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The endogenous vitamin D content of bovine milk: influence of season

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A liquid chromatographic technique has been developed to estimate cholecalciferol at endogenous levels, in the milk of cows exclusively and extensively grazed on pasture.

The temporal variation in vitamin D content ranges between 0·06 (winter) and 0·23 iu/g fat (summer) and, by comparison with the radically different husbandry practices of the northern hemisphere, this study has revealed and confirmed the dominant influence of solar radiation on the levels of this secosteroid secreted in the milk of lactating animals.

INTRODUCTION

Circulating levels of the physiologically antirachitic hydroxylated metabolites of cholecalciferol are known to be primarily dependent on levels of the parent pro-hormone, vitamin D_3. Adequate intestinal absorption from the diet partly satisfies this requirement, although efficiency is generally poor in comparison with the other fat-soluble vitamins.

A far more efficient mechanism has evolved through the apparently unregulated action of ultraviolet light on the skin, which generates the major supply of the vitamin (Webb *et al.*, 1988; Collins & Norman, 1991). Once available, entry of vitamin D_3 (and its metabolites) from circulating blood plasma into milk, occurs via the interaction of a D-binding protein and the cytosolic protein, actin, during lactation (McDermott *et al.*, 1985). Most reports indicate a dominating influence of solar radiation, with higher levels of cholecalciferol in milk of mammals exposed consistently to light as compared to those confined indoors (Renner, 1983; Thompson & Hidiroglou, 1983; Greer *et al.*, 1984; Ball, 1988), although one author found no significant difference between summer and winter milk (Scott, 1984).

Vitamin D is an essential micronutrient in certain human groups. Normal infants will generally satisfy their requirements through suckling, since human breast milk is a rich source of this nutrient, provided that the mother's nutritional status and exposure to light are both adequate. In contrast, mature bovine milk is comparatively low in vitamin D, with the consequence that supplementation is required when breast milk substitutes based on cows' milk are formulated for infants. Supplementation of whole milk is also practised in order to furnish the dietary needs of other at-risk groups within the community, although this is still a somewhat controversial practice.

Although there are several reports reviewing the influence of both diet and UV exposure on vitamin D content in both human and bovine milk, and despite the significance of establishing its level in nutritional milk products (Holick *et al.*, 1992), very few studies have examined the temporal variability of this vitamin in seasonally representative samples of cows' milk. This may partly be a consequence of the complex herd management regimes practised in the northern hemisphere, where most studies on bovine milk composition have been performed. However, a major contributing factor has undoubtedly been the difficulty in the determination of this vitamin at endogenous levels in complex food matrices such as milk.

The aim of this study has been to extend the current analytical techniques and apply them to a comprehensive temporal survey of endogenous vitamin D in bovine milk derived from animals grazed exclusively on pasture, and where herd lactation is synchronised to commence in early spring. Thus, true seasonal influences may be distinguished from the potential extraneous impacts of other factors (e.g. feedstocks and husbandry management), and data assessed and compared with the various *ad hoc* indicative levels reported in the literature.

MATERIALS AND METHODS

Apparatus

Semipreparative high-performance liquid chromatography (HPLC)

Model 510 pump, U6K injector, Resolve silica (5 μm) Rad-Pak column fitted in an 8 × 10 Radial Compression Module, silica insert in a Guard-Pak module and 745

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Data Module from Waters (Milford, MA, USA). A PU4025 variable wavelength ultraviolet (UV) detector was used from Pye Unicam (Cambridge, UK).

Analytical HPLC
Model PU4100 quaternary pump, PU4110 ultraviolet/visible (UV/VIS) detector from Pye Unicam equipped with a Rheodyne 7125 injector. A Resolve C_{18} (5 μm) Rad-Pak column fitted in an 8 × 10 RCM was coupled with a C_{18} Guard-Pak insert and mounted within a column oven maintained at 30°C. An HP 1040A diode-array detector was also used during validation studies (Hewlett Packard, Palo Alto, CA, USA).

Solid-Phase Extraction
Sep-Pak silica cartridges (Waters).

