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The endogenous vitamin K_1 content of bovine milk: temporal influence of season and lactation

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A liquid chromatographic technique was applied to the estimation of phylloquinone at endogenous levels in the milk of cows exclusively grazed on pasture. The temporal variation ranged between 3.1 and 6.9 μg/litre (mean, 4.4 μg/litre) across the production season and was independent of milk-fat content. A single animal was also studied serially over 35 days lactation. High levels were measured in early colostrum (>20 μg/litre), while normal levels (4.0-6.6 μg/litre) were established beyond day 5 transition milk. Cows' milk was confirmed as containing higher vitamin K content, as compared to human milk (2.7±0.3 μg/litre).

INTRODUCTION

Vitamin K_1 (phylloquinone) has been identified as a cofactor in the post-translational modification of the calcium-binding proteins involved in antihaemorrhagic activity, and, more speculatively in calcium homeostasis (Suttie, 1991; Shearer, 1993). It is ubiquitous within green plants, which constitute the major dietary source of this vitamin. The related vitamin K_2 (menaquinones) are of bacterial origin and are thus found primarily within the human intestine and bovine rumen (Cremin & Power, 1985). Recent evidence suggests that the menaquinones play a significant role in contributing to human requirements for this vitamin (Conly & Stein, 1992). This information would tend to support the common prophylactic use of vitamin K_1 in the human neonate, a practice consistent with both an inadequate colonisation of intestinal microflora and the insufficient vitamin K_1 content of mothers' milk.

Reliable quantitative estimations of vitamin K_1 in mammalian milks are only recently becoming available, since earlier studies were based mainly on relatively crude bioassay techniques (Suttie, 1991). The advent of high performance liquid chromatography (HPLC) has facilitated its accurate measurement in many biological tissues, including milk, although much less attention has been given to the menaquinones (Shearer, 1993). HPLC has also allowed for the increasingly reliable estimation of vitamin K in foods, and a provisional data base has recently been reported (Booth *et al.*, 1993).

Several HPLC methods have been developed for the estimation of vitamin K_1 in supplemented infant formulas (Barnett *et al.*, 1980; Haroon *et al.*, 1982; Bueno & Villalobos, 1983; Sato *et al.*, 1985; Hwang, 1985; Schneiderman *et al.*, 1988). The majority of these reports cite the successful use of UV detection at the elevated levels present in these foods, provided adequate cleanup strategies are incorporated in the sample scheme. Fluorescence and electrochemical detection techniques have also been advocated in studies at the lower endogenous levels found in milk, foods and oils, in recognition of their inherently greater specificity and sensitivity as compared to UV (Sato *et al.*, 1985; Isshiki *et al.*, 1988; Canfield *et al.*, 1990; Canfield *et al.*, 1991; Lambert *et al.*, 1992; Moussa *et al.*, 1994; Booth *et al.*, 1994). Nevertheless, a few reports have retained the use of UV detection, even at these endogenous levels (Haroon *et al.*, 1982; Fournier *et al.*, 1987*b*).

As a biological fluid, the composition of mammalian milk is not constant, but is influenced by species, breed, lactational maturity and diet. Most reports cited above have indicated typical levels only in human or cows' milk. Data related to the influence of season on the vitamin K content of bovine milk are limited (Haroon *et al.*, 1982; Fournier *et al.*, 1987*b*), while for human milk, the significance of lactational maturity has been evaluated (Canfield *et al.*, 1991; Lambert *et al.*, 1992). The extensive utilisation of cows' milk in the production of vitamin K supplemented infant formulas emphasises the need for further such studies.

We have recently developed a method for the estimation of vitamin K_1 in infant formulas, which has also been demonstrated to be successful at the natural levels present in milk (Indyk *et al.*, 1995). An initial enzymatic

sample hydrolysis scheme, followed by semi-preparative normal phase HPLC fractionation facilitates the final analysis by reversed-phase LC-UV with quantitation by the internal standard technique. Routine use of dual wavelength monitoring ensures peak purity, while retaining the advantages of simplicity and robustness inherent to UV detection.

