JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Vitamin D_3 Fortification, Quantification, and Long-Term Stability in Cheddar and Low-Fat Cheeses

DENNIS WAGNER,*,†,‡ DÉRICK ROUSSEAU,§ GLORIA SIDHOM,|| MICHEL POULIOT,⊥ PIERRE AUDET,⊥ AND REINHOLD VIETH†,‡

Department of Nutritional Sciences, University of Toronto, 150 College Street, Toronto, Ontario M5S 3E2, Canada, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada, Department of Chemistry and Biology, Ryerson University, 350 Victoria Street, Toronto, Ontario M5B 2K3, Canada, Department of Clinical and Chemical Pathology, National Research Centre, Post Office Box 5216, 11771 Heliopolis West, Cairo, Egypt, and Agropur Coopérative, 510 Principale Street, Granby, Québec J2G 7G2, Canada

Considering the widespread insufficiency of vitamin D, the fortification of additional foods with vitamin D is warranted. The objective of this research was to assess the feasibility of vitamin D_3 fortification in natural hard cheeses. We examined the recovery, distribution, long-term retention, and heat stability of the vitamin in industrially made fortified Cheddar and low-fat cheeses. The results indicated that the vitamin D_3 did not degrade during processing, over 1 year of ripening (3−8 °C), or after thermal treatment at 232 °C for 5 min. Vitamin D_3 recovery in the fortified Cheddar and low-fat cheeses were, respectively, 91 and 55% of the vitamin D_3 added to the milk used to make each cheese. The remaining vitamin D_3 was entrained in the whey. The vitamin D_3 was uniformly distributed throughout the blocks of cheese. The fortification process did not alter the yield, chemical composition, or flavor of the Cheddar cheese. We conclude that industrially manufactured Cheddar and low-fat cheeses are suitable for vitamin D_3 fortification.

KEYWORDS: Vitamin D; fortification; Cheddar; cheese; dairy food; stability

INTRODUCTION

Vitamin D plays a vital role in bone metabolism and many cellular and immunological processes. Low levels of vitamin D have been associated with several chronic and infectious diseases, including cancer (*1*), multiple sclerosis (*2*), diabetes (*3*), cardiovascular disease (*4*), osteoporosis (*5*), and microbial infections (*6*). A growing number of studies have reported widespread vitamin D deficiency in apparently healthy populations worldwide (*7*).

The diet contains very few natural sources of vitamin D. North American populations require fortified foods and dietary supplements to meet their vitamin D needs during times of insufficient sunlight. Vitamin D fortification is mandatory in Canada for beverage milk (100 IU/250 mL) and margarine (53 IU/10 g) (*8*) and optional in the United States for milk, breakfast cereals, and calcium-fortified fruit juices (40−140 IU/serving) (*9*). However, current North American fortification practices are ineffective in preventing vitamin D insufficiency because fortified foods provide inadequate amounts of vitamin D and are often under-fortified (*10, 11*). Furthermore, milk consumption has declined significantly since the 1980s (*12*), and lactose intolerance is a common problem (*13*), particularly in populations that are at the greatest risk of vitamin D deficiency (e.g., Blacks, Asians, and Aboriginals).

Information on vitamin D fortification in foods other than milk is limited. The stability of vitamin D has been reported in bread (*14*), processed dairy products (*15*), and orange juice (*16*). However, individual studies have often proven contradictory, and it appears that vitamin D stability depends upon the composition, processing, and local environment (e.g., pH, A_w, and temperature) of the food.

In North America, industrial milk destined for cheese manufacture is not fortified with vitamin D and fortification of the final product is not required (*17*). Cheese is an ideal candidate for vitamin D fortification for several reasons: (i) the milkfat in cheese may enhance the stability and absorption of vitamin D (*18*); (ii) cheese is a good source of calcium and can be consumed by many lactose-intolerant individuals because it may contain negligible amounts of lactose; (iii) cheese is a widely consumed food in North America (*19*); and (iv) cheese production is expected to grow with the development and

* To whom correspondence should be addressed. Telephone: 416-586-4800 ext. 2726. Fax: 416-586-8628. E-mail: dennis.wagner@utoronto.ca.
† University of Toronto.
‡ Mount Sinai Hospital.
§ Ryerson University.
|| National Research Centre.
⊥ Agropur Coopérative.

10.1021/jf801316q CCC: $40.75
Published on Web 08/13/2008

consumption of new varieties (*12*). For these reasons, we chose to fortify Cheddar cheese and a low-fat alternative with vitamin D_3 (cholecalciferol), which is more stable and potent compared to vitamin D_2 (*20*).

To date, three studies have examined vitamin D_3 fortification in cheese. Upreti et al. (*21*) found no losses of vitamin D_3 during the manufacture of pasteurized processed cheese or over 9 months of storage. Banville et al. (*22*) reported 40−60% recovery of vitamin D_3 in Cheddar cheese, depending upon the fortification method used, attributing the losses to breakdown by lactic acid production and/or oxidation. In contrast, Kazmi et al. (*15*) described a highly reproducible method, which produced near total recovery of vitamin D_3 in a laboratory-scale Cheddar-cheese-like matrix with a small portion lost in the whey. The latter two studies had important limitations. Given that Cheddar is often aged for years, the stability of vitamin D_3 in the cheeses was not monitored over a ripening period sufficiently long to be industrially relevant. As well, in previous studies, the cheeses were not manufactured at an industrially relevant scale, making it difficult to generalize their findings to actual production conditions.

