Journal of Food Composition and Analysis 22 (2009) 472–478

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Article

Vitamin D metabolites in bovine milk and butter

Jette Jakobsen *, Erling Saxholt

National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark

ARTICLE INFO

Article history:
Received 1 November 2007
Received in revised form 23 May 2008
Accepted 16 January 2009

Keywords:
Vitamin D_3
Vitamin D_2
25-hydroxyvitamin D_3
25-hydroxyvitamin D_2
Vitamin D in dairy products
Fat
Season
Organic
Food data quality
Food analysis
Food composition

ABSTRACT

The main goal of this study was to establish the content of vitamin D_3, 25-hydroxyvitamin D_3 ($25OHD_3$), vitamin D_2, and 25-hydroxyvitamin D_2 ($25OHD_2$) in dairy products. Composite samples of milk, cream, and butter (1.5–80% fat), and organic milk (3.5% fat) were analyzed. Each composite sample was sampled over 3 months and the sample comprised 12 units (range 8–14). The average content of vitamin D_3 and $25OHD_3$ were 4.6–196 ng/100 g and 4.2–96 ng/100 g, respectively. For vitamin D_2 the values, in milk and butter, were 3.4 and 61 ng/100 g, respectively, and for $25OHD_2$ they were 3.1 and 58 ng/100 g, respectively. Statistically significant effects of food product ($p < 0.0001$) and season ($p < 0.05$) on the content of vitamin D_3 and $25OHD_3$ were shown. Vitamin D_3 and $25OHD_3$ were significantly associated with fat ($R = 0.88$, $p < 0.0001$ and $R = 0.96$, $p < 0.0001$, respectively). Differences were observed between organically and conventionally produced milk. Implementation of these values into Food Databanks has to be done carefully, due in part to a lack of consensus on the relative bioactivity between the vitamin D active compounds, and partly because quantification of dihydroxy vitamin D compounds were not included in this study.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Vitamin D is an essential nutrient for maintaining a satisfactory calcium homeostasis for proper development and maintenance of bone (Holick, 2004). During summer, the primary source of vitamin D for a person exposed to sunlight is the metabolism of 7-dehydrocholesterol to pre-vitamin D in the skin by UV-B radiation, whereas vitamin D in food is the secondary source. In winter, oral intake of vitamin D may be the primary source as the UV-B-related synthesis in the skin is limited at latitudes above 35° (Holick, 2004). Similarly, oral intake of vitamin D is the primary source all the year round for people not exposed to sunlight, e.g. due to clothing.

Data derived from reliable, accurate, and precise methods performed on samples representative for the products of concern, are essential for the calculation of dietary intake of vitamin D from food.

The Danish Dietary Survey showed that milk products contribute 10% of the dietary intake of vitamin D (Lyhne et al., 2005). This calculation is based on the content of vitamin D in a very limited number of food samples analyzed from 1961 to 1976 with the biologic assay (Søndergaard and Leerbeck, 1982; DFCD, 2005; Lyhne et al., 2005). Similarly, imprecision concerning vitamin D may occur in Food Composition Tables if the content of $25OHD_3$ is not included (Deharveng et al., 1999).

Vitamin D metabolism is responsible for the different vitamin D active compounds present in milk. In mammals the parent vitamin D compounds, cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), are hydroxylated in the liver to 25-hydroxyvitamin D_3 ($25OHD_3$) and 25-hydroxyvitamin D_2 ($25OHD_2$) and are further hydroxylated in the kidney to the metabolic active forms 1,25-dihydroxyvitamin D_3 and 1,25-hydroxyvitamin D_2, as well as to other dihydroxyvitamin D components (Holick, 2004). In milk, the major vitamin D activity derives from vitamin D_3, vitamin D_2, $25OHD_3$ and $25OHD_2$ (Hollis et al., 1981; Reeve et al., 1982; Kunz et al., 1984; Parvianinen et al., 1984; Takeuchi et al., 1988). However, in vitamin D fortified milk the major vitamin D activity will only be the vitamin D components added, e.g. in the United States the optional fortification is 10–15 μg vitamin D per liter (Murphy et al., 2001).

The original standard methods for measuring vitamin D were biological assays using the ability of vitamin D to cure rickets in vitamin D deficient rats (US Pharmacopoeia, 1955; Ph. Nord, 1964). These methods are, however, time consuming and imprecise, and cannot distinguish between the different forms of vitamin D. In 2000, a chemical method for the determination of vitamin D_3 and vitamin D_2 was set as a European standard (CEN, 2000). It is essential for food composition data to include the determination of

* Corresponding author. Tel.: +45 72 34 74 15.
E-mail address: jeja@food.dtu.dk (J. Jakobsen).

0889-1575/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jfca.2009.01.010

$25OHD_3$ as well as $25OHD_2$, as both these compounds have vitamin D activity (Blunt et al., 1968; Suda et al., 1970).

Investigations into vitamin D activity in milk have used an in-house protein-binding assay (Hollis et al., 1981; Kunz et al., 1984; Parvianinen et al., 1984) or specific HPLC (Reeve et al., 1982; Takeuchi et al., 1988; Mattila et al., 1995). However, except for Mattila et al. (1995), the number of samples analyzed was limited or part of a feeding trial, and therefore not suitable as source of food composition data.

