A Survey of Vitamin A and D Contents of Fortified Fluid Milk in Ontario

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ABSTRACT

High performance liquid chromatographic methods for measuring the concentration of vitamins A and D in fluid milk were validated and used to assess the level of these nutrients in Ontario retail milk samples. Thirteen and fifteen fortified milk samples were tested for vitamins A and D, respectively. Repeatability relative standard deviation values for vitamins A and D in milk were generally less than 10%. Recoveries varied from 87 to 107%. Vitamin D results indicated that only 20% of skim, 40% of 2% fat milk, and 20% of whole milk contained the recommended levels, whereas 46% of skim, and 77% of 2% fat milk had the required levels of vitamin A. The results indicate that vitamin level varies widely in Ontario retail milk.
(**Key words:** vitamin A, vitamin D, fortification, milk)

Abbreviation key: BHT = 2, 6-di-ter-butyl-4-methylphenol, **CFIA** = Canadian Food Inspection Agency, **USFDA** = United States Food and Drug Administration.

INTRODUCTION

The Canadian Food and Drug Act (9) mandates that all fluid milk sold should contain vitamin D 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**) (formerly The Health Protection Branch of Health and Welfare Canada) has simplified the regulation by requiring all fortified milk to contain between 31.7 to 51.6 IU of vitamin D/100 ml. Although whole milk fortification with vitamin A is optional, it is mandatory for reduced-fat and skim milks, such that a reasonable daily intake of milk contains not less than 1200 and not more than 2500 IU (in CFIA format, 127 to 322 IU of vitamin A/100 ml). This requirement, which has been in place for decades, has resulted in a dramatic reduction of disorders caused by deficiencies of these vitamins.

Over-fortification with vitamin D can cause intoxification (4,15); it may also result in soft tissue damage and kidney failure. Under-fortification can cause deficiency diseases such as rickets in the young and osteomalacia and possibly osteoporosis in the elderly. Besides the previously known and well-publicized effect of this vitamin on bone development and structure maintenance, newer findings are indicating cancer-preventing properties. Quite recently, on the basis of epidemiological studies, it has been proposed that vitamin D deficiency is a risk factor for prostate cancer. An in vitro culture system showed that prostate cells have receptors for vitamin D, and that it is growth inhibitory (19). A role for vitamin D in prostate cancer prevention or therapy is thus suggested. Other studies have shown that 30 to 40% of patients with hip fractures are deficient in vitamin D (11, 21).

Variations of vitamin concentrations have been reported for fortified whole and partially skimmed milk products. In 1991 and 1993, milk purchased from supermarkets in western Canada and across the US were tested. Seven of ten milk samples contained less than 80% of the amount of vitamin D that was listed on the label, and half had less than 50%. Also, 14% of the skim milk samples contained no detectable vitamin D (6, 13). Upon testing 158 fortified skim milk samples, Tanner et al. (22) reported that only 15.8% of the samples had vitamin concentrations within 81 to 120% of the label claims. Henderson and Wickroski (10) found wide variation between the amount of vitamin D detected in the milk and the amount declared on the label. This discrepancy between label values and measured amounts of vitamin D_3 in skim milk may be due to light degradation as concluded by Renken and Warthesen (20) or to other errors in manufacturing practices. The margin between the nutritionally desirable intake and the harmful excess is considered to be very small (7), therefore, it is essential that errors in fortification be closely monitored. Vitamin D deficiency is considered the "silent epidemic" with everyone in North America being vulnerable to it especially between October and March (11,12). Vitamin D nutritional status in Ontario com-

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pared to American standards can best be considered as suboptimal according to Reinhold Veith (25; personal communication). He estimates that half of Ontarians are marginal for vitamin D deficiency, and he advocates a daily vitamin D intake of 400 IU for infants and 800 IU for adults to maintain optimal plasma levels of 25-OH vitamin D.

Currently, milk processing plants rely largely 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 vitamins A and D, but without actual analysis, it is difficult to monitor vitamin losses during processing.

Numerous studies have been conducted to develop satisfactory methods for the analysis of vitamins A and D in milk (1, 5, 14, 17, 18, 23, 24, 26, 27). These continued attempts at developing a better method are indicative of the complexity of vitamin D assay in particular. Ball (3) reviewed and compiled HPLC methods for the isolation and determination of fat-soluble vitamins in foods.

