipurpose vat can be heated using various sources of energy (e.g. electrical, steam, or gas) and this type of versatility makes this type of processing equipment very popular with manufacturers. For cooling, mains water is used or a closed circuit cooling system circulating chilled water is employed. However, if in-tank cooling is used for cooling the yogurt, a slow moving agitator is operated to mix the coagulum gently and assist cooling.

1.2.1.3 Ice cream

Ice cream is a frozen mixture of milk, cream, sweeteners, stabilizers, emulsifiers and flavoring. Other ingredients such as egg products, colorings and starch hydrolysates may also be added. This mixture called the "mix", is pasteurized and homogenized before freezing. Freezing involves rapid removal of heat while agitating vigorously to incorporate air, thus imparting the desired smoothness and softness of the frozen product (Marshall and Arbuckle, 1996).

Ice cream is defined by government standards as containing no less than 10% (w/w) milk fat and as high as 16% in some premium ice cream brands, between 9-12% milk-solids-non-fat, which contains proteins (caseins and whey proteins) and carbohydrate (lactose) found in milk. Sweeteners, usually a combination of sucrose and glucose, based on corn syrup sweeteners, stabilizers and emulsifiers represent 12-16% of the final weight (www.foodsci.uoguelph.ca/dairyedu/icform.html).

1.2.1.3.1 Ice cream manufacture

Ice cream processing operations can be divided into two stages - mix manufacture and freezing operations, as shown in Figure 5. Ingredients are chosen on the basis of desired quality, availability and cost. The ingredients are blended together prior to pasteurization (Goff, 1997).

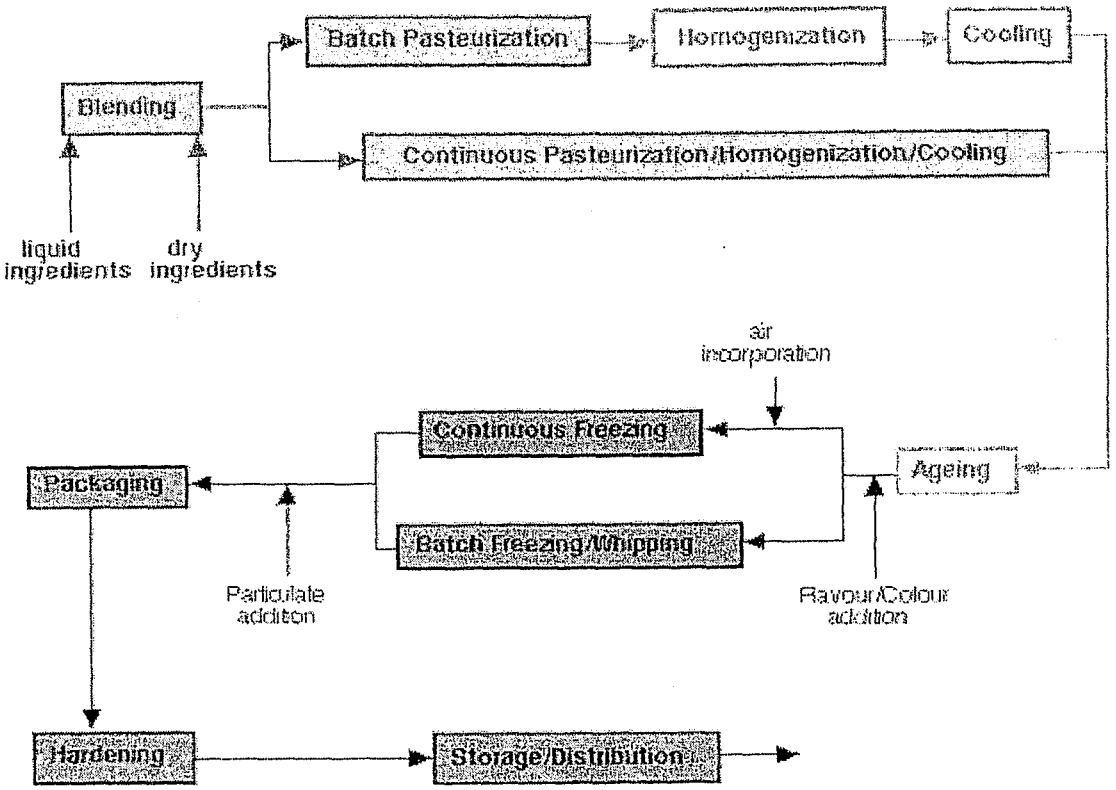

FIGURE 5: Process flow diagram for ice cream manufacturing (Goff, 1997)

Ice cream manufacture consists of several unit operations: combination and blending of ingredients, batch or continuous pasteurization, homogenization and ageing of mix. The mix is first pasteurized, so as to kill pathogens that may be present, namely; *Mycobacterium tuberculosis, Salmonella, Staphylococcus aureus, Listeria monocytogenes,* and others that cause human disease. In addition, the role of pasteurization in colloid development is to melt the fat for proper homogenization (Marshall and Arbuckle, 1996; and Goff, 1997).

The mix is then homogenized, which forms the fat emulsion by breaking down or reducing the size of the fat globules found in milk or cream to less than 1 μm. Homogenization is responsible for emulsion formation by forcing hot mix through a small orifice under moderate pressures, depending on mix composition. In milk containing 3.5% fat, homogenization reduces the average diameter of fat globule from 3.3 to 0.4 μm, decreases the maximum diameter from 10.0 to 2.0 μm, increases surface area from 0.08 to 0.75 m^2/ml, and increases the number of globules from 0.015 to 12/μm^3 (Marshall and Arbuckle, 1996). The actual mechanism of fat disruption during homogenization is due to turbulence, cavitations and the velocity gradients created (Pandolfe, 1982). Homogenization also helps to produce a smooth product when frozen.

After homogenization, ageing for about 4 hours at 2-4°C allows fat to cool down and crystallize, and for the protein and stabilizers to fully hydrate (some

viscosity increase occurs during the ageing period), and to produce a smoother and better quality product (Iversen and Pedersen, 1982). The whipping qualities of the mix are also improved with ageing.

Following mix processing, the mix is drawn into a mixing tank where any liquid flavors, fruit purees, or colors are added. The mix then enters into a dynamic freezing process, where a portion of the water freezes. The barrel freezer is a scraped-surface, tubular heat exchanger, which is jacketed with a refrigerant such as ammonia or freon. Mix is pumped through this freezer and is drawn off the other end in a matter of 30 seconds, (or 10 to 15 minutes in the case of batch freezers) with ~50% of its water frozen. There are rotating blades inside the barrel that keep the ice scraped off the surface of the freezer and as well as dashers that assist in whipping the mix and with air incorporation. Ice cream contains a considerable amount of air, up to half of its volume (called overrun), which provides the characteristic lightness of the product. As the ice cream is drawn, particulate matter (fruits, nuts, candy, etc.) is added to the semi-frozen slurry. Soft serve ice cream is drawn into cones at this stage, while hard serve ice cream is packaged and placed into a blast freezer at -20°C to -30°C for subsequent hardening (where most of the remaining water freezes). Below about -25°C, ice cream is stable for an indefinite period of time without danger of ice

crystal formation. However, above this temperature; ice crystal growth is possible with the rate of crystal growth depending on storage temperature.

1.2.1.3.2 Ice cream making on industrial scale

A typical modern ice cream operation consists of a two-tank blending system and a dry-ingredient blender to combine liquid and dry mix ingredients. Desired amounts of dairy ingredients including milk, cream, condensed milk, skim or whole milk can be combined with sweeteners by volume through the use of meters in the supply lines. Stabilizers, emulsifiers, and small amounts of other ingredients, such as whey solid, may then be introduced through the dry ingredient blender. Adequate soak time must be provided to ensure complete hydration and blending of the stabilizers, which usually takes 10 to 20 minutes. The mix is then pumped to the pasteurizer, where it is pasteurized at 80°C for 25 sec. The mix is then transferred to the mix storage tank for ageing prior to flavoring and freezing.

Aged mix is pumped from the storage tanks to the flavor tanks, where coloring and flavoring ingredients are incorporated. The flavor tanks are equipped with positive displacement pumps used as meters to transfer controlled quantities of mix to the tank. After flavoring, the mix is pumped to ice cream freezers and the frozen ice cream is discharged to the fillers. Large ice

cream operations use continuous freezers, whereas some small operations and soft-serve ice cream makers use batch freezers.

After freezing and packaging, ice cream is transferred to a hardening room to complete the freezing operation. The hardening room is maintained at -25 to -30°C. A number of mechanized and automated hardening systems have been utilized to some degree in the industry.

2. VITAMIN D AND PROCESSING

Interest in fortification of foods with vitamin D has received a great deal of attention because of the role of vitamin D in the prevention of rickets, osteoporosis and osteomalacia. This has led to marketing of a number of vitamin D-supplemented foods, including fluid milk, hard and soft margarine, infant formula and recently, yogurt. Consumption of total dairy products in 2002 in Canada was similar to that in 1992, although shifts within this commodity group were apparent, including decreased consumption of fluid milk and increased consumption of cheese and other dairy products. While consumption of fluid milk decreased by 9%, consumption of both skim and 1% milk increased; 1% milk increased by 67% from 1992 to 2002; skim milk consumption increased by 40%, from almost 5 liters per person in 1992 to almost 7 liters per person in 2002. Consumption of 2% milk decreased by 26% from 40 liters per person in 1992 to 30 liters per person in 2002. Consumption of standard (whole) milk decreased by 28%, from 14 liters per person in 1992 to 10 liters per person in 2002. On the other hand, consumption of cheese increased by 5%. A 20% increase in consumption of 'variety cheese', from 4 kg per person in 1992 to almost 5 kg per person in 2002 was noted. Consumption of other dairy products increased by 18%. Among other dairy products, consumption of yogurt increased by 81%

from 1992 to 2002 (http://www.hc-sc.gc.ca/hpfb-dgpsa/onpp-bppn/review_food_supply_e.pdf).

Given the shift in consumption patterns, foods other than fluid milk will need to be fortified with vitamin D. It is the purpose of this research work to develop ways of incorporating vitamin D to dairy products other than fluid milk, namely Cheddar cheese, yogurt and ice cream. These products do not normally contain vitamin D, although vitamin D-fortified yogurt has recently appeared on the Canadian market.

2.1 How does one incorporate vitamin D in these products?

2.1.1 Cheese

Banville *et al.* (2000) presented a study on the fortification of vitamin D_3 in Cheddar cheese, using different methods of fortification. They entrapped liposoluble or hydro-soluble vitamin D_3 preparations in milk fat or liposomes, respectively, before cheesemaking, and compared this with an emulsified water-soluble vitamin D_3 premix (Vitex D). The composition of the cheese fortified with vitamin D_3 was compared with an unfortified controlled cheese. Vitamin D_3 retention and stability was also measured in cheese curd for seven months. Preliminary results indicated that vitamin D_3 was stable after 3-5 months of ripening. Approximately 40-60% of vitamin D_3 was recovered in the cheese

depending on the fortification method. Lost portions of vitamin D_3 were not found in the whey fraction as determined by HPLC; the loss was attributed to different factors including fermentation by lactic acid bacteria, acidification or oxidation that may be involved in destabilizing the vitamin during cheese making.

Upreti *et al.* (2002) fortified pasteurized process cheese with vitamin D_3. Results of this study showed that there was no loss of vitamin D_3 during cheese manufacture, and that the vitamin was uniformly distributed within the product. No losses occurred during storage of the fortified cheeses over a 9-month period at 21 to 29°C and 4 to 6°C. There was a ~25-30% loss of the vitamin when cheeses were heated in an oven at 232°C. There were no differences between the water and fat-dispersible forms of the vitamin.

2.1.2 Yogurt

No research is currently available on the fortification of yogurt with vitamin D_3. Fortification of yogurt by vitamins is targeted at children and has been marketed in some countries, including Canada. The stability of vitamin A and C in yogurt was evaluated by Ilic and Ashoor (1988). The concentration of both vitamins decreased during storage. This effect was minimized by using water-miscible beadlets of β-carotene (Parker, 1996).