This study had four goals. The first goal was to obtain a quantitative detection limit as low as 5 ng/100 g for vitamin D_3, $25OHD_3$, vitamin D_2, and $25OHD_2$ in dairy products. The second goal was to investigate the effect of season, fat content, and organic versus conventional farming on the vitamin D content of dairy products. The third goal was to establish new data for the content of the vitamin D metabolites in milk and butter, and the fourth goal to compare these specific data with the amounts obtained by the biological assay.

2. Materials and methods

2.1. Sampling protocol

All samples were produced and sampled between February 2002 and January 2003. Sampled food products included semi-skimmed milk (1.5% fat), whole milk (3.5% fat), cream (13% fat, except for last quarter when industry standard changed to 9% fat), whipping cream (38% fat), butter (80% fat), and organic whole milk (3.5%). We stratified the sampling according to the market share in Denmark using information from the Danish Dairy Board, Arla Foods (the major dairy in Denmark, which holds a market share of 79–98%) and websites of the other Danish dairies. We sampled milk and cream solely from dairies in Arla Foods, while butter and organic whole milk was sampled from three and five different dairies, respectively.

Arla Foods collaborated in the sampling of their own product. At each of their dairies, a contact person collected the samples. For products from other dairies we purchased the samples in Copenhagen area supermarkets, and likewise in the few cases if direct sampling at Arla Foods' dairies failed. Each unit consisted of 1 L of milk (semi-skimmed, whole, organic whole), 1/4 L of cream, or 250 g butter. All units were kept at a maximum of 5 °C during transport.

In the laboratory composite samples were stored in brown glass bottles. Each composite sample consisted of subsamples of each unit collected. The subsamples were 125 g semi-skimmed milk, 100 g whole milk, 50 g cream 13% fat (9% fat), 30 g whipping cream, or 15 g butter. The air was replaced by nitrogen, and the samples were stored at −20 °C for a maximum of 2 years until analysis, but the second analysis of some of the samples were run after up to 5 years of storage.

2.2. Analytical method

The analytical method and the equipment used to determine vitamin D_3 and $25OHD_3$ in milk, cream and butter were previously described for the analysis of meat and liver (Jakobsen et al., 2004; Jakobsen et al., 2007). Briefly, 200 ng each of vitamin D_2 and $25OHD_2$ were added as an internal standard to the test portion and saponified with ethanolic potassium hydroxide. The unsaponifiable matter was extracted with diethyl-ether:petroleum ether (1:1). This solution was purified on a silica solid-phase extraction column. Subsequently, clean-up was performed on two preparative HPLC-procedures, first, a silica and amino column and second, a cyano-column. Finally, the separation, detection and quantitation were performed on an analytical HPLC-system with a reversed phase column and DAD-detector and UV-detector for detection and quantification, respectively.

We made some modifications to the method partly to enhance the quantitative limit and partly to include quantitation of vitamin D_2 and $25OHD_2$. For the identification by DAD-detector, a minimum of 1 ng was injected, which corresponded to at least a content of 4 ng in the test portion. This was obtained by freeze-drying (Christ Beta 1-8, Martin Christ Gefriertrochnungsanlagen GnbH, Osterode, D) the milk, and using 20–30 g as test portions, while 30, 25, and 10 g test portions were used for cream (13/9% fat), cream (38% fat), and butter, respectively. Furthermore, the dilution volumes in the clean-up step were minimized to 200–400 μL, while the injection volumes were to the maximum of 150 μL.

Test portions of freeze-dried whole milk and butter were analysed without addition of the internal standards. To calculate the content of vitamin D_2 and $25OHD_2$, the samples were analysed without the addition of the internal standards, and afterwards the relative peak area of vitamin D_2/vitamin D_3 and $25OHD_2/25OHD_3$ were multiplied by the content of vitamin D_3 and $25OHD_3$, respectively.

Validity of the method for vitamin D_3 was assessed by analysis of a certified reference material of 0.143 ± 0.008 mg vitamin D_3/kg (Milk Powder, CRM 421, IRMM, Geel, B). The quality control included recovery tests for vitamin D_3 and $25OHD_3$ added in equivalent amount as in the test portion, and analyses of house-reference materials of freeze dried whole milk and butter analysed regularly, i.e. every fifth sample, throughout the study period.

All analyses were made in duplicate, and were performed after completion of sampling. The capacity for the extraction comprised only two samples a day. Based on the results obtained in this study the method became accredited according to ISO17025 (ISO, 2000).

2.3. Data analysis

To test the effect of season and food products on each vitamin D compound, a regression analysis was performed. In the regression model vitamin D_3 and $25OHD_3$ were dependent variables, and season and food products independent variables. To test the effect of fat content, the organic milk was excluded from the dataset. Association between determinants and variables were assessed with Pearson's correlation coefficients. Data are expressed as mean and standard deviation ($x \pm$ SD). SAS version 9.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses, with a significance level of 0.05.

3. Results

3.1. Performance of the analytical method

Chromatograms at 265 nm for vitamin D and 25OHD are shown in Figs. 1 and 2, respectively.

