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The determination of vitamin D_3 and 25-hydroxyvitamin D_3 in early lactation and seasonal bovine milk

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ABSTRACT

There is a need to account for the content of 25-hydroxyvitamin D_3 (25OH-D_3) in foods to more accurately estimate dietary vitamin D intake, given its higher biological activity. A high-performance liquid chromatography–tandem mass spectrometry method was applied to the determination of vitamin D_3 and 25OH-D_3 in bovine milk obtained during early lactation and over the course of a full milking season. In this seasonal study of bovine milk, vitamin D_3 levels ranged from 167 ng L^{-1} in winter to 615 ng L^{-1} in summer, whereas the content of 25OH-D_3 in bovine milk was <50 ng L^{-1} and showed little variation. This study will provide manufacturers with data concerning endogenous vitamin D content that will enhance formulation capability related to the production of bovine-milk-based paediatric products.

1. Introduction

For humans, vitamin D_3 (cholecalciferol) is obtained from both animal-based food intake and exposure of subcutaneous 7-dehydrocholesterol to ultraviolet (UV-B) radiation. In general, humans derive the majority of their vitamin D requirement from the latter route, as unsupplemented foods contain only modest levels. Vitamin D is transported to the liver via a specific binding protein and is metabolised to 25-hydroxyvitamin D_3 (25OH-D_3), which is the major circulating and storage form of vitamin D (Horst, Goff, & Reinhardt, 1994). Indeed, it is now accepted that all higher mammals possess the same conversion pathway of vitamin D_3 to 25OH-D_3 (Heaney & Armas, 2015).

In view of its higher biological activity, recent literature has increasingly focused on the need to account for the content of 25OH-D_3 in foods to more accurately estimate dietary vitamin D in intake surveys, despite the uncertainty regarding the biological potency of 25OH-D_3 relative to that of vitamin D_3, which is variously reported to be 1.5–5.0 (Jakobsen, 2007; Ovesen, Brot, & Jakobsen, 2003). In mammalian milk, vitamin D activity derives predominantly from vitamin D_3 and 25OH-D_3, although the related ergocalciferol forms may also contribute to a minor degree. Any estimate of the contribution to the dietary intake of vitamin D from milk products will therefore be an underestimate if the content of 25OH-D_3 is excluded (Jakobsen & Saxholt., 2009).

Despite the historical dominance of liquid chromatography-ultraviolet (LC–UV) and immunological techniques, LC–mass spectrometry (LC–MS) methods for the determination of vitamin D and its metabolites in milk and foods are becoming increasingly prevalent because of their inherent advantages of sensitivity and selectivity (Kasalová, Aufartová, Krčmová, Solichová, & Solich, 2015; Perales, Alegría, Barberá, & Farré, 2005). Typically, these LC–MS methods rely on either saponification or protein precipitation and lipid extraction, followed by a solvent concentration step to achieve sufficient sensitivity during analysis (Kasalová et al., 2015; Perales et al., 2005; Trenerry, Plozza, Caridi, & Murphy, 2011). Recently, the use of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) as a Diels–Alder derivatisation reagent has been demonstrated to overcome the relatively poor ionisation efficiency of vitamin D and is rapidly becoming a popular technique for the analysis of vitamin D and its metabolites in milk (Gill, Zhu, & Indyk, 2015; Gomes, Shaw, Whitfield, & Hewavitharana, 2015).

Although the vitamin D content of bovine milk is relatively low, milk is considered to be both a significant dietary source and an excellent vector for fortification for populations at risk of deficiency. Currently, there are limited data regarding the influence of lactation and season on the contents of vitamin D_3 and 25OH-D_3 in bovine milk. Therefore, there is a need to evaluate the contribution of these two innate forms to the vitamin D activity of supplemented paediatric and adult nutritional products that are manufactured predominantly from bovine milk. The aim of the present study was to provide knowledge of the temporal variation in the endogenous contents of vitamin D_3 and 25OH-D_3 in bovine milk that may be

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used to improve the formulation of bovine-milk-based nutritional products.