The relative availability of vitamins in yogurt is much more difficult to assess because, unlike minerals, many vitamins are sensitive to the conditions of processing. Thus, the method of fortification, for example, the addition of milk powder or membrane processing, the heat treatment of the milk base, the strains of starter bacteria used and the conditions of fermentation can all alter the concentrations of the more important vitamins (Noh *et al.*, 1994). The fortification of yogurt with vitamins such as vitamin A and C is possible and losses over the period of two weeks storage are ~50% (Tamime and Robinson, 1999).

Some relevant aspects of the vitamin content of yogurt have been reported by Rao and Shahani (1987). It is interesting to note that certain B group vitamins are synthesized by the starter cultures. Kneifel *et al.* (1989) monitored these vitamins in yogurt during fermentation and observed that by using short (i.e. 3-4 hours) incubation at 42°C, the starter culture enriched the vitamins during fermentation by more than 20%.

In the fortification of yogurt with vitamin D_3, vitamin D_3 may be added to the fluid milk prior to pasteurization and prepared as outlined in Figure 6.

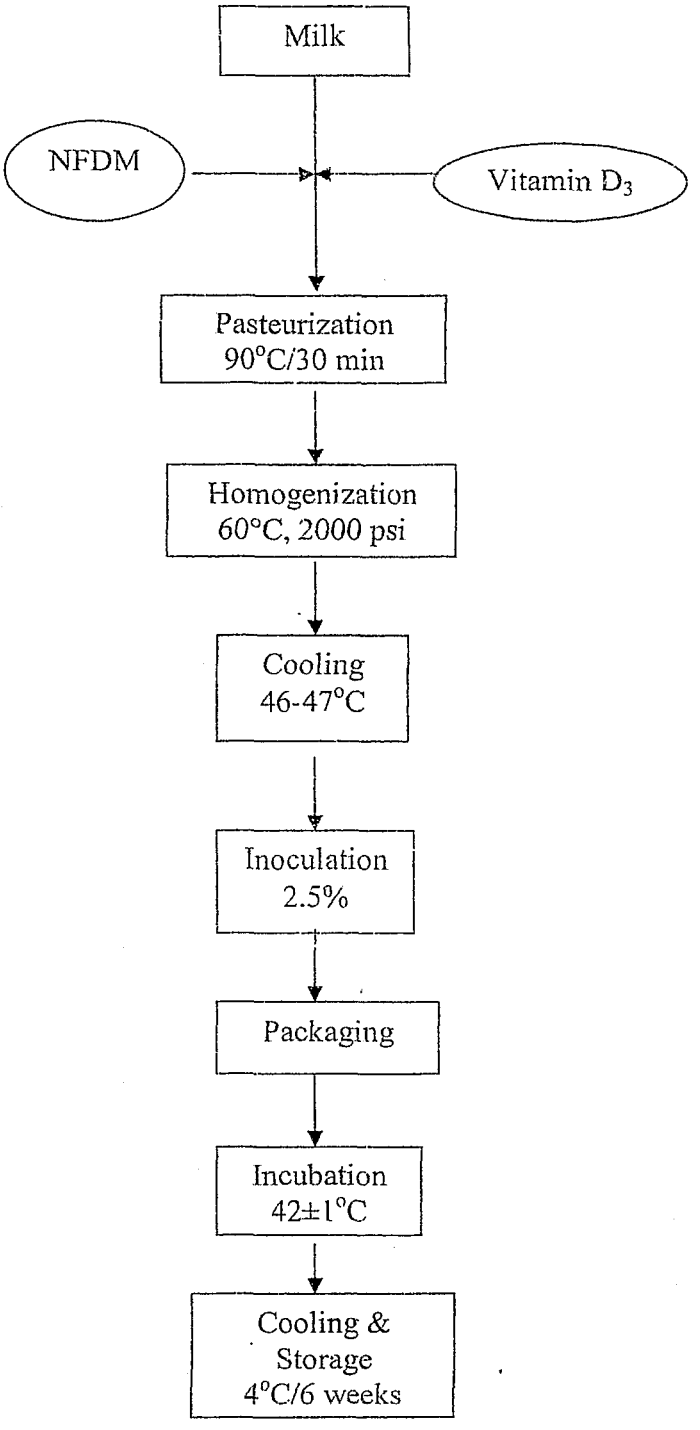

FIGURE 6: Proposed flow diagram for preparation of the fortified yogurt with Vitamin D_3

2.1.3 Ice cream

Like milk, ice cream is an important source of several vitamins, the content depending primarily on the amount of milk solids and the weight of a serving. The fat-soluble vitamins, A, D, E, and K, are contained in the fat and are absent in unfortified nonfat products. However, no research has been performed regarding the fortification of ice cream with vitamin D_3 and no such ice cream product is available on the Canadian market.

3. STABILITY OF VITAMIN D

There is a question whether vitamin D variability reported in the literature is due to inconsistencies in fortification or to instability of the vitamin itself. Early studies on its stability in water and milk with a biological assay were reported by Supplee *et al.* (1936). They reported that propylene glycol solutions of vitamin D were unstable when diluted with water, but stable when diluted with milk. More studies on the stability of propylene glycol solutions diluted with milk were reported by Huber and Barlow (1943). Using bioassays, they found no evidence of vitamin D deterioration in milk samples held for 8 days at refrigerated condition. In canned condensed milk stored for 6 months at 40°C or 15 months at room temperature (~23°C), Renken and Warthesen (1993) found that vitamin D was stable.

Cremin and Power (1985) showed that vitamin D was unstable to oxidation, light, and acid. Kutsky (1981) reported that vitamin D was unstable to oxidation and light, but stable to acid and alkali. Pike and Brown (1984) reported that vitamin D was unstable to irradiation and acid while remaining stable to oxidation and alkali. Kreutler (1980) reported that vitamin D was remarkably stable and that exposure to light, heat and oxygen.

Although information on the stability of vitamin D in foods is quite limited, the general assumption is that its stability is high (Kilcast, 1994).

However, individual studies have provided quite contradictory data and it appears that the stability of this vitamin is strongly dependent on the processing techniques used and the environmental conditions (Matilla *et al.*, 1999). In addition, various research groups have reached different conclusions regarding the stability of vitamin D in foods. This is perhaps due to inconsistent, and in some cases, poorly-controlled analytical methods.

Mawer and Gomes (1994) determined the contents of vitamin D and its metabolites in raw and cooked meat and found it to be very stable during cooking. On the other hand, Bennink and Ono (1982) reported 35-40% losses during cooking. No losses of vitamin D were found during pasteurization and sterilization of milk or during production of dried and evaporated milk (Davidek *et al.*, 1990). Furthermore, exposure to air did not affect the stability of vitamin D in milk, but some losses occurred when the milk was exposed to light (Renken and Warthesen, 1993). The loss of vitamin D could be explained by oxidation reactions in which light acted as catalyst.

4. OBJECTIVES AND HYPOTHESES

The objectives of the present study are:

a) To fortify Cheddar cheese, yogurt and ice cream with vitamin D_3.

b) To develop reproducible methods for the estimation and fortification of vitamin D_3 in Cheddar cheese, yogurt and ice cream.

c) To determine the retention of vitamin D_3 in these products during and after processing.

d) To examine the stability and shelf life of vitamin D_3 in these products after fortification.

The hypotheses for the research study are as follows:

Since vitamin D_3 is liposoluble, fortification should be straightforward in high fat products such as Cheddar cheese (33-35% milk fat) and ice cream (10-16% milk fat). On the other hand, in plain low-fat yogurt, fortification may be hampered. As vitamin D_3 is quite stable in fats, it is inferred that it will show very minor or no loss over the certain period of storage time in Cheddar cheese and ice cream compared to the yogurt. However, it is possible that the effects of time, temperature and pH may provoke degradation of vitamin D_3 and these effects must be characterized.

5. MATERIALS AND METHODS

5.1 Materials

Unfortified, pasteurized and homogenized milk was obtained from Parmalat Canada (London, Ontario). Crystalline vitamin D_3 (cholecalciferol, Sigma Chemicals, Saint Louis, MO, USA) was provided by Dr. R. Vieth at Mt. Sinai Hospital. A water-soluble emulsion of vitamin D_3 containing propylene glycol and polysorbate 80 (Vitex D, 205,000 IU/ml $\approx$ 5125 μg/ml) was provided by Kingsway Chocolate Ltd (Mississauga, Ontario, Canada). Rennet (from *Mucor miehei*) was purchased from a local distributor (Sigma Chemicals, St. Louis, Missouri, USA). The mesophilic culture for cheese making was made in the laboratory (cultured butter milk was ripened for 8 hours at room temperature). Yogurt with active cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) was purchased from the local grocery store to use as a starter culture for yogurt manufacture. Vanilla extract and whipped cream were also purchased from the grocery store. Potassium hydroxide was obtained from Fisher Scientific (Fisher Scientific, Fair Lawn, New Jersey, USA). All chemical reagents of HPLC grade were used for extraction and chromatography (Fisher Scientific, Fair Lawn, New Jersey).

5.1.1 Vitamin D_3 stock solution

Crystalline vitamin D_3 (100 mg) was dissolved in 10 ml ethanol to yield a stock solution of 400,000 IU/ml (0.026 mmoles of vitamin D_3 per ml)

5.1.2 Fortification of milk with crystalline vitamin D_3

One (1) ml of stock solution was homogenized with 3 ml of melted butter to yield a final concentration of 100,000 IU/ml or 6500 nmol/ml. Unfortified milk was then heated to 50°C in a water bath. The fortified butter emulsion was added to the milk to get the desired concentration of vitamin D required for each product.

5.1.3 Fortification of milk with Vitex D

Vitex D is a water-soluble emulsion of vitamin D_3. Two (2) ml of Vitex D were dispersed in 2 ml of butter to get the final concentration of 100,000 IU/ml or 6500 nmol/ml. It was then homogenized for 5 minutes. Unfortified milk was heated to 50°C in a water bath. The fortified butter emulsion was incorporated into the milk to reach the desired concentration of milk required for each product.

5.2 Manufacturing techniques

Cheese, yogurt and ice cream were processed on bench top equipment in the food science laboratory at Ryerson University.

5.2.1 Cheddar cheese

5.2.1.1 Preparation of vitamin D_3-supplemented milks for cheese

5.2.1.1.1 With crystalline vitamin D_3

Unfortified pasteurized and homogenized milk (2 kg) at 50°C was enriched with 2 ml of crystalline vitamin D_3 butter emulsion to give a final concentration of 100,000 IU/kg or 6500 nmoles/kg. Fortified milk was then stirred and homogenized on a lab scale homogenizer for five minutes to distribute the vitamin D_3 uniformly throughout the milk. The milk was refrigerated overnight after drawing samples for vitamin D_3 analysis.

5.2.1.1.2 With water-soluble emulsion of vitamin D_3 (Vitex D)

Milk was heated to 50°C on a hot plate. One (1) ml of fortified butter emulsion with a water-soluble emulsion of vitamin D_3 (Vitex D) was added to the fluid milk (2 kg) to give a final concentration of 50,000 IU/kg or 3250 nmoles/kg. Vitamin D_3-enriched milk was then mixed on a hot plate stirrer and then

homogenized for five minutes. The samples of fortified milk were taken for vitamin D_3 quantification and then refrigerated overnight.