The extensive pasture grazing and synchronised calving practices common in New Zealand, offers the rare possibility of studying temporal variability of endogenous vitamin content free from the potential extraneous impacts of other husbandry factors. We report here a survey of the lactational and seasonal content of vitamin K_1 in bovine milk, and further report typical levels found in pooled human milk.

MATERIALS AND METHODS

Apparatus and reagents have been described elsewhere (Indyk *et al.*, 1995).

Standards included a vitamin K_1 working standard (2.5 μg/ml), a cholesteryl phenylacetate internal standard (0.1 mg/ml) and a single level calibration standard (vitamin K_1, 2.5 μg/ml: cholesteryl phenylacetate, 1.0 mg/ml).

Sample collection

Whole milk powders were sourced at monthly intervals from a central processing site and were representative of at least 100 predominantly Friesian–Jersey cross supply herds, with each herd consisting of 150–200 cows. Milk powders were spray-dried from pooled, pasteurised milk under a low to medium heat schedule following fat standardisation.

Raw milk was collected from a single 4-year-old Jersey–Friesian cross (3rd calving) between days 1 and 35 post partum, and held frozen until required for analysis. Other fluid milks included pooled, raw herd whole and skim milks from the factory refrigerated holding silos and commercial pasteurised and homogenised whole and skim milks obtained from retail outlets.

Pooled, raw human milk was obtained from five mothers (1–6 months post partum). All liquid milks were tempered at 37°C for 15 min with gentle agitation prior to sampling.

Estimation of fat content

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

Vitamin K determination

Milk powder (3.00 g) and liquid milk samples (3.00–15.00 g, dependent on fat content) were subjected to analysis for vitamin K at endogenous levels as described elsewhere (Indyk *et al.*, 1995).

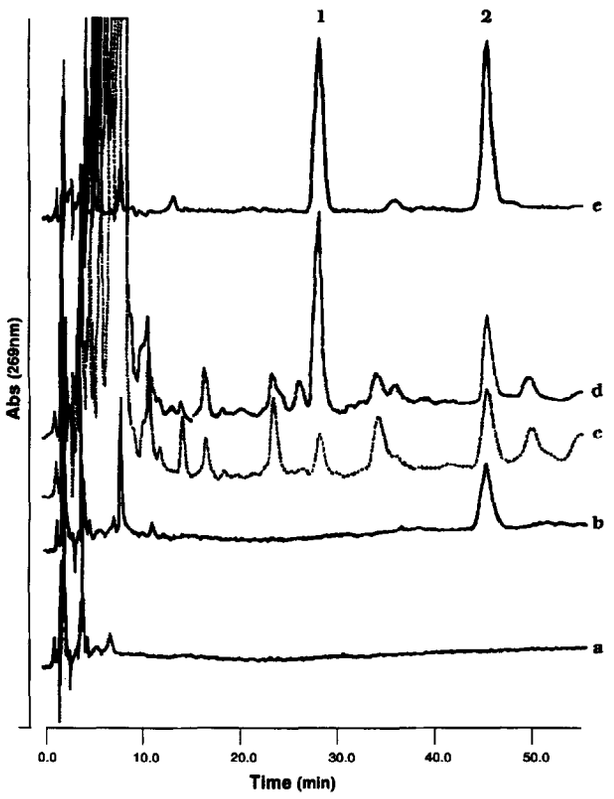

Fig. 1. Analytical reversed-phase chromatography of (a) reagent blank, (b) skim milk, (c) whole milk, (d) spiked whole milk, and (e) standards. Conditions: column, 5 μm C_{18} 'Resolve'; mobile phase, methanol:isopropanol:ethylacetate: water (450:350:145:135); flow rate, 2.0 ml/min; injection volume, 20 μl; detection, 269 nm (0.002 aufs). Peak 1, vitamin K_1 (*cis*+*trans*); peak 2, cholesteryl phenylacetate.

RESULTS

Figure 1 illustrates the analytical chromatography subsequent to semi-preparative fractionation. Human milk extracts were qualitatively similar, with only minor differences in elution profile apparent.