The objectives of the present study were 1) to validate a method obtained from Cornell Food Science Department for analysis of vitamin D in fluid milk and to validate a modified version of the method for vitamin A analysis found in Standard Methods for the Examination of Dairy Products (21) and 2) to determine the level of vitamins A and D in randomly selected commercial milk samples processed in Ontario.

MATERIALS AND METHODS

The reagents used were all trans retinol palmitate, (Sigma R-3375) all trans retinol acetate (Sigma R-3250) vitamin D_2 (Ergocalciferol; Sigma E-5750) vitamin D_3 (Cholecalciferol; Sigma C-9756) KOH, sodium sulfate anhydrous, pyrogallol (Sigma P-2923), 2,6-di-ter-butyl-4-methylphenol (**BHT**), HPLC-grade hexane with 0.005% BHT, chloroform, acetonitrile, methanol with 0.005% BHT, ethyl acetate, absolute ethanol refluxed with silver nitrate (5 g/L) and KOH (10 g/L) overnight and distilled, 10% formaldehyde, 1% ethanolic pyrogallol solution (freshly prepared), 5% aqueous KOH, and 55% aqueous ethanol.

Vitamin D_3 and Vitamin D_2 Standard Solutions

Stock solutions of roughly 200 μg/ml in methanol were prepared by dissolving 20 mg of vitamins D_3 and D_2 separately in 100 ml of methanol. Daily working standards (2 μg/ml in methanol) were prepared by diluting 1.0 ml of the stock solution to 100 ml with methanol. The concentrations of all working solutions were determined by measuring absorbance at 264 nm and dividing by the specific absorption coefficient E(1%/1cm) of 460 and 485 for D_2 (internal standard) and D_3, respectively. The concentration range of the calibration standard was 0.09 to 0.50 μg/ ml.

Vitamin A Palmitate and Acetate Standard Solutions

Stock solutions (100 μg/ml) of retinol palmitate and acetate (internal standard) were prepared separately in 100 ml of absolute ethanol. Daily working standards (5 μg of retinol palmitate/ml and 1 μg of retinol acetate/ml) were prepared by diluting 5 ml of retinol palmitate and 1 ml of retinol acetate stock solutions to 100 ml with ethanol, respectively. The concentration of working standards were determined by measuring absorbance at 328 nm and dividing by the specific absorption coefficients E(1%/1cm) of 975 for retinyl palmitate and 1565 for acetate. The concentration range of the calibration standard was 0.09 to 2.0 μg/ml.

Apparatus

The HPLC system (Waters, Milford, MA) consisted of a 600E multisolvent delivery module, an autosampler (model 717 plus), a diode array detector (model 996), Millennium data acquisition and analysis software, a vitamin D analytical column (Vydac 201TP54 C-18 5-μm reversed-phase; 250 mm $\times$ 4.6 mm i.d.) with a 5-μm guard column (Vydac 201TP C-18 10 μm reversed-phase 10 mm $\times$ 4.6 mm i.d.), and a vitamin A Column (5-μm LiChrosorb Si 60; 5 μm; 250 mm $\times$ 4 mm i.d.) with a guard column (5-μm LiChrosorb Si 60; 4 mm $\times$ 4 mm i.d.). Also used were a rotary evaporator with thermostated water bath and nitrogen supply, a diode array spectrophotometer (HP 8452A; Hewlett Packard), and solid phase extraction columns (extract-clean Si 3 ml, 200 mg; Alltech,), 300-μl vial inserts.

Vitamin D Assay Procedure

Saponification.We pipetted 15 ml of homogenized milk into a 50-ml actinic flask. We added 15 ml of 1.0% ethanolic pyrogallol and 100 μl of internal standard solution of vitamin D_2. We placed the flask in an ice bath and, under a stream of nitrogen, added 6.0 g of

KOH and swirled the flask intermittently until all KOH was dissolved. We stoppered the flask, and let it stand overnight in the dark at room temperature.