5.2.1.2 Cheddar cheese making procedure

The refrigerated vitamin D_3 fortified milk was heated at 32.5°C in a beaker. The mesophilic starter culture was added to the milk and allowed to age for one hour. After 60 minutes of ripening, rennet was added and stirred for 5 minutes. The milk was allowed to sit for two hours at 32.5°C in a water bath until a firm curd was obtained. The curd was then cut and left for 15 minutes. Afterwards, the temperature of water bath was slowly raised from 32.5°C to 39°C over 40 minutes with stirring. This slightly elevated temperature helped to separate the whey from the curd. The whey was then drained when the pH reached 6.0. The curd was milled and salted, and then wrapped in cheesecloth and pressed for 30 hours at room temperature to remove the remaining whey from the curd. A laboratory-designed press was used (pressure of 5 kg for 1 hour, 10 kg for 2 hours, and 20 kg for 27 hours). After pressing, the curd yielded a cheese block. The cheese block was dried for 6-8 hours at room temperature and kept in a refrigerator at 4°C after vacuum packaging. The moisture of cheese blocks was measured right after cheese making. The Cheddar cheese was aged for three months at 4°C, and the analysis to quantify vitamin D content was made at regular time intervals.

5.2.1.3 Cheese yield and moisture

The cheese yield was obtained by accurately weighing the amount of milk and cheese immediately after pressing. Moisture content of all batches was also determined by heating a cheese sample in an oven at 100°C for four h.

5.2.2 Yogurt

5.2.2.1 Fortification of Yogurt milk

5.2.2.1.1 With crystalline vitamin D_3

Unfortified milk was heated to 50°C on a hot plate and mixed with 0.5 ml of crystalline vitamin D_3 butter emulsion (previously mentioned) to get the final concentration of 50,000 IU/kg or 3250 nmol/kg of vitamin D_3. It was then mixed and homogenized for proper distribution of the vitamin in the milk. The fortified milk was used to make yogurt.

5.2.2.1.2 With water-soluble emulsion of vitamin D_3

One (1) kg of unfortified milk was taken and heated at 50°C in a glass beaker using a laboratory stirrer/hot plate, then 0.5 ml of the Vitex D-fortified butter emulsion (as previously mentioned) was added into the milk to obtain a final concentration of 50,000 IU/kg or 3250 nmoles per kg. Homogenization and mixing was carried out in the same manner as it was done with crystalline form

to ensure that the vitamin D was uniformly distributed. This milk was used to manufacture yogurt.

5.2.2.2 Yogurt manufacturing

Plain set type fortified yogurt was processed on benchtop equipment, composed of fortified vitamin D_3 whole milk (3.25% fat content) and a culture. Commercial yogurt containing *Streptococcus thermophilus* (ST) and *Lactobacillus bulgaricus* (LB) was used as a starter culture. Vitamin D_3 fortified milk was heated to 85°C in a glass beaker for 30 minutes and cooled down to 45°C in an ice water bath. Three samples were taken to quantify vitamin D_3 content in the fortified milk. The starter culture was added to inoculate the milk at 43°C. The mixing of the starter culture was performed manually. The inoculated milk was then transferred to a clean yogurt plastic container and incubated at 33°C for 6 hours until the firm curd was obtained and the pH reached 4.3. Samples were then cooled to 4°C and kept refridgerated. The samples were analyzed weekly for vitamin D_3 content. All samples were analyzed in triplicates.

5.2.3 Ice cream

5.2.3.1 Vitamin D_3 supplementation to ice cream mix

5.2.3.1.1 With crystalline vitamin D_3

The ice cream mix (50°C) was fortified with 0.5 ml of crystalline vitamin D_3 butter emulsion to obtain the final concentration of 50,000 IU/kg or 3250 nmoles/kg. The mix was then homogenized and mixed at low heat for uniform distribution of the vitamin.

5.2.3.1.2 With water-soluble vitamin D_3 emulsion (Vitex D)

On (1) ml of fortified butter emulsion of Vitex D was incorporated into the custard mix at 50°C to yield the final concentration of 100,000 IU/kg or 6500 n moles/kg in the ice cream. The mix was vigorously mixed by continuously stirring for 15 minutes at low heat and then homogenized on a lab homogenizer for ~5 minutes.

5.2.3.2 Ice cream manufacture

Ice cream was made using a DeLonghi ice cream maker (DeLonghi, Toronto, Canada). The ice cream was formulated to contain 200g of milk, 200g of whipped cream, and 100g of granulated sugar, 25g of egg yolk and 8 g of vanilla extract. Unfortified milk and whipped cream were mixed and scalded at 100°C

on a hot stirrer plate (brought slowly up to boiling in a beaker). The egg yolk and sugar were mixed together manually in a separate beaker until a thick creamy mass was obtained; it was then poured into the mix containing milk and whipped cream, whilst continuously stirring. The mix was then heated gently to 80°C and stirred until the mix thickened. After this, the ice cream mix was removed from the hot plate and cooled down to 50°C. At this point, the mix was fortified with vitamin D_3 as described above. After fortification, the mix samples were taken to quantify vitamin D_3 and the mix was kept in a refrigerator for one hour to bring down the temperature to 4°C. The mix was then poured into an ice cream maker to generate the ice cream. Ice cream making was carried out in the ice cream maker. Batches of ice cream were stored at -25°C. Ice cream samples were analyzed for vitamin D_3 content after week 0,1,2 and 4 in triplicates.

5.2.3.3 Ice cream yield and overrun calculation

The ice cream yield was calculated by measuring the weight of ice cream mix before aeration and the final ice cream. Overrun was also determined with the help of following equation:

$$\frac{(Volume\ of\ ice\ cream) - (Volume\ of\ ice\ cream\ mix)}{(Volume\ of\ ice\ cream\ mix)} \times 100$$

5.3 Analytical methods

5.3.1 Sample preparation

Vitamin D_3 content was determined in whole milk, Cheddar cheese, cheese whey, yogurt, ice cream mix and ice cream. The semi solid nature of cheese, yogurt and ice cream required an additional sample preparation step prior to saponification to enhance vitamin D recovery; the fluid milk and cheese whey did not require any additional step. The following ratios of distilled water and fortified dairy samples were used for sample preparation.

5.3.1.1 Milk samples

Fluid milk was used without adding any water for the extraction of vitamin D_3 content. One (1) g of fluid milk was used for the extraction of vitamin D_3.

5.3.1.2 Cheese samples

One (1) g of cheese was drawn from the cheese block. It was then mixed with 4 g of distilled water using a mortar and pestle. One (1) g of sample was used for the extraction of vitamin D_3 in Cheddar cheese.

5.3.1.3 Whey samples

One (1) g of whey was taken for saponification and extraction of vitamin D_3 without adding any distilled water.

5.3.1.4 Yogurt samples

Yogurt was homogenized with water in the proportion of 1:2 (yogurt:water mixture) using a lab blender. One (1) g of sample was used for extraction.

5.3.1.5 Ice cream mix samples

The ice cream mix before freezing was diluted with distilled water in a ratio of 1:3 (ice cream mix:water). Sample size for extraction was 1 g.

5.3.1.6 Ice cream samples

Ice cream was melted at room temperature and then dissolved in distilled water in the proportion of 1:3 (ice cream:water). Vitamin D_3 was extracted from a 1 g ice cream sample.

5.3.2 Saponification and extraction

The saponification and extraction method was the same for all samples. One (1) g of sample was weighed into a 10 ml test tube. A 0.5 ml sample of 60% aqueous KOH was added to the tube which was nitrogen-filled to remove

oxygen. The tube was capped, shaken, and transferred to a water bath at 70°C for 30 minutes (Upreti *et al.*, 2002). The tube was shaken once after 5 minutes. After 30 minutes, the tube was cooled in an ice-water bath for 10 minutes (Figure 7).

After saponification and subsequent cooling, 3.75 ml of a methanol:chloroform mixture (2:1) was added to the tube and vortexed. A further 1.25 ml of chloroform was added to the tube to extract vitamin D_3, and once again vortexed. The sample was then centrifuged for 10 minutes at 4°C. A clear chloroform layer was separated out at the bottom of the tube. The chloroform layer was taken out using a glass syringe and transferred to an evaporation vial. The chloroform extract was dried under nitrogen, reconstituted in 2 ml of mobile phase, and left undisturbed for at least 15 minutes at room temperature. The reconstituted extract was then filtered through a 0.45 μm filter (13mm Durapore membrane Disposable Hydrophilic Filter, Millipore, Carrigtwohill, Co. Cork, Ireland) and injected into the vial for HPLC analysis.

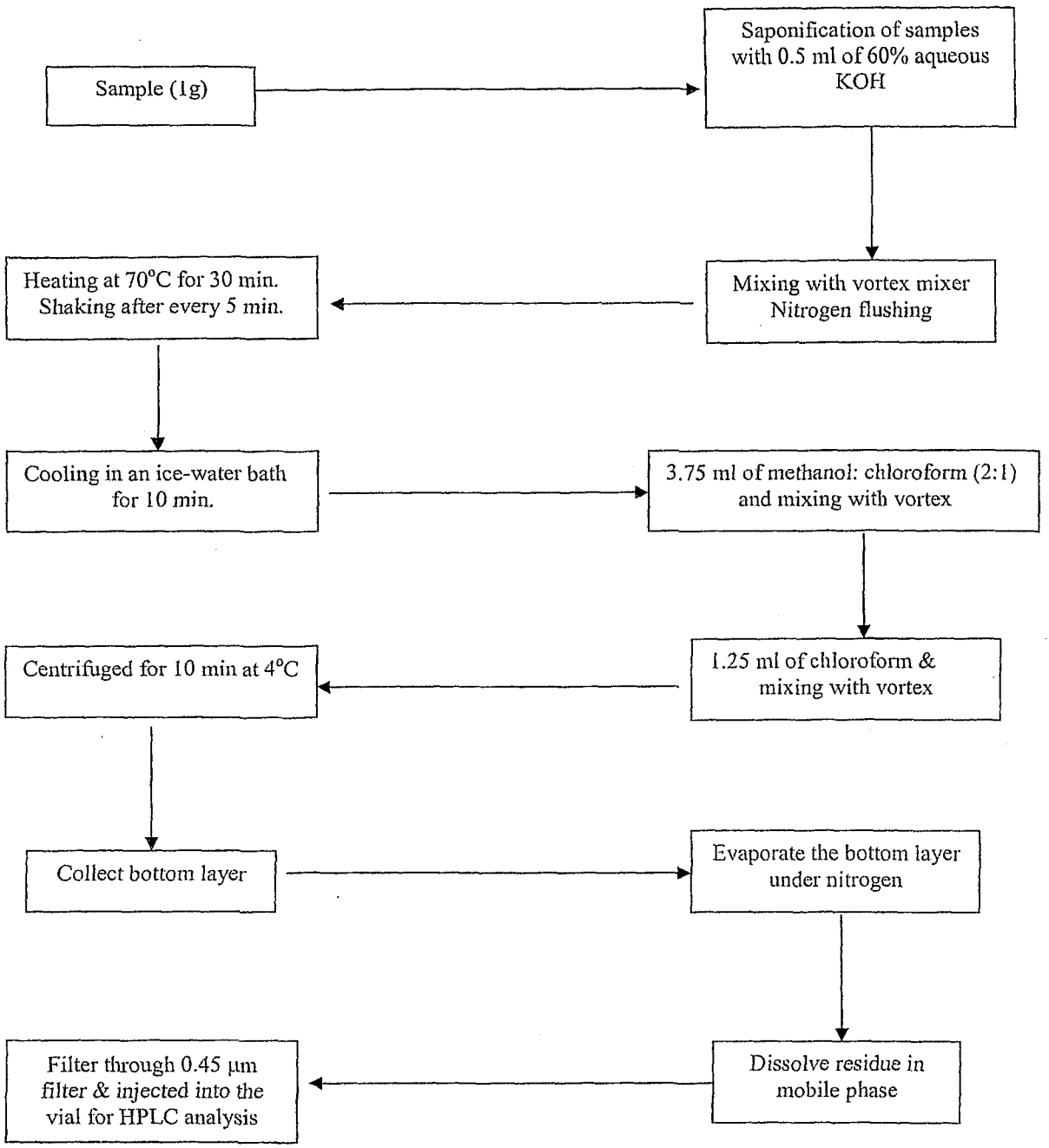

FIGURE 7: Saponification and extraction procedure of vitamin D_3 for whole milk, Cheddar cheese, cheese whey, yogurt and ice cream and ice cream mix samples.

