Title:

Multiple Vitamin K Forms Exist in Dairy Foods

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A running title: Vitamin K content of dairy foods

Footnotes:

Abbreviations: AI, adequate intakes; LAB, lactic acid bacteria; MK, menaquinones; PK, phylloquinone; USDA, United States Department of Agriculture.

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Abstract

Background: The plant-based form of vitamin K (phylloquinone, PK, vitamin K1) has been well-quantified in the U.S. diet. Menaquinones (MK, vitamin K2) are another class of vitamin K compounds that differ from PK in the length and saturation of their side chain, but have not been well characterized in foods.

Objectives: The objectives of this study were to: 1) quantify PK and the different forms of MK (MK4 through MK13) in milk, yogurt, Greek yogurt, creams and cheeses; and 2) compare the MK contents of full-fat, reduced-fat and non-fat dairy products.

Method: All dairy samples were either obtained from USDA National Food and Nutrient Analysis Program or purchased from retail outlets. PK and MK concentrations in these dairy products were quantified by mass spectrometry technology.

Results: Full fat dairy products contained appreciable amounts of MK, primarily in the forms of MK9, MK10 and MK11. We also measured modest amounts of PK, MK4, MK8 and MK12 in these products. In contrast, there was little MK5-7 or MK13 detected in the majority of dairy products. The total vitamin K contents of soft cheese, blue cheese, semi-soft cheese and hard cheese were 506±63, 440±41, 289±38 and 282±5.0 µg/100 g, respectively. Non-fermented cheeses, like processed cheese, contained lower amounts of vitamin K (98±11 µg/100 g). Reduced fat or fat free dairy products contained ~5-22% of the vitamin K found in full fat equivalents. For example, total vitamin K contents of full fat milk (4% fat), 2% fat milk, 1% fat milk and non-fat milk were 38.1±8.6, 19.4±7.7, 12.9±2.0 and 7.7±2.9 µg/100 g, respectively.

Conclusions: To the best of our knowledge, this is the first report of MK contents of U.S. dairy products. Findings indicate that the amount of vitamin K contents in dairy products is high and proportional to the fat content of the product.

Key words: vitamin K, menaquinones, dairy products, fermented, reduced fat, phylloquinone

Introduction

Dietary sources of vitamin K are found in two natural forms: phylloquinone (PK; vitamin K1) and menaquinones (MK; vitamin K2). All forms of this fat-soluble vitamin share the common structure, 2-methyl-1,4-napthoquinone. The MK differ in structure from PK in their 3-substituted lipophilic side chain, and are designated by the number of isoprenoid units, i.e. MK-n. MK with up to 13 isoprenoid units have been identified (1). Whereas PK is widely distributed in the food supply, MK forms appear to be limited to animal products and fermented foods (2). As an essential vitamin, vitamin K plays a role as an enzyme co-factor necessary for the modification of glutamic acid residues to γ-carboxyglutamic acid residues in specific proteins, referred to as vitamin K-dependent proteins (3). The vitamin K-dependent proteins, matrix Gla-protein, osteocalcin and gas-6, have been implicated in tissue calcification, bone metabolism and cell cycle regulation (4-6). Vitamin K has multiple roles independent of its known biochemical function as an enzyme cofactor, like anti-inflammation (7), a ligand for steroid and xenobiotic receptor (8).

The current U.S. recommendation for intakes of vitamin K are 90 and 120 μg/day for women and men, respectively. These guidelines are termed adequate intakes (AI) due to insufficient data regarding vitamin K metabolism and lack of a robust biomarker to generate precise dietary recommendations (9). The AI is based on usual PK intakes, and does not take into account the potential dietary contribution of other forms of vitamin K. Very little is known about the contribution of dietary MK to overall vitamin K nutrition, and although it has been stated that approximately 50% of the daily requirement for vitamin K is supplied by gut bacteria through the production of MK (1), there is little evidence to support this estimate. Estimated intakes of PK and MK in dairy-producing countries in Western Europe suggest that between 10% and 25%

of total vitamin K intake are provided by MK, primarily from dairy sources (10, 11). However, MK have not been systematically analyzed in U.S. foods nor have MK been included in total vitamin K intake estimated in the U.S. population so these observations have yet to be substantiated outside of Western Europe.