Other apparatus included an R2 horizontal orbital shaker (New Brunswick Sci., Edison, NJ, USA) and an RE111 rotary evaporator (Buchi, Switzerland).

Reagents

Solvents
Methanol, hexane, ethyl acetate, tetrahydrofuran and isopropyl alcohol, all LC grade, diethyl ether and ethanol, AR grade (BDH or equivalent). High quality water (≥18 MΩ) was used throughout.

Ethanolic potassium hydroxide solution
Potassium hydroxide pellets (300 g), AR (BDH or equivalent), were dissolved in water (100 ml) and made up to 1 litre with ethanol containing pyrogallol (1%, w/v).

Phenolphthalein solution
Phenolphthalein (0·5 g) dissolved in ethanol (100 ml).

Extraction solvent
Hexane–diethyl ether (9 : 1, v/v).

Anhydrous sodium sulphate
This compound was of AR grade (BDH).

Vitamin D_2 stock solution
Vitamin D_2 (30 mg) (USP, Rockville, MD, USA) were accurately weighed and made up to volume (100 ml) with methanol. This was stored in a freezer for no longer than 4 months. Exact concentrations were determined, after dilution (1 : 100), spectrophotometrically at 265 nm (extinction coefficient $E^{1\%}_{1cm}$ = 460).

Vitamin D_2 working solution (internal standard)
Vitamin D_2 stock was diluted in methanol to *c.* 0·05 μg/ml. This was prepared fresh for each analysis.

System suitability standard
A mixed vitamin D_2 and D_3 (USP) standard solution was prepared (*c.* 0·05 μg/ml each).

Semi-preparative mobile phase
Hexane–isopropyl alcohol, isocratic (99 : 1, v/v).

Analytical mobile phase
Methanol–tetrahydrofuran–water, isocratic (93 : 2 : 5, v/v/v).

Milk samples

Milk was sampled from the reception silo (at 5 to 6°C) of a central milkpowder processing site and was representative of at least 100 predominantly Friesian and Jersey-cross herds. Each herd consisted of between 150 and 200 cows. Sampling was performed on the same day each month with analysis following immediately.

Sample preparation

All procedures were performed under subdued lighting.

Fresh, pooled herd milk (100 ml), ethanolic potassium hydroxide (250 ml) and vitamin D_2 internal standard (1·0 ml) were measured into an Erlenmeyer flask (1 litre) and flushed with nitrogen, and the flask was stoppered and shaken overnight at ambient temperature (16 h). Each sample was routinely assayed in duplicate.

The contents were transferred to a separating funnel (2 litres) with water (2 × 50 ml) and extracted vigorously with extraction solvent (500 ml). A second similar extraction was performed on the aqueous phase following separation (15 min) and the combined organic phase was washed repetitively with water until neutral to phenolphthalein. The extract was filtered through anhydrous sodium sulphate (*c.* 25 g), evaporated to near dryness at 40°C on rotary evaporator and dissolved in dry hexane (1 ml).

A silica Sep-Pak cartridge was prepared by passing hexane (dried over sodium sulphate, 10 ml) to waste. The sample extract was loaded and eluted to waste with hexane–ethyl acetate (90 : 10 v/v, 3 ml). A vitamin-rich fraction was eluted into a tapered centrifuge tube with hexane–ethyl acetate (80 : 20 v/v, 5 ml) and evaporated under nitrogen to *c.* 100 μl.

Semi-preparative HPLC fractionation

Following determination of the critical elution volume with a vitamin D standard, the entire crude sample extract (*c.* 100 μl) was injected at 1·0 ml/min and monitored at 265 nm (0·04 absorbance unit full-scale, aufs). A vitamin D fraction was collected in a tapered centrifuge tube, between 2 min before and 2 min after the previously established peak retention time. This was then evaporated to dryness under nitrogen and dissolved immediately in methanol (100 μl).