5.3.3 Vitamin D_3 analysis by HPLC

For this research work, reverse phase HPLC system was used for quantification of vitamin D_3 in milk, cheese, yogurt and ice cream. All HPLC experiments were performed at Mount Sinai Hospital in the vitamin D research lab. Vitamin D_3 was measured using two detectors set at wavelengths of 254 nm and 228 nm. The retention time was determined by running the vitamin D_3 standard solution in the mobile phase. Vitamin D_3 was identified on the chromatogram on the basis of its retention behavior with respect to reference compounds. It was quantified on the basis of adsorption area of the peak, using an external standard solution. A C18 column was used throughout the HPLC analysis. Operating conditions were: ambient temperature of ~23°C; mobile phase was methanol:acetonitrile:water (49.5:49.5:1 v/v); flow rate was 0.3 ml/min; and the injection volume was 100 µL. The area of the vitamin D_3 peak was used as an index of concentration of vitamin D_3 in the sample.

5.3.3.1 Standard curve and assessment of method

Vitamin D_3 was estimated in dairy products with the help of an external standard. Different concentrations from 25-125 IU/ml of vitamin D_3 in ethanol were used to quantify the vitamin. A standard solution of vitamin D_3 was run at HPLC after every 15 samples to estimate the accurate amount of vitamin D_3 in

dairy products. The standard curve obtained at different concentrations is shown in Figure 8. The coefficients of variation (c.v.) calculated at 25, 50, 75, 100 and 125 IU were 7.5%, 5.15%, 5.04%, 4.37% and 5.06%, respectively. The r^2 value of 0.9974 indicated that the fit explains 99.74% of the total variation in the data about the average.

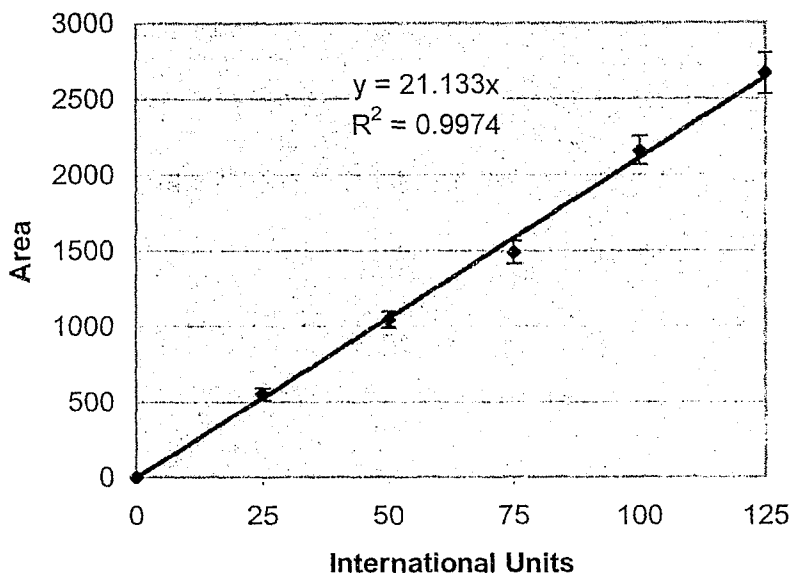

FIGURE 8: Standard curve for vitamin D_3 standard solutions used for the estimation of vitamin D_3 content in dairy products.

5.4 Statistical analysis

One-way ANOVA and T-tests were used to assess statistically significant differences in fortification, treatments, storage stability and yield. Statistical analyses were made at a level of significance of $P < 0.05$. A statistical analysis software (SPSS version 8.0, SPSS, Chicago, IL, USA) was used to perform all statistical analyses (Appendix A1 and A2).

The T-test for comparing group means is appropriate when the independent variable consists of two levels of a variable (i.e., when there are two and only two groups). If we have 3 treatments to compare (e.g., A, B, C) then 3 separate T-tests (comparing A with B, A with C, and B with C) would be required. With 7 treatments, 21 separate T-tests would be required. This would be time-consuming, but more importantly, it would be inherently flawed because in each T-test, thre is a 5% chance that the conclusion is wrong (at alpha = 5%). With 21 tests, by probability, one test would give a false result. Analysis of variance (ANOVA) overcomes this problem by enabling detection of significant differences between the treatments as a whole (Polit, 1996).

6.0 RESULTS AND DISCUSSION

The results of this study will be presented and discussed in two parts. Section 6.1 focuses on the methodology developed for the determination of vitamin D_3 in whole milk, Cheddar cheese, whey, yogurt, ice cream and the ice cream mix. Section 6.2 focuses on the recovery and stability of vitamin D_3 in these dairy products.

6.1 Methodology development for vitamin D_3 determination

Vitamin D_3 is usually determined in three different types of samples, namely human fluids, processed food and pharmaceuticals. The determination of vitamin D_3 in foods, which can be liquid, semi-solid or solid in nature, is generally determined using conventional large-scale extraction methods, which consist of a solid-liquid extraction, followed by solid phase extraction. The procedure usually involves 3 main steps: saponification, extraction, and quantitation via HPLC. Saponification followed by extraction is the most widely used sample preparation method for fat-soluble vitamins in food samples. Saponification removes the bulk of the fat and facilitates extraction by releasing fat-soluble vitamins from the food matrix. Factors to be optimized in any saponification method are sample size, base concentration, saponification temperature and time, composition of the saponified solution before extraction

and extraction solvents. Saponification can be carried out at high temperature for a short period of time or overnight at ambient temperature. Selection of an antioxidant and removal of oxygen by nitrogen are also important. Fat-soluble vitamins are sensitive to extreme pH, oxygen, light and heat (Turner *et al.*, 2001).

It is still quite common to use conventional large-scale extraction methods when analyzing vitamin D in foods. These methods are, however, laborious and consume large amounts of reagents (Upreti *et al.*, 2002, Renken and Warthesen, 1993, Sliva *et al.*, 1992, Bekhof and van den Bedem, 1988). There have been a few small-scale extraction reported, but these have been for the simultaneous extraction of β-carotene, tocopherol and all-*trans*-retinol from dairy products (Indyk, 1988; Panfili *et al.*, 1994), and not vitamin D. In term of solvents used, vitamin D_3 has been extracted from food using different organic solvents such as petroleum ether, diethyl ether (Renken and Warthesen, 1993 and Upreti *et al.*, 2002), hexane (Sliva *et al.*, 1992) and chlorobutane (Bekhof and van den Bedem, 1988).

When this research work was started, the most difficult task was to find an appropriate method to extract vitamin D_3 in Cheddar cheese, yogurt and ice cream, as there was no existing method. Several large-scale methods were available for the extraction of vitamin D content from other dairy products (e.g., fluid milk, milk powder, infant formula, pasteurized process cheese), but no

small-scale extraction methods were. The only similar study was made by Banville *et al.* (2000), who compared different fortification methods for fortifying Cheddar cheese with vitamin D. However, they did not report their extraction method. Samples were likely analyzed by an outside laboratory.

Selection of an effective extraction method is probably the single most important factor in achieving high performance and reproducible results. Given the extraction methods for vitamin D reported in the literature, a newcomer in this field is faced with the following question: which extraction method is the best for a given application? This question led us to characterize the available extraction methods, so that the most promising methods could be selected.

Given that several papers published methods on the extraction of vitamin D from different foods, it was impossible to evaluate all of the available methods. Therefore, the three most common large-scale methods of vitamin D extraction from dairy products were considered. The first method was presented by Bekhof and van den Bedem (1988) on the extraction of vitamin D in fortified milk, milk powder and infant formula; the second method was reported by Renken and Warthesen (1993) on vitamin D estimation in fortified fluid milk, and the third method was developed by Upreti et al. (2002) for the extraction of vitamin D_3 in pasteurized process cheese.

Upreti *et al.* (2002) developed a method to estimate vitamin D_3 in pasteurized process cheese based on the modifications in the method of Renken and Warthesen (1993), who estimated vitamin D content in milk. Vitamin D_3 was estimated using alkaline saponification with KOH followed by extraction with petroleum ether:diethyl ether (90:10 v/v) and HPLC in both methods. Changes were made in the saponification method and quantities of chemical reagents used, according to the estimated fat content in the cheese, otherwise the two methods were identical. In the method of Upreti *et al.* (2002), a 60% aqueous KOH solution was used to saponify the cheese sample at 70°C for 30 minutes whereas in the other method (Renken and Warthesen, 1993), 5% aqueous KOH solution for skim milk samples and 35% KOH solution (w/v in 85% ethanol) for whole milk samples were used with overnight saponification at ambient temperature (~23°C). Overnight saponification at ambient temperature is a preferred way of removing the fat components (Indyk and Woollard, 1985), as it reduces the thermal isomerization of vitamin D_3 to its previtamin form (Keverling Buisman *et al.*, 1968). These modifications were made due to the high fat levels in cheese as compared to the fluid milk, which requires a stronger KOH solution to hydrolyze the fat content in the processed cheese. Upreti *et al.* (2002) reported a recovery of ~ 98-106% vitamin D_3 in processed cheese samples using their extraction method, while Renken and Warthesen (1993) and Bekhof and van

den Bedem (1988) reported the overall recoveries of 93% and 91.4% in dairy samples, respectively.

For this research work, we required a method that could be used for both types of products, liquid (fluid milk) and semi solid products (Cheddar cheese, yogurt and ice cream). Thus, the method of Renken and Warthesen (1993) for quantifying vitamin D_3 in the fluid milk and the method of Upreti *et al.* (2002) to determine vitamin D_3 in semi-solid products were selected. The reliability of the method of Renken and Warthesen (1993) was determined by extracting vitamin D_3 from whole milk samples. It was then decided to use the heated saponification method (Upreti *et al.*, 2002) instead of overnight saponification method (Renken and Warthesen, 1993) to speed up the process of extraction and to get the samples analyzed quickly.

Two types of fluid milk samples were analyzed using this method, one fortified with crystalline vitamin D_3 and other fortified with Vitex D. The same method of milk fortification with vitamin D_3 was used as discussed in sections 5.1.2 and 5.1.3 for the fortification of milk.

TABLE 1: Recovery of vitamin D_3 in whole milk using the extraction method of Renken and Warthesen (1993)

Sample	Type of Vitamin D_3	Fortified Value (IU/g)	Recovered Value (IU/g)	Recovery (%)	CV (%)
Whole Milk	Vitex D	10	8.533 ± 0.503	85.33 ± 5.03	5.90
Whole Milk	Crystalline	10	79.0 ± 0.361	79.00 ± 3.61	4.564

The results of the recovery tests, obtained for both types of vitamin D_3 (crystalline and Vitex D) added to unfortified whole milk samples are listed in Table 1. All samples were analyzed in triplicate.

Using this method, recoveries ranged between 79-85% ($P>0.05$) (Table 1). When we compared these results with Renken and Warthesen (1993) results, our results were quite lower, indicating some losses of vitamin D_3 content during the extraction process.

These losses of vitamin D_3 during extraction may be due to the following factors:

- laboriousness of the extraction technique, which involves much handling.
- degradation of vitamin D_3 due to oxidation. This oxidation might have occurred due to laborious multiple extractions with organic solvents, which are necessary for complete recovery.
- thermal isomerization of vitamin D_3 might have occurred during heated saponification method (70°C for 30 minutes)

In order to investigate these losses, the whole milk samples were saponified overnight at ambient temperature (~23°C) using the extraction technique of Renken and Warthesen (1993). The method of Bekhof and van den Bedem (1988) was not considered given its low recovery as compared to this method. Saponification performed at a high temperature for a short period or at ambient temperature overnight had no influence on recovery. Higher concentrations of vitamin D added to the whole milk (e.g., 50 and 100 IU/g) showed that the initial concentration was above the detection limit of the HPLC. The results thus obtained are shown in Table 2.