The need to analyze MK in commonly consumed foods is timely because observational data from dairy-producing countries in Europe suggest that intakes of MK present in dairy products have stronger associations with heart health benefits compared with PK intakes (12). MK data for commonly consumed foods from other countries are critical for determining if these observations are generalizable. Furthermore, the food composition data applied to these few observational studies, almost exclusively from the Netherlands, predominantly represent full-fat dairy products. Low fat and non-fat dairy products are recommended as part of a healthy diet in the U.S., to reduce risk of cardiovascular disease and associated co-morbidities (13). The impact of reducing the fat content of dairy products on MK content is unknown.

Advances in mass spectrometry methodology have provided an ability to quantify multiple forms of vitamin K (PK and MK) in various matrices, allowing us for the first time to explore the MK content in the U.S. food supply (14). The purpose of this study was to quantify the content of multiple forms of vitamin K content in various dairy products including yogurt, cheeses, milk, and milk-based products, and examine the effect of fat content on the distribution and concentration of vitamin K forms in those products.

Methods

Fifty (50) of the dairy samples used in this study were provided by United States Department of Agriculture (USDA) Nutrient Data Laboratory, which conducts the National Food and Nutrition Analysis Program (15). The nationally-collected dairy samples were first delivered to the Food Analysis Laboratory Control Center at Virginia Tech in Blacksburg, Virginia, for preparation of aliquots, and then delivered frozen on dry ice to the Vitamin K Laboratory at Tufts University and stored at -80^{o}C until analysis. The National Food and Nutrition Analysis Program infrastructure incorporates a nationally-representative sampling approach (15, 16), approved analytical methods, and a rigorous quality assurance scheme. In addition, 148 dairy samples used in this study were purchased in 2016 from retail outlets that have substantial annual sales in order to capture the diversity of products available in Boston (MA, USA) area. Appropriate containers were used to maintain refrigeration during the transport to the laboratory. All samples collected by our laboratory were composited, aliquoted and stored at -80^{o}C before analysis. Shelf life date, analysis date, brand name and fat content were recorded. We used available information from the manufacturers to determine fat content (i.e. full fat, reduced fat, etc.).

The dairy products were grouped in categories based on dairy types and fat content (**Table 1**): milk, yogurts, Greek yogurts, kefirs, creams, processed cheeses, fresh cheeses, blue cheeses, soft cheeses, semi-soft cheeses and hard cheeses. Aside from processed cheese, all other types of cheeses included at least two different brands and different lots.

All cheese sample aliquots (about 10g) were frozen by liquid nitrogen and manually ground into a powder using a mortar and pestle. Approximately 0.05-0.2g of sample was used for analysis. The procedures for vitamin K extraction and sample purification have been previously described (14). PK and MK4-13 concentrations were measured by LC-MS, using deuterium-labeled PK as an internal standard (Sigma Aldrich, St. Louis, MO) and synthesized PK, MK4-MK13 as calibration standards (14).

The effect of dairy product fat content (full fat, fat-free/reduced) on concentrations of total vitamin K, PK, and all detectable MK were analyzed by two-sample T-test. Given the smaller sample size, the vitamin K content of cream products (heavy/whipping cream, half and half, and light cream) was examined by general linear model, with heavy/whipping cream as the reference group. Significance was determined by $P < 0.05$, and all analyses were carried out using SAS v 9.4 (Cary, NC). Data are reported as means (expressed as µg/100g wet weight) ± SEM.

Results

Dairy products obtained from the USDA Nutrient Data Laboratory and those purchased from retail outlets contained appreciable amounts of MK, primarily in the forms of MK9, MK10 and MK11. Together these three MK account for about 90% of total vitamin K in dairy foods.