Solvent Extraction. We transferred the contents of the flask into a 125-ml actinic separatory funnel. We rinsed the flask with 15 ml of water followed sequentially by 5 ml of absolute ethanol and 45 ml of hexane and then transferred the liquid into a separatory funnel after each rinse. We shook the funnel vigorously, and let it stand until the phases separated. We transferred the aqueous layer into the original flask and the hexane layer into a 250 ml actinic separatory flask, repeated the extraction twice with 45 ml of hexane each time, and then transferred the hexane layer to the 250-ml separatory funnel. We washed the pooled hexane layers with 50 ml of 5% KOH, followed by 100 ml of water twice and once with 100 ml of 55% aqueous ethanol and discarded the lower aqueous layer each time. We transferred the hexane layer into a 250-ml round-bottom flask and evaporated the hexane to dryness at 40°C. We added 5 ml of absolute ethanol to facilitate the complete removal of trace water and evaporated again to dryness. Immediately we added 5 ml of hexane to the flask and swirled and transferred it to a 50-ml centrifuge tube. We washed the flask twice, first with 3 ml of hexane and then with 1 ml of hexane; the washes were added to the centrifuge tube.

Solid Phase Extraction. We concentrated the hexane fraction to 1 ml under nitrogen then mixed vigorously on a vortex for 30 s. We conditioned the silica cartridge with 1 to 2 volumes of hexane (~ 5 ml). Next, we transferred the hexane fraction to the column using Pasteur pipette and allowed it to pass through by gravity feed. We rinsed the 50-ml centrifuge tube with 1 ml of hexane and transferred it onto the column. We washed the column with 1.0 ml hexane:chloroform (22:78, vol/vol) and then discarded the hexane. Vitamins D_3 and D_2 were eluted from the silica cartridge with 1.0 ml methanol into a clean, 15-ml graduated centrifuge tube. We removed the solvent under a gentle stream of nitrogen on a water bath at 30°C. The residue was immediately dissolved in 600 μl of methanol. We filtered the sample extract through a 0.22-μm syringe filter into 300-μl vial inserts and injected 100 μl onto the column for HPLC analysis.

HPLC Condition

Chromatography was done with a column of 4.6×250 mm Vydac TP201 C18, 5μ; a detector and a photodiode array at 264 nm. (Table 1).

Table 1. HPLC conditions with a linear gradient program after solid phase extraction.

Time (min)	Flow (ml)	% Methanol	% Acetonitrile: chloroform: ethyl acetate (88:4:8 vol/vol/vol
Initial	1.0	0	100
24.00	1.0	0	100
28.00	3.0	100	0
30.00	3.0	100	0
31.00	3.0	0	100
33.00	3.0	0	100
34.00	1.0	0	100
36.00	0.0	0	100

Method Validation

Validation was done with unfortified, whole, raw milk containing vitamin D_3 at five levels of fortification: 0X, 0.25X, 0.5X, 1X, and 2X (where X represented the expected level in fortified milk). The concentration range was 0.0, 10.6, 21.2, 42.4, and 84.8 IU/100 ml. The study consisted of six sets of determinations. Each set was composed of five samples, one for each level of fortification. Each set was tested by the same analyst on different days.

Vitamin A Assay Procedure

The HPLC method in Standard Methods for the Examination of Dairy Products (21) was used for vitamin A assay with minor modifications, such as substituting hexane:chloroform (92:8 vol/vol) as the mobile phase for wet hexane and the use of retinyl acetate as an internal standard.

Method Validation

The method was validated with unfortified, whole, raw milk containing vitamin A (retinol palmitate) at five levels: 0X, 0.25X, 0.5X, 1X, and 2X (where X represented the normal level) that corresponded to 0.0, 52.8, 105.6 , 211.2, and 422.4 IU/100 ml addition. The study consisted of six sets of determinations. Each set was composed of five samples, one for each level of addition. Each set was tested by the same analyst on different days.

Proficiency Samples

We participated in a collaborative study, organized by the US Food and Drug Administration (**USFDA**) on the analysis of pasteurized and homogenized milk samples. Participating laboratories were allowed to use their own methods. The USFDA proficiency samples were also analyzed quarterly; each sample set had four samples of the same milk type fortified at various levels of vitamins A and D. Samples were analyzed on the day of receipt.