TABLE 2: Recovery of vitamin D_3 in whole milk at high concentrations of vitamin D_3 using the extraction method of Renken and Warthesen (1993)

Sample	Type of Vitamin D_3	Fortified Value (IU/g)	Recovered Value (IU/g)	Recovery (%)	CV (%)
Whole Milk	Vitex D	50	43.42 ± 0.68	86.84 ± 1.36	1.56
Whole Milk	Crystalline	100	83.94 ± 2.24	83.94 ± 2.24	2.67

Results obtained were not substantially different from previous experiments, indicating some losses during the extraction process may be due to human error or as-yet-undetermined factors. Consequently, it was desirable to

use a method capable of more rapidly and precisely quantifying vitamin D_3 in both liquid and semi-solid products.

The aim was to develop a method that was fast, effective, and that enabled the processing of a large number of samples quickly.

6.1.1 Method development

The method of Bligh and Dyer (1959) for total lipid extraction was considered. This method was used for lipid extraction from biological materials, later modified by Vieth and Fraser (1979), for the quantification of vitamin D in rat kidney mitochondria. Lipids are a diverse group of biological substances made up primarily of non-polar compounds (triglycerides, diglycerides, monoglycerides and sterols) and more polar compounds (free fatty acids, phospholipids and sphingolipids). Given that lipids may bind other molecules, combined with the ability of solvent mixtures to solubilize different lipid classes, has led to the concept of "total lipid extract" and "extractable lipids". Solvents used for lipid extraction should have a high solubility for all lipid compounds and be sufficiently polar to remove them from their binding sites. Chloroform/methanol mixtures apply well as was recognized by Folch *et al.* (1957). This approach was adapted by Bligh and Dyer (1959) resulting in a method which has become the standard method for total lipid determination for decades. In the method of Bligh and Dyer (1959), optimum lipid extraction

should result when the sample is homogenized with a mixture of chloroform and methanol which, when mixed with the water in the sample, should yield a monophasic solution. The resulting homogenate can then be diluted with chloroform to produce a biphasic system, the chloroform layer of which should contain the lipids and the methanol-water layer which contains non-lipids. Hence, a purified lipid extract should be obtained when the chloroform layer is isolated.

As vitamin D_3 is a non-polar, liposoluble vitamin, it has a strong binding affinity with triglycerides and is soluble in chloroform. Cow's milk has 97.5% triglycerides, 0.36% diglycerides, 0.027% monoglycerides, 0.31% cholesterol, 0.027% free fatty acids and 0.8% total phospholipids. Given this compositional variety, modifications were necessary to make this technique viable for vitamin D extraction in dairy products.

In order to tackle this problem, an additional saponification step was incorporated prior to extraction. Saponification was performed as per Upreti *et al.* (2002). Secondly, quantification of vitamin D_3 on small-scale is difficult due to typically low vitamin D_3 concentrations found in foods. In large samples (~35-50g), a sufficient concentration of vitamin D is present to be detected via HPLC (Parrish, 1979). To address this problem in small samples (1g), high concentrations of vitamin D_3 were incorporated into the fluid milk, and as a

result, into the cheese, yogurt and ice cream. Thirdly, a sample preparation step prior to saponification was made when cheese, yogurt and ice cream samples were analyzed. The method of Bligh and Dyer (1959) and Vieth and Fraser (1979) can be applied to any sample containing 80% water content. Thus, in the extraction of materials that do not contain 80% water, it is necessary to add distilled water to the sample. Therefore, the semi-solid nature of Cheddar cheese, yogurt and ice cream and ice cream mix required an additional sample preparation step by adding distilled water prior to saponification to enhance vitamin D_3 recovery. The fluid milk and cheese whey did not require any additional step of sample preparation, as milk contains ~80% water. Different amounts of distilled water were added depending on the type of product to be analyzed. This sample preparation helps to extract the analytes, so as to obtain a measurable response in a chromatographic system. When the proper proportions of water are added to the samples, the system separates the chloroform layer from methanol-water layer. The proportions may be chosen in such a manner that the lower layer will be practically 100% chloroform and the upper layer nearly all methanol-water.

In summary, the developed extraction procedure presented here is partly based on some previous work (Bligh and Dyer, 1959 and Vieth and Fraser, 1979 and Upreti *et al.*, 2002). The procedure was modified to enable extraction of

vitamin D_3 from whole milk, Cheddar cheese, cheese whey, yogurt, ice cream and ice cream mix with different vitamin D_3 levels.

6.1.2 Implementation of developed method

Samples of whole milk were fortified with 50 IU/g and 100IU/g of Vitex D and crystalline Vitamin D_3, respectively. Vitamin D_3 from fortified milk samples was extracted using the developed extraction method. The results obtained from the developed method are listed in Table 3.

TABLE 3: Recovery of vitamin D_3 in whole milk using the developed method

Sample	Type of Vitamin D_3	Fortified Value (IU/g)	Recovered Value (IU/g)	Recovery (%)	CV (%)
Whole Milk	Vitex D	50	52.47 ± 4.88	104.93 ± 9.77	9.3
Whole Milk	Crystalline	100	96.77 ± 9.74	96.77 ± 9.74	10.06

The data showed high recovery of vitamin D_3 (~97-105%) ($P>0.05$) using this method, which was substantially higher than the method of Renken and Warthesen (1993). These results demonstrated that the developed method was suitable for the extraction of vitamin D_3 from whole milk. Since this extraction technique is rapid, no degradation of vitamin D_3 was observed during the extraction process. As a result, this method was used for the determination of

vitamin D_3 for all samples of whole milk, Cheddar cheese, whey, yogurt, ice cream and ice cream mix.

6.1.3 Comparison of two-extraction methods in vitamin D_3 fortified fluid milk

Two methods were tested to determine vitamin D_3 in the fortified fluid milk, namely, the method of Renken and Warthesen (1993) and the modified method. The recoveries of vitamin D_3 obtained by the Renken and Warthesen (1993) method were lower than the developed method. In addition, the method of Renken and Warthesen (1993) was very laborious and time consuming as it took 4-5 hours to process one sample due to the larger sample size and the multiple extractions with large volumes of organic solvents. In contrast, the developed method was rapid and cost effective, as it took only 2 hours to process a set of extraction experiments simultaneously. This method was also cost effective due to low reagents consumption and less labour cost required to process vitamin D_3 extraction from the dairy samples.

6.1.4 Method development for quantification of vitamin D_3 in dairy samples

Quantification of vitamin D_3 was done by reverse phase HPLC. In the case of vitamin D compounds, the use of a UV detector is preferred as it allows the use of vitamin D_2 as an internal standard for vitamin D_3 (Mattila, 1995). Vitamin D_3 was measured with two absorbance detectors at 254 nm and 228 nm.

Vitamin D_3 was eluted at approximately 12.5 minutes by running a vitamin D_3 standard solution with a mobile phase of a 90:10 v/v mixture of acetonitrile:methanol (Renken and Warthesen, 1993). When this mobile phase was used with dairy samples without any internal standard, the peak eluted at the same time but the area was increased significantly, indicating the presence of an interfering substance that eluted at the same time as vitamin D_3. In order to remove this interfering compound, dairy samples were filtered twice with 0.45μm hydrophilic filters before injection into the column, but the interfering peak could not be separated or avoided. This interfering compound was possibly cholesterol (Ball, 1992).

It was impossible to accurately quantify vitamin D_3 without separating the interfering peak from the vitamin D_3 peak. Therefore, after using different combinations, a 49.5:49.5:1 v/v mixture of acetonitrile:methanol:H_2O was considered to be the best mobile phase to separate the interfering peak from the vitamin D_3 peak. With this mobile phase, the retention time was shifted to 10 minutes from 12.5 due to the less polarity of mobile phase as compare to the previous one used, and a clear separate peak of vitamin D_3 was obtained.

The determination of vitamin D_3 was achieved by using the external standard method owing to the difficulty encountered in selecting an adequate internal standard for vitamin D_3. Vitamin D_2 is considered to be an ideal choice

to determine vitamin D_3 using HPLC because it is virtually identical in its physiochemical properties (Indyk and Woollard, 1985). Vitamin D_2 standards run with a mobile phase of 49.5:49.5:1 methanol:acetonitrile:H_2O yielded a peak at 9.00 minutes (i.e. just prior to vitamin D_3 peak). When vitamin D_2 was incorporated into the cheese, yogurt and ice cream samples prior to saponification, the peak eluted with a greater absorption area i.e. vitamin D_2 and D_3 peaks were combined.

Some measures were taken to rectify this problem, such as changing the mobile phase for the early elution of vitamin D_2 peak, changing the detector wavelength to 280 nm. However, the latter also affected the sensitivity of vitamin D_3 (Ball, 1992; Indyk and Woollard, 1985). Other changes included modifying the mobile phase by eliminating H_2O, and changing the combination to 40:60 methanol:acetonitrile. These modifications did not completely eliminate the interfering peak. As a result, an external standard of vitamin D_3 was employed to quantify vitamin D_3 in the cheese, yogurt and ice cream samples.

6.2 Recovery of vitamin D_3 in Cheddar cheese

The concentration of vitamin D_3 in fluid milk was 2.229 ± 0.191 μg/g for crystalline vitamin D_3 dissolved in ethanol and 1.44 ± 0.06 μg/g for Vitex D. The main concern in fortification of cheese with vitamin D is how much of it can be recovered in the final cheese and whey. The total recovery of crystalline vitamin D and Vitex D in the final Cheddar cheese mass was 18.134 ± 0.975 μg /g (86.703% ± 1.859) and 12.309 ± 0.858 μg /g (90.413% ± 3.488) respectively (Table 4). An example of recovery calculation in Cheddar cheese is shown in appendix A3.

TABLE 4: Calculated recovery of vitamin D_3 in Cheddar cheese and whey from fortified whole milk

	CRYSTALLINE VITAMIN D_3	VITEX D
Milk (μg/g)	2.229 ± 0.191	1.44 ± 0.06
Cheese (μg/g)	18.134 ± 0.975	12.309 ± 0.858
Whey (μg/g)	0.023 ± 0.003	0.0109 ± 0.006
Recovery (%) in cheese	86.703 ± 1.859	90.413 ± 3.488
Recovery (%) in whey	8.727 ± 1.274	6.537 ± 3.01
Total Recovery (%)	95.427 ± 3.117	96.967 ± 0.936

Recovery of vitamin D_3 added to the milk and recovered from the Cheddar cheese mass and the cheese whey did not differ significantly ($P>0.05$), indicating that the amount of vitamin D_3 lost during the manufacture of Cheddar cheese was 3-5%. A low concentration of vitamin D was detected in the whey. With crystalline vitamin D, 0.023 ± 0.003 μg /ml and for Vitex D, 0.0109 ± 0.006μg /ml was found. Contrary to previous reports (Banville *et al.* 2000), a ~ 6.5-8.7% recovery of vitamin D_3 in the whey was obtained. Banville *et al* (2000) reported no detection of the vitamin in the cheese whey.

Recovery of vitamin D shows that the processing conditions necessary for Cheddar cheese production (outlined in the literature review) were not detrimental to vitamin D_3 content as no significant losses were observed during cheese making. Recoveries of ~95-97% ($P>0.05$) using either form of the vitamin were obtained.

Our results substantially differ from the Banville *et al.* (2000), who reported that ~39, 57 and 59% of vitamin D added to Cheddar cheese as liposomes, Vitex D or vitamin D in cream, respectively, was either lost in the whey or destroyed during cheese making process. Since they added a low concentration of vitamin D to the milk for Cheddar cheese making, no vitamin D would have been found in the whey as it would be below the detection limit of the analytical method. The other factors they attributed to the loss of vitamin D

in cheese making were fermentation by lactic acid bacteria, acidification and oxygen. In contrast, our results showed only 3-5% losses of vitamin D_3 in cheese making.

