Estimation and Fortification of Vitamin D₃ in Pasteurized Process Cheese¹

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ABSTRACT

The objective of this study was to develop methods for the estimation and fortification of vitamin D₃ in pasteurized Process cheese. Vitamin D₃ was estimated using alkaline saponification at 70°C for 30 min, followed by extraction with petroleum ether:diethyl ether (90:10 vol/vol) and HPLC. The retention time for vitamin D₃ was approximately 9 min. A standard curve with a correlation coefficient of 0.972 was prepared for quantification of vitamin D₃ in unknown samples. In the second phase of the study, pasteurized Process cheeses fortified with commercial water- or fat-dispersible forms of vitamin D₃ at a level of 100 IU per serving (28 g) were manufactured. There was no loss of vitamin D₃ during Process cheese manufacture, and the vitamin was uniformly distributed. No losses of the vitamin occurred during storage of the fortified cheeses over a 9-mo period at 21 to 29°C and 4 to 6°C. There was an approximately 25 to 30% loss of the vitamin when cheeses were heated for 5 min in an oven maintained at 232°C. Added vitamin D₃ did not impart any off flavors to the Process cheeses as determined by sensory analysis. There were no differences between the water- and fat-dispersible forms of the vitamin in the parameters measured in fortified cheeses.

(**Key words:** Process cheese, vitamin D₃, fortification)

INTRODUCTION

The importance of vitamin D in the human diet has been recognized since the early 1900s (McCollum et al., 1922; Mellanby, 1944). Vitamin D-fortified fluid milk

was introduced in 1931 (Olson and Wallis, 1935) and has since been a significant source of this vitamin. Unfortunately, recent studies have reported a possible resurgence of childhood rickets in the United States and United Kingdom (Rani Pal and Shaw, 2001; Tomashek et al., 2001). Hypovitaminosis D has been reported in school children (Fuleihan et al., 2001) as well as in the elderly population (McBean, 1998; Thomas et al., 1998). Census projections indicate that by 2050, the proportion of individuals over the age of 65 yr in the United States will rise to 20% (U.S. Department of Commerce, Economics, and Statistics Administration, Bureau of Census, 1995). The consumption of fluid milk, and hence, the intake of vitamin D, has also declined. In 1999, the per capita fluid milk consumption for the 13-to-17-yr age group was 87 L, whereas it was 35 L in the 53-to-59-yr age group (International Dairy Foods Association, 2000a). On the other hand, the consumption of cheese has steadily increased by almost 100% since 1980 (International Dairy Foods Association, 2000b).

This change in demographics and, consequently, food consumption patterns, suggests a need to expand the range of foods that can provide the essential amounts of vitamin D in the daily diet. Fluid milk by itself does not supply the required amounts of vitamin D to the elderly because of reduced consumption. Cheese may be an excellent vehicle for the delivery of this vitamin to most age groups. Fortification of natural cheeses, such as Cheddar, with consistent levels of vitamin D is difficult (Banville et al., 2000), so fortification of Process cheese may be a better alternative, provided no degradation of the vitamin occurs during manufacture and it is evenly distributed in the cheese mass.

Vitamin D may have toxic effects above a certain threshold concentration. It is therefore also important to develop an adequate method to estimate vitamin D in Process cheeses. The objectives of this study were to develop a technique for manufacturing pasteurized Process cheese fortified with vitamin D₃ and to develop a procedure for the quantification of vitamin D₃ in fortified cheeses. Vitamin D₃ was selected as it has been

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widely accepted for fortification of food, though both vitamin D₂ and D₃ have equal biological potency (Greenbaum, 1973).

MATERIALS AND METHODS

Method Development

The method developed to estimate vitamin D₃ in pasteurized Process cheese was based on modifications of methods for the estimation of vitamin D in milk (Renken and Warthesen, 1993), and simultaneous determination of tocopherols, carotenes, and retinol in Italian cheeses (Panfili et al., 1994). All glassware was rinsed with chloroform:methanol (2:1 vol/vol) before use.