Analytical HPLC

Compliance with the requirement for a resolution factor of greater than 1·5 between vitamins D_2 and D_3 was established through prior injection of the system suitability standard. Following this procedure, the entire extract isolated from semi-preparative fractionation was injected at 1·0 ml/min and routinely monitored at 265 nm

(0·003 aufs). Quantitation was by internal standard methodology and peak height measurement.

The column was purged with methanol between injections allowing sufficient time for re-equilibration with the mobile phase.

Estimation of fat content

Milk samples (10 ml) were analysed for their fat content by the Rose-Gottlieb reference method (AOAC, 1984).

Solar radiation

Data were obtained as the average daily solar radiation (MJ/m^2) per month (National Institute of Water and Atmospheric Research, Wellington, NZ).

RESULTS

Figure 1 shows a chromatogram of a typical fluid milk sample obtained under analytical reversed-phase conditions, subsequent to the described fractionation procedures.

There was no evidence of chromatographic interference coincident with vitamin D_2, as demonstrated with samples assayed without addition of internal standard. In addition, both putative calciferol peaks followed exactly the elution characteristics of authentic standards under modified isocratic conditions, and with no effect on quantitation. Several samples were also monitored under dual wavelength conditions (265 and 280 nm), with equivalent results, supporting the integrity of peak identity and purity.

Peak identity was also confirmed in pooled extracts with on-line diode-array detection. Figure 2 illustrates the spectral equivalence of the alleged cholecalciferol peak to that of the authentic compound.

Linearity of detector response has been evaluated and confirmed for both vitamins D_2 and D_3 over the range encountered in milk, at both endogenous and supplemental levels. The limit of detection (3 × noise) has been established at 2 ng on-column, which facilitates reliable estimation in samples containing almost one order of magnitude less cholecalciferol than the lowest concentration found.

Overall recoveries throughout the entire procedure were generally greater than 80% estimated against the internal standard, since both calciferols are equivalent in physicochemical properties, while replicate analyses of a control sample revealed a between-run relative standard deviation of 2·7% ($n = 4$).

Preliminary experiments confirmed the absence of thermal conversion to previtamin D during ambient saponification and, therefore, measurement of this congener was not incorporated into the estimation of vitamin D_3.

Seasonal variation of cholecalciferol in fluid whole milk was assessed by the described technique and is represented graphically in Fig. 3 on both an 'as-is' and fat-normalised basis. This latter illustrates the independence in temporal variation of vitamin D (of fat secretion). Further, the annual variation in solar flux has been superimposed, clearly indicating close coincidence with the estimated vitamin D content.

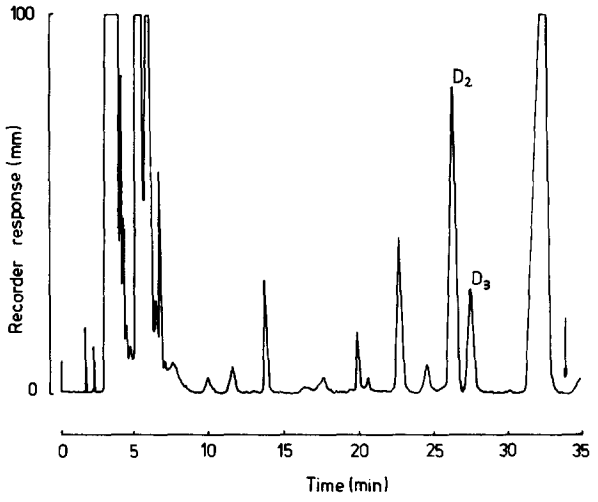

Fig. 1. Chromatogram of endogenous cholecalciferol in bovine milk. Column, 5 μm C_{18} Rad-Pak; mobile phase, methanol–tetrahydrofuran–water (93 : 2 : 5, v/v/v); flow rate, 1·0 ml/min; detection, 265 nm (0·003 aufs); injection volume, 100 μl. Arrow indicates column purge initiation (methanol, 100% at 2·5 ml/min).