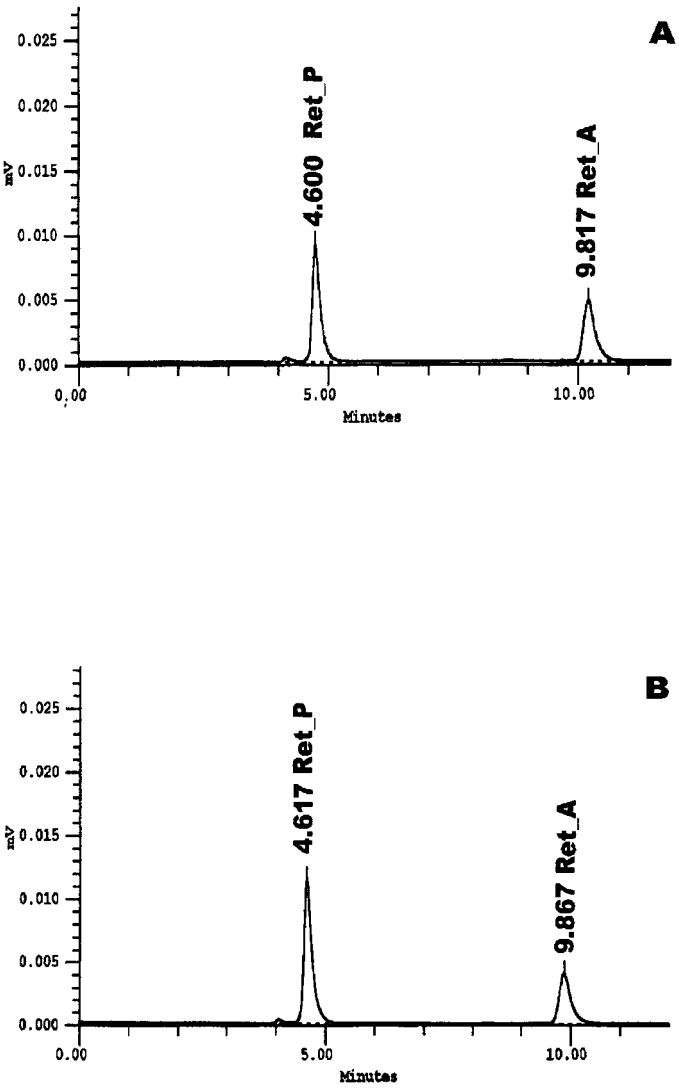

Figure 1. Typical liquid chromatogram of retinol palmitate (Ret_P) and (internal standard) retinol acetate (Ret_A) in A) working standards and B) extract of commercially fortified milk with added (Ret_A).

Ontario Retail Milk Samples

A total of 54 commercial milk samples from 18 milk processing plants across Ontario was tested for vitamins A and D. They included 18 samples of whole milk, 18 samples of milk with 2% fat content, and 18 samples of skim milk selected with as much randomness in processor location as was feasible. Samples were refrigerated upon receipt until analyzed, with all operations done under reduced light. Samples 1 to 10 were tested for vitamins A and D. Although testing took place on different dates, three more samples were tested for vitamin A and five more samples were tested for vitamin D. All the samples were analyzed (in duplicate) before the expiration date.

RESULTS AND DISCUSSION

The selection of the HPLC methods was based on precision, accuracy, and suitability for routine monitoring of vitamins A and D in fluid milk. Figure 1 illustrates a chromatogram for A) retinol palmitate and acetate standards and B) a typical extract of a 2% reduced-fat milk sample. Table 2 lists the validation data obtained by augmenting whole fluid milk samples with known amounts of retinol palmitate (0.0, 52.8, 105.6, 211.2, or 422.4 IU/100 ml). This test was repeated on 6 separate d. The analysis of six replicates gave RSD_r of less than 10% as shown in Table 2 with mean recoveries from 92 to 102%. The regression analysis of response versus concentration showed linearity ($R^2 = 0.99$) from 0.4 to 3.0 μg/ml. For the quantitative estimation of vitamin D in fluid milk, an unpublished method obtained from Cornell Food Science Department, was adopted. It provided a quick and efficient clean-up procedure, with well-separated vitamin D_2 and D_3 peaks, which permitted the use of one or the other as internal standard. Because the procedure had multiple steps, an internal standard was used to control for any loss of vitamin D that might have occurred during extraction. According to Johnsson and Hessel (16), use of an internal standard should eliminate the effect of previtamin

Table 2. Method validation data for vitamin A in unfortified raw milk augmented with 52.8, 105.6, 211.2, or 422.4 IU/100 ml of retinol palmitate (basal level ranged from 86 to 103 IU/100 ml).