Saponification and extraction. Cheese plugs were drawn from blocks of pasteurized Process cheese, shredded and mixed together. A representative sample (4 to 5 g) was transferred to a 250-ml cork-stoppered, low-actinic Erlenmeyer flask. To this, 20 ml of 60% aqueous KOH, 20 ml of 95% ethanol, 10 ml of 1% NaCl solution, and 50 ml of ethanolic pyrogallol (6% wt/vol in 95% ethanol) were added. The flask was flushed with nitrogen to remove oxygen, capped, and placed in a waterbath at 70°C for 30 min with occasional stirring. The saponified mass was cooled in an ice bath and 15 ml of NaCl solution (1% wt/vol) was added.

The saponified sample was poured into a 250-ml separatory funnel (A). The emptied flask was rinsed sequentially with 15 ml of distilled water, 5 ml of ethanol, and 20 and 15 ml of petroleum ether:diethyl ether mixture (90:10 vol/vol). The rinsings were poured into the separatory funnel (A) and thoroughly mixed by inverting the funnel 50 times. The funnel was left undisturbed until the ether layer was distinctly separated. The bottom aqueous layer was drained into another 250-ml separatory funnel (B), and the ether layer in the separatory funnel (A) was left undisturbed. Five milliliters of ethanol and 35 ml of petroleum ether:diethyl ether mixture (90:10 vol/vol) were added to the separatory funnel B. The contents were mixed by inverting the funnel 50 times, and then left undisturbed for 15 min until a clear ether layer emerged. The aqueous layer was drained, and the ether layer was mixed with the ether extract in separatory funnel A. The emptied separatory funnel (B) was rinsed with 50 ml of ice water, which was then poured in the separatory funnel (A). It was then mixed and left standing for at least 15 min before draining the aqueous layer. The ether extract in the separatory funnel (A) was washed using 50 ml of ice water each time, until the extract became neutral. A few drops of 1% phenolphthalein were added to the last wash to ensure neutrality. Up to three washings were required to obtain a neutral extract. Emulsions could form when soaps (potassium salts of fatty acids),

water, and nonpolar solvents, such as petroleum ether, are shaken vigorously. If stable emulsions formed during washings, 1% cold saline was used as an alternative for ice water. The neutral ether extract was transferred to a 250-ml round-bottom flask and evaporated in a Haake Buchler rotary evaporator (Haake Buchler Instruments Inc., Saddle Brook, NJ) at approximately 40°C and later completely dried under nitrogen.

Solid phase extraction. Three milliliters of hexane was added to the dried contents of the round-bottom flask, swirled vigorously to mix well, and left undisturbed for 5 min. The reconstituted extract was transferred to a 1-ml Bond Elute SPE silica cartridge (Varian Inc., Harbor City, CA), which had been preconditioned with 5 ml of hexane. Low vacuum (490 to 1471 kPa) was used to aid elution. Two more 1-ml increments of hexane were added separately to the flask and individually transferred to the cartridge. Vitamin D₃ from the cartridge was eluted with 5 ml of hexane:chloroform mixture (21.5:78.5 vol/vol). The eluent was dried under nitrogen, reconstituted in 2-ml acetonitrile, and left undisturbed for at least 10 min. The reconstituted extract was then filtered through a 0.45-μm filter (13 mm GD/X Disposable Filter Device, Whatman Inc., Clifton, NJ) and injected into the HPLC column.