The analytical methodology utilising saponification, liquid extraction, PTAD derivatisation, and LC–MS/MS reported previously (Gill et al., 2015) for vitamin D_3 was therefore extended and validated in this study to include the quantitation of 25OH-D_3 in bovine milk.

2. Materials and methods

2.1. Apparatus

A Nexera X2 UHPLC system consisting of two LC-30AD pumps, an SIL-30AC autosampler, a CTO-20AC column oven, a CBM-20A control module, and a DGU-20A5R degasser unit (Shimadzu, Kyoto, Japan) was used and incorporated a Kinetex 50 mm × 2.1 mm, 2.6 μm core-shell reverse-phase C_{18} column (Phenomenex, Torrance, CA, USA). MS was performed using a 6500 QTrap triple quadrupole detector (ABSciex, Foster City, CA, USA). Analyst software (ABSciex) version 1.6 was used for instrument control and data processing.

2.2. Reagents

Potassium hydroxide, pyrogallol, LC-grade ethanol, acetone, and isooctane, and LC–MS grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Vitamin D_3, formic acid, and PTAD were supplied by Sigma-Aldrich (St. Louis, MO, USA). Stable isotope-labelled (SIL) vitamin D_3 (26,26,26,27,27,27-d_6 cholecalciferol) was supplied by Chemaphor Services (Ottawa, Canada). SIL 25OH-D_3 (26,26,26,27,27,27-d_6 25-hydroxycholecalciferol) was supplied by Isosciences (King of Prussia, PA, USA). Water was purified to 18.2 MΩ resistivity using a Barnstead Genpure water system (Thermoscientific, Waltham, MA, USA).

2.3. Standards

A SIL vitamin D_3 stock standard (~10 μg mL^{-1}) and a SIL 25OH-D_3 stock standard (~10 μg mL^{-1}) were prepared by dissolving the contents of 1 mg vials into separate 100 mL volumetric flasks and made to volume with ethanol; aliquots (~1.3 mL) of these solutions were stored in vials at −80 °C. A mixed SIL internal standard solution was prepared by diluting 100 μL of SIL vitamin D_3 stock standard and 100 μL of SIL 25OH-D_3 stock standard in 10 mL of acetonitrile. A non-labelled (NL) vitamin D_3 stock standard (~1 mg mL^{-1}) was made by dissolving 50 mg of vitamin D_3 in 50 mL of ethanol; this solution was stored at <−15 °C. A NL vitamin D_3 intermediate standard was prepared fresh each run by diluting 1 mL of the vitamin D_3 stock standard to 100 mL with ethanol. The accurate concentration of this solution was determined by UV absorbance measurement ($E^{1\%}_{1cm}$: 479.9 dL g^{-1} cm^{-1} at 265 nm) (Mattila, Piironen, Uusi-Rauva, & Koivistoinen, 1995). A NL 25OH-D_3 stock standard (~50 μg mL^{-1}) was made by dissolving the contents of a 5 mg vial of 25OH-D_3 in 100 mL of ethanol; aliquots (~1.3 mL) of this solution were stored in vials at −80 °C. The concentration was determined by UV absorbance measurement ($E^{1\%}_{1cm}$: 463.9 dL g^{-1} cm^{-1} at 265 nm) (Mattila et al., 1995) of a 1:10 mL dilution of the stock standard with ethanol. A mixed NL working standard was prepared by diluting 0.1 mL of NL vitamin D_3 intermediate standard and 0.1 mL of NL 25OH-D_3 stock standard in 10 mL of acetonitrile. Calibration standards (NL vitamin D_3: 0.04–5 ng mL^{-1}, NL 25OH-D_3: 0.2–25 ng mL^{-1}) were made by diluting 250 μL of mixed SIL internal standard solution and 10, 50, 250, 500, or 1250 μL of mixed NL working standard into separate 25 mL volumetric flasks. To each volumetric flask, 5 mL acetonitrile and 75 μL PTAD solution (10 mg mL^{-1} in acetone) were added before making each to volume with acetonitrile.