Response factors for vitamin D_3/vitamin D_2 and $25OHD_3$/$25OHD_2$ were 0.94 and 0.98, respectively. The quantitation limits for the components by the UV-detector depend on the test portion and were for all components in the range 1.3–40 ng/100 g. The identification limits by DAD-detector was approximately twice as high. The results ($n = 3$) for the certified reference material, whole milk powder, 0.140 ± 0.004 mg vitamin D_3/kg was satisfactory compared to certification. The recovery test performed in all food products ($n = 7$) showed a recovery for vitamin D_3 at $95.4\% \pm 2.4\%$ and for $25OHD_3$ at $97.1\% \pm 4.3\%$. The precision assessed by duplicate analysis of the samples ($n = 24$) for vitamin D_3 and $25OHD_3$ showed a CV of 6.9% and 12.1%, respectively. In the house-reference materials of butter, which contained 232 ng vitamin D_3/100 g and 101 ng $25OHD_3$/100 g, the precision in terms of CV% was 7.6% ($n = 10$) and

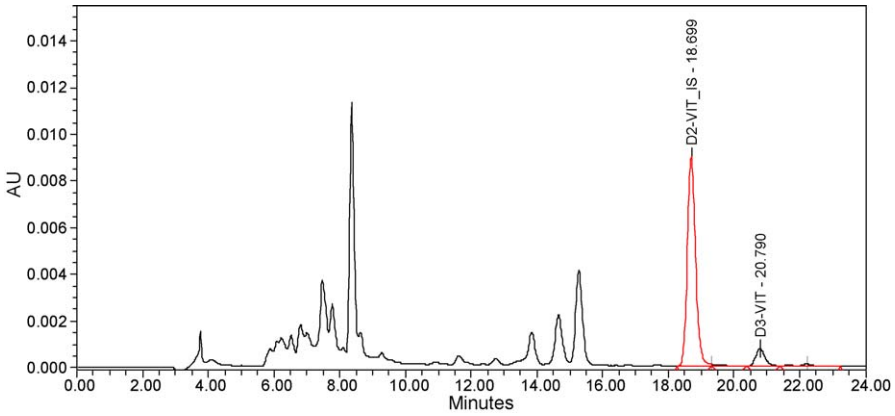

Fig. 1. HPLC-chromatogram of freeze-dried whole milk, 106 ng vitamin D_3/100 g detected at 265 nm in absorption unit (AU). Abbreviation: D_2-VIT_IS, vitamin D_2 added as internal standard; D_3-VIT, vitamin D_3 in test sample.

7.4% ($n = 9$), respectively. While for freeze-dried whole milk with a content of 63 ng vitamin D_3/100 g and 58 ng 25OHD$_3$/100 g the precision in terms of CV% was 3.7% ($n = 8$) and 7.4% ($n = 11$), respectively. The pooled estimates for the precision for vitamin D_3 was CV = 6.6% and for 25OHD$_3$ CV = 10.3%. CV for vitamin D_2 and 25OHD$_2$ assessed by duplicate analysis of butter and freeze-dried whole milk was 14% for both.

3.2. Composite samples

The composite samples comprised 12 ± 2 units (range 4–14) were produced for samples collected from February to April, May to July, August to October or November to January. The specific number of units for each composite sample is presented in Table 1.

3.3. Vitamin D_3 and 25OHD$_3$

The analytical results for vitamin D_3 and 25OHD$_3$ in each of the 24 composite samples, and the mean content for each of the food products are presented in Table 1. The results showed that food product ($p < 0.0001$) and season ($p < 0.05$) significantly affected the content of vitamin D_3 as well as 25OHD$_3$.

The quantitative effect of the fat content on the content of vitamin D_3 and 25OHD$_3$ was performed in the non-organic product, i.e. semi-skimmed milk (1.5% fat), whole milk (3.5% fat), cream 9/13% fat and 38% fat and butter with 80% fat. The content of vitamin D_3 and 25OHD$_3$ was significantly associated with the content of fat ($R = 0.88$; $p < 0.0001$, and $R = 0.96$; $p < 0.0001$, respectively). For each season the dependence on fat for vitamin D_3 and 25OHD$_3$ was estimated by linear regression in all 20 composite samples. The results are shown in Table 2.

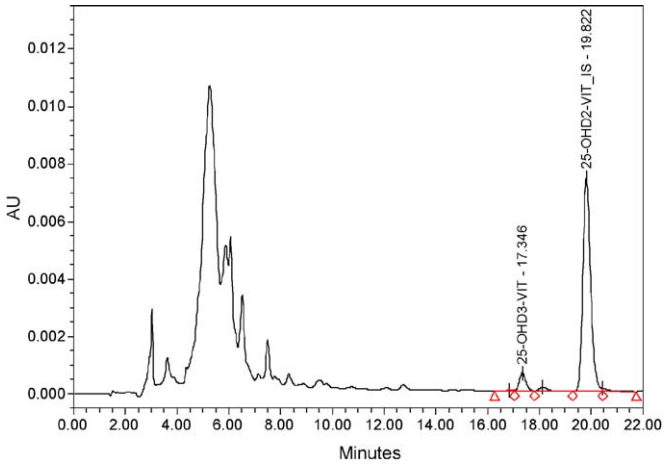

Fig. 2. HPLC-chromatogram of freeze-dried whole milk, 66 ng 25OHD$_3$/100 g detected at 265 nm in absorption unit (AU). Abbreviation: 25OHD$_2$-VIT_IS, vitamin 25OHD$_2$ added as internal standard; 25-OHD$_3$-VIT, vitamin 25OHD$_3$ in test sample.

3.4. Vitamin D_2 and 25OHD$_2$

The content of vitamin D_2 and 25OHD$_2$ were quantitated in whole milk and butter, see Table 3.