HPLC. The HPLC system used for quantification of vitamin D₃ consisted of a model 6000A Solvent Delivery System, a model 440 absorbance detector at 254 nm, a model QA-I Data System Integrator (Waters Associates, Milford, MA), and a Rheodyne fixed-volume loop injector. The analytical column (Discovery C₁₈, Cat# 504955, 4.6 mm i.d. × 15 cm, 5-μm particle size) was protected by a 4.6 mm i.d. × 5 cm, 5-μm particle size, guard column (Discovery C₁₈ Cat# 504947). Operating conditions were: ambient temperature was approximately 23°C; mobile phase was methanol:acetonitrile (70:30 vol/vol); flow rate was 1.0 ml/min; and injection volume was 100 μl. Areas of vitamin D₃ peaks were used as an index of concentration of vitamin D₃ in the sample.

Standard curve. A standard curve of vitamin D₃ was prepared by adding 1 ml of vitamin D₃ standard solution (preparation discussed later) to approximately 5 g of shredded, unfortified, pasteurized Process cheese at six levels: 0, 4.46, 8.93, 17.86, 26.79, and 35.71 IU per 5 g of cheese. These samples were analyzed using the above procedure. The area of the vitamin D₃ peak was adjusted by deducting the area in the control (cheese sample without added vitamin D₃) and plotted against a known concentration of vitamin D₃ (i.e., amount actually added). The standard curve was replicated five times.

Calculations. The amount of vitamin D₃ (IU) per 28 g (one serving) of cheese was

$$\frac{(((\text{vitamin D}_3 \text{ peak area}) - 506.07) / 562.53) \times 28}{\div \text{sample weight in grams}}$$

where 506.07 is the y-intercept, and 562.53 is the slope of the regression equation.

Preparation of standard solutions. Vitamin stock solution was prepared by dissolving 50 mg of crystalline vitamin D₃ (cholecalciferol) or vitamin D₂ (ergocalciferol) (Sigma Chemical Co., St. Louis, MO) in 100 ml of acetonitrile in a volumetric flask. Five milliliters of this solution was diluted with acetonitrile to 100 ml in another volumetric flask to obtain an intermediate solution (25 µg/ml). Work solutions of required strengths (i.e., 0, 4.46, 8.93, 17.86, 26.79, and 35.71 IU/ml) were obtained by diluting the intermediate solution with acetonitrile. Solutions were stored at 4°C. One microgram of vitamin D = 40 IU.

Pasteurized Process Cheese Manufacture

Preparation of fortifying solution of vitamin D₃. Vitamin D₃ for fortification of foods is commercially available in water- and fat-dispersible forms. Cheeses were made using both forms to assess their relative potential. A known amount of water-dispersible vitamin D₃ (dry vitamin D₃ 100 GFP Kosher, BASF Corp., Edison, NJ) was dissolved in distilled water. Dilutions were made in distilled water such that 1 ml of fortifying solution corresponded to the vitamin D₃ fortification level for 0.454 kg (1 lb) of cheese. For example, 1.53 g of 106,000 IU/g of water-dispersible vitamin D₃ fortificant was dissolved in 100 ml of distilled water, and 1 ml of this solution was added to 0.454 kg of cheese to obtain 100 IU/28 g of vitamin D₃ in cheese.

Similarly, a known amount of fat-dispersible vitamin D₃ (vitamin A/D₃ palmitate 1.0/0.2 mIU/g1 in corn oil, BASF Corp.) was dissolved in corn oil. Dilutions were made in corn oil such that 1 ml of fortifying solution corresponded to the vitamin D₃ fortification level for 1.362 kg of cheese. A lower dilution was used for fat-dispersible vitamin D₃ to ensure minimal addition of corn oil to the final cheese.