Our aim was to assess the feasibility of larger scale fortification of natural hard cheeses with vitamin D_3 and to confirm the stability of the vitamin D with cooking. We examined the recovery, distribution, long-term retention, and heat stability of the vitamin in industrially made fortified Cheddar and low-fat cheeses.

MATERIALS AND METHODS

Vitamin D_3 Fortification Procedure. *Fortification of Milk.* The milk fortification phase of this work was conducted at the Food Research and Development Centre (FRDC) (Saint-Hyacinthe, Québec, Canada). Raw skim [0.8% fat (w/w)] and whole [3.8% fat (w/w)] milks were obtained from an Agropur dairy farm and delivered to the FRDC; these milks were nonhomogenized, unfortified, and unpasteurized. The fortifying solution used was a vitamin D_3 premix (205 000 IU mL^{-1}) (Kingsway Chocolate Co. Ltd., Mississauga, Ontario, Canada), consisting of a concentrated water-dispersible liquid preparation of vitamin D_3 blended in a food-grade emulsifier base. We chose this product because it is used in the dairy industry to fortify milk and it has been shown to exhibit superior recovery and retention of vitamin D_3 in processed dairy products compared to crystalline vitamin D_3 dissolved in ethanol (*15*). The vitamin premix was stored at 3−8 °C and stirred thoroughly before usage.

The preliminary production step involved the addition of the premix to whole milk or skim milk destined for regular-fat Cheddar or low-fat cheese manufacture. The amount of premix added to the cheesemilks was based on (i) the estimated vitamin D_3 recovery in the industrially made cheeses [i.e., ∼80% for Cheddar cheese and ∼70% for low-fat cheese (w/w)], (ii) the desired vitamin D_3 content of the cheeses (i.e., 28 000 IU vitamin D_3 per 30 g serving), and (iii) the expected yield of the cheeses (∼10% for Cheddar and ∼7% for low-fat). For whole milk fortification, 180 mL of the premix was diluted with 900 mL of whole milk drawn from a vat containing 350 kg of whole milk (1:5 vitamin D_3/whole milk ratio). The mixture was then returned to the original volume of milk and stirred thoroughly by a high-speed agitator mounted on the vat, until just before a vortex appeared in the milk. This mixing procedure was repeated 3 times before pasteurizing the milk. The same procedure was used for the fortification of skim milk. The final expected vitamin D_3 concentration of each cheesemilk was ∼100 IU/g.

Cheese Manufacture. Cheese production was also carried out at the FRDC. Single batches of three different types of cheese (∼20 kg each) were made: (1) unfortified regular-fat Cheddar cheese (used as the control), (2) fortified regular-fat Cheddar cheese, and (3) fortified low-fat cheese. All cheeses were manufactured by Agropur using typical industrial cheesemaking methodologies.

All cheesemilks, with or without vitamin D_3, were pasteurized (72 °C for 16 s) prior to cheesemaking. All cheeses were made from nonhomogenized raw milks. For Cheddar cheese manufacture, pasteurized whole milk (210 kg) was collected at 32 °C and 26.25 mL of calcium chloride solution (0.0125% w/w) was added to it. The cheesemilk was then inoculated with 1.09 kg of mesophilic starter culture (0.52% w/w, Starter EXP5, Agropur collection). After 60 min of curing at 32 °C under constant stirring, 21 mL of rennet (0.01% w/w, Chymax, double strength, Hansen's) was added to the mixture and stirred. Coagulation, pH, and temperature were monitored throughout the process. The curd was cut 30 min after renneting, stirred gently for 15 min, and heated from 32 to 38 °C in 30 min. It was then stirred at 38 °C until the pH was 6.1, after which the whey was drawn. Afterward, the curd was divided into two parts and baked in the vat at 38.5 °C (cheddaring). During cheddaring, the cheese mass was cut into pieces and turned every 20 min. The curd was milled when a pH of 5.4 was reached. It was then salted (2.4% w/w), wrapped in cheesecloth, and transferred into two rectangular molds. Each mold contained ∼10 kg of curds. The salted curd was pressed at room temperature at an applied pressure of 15 psi for 35 min and then at 40 psi for 3 h. After pressing, the pressed whey was drawn. The pressed cheeses were vacuum-packaged and stored in a refrigerator (3−8 °C). A similar procedure was employed for the manufacture of fortified low-fat cheese, with two additional steps: predrawing and curd washing. Because of its proprietary nature, further details regarding the manufacturing procedures for the low-fat cheese are not available for publication.

Analysis. *Sampling of Milk, Whey, and Cheese for Vitamin D_3 Analysis.* Milk and whey samples (80 mL) were collected at various stages of fortified cheese manufacture to track vitamin D_3 content throughout production. We obtained samples of whole and skim milk at three points: (1) before the addition of vitamin D_3, (2) after the addition of vitamin D_3 but before pasteurization, and (3) after the addition of vitamin D_3 and after pasteurization. Whey was collected at two stages of cheese manufacture: (1) after milk curdling and drawing of the whey (total whey) and (2) after pressing of the cheese (pressed whey). Milk and whey samples were stored at 3−8 °C until they were analyzed for vitamin D_3 content (as described below).

After production, the cheeses were cut into smaller 2 kg blocks, vacuum-packaged in sterile plastic food bags, and stored at 3−8 °C. At various storage intervals (0.5, 3, 6, 9, and 12 months), we randomly selected a new cheese block and assayed it for vitamin D_3 to assess the storage stability and distribution of vitamin D_3 in the cheeses. These experiments were performed in triplicate.