2.4. Sample collection

Early lactation samples included raw colostrum, transitional milk, and mature milk (mid-flow, same quarter) acquired at ten intervals from a single Jersey cow (4th calving) over the first 30 days postpartum. Seasonal milk samples were acquired from a bulk composite herd milk processing silo (prior to pasteurization) across the 2014–2015 season at a frequency of one per fortnight. For each of the early lactation or seasonal milk samples, duplicate 20.0 g samples were accurately weighed into 50 mL disposable centrifuge tubes and were stored at −80 °C until analysis.

2.5. Sample preparation

Sample preparation was based upon the extraction technique described in AOAC Official Method 2002.05 (AOAC, 2002; Staffas & Nyman, 2003) combined with the PTAD derivatisation method described previously (Gill et al., 2015). A frozen liquid milk sample was defrosted and poured into a 250 mL amber conical flask. The centrifuge tube was rinsed twice with 20 mL of ethanolic pyrogallol solution (1%, w/v) into the conical flask and 0.5 mL of mixed SIL internal standard and 8 mL of potassium hydroxide solution (50%, w/v) were added. The flask was then capped and mixed for 10 min on an orbital shaker. The sample was placed in a water bath at 70 °C for 1 h, with regular mixing every 15 min.

After cooling to room temperature, the contents of the flask were transferred into a 250 mL separating funnel. The flask was rinsed twice with 20 mL aliquots of water, once with 30 mL of water:ethanol (2:1, v/v), and twice with 20 mL aliquots of isooctane and each was sequentially transferred into the separating funnel. The separating funnel was then capped tightly and mixed on a horizontal shaker for 10 min. An additional 80 mL of water was added to the separating funnel, which was then inverted 10 times before draining the lower aqueous layer to waste.

The upper isooctane layer was drained dropwise through anhydrous sodium sulphate (~25 g in a filter paper) into a round-bottom flask. The extract was evaporated to dryness under nitrogen gas, reconstituted in 5 mL of isooctane, and transferred to a 15 mL disposable centrifuge tube containing 75 μL of PTAD solution (10 mg mL^{-1} in acetone). The tube was vortex mixed and was then allowed to stand in the dark for 5 min to allow the derivatisation reaction to complete. A 1 mL aliquot of acetonitrile was added to the centrifuge tube and vortex mixed, prior to centrifugation at 2000× *g* for 5 min. A 0.5 mL aliquot of the lower acetonitrile layer was transferred to a 2 mL Eppendorf vial containing 167 μL of water. After vortex mixing, the extract was syringe filtered into an LC vial ready for analysis.

2.6. LC–MS analysis

High-pressure binary gradients utilising mobile phase A (0.1%, v/v, formic acid) and mobile phase B (100%, methanol), were formed as described in Table 1. A switching valve was timed to divert the column eluate to the ion source between 1.5 and 4.9 min, with the flow otherwise directed to waste. Analyte detection was achieved by electrospray ionisation in positive mode and multiple reaction monitoring (MRM) under the MS conditions presented in Tables 2 and 3. Fragmentation of PTAD adducts of vitamin D_3 $[M+H]^+$, 25OH-D_3 $[M+H-H_2O]^+$, and their corresponding SIL forms produce a common major fragment of *m/z* 298.1 (Fig. 1; Abernethy, 2012).

Table 1
Chromatographic gradients.[a]

Time (min)	Flow rate (mL min^{-1})	Mobile phase composition	
		%A	%B
0.0	0.6	50	50
3.3	0.6	0	100
3.7	1.0	0	100
4.8	1.0	0	100
4.9	0.6	50	50

[a] Mobile phase A, 0.1% (v/v) formic acid; mobile phase B, methanol. Oven temperature was 40 °C.

Table 2
Ion source parameters for mass spectrometry.

Source/gas parameter	Value
Source	ESI$^+$
Curtain gas	30
Nebuliser gas GS1	40
Heater gas GS2	40
Collision gas	N_2
Temperature	300 °C
CID[a] gas	Medium
Ion spray voltage	5500 V

[a] CID, collision-induced dissociation.