Chromatographic interferences were absent as demonstrated with both a reagent blank and lipid-free skim milk. The identity and purity of both phylloquinone and cholesteryl phenylacetate were routinely verified by comparison of retention times and spectral absorbance ratios (269:277 nm) against standards.

Peak integrity was also confirmed in pooled whole milk extracts with on-line diode-array spectral comparison against authentic standards as shown in Fig. 2.

Spectral resolution of the characteristic naphthoquinone nucleus is diminished at low wavelengths, due to a solvent effect of the relatively polar mobile phase.

Critical validation parameters are as reported elsewhere, with respect to linearity, sensitivity, recovery and precision (Indyk *et al.*, 1995).

A number of milks were evaluated for their endogenous vitamin K_1 content and the data collated in Table 1 on both an "as-is" and fat normalised basis.

One of the whole milk powders was also analysed by a previously published clinical HPLC procedure utilising redox-electrochemical detection (Hart *et al.*, 1985),

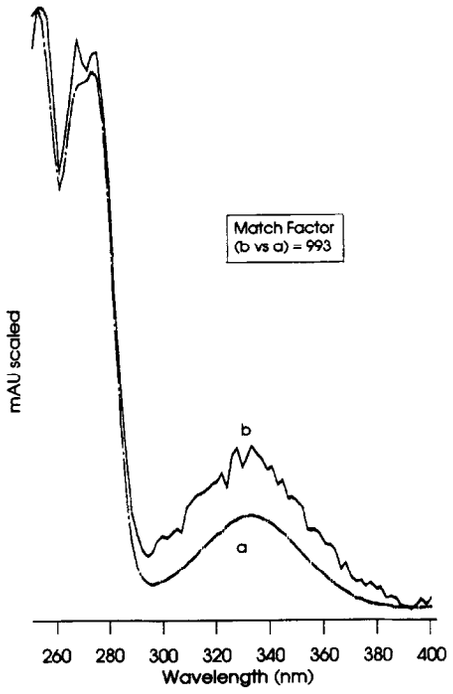

Fig. 2. Photodiode-array detection of a pooled milk extract and authentic phylloquinone. Peak apex spectral scan (normalised) of (a) vitamin K_1 standard vs (b) putative phylloquinone in milk fraction. Chromatographic conditions as for Fig. 1. (Match Factor $= 1000\ r^2$, where r^2 is least-square-fit coefficient of all absorbances at the same wavelength).

with equivalent results (2.9 vs. 2.7 μg/100 g). Fat-normalised data also reveal that vitamin K was conserved quantitatively in whole milk powder produced from the spray-drying of pasteurised milk.

Data was collected from fluid milk of a single cow between days 1 and 35 post partum as shown graphically in Fig. 3.

Parturition appears to be associated with a significant increase in phylloquinone, followed by a rapid decrease to comparatively consistent levels more representative of normal milk. The significantly higher vitamin K content of early colostrum milk may be partially rationalised as due to its elevated fat content, as shown in the lipid-normalised plot.

Table 1. Phylloquinone contents of various milks[a]

Milk type	K_1 (μg/100 g)	K_1 (μg/g fat)
Whole milk powder	2.9[b] (5.5)	0.11
Whole milk powder	3.3 (5.2)	0.12
Whole milk powder	3.6 (5.7)	0.13
Whole milk powder	4.5 (5.9)	0.16
Raw silo milk	0.44 (5.5)	0.10
Pasteurised domestic milk	0.50 (5.3)	0.15
Raw human milk	0.27 (4.8)	0.06
Skim milk	0	-

[a] Means of triplicate determination. RSD (%) in parentheses.
[b] This sample measured as 2.7 μg/100 g (mean of duplicate) by the LC-ECD method of Hart *et al.* (1985).