DISCUSSION

Biological tissues, including milk, generally contain very low concentrations of vitamin D and highly specific and sensitive assays are, therefore, required for its estimation. Metabolite levels in plasma have generally been assessed by saturation analysis, using either receptor protein, binding protein or antibody-based techniques (Coldwell *et al.*, 1987). Usually, however, prior purification is mandatory in order to overcome specificity problems.

In foods, traditional biological assays including the rat line test, chick assay and growth rate are still utilised in the estimation of parental vitamin D (Collins & Norman, 1991). Currently, however, the most prevalent techniques, particularly at supplemental levels, rely on on-line UV detection of the triene chromophore subsequent to HPLC separation (Ball, 1988). Multidimensional chromatography is commonly recommended in order to overcome the relative lack of sensitivity and selectivity of UV detection during the analytical stage. Quantification has also been achieved at both elevated and natural levels in human and bovine milk by off-line competitive protein binding following prior chromatographic purification (Kunz *et al.*, 1984; Parviainen *et al.*, 1984; McDermott *et al.*, 1985; Van den Berg *et al.*, 1986). Such methods, however, suffer the limitations of lack of congener specificity and the requirement of additional specialised techniques. Recent developments in fluorescence detection have been reported for plasma assay, but currently retain the requirement for multidimensional