Table 3
Compound parameters for mass spectrometry.[a]

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)
PTAD-D_3	560.4	298.1	130	10	21	18
PTAD-25OH-D_3	558.4	298.1	86	10	23	24
PTAD-d_6-D_3	566.4	298.1	130	10	21	18
PTAD-d_6-25OH-D_3	564.4	298.1	86	10	45	24

[a] Abbreviations are: PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; D_3, vitamin D_3; d_6-D_3, hexadeuterated vitamin D_3; 25OH-D_3, 25-hydroxyvitamin D_3; d_6-25OH-D_3, hexadeuterated 25-hydroxyvitamin D_3; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential. Dwell time was 120 ms.

3. Results and discussion

Despite advances in analytical methodology, the quantitation of vitamin D_3 and 25OH-D_3 in milk remains a challenge because of low levels, instability to heat and light, and an abundance of potentially interfering endogenous milk components.

3.1. Method optimisation

Two epimers, 6S and 6R, are produced by derivatisation of vitamin D, as the PTAD reagent reacts with the *cis*-diene moiety from both the α-side and the β-side; the ratio of 6S:6R is approximately 4:1 (Shimizu & Yamada, 1994). Using the described chromatographic conditions, the 6S and 6R epimers of vitamin D_3 co-elute; however, the shoulder on the front of the 25OH-D_3 peak is indicative of partial resolution of the two epimers for the hydroxylated metabolite (Fig. 2).

3.2. Method validation

To evaluate the reliability of the method changes made for this study, analytical recovery and precision were determined. Mean recovery was measured by spiking an unfortified liquid whole milk sample with vitamin D_3 (0.45 μg L^{-1}) and 25OH-D_3 (0.34 μg L^{-1}) and were calculated as 83% (74–92%) for vitamin D_3 and 111% (104–120%) for 25OH-D_3, which are within expected recovery ranges at these concentrations (70–120%) as recommended by AOAC International (AOAC, 2016). Precision as repeatability was estimated as the relative standard deviation (RSD_r) of four replicate results and were estimated as11% RSD_r for vitamin D_3 and 10% RSD_r for 25OH-D_3, consistent with repeatability values typical at these analyte concentrations (AOAC, 2016).

3.3. Application to bovine milk

There is consensus that the vitamin D_3 and 25OH-D_3 levels in both bovine milk and human milk are significantly lower than those in circulating plasma, suggesting a low efficiency mechanism of expression in milk (Kasalová et al., 2015; Ovesen et al., 2003). Nonetheless, milk is recognised as an important dietary source of vitamin D, and the described analytical methodology was therefore applied to determining the temporal variability of the vitamin D_3 and 25OH-D_3 contents in bovine milk. The composition of ruminant milk is generally influenced by the physiological stage of lactation and, although colostrum is withheld from entering the

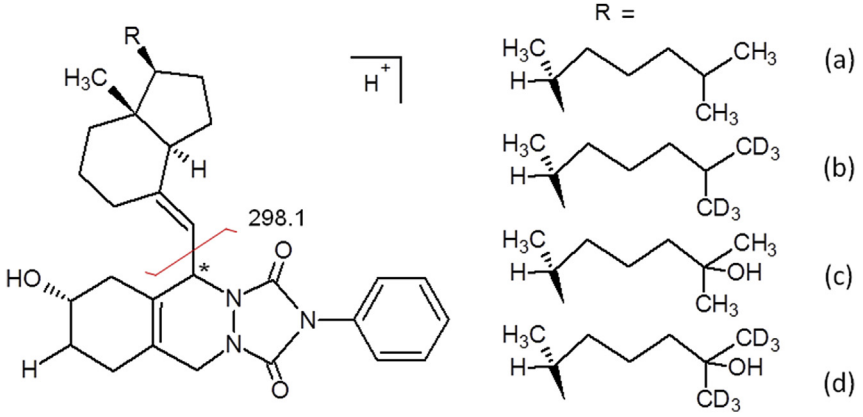

Fig. 1. Structures of 4 phenyl 1,2,4 triazoline 3,5 dione (PTAD) adducts of (a) vitamin D_3, (b) 26,26,26,27,27,27-d_6 vitamin D_3, (c) 25-hydroxyvitamin D_3, and (d) 26,26,26,27,27,27-d_6 25OH-D_3.