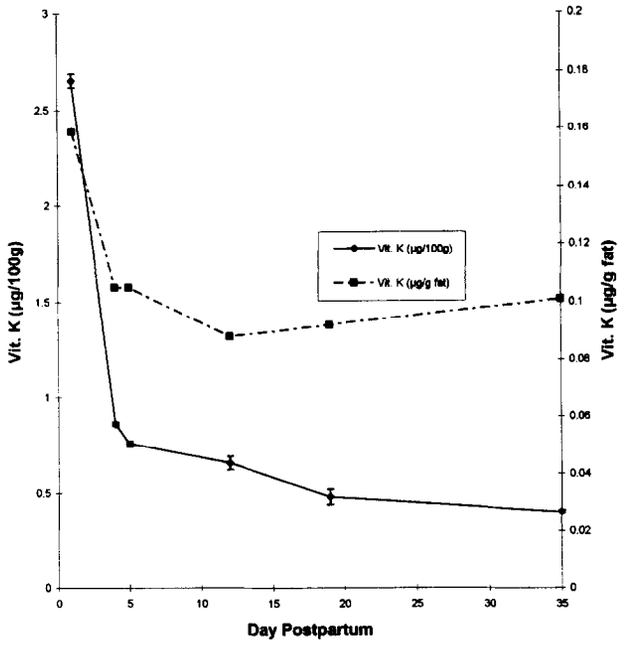

Fig. 3. Phylloquinone content of colostrum, transitional and mature milk from a single lactating cow. Mean of duplicates (individual values denoted by error bars).

Temporal variation of bulk bovine whole milk powder across an entire production season was assessed by the described technique and is represented graphically in Fig. 4 on both an 'as-is' and fat-normalised basis. This latter data illustrates the relative independence of phylloquinone levels in mature milk from that of fat content.

A consistent decrease in vitamin K_1 content is evident with continued milk collection, following the synchronised calving regime practiced during the southern hemisphere spring period.

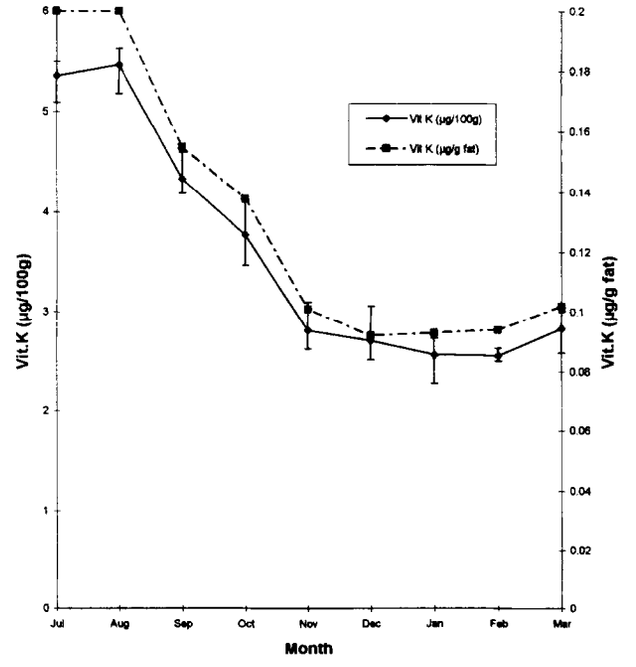

Fig. 4. Phylloquinone content of bulk bovine whole milk powder across the season. Mean of triplicate samples (error bars denote data range).

DISCUSSION

Despite the overwhelming benefits of breast-feeding, sub-optimal levels of vitamin K_1 in human milk have been implicated as the major risk factor in the etiology of hemorrhagic disease of the newborn. In view of this, and the increasingly significant usage of cows' milk based infant formulas, it is now recognised as essential that reliable data exists on dietary phylloquinone available to infants.

Transport of vitamin K from the liver to non-hepatic tissues such as the mammary gland, remains a poorly understood phenomenon. Most have suggested a non-specific lipoprotein transfer mechanism (Shearer, 1993), although the presence of a possible specific binding protein has also been recently suggested (Fournier *et al.*, 1987*a*). It has been noted that most reported serum levels seem generally lower than the limited data available for human milk, which may suggest an active concentration transfer mechanism across the basal membrane (Canfield & Hopkinson, 1989).