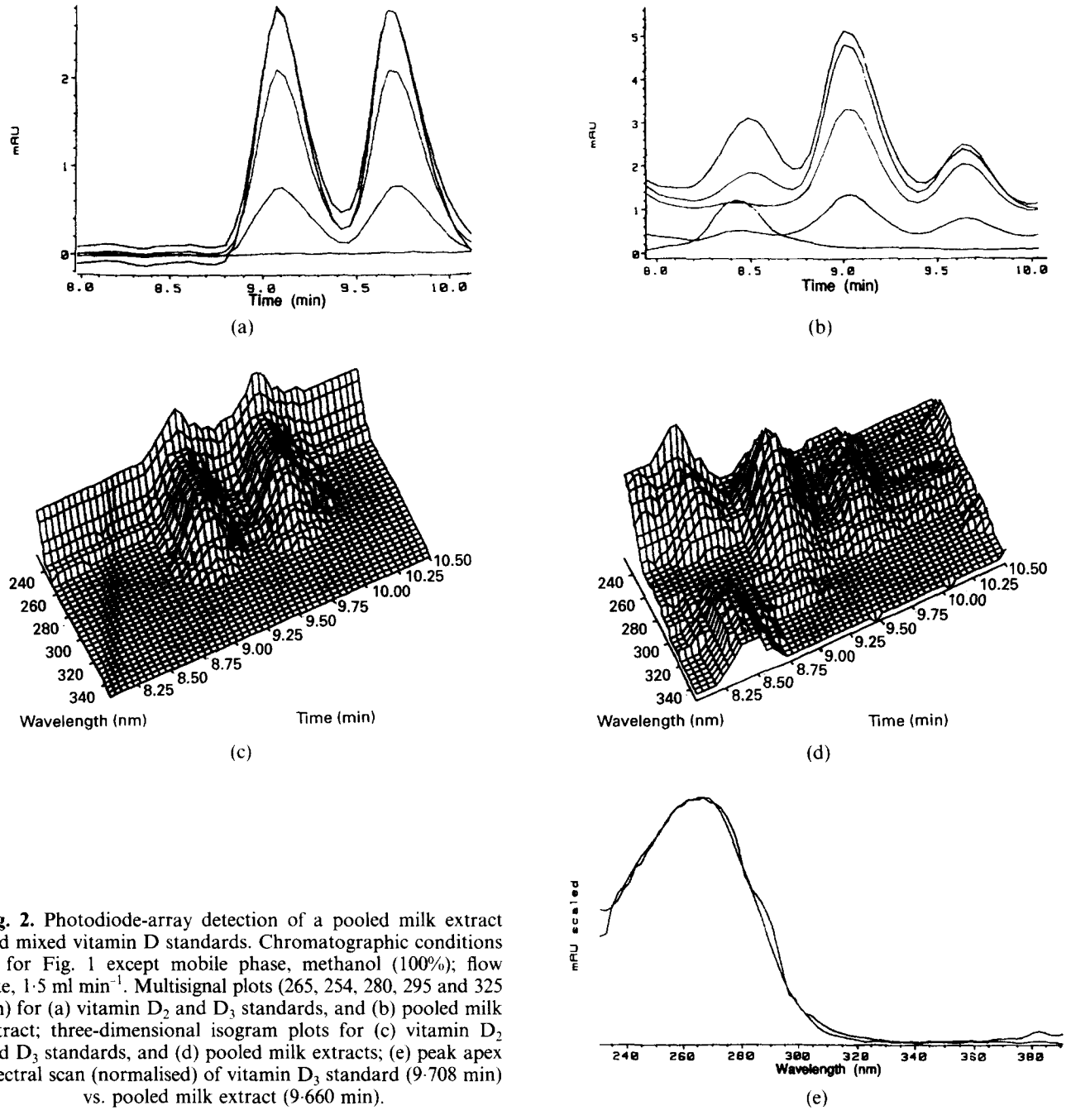

Fig. 2. Photodiode-array detection of a pooled milk extract and mixed vitamin D standards. Chromatographic conditions as for Fig. 1 except mobile phase, methanol (100%); flow rate, 1·5 ml min^{-1}. Multisignal plots (265, 254, 280, 295 and 325 nm) for (a) vitamin D_2 and D_3 standards, and (b) pooled milk extract; three-dimensional isogram plots for (c) vitamin D_2 and D_3 standards, and (d) pooled milk extracts; (e) peak apex spectral scan (normalised) of vitamin D_3 standard (9·708 min) vs. pooled milk extract (9·660 min).

purification (Shimada & Kobayashi, 1991; Shimizu *et al.*, 1991). Additional derivatisation steps, as well as the potential for epimer formation, probably mediate against this technique at present. Some progress has also been reported in redox electrochemical detection, although currently limited to elevated levels (Hart *et al.*, 1992; Hasegawa, 1992). Although gas chromatography–mass spectrometry (GC–MS) has been regarded as a definitive technique for vitamin D secosteroids, there are many practical difficulties associated with its routine application and it is not yet widely employed (Adachi & Kobayashi, 1979; Coldwell *et al.*, 1987). The advent of coupled liquid chromatography–mass spectrometry offers the promise of a simpler definitive technique as compared to GC–MS, although current progress is hindered due to (thermospray) interface instability (Watson *et al.*, 1991).

Despite the inherent limitations of UV-based detection and quantitation, it has been successfully applied amongst the relatively few previous reports of parental vitamin D estimation in mammalian milk at endogenous levels (Reeve *et al.*, 1982; Thompson & Hidiroglou, 1983; Okano *et al.*, 1984; Scott *et al.*, 1984; Takeuchi *et al.*, 1988). The success of this strategy is absolutely dependent on adequate concentration and purification through multidimensional chromatography prior to quantitation and extends, to low levels, the analytical philosophy utilised in assays at higher levels in milks and foods (Indyk & Woollard, 1985; Johnsson & Hessel, 1987; Ball, 1988; AOAC, 1990).

The method described in this study has incorporated both solid-phase extraction and normal-phase semi-preparative purification stages prior to the chromatographic separation of vitamin D_3 and the internal