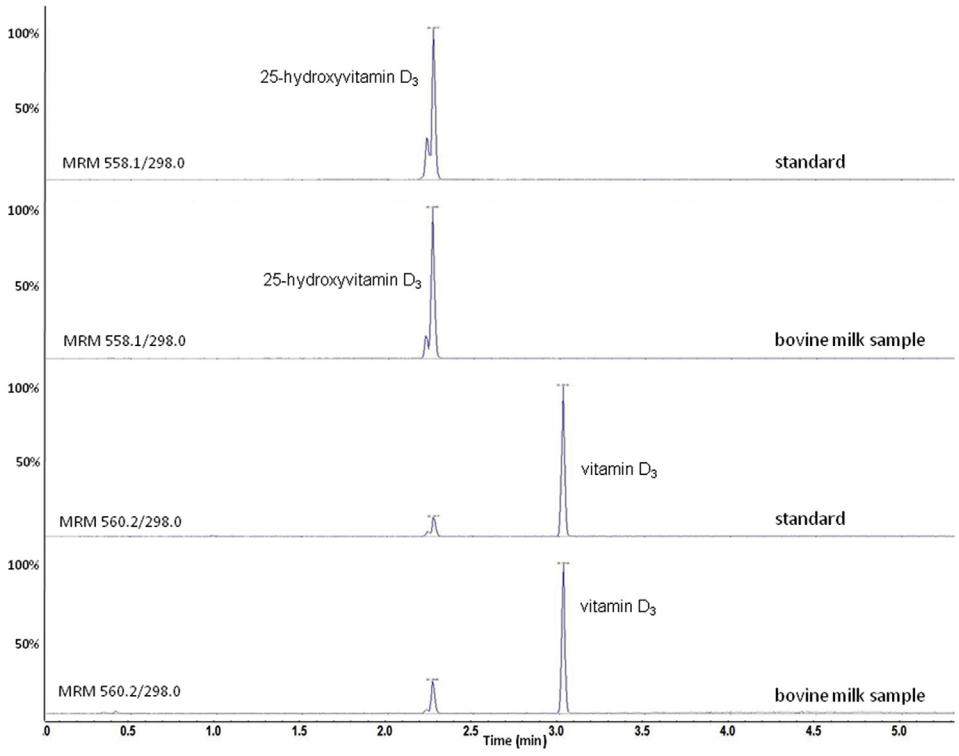

Fig. 2. Chromatograms of 4 phenyl 1,2,4 triazoline 3,5 dione (PTAD) adducts of vitamin D_3 and 25-hydroxyvitamin D_3 in a standard and a bovine milk sample.

commercial bovine milk supply, it was considered to be worthwhile to investigate the distribution of the two forms of vitamin D_3 during the transition from colostrum to mature milk. The described analytical method was applied during this transition in a single pasture-fed lactating cow over 28 d subsequent to parturition (Table 4; Fig. 3).

There was a significant increase in vitamin D_3 during the initial postpartum colostral phase, followed by a decline to a relatively stable content following the transition to mature milk, whereas the 25OH-D_3 content remained essentially constant across the entire 28-day period, with the vitamin D_3 to 25OH-D_3 ratio ranging from 1.1 to 2.5. Although this study was limited to a single animal, the quantitative data for mature bovine milk are consistent with the current literature (Jakobsen & Saxholt, 2009; Kunz, Niesen, von Lilienfeld-Toal, & Burmeister, 1984; Mattila et al., 1995; McDermott, Beitz, Littledike, & Horst, 1985) derived from the application of modern specific techniques, although a recent review of several studies has reported a considerable variability in the contents of vitamin D_3 and 25OH-D_3 in mature bovine milk (Schmid & Walther, 2013). Such variability has generally been rationalised based on the influence of feedstock, breed, season, sunlight, lactation, and fat content, with method diversity significantly impacting reported levels. The few studies that have specifically investigated the early lactation temporal content of these vitamin D compounds in bovine milk utilised earlier analytical chromatographic techniques, with an order of magnitude range in the results (Kunz et al., 1984; McDermott et al., 1985; Okano, Yokoshima, & Kobayashi, 1984). The content of native vitamin D_3 in bovine milk is generally reported to be low, in most studies less than 200 ng L^{-1}, and currently there is no consensus on whether vitamin D_3 or 25OH-D_3 is the predominant form (Ovesen et al., 2003).