Fortified Process cheese manufacture. Two- and 6-mo-old commercial Cheddar cheeses in the form of 18-kg blocks (Valley Queen Cheese Factory, Inc., Milbank, SD) were shredded (model PD-35, 48-mm rotary blade; Hobart Corp., Troy, OH). Equal amounts (5.5 kg each) of the 2- and 6-mo-old shredded cheeses were blended and heated with direct steam injection in a lay-down single-auger Process cheese cooker (model 84-062, Damrow, Fond du Lac, WI). Calculated amounts of butter oil were added to adjust for the final moisture content to 38.5%. Steam condensation allowance was 7.29%. Disodium phosphate duohydrate (Rhodia Inc., Chicago

Heights, IL) was used as an emulsifier at the rate of 3%. The amount of fortifying vitamin solution to be added was determined based on the final expected yield of cheese (13.62 kg), and added to the cheese along with other ingredients. The cheese mass was heated to 79°C, held for 1 min, and immediately packed hot in 2-kg plastic containers. The containers were turned upside down and transferred to a storage room at 4 to 6°C. The next day, cheese containers were split into two batches. One batch was stored at 4 to 6°C and the other at 21 to 29°C for 5 mo. There were three Process cheese treatments: control, cheese fortified with water-dispersible vitamin D₃, and cheese fortified with fat-dispersible vitamin D₃.

Analysis

Composition. Fat in base and Process cheeses was estimated by the Mojonnier method (Atherton and Newlander, 1977). Moisture content of cheeses was determined using a moisture balance (model MB200; Ohaus Corp., Florham Park, NJ) (Crosser and Mistry, 1991).

Vitamin D₃. Process cheese for each treatment replicate was packed in five to six containers (approximately 2 kg each). Of these, three containers were randomly selected and analyzed for vitamin D₃ as outlined above to assess uniformity of distribution in the cheese mass.

As Process cheese is a long-shelf-life product, and is also stored at room temperature, it was important to study the stability of vitamin D₃ during room-temperature and refrigerated storage. Of the 3 containers analyzed for distribution of vitamin D₃, one container was stored at room temperature and another was refrigerated. Samples were drawn from each of these containers throughout the study at 1-d, and 1-, 3-, 5-, and 9-mo periods for vitamin analysis.

Heat stability of vitamin D₃ in Process cheeses was assessed by heating a cheese disk (5-mm thick and 39-mm diameter) in a petri dish for 5 min in a hot-air oven maintained at 232°C as done in the Schreiber cheese-melting test (Kosikowski and Mistry, 1997). Melted cheese was shredded and analyzed for vitamin D₃. The vitamin values obtained were adjusted for moisture-evaporation losses during heating, and compared to the original values of vitamin D₃ in cheese that were not heat-treated.

Sensory evaluation. A Triangle test was conducted to identify flavor attributes in the vitamin D₃ fortified cheeses (Stone and Sidel, 1985). The judges were provided with three samples, two of which were the same, and asked to identify the odd sample. The order of samples in each set was randomized. The experienced panel consisted of four judges. Data obtained were analyzed

statistically using the probability tables for the Triangle test (Stone and Sidel, 1985). Cheeses were maintained at 4 to 5°C prior to evaluation.

Statistical analysis. Process cheeses were manufactured in three replicate trials. A chi-squared test was used to analyze the data for distribution of water- and fat-dispersible vitamin D₃ among containers. Storage stability of vitamin D₃ in cheeses was analyzed using the repeated measures design in SAS (1990). ANOVA was conducted to determine the significance of differences among means of vitamin D₃ content of cheeses with or without heat treatment. All data were analyzed at a significance level of 5%. Statistical parameters were also calculated to assess the precision and accuracy of the analytical method.

RESULTS AND DISCUSSION

Method to Estimate Vitamin D₃ in Process Cheese

Techniques using HPLC to estimate vitamin D₃ in foods, including dairy foods such as fortified fluid milk, condensed milk, infant formulas, and dried whole and skim milk, have been developed, but published methods specifically for cheese are lacking.

Sampling and saponification. Obtaining a representative sample for vitamin D₃ analysis is difficult because of very low concentrations of the vitamin in the product. It has been suggested that the appropriate size of the sample to be used for analysis should be calculated from the vitamin D₃ declaration on the food product (Parrish, 1979). Based on these guidelines, a cheese sample weight of 4 to 5 g was selected to have a sufficient quantity of vitamin D₃ in the final extract to be quantified using HPLC.