	Retinol palmitate			
	52.8 IU/100 ml	105.6 IU/100 ml	211.2 IU/100 ml	422.4 IU/100 ml
Mean[1] ± SD	48.5 ± 4	106.7 ± 8	215 ± 19	419 ± 35
% Recovery[2]	89 ± 8	101 ± 7	102 ± 9	99 ± 8
RSD, [3] %	8.8	7.4	8.9	8.4

[1]n = 6.

[2]Overall mean recovery calculated on the basis of internal standard (retinyl acetate).

[3]Day-to-day repeatability.

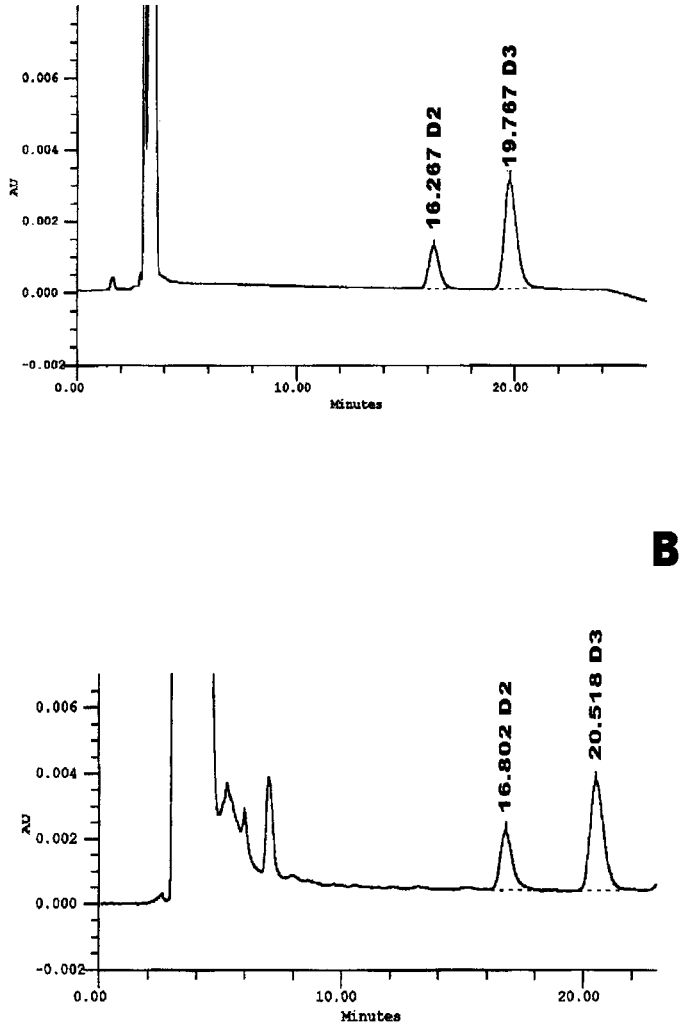

Figure 2. HPLC chromatogram of vitamins (internal standard) and D_3, in A) working standards and B) commercially fortified milk sample with added vitamin D_2. AU = absorbance unit.

formation, because the rate of isomerization of vitamin D_2 and D_3 are nearly equal, and therefore, the ratio of the two forms at a given temperature are constant. A combination of an SPE clean up column and a Vydac analytical column provided excellent baseline separation of vitamins D_2 and D_3 peaks, free from interfering peaks. Peak purity and identity were established with the PDA detector, by using its peak-matching capability. Chromatograms of vitamins D_2 and D_3 standards and extracts for a milk sample are in Figure 2. The figure shows a good baseline separation of these isomers for the standards and the milk sample. The average retention times were 16.9 and 20.2 min, respectively. The recoveries of added vitamin to whole fluid milk fortified at 0.0, 10.6, 21.2, 42.4, or 84.4 IU/100 ml were measured at the specified levels on 6 different d. The mean recoveries of added vitamin D_3 varied from 87 to 94%, whereas day-to-day repeatability (% RSD_r) values were generally less than 10% (Table 3). The regression analysis of peak area versus concentration showed linearity ($R^2 = 0.999$) over the tested range. The collaborative study organized by the USFDA was used to validate the methods for the analysis of these vitamins in processed fluid milk. The data in Tables 4 and 5 show that the methods are equally applicable to pasteurized and homogenized samples. The USFDA's vitamin A and D proficiency test program led to laboratory certification for the analysis of these vitamins in milk sold under the National Conference on Interstate Milk Shipments.