Dairy husbandry in New Zealand exploits an extensive pasture-grazing regime, which facilitates the investigation of the natural

Table 4
Vitamin D_3 and 25OH-D_3 in bovine milk over the first month after parturition.[a]

Day after parturition	Vitamin D_3 (ng kg^{-1})	25OH-D_3 (ng kg^{-1})
0	55.5 (4.3)	51.2 (25.8)
1	129.1 (31.9)	52.5 (26.7)
2	91.7 (8.0)	50.4 (25.6)
3	102.7 (3.1)	51.2 (26.7)
5	72.8 (8.2)	48.8 (26.0)
7	73.9 (3.1)	48.7 (25.1)
14	61.2 (3.4)	48.0 (25.6)
21	68.1 (0.6)	48.4 (25.3)
28	89.7 (2.7)	48.8 (25.5)

[a] Values are the mean (with standard deviation in parentheses) of duplicate results.

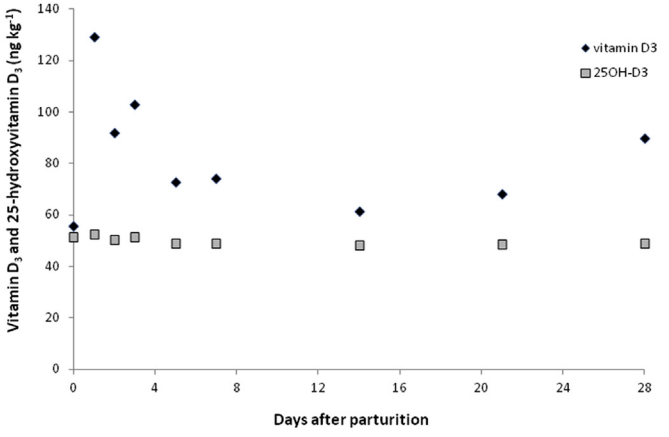

Fig. 3. Vitamin D_3 (◆) and 25-hydroxyvitamin D_3 (■) in bovine milk from a single cow over the first month after parturition.

seasonal changes in herd milk. In contrast to dairy herd feeding practices elsewhere, the absence of a dietary source of vitamin D_2 results in vitamin D_3 as the dominant form expressed in the New Zealand bovine milk supply. The vitamin D_3 and 25OH-D_3 contents in bulk raw bovine herd milk were therefore quantified over the 2014–2015 production season (Table 5; Fig. 4).

There was a significant influence of season on the vitamin D_3 levels, which correlates with temporal exposure of the skin to UV-B solar radiation with a summer maximum (NIWA, 2016), an observation that is consistent with previous studies (Jakobsen & Saxholt., 2009; Jakobsen et al., 2015; Kurmann & Indyk, 1994). Vitamin D_3 levels ranged from a winter low of 16.7 ng dL^{-1} to a summer high of 615 ng L^{-1}, which can be compared with the seasonal range of 100–300 ng L^{-1} that was reported in an earlier New Zealand study that utilised HPLC–UV methodology (Kurmann & Indyk, 1994). In contrast, the content of 25OH-D_3 in bovine milk was essentially constant and revealed no apparent seasonal dependence.

The limited number of previous studies of vitamin D_3 and 25OH-D_3 in bovine milk and human milk have reported variable contents, and differing temporal trends for each vitamer have also been noted (Ala-Houhala, Koskinen, Parviainen, & Visakorpi, 1988; Jakobsen & Saxholt., 2009; Jakobsen et al., 2015; Kunz et al., 1984; Parviainen, Koskinen, Ala-Houhala, & Visakorpi, 1984; Takeuchi et al., 1988). The entry of bovine circulatory plasma vitamin D_3 and 25OH-D_3 into milk during lactation has been confirmed to be mediated via a vitamin D binding protein and the cytosolic actin protein (Schmid & Walther, 2013). Although not yet confirmed for the ruminant bovine, 25OH-D_3 does not transfer across the secretory mucosa of the human mammary gland as efficiently as vitamin D_3, leading to the conclusion that, without maternal vitamin D dietary supplementation, human breast milk may contain vitamin D activity that is insufficient to meet the needs of the infant (Heaney & Armas, 2015; Hollis, Pittard, & Reinhardt, 1986; Kovacs, 2008). In the present study, the observation of significant lactation and seasonal trends in the expression of vitamin D_3 in bovine milk relative to the essentially invariant 25OH-D_3 may support the apparent differences in secretory mucosal transfer efficiency between these vitamers.