The vitamin K content of different cheeses had significant variability in total vitamin K concentrations, ranging from 40 µg/100g to values up to 850 µg/100g (**Figure 1**). All forms of cheese contained MK9, MK10 and MK11. We also measured modest amounts of PK, MK4, MK7, MK8 and MK12 in these samples. In contrast, there was little MK5, MK6 or MK13 detected in the majority of cheese products. The total vitamin K content varied by cheese type, with soft cheese having the highest concentration, followed by blue cheese, semi-soft cheese and hard cheese (506±63, 440±41, 289±38 and 282±5.0 µg/100 g, respectively) (**Supplemental Table 1**). Non-fermented cheeses, like processed cheese, contained lower amount of vitamin K (98±11 µg /100 g). There was considerable diversity in vitamin K forms among fresh, semi-soft, blue and soft cheeses, but not in hard and processed cheeses. Soft cheeses and hard cheeses had a similar vitamin K pattern with high MK9 and MK10, with blue and semi-soft cheeses sharing a similar pattern dominated by MK9 and MK11.

Milk and yogurt products were also measured. The vitamin K concentrations of full fat (4% fat), 2% fat, 1% fat and fat free milk varied by fat content (**Figure 2**). Mean total vitamin K content of full fat milk, 2% fat milk, 1% fat milk and fat free milk was 38.1±2.7, 19.4±2.4, 12.9±0.6 and 5.1±0.9 μg/100 g, respectively. Both total vitamin K and individual MK concentrations in the full fat milk were significantly higher than 2% milk products ($P<0.05$). PK was only detected in the full fat milk. MK5-8 and MK12-13 were not detected in any milk samples. Fat free milk contained only a minimal amount of MK9 and MK11.

Regular and Greek yogurt with full fat (%±SEM, 4.6±0.5 and 4.0±0.2, respectively) had similar vitamin K concentrations as full fat milk (4% fat) (**Table 2**). Surprisingly, neither MK nor PK were detected in fat free yogurt. Low fat kefir (n=4) contained 10.2±0.3μg total vitamin K/100g, of which only MK9 and MK11 were detected.

Additional dairy products were examined including cottage cheese, cheddar cheese and cream (full fat and reduced fat) and found to have unique distribution of vitamin K forms (**Table 3**). 4% fat cottage cheese had significant higher MK8, MK9 and MK11 concentrations than reduced-fat cottage cheese. Reduced-fat cheddar cheese contained only 17% of total vitamin K content when compared to full fat cheddar cheese. MK9, MK10 and MK11 in reduced-fat cheddar cheeses decreased by 87.1%, 96.5%, and 61.4% of full fat cheddar cheeses, respectively. Reduced fat cream products had less vitamin K content overall.

Discussion

Current dietary guidelines recommend a diet containing high quality dairy foods (17). Dairy does not contain appreciable amounts of PK, hence dairy has not historically been considered a rich dietary vitamin K source. However, our data indicate that U.S. dairy products are a good dietary source of MK. MK9 was the major form quantified in the dairy samples, which is consistent with the findings of others (18, 19). However, through use of a sensitive LC-MS assay (14), we were able to extend that analysis to include measurement of MK11 through MK13, and our data indicated five to ten-fold higher MK9 and MK10 content in dairy products than previously reported (19), albeit in different dairy products, and in particular, artisan cheeses. We do not currently have an explanation for the higher concentrations reported here.

The large diversity of vitamin K forms among dairy products may be related to the microbial species used in the production of fermented dairy products. MK are synthesized by bacteria, including many found in the fermented foods. In particular, lactic acid bacteria (LAB) are widely used in dairy and fermented food industries (20, 21). LAB include a large number of *cocci* and *bacilli*, such as species of the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (22). Most of the cheese products contained LAB species as starters, which are reported to be the source of various MK forms (23). *Staphylococcus*, *Hafnia*, and *Arthrobacte* and other bacteria that are used in the surface ripening of certain cheeses may be the reason for their corresponding high MK values (2). However, the presence of MK in non-fermented products such as milk are largely unexplained, and could relate to the microbial content of the highly specialized ruminant digestive system (24). Kefir and yogurt have short fermentation time, which may explain their low MK content. Further investigation of the microbial composition of the different fermented dairy products is needed to interpret the diversity of MK forms.