High recoveries of vitamin D_3 in Cheddar cheese were obtained. It is proposed that some vitamin D_3 molecules are bound to the cheese whey and the rest bound with Cheddar cheese as cheese whey contains 6% of the total fat and 94% remains in the Cheddar cheese mass. This approach seems quite reasonable when the recovery results are considered - ~6.5-8.7% vitamin D_3 was found in the cheese whey and ~90% was found in the Cheddar cheese. Moreover, β-lactoglobulin (ß-LG), present in whey, has a binding affinity with vitamin D_3. Wang *et al.* (1997) reported the affinity of β-LG to vitamin D_3. Most evidence points toward the calyx formed by β-barrel in ß-LG as the hydrophobic binding site (Chen *et al.*, 1993, Cho *et al.*, 1994 and Papiz *et al.*, 1986). However, a surface hydrophobic pocket formed by the α-helix and the surface of the barrel also exists (Monaco *et al.*, 1987).

On the basis of the above studies and experimental results obtained from this study, we conclude that vitamin D_3 has a strong binding affinity with the Cheddar cheese mass and also some binding affinity with the cheese whey.

6.2.1 Yield and moisture content of Cheddar cheese

The cheese yield and moisture content were measured for all batches. The actual yields for experimental cheeses were found to be 10.49 ± 0.22% ($P>0.05$) as shown in appendix A4. The yield obtained was according to the standard yield of Cheddar cheese that should be ~10%. The moisture content of all cheese batches was also determined. Cheese samples were drawn immediately after pressing and kept in an oven for four hours at 100°C. The moisture content of these cheeses were 39.16 ± 0.32% ($P>0.05$).

6.3 Stability of vitamin D_3 in Cheddar cheese

Changes in vitamin D_3 concentration of the Cheddar cheese over a three-month ripening period were observed. No significant differences in the concentration of vitamin D_3 incorporated as Vitex D were observed ($P>0.05$), but a deterioration of ~ 7% over three months for the crystalline form was seen (Table 5) ($P<0.05$). These results tend to the same conclusion as Banville *et al.* (2000), who found a reduction in vitamin D_3 content after 3 months in fortified Cheddar cheese containing liposomes and 5 months for cheese made with Vitex D or vitamin D in cream. Conversely, Upreti *et al.* (2002) reported no loss of vitamin D in processed cheese after 9 months at room and refrigerator temperature.

TABLE 5: Calculated vitamin D_3 retention in cheese blocks during three months ripening at refrigerated temperature.

Time (Weeks)	Crystalline Vitamin D_3 in nmoles/g	Recovery %	Vitex D in nmoles/g	Recovery %
0	44.158 ± 2.842	100	31.311 ± 1.491	100
2	42.090 ± 5.045	95.177 ± 6.487	30.445 ± 1.070	97.235 ± 1.241
4	40.873 ± 4.352	92.420 ± 6.38	31.520 ±0.858	100.665 ± 1.379
8	41.489 ± 4.932	93.835 ± 5.597	30.681 ± 0.900	97.988 ± 2.148
12	41.418 ± 2.905	93.711 ± 3.764	31.606 ± 1.784	100.942 ± 0.844

Data from this study show that vitamin D_3 had good stability in experimental cheeses during 3 months' ripening, with a recovery of ~93% and 100% (Table 5) for cheese containing crystalline vitamin D_3 and Vitex D, respectively. The conditions used for cheese ripening, which involve the absence of air (vacuum packaging) and low storage temperature in the dark, might explain the good stability.

The stability of vitamin D_3 during storage was higher in cheese containing Vitex D. Since Vitex D is in droplet form, the protective effect of vitamin D_3 from oxidation, pH and acidification could be higher than the crystalline form. The lower recovery of crystalline vitamin D_3 in cheese when added to milk by

homogenizing with butter indicates that this method is perhaps not be the ideal method of incorporating vitamin D. However, in crystalline form, vitamin D's stability will depend on the physiochemical state of the surrounding media, particularly in complex environments such as cheese and other dairy products. Cheese ripening involves a complex series of microbiological and biochemical events. As the Cheddar matures, it loses moisture and its texture becomes drier and more crumbly. The sharpness in taste is the result of increased levels of salts and acids that naturally intensify during the ripening. This acidity increase during ripening may be a factor in vitamin D_3 degradation during ripening.

6.4 Recovery of vitamin D_3 in yogurt

Yogurt is an acidified, coagulated product obtained from milk by fermentation with lactic acid producing bacteria. During bacterial growth and conversion of lactose to lactic acid, vitamin D_3 did not affect the incubation time required for the yogurt mixes to reach pH 4.3. All batches reached pH 4.24 ± 0.015 after 6 hours. Further acid production during storage time was also similar for all experimental yogurts. The pH values of fortified yogurt samples reached 4.18 ± 0.02 after one week and 4.07 ± 0.02 after 4 weeks as shown in Figure 9.

The concentration of vitamin D in the initial milk used for yogurt production was 1.478 ± 0.066 μg/g for crystalline and 1.482 ± 0.032 μg/g for Vitex

D. All fortified yogurt samples were analyzed immediately after manufacture to calculate the recovery of vitamin D_3 and to estimate the loss of the vitamin during manufacture. As shown in Table 6, the recovery of vitamin D_3 in yogurts after manufacture containing crystalline vitamin D and Vitex D were 1.425 ± 0.030 µg/g (96.613% ± 5.138) and 1.449 ± 0.0.19 µg/g (97.757% ± 1.861), respectively. No significant difference in the recovery of vitamin D_3 was observed between the two methods ($P>0.05$). The recovery calculations are shown in appendix A5.

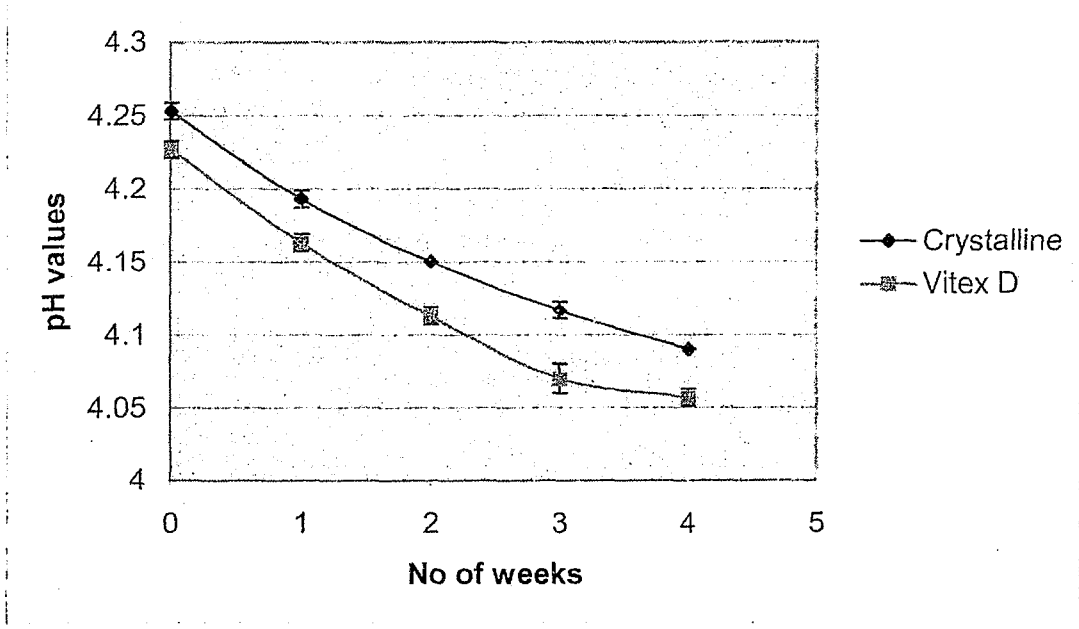

FIGURE 9: pH profile of yogurt samples over four weeks for both crystalline and Vitex D.

Recovery data indicate that there was no loss of vitamin D_3 during the manufacture of yogurt (Table 6).

Conflicting reports have been published regarding factors affecting the stability of vitamin D during the manufacture of fortified foods. Cremin and Power (1985) stated that vitamin D was unstable to light, oxidation and acid whereas Kutsky (1981) reported that vitamin D is susceptible to oxidation and light but stable to acid and alkali. Pike and Brown (1984) observed instability of vitamin D to irradiation and acid. However, the stability of vitamin D_3 in yogurts has never been reported in the literature.

In view of all these factors, potential losses of vitamin D were assessed during the manufacture of yogurt. During yogurt manufacture, a ~2-3% loss of vitamin D_3 was observed, this loss might have occurred due to the rapid decrease of pH from 6.7 to 4.3 during fermentation. It shows that acid production probably had little effect on the stability of vitamin D_3 during manufacture as these results are well within experimental error.

TABLE 6: Recovery of vitamin D_3 in yogurt from fortified whole milk.

	CRYSTALLINE VITAMIN D_3	VITEX D
Milk μg/g	1.478 ± 0.066	1.482 ± 0.032
Yogurt μg/g	1.425 ± 0.03	1.449 ± 0.019
Recovery in %	96.613 ± 5.138	97.757 ± 1.861

6.4.1 Distribution of vitamin D_3 in yogurt

Because of the gel-like structure of yogurt, it was extremely important to determine how well the vitamin D was distributed in the yogurt samples. With a non-uniform distribution, some portions of the yogurt would have higher concentrations of vitamin D than other areas. In order to ensure that the vitamin D_3 content was uniformly distributed within the yogurt samples, samples for quantification of vitamin D_3 were drawn from three different places of each yogurt sample container. There was no detectable difference among samples for both types of vitamin D_3 ($P>0.05$). It also implies that the method used for fortification is feasible for yogurt manufacture under actual production conditions.

6.5 Stability of vitamin D_3 in yogurt

Quantification of vitamin D_3 in the yogurt samples was performed after 0,1,2,3, and 4 weeks. A high retention was obtained (95-102%) ($P>0.05$) (Table 7). The vitamin D_3 retention data sheet over four weeks is available in appendix A6.

In contrast to Cheddar cheese, the crystalline vitamin D_3 in yogurt showed no degradation during the same storage period (i.e., after 4 weeks) indicating that the notable differences in structure between yogurt and Cheddar cheese render the former more suitable matrix for fortification with crystalline vitamin D_3. The stability of Vitex D in both products did not differ substantially.

On the basis of our results, we conclude that vitamin D_3 had good stability in all experimental yogurts containing crystalline vitamin D_3 and Vitex D under the storage conditions used.

TABLE 7: Retention of vitamin D_3 in experimental yogurts during four weeks of storage at refrigerated temperature (4°C)

TIME (WEEKS)	CRYSTALLINE VITAMIN D_3 (nmoles/g)	Recovery %	VITEX D (nmoles/g)	Recovery %
0	3.706 ± 0.098	100	3.766 ± 0.103	100
1	3.769 ± 0.195	101.710 ± 3.519	3.858 ± 0.154	102.457 ± 3.765
2	3.677 ± 0.248	99.218 ± 0.708	3.658 ± 0.232	97.143 ± 3.526
3	3.648 ± 0.078	98.461 ± 2.600	3.578 ± 0.237	95.025 ± 3.598
4	3.783 ± 0.283	102.116 ± 4.226	3.766 ± 0.489	99.938 ± 5.668

6.6 Recovery of vitamin D_3 in ice cream

Ice cream is a complex colloidal system that consists of air bubbles, fat globules, ice crystals and an unfrozen serum phase. During manufacture, vitamin D_3 was added to the mix at which point the ice cream was made. Vitamin D content in ice cream determined, using HPLC, was 1.412 ± 0.041 μg/g with crystalline and 2.626 ± 0.105 μg/g with Vitex D, with retention of 99.781% ± 0.144 and 99.328% ± 0.977, respectively ($P>0.05$) (Table 8). As a result, there was no loss of vitamin D_3 during the ice cream making process. An example of recovery calculation is shown in appendix A7.

TABLE 8: Calculated recovery of vitamin D_3 in ice cream after processing from fortified ice cream mix.