Table 5
Vitamin D_3 and 25OH-D_3 in bovine milk from a bulk silo over a season.[a]

Month	Vitamin D_3 (ng kg^{-1})	25OH-D_3 (ng kg^{-1})
August	167.3 (42.2)	37.6 (3.0)
September	277.9 (45.7)	36.1 (2.2)
October	488.0 (289.4)	38.2 (2.2)
November	534.9 (79.1)	37.6 (0.0)
December	614.9 (–)	41.8 (4.4)
January	625.7 (45.1)	38.2 (2.2)
February	579.7 (60.5)	37.1 (0.7)
March	619.0 (13.6)	38.7 (1.5)
April	426.6 (103.5)	39.2 (0.7)

[a] Values are the mean (with standard deviation in parentheses) of duplicate results, except for vitamin D_3 in December, which is a single result.

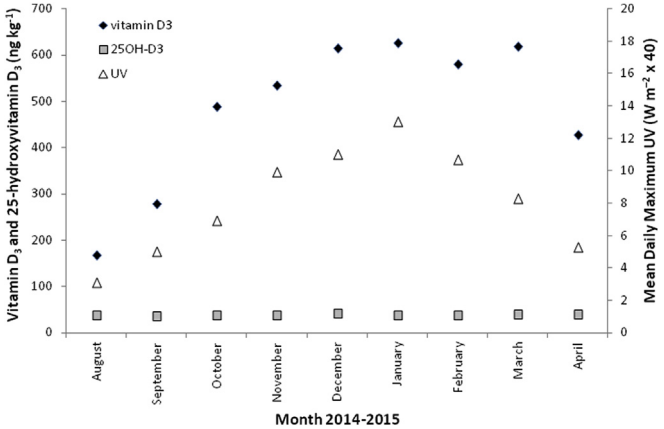

Fig. 4. Vitamin D_3 (◆) and 25-hydroxyvitamin D_3 (■) in bovine milk from a bulk silo over the 2014–2015 season, with the mean daily maximum UV light (△) also shown.

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References

Abernethy, G. A. (2012). A rapid analytical method for cholecalciferol (vitamin D3) in fortified infant formula, milk and milk powder using Diels–Alder derivatisation and liquid chromatography–tandem mass spectrometric detection. *Analytical Bioanalytical Chemistry, 403*, 1433–1440.

Ala-Houhala, M., Koskinen, T., Parviainen, M. T., & Visakorpi, J. K. (1988). 25-Hydroxyvitamin D and vitamin D in human milk: Effects of supplementation and season. *American Journal of Clinical Nutrition, 48*, 1057–1060.

AOAC. (2002). *Determination of cholecalciferol (vitamin D3) in selected foods. Official methods of analysis, 2002.05*. Gaithersburg, MD, USA: AOAC International.

AOAC. (2016). *Guidelines for dietary supplements and botanicals. Official methods of analysis*. Appendix K. Gaithersburg, MD. USA: AOAC International.

Gill, B. D., Zhu, X., & Indyk, H. E. (2015). A rapid method for the determination of vitamin D_3 in milk and infant formula by liquid chromatography/tandem mass spectrometry. *Journal AOAC International, 98*, 431–435.

Gomes, F. P., Shaw, P. N., Whitfield, K., & Hewavitharana, A. K. (2015). Simultaneous quantitative analysis of eight vitamin D analogues in milk using liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta, 891*, 211–220.

Heaney, R. P., & Armas, L. A. G. (2015). Quantifying the vitamin D economy. *Nutrition Reviews, 73*, 51–67.