The complications involved in extracting vitamin D₃ from the cheese sample matrix are due to the presence of large amounts of fat and protein in the cheese and the coating material used for stabilizing vitamin D₃ in its fortificant form (Ball, 1992; Parrish, 1979). In general, alkaline hydrolysis or saponification provides an effective means of removing glycerides from the food sample in an efficient way. Saponification can be carried out at ambient temperature (Renken and Warthesen, 1993) or by refluxing (i.e., at a higher temperature) (Panfili et al., 1994). Although both methods have been used for vitamin D₃ analysis, overnight saponification at ambient temperature is a preferred means of removing the bulk of the lipid components (Indyk and Woollard, 1985a). This method avoids thermal isomerization of vitamin D₃ to its previtamin form (Keverling Buisman et al., 1968).

Given the complexity of the cheese matrix, we used heated saponification (Panfili et al., 1994), and quanti-

ties of chemical reagents were modified according to the estimated fat content in the cheeses. The potential effect of isomerization was nullified by employing the method for the standard curve (discussed later). Another concern for heat involved in saponification was the loss of vitamin D₃ due to oxidation. Using a nitrogen-flushed flask and pyrogallol as an antioxidant prevented this loss (Ball, 1992). The omission of nitrogen has been reported to reduce vitamin D recoveries by 10 to 20% (Indyk and Woollard, 1984).

Details pertaining to ether extraction and emulsion formation and solid-phase extraction have been discussed (Parrish, 1979; Renken and Warthesen, 1993) elsewhere.

HPLC. Separation of vitamin D₃ was conducted by chromatographic separation, which was run isocratically, keeping the volumetric flow the same throughout the run. It was subsequently quantified with an absorbance detector at 254 nm. Identification of compounds by HPLC is established by their respective retention times on the analytical column. With a mobile phase consisting of a 90:10 mixture of acetonitrile: methanol, as adopted from Renken and Warthesen (1993), vitamin D₃ eluted at approximately 13.5 min (Figure 1a). When this mobile phase was used with a cheese sample with no externally added vitamin D₃, a peak eluted at the same time (13.5 min) (Figure 1b). In a cheese sample with vitamin D₃ added, an increase in the area of this peak was observed (Figure 1c), indicating the overlap of the vitamin D₃ peak with the interfering peak.

It was important to separate the interfering peak from the vitamin D₃ peak for accurate quantitation. After a number of combinations by trial and error, it was determined that a 70:30 methanol:acetonitrile ratio permitted such a separation (Figure 2a and 2b). With the same mobile phase, the retention time of vitamin D₃ on the column was reduced to approximately 9 min (Figure 2c).

Quantification of vitamin D₃ in unknown samples. Vitamin D₂ is an ideal choice for an internal standard for the quantification of vitamin D₃ using HPLC because it is virtually identical in its physicochemical properties (recovery, wavelength for maximum absorbance $\lambda_{\max}$, and extinction coefficient $E_{1\text{cm}}^{1\%}$) (Indyk and Woollard, 1985b). With 70:30 methanol:acetonitrile as the mobile phase, the vitamin D₂ standard eluted at 8.5 min (i.e., just prior to the vitamin D₃ peak) (Figure 3a). When vitamin D₂ was added to a cheese sample as an internal standard before saponification, it eluted with an interfering peak (Figure 3b). This interfering compound was possibly cholesterol (Ball, 1992).