4. Discussion

4.1. Analytical method

The quantitative limits by the DAD-spectrum for each of the vitamin D active compounds were approximately one fifth of those obtained by Mattila et al., 1995. Additionally, in the present study DAD-spectra were used for the identification of the compounds, while the more sensitive UV-detector was used for the quantitation. The trueness and precision for vitamin D_3 is acceptable for the method according to CEN-standard (CEN, 2000). For 25OHD$_3$, either a standard method or a proficiency-testing program is available. However, concerning the trueness, the acceptable results of the recovery test in milk are confirmed by satisfactory results in a proficiency-testing program for 25OHD$_3$ in serum (DEQAS, Charing Cross Hospital, London, UK) with a similar HPLC-method for 25OHD$_3$ in serum (Jakobsen et al., 2008). The precision for 25OHD$_3$, vitamin D_2, and 25OHD$_2$ was higher than for vitamin D_3, but comparable with a similar study in milk (Mattila et al., 1995).

The amount of the internal standard was increased to be at least 50 times the content of vitamin D_2 in the test sample. Due to this, the quantitated amount of vitamin D_3, and to a lesser extent 25OHD$_3$, show at maximum a bias of 2%; which, compared with the analytical data for the method, is acceptable.

4.2. Vitamin D depends on season

The statistically significant dependence on season was similar to the results from the former Danish Survey for vitamin D in butter. In 1961–1967, the highest content of vitamin D assessed by the biological assay was in May and June (Søndergaard and Leerbeck, 1982). In contrast, Mattila et al., 1995 found a lower content of vitamin D_3, 25OHD$_3$, and vitamin D_2 in butter and whipping cream sampled in May than in October. An explanation for this difference could be the difference in latitude, as Finland is situated at higher latitudes than Denmark (Holick, 2004).

Table 1
Content of vitamin D_3 and $25OHD_3$ (ng/100 g) in the composite samples and the mean values of all the samples.

Food	Fat[a] (%)	Season (months)	Composite samples			Mean[b] ± SD	
			Units[c]	Vitamin D_3	$25OHD_3$	Vitamin D_3	$25OHD_3$
Milk (semi-skimmed)	1.5	Feb–April	11	4.1	3.8*	4.6 ± 1.5a	4.2 ± 0.3a
Milk (semi-skimmed)	1.5	May–July	14	6.6	4.4*		
Milk (semi-skimmed)	1.5	Aug–Oct	12	4.9	4.5*		
Milk (semi-skimmed)	1.5	Nov–Jan	12	2.9	4.0		
Milk (whole)	3.5	Feb–April	11	7.7	6.3	9.2 ± 3.0a	7.5 ± 1.3a
Milk (whole)	3.5	May–July	14	13.5	8.3		
Milk (whole)	3.5	Aug–Oct	12	8.9	8.9		
Milk	13.0	Feb–April	9	26.3	20.2*	44 ± 19a	27 ± 6b
Cream (coffee)	13.0	May–July	12	63.7	31.3		
Cream (coffee)	13.0	Aug–Oct	11	43.5	28.2*		
Cream (coffee)	9.0	Nov–Jan	12	21.1	15.7*		
Cream (whipping)	38.0	Feb–April	11	62.3	47.6	94 ± 4b	59 ± 10c
Cream (whipping)	38.0	May–July	14	152	70.3		
Cream (whipping)	38.0	Aug–Oct	12	95.9	64.2		
Cream (whipping)	38.0	Nov–Jan	12	66.6	55.1		
Butter	80.0	Feb–April	11	135	81.0	196 ± 95c	96 ± 18d
Butter	80.0	May–July	8	336	102		
Butter	80.0	Aug–Oct	12	177	119		
Butter	80.0	Nov–Jan	11	137	81.5		
Milk (whole, organic)	3.5	Feb–April	10	3.1*	3.6*	7.6 ± 7.5a	5.7 ± 2.2a
Milk (whole, organic)	3.5	May–July	14	18.6	8.1		
Milk (whole, organic)	3.5	Aug–Oct	11	6.2	7.0		
Milk (whole, organic)	3.5	Nov–Jan	12	2.6	4.0		

Mean values within a column with unlike letters were significantly different ($p < 0.05$).
Asterisks show results below the identification limit for the DAD-detector, but higher than the quantitation limit for the UV-detector.
[a] Content of fat from the nutritional information on the packages.
[b] Means of the four composite samples.
[c] Units in the composite samples.

The effect of season may be explained by the feeding practice in summer, i.e. the cows grazing. In New Zealand, in the southern hemisphere, the seasonal variation for milk from dairy cows grazing during summer, and without supplementation of vitamin D_3 during winter, showed a similar highest content of vitamin D_3 in the summer (Kurmann and Indyk, 1994). However, the effect of season may decrease due to changes in husbandry practices. In 2003, 74% of the Danish conventional farmers used grazing for their dairy cows, which in 2007 had decreased to 50% (DLR, 2007). The observed effect of season was not surprising as a similar association between vitamin D status and sun-habits was observed in humans (Brot et al., 2001).

4.3. Vitamin D depends on fat content

Analysis for fat was not performed, due to the very strictly regulated and the high degree of control of fat in dairy products. Therefore the nutrition label for fat was taken as the content of fat. Vitamin D_3 and $25OHD_3$ depend significantly on the content of fat. The estimations for the dependence on fat show that vitamin D_3 is almost twice as sensitive to fat compared to $25OHD_3$, while $25OHD_3$ is present in even fat-free products, which may be explained by the difference in polarity of vitamin D_3 and $25OHD_3$. A similar relative difference between the two vitamin D compounds was observed in pork (Jakobsen et al., 2007).