After 12 months of ripening, we tested the heat stability of vitamin D_3 in the fortified cheeses. To do so, four 20 g slices were cut from randomly selected 2 kg blocks of the fortified cheeses. Each slice was placed in a separate glass dish and heated in a conventional kitchen oven (Maytag, Toronto, Ontario, Canada) at two different temperatures until the cheese melted: (1) 232 °C for 5 min, as per Schreiber's cheese meltability test (*23*), and (2) 100 °C for 12 min. We tested the lower temperature because the 232 °C setting can be excessive because hard cheeses usually melt at temperatures below 100 °C. For example, when baking pizza, the cheese temperature rarely exceeds 100 °C (*24*). After 30 min of cooling, the melted cheeses were shredded and 0.2 g samples were analyzed for vitamin D_3, as described below. Moisture losses caused by evaporation were measured with a HB43 halogen moisture analyzer (Mettler Toledo, Greifensee, Switzerland), and reported vitamin D_3 concentrations were adjusted accordingly. The heat stability tests were all performed in triplicate.

Procedure for Analysis of Vitamin D_3 Content. Samples (1 g) of fluid milk and whey were used as for saponification and lipid extraction, as per Kazmi et al. (*15*). For the cheeses, three random sites were drawn from 2 kg cheese blocks and shredded with a fine cheese grater. Samples (200 mg) of the shredded cheese were weighed into 10 mL test tubes and mixed with 0.8 mL of distilled water.

All milk, whey, and cheese−water samples (1 g) were heat-saponified with 0.5 mL of aqueous potassium hydroxide (KOH) (60% w/v), and the lipids were extracted with 3.75 mL of methanol/chloroform, according to the method of Kazmi et al. (*15*), with one modification: after heated saponification and subsequent cooling, 25-hydroxyvitamin D [25(OH)D] (Sigma, St. Louis, MO) was added to the mixtures to serve as an internal standard.

Vitamin D_3 was quantified using an Agilent 1100 series isocratic HPLC, equipped with detectors set at 266 and 228 nm. Absorbance data were recorded to a computer, and ChemStations software (Agilent, Mississauga, Ontario, Canada) was used to integrate the peak areas. The retention time of vitamin D_3, 25(OH)D internal standard, and instrument calibration were established using known concentrations of crystalline vitamin D_3 and 25(OH)D in mobile phase ($1-35\ \mu g\ mL^{-1}$). A C_{18} HPLC column (5 μm particles, 4.6 mm i.d., 20 cm length) (Grace Vydac, Toronto, Ontario, Canada) was used throughout the HPLC analysis. Operating conditions were: ambient temperature (21 °C); mobile phase, methanol/acetonitrile/water (49.5:49.5:1, by vol); flow rate, 1 mL min^{-1}; and injection volume, 50 μL. By recording absorptions at both 228 and 266 nm, the method provided additional confirmation for the presence of vitamin D_3, because the peak height for vitamin D compounds at 266 nm is twice as large as at 228 nm. Areas of vitamin D_3 peaks were used as an index of vitamin D_3 concentrations in the samples.

Chemical Composition of Milks, Wheys, and Cheeses. Chemical and microbiological analyses were carried out by Agropur at their accredited Central Analysis Laboratory (Granby, Québec, Canada). The fat content of the milks and wheys was determined by the Mojonnier method (*25*), and cheese fat was measured by the Babcock method (*26*). Total protein in milks, wheys, and cheeses was determined by the Kjeldahl method (*25*). Solids content of milks, wheys, and cheeses was determined by the Mojonnier methods (*25*). Moisture in milks, wheys, and cheeses was measured gravimetrically (100 °C for 4 h) following the Association of Official Analytical Chemists (AOAC) procedure (*25*). Salt content of the cheeses was determined by the Volhard method (*25*). Calcium content of the cheeses was measured by atomic absorption spectroscopy (*25*). Microbial analyses (total coliforms, staphylococci, yeasts, and molds) in the cheeses were performed using standard laboratory methods (*25*). All assays pertaining to chemical composition were performed in singleton for the milks and wheys and in duplicate for the cheeses (with the exception of calcium).

After 2 months of cheese ripening, volunteer taste panellists (untrained) were asked to informally evaluate the effect of the added vitamin D on the flavor of the fortified Cheddar compared to the control cheese. This was not a sophisticated evaluation of cheese per se but rather a subjective taste test to address the simple question of whether vitamin D altered the taste of the cheese. To do this, 10 volunteers each consumed two samples of Cheddar cheese (one fortified and the other unfortified) without knowing which of the samples contained vitamin D. The volunteers drank a glass of water in between each sampling to rinse the palate. After having consumed both cheese samples, they were asked if the samples tasted differently from one another and their responses were recorded. Cheese flavor was also assessed on the basis of detecting off- or bad flavors by Agropur personnel (head cheesemaker and technologist). All sensory evaluations were performed in duplicate.

Statistical Analyses. The results are presented as means ± standard deviations (SDs). All data were analyzed with SPSS software (version 13.0; SPSS, Inc., Chicago, IL). Differences in the chemical composition and recoveries of the cheeses were analyzed with one-way analysis of variation (ANOVA). Differences in vitamin D_3 content of the fortified milks, cheeses, and wheys were analyzed with *t* tests. Storage stability and distribution of vitamin D_3 within the fortified cheeses were statistically analyzed with repeated-measures ANOVA. Paired *t* tests were used to assess differences in vitamin D_3 content in cheesemilks resulting from pasteurization. Differences in the vitamin D_3 content of cheeses with or without heat treatment were analyzed with paired *t* tests. The criterion for statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Milk and Whey Composition. The cheesemilks and wheys were analyzed to determine whether the fortification process would alter their yield or chemical composition. **Table 1** shows the fat, protein, and solids content in the cheesemilks and wheys with and without vitamin D_3 fortification.