Remedies recommended for removing cholesterol include changing the mobile phase to delay the elution

Figure 1. a) Elution of vitamin D₃ (arrow); b) Process cheese with no vitamin D₃ showing the presence (arrow) of interfering peak at 13.5 min; c) Process cheese with vitamin D₃ added shows a spiked peak (arrow) at 13.5 min. The mobile phase was a 90:10 mixture of acetonitrile:methanol.

of the vitamin D₂ peak, precipitation of cholesterol, or changing the detector wavelength to 280 nm. The latter also reduced the sensitivity to vitamin D₃ (Ball, 1992; Indyk and Woollard, 1985a). Of the above steps, only precipitation with digitonin and freezing ethanolic precipitation (Eisses and deVries, 1969; Ball, 1992) demonstrated some potential. Unfortunately, the interfering peak was not completely eliminated. Therefore, a standard curve with external standards rather than an internal standard was employed for quantitation.

Standard curve and assessment of method. The correlation coefficient for the standard curve was 0.9722 (Figure 4). In the development of the standard curve,

Figure 2. a) Process cheese with no vitamin D₃ showing the absence (arrow) of a peak at 9 min; b) Process cheese with vitamin D₃ added showing the vitamin D₃ peak (arrow) at 9 min; c) elution of vitamin D₃ (arrow) at 9 min. The mobile phase was a 70:30 mixture of methanol:acetonitrile.

each cheese sample was treated as an unknown. This should have nullified any problems associated with thermal isomerization of vitamin D₃ and variability in recoveries due to multiple steps. The range of vitamin concentrations was such that the anticipated fortification level in Process cheese fell at approximately midpoint.

Six replicate determinations of vitamin D₃ in a sample of cheese fortified at a level of 100 IU/28 g were conducted to assess the method for accuracy and precision (Table 1). In a separate study, 18 batches of Process cheeses were made with vitamin D₃ fortification level of 200 IU/28 g. Samples were analyzed for vitamin D₃ using the method reported here (Table 1).

Applicability of method to other cheeses. Commercial Cheddar, Mozzarella, and Cottage cheeses were also analyzed for vitamin D₃ using this method. No

Figure 3. a) Vitamin D₂ (arrow) elutes at 8.5 min; b) Process cheese with added vitamin D₂ showing the presence of interfering peak (arrow) at 8.5 min. The mobile phase was a 70:30 mixture of methanol:acetonitrile.

peak coincided with the elution of vitamin D₃. Thus, the method described above could potentially be used to quantify vitamin D₃ in other cheeses as well.

Fortified Process Cheese Manufacture

The pasteurized Process cheeses had an average fat content of 35.4% and moisture content of 39.9%, and there were no differences ($P \geq 0.05$) between the treatments.

Recovery of vitamin D₃ in cheese. There were no differences ($P \geq 0.05$) between expected and analyzed

Table 1. Statistical characterization of analytical method.

	Value
Expected value	100 IU
Mean value ¹	114.34 IU
SD	17.96 IU
CV ²	15.71%
95% confidence interval	114.34 $\pm$ 20.64 IU
Expected value	200 IU
Mean value ³	200.14 IU
SD	16.11 IU
CV ²	8.05%
95% confidence interval	200.14 $\pm$ 6.61 IU

¹Mean of six cheese samples.

²CV (%) = (SD/mean) $\times$ 100.

³Mean of 18 samples.

Table 2. Recovery of vitamin D₃ during pasteurized Process cheese manufacture.¹

Treatment ²	Vitamin D ₃ (IU/28g)	
	Expected level	Analytical values
WD	100 ^a	106.20 ^a
FD	100 ^a	98.05 ^a

^aMeans do not differ ($P \geq 0.05$).

¹Mean of three replicates.

²Treatment: WD = water-dispersible vitamin D₃-fortified cheese, FD = fat-dispersible vitamin D₃ fortified cheese.

amounts of vitamin D₃ in the cheeses, indicating that there was no vitamin D₃ loss during the manufacture of the pasteurized Process cheese (Table 2).