Several previous studies of mature, post-translational human milk indicate levels in the range of 0–6.5 μg/litre (overall mean: 2.3 μg/litre) (Shearer *et al.*, 1980; Miyaji, 1982; Haroon *et al.*, 1982; Sato *et al.*, 1985; Isshiki *et al.*, 1988; Canfield *et al.*, 1990; Canfield *et al.*, 1991; Lambert *et al.*, 1992), although Fournier *et al.* (1987*b*) suggests the much higher levels of 4.9-15.7 μg/litre. Our own lactational mature human milk of 2.7 ± 0.3 μg/litre are in excellent agreement with the above cited mean and confirm the growing consensus view that human milk contains appreciably less vitamin K than cow's milk.

Previous studies of the phylloquinone content of mature, fluid cows' milk have shown variation in reported values. These range from 1.5 to 11.7 μg/litre (overall mean: 5.6 μg/litre) (Shearer *et al.*, 1980; Haroon *et al.*, 1982; Sato *et al.*, 1985; Isshiki *et al.*, 1988; Booth *et al.*, 1994) while much higher values of 7.5-37.7 μg/litre were reported by Fournier *et al.* (1987*b*). Our own results of 3.6-5.6 μg/litre (mean: 4.5 μg/litre) agree well with the majority of the above cited data.

Although there have been a few studies of the effect of lactational maturity of human milk (reviewed by Canfield & Hopkinson, 1989), there have been no equivalent surveys for bovine milk. In this study, a single donor cow indicated significantly higher levels of vitamin K_1 in very early colostrum (within 6 h parturition) compared to both transitional and mature milk. This observation, although partially rationalised by the three-fold increase in fat content of colostrum compared to mature milk, agrees with the data of Von Kries *et al.*, 1987, who demonstrated a significantly higher vitamin K content in human colostrum milk over the first 24 h.

The progression to mature milk is coincident with expression of relatively constant vitamin K levels (4.0–6.6 μg/litre) consistent with those of both commercial milks and previously cited literature values. It is unlikely that seasonal effects will distort this short-term (35 days) data, with the consequence that these observations possibly reflect true physiological changes in response to the commencement of lactation in the dairy cow.

Of the few surveys reporting values for the phylloquinone content of bovine milk, only two have alluded to possible seasonal effects. Haroon *et al.* (1982) saw no evidence of temporal variation in Friesian milk over 12 months, (mean, 4.9 μg/litre, range, 3.6–8.9 μg/litre), while Fournier *et al.* (1987*b*) reported a five-fold difference across the year (mean, 19.7 μg/litre; range, 7.5–37.7 μg/litre). Apart from the significant quantitative differences between these data, any such attempts to isolate a true seasonal influence on the vitamin K content of bovine milk will have been compromised by the artificial feeding regimes practiced in Europe. Thus, milk supply can be expected to reflect the substantial dietary variations inherent to both dry-feeding and over-wintering dairy practices. By contrast, the present study has exploited the attributes of extensive pasture grazing. The observation of a spring maximum and autumn minimum, with a mean of 3.5 μg/100 g whole milk powder and range of 2.5-5.5 μg/100 g (equivalent to 4.4 and 3.1–6.9 μg/litre fluid milk, respectively) is therefore uncomplicated by potentially extraneous and artificial factors. This seasonal trend is likely to incorporate both an expression of forage variability, and the superimposition of a physiological lactation trend due to the synchronised herd calving regime commencing in early spring.

It should be noted also that vitamin K levels, when expressed on a constant fat basis, show equivalence between the individual animal trial and the seasonal survey of milk powder (0.1–0.2 μg/g fat). This suggests that the vitamin is relatively stable to the thermal stresses involved in spray-drying, a property reported previously (Indyk, 1988). Further, the results indicate an absence of significant correlation between phylloquinone and neutral lipid content (with the possible exception of early colostral milk), a conclusion in agreement with that of others (Canfield & Hopkinson, 1989; Fournier *et al.*, 1987*b*; Canfield *et al.*, 1991; Lambert *et al.*, 1992).