4.4. Organic versus conventional

The whole milk delivered from cows conventionally or organically fed shows no significant difference with respect to the content of vitamin D_3. This is probably due to the high amount of vitamin D_3 in the organic composite sample taken between May and July, as all other organically produced samples show lower content of vitamin D_3 than similar conventional samples. For $25OHD_3$ a similar trend was observed, but the limited number of samples hinders statistical significance of the difference. Even with this lack of significant difference, the visible difference can be explained in the difference in feeding methods between the two farming types.

In Denmark, conventional farmers are advised to add 475 μg vitamin D_3/day/dairy cow if the cows are staying inside, while the norm is only 300 μg vitamin D_3/day/dairy cow (Studsholm et al., 1999; Møller and Aaes, 2004). Furthermore, in summer the producers of organic milk, if possible, shall take their cows on grass

Table 2
For each season and for the whole year the dependence on fat for vitamin D_3 and $25OHD_3$ estimated by linear regression.

Season (months)	Composite samples[a]	Fat (%)	Vitamin D_3[b] (ng/100 g)	$25OHD_3$[c] (ng/100 g)
February–April	5	1.5–80	1.6 × %fat + 2.2a	0.98 × %fat + 5.0a
May–July	5	1.5–80	4.2 × %fat + 1.3b	1.2 × %fat + 9.4b
August–October	5	1.5–80	2.2 × %fat + 6.8ab	1.4 × %fat + 5.7b
November–January	5	1.5–80	1.7 × %fat + 2.2a	1.0 × %fat + 5.7a
The whole year	20	1.5–80	2.4 × %fat + 3.3	1.2 × %fat + 6.6

[a] Only composite samples of conventionally produced products included.
[b] Coefficient of determination (R^2) for each of the four seasons were 0.999, 0.998, 0.991, and 1.000, respectively. While for the whole year the value was 0.752.
[c] Coefficient of determination (R^2) for each of the four seasons were 0.988, 0.966, 0.995, and 0.962, respectively. While for the whole year the value was 0.926.
Within a column unlike letter indicate significantly different dependence of vitamin D_3 and $25OHD_3$ on fat content ($p < 0.05$).

Table 3
Content of vitamin D_2 and $25OHD_2$ (ng/100 g) in whole milk and butter.

Food	Fat[a] (%)	Season (months)	Composite samples			Mean[b] ± SD	
			Units[c]	Vitamin D_2	$25OHD_2$	Vitamin D_2	$25OHD_2$
Milk (whole)	1.5	Feb–April	11	2.0*	2.9*	3.4 ± 1.2	3.1 ± 0.7
Milk (whole)	1.5	May–July	14	4.7*	2.3*		
Milk (whole)	1.5	Aug–Oct	12	4.2*	4.0*		
Milk (whole)	1.5	Nov–Jan	12	2.9*	3.3*		
Butter	80.0	Feb–April	11	56*	53*	61 ± 5.0	58 ± 23
Butter	80.0	May–July	14	58*	35*		
Butter	80.0	Aug–Oct	12	63*	54*		
Butter	80.0	Nov–Jan	12	67	90		

Asterisks show results below the identification limit for the DAD-detector, but higher than the quantitation limit for the UV-detector.
[a] Content of fat from the nutritional information on the packages.
[b] Means of the four composite samples.
[c] Units in the composite samples.

from April 15 to November 1, while only 74% of the farmers of conventional milk give their cows admission to grazing (PD, 2000; DLR, 2007). The high degree of dependence on the vitamin D content of UV-B radiation (290–315 nm) may explain the rather low vitamin D_3 content in the composite sample from August–October, as no production of vitamin D_3 through skin is possible in October in Denmark (Holick, 2004).

The apparent difference in vitamin D_3 and $25OHD_3$ between the conventionally and the organically produced milk due to feeding, whether exposed to sunshine or given synthetic vitamin D_3, has previously been shown in cow's milk, pork, egg, and salmon (Hollis et al., 1981; Mattila et al., 1999; Graff et al., 2002; Jakobsen et al., 2007).

4.5. Specific data for the content of vitamin D metabolites in milk

Systematic studies of vitamin D, and especially vitamin D_3, $25OHD_3$, vitamin D_2, and $25OHD_2$ are limited to Mattila et al., 1995. As previously mentioned for butter and whipping cream, the seasonal variation was different between the two studies, but the content of vitamin D_3, $25OHD_3$, and vitamin D_2 were at a similar level.

The calculation of the vitamin D activity derived from the quantitated vitamin D compounds is difficult as the studies investigating the relative bioactivity of these compounds are limited.

Though a relative bioactivity of $25OHD_3$ compared to vitamin D_3 is often regarded to be 5 (IOM, 1997; FSA, 2002; DFCD, 2005), no consensus has been established (Ovesen et al., 2003), and documentation for this factor seems to be lacking (Jakobsen, 2007). By the standardized biological method for quantitation of vitamin D, the bioactivity of $25OHD_3$ compared to vitamin D_3 was assessed to 1.4, 1.7 and 2 (Blunt et al., 1968; Miravet et al., 1976; Leerbeck, 1977). Today, vitamin D status, i.e. $25OHD_3$ in plasma, is an accepted biomarker for dietary intake of vitamin D in the absence of sunlight (SCF, 2006). In pigs, which from weaning to slaughter were fed either vitamin D_3 or $25OHD_3$ as a vitamin D source, the effect on vitamin D status was similar for the two compounds (Jakobsen et al., 2007). Based on these four studies the relative bioactivity of $25OHD_3$ compared to vitamin D_3 is estimated as the average value of 1.5.