Point of concern in fortification of foods is how much fortificant can be recovered in the final product (i.e., how much fortificant is destroyed during the process of manufacture of the product). The destruction of vitamin D₃ during cheese manufacture could be due to oxidation, low pH, or heat. Destabilization of vitamin D has been reported during Cheddar cheese manufacture, possibly due to fermentation by lactic acid bacteria, acidification, and oxidation (Banville et al., 2000). Conflicting reports have been published regarding factors affecting the stability of vitamin D. Cremin and Power (1985) stated that vitamin D was unstable to oxidation, light, and acid whereas Kutsky (1981) reported that vitamin D was susceptible to oxidation and light, but stable to acid and alkali. Pike and Brown (1984) reported that vitamin D was unstable to irradiation and acid, but stable to oxygen and alkali. Kreutler (1980) reported that vitamin D was remarkably stable to light, heat, and oxygen. Renken and Warthesen (1993) reported a slight loss of vitamin D₃ in fortified milk upon exposure to light.

Assessing potential losses during pasteurized Process cheese manufacture is particularly important because of the use of pasteurization temperatures and possible interactions with other cheese components. Our results suggested that the manufacturing conditions of Process cheese were not detrimental to vitamin D₃.

Distribution of vitamin D₃ in cheese. Because of the low ratio of vitamin D₃ to cheese (1:11,200,000), it was important to determine how well the vitamin was distributed in the cheese mass. If it is not uniformly distributed, some portions of cheese will have higher vitamin D₃ than others, creating a potential for toxicity when consumed. Vitamin D₃ content was analyzed in three containers from each treatment replicate and the variability among the containers was determined using the chi-square test. There was no detectable difference

Table 3. Distribution of vitamin D₃ in pasteurized Process cheese.

Treatment ¹		Vitamin D ₃ (IU/28g)		
		Container 1	Container 2	Container 3
WD	Rep 1	99.48 ^a	115.27 ^a	109.41 ^a
	Rep 2	97.82 ^a	104.62 ^a	85.40 ^a
	Rep 3	113.51 ^a	113.43 ^a	116.88 ^a
FD	Rep 1	99.97 ^a	87.78 ^a	83.42 ^a
	Rep 2	88.07 ^a	71.47 ^a	92.23 ^a
	Rep 3	123.39 ^a	116.18 ^a	119.96 ^a

^aMeans do not differ ($P \geq 0.05$).

¹Treatment: WD = water-dispersible vitamin D₃-fortified cheese; FD = fat-dispersible vitamin D₃-fortified cheese.

among containers ($P \geq 0.05$) for both types of vitamin D₃ preparations (Table 3). These results demonstrated that vitamin D₃ was uniformly distributed in cheese, indicating the feasibility of the method for fortification of pasteurized Process cheese under actual production conditions. This was because of the vigorous mixing that occurs in the cheese cooker during Process cheese manufacture.

Heat stability of vitamin D₃ in cheese. One of the popular applications of pasteurized Process cheese is in ready-to-eat or ready-to-serve meals/foods, on pizzas, and in other foods, which are baked at high temperatures to melt the cheese. The stability of vitamin D₃ in the fortified cheeses under such conditions of heat treatment was tested. Approximately 25 to 30% vitamin D₃ was lost in pasteurized Process cheeses fortified with both water- and fat-dispersible vitamin D₃, when heated for 5 min in an oven maintained at 232°C ($P \geq 0.05$) (Table 4). This lower recovery of vitamin D after the oven treatment could be destruction or lack of recovery due to binding to the protein or entrapment.

Vitamin D in natural or fortified foods has been reported to be stable during pasteurization at 62.8°C for 30 min, or sterilization at 115.6°C for 15 min (Krauss et al., 1933), as employed for evaporated milk. Vitamin D in milk was unaffected by pasteurization, boiling, or

sterilization (Hartman and Dryden, 1974). More research is needed to determine what levels of heat treatments can deteriorate vitamin D₃ activity in cheese, both chemically and biologically.