Two recent studies have reported seasonal data for endogenous vitamins in bovine milk (Indyk *et al.*, 1993; Kurmann & Indyk, 1994). The fat soluble group have all been shown to exhibit marked seasonality, with vitamin D levels dominated by solar exposure, and the antioxidant vitamins A, E and ß-carotene largely influenced by seasonal feed quality. The temporal variation of vitamin K reported here, is of similar magnitude (ca. two-fold) and shares an apparent independence from milk fat content. While the direction of phylloquinone variation is broadly superimposable with that of the antioxidant vitamins, it is not yet possible to distinguish between the relative influences of feed quality and lactational maturity. A full understanding will also require further progress to be made in identifying the mechanisms of vitamin K transport and expression into milk. At present, the consensus view suggests that vitamins A and D are mediated via specific binding proteins, while E, K and ß-carotene appear to be carried by non-specific serum lipoproteins (Canfield & Hopkinson, 1989; Shearer, 1993).

CONCLUSION

A recently developed analytical method has been applied to the evaluation of seasonal and lactational vitamin K levels in bovine whole milk. We report a significant temporal variation, which is possibly a function of both feed composition and lactational maturity, and appears independent of milk fat content. We also confirm that phylloquinone levels in human milk are lower than those found in cows' milk.

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REFERENCES

AOAC (1984). Fat in milk; final action. In *AOAC Official Methods of Analysis*, 14th edn. American Association of Analytical Chemists, Washington, DC, Method 16.064.

Barnett, S. A., Frick, L. W. & Baine, H. M. (1980). Simultaneous determination of vitamins A, D_2 or D_3, E, and K_1 in infant formulas and dairy products by reversed-phase liquid chromatography. *Anal. Chem.*, **52,** 610–14.

Booth, S. L., Sadowski, J. A., Weihrauch, J. L. & Ferland, G. (1993). Vitamin K_1 (phylloquinone) content of foods: A provisional table. *J. Food Comp. Anal.*, **6,** 109–20.

Booth, S. L., Davidson, D. W. & Sadowski, J. A. (1994). Evaluation of an HPLC method for the determination of phylloquinone (Vitamin K_1) in various food matrices. *J. Agric. Food Chem.*, **42,** 295–300.

Bueno, M. P. & Villalobos, M. C. (1983). Reverse phase HPLC determination of vitamin K_1 in infant formulas. *J. Assoc. Off. Anal. Chem.*, **66,** 5, 1063–6.

Canfield, L. M. & Hopkinson, J. M. (1989). Review: State of the art vitamin K in human milk. *J. Pediat. Gastroenterol. Nutr.*, **8,** 4, 430–41.

Canfield, L. M., Hopkinson, J. M., Lima, A. F., Martin, G. S., Sugimoto, K., Burr, J., Clark, L. & McGee, D. L. (1990). Quantitation of vitamin K in human milk. *Lipids*, **25,** 7, 406–11.

Canfield, L. N., Hopkinson, J. M., Lima, A. F., Silva, B. & Garza, C. (1991). Vitamin K in colostrum and mature human milk over the lactation period — a cross-sectional study. *Am. J. Clin. Nutr.*, **53,** 3, 730–5.

Conly, J. N. & Stein, X. (1992). The production of menaquinones (vitamin K_2) by intestinal bacteria and their role in maintaining coagulation homeostasis. *Prog. Food Nutr. Sci.*, **16,** 307–43.

Cremin, F. M. & Power, P. (1985). Vitamins in bovine and human milks. In *Developments in Dairy Chemistry*, ed. P.F. Pox. Elsevier, London, pp. 356–60.

Fournier, B., Leclercq, M., Audigier-Petit, C., Letoublon, R., Got, R. & Frot-Coutaz, J. (1987*a*). Vitamin K_1 binding protein in milk. *Int. J. Vit. Nutr. Res.*, **57,** 145–50.

Fournier, B., Sann, L., Guillaumont, M. & Leclercq, M. (1987*b*). Variations of phylloquinone concentration in human milk at various stages of lactation and in cows' milk at various seasons. *Am. J. Clin. Nutr.*, **45,** 551–8.