The results of vitamin A content in different types of Ontario retail milk are shown in Table 6. The Health Protection Branch of Health and Welfare Canada (9) (now the CFIA) set 80% as minimum national level of compliance by all processors for fortified, skimmed milk and partly skimmed milk. Of the samples analyzed in this project, only 38% of the whole milk samples assayed had vitamin A levels within 127 to 322 IU/100 ml of milk. For skim and partially skimmed milk we found that only 46% of skim milk, tested had satisfactory levels of vitamin A. The low levels observed may be due to the method of vitamin addition and mixing or distribution during processing (22). On the other hand,

Table 3. Method validation data for vitamin D_3 in unfortified raw milk augmented with 0, 10.6, 21.2, 42.4, or 84.4 IU/100 ml of vitamin D_3 (basal level was zero).

	Vitamin D_3			
	10.6 IU/100 ml	21.2 IU/100 ml	42.4 IU/100 ml	84.4 IU/100 ml
Mean[1] ± SD	9.4 ± 0.8	18.4 ± 2.4	40 ± 2	77 ± 4.4
% Recovery[2]	89 ± 8	87 ± 11	94 ± 5	91 ± 5
RSD,[3] %	9.0	13.0	5.2	5.7

[1]n = 6.

[2]Overall mean recovery calculated on the basis of internal standard (vitamin D_3).

[3]Day-to-day repeatability.

Table 4. Summary of statistics for vitamin A (retinol palmitate) analysis in whole, 2% fat, and skim milks from US Food and Drug Administration proficiency test samples.

	Milk type		
	Whole	2% Fat	Skim
Range (IU/100 mL)	200–420	50–420	0–420
SD	12	2.7	3.5
Mean recovery[1]	92%	95%	93%
n	17	10	10
% RSD_r[2]	5.6	1.2	1.5

[1]Overall mean recovery calculated using sample one of each proficiency set.

[2]Day-to-day repeatability.

the levels of vitamin A in 2% fat milk were much better and were close to the satisfactory level. Our analysis revealed that none of the samples tested for vitamin A contained more than the maximum recommended limit. In all, 22 of the 39 samples (56%) tested for vitamin A content were in compliance, whereas 17 (44%) were below the required level. A survey conducted by Tanner et al. (22) found that in Oregon approximately 75% of the whole milks assayed contained less than 80% of the label claim for vitamin A. In contrast 80% of the whole milks assayed across the US for vitamin A contained more than 120% of the label claim. All data for Ontario reatail milk were corrected for recovery with a mean recovery of 93% for vitamin A and 94% for vitamin D.

In the US vitamin D fortification in milk is optional; however, fortified milk must contain 400 IU/0.946 ml, and this must be in the label, but in Canada, it is not required to declare the level of vitamins on the label. However, the processors must adhere to good manufacturing practices for vitamin addition to fluid milk, which should, in theory, ensure satisfactory fortification levels in the Canadian retail milk supply. Good manufacturing practices require that analysis be done on all enriched products at least monthly and more frequently if significant deviations are encountered.

Table 5. Summary of statistics for vitamin D_3 analysis in whole, 2% fat, and skim milks from US Food and Drug Administration proficiency test samples.

	Milk type		
	Whole	2% Fat	Skim
Range (IU/100 mL)	0–42	0–42	0–85
SD	2.9	1.9	1.6
Mean recovery[1]	97%	92%	93%
n	17	10	10
% RSD_r[2]	8.5	5.8	6.2

[1]Overall mean recovery calculated using sample one of each proficiency set.

[2]Day-to-day repeatability.

Table 6. Vitamin A content in whole, 2% fat, and skim milks.