	CRYSTALLINE VITAMIN D_3	VITEX D
Custard (μg/g)	1.412 ± 0.041	2.626 ± 0.105
Ice cream (μg/g)	1.409 ± 0.039	2.608 ± 0.079
Recovery in %	99.781 ± 0.144	99.328 ± 0.977

Ice cream generally has 10-16% milk fat, depending on the type of ice cream. During ice cream manufacture, vitamin D_3 may likely bind with fat molecules. As one-third or more of the volume of ice cream usually consists of

air bubbles, it is conceivable that vitamin D, which may be sensitive to both air and light, may break down (Renken and Warthesen, 1993). In ice cream, air bubbles are almost completely covered with fat globules. However, there was no evidence found that aeration of ice cream during processing affected vitamin D stability in the present study.

Our results suggest that the manufacturing conditions of ice cream have no adverse effects on the recovery of vitamin D_3.

6.6.1 Ice cream yields and overrun calculation

The ice cream yield was found to be 100% ($P>0.05$); there was no loss during manufacturing. The density of the mix was measured to determine the volume used for ice cream making. The density of mix was found to be ~1.1 kg/L. Overrun was also calculated on the basis of ice cream and ice cream mix volume (Marshall and Arbuckle, 1996). Overrun was 50% ($P>0.05$) in all ice cream batches (appendix A8).

6.7 Stability of vitamin D_3 in ice cream

Ice cream samples were kept for four weeks at –25°C in a blast freezer. These samples, analyzed at time 0, 1, 2 and 4 weeks, showed no degradation in vitamin D_3, indicating a high retention 98-100% ($P>0.05$) (Table 9). The stability

calculations for vitamin D_3 in ice cream over 4 weeks storage is shown in appendix A9.

No significant differences were found between the two addition methods indicating the viability of these methods in ice cream ($P > 0.05$). The high stability of vitamin D_3 in ice cream was presumably due to the storage temperature (-25°C) and the fact that samples were not exposed to light. Low storage temperatures play an important role in the stability of vitamin D_3 in ice cream. Below -4°C, all chemical reactions start slow down and are mostly halted at -25°C. On the basis of our results, it is concluded that vitamin D_3 is very stable during storage; however, proper storage conditions should be maintained.

TABLE 9: Retention of vitamin D_3 in ice cream during four weeks of storage at -25°C in a blast freezer.

TIME (WEEKS)	CRYSTALLINE VITAMIN D_3 in n moles/g	Recovery %	VITEX D in n moles/g	Recovery %
0	3.663 ± 0.107	100	6.781 ± 0.304	100
1	3.650 ± 0.187	99.671 ± 0.481	6.722 ± 0.212	99.161 ± 1.840
2	3.687 ± 0.123	100.726 ± 2.799	6.750 ± 0.181	99.577 ± 1.719
4	3.612 ± 0.131	98.658 ± 1.723	6.741 ± 0.228	99.430 ± 0.868

7. CONCLUSION

The fortification and retention of vitamin D_3 in Cheddar cheese, yogurt and ice cream was estimated, using two forms of the vitamin - crystalline and emulsified (Vitex D). Over 90% retention was observed in Cheddar following fortification with either form. Low amounts of vitamin D_3 were also recovered in the cheese whey. Stability of vitamin D_3 in cheese samples differed in both forms of vitamin D_3 over a period of three months - stability was higher in cheese samples containing Vitex D.

In yogurt, there was no detectable difference observed between both forms of vitamin D_3 following fortification. Vitamin D_3 was stable in experimental yogurts during 4 weeks of storage at refrigeration regardless of vitamin D_3 type.

Likewise, no vitamin D_3 was lost during the manufacture of ice cream with either form of the vitamin. Storage stability monitored every week for up to 4 weeks at -25°C showed no deterioration, suggesting ice cream may be an excellent vehicle to transport vitamin D to the consumer market.

In conclusion, given the demonstrated stability of vitamin D_3 during storage, these fortified dairy products may play a promising role as a carrier for supplying this vitamin to the Canadian population. Results showed that the

processing involved in these product making had no detrimental effect on its stability.

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9. APPENDICES

Appendix A1

Retention of vitamin D in ice cream over 1 month

ANOVA

	Sum Square	df	Mean	F	Sig
Between	.218	14	.016	.640	.810
Within	.731	30	.024		
Total	.949	44			

F= ratio between variances between groups and variance within the group

In above table, no significant difference (P>0.05) found in ice cream samples over one month when fortified with crystalline vitamin D_3. The P value obtained was 0.810 indicating no significant difference.

Retention of crystalline Vit. D in Cheddar cheese during 3 months

ANOVA

REPLICAT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	419.910	14	29.994	2.805	.009
Within Groups	320.771	30	10.692		
Total	740.681	44			

In above table, there is a significant difference (P<0.05) found in Cheddar cheese samples over three months when fortified with crystalline vitamin D_3. The P value obtained was 0.009, indicating significant difference.

Appendix A2

T-Test analysis for large-scale extraction method during methodology development.

Group Statistics

	SAMPLE	N	Mean	Std. Deviation	Std. Error Mean
RAPLICAT	Large scale crystalline	3	79.0000	3.60555	2.08167
	Large scale Vitex D	3	85.3333	5.03322	2.90593

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
RAPLICA	Equal variance assumed	.272	.629	-1.772	4	.151	-6.3333	3.57460	6.25802	3.59135
	Equal variance not assumed			-1.772	3.625	.159	-6.3333	3.57460	6.67662	4.00996

The above data show no significant difference between two means (i.e, P>0.05).

Appendix 3

Recovery calculation of vitamin D in Cheddar cheese

Date	Sample	RT	Area	IU/Inj	Moles/Inj	IU/g	No of moles
		Minutes			(n moles)		per gm
26/04/2004	*Standard 46*	*10.483*	1949.975	*10*	*0.65*	100	6.50
26/04/2004	Milk Cheese 1 (	10.23	1160.96	5.95	0.39	59.5	3.87
26/04/2004	Milk Cheese 2 (	10.02	1118.29	5.73	0.37	57.3	3.73
26/04/2004	Milk cheese 3 (	10.3	1151.51	5.91	0.38	59.1	3.84
26/04/2004	**STD. DEV**	**0.146**	**22.411**	**0.115**	**0.007**	**1.172**	**0.075**
26/04/2004	**AVERAGE**	**10.183333**	**1143.59**	**5.86**	**0.38**	**58.63**	**3.81**
26/04/2004	Whey 1(8)	10.4	102.881	0.53	0.03	7.9	0.34
26/04/2004	Whey 2(8)	10.38	100.212	0.51	0.03	7	0.33
26/04/2004	Whey 3(8)	10.4	118.328	0.61	0.04	6.1	0.39
26/04/2004	**STD. DEV**	**0.012**	**9.780**	**0.050**	**0.003**	**0.900**	**0.033**
26/04/2004	**AVERAGE**	**10.393**	**107.140**	**0.549**	**0.036**	**7.000**	**0.36**
26/04/2004	Cheese 1 (8)	10.47	973.06	4.99	0.32	499.01	32.43
26/04/2004	Cheese 2 (8)	10.28	989.389	5.07	0.33	507.39	32.98
26/04/2004	Cheese 3 (8)	10.23	950.51	4.87	0.32	487.45	31.68
26/04/2004	**STD. DEV**	**0.1266**	**19.5223**	**0.1001**	**0.0065**	**10.0116**	**0.651**
26/04/2004	**AVERAGE**	**10.327**	**970.986**	**4.979**	**0.324**	**497.948**	**32.36**

Total volume of milk = 2045 g

Conc of vitamin D in milk = 3.81x2045=7791.45 nmoles or 7.791 umoles

Total weight of whey = 1805 gms

Conc of vitamin D in whey = 0.36x1805 = 649.80 nmoles or 0.650 umoles

Total weight of cheese = 215 gms

Conc of vitamin D in cheese = 32.36x215=6957.40 nmoles or 6.957 umoles

Total vitamin D added into the milk = 7791.5 nmoles

Total vitamin D recovered = 649.80+ 6957.40 = 7607.20 nmoles

Retention of vitamin D in cheese = (6957.40/7791.45)x100= 89.29%

Retention of vitamin D in whey = (649.80/7791.45)x100= 8.34%

Total recovery obtained = 89.29 + 8.34 = 97.63%

Appendix A4

Example of mass balance and yield calculation for Cheddar cheese

Total weight of milk = 2045 g

Total weight of cheese whey = 1805 g

Weight of final cheese block = 214 g

Mass balance for cheddar cheese was performed with the help of following formula:

Total weight of milk= Total weight of cheese whey + Weight of final cheese block

$$2045\text{g} = 1805 + 214 = 2019\ \text{g}$$

Approximately 25 g of milk was evaporated/ lost during cheese making process

Yield was calculated as follows:

$$\textit{Yield of cheese} = \frac{\textit{Weight of cheese block}}{\textit{Total weight of milk}} \times 100$$

$$= \frac{214}{2045} \times 100 = 10.46\%$$

Appendix 5

Recovery calculation of vitamin D in yogurt

Date	Sample ID	Time Days	RT	Area	IU/Inj	IU per gm	Moles/Inj n moles	Moles/gm n moles
→ 15-Jun	Stanadard 76		10.03	2142.248	10	100	0.65	6.5
15-Jun	Milk yogurt 1(13)	week 0	10.170	1299.472	6.066	60.659	0.394	3.943
15-Jun	Milk yogurt 2(13)	week 0	10.070	1273.745	5.946	59.458	0.386	3.865
15-Jun	Milk yogurt 3(13)	week 0	10.110	1255.215	5.859	58.593	0.381	3.809
	Std. Dev.		0.050	22.226	0.104	1.037	0.007	0.067
	Average		10.117	1276.144	5.957	59.570	0.387	3.872
15-Jun	Yogurt 1(13)	week 0	10.180	602.732	2.814	56.271	0.183	3.658
15-Jun	Yogurt 2(13)	week 0	10.230	644.973	3.011	60.215	0.196	3.914
15-Jun	Yogurt 3(13)	week 0	10.200	625.333	2.919	58.381	0.190	3.795
	Std. Dev.		0.025	21.138	0.099	1.973	0.006	0.128
	Average		10.203	624.346	2.914	58.289	0.189	3.789
→ 25-Jun	Standard 82		10.28	2249.05	10	100	0.65	6.5
	Yogurt 4(13)	week 1	10.200	669.181	2.975	59.508	0.193	3.868
	Yogurt 5(13)	week 1	10.180	683.913	3.041	60.818	0.198	3.953
	Yogurt 6(13)	week 1	10.220	651.095	2.895	57.900	0.188	3.763
	Std. Dev.		0.020	95.000	0.073	1.462	0.005	0.095
	Average		10.200	668.063	2.970	59.408	0.193	3.862
→29-Jun	Standard 85	week 2	10.22	2270.865	10	100	0.65	6.5
	Yogurt 7(13)	week 2	10.140	674.098	2.968	59.369	0.193	3.859
	Yogurt 8(13)	week 2	10.080	588.902	2.593	51.866	0.169	3.371
	Yogurt 9(13)	week 2	10.100	720.302	3.172	63.439	0.206	4.124
	Std. Dev.		0.042	66.657	0.294	5.871	0.019	0.382
	Average		10.110	661.101	2.911	58.225	0.189	3.785
→ 4-Jul	Standard 90	week 3	10.15	2163.251	10	100	0.65	6.5
4-Jul	Yogurt 10(13)	week 3	9.920	598.725	2.768	55.354	0.180	3.598
4-Jul	Yogurt 11(13)	week 3	9.970	618.049	2.857	57.141	0.186	3.714
4-Jul	Yogurt 12(13)	week 3	9.930	595.294	2.752	55.037	0.179	3.577
	Std. Dev.		0.026	12.268	0.057	1.134	0.004	0.074
	Average		9.940	604.023	2.792	55.844	0.181	3.630
→ 10-Jul	Standard 92	Week 4	10.4	2388.828	10	100	0.65	6.5
10-Jul	Yogurt 13(13)	Week 4	10.230	640.836	2.683	53.653	0.174	3.487
10-Jul	Yogurt 14(13)	Week 4	10.080	723.655	3.029	60.587	0.197	3.938
10-Jul	Yogurt 15(13)	Week 4	10.120	700.025	2.930	58.608	0.190	3.810
	Std. Dev.		0.078	42.663	0.179	3.572	0.012	0.232
	Average		10.143	688.172	2.881	57.616	0.187	3.745