For vitamin D_2 compared to vitamin D_3 it is assumed that these compounds act similarly, though human intervention studies have shown a relatively lower activity of vitamin D_2 (Trang et al., 1998; Armas et al., 2004). However, recently results from Holick et al. (2008) showed no difference.

Investigation of the bioactivity of $25OHD_2$ has been tested only in the rat line test to be 1.5 times higher than for vitamin D_2 (Suda et al., 1970).

Calculation of the contribution to the vitamin D activity in butter from the four compounds quantitated was performed with these values. The results are shown in Fig. 3.

The metabolites 1,25-dihydroxyvitamin D_3, 24,25-dihydroxy vitamin D_3, and 25,26-dihydroxyvitamin D_3 were shown to express vitamin D activity (Boyle et al., 1973; Tanaka et al., 1973; Miravet et al., 1976).

The lack of composition data for dihydroxy vitamin compounds, and the lack of consensus on the relative bioactivity among the vitamin D active compounds, make accurate calculation of dietary intake difficult. However, until specific factors have been established, the new specific data for all food items will improve the ability for human intervention studies to find an association between dietary intake and vitamin D status (Andersen et al., 2005). At present, data obtained by biological assay hampers such calculation due to the different amount of dihydroxy vitamin D compounds in food items.

4.6. Comparison between new specific data and old data obtained by biological assay

Johnsson and Hessel (1987) compared vitamin D_3 determined by HPLC and by a biological assay in low fat milk, porridge, gruel, and infant formula, and found no difference between the two methods. However, the amount of 25-hydroxy- and dihydroxyvitamin D compounds in those samples are regarded as very limited. An optimal method to calculate the contribution from dihydroxy vitamin D compounds would be to run the biological

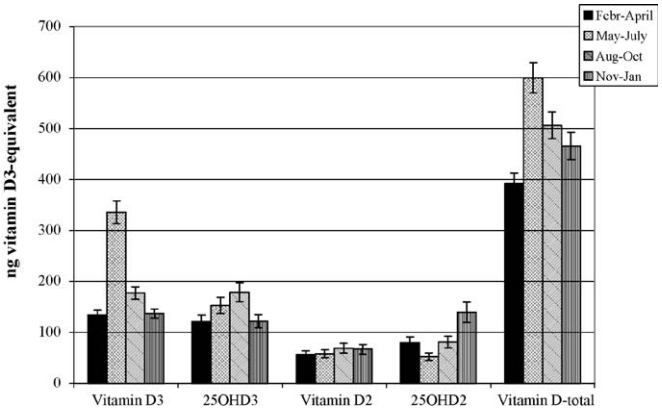

Fig. 3. Calculation of vitamin D activity expressed by the content ($x \pm SD$) of vitamin D_3, $25OHD_3$, vitamin D_2, and $25OHD_2$ in the composite samples of butter. The calculation of vitamin D-total is based on the relative bioactivity factor compared to vitamin D_3 of 1.5, 1 and 1.5 for $25OHD_3$, vitamin D_2, and $25OHD_2$, respectively. See text in Section 4.5.

assay in vitamin D deficient rats on samples included in this study. However, to our knowledge no laboratory is currently using the biological assay. An alternative is to compare the results for a well-defined food analyzed by the biological assay and the present method. Butter is a well-described food, and data assessed by the biological assay is available (Søndergaard and Leerbeck, 1982). Especially the average content of vitamin D at 900 ng vitamin D/100 g in the samples collected in Denmark in May–July during the years 1961–1967 may be compared to the results found in this study. The season May–July is chosen as the dairy cows grazed in the 1960s as well as in 2002. However, the husbandry practice of the 1960s was similar to the organic production in 2002, i.e. all dairy cows were grazing during the summer. The results from the 2002s have to be adjusted from 74% to 100% grazing, i.e. in butter, 454 ng vitamin D_3/100 g butter.

In the comparison between specific chemical data and vitamin D activity assessed by biological assay, the method to assess the relative bioactivity between the specific vitamin D components should be similar to the method used for the quantitation of vitamin D in food. By the standardized biological method performed in rats the bioactivity of $25OHD_3$ compared to vitamin D_3 was assessed to 1.4, 1.7 and 2 (Blunt et al., 1968; Miravet et al., 1976; Leerbeck, 1977), which give an average of 1.7. Similarly, the relative bioactivity for vitamin D_2 compared to vitamin D_3 is regarded as 1, while $25OHD_2$ is 1.5 times as biologically active as vitamin D_3 (Suda et al., 1970).

With these assumptions, and use of the above-mentioned relative bioactivity factors, the vitamin D activity expressed by the content of vitamin D_3, $25OHD_3$, vitamin D_2, and $25OHD_2$ is calculated to represent 740 ng vitamin D equivalent/100 g. The difference between the vitamin D activity in butter in the 1960s and in 2002 may be regarded as the contribution from dihydroxy vitamin D metabolites, i.e. 160 ng vitamin D equivalent or 18% of the vitamin D activity. This rough estimation shows the necessity to include quantitation of dihydroxy vitamin D compounds in the chemical methods in order not to risk a difference between new and old data due to analytical method.

5. Conclusion

Specific contents of vitamin D_3, $25OHD_3$, vitamin D_2, and $25OHD_2$ have been established in milk, cream, and butter. The content of vitamin D is strongly affected by the content of fat and the season. Differences were observed between organically and conventionally produced milk.