The fortification process did not change the yield or chemical composition of the whole and skim milks. Similarly, these properties were not altered by fortification in the Cheddar wheys. Our results indicated that vitamin D_3 fortification did not appreciably affect the yield or chemical composition of milk or whey in Cheddar cheesemaking. These findings are similar to those of Banville et al. (*22*), who found no differences in the chemical composition of control and experimental milks and wheys when using a similar vitamin D_3 emulsion to the one that we used.

Table 1. Chemical Composition of Milks and Wheys[a]

products	unfortified Cheddar cheese	fortified Cheddar cheese	fortified low-fat cheese
	Unfortified Milk		
mass (kg)	350.00	350.00	350.00
fat (% w/w)	3.83	3.83	0.78
protein (% w/w)	3.29	3.30	3.51
solids (% w/w)	12.48	12.41	9.67
	Fortified Milk		
mass (kg)	N/A[b]	350.00	350.00
fat (% w/w)	N/A[b]	3.86	0.80
protein (% w/w)	N/A[b]	3.29	3.54
solids (% w/w)	N/A[b]	12.46	9.71
	Total Whey		
mass (kg)	183.45	181.60	242.30
fat (% w/w)	0.52	0.37	0.14
protein (% w/w)	0.90	0.80	0.96
solids (% w/w)	6.98	6.18	6.71
	Pressed Whey		
mass (kg)	1.84	1.57	4.50
fat (% w/w)	3.02	2.47	0.11
protein (% w/w)	1.03	1.08	1.02
solids (% w/w)	15.82	13.43	8.47

[a] Statistical significance testing not performed because values were measured once. [b] Does not apply to unfortified Cheddar cheese.

Table 2. Yield, Recoveries, and Chemical Composition of Cheeses[a]

	unfortified Cheddar cheese	fortified Cheddar cheese	fortified low-fat cheese
mass (kg)	21.68	21.63	18.30
% yield	10.33	10.30	7.62
fat (% w/w)	33.00 ± 1.41[b] a	33.00 ± 0.03 a	7.00 ± 0.03 b
% recovery[c]	88.96 ± 3.81 a	89.21 ± 0.08 a	66.72 ± 0.27 b
protein (% w/w)	24.39 ± 0.31 a	24.41 ± 0.16 a	34.55 ± 0.51 b
% recovery[c]	76.53 ± 0.98 a	76.42 ± 0.48 a	75.05 ± 1.11 a
solids (% w/w)	62.02 ± 0.52 a	62.72 ± 0.48 a	49.6 ± 0.61 b
% recovery[c]	51.31 ± 1.54 a	51.89 ± 1.44 a	38.75 ± 1.80 b
moisture (% w/w)	37.98 ± 0.01 a	37.28 ± 0.35 a	50.40 ± 0.43 b
salt (% w/w)	1.63 ± 0.10 a	1.70 ± 0.04 a	1.93 ± 0.06 a
calcium (ppm)	7256.00	7646.00	8541.00

[a] Mean of two replicates (unless indicated otherwise). Means within a row with different letters differ ($p < 0.05$); the absence of a letter indicates that statistical testing was not performed because the value was measured once. [b] Mean ± standard deviation (all such values). [c] X recovery in cheese (%) = (cheese X × kg of cheese/milk X × kg of milk) × 100, where X = fat, protein, or solids.

Cheese Composition. The chemical composition of the fortified Cheddar and low-fat cheeses conformed to the legal standards of identity described in Food and Drug Regulations of Health Canada (*8*). The yields, recoveries, and chemical compositions of the fortified and unfortified Cheddars were not significantly different ($p > 0.60$) (**Table 2**). The low-fat and Cheddar cheeses differed substantially in their yield, recoveries, and chemical composition, as is expected in industrial cheesemaking (*27*). Microbiological analysis showed that the total counts of coliforms, staphylococci, yeast, and mold were well below the maximum allowable levels in all cheeses (data not shown).

Table 3. Vitamin D_3 Concentrations in Fortified Milks, Wheys, and Cheeses[a]

	fortified Cheddar cheese	fortified low-fat cheese
milk (IU/g)		
before pasteurization	94.45 ± 1.20[b] a	93.83 ± 2.95 a
after pasteurization	94.53 ± 0.85 a	94.38 ± 2.24 a
cheese (IU/g)	833.88 ± 19.83 a	676.09 ± 11.09 b
whey (IU/g)		
total	17.56 ± 1.62 a	48.78 ± 5.05 b
pressed	44.33 ± 5.21 a	32.57 ± 3.35 b
recovery (%) in cheese[c]	90.90 ± 2.16 a	54.96 ± 0.67 b
recovery (%) in whey[d]		
total	16.07 ± 1.49 a	52.33 ± 5.42 b
pressed	0.35 ± 0.04 a	0.65 ± 0.07 b
total recovery (%)[e]	107.32 ± 3.10 a	107.76 ± 4.57 a

[a] Mean of six replicates. Means within a row with different letters differ ($p < 0.05$). [b] Mean ± standard deviation (all such values). [c] Recovery in cheese (%) = (vitamin D_3 in cheese × kg of cheese/vitamin D_3 in milk × kg of milk) × 100. [d] Recovery in whey (%) = (vitamin D_3 in whey × kg of whey/vitamin D_3 in milk × kg of milk) × 100. [e] Total recovery (%) = (recovery in cheese + recovery in total whey + recovery in pressed whey).