Recovery calculation of vitamin D in yogurt

No of moles of vitamin D in milk/gm=3.872 n moles

No of moles of vitamin D in yogurt/gm= 3.789 n moles

Retention of vitamin D in yogurt= 3.789/3.872x100= 97.856%

Appendix A6

Stability calculation of vitamin D in yogurt over 4 weeks

Date	Sample ID	Time Days	RT	Area	IU/Inj	IU per gm	Moles/Inj n moles	Moles/gm n moles
→ 15-Jun	Stanadard 76		10.03	2142.248	10	100	0.65	6.5
15-Jun	Milk yogurt 1(13)	week 0	10.170	1299.472	6.066	60.659	0.394	3.943
15-Jun	Milk yogurt 2(13)	week 0	10.070	1273.745	5.946	59.458	0.386	3.865
15-Jun	Milk yogurt 3(13)	week 0	10.110	1255.215	5.859	58.593	0.381	3.809
	Std. Dev.		0.050	22.226	0.104	1.037	0.007	0.067
	Average		10.117	1276.144	5.957	59.570	0.387	3.872
15-Jun	Yogurt 1(13)	week 0	10.180	602.732	2.814	56.271	0.183	3.658
15-Jun	Yogurt 2(13)	week 0	10.230	644.973	3.011	60.215	0.196	3.914
15-Jun	Yogurt 3(13)	week 0	10.200	625.333	2.919	58.381	0.190	3.795
	Std. Dev.		0.025	21.138	0.099	1.973	0.006	0.128
	Average		10.203	624.346	2.914	58.289	0.189	3.789
→ 25-Jun	Standard 82		10.28	2249.05	10	100	0.65	6.5
	Yogurt 4(13)	week 1	10.200	669.181	2.975	59.508	0.193	3.868
	Yogurt 5(13)	week 1	10.180	683.913	3.041	60.818	0.198	3.953
	Yogurt 6(13)	week 1	10.220	651.095	2.895	57.900	0.188	3.763
	Std. Dev.		0.020	95.000	0.073	1.462	0.005	0.095
	Average		10.200	668.063	2.970	59.408	0.193	3.862
→29-Jun	Standard 85	week 2	10.22	2270.865	10	100	0.65	6.5
	Yogurt 7(13)	week 2	10.140	674.098	2.968	59.369	0.193	3.859
	Yogurt 8(13)	week 2	10.080	588.902	2.593	51.866	0.169	3.371
	Yogurt 9(13)	week 2	10.100	720.302	3.172	63.439	0.206	4.124
	Std. Dev.		0.042	66.657	0.294	5.871	0.019	0.382
	Average		10.110	661.101	2.911	58.225	0.189	3.785
→ 4-Jul	Standard 90	week 3	10.15	2163.251	10	100	0.65	6.5
4-Jul	Yogurt 10(13)	week 3	9.920	598.725	2.768	55.354	0.180	3.598
4-Jul	Yogurt 11(13)	week 3	9.970	618.049	2.857	57.141	0.186	3.714
4-Jul	Yogurt 12(13)	week 3	9.930	595.294	2.752	55.037	0.179	3.577
	Std. Dev.		0.026	12.268	0.057	1.134	0.004	0.074
	Average		9.940	604.023	2.792	55.844	0.181	3.630
→ 10-Jul	Standard 92	Week 4	10.4	2388.828	10	100	0.65	6.5
10-Jul	Yogurt 13(13)	Week 4	10.230	640.836	2.683	53.653	0.174	3.487
10-Jul	Yogurt 14(13)	Week 4	10.080	723.655	3.029	60.587	0.197	3.938
10-Jul	Yogurt 15(13)	Week 4	10.120	700.025	2.930	58.608	0.190	3.810
	Std. Dev.		0.078	42.663	0.179	3.572	0.012	0.232
	Average		10.143	688.172	2.881	57.616	0.187	3.745

Stability calculation of vitamin D in yogurt over 4 weeks

After week 1 = 3.862/3.789x100 = 101.92%

After week 2 = 3.785/3.789x100 = 99.89%

After week 3 = 3.630/3.789x100 = 95.80%

After week 4 = 3.745/3.789x100 = 98.83%

Appendix A7

Recovery calculation of vitamin D in ice cream

Date	Sample ID	Time Days	RT	Area	IU/Inj	IU per gm	Moles/Inj n moles	Moles/gm n moles
31-May	Standard 61		10.53	2150.684	10	100	0.65	6.5
31-May	Custard 1(2)	week 0	10.15	195.489	0.909	54.538	0.059	3.545
31-May	Custard 2(2)	week 0	10.2	192.806	0.896	53.789	0.058	3.496
31-May	Custard 3(2)	week 0	10.18	205.213	0.954	57.251	0.062	3.721
	Std. Dev.		0.025	6.528	0.030	1.821	0.002	0.118
	Average		10.17667	197.836	0.920	55.192	0.060	3.588
31-May	Ice cream 1(2)	week 0	10.22	199.082	0.926	55.540	0.060	3.610
31-May	Ice cream 2(2)	week 0	10.22	200.512	0.932	55.939	0.061	3.636
31-May	Ice cream 3(2)	week 0	10.23	193.26	0.899	53.916	0.058	3.505
	Std. Dev.		0.006	3.841	0.018	1.072	0.001	0.070
	Average		10.223	197.618	0.919	55.132	0.060	3.584
2-Jun	Standard 64	week 1	10.167	2118.83	10	100	0.65	6.5
2-Jun	Ice cream 4(2)	week 1	9.87	186.574	0.881	52.833	0.057	3.434
2-Jun	Ice cream 5(2)	week 1	10.15	198.861	0.939	56.312	0.061	3.660
2-Jun	Ice cream 6(2)	week 1	10.1	197.607	0.933	55.957	0.061	3.637
	Std. Dev.		0.149	6.761	0.032	1.915	0.002	0.124
	Average		10.04	194.347	0.917	55.034	0.060	3.577
2-Jun	Standard 64	week 2	10.167	2118.83	10	100	0.65	6.5
2-Jun	Ice cream 7(2)	week 2	10.15	179.72	0.848	50.892	0.055	3.308
2-Jun	Ice cream 8(2)	week 2	10.22	199.253	0.940	56.423	0.061	3.668
2-Jun	Ice cream 9(2)	week 2	10.17	216.381	1.021	61.274	0.066	3.983
	Std. Dev.		0.036	18.344	0.087	5.194	0.006	0.338
	Average		10.18	198.451	0.937	56.196	0.061	3.653
2-Jun	Standard 64	week 4	10.167	2118.83	10	100	0.65	6.5
2-Jun	Ice cream 10(2)	week 4	10.18	201.307	0.950	57.005	0.062	3.705
2-Jun	Ice cream 11(2)	week 4	10.08	198.338	0.936	56.164	0.061	3.651
2-Jun	Ice cream 12(2)	week 4	10.07	182.49	0.861	51.677	0.056	3.359
	Std. Dev.		0.061	10.116	0.048	2.865	0.003	0.186
	Average		10.11	194.045	0.916	54.949	0.060	3.572

Recovery calculation of vitamin D in ice cream

No of moles of vitamin D in ice cream mix custard/gm=3.588 n moles

No of moles of vitamin D in ice cream/gm= 3.584 nmoles

Recovery of vitamin D in ice cream= 3.584/3.588x100 = 99.88%

Appendix A8

Example of overrun calculation for the ice cream

Density of mix was calculated with the help of following equation:

$$Density\ of\ mix = \frac{Mass\ of\ mix}{Volume\ of\ mix}$$

Where,

Mass of (50 ml) mix = 54.95 g
Volume of mix = 50 ml

$$Density\ of\ mix = \frac{54.95}{50} = 1.099\ \text{g/ml or} \sim 1.1\ \text{kg/L}$$

Overrun was calculated as follows:

$$\frac{(Volume\ of\ frozen\ mixture) - (Volume\ of\ unfrozen\ mixture)}{(Volume\ of\ unfrozen\ mixture)} \times 100$$

Where,

Volume of ice cream after overrun = 750 ml
Volume of ice cream mix= 500 ml

$$Overrun = \frac{(750) - (500)}{(500)} \times 100 = 50\%$$

Appendix A9

Stability calculation of vitamin D in ice cream over 4 weeks

Date	Sample ID	Time Days	RT	Area	IU/Inj	IU per gm	Moles/Inj n moles	Moles/gm n moles
31-May	Standard 61		10.53	2150.684	10	100	0.65	6.5
31-May	Custard 1(2)	week 0	10.15	195.489	0.909	54.538	0.059	3.545
31-May	Custard 2(2)	week 0	10.2	192.806	0.896	53.789	0.058	3.496
31-May	Custard 3(2)	week 0	10.18	205.213	0.954	57.251	0.062	3.721
	Std. Dev.		0.025	6.528	0.030	1.821	0.002	0.118
	Average		10.17667	197.836	0.920	55.192	0.060	3.588
31-May	Ice cream 1(2)	week 0	10.22	199.082	0.926	55.540	0.060	3.610
31-May	Ice cream 2(2)	week 0	10.22	200.512	0.932	55.939	0.061	3.636
31-May	Ice cream 3(2)	week 0	10.23	193.26	0.899	53.916	0.058	3.505
	Std. Dev.		0.006	3.841	0.018	1.072	0.001	0.070
	Average		10.223	197.618	0.919	55.132	0.060	3.584
2-Jun	Standard 64	week 1	10.167	2118.83	10	100	0.65	6.5
2-Jun	Ice cream 4(2)	week 1	9.87	186.574	0.881	52.833	0.057	3.434
2-Jun	Ice cream 5(2)	week 1	10.15	198.861	0.939	56.312	0.061	3.660
2-Jun	Ice cream 6(2)	week 1	10.1	197.607	0.933	55.957	0.061	3.637
	Std. Dev.		0.149	6.761	0.032	1.915	0.002	0.124
	Average		10.04	194.347	0.917	55.034	0.060	3.577
2-Jun	Standard 64	week 2	10.167	2118.83	10	100	0.65	6.5
2-Jun	Ice cream 7(2)	week 2	10.15	179.72	0.848	50.892	0.055	3.308
2-Jun	Ice cream 8(2)	week 2	10.22	199.253	0.940	56.423	0.061	3.668
2-Jun	Ice cream 9(2)	week 2	10.17	216.381	1.021	61.274	0.066	3.983
	Std. Dev.		0.036	18.344	0.087	5.194	0.006	0.338
	Average		10.18	198.451	0.937	56.196	0.061	3.653
2-Jun	Standard 64	week 4	10.167	2118.83	10	100	0.65	6.5
2-Jun	Ice cream 10(2)	week 4	10.18	201.307	0.950	57.005	0.062	3.705
2-Jun	Ice cream 11(2)	week 4	10.08	198.338	0.936	56.164	0.061	3.651
2-Jun	Ice cream 12(2)	week 4	10.07	182.49	0.861	51.677	0.056	3.359
	Std. Dev.		0.061	10.116	0.048	2.865	0.003	0.186
	Average		10.11	194.045	0.916	54.949	0.060	3.572

Stabili[illegible] calculation of vitamin D in ice cream over 4 weeks

After week 1 = 3.577/3.584x100 = 99.80%

After week 2 = 3.653/3.584x100 = 101.92%

After week 4 = 3.572/3.584x100 = 99.66%

Appendix A10

Cheddar cheese with vitamin D_3 added shows a peak (arrow) at 10.0 min. The mobile phase was a 49.5:49.5:1 mixture of methanol:acetonitrile:H_2O.