The implementation of the values into Food Databanks has to be done carefully partly because of the lack of consensus on the relative bioactivity between the vitamin D active compounds, and partly because quantitation of dihydroxy vitamin D compounds was not included in this study.

Acknowledgements

The authors thank Kristine Thorslund, ArlaFoods, for the assistance with the sampling at ArlaFoods dairies and Kirsten Pinndal and Heddi Koch Jacobsen from the National Food Institute, Technical University of Denmark for technical skills in the analysis.

References

Andersen, R., Molgaard, C., Skovgaard, L.T., Brot, C., Cashman, K.D., Chabros, E., Charzewska, J., Flynn, A., Jakobsen, J., Karkkainen, M., Kiely, M., Lamberg-Allardt, C., Moreiras, O., Natri, A.M., O'brien, M., Rogalska-Niedzwiedz, M., Ovesen, L., 2005. Teenage girls and elderly women living in northern Europe have low winter vitamin D status. European Journal of Clinical Nutrition 59, 533–541.

Armas, L.A.G., Hollis, B.W., Heaney, R.P., 2004. Vitamin D-2 is much less effective than vitamin D-3 in humans. Journal of Clinical Endocrinology and Metabolism 89, 5387–5391.

Blunt, J.W., Tanaka, Y., Deluca, H.F., 1968. Biological activity of 25-hydroxycholecalciferol A metabolite of vitamin D3. Proceedings of the National Academy of Sciences of the United States of America 61, 1503–1516.

Boyle, I.T., Omdahl, J.L., Gray, R.W., Deluca, H.F., 1973. Biological-activity and metabolism of 24,25-dihydroxyvitamin-D D. Journal of Biological Chemistry 248, 4174–4180.

Brot, C., Vestergaard, P., Kolthoff, N., Gram, J., Hermann, A.P., Sorensen, O.H., 2001. Vitamin D status and its adequacy in healthy Danish perimenopausal women: relationships to dietary intake, sun exposure and serum parathyroid hormone. British Journal of Nutrition 86 (Suppl 1), S97–103.

CEN, 2000. EN12821 Foodstuffs—determination of vitamin D by high performance liquid chromatography—measurement of cholecalciferol (D_3) and ergocalciferol (D_2). European Committee for Standardization, Rue de Stassart, 36, B-1050 Bruxelles.

Deharveng, G., Charrondiere, U.R., Slimani, N., Southgate, D.A.T., Riboli, E., 1999. Comparison of nutrients in the food composition tables available in the nine European countries participating in EPIC. European Journal of Clinical Nutrition 53, 60–79.

DFCD, 2005. Danish Food Composition Databank. http://www.foodcomp.dk/fcdb_useofdata.asp.

DLR, 2007. Dansk Landbrugsrådgivnings Landscenter, Skejby, Denmark. Retrieved 2007-10-12. http://www.kvaeg.dk/dyrevelfaerd/faktaomKoerPaaGraes.

FSA, 2002. Food Standards Agency, McCance and Widdowson's The Composition of Foods, Sixth Summary edition. Royal Society of Chemistry, Cambridge.

IOM (Institute of Medicine), 1997. Vitamin D, Chapter 7. In: Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D and Fluoride, The National Academy Press, Food and Nutrition Board, Washington, DC. , In: http://www.nap.edu.

Graff, I.E., Høje, S., Totland, G.K., Lie, O., 2002. Three different levels of dietary vitamin D_3 fed to first-feeding fry of Atlantic salmon (*Salmo salar* L.): effect on growth, mortality, calcium content and bone formation. Aquaculture Nutrition 8, 103–111.

Holick, M.F., 2004. Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. American Journal of Clinical Nutrition 79, 362–371.

Holick, M.F., Biancuzzo, R.M., Chen, T.C., Klein, E.K., Young, A., Bibuld, D., Reitz, R., Samameh, W., Ameri, A., Tannenbaum, A.D., 2008. Vitamin D_2 is as effective as vitamin D_3 in maintaining circulation concentrations of 25-hydroxy vitamin D. Journal of Clinical Endocrinology & Metabolism 93 (3), 677–681.

Hollis, B.W., Roos, B.A., Draper, H.H., Lambert, P.W., 1981. Vitamin-D and its metabolites in human and bovine-milk. Journal of Nutrition 111, 1240–1248.

ISO, 2000. EN ISO/IEC 17025. CEN, Central Secretariat: rue de Stassart, 36 B-1050 Brussels.

Jakobsen, J., Clausen, I., Leth, T., Ovesen, L., 2004. A new method for the determination of vitamin D-3 and 25-hydroxyvitamin D-3 in meat. Journal of Food Composition and Analysis 17, 777–787.

Jakobsen, J., 2007. Bioavailability and bioactivity of vitamin D_3 active compounds—which potency should be used for 25-hydroxyvitamin D_3? International Congress Series 1297, 133–142.

Jakobsen, J., Maribo, H., Bysted, A., Sommer, H.M., Hels, O., 2007. 25-Hydroxyvitamin D_3 affect vitamin D status similar to vitamin D_3 in pigs —but the meat produced have a lower content of vitamin D. British Journal of Nutrition 98, 908–913.

Jakobsen, J., Bysted, A., Andersen, R., Brot, C., Bügel, S., Cashman, K., Teucher, B., Walscyk, T., Ovesen, L., 2008. Vitamin D status assessed by a simple and reliable HPLC-method—within and between variation in subjects supplemented with vitamin D3. Scandinavian Journal of Clinical Investigation, Epub Ahead 21 Oct. doi:10.1080/00365510802471570.