The flavor of the fortified and unfortified Cheddar cheeses after 2 months of ripening were indistinguishable by volunteer taste testers and characterized as "good, not intense, with a light bitterness" by Agropur personnel. This observation can be explained by the negligible quantity of vitamin D_3 added to the cheeses. Furthermore, vitamin D is commonly added to milk and other foods without imparting off-flavors. Expectedly, the flavor of the fortified low-fat cheese was different and distinguishable from the Cheddars and classified as "weak, not intense, with a light bitterness" by Agropur staff. Our results demonstrated that vitamin D_3 fortification did not affect the yield, recoveries, chemical composition, or flavor of the Cheddar cheese.

A limitation of this study was that an unfortified low-fat cheese was not produced because it was unfeasible for us to do so. Therefore, without a control to serve as a comparison, we could not specifically determine whether the fortification process affected the yield, chemical composition, or flavor of the low-fat cheese. However, Agropur advised us that these attributes were very similar to those pertaining to previous lots of unfortified low-fat cheese. There is also no plausible way that the milligram amounts of vitamin D would have affected the chemical or palatable quality in the final product.

Vitamin D_3 Recovery in the Fortified Cheeses. After 2 weeks of ripening, the mean vitamin D_3 concentration was 833.88 ± 19.83 IU/g in the fortified Cheddar cheese and 676.09 ± 11.09 IU/g in the fortified low-fat cheese ($p < 0.001$), with vitamin D_3 recoveries of 91 and 55% in the cheeses ($p < 0.001$), respectively (**Table 3**). The remaining vitamin D_3 was entrained into the wheys. These results are very similar to those of Kazmi et al. (*15*), who documented 90% recovery of vitamin D_3 in a laboratory-scale Cheddar cheese-like matrix and 7−9% loss into whey. In contrast, Banville et al. (*22*) reported vitamin D_3 losses of ~38, 57, and 59% when Cheddar cheese was fortified with vitamin D_3 via liposomes, emulsions, or cream solutions, respectively. Moreover, we and Kazmi et al. (*15*) detected a small amount of vitamin D_3 in the whey, whereas Banville et al. (*22*) did not. Those authors attributed vitamin D_3 losses to degradation during the cheesemaking process. We found that vitamin D_3 was not destroyed by cheesemaking because the full amount of added vitamin D_3 was recovered (cheese plus whey). These inconsistencies are likely related to the vitamin D_3 extraction technique used by Banville et al. (*22*). We used the method of Kazmi et al. (*15*) and modified it slightly to optimize extraction efficiency in difficult-to-extract cheese samples. The actual cheesemaking methodologies employed in our study were similar to the ones used by Banville et al. (*22*); therefore, it is unlikely that the observed differences in recovery between the studies were the result of the processing techniques used.

To our knowledge, the fortification of reduced-fat cheese with vitamin D_3 has never been reported. The lower vitamin D_3 content in the fortified low-fat cheese is consistent with a lower fat recovery ($p < 0.05$, **Table 2**) and a lower vitamin D_3 recovery ($p < 0.001$) in the cheese, as well as higher loss in both the total ($p < 0.001$) and pressed ($p = 0.002$) wheys compared to the fortified Cheddar (**Table 3**). Given its fat-soluble nature, the vitamin D_3 was retained better in the Cheddar cheese, which had a near 5-fold higher fat content than the low-fat cheese (**Table 2**). Speculatively, in the low-fat cheese, more vitamin D_3 may have been entrained into the fat present (0.11% w/w) in the whey. Furthermore, vitamin D_3 has been shown to bind to β-lactoglobulin (β-LG), which is a key whey protein (*28, 29*). During the production of fortified low-fat cheese, where the vitamin D_3 is missing most of its protective fat matrix, the vitamin D_3 may be stabilized by β-LG and other whey proteins that protect it from an otherwise polar environment. Overall, the vitamin D_3 recovery in the fortified low-fat cheese (55%) was high and, if necessary, might be improved by microencapsulating the vitamin D_3 in liposomes, as performed by Banville et al. (*22*).

When our results are taken together, they demonstrate that the vitamin D_3 added to fluid milk is fully recovered in the cheese and whey. Although there were higher vitamin D_3 losses in the whey in the fortified low-fat cheese, its recovery in the cheese was considered acceptable. Therefore, we conclude that the conditions used in the manufacturing of fortified Cheddar and low-fat cheeses were not detrimental to vitamin D_3.

Our protocol had limitations. First, we did not repeat the cheese fortification experiments (i.e., only a single batch of each cheese was made). This was because the cheeses were manufactured industrially, which makes replicating the experiment less feasible compared to a laboratory-scale production. Furthermore, our previous research obtained 90% (± 3% SD between preparations) recovery of vitamin D_3 in laboratory-scale Cheddar cheese (*15*), and the present 91% recovery of vitamin D from Cheddar cheese on a larger scale is essentially identical. In essence, the present study can be thought of as an extension of this older "proof-of-principle" investigation, moving from the laboratory scale to a more industrially relevant setting. Even so, it will be important for others to assess recovery of vitamin D in their applications. Another limitation is that the amount of vitamin D_3 used for fortification was substantially higher than what would be added to foods. Our rationale for this high level was mainly methodological; the fortified cheeses were used for a clinical trial, investigating the bioavailability of vitamin D_3 from the cheeses (*30*). We chose a vitamin D_3 dose that was safe and effective (*31*) because this would produce increases in vitamin D status (measured as serum 25-hydroxyvitamin D) that would be large enough to permit detection of potential differences in bioavailability. Our extraction method is indeed capable of detecting lower concentrations of vitamin D_3 that are closer to legal fortification limits. There is no reason to suspect that a lower fortification level would have changed the outcome of the research presented here.