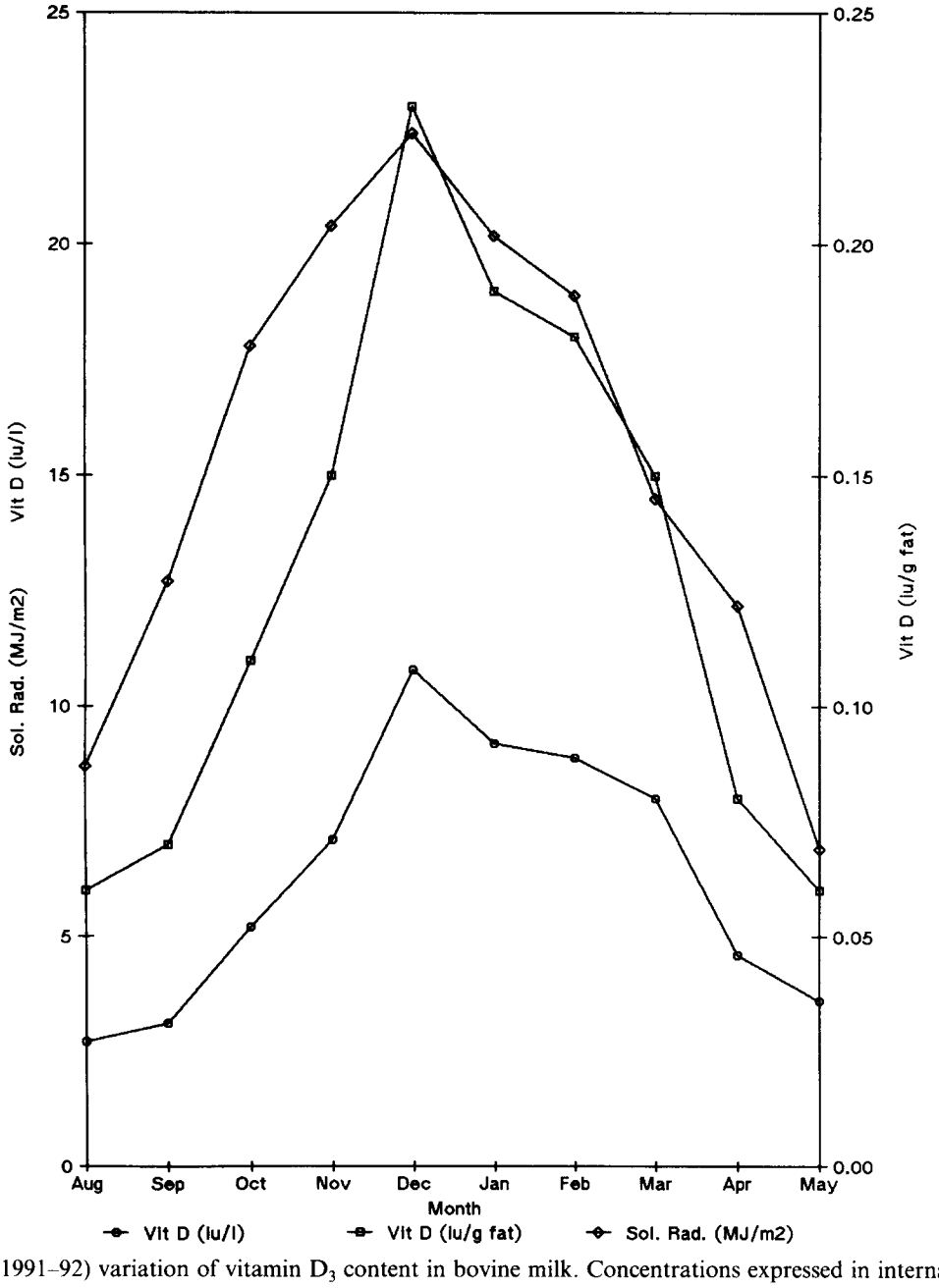

Fig. 3. Seasonal (1991–92) variation of vitamin D_3 content in bovine milk. Concentrations expressed in international units (iu).

standard (vitamin D_2) under reversed-phase analytical conditions. Ambient saponification has been chosen both to facilitate sufficient sample volumes and avoid potential conversion to previtamin D_3 during the analysis. In addition to the usual validation criteria, the diagnostic attributes of the photodiode-array system have demonstrated chromatographic integrity of the putative endogenous cholecalciferol peak.