Johnsson, H., Hessel, H., 1987. High performance liquid chromatographic determination of cholecalciferol (vitamin D_3) in food—a comparison with a bioassay method. International Journal Vitamin Nutrition Research 57, 357–365.

Kunz, C., Niesen, M., Vonlilienfeldtoal, H., Burmeister, W., 1984. Vitamin-D, 25-hydroxy-vitamin-D and 1,25-dihydroxy-vitamin-D in cows milk infant Formulas and breast-milk during different stages of lactation. International Journal for Vitamin and Nutrition Research 54, 141–148.

Kurmann, A., Indyk, H., 1994. The endogenous vitamin-D content of bovine-milk—Influence of season. Food Chemistry 50, 75–81.

Leerbeck, E., 1977. Biologisk aktivitet af 25-hydroxyvitamin D_3. Report 23. National Food Agency, Søborg, Denmark.

Lyhne, N., Christensen, T., Groth, M. V., Fagt, S., Biltoft-Jensen, A., Hartkopp, H., Hinsch, H.-J., Matthiessen, J., Møller, A., Saxholt, E., Trolle, E., 2005. Danskernes kostvaner 2000–2002—Hovedresultater. Danmarks Fødevareforskning, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.

Mattila, P.H., Piironen, V.I., Uusirauva, E.J., Koivistoinen, P.E., 1995. Contents of cholecalciferol, ergocalciferol, and their 25-hydroxylated metabolites in milk-products and raw meat and liver as determined by HPLC. Journal of Agricultural and Food Chemistry 43, 2394–2399.

Mattila, P., Lehikoinen, K., Kiiskinen, T., Piironen, V., 1999. Cholecalciferol and 25-hydroxycholecalciferol content of chicken egg yolk as affected by the cholecalciferol content of feed. Journal of Agricultural and Food Chemistry 47, 4089–4092.

Miravet, L., Redel, J., Carre, M., Queille, M.L., Bordier, P., 1976. Biological-activity of synthetic 25,26-dihydroxycholecalciferol and 24 25-dihydroxycholecalciferol in vitamin-D-deficient rats. Calcified Tissue Research 21, 145–152.

Murphy, S.C., Whited, L.J., Rosenberry, L.C., Hammond, B.H., Bandler, D.K., Boor, K.J., 2001. Fluid milk vitamin fortification compliance in New York State. Journal of Dairy Science 84, 2813–2820.

Møller, J., Aaes, O., 2004. KvægInfo: Ny aftale om typemineralblandinger. Rapport 1293. Dansk Landbrugsrådgivnings Landscenter, Skejby, Denmark.
Ovesen, L., Brot, C., Jakobsen, J., 2003. Food contents and biological activity of 25-hydroxyvitamin D: a vitamin D metabolite to be reckoned with? Annals of Nutrition and Metabolism 47, 107–113.
Parvianinen, M.T., Koskinen, T., Ala-Houlala, M., Visakorpi, J.K., 1984. A method for routine estimation of vitamin D activity in human and bovine milk. Acta Vitaminology Enzymology 6, 211–219.
PD, 2000. Guidance on organic farming. The Danish Plant Directorate, Skovbrynet 20, DK-2800 Lyngby, Denmark.
Ph. Nord, 1964. The Danish Pharmacopoea Commission. Biologica assessment of vitamin D activity. Pharmacopoea Nordica, vol. IV. Nyt Nordisk Forlag Arnold Busck, Copenhagen, Denmark, pp. 95a–100.
Reeve, L.E., Jorgensen, N.A., Deluca, H.F., 1982. Vitamin-D compounds in cows milk. Journal of Nutrition 112, 667–672.
SCF, 2006. Opinion of the scientific committee on food on the tolerable upper intake level of vitamin D. European Commission, Health and Consumer Protection Directorate-General, B-1049 Bruxelles.
Søndergaard, H., Leerbeck, E., 1982. The content of vitamin D in Danish Foods. Report 69. Statens Levnedsmiddelinstitut, Mørkhøj Bygade 19, DK-2860 Søborg.
Studsholm, F., Aaes, O., Madsen, J., Kristensen, V.F., Hvelplund, T., Østergaard, S., 1999. Danske fodernormer til kvæg. Rapport 84. Landsudvalget for Kvæg, Skejby, Denmark.
Suda, T., Deluca, H.F., Tanaka, Y., 1970. Biological activity of 25-Hydroxyergocalciferol in Rats. Journal of Nutrition 100, 1049–1052.
Takeuchi, A., Okano, T., Tsugawa, N., Katayama, M., Mimura, Y., Kobayashi, T., Kodama, S., Matsuo, T., 1988. Determination of vitamin-D and its metabolites in human and cows milk. Journal of Micronutrient Analysis 4, 193–208.
Tanaka, Y., Frank, H., Deluca, H.F., 1973. Biological-activity of 1 25-dihydroxyvitamin D_3 in rat. Endocrinology 92, 417–422.
Trang, H.M., Cole, D.E.C., Rubin, L.A., Pierratos, A., Siu, S., Vieth, R., 1998. Evidence that vitamin D-3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D-2. American Journal of Clinical Nutrition 68, 854–858.
U.S. Pharmacopoeia, 1955. Method of Assay for Vitamin D, 5th rev. Mack Publishing Co., Easton, PA.