Table 4. Stability and Distribution of Vitamin D_3 in Fortified Cheeses through 1 Year of Refrigerated Storage[a]

type of cheese (cheese block)	storage period: 14 days (block 1)	15 days (block 2)	3 months (block 3)	6 months (block 4)	9 months (block 5)	12 months (block 6)
fortified Cheddar cheese (IU/g)	831.64 ± 18.91[b] a	836.12 ± 24.71 a	829.60 ± 41.63 a	833.68 ± 22.09 a	827.57 ± 39.11 a	830.21 ± 34.14 a
fortified low-fat cheese (IU/g)	678.55 ± 12.22 b	673.60 ± 11.82 b	665.25 ± 15.62 b	669.72 ± 8.13 b	674.78 ± 15.59 b	670.45 ± 16.01 b

[a] Mean of three replicates. Means within a row with different letters differ ($p < 0.05$); there were no differences across storage periods or cheese blocks. [b] Mean ± standard deviation (all such values).

Long-Term Stability of Vitamin D_3 in the Fortified Cheeses. Given that cheese is often aged for long periods of time (up to 4 or 5 years in some cases), it was important to characterize the storage stability of vitamin D_3 in the fortified cheeses. **Table 4** shows the vitamin D_3 content of the fortified Cheddar and low-fat cheeses over a 1 year ripening period. During this time, the cheeses evolved from a mild (<3 months) to a medium (6 months) to an old (>12 months) age classification. The vitamin D_3 concentrations in the fortified cheeses were not significantly different at any of the time points ($p > 0.30$), indicating that the vitamin D_3 was stable during ripening. To our knowledge, the long-term stability of vitamin D_3 in fortified cheeses has never been reported in the literature. Kazmi et al. (*15*) and Banville et al. (*22*) followed the vitamin D_3 content in fortified Cheddar cheese over shorter storage periods and found that vitamin D_3 was stable for 3−5 months. However, in the latter study, the vitamin D_3 content in the fortified cheese decreased by 16% after 5 months of ripening. Similar to their recovery results, these losses of vitamin D_3 were probably due to an inferior vitamin D_3 extraction method. Conversely, Upreti et al. (*21*) reported no loss of vitamin D_3 in processed cheese during refrigerated storage for 9 months.

Distribution of Vitamin D_3 in the Fortified Cheeses. It was important to determine how well the vitamin D_3 was distributed in the cheese mass. In theory, the consumption of large amounts of cheese with an uneven vitamin D_3 distribution could create a potential for toxicity. Therefore, we analyzed the vitamin D_3 content in six different randomly selected 2 kg cheese blocks cut from 20 kg batches. These cheese blocks were sampled at three random sites after 2 weeks of ripening and every 3 months thereafter (**Table 4**). There were no significant differences in vitamin D_3 concentration in any of the fortified cheese blocks shortly after production or during storage ($p > 0.30$). These results indicated that the vitamin D_3 was evenly distributed in the fortified cheeses, which agrees with the findings of Upreti et al. (*21*). The even distribution of vitamin D_3 in the cheese mass was probably facilitated by the easily dispersible emulsified form of vitamin D_3 that was used to fortify the milk, the constant mixing that occurs in the cheese vats during cheesemaking, and the uniform distribution of fat in the cheese.

Heat Stability of Vitamin D_3 in the Pasteurized Milks and Cheeses. Prior to cheesemaking, we collected samples of fortified milk before and after pasteurization to determine whether the pasteurization conditions affected vitamin D_3 content. Pasteurization (72 °C for 16 s) did not affect the vitamin D_3 concentrations in either of the fortified cheesemilks ($p > 0.50$) (**Table 3**). Similarly, Krauss et al. (*33*) reported that vitamin D in natural or fortified foods was stable during pasteurization at 62.8 °C for 30 min or sterilization at 115.6 °C for 15 min.

Hard cheeses are commonly added to a variety of different foods, including pizzas, hamburgers, and pasta. In many cases, the cheese is melted at high temperatures. Therefore, we tested the stability of vitamin D_3 in the fortified cheeses under practical heat treatments. After adjusting for minor moisture losses because of evaporation that occurred at 232 °C, we found no changes in vitamin D_3 content after heating the cheeses at 100 and 232 °C (**Table 5**). These findings are different from those of Upreti et al. (*21*), who reported vitamin D_3 losses of ∼25−30% in fortified process cheese heated for 5 min at 232 °C. Their lower recoveries could have two explanations. First, the manufacture of processed cheese is substantially different from the production of natural cheeses, such as Cheddar. With processed cheese, the vitamin D_3 may not be incorporated into the protective fat matrix as well as with natural cheeses, leaving the vitamin D_3 more exposed and susceptible to heat degradation. Second, the heat treatment may have caused the vitamin D_3 in the processed cheese to become more bound or entrapped to protein, making it much more difficult to extract the vitamin D_3 using their method. Our results showed that vitamin D_3 is stable in fortified Cheddar and low-fat cheeses even after heating the cheeses to 232 °C for 5 min.