Sample	Whole milk[1]		2% Fat		Skim milk	
	(IU/100 ml)					
1	87	U	98	U	57	U
2	87	U	265	S	78	U
3	271	S	96	U	150	S
4	254	S	134	S	0	U
5	95	U	232	S	166	S
6	186	S	211	S	185	S
7	103	U	10	U	54	U
8	131	S	148	S	104	U
9	114	U	147	S	102	U
10	121	U	247	S	234	S
11	110	U	267	S	244	S
12	125	U	212	S	164	S
13	154	S	221	S	159	S

[1]S = satisfactory level, >127 to 322< IU/100 ml; U = unsatisfactory level, <127 or 322> IU/100 ml.

Vitamin D_3 content in whole, 2% fat, and skim milks are illustrated in Table 7. Only 20% of whole milk samples contained the recommended levels of vitamin D. The majority of the samples were overfortified, whereas 27% were underfortified. Almost identical results were obtained for the skim milk in which 80% of the samples were either over- or underfortified, whereas only 47% of 2% fat milk samples were within the specified range. Thirteen of the 45 milk samples (29%) that were assayed for vitamin D contained the level stipulated by federal guidelines (31.7 to 51.6 IU/100 ml). Sixteen (36%) of the milk samples were above the range, whereas 16 (36%) were below the recommended level. Chen et al. (6) reported a similar trend. Two samples of whole milk, one sample of 2% fat milk, and one sample of skim milk contained no detectable levels of vitamin D. This finding is of concern because the Canadian

Table 7. Vitamin D content in whole, 2% fat, and skim milks.

Sample	Whole milk[1]		2% Fat		Skim milk	
	(IU/100 ml)					
1	0.0	U	0	U	0.0	U
2	0.0	U	97.6	U	61.8	U
3	64.4	U	66.7	U	60.2	U
4	47.3	S	26.2	U	34.2	S
5	16.4	U	25.1	U	51.3	S
6	20.7	U	52.2	S	57.6	U
7	39.6	S	39.8	S	39.1	S
8	85.3	U	27.8	U	30.0	U
9	61.3	U	43.6	S	25.8	U
10	34.9	S	16.9	U	28.9	U
11	58.2	U	33.3	S	64.4	U
12	77.3	U	15.6	U	13.3	U
13	75.1	U	48	S	97.8	U
14	55.1	U	39.6	S	29.8	U
15	74.4	S	41.1	S	89.9	U

[1]S = satisfactory level, >31.7 to 51.6< IU/100 ml; U = unsatisfactory level< 31.7 or 51.6> IU/100 ml.

federal guidelines state that "the finding of consistently low levels of vitamin D in milk" should be considered a potential health hazard. The lack of vitamin D in milk can eventually have detrimental effects on children and the elderly. Health Canada started a compliance improvement program for Canadian dairies in 1990, with the intent of raising the compliance levels for vitamin D fortification up to about 85% by 1995.

The result of this study, indicates that a portion of the milk marketed in Ontario does not contain the recommended level of vitamins, which is similar to findings of an earlier study in the US conducted by the USFDA (22) and most recently Holick et al. (13). To ensure adequate vitamin fortification of retail milk, it is essential that a rigorous monitoring program be instituted.

CONCLUSIONS

The methods used were simple and reproducible for the analysis of vitamins A and D in fluid milk. We separated analyte from interfering substances and identified them, and we achieved reasonable measure of precision and accuracy. The reliability of these procedures should encourage more frequent testing so that the wide variation observed in the vitamin content of Ontario retail milk could be easily monitored.