Storage stability of vitamin D₃ in cheese. There was no detectable deterioration of vitamin D₃ in fortified cheeses during 9 mo at room and refrigerated temperatures ($P \geq 0.05$) (Table 5). The temperature during room temperature storage varied from 21 to 29°C, thus simulating a situation that may be encountered in the commercial handling and distribution of this product.

Steenbock and Wirick (1930) reported that irradiated butterfat stored at room and refrigerated temperatures had stable vitamin D activity up to 9 mo. Krieger and Scott (1938) observed little or no loss of vitamin D potency of evaporated milk at a storage temperature of 4.4 to 43.3°C for 2 to 3 yr. No degradation of vitamin D was observed when canned condensed milk was stored at 40°C for 6 mo and at 23°C for 15 mo (Huber and Barlow, 1943). Banville et al. (2000) reported a reduction in the vitamin D content of fortified Cheddar

Table 4. Heat stability of vitamin D₃ in pasteurized Process cheese.¹

Treatment ²	Vitamin D ₃ (IU per 28 g)	
	Before heat treatment	After heat treatment ³
WD	113.44 ^a	87.21 ^b
FD	111.84 ^a	89.63 ^b

^{a,b}Means in a row with a common superscript do not differ ($P \geq 0.05$).

¹Mean of three replicates.

²Treatment: WD = water-dispersible vitamin D₃-fortified cheese; FD = fat-dispersible vitamin D₃-fortified cheese.

³Heat treatment: 5 min in oven maintained at 232°C.

Figure 4. Standard curve for vitamin D₃ in Process cheese.

Table 5. Storage stability of vitamin D₃ in pasteurized Process cheese.¹

Treatment ²	Storage temperature (°C)	Storage period				
		1 d	1 mo	3 mo	5 mo	9 mo
		(Vitamin D ₃ (IU/28g))				
WD	21 to 29	112.26 ^a	97.46 ^a	107.26 ^a	100.63 ^a	80.00 ^a
WD	4 to 6	102.75 ^a	104.49 ^a	113.44 ^a	108.32 ^a	99.59 ^a
FD	21 to 29	97.13 ^a	108.65 ^a	101.24 ^a	92.00 ^a	95.97 ^a
FD	4 to 6	98.73 ^a	107.82 ^a	111.84 ^a	100.34 ^a	101.73 ^a

^aMeans do not differ ($P \geq 0.05$).

¹Mean of three replicates.

²Treatment: WD = water-dispersible vitamin D₃-fortified cheese; FD = fat-dispersible vitamin D₃-fortified cheese.

cheese during 7 mo of ripening, though stability varied between treatments.

Sensory attributes of fortified cheese. Fortification of pasteurized Process cheese with vitamin D did not ($P \geq 0.05$) have any effect on the flavor of cheese as indicated by the inability of judges to consistently pick the fortified cheese sample as an odd one out (data not shown). This could be expected due to the negligible quantities of vitamin D₃ added to the relatively larger quantities of final product. Also, vitamin D is commonly added to milk without imparting off flavors.

CONCLUSIONS

Pasteurized Process cheeses successfully fortified with vitamin D₃ were manufactured. There was no loss of the vitamin during processing nor losses during storage at room or refrigeration storage. The analytical method developed to estimate of vitamin D₃ in Process cheese can be used satisfactorily as a tool for monitoring vitamin D₃ content of fortified cheeses. Stability of vitamin D₃ during storage will help pasteurized Process cheeses play a promising role as a vehicle for supplying this micronutrient throughout its shelf life. The popularity of Process cheese and its fortification with vitamin D₃ will help address the deficiency of this nutrient in diverse populations that have limited fluid milk consumption. Relative stability of vitamin D₃ in cheeses during severe heat-treatment applications suggests that this vitamin can still be supplied in the diet under cooking applications. Ongoing studies assessing the bioavailability of vitamin D from the fortified cheeses will be valuable to help validate this approach of supplying vitamin D to consumers.

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