Table 5. Heat Stability of Vitamin D_3 in Fortified Cheeses[a]

type of cheese	before heat treatment	after heat treatment[b]: 100 °C	232 °C
fortified Cheddar cheese (IU/g)	830.21 ± 34.14[c] a	820.99 ± 43.19 a	822.79 ± 10.52 a
fortified low-fat cheese (IU/g)	670.45 ± 16.01 b	672.19 ± 9.81 b	673.26 ± 8.80 b

[a] Mean of three replicates. Means within a row with different letters differ ($p < 0.05$). [b] Heat treatment: (1) 5 min in an oven maintained at 232 °C and (2) 12 min in an oven maintained at 100 °C. [c] Mean ± standard deviation (all such values).

Applicability to Other Cheeses. On the basis of our results, we can infer that vitamin D_3 fortification would also be feasible in other cheeses for the following reasons. First, we successfully fortified Cheddar and low-fat cheeses, which substantially differed in chemical composition and processing methods. In a way, these cheeses represent "extremes" in the hard cheese classification, and our findings can potentially be generalized to other hard and semi-hard cheeses (e.g., Mozzarella, Swiss, etc.). Second, our vitamin D_3 fortification and extraction methods were optimized for cheese and other processed dairy products, including yogurt and ice cream (*15*). Therefore, the method described above can be used to measure vitamin D_3 in other cheeses as well. Lastly, Upreti et al. (*21*) showed that pasteurized processed cheese could also be fortified with vitamin D_3, without losses of the vitamin over 9 months of storage. However, confirmation of vitamin D stability in cheeses where added microorganisms may metabolize the vitamin (e.g., in Brie or Roquefort), would be necessary to confirm the encompassing nature of this claim.

SUMMARY

We have successfully fortified natural hard cheeses with vitamin D_3. Our results indicate that the vitamin does not degrade in fortified Cheddar and low-fat cheeses during processing, over 1 year of ripening, or after baking at high temperatures.

Furthermore, the vitamin D_3 is uniformly distributed in the cheeses, and it does not alter yield or chemical composition. In the production of both cheeses, the whey retains a certain portion of the vitamin D_3. Future research could explore ways to minimize vitamin D losses into the whey, particularly in reduced-fat cheeses.

Our findings clearly demonstrate that Cheddar and low-fat cheeses are suitable foods for vitamin D_3 fortification. Furthermore, we have recently completed a bioavailability study that demonstrated that these fortified cheeses raise vitamin D status as effectively as a vitamin D supplement (*30*). These findings will help to validate this approach for expanding the dietary sources of this essential nutrient for consumers. This will ultimately increase vitamin D intakes in the population, which are currently too low, and could help bring about the public health benefits that result from a greater consumption of vitamin D.

ACKNOWLEDGMENT

We sincerely thank Agropur Cooperative and their staff for making and supplying the fortified cheeses.

LITERATURE CITED

(1) Lappe, J. M.; Travers-Gustafson, D.; Davies, K. M.; Recker, R. R.; Heaney, R. P. Vitamin D and calcium supplementation reduces cancer risk: Results of a randomized trial. *Am. J. Clin. Nutr.* **2007**, *85* (6), 1586–1591.

(2) Munger, K. L.; Levin, L. I.; Hollis, B. W.; Howard, N. S.; Ascherio, A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *J. Am. Med. Assoc.* **2006**, *296* (23), 2832–2838.

(3) Hypponen, E.; Laara, E.; Reunanen, A.; Jarvelin, M. R.; Virtanen, S. M. Intake of vitamin D and risk of type 1 diabetes: A birth-cohort study. *Lancet* **2001**, *358* (9292), 1500–1503.

(4) Wang, T. J.; Pencina, M. J.; Booth, S. L.; Jacques, P. F.; Ingelsson, E.; Lanier, K.; Benjamin, E. J.; D'Agostino, R. B.; Wolf, M.; Vasan, R. S. Vitamin D deficiency and risk of cardiovascular disease. *Circulation* **2008**, *117*, 503–511.

(5) Chapuy, M. C.; Arlot, M. E.; Duboeuf, F.; Brun, J.; Crouzet, B.; Arnaud, S.; Delmas, P. D.; Meunier, P. J. Vitamin D_3 and calcium to prevent hip fractures in the elderly women. *N. Engl. J. Med.* **1992**, *327* (23), 1637–1642.

(6) Liu, P. T.; Stenger, S.; Li, H.; Wenzel, L.; Tan, B. H.; Krutzik, S. R.; Ochoa, M. T.; Schauber, J.; Wu, K.; Meinken, C.; Kamen, D. L.; Wagner, M.; Bals, R.; Steinmeyer, A.; Zugel, U.; Gallo, R. L.; Eisenberg, D.; Hewison, M.; Hollis, B. W.; Adams, J. S.; Bloom, B. R.; Modlin, R. L. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **2006**, *311* (5768), 1770–1773.

(7) Holick, M. F. Vitamin D deficiency. *N. Engl. J. Med* **2007**, *357* (3), 266–281.

(8) Health Canada. Consolidation of the Food and Drugs Act and the Food and Drug Regulations. 2007.

(9) U.S. Food and Drug Administration. Direct food substances affirmed as generally recognized as safe. 2008.

(10) Faulkner, H.; Hussein, A.; Foran, M.; Szijarto, L. A survey of vitamin A and D contents of fortified fluid milk in Ontario. *J. Dairy Sci.* **2000**, *83* (6), 1210–1216.