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REFERENCES

1 Agarwal, V. K. 1988. Liquid chromatographic determination of vitamin D in fortified milk J. Assoc. Off. Anal. Chem. 71:19–22.
2 American Public Health Association. 1992. Standard Methods for the Examination of Dairy Products. 16th ed. American Public Health Assoc., Washington, DC.
3 Ball, G.F.M. 1996. Determination of the fat-soluble vitamins in foods by high- performance liquid chromatography. Pages 601–647 *in* Handbook of Food Analysis. Vol. 1 Marcel Dekker, New York, NY.
4 Blank, S., K. S. Scanlon, T. H. Sinks, S. Lett, and H. Falk. 1995 An outbreak of hypervitaminosis D associated with the overfortification of milk from a home delivery dairy. Am. J. Publ. Health 855:656–659.
5 Bui, M. H. 1987. Sample preparation and liquid chromatographic determination of vitamin D in food products. J. Assoc. Off. Anal. Chem. 70:802–805.
6 Chen, T. C., H. Heath, and M. F. Holick. 1993. An update on the vitamin D content of fortified milk from the United States and Canada. N. Engl. J. Med. 329:1507.
7 Committee on Nutrition. 1967. The relation between infantile hypercalcemia and vitamin D- public health implications in North America. Pediatrics 40:1050–1061.
8 Food Directorate, Health Protection Branch. 1996. Vitamin Addition to Fluid Milk. Federal Doc. FGN1. Health and Welfare Canada, Ottawa, ON, Canada.
9 Health and Welfare Canada. 1976. The Food and Drug Act and Regulations. Info. Canada, Ottawa, ON, Canada.
10 Henderson, S. K., and A. F. Wickroski. 1978. Reverse-phase-high pressure liquid chromatographic determination of vitamin D in fortified milk. J. Assoc. Off. Anal. Chem. 61:1130–1134.
11 Holick, M. F. 1996. Vitamin D and bone health. J. Nutr. 126(Suppl. 4):1159S–1164S.
12 Holick, M. F. 1997. Nutr. Action Health Lett. Oct.:3–6.
13 Holick, M. F., Q. Shao, W. W. Liu, T. C. Chen. 1992. The vitamin D content of fortified milk and infant formula N. Engl. J. Med. 326:1178–1181.
14 Indyk, H., and D. C. Woollard. 1985. The determination of vitamin D in milk powder and infant formulas by HPLC. J. Micronutr. Anal. 1:121–141.
15 Jacobus, C. H., M. F. Hollick, Q. Shao, T. C. Chen, I. Holm, J. M. Kolodny G.E.-H., Fuleihan, and E. W. Seeley. 1992. N. Engl. J. Med. 326:1173–1177.
16 Johnsson, H., and H. Hessel. 1987. HPLC Determination of cholecalciferol (Vitamin D) in food. Int. J. Vitam. Nutr. Res. 59:357–365.
17 Muniz, J. F., C. T. Wehr, and H. M. Wehr. 1982. Reverse-phase liquid chromatographic determination of vitamin D_2 and D_3 in milk. J. Assoc. Off. Anal. Chem. 65:791–797.
18 O'Keefe, S. F., and P. A. Murphy. 1988. Rapid determination of vitamin D in fortified skim milk. J. Chromatogr. 445:305.
19 Peehl, D. M., R. J. Skowronski, and D. Feldman. 1995. Vitamin D and prostate cancer. Adv. Exp. Med. Biol. 375:53–63.
20 Renken, S. A., and J. J. Warthesen. 1993. Vitamin D stability in milk. J. Food Sci. 58:502–555.
21 Sokoloff, L. 1978. Occult osteomalacia in American (U.S.A.) patients with fracture of the hip. Am. J. Surg. Pathol. 2:21.
22 Tanner, J. T., J. Smith, P. Defibaugh, G. Angyal, M. Villalobos, M. P Bueno, E. T. McGarrahan, H. M. Wehr, J. F. Muniz, and B.W. Hollis. 1988. Survey of vitamin content of fortified milk. J. Assoc. Off. Anal. Chem. 71:607–610.
23 Thompson, J. N., G. Hatina, and W. B. Maxwell. 1980. HPLC determination of vitamin A in margarine, milk, partially skimmed milk and skimmed milk. J. Assoc. Off. Anal. Chem. 63:894–898.
24 Thompson, J. N., G. Hatina, W. B. Maxwell, and S. Duval. 1982. High- performance liquid chromatographic determination of vitamin D in fortified milks margarine, and infant formulas. J. Assoc. Off. Anal. Chem. 65:624–631.
25 Veith, R. 1999. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. Am. J. Clin.Nutr. 69:842–856.
26 Wickroski, A. F. 1984. Improved reversed phase liquid chromatographic determination of vitamins A & D in fortified milk J. Assoc. Off. Anal. Chem. 67:62-65.
27 Woolland, D. C., and H. Indyk. 1986. The HPLC analysis of vitamin A isomers in dairy products and their significance in biopotency estimation. J. Micronutr. Anal. 2:125–146.