The assay protocol has been applied to a survey of endogenous vitamin D content and temporal variation in the milk of exclusively pasture-grazed cows. Clear evidence of a positive seasonal correlation with exposure to solar radiation has been obtained, with maximum levels coincident with the southern hemisphere summer flux and four times the minima observed during winter. Significantly, vitamin D levels appear independent of the proportion of fat in milk, which in this survey has been shown to vary as with the expected trend in such herds (Hawke & Taylor, 1983). Such seasonal fluctuations in vitamin D have been previously noted on an *ad hoc* basis in northern hemisphere studies, although under conditions of herd management where animals are routinely confined indoors and fed dry rations over winter (Renner, 1983; Thompson & Hidiroglou, 1983; Ball, 1988), while one study revealed no significant seasonality (Scott *et al.*, 1984).

Further evidence for the positive association with light exposure has accrued from studies of human breast milk (Greer *et al.*, 1984; Ala-Houhala *et al.*, 1988) and skin (Webb *et al.*, 1988). An important attribute of the present study is the absence of extraneous factors, including artificial diets, vitamin supplements and enclosure, from the inherent annual variations experienced by animals extensively grazed year-round. Under this regime, it is also common to synchronise calving in the early spring, with the consequence that lactational maturity follows milk collection across the season. In this context, it has been reported that there

is little variation in vitamin D_3 content in bovine colostrum and later milk (Okano *et al.*, 1984), and it is, therefore, likely that the temporal trend reported here is dominated entirely by seasonal UV flux.

The predominant antirachitic sterols in bovine (and human) milk are vitamin D_3 and 25-hydroxyvitamin D_3, which together account for the majority of total activity. The fat-soluble calciferols now appear likely to provide the only bioactive source in view of the absence of confirmatory evidence for a putative water-soluble sulphate form.

Both relative and absolute contributions of these two compounds vary considerably within the literature and probably reflect wide variations in both experimental design and analytical methodology. Thus, levels of parental vitamin D_3 in bovine milk have variously been reported within the overall range of 1·7 to 80 iu/litre. These reports seem to be classifiable into low (1·7–3·5 iu/litre: Hollis *et al.*, 1981; Kunz *et al.*, 1984; McDermott *et al.*, 1985), medium (8–17 iu/litre: Reeve *et al.*, 1982; Parviainen *et al.*, 1984; Scott *et al.*, 1984; Van den Berg *et al.*, 1986; Takeuchi *et al.*, 1988) and high ranges (19–80 iu/litre; Adachi & Kobayashi, 1979; Renner, 1983; Thompson & Hidiroglou, 1983; Okano *et al.*, 1984). Vitamin D levels across the season, as presented in the present work (2·7–10·8 iu/litre), fall within the lower of the literature range of values and thus may be presumed to indicate methodological specificity, although they may also reflect the unsupplemented livestock feeding regime as compared to a few of the above cited studies.

Bovine milk is commonly utilised as a vehicle for supplementation with vitamin D, both in the management of community health and as an alternative to human breast milk in infant nutrition. Fortification is generally required in view of the comparatively low levels of endogenous vitamin relative to the Recommended Dietary Allowance. This is a still somewhat controversial subject, with some questioning the efficacy of dietary absorption (Packard, 1982), the possible preference of supplementation with 25-hydroxy vitamin D_3 in order to resemble more accurately human milk (Kunz *et al.*, 1984), and intermittent problems with faulty fortification practices, leading to intoxication or deficiency (Holick *et al.*, 1992).

Nevertheless, the practice of providing supplemented bovine milk products is unlikely to change in view of the substantial community benefits accruing from their consumption. It is important, therefore, from the perspective of nutritional science, to establish with greater accuracy the basal level and seasonal fluctuation in endogenous vitamin D in raw milk. The data presented here, therefore, should assist those responsible for compliance with the regulatory requirements of fortification as currently practised.

CONCLUSIONS

A recent paper has reported seasonal data for a wide range of endogenous micronutrients present in bovine milk powder (Indyk *et al.*, 1993). The present report is intended to extend this survey by inclusion of the nutritionally important and analytically challenging vitamin D in milk derived from pasture-fed dairy cows. Unlike vitamins A and E, whose concentrations in milk are largely influenced by seasonal feed quality, the dominating influence of solar exposure has been confirmed in the case of cholecalciferol, resulting in a temporal trend which, although different, is nevertheless also a function of season.

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