Hollis, B. W., Pittard, W. B., 3rd, & Reinhardt, T. A. (1986). Relationships among vitamin D, 25-hydroxyvitamin D, and vitamin D-binding protein concentrations in the plasma and milk of human subjects. *Journal of Clinical Endocrinology and Metabolism, 62*, 41–44.

Horst, R. L., Goff, J. P., & Reinhardt, T. A. (1994). Calcium and vitamin D metabolism in the dairy cow. *Journal of Dairy Science, 77*, 1936–1951.

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., Jensen, S. K., Hymøller, L., Andersen, E. W., Kaas, P., Burild, A., et al. (2015). Artificial ultraviolet B light exposure increases vitamin D levels in cow plasma and milk. *Journal of Dairy Science, 98*, 6492–6498.

Jakobsen, J., & Saxholt, E. (2009). Vitamin D metabolites in bovine milk and butter. *Journal of Food Composition and Analysis, 22*, 472–478.

Kasalová, E., Aufartová, J., Krčmová, L. K., Solichová, D., & Solich, P. (2015). Recent trends in the analysis of vitamin D and its metabolites in milk—A review. *Food Chemistry, 171*, 177–190.

Kovacs, C. S. (2008). Vitamin D in pregnancy and lactation: Maternal, fetal, and neonatal outcomes from human and animal studies. *American Journal of Clinical Nutrition, 88*, 520S–528S.

Kunz, C., Niesen, M., von Lilienfeld-Toal, H., & Burmeister, W. (1984). Vitamin D, 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D in cow's 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. E. (1994). The endogenous vitamin D content of bovine milk: Influence of season. *Food Chemistry, 50*, 75–81.

Mattila, P. H., Piironen, V. I., Uusi-Rauva, 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 Agriculture and Food Chemistry, 43*, 2394–2399.

McDermott, C. M., Beitz, D. C., Littledike, E. T., & Horst, R. L. (1985). Effects of dietary vitamin D_3 on concentrations of vitamin D and its metabolites in blood plasma and milk of dairy cows. *Journal of Dairy Science, 68*, 1959–1967.

NIWA. (2016). *CliFlo: NIWA's National climate database on the web*. http://cliflo.niwa.co.nz/Retrieved 16-June-2016.

Okano, T., Yokoshima, K., & Kobayashi, T. (1984). High-performance liquid chromatographic determination of vitamin D_3 in bovine colostrum, early and later milk. *Journal of Nutritional Science and Vitaminology, 30*, 431–439.

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.

Parviainen, M. T., Koskinen, T., Ala-Houhala, M., & Visakorpi, J. K. (1984). A method for routine estimation of vitamin D activity in human and bovine milk. *Acta Vitaminologica Enzymologica, 6*, 211–219.

Perales, S., Alegría, A., Barberá, R., & Farré, R. (2005). Review: Determination of vitamin D in dairy products by high performance liquid chromatography. *Food Science and Technology International, 11*, 451–462.

Schmid, A., & Walther, B. (2013). Natural vitamin D content in animal products. *Advances in Nutrition, 4*, 453–462.

Shimizu, M., & Yamada, S. (1994). New fluorescence-labeling reagent targeting conjugated dienes: Application to the fluorometric analysis of vitamin D and A metabolites. *Vitamins (Japan), 68*, 15–30. cited in: Ding, S., Schoenmakers, I., Jones, K., Koulman, A., Prentice, A., & Volmer, D. A. (2010). Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. *Analytical and Bioanalytical Chemistry, 398*, 779–789.

Staffas, A., & Nyman, A. (2003). Nordic committee on food analysis. Determination of cholecalciferol (vitamin D3) in selected foods by liquid chromatography: NMKL collaborative study. *Journal of AOAC International, 86*, 400–406.

Takeuchi, A., Okano, T., Tsugawa, N., Katayama, M., Mimura, Y., Kobayashi, T., et al. (1988). Determination of vitamin D and its metabolites in human and cow's milk. *Journal of Micronutrient Analysis, 4*, 193–208.

Trenerry, V. C., Plozza, T., Caridi, D., & Murphy, S. (2011). The determination of vitamin D_3 in bovine milk by liquid chromatography mass spectrometry. *Food Chemistry, 125*, 1314–1319.