(11) Vieth, R.; Cole, D. E.; Hawker, G. A.; Trang, H. M.; Rubin, L. A. Wintertime vitamin D insufficiency is common in young Canadian women, and their vitamin D intake does not prevent it. *Eur. J. Clin. Nutr.* **2001**, *55* (12), 1091–1097.

(12) Putnam, J.; Allshouse, J. Trends in U.S. per capita consumption of dairy products, 1909−2001. *Amber Waves* **2003**, 12–13.

(13) Vesa, T. H.; Marteau, P.; Korpela, R. Lactose intolerance. *J. Am. Coll. Nutr.* **2000**, *19* (supplement 2), 165S–175S.

(14) Natri, A. M.; Salo, P.; Vikstedt, T.; Palssa, A.; Huttunen, M.; Karkkainen, M. U.; Salovaara, H.; Piironen, V.; Jakobsen, J.; Lamberg-Allardt, C. J. Bread fortified with cholecalciferol increases the serum 25-hydroxyvitamin D concentration in women as effectively as a cholecalciferol supplement. *J. Nutr.* **2006**, *136* (1), 123–127.

(15) Kazmi, S. A.; Vieth, R.; Rousseau, D. Vitamin D fortification and quantification in processed dairy products. *Int. Dairy J.* **2007**, *17* (7), 753–759.

(16) Tangpricha, V.; Koutkia, P.; Rieke, S. M.; Chen, T. C.; Perez, A. A.; Holick, M. F. Fortification of orange juice with vitamin D: A novel approach for enhancing vitamin D nutritional health. *Am. J. Clin. Nutr.* **2003**, *77* (6), 1478–1483.

(17) Calvo, M. S.; Whiting, S. J.; Barton, C. N. Vitamin D fortification in the United States and Canada: Current status and data needs. *Am. J. Clin. Nutr.* **2004**, *80* (supplement 6), 1710S–1716S.

(18) Holmberg, I.; Aksnes, L.; Berlin, T.; Lindback, B.; Zemgals, J.; Lindeke, B. Absorption of a pharmacological dose of vitamin D_3 from two different lipid vehicles in man: Comparison of peanut oil and a medium chain triglyceride. *Biopharm. Drug Dispos.* **1990**, *11* (9), 807–815.

(19) Buzby, J. Cheese consumption continues to rise. *Amber Waves* **2005**, 4–5.

(20) Trang, H. M.; Cole, D. E.; Rubin, L. A.; Pierratos, A.; Siu, S.; Vieth, R. Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. *Am. J. Clin. Nutr.* **1998**, *68* (4), 854–858.

(21) Upreti, P.; Mistry, V. V.; Warthesen, J. J. Estimation and fortification of vitamin D_3 in pasteurized process cheese. *J Dairy Sci.* **2002**, *85* (12), 3173–3181.

(22) Banville, C.; Vuillemard, J. C.; Lacroix, C. Comparison of different methods for fortifying Cheddar cheese with vitamin D. *Int. Dairy J.* **2000**, *10*, 375–382.

(23) Kosikowski, F. V. *Cheese and Fermented Milk Foods*, 2nd ed.; F. V. Kosikowski and Associates: Brooktondale, NY, 1977.

(24) Muthukumarappan, K. Short communication: Modified schreiber test for evaluation of mozzarella cheese meltability. *J. Dairy Sci.* **1999**, *82*, 1068–1071.

(25) Association of Official Analytical Chemists (AOAC). *Official Methods of Analysis*, 18th ed.; AOAC: Arlington, VA, 2005.

(26) Wehr, M. H.; Frank, J. F. *Standard Methods for the Examination of Dairy Products*; 17th ed.; American Public Health Association: Washington, D.C., 2004.

(27) Mistry, V. V. Low fat cheese technology. *Int. Dairy J.* **2001**, *11* (4), 413–422.

(28) Forrest, S. A.; Yada, R. Y.; Rousseau, D. Interactions of vitamin D_3 with bovine β-lactoglobulin A and β-casein. *J. Agric. Food Chem.* **2005**, *53* (20), 8003–8009.

(29) Wang, Q.; Allen, J. C.; Swaisgood, H. E. Binding of vitamin D and cholesterol to β-lactoglobulin. *J. Dairy Sci.* **1997**, *80* (6), 1054–1059.

(30) Wagner, D.; Sidhom, G.; Whiting, S. J.; Rousseau, D.; Vieth, R. Equivalent bioavailability of vitamin D from fortified cheeses and supplement in adults. *J. Nutr.* **2008**, *138*, 1365–1371.

(31) Vieth, R.; Chan, P. C.; MacFarlane, G. D. Efficacy and safety of vitamin D_3 intake exceeding the lowest observed adverse effect level. *Am. J. Clin. Nutr.* **2001**, *73* (2), 288–294.

(32) Parrish, D. B. Determination of vitamin D in foods: A review. *Crit. Rev. Food Sci. Nutr.* **1979**, *12* (1), 29–57.

(33) Krauss, W. E.; Erb, J. H.; Washburn, R. G. Studies on the nutritive value of milk. II. The effect of pasteurization on some of the nutritive properties of milk. *Ohio Agric. Exp. Stn. Bull.* **1933**, *518*, 1–33.

Received for review April 27, 2008. Revised manuscript received July 11, 2008. Accepted July 18, 2008. This work was supported by grants from the Dairy Farmers of Canada and the Natural Sciences and Engineering Research Council of Canada (NSERC).

JF801316Q