Haroon, Y., Shearer, M. J., Rahim, S., Gunn, W. G., McEnery, G. & Barkham, P. (1982). The content of phylloquinone (vitamin K_1) in human milk, cows' milk and infant formula foods determined by HPLC. *J. Nutr.*, **112,** 6, 1105–17.

Hart, J. P., Shearer, M. J. & McCarthy, P. T. (1985). Enhanced sensitivity for the determination of endogenous phylloquinone (vitamin K_1) in plasma using HPLC with dual-electrode electrochemical detection. *Analyst*, **110,** 1181–4.

Hwang, S-M. (1985). Liquid chromatographic determination of vitamin K_1 *trans*- and *cis*-isomers in infant formula. *J. Assoc. Off. Anal. Chem.*, **68,** 4, 684–9.

Indyk, H. (1988). Liquid chromatographic study of vitamin K_1 degradation: possible nutritional implications in milk. *Milchwissenschaft*, **43,** 8, 503–7.

Indyk, H. E., Lawrence, R. & Broda, D. (1993). The micronutrient content of bovine whole milk powder: influence of pasture feeding and season. *Food Chem.*, **46,** 389–96.

Indyk, H., Littlejohn, V. C., Lawrence, R. J. & Woollard, D. C. (1995). Liquid chromatographic determination of vitamin K_1 in infant formulas and milk. *J. Assoc. Off. Anal. Chem.*, **78,** 719–23.

Isshiki, H., Suzuki, Y., Yonekubo, A., Hasegawa, H. & Yamamoto, Y. (1988). Determination of phylloquinone and menaquinone in human milk using HPLC. *J. Dairy Sci.*, **71,** 627–32.

Kurmann, A. & Indyk, H. (1994). The endogenous vitamin D content of bovine milk: influence of season. *Food Chem.*, **50,** 75–81.

Lambert, W. E., Vanneste, L. & De Leenheer, A. P. (1992). Enzymatic sample hydrolysis and HPLC in a study of phylloquinone concentration in human milk. *Clin. Chem.*, **38,** 9, 1743–8.

Miyaji, Y. (1982). Study on the idiopathic vitamin K deficiency of young infants. I. Levels of vitamin K in breast milk and formula milk. *Acta Paediatr. Jpn.*, **24,** 4, 506.

Moussa, F., Depasse, F., Lompret, V., Hautem, J.-Y., Girardet, J.-P., Fontgaine, J.-L. & Ayrnard, P. (1994). Determination of phylloquinone in intravenous fat emulsions and soybean oil by HPLC. *J. Chromatogr. A.*, **664,** 189–94.

Sato, T., Yahiro, M., Shimoda, K., Asai, Y. & Hamamoto, M. (1985). A method for the determination of vitamin K in milk, milk powder and vegetable oil by HPLC. *J. Jpn. Soc. Nutr. Food Sci.*, **38,** 6, 451–8.

Schneiderman, M. A., Sharma, A. K., Mahanama, K. R. R. & Locke, D. C. (1988). Determination of vitamin K_1 in powdered infant formulas, using supercritical fluid extraction and liquid chromatography with electrochemical detection. *J. Assoc. Off. Anal. Chem.*, **71,** 4, 815–17.

Shearer, M. J., McEnery, G., Allan, V. E. & Barkhan, P. (1980). Assay of vitamin K_1 (phylloquinone) by HPLC: values for human and cows' milk. *Proc. Nutr. Soc.*, **39,** 49A.

Shearer, M. J. (1993). Vitamin K. In *Encyclopaedia of Food Science, Food Technology and Nutrition*, eds R. Macrae, R. K. Robinson & M. J. Sadler. Academic Press, London, pp. 4804–16.

Suttie, J. W. (1991). Vitamin K. In *Handbook of Vitamins*, ed. L. J. Machlin. Marcel Dekker, New York, pp. 145–94.

Von Kries, R., Shearer, M., McCarthy, P. T., Huag, M., Harzer, G. & Gobel, U. (1987). Vitamin K_1 content of maternal milk: Influence of the state of lactation, lipid composition and vitamin K supplements given to the mother. *Pediatr. Res.*, **22,** 513–17.