Our study demonstrates that dairy products are a significant source of MK, and that the MK content varies by fat content of the dairy product. This differs from the conclusions of

Manoury *et al* (19), who did not find an overall association between MK9 content and fat content of fermented dairy products. Whereas the latter study conducted a single correlation analysis between MK9 content and fat content across all fermented dairy products, we compared the individual MK contents with different levels of fat within the same dairy product, which may explain why we were able to detect a consistent reduction in MK content with reduction in fat content. Currently reduced-fat milk, and yogurt are the most commonly consumed dairy product in the U.S. In 2015, whole fat milk accounted for 33% of milk sales with the remaining 67% of milk purchased as reduced-fat milk (2%, 1% and nonfat) (25). There is a recent trend of increasing full fat milk and cheese consumption (25, 26), fueled by recent evidence that individuals consuming full-fat dairy products (measured by plasma dairy fat biomarkers) had at least 43% lower risk of developing diabetes over the course of 15 years, compared with people who opted for low-fat dairy products (27, 28). Moreover, they found greater intake of high-fat dairy products, but not low-fat dairy products, was associated with less weight gain in the Women's Health Study (29). The nutrient components contributing to these beneficial effects have yet to be identified, and our observations suggest that MK warrants consideration.

Although MK bioavailability has not been studied using stable isotopes, bacterially-produced MK7 isolated from a food source (natto, a fermented soybean product) can be absorbed and is attributed to multiple health benefits, including bone and cardiovascular health (30, 31). More recently, studies have demonstrated that consumption of dairy products fortified with individual MK forms are absorbed and may have greater bioactivity than MK delivered in supplement form (30, 31). However, current understanding of fat-soluble MK absorption, transport, and bioactivity is limited. As reviewed elsewhere (32), most MK forms are not normally detected in circulation unless administered in supplement form. As our data indicate

MK forms are more abundant in commonly-consumed foods in the U.S. diet than previously recognized. It is critical that a more complete understanding of MK absorption and transport be developed in order to refine dietary recommendations for this nutrient. Collectively these data highlight major gaps that still existing in our understanding of the role of MK forms in vitamin K metabolism and its contribution to human health.

Our study was limited by the reliance on food labels for fat content instead of direct measurement of fat content. Whereas the samples obtained from the National Food and Nutrition Analysis Program were geographically representative of the U.S. diet, those purchased in the Boston region were not. However, those purchased locally were selected from retail outlets that had national representation. Strengths of the study included using a highly sensitive and validated LC-MS method to quantify all MK forms in a variety of dairy product types, and direct comparison of full fat and reduced fat dairy products of the same brand and food type. Future studies are needed to compare relative bioavailability and contribution of these individual MK to health outcomes.

In summary, our results demonstrate that commonly-consumed dairy products in the U.S. diet contain appreciable amounts of multiple vitamin K forms which are directly related to fat content. Additional research is necessary to determine the role of microbes used in production of dairy products, and their impact on MK content. There is also a need to determine the relative bioavailability of all MK forms given their abundance in the U.S. diet.

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X.F. and X.S. conducted research; X.F. and S.G.H analyzed data; X.F. and S.G.H. wrote the paper; X.F., S.G.H., D.B.H., J.P.K., B.E.F., and S.L.B. reviewed the data, aided in interpretation

of results and reviewed manuscript; S.L.B had primary responsibility for final content. All authors read and approved the final manuscript.

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Table 1. The analyzed dairy products.

Dairy	n	Type
Milk	43	Full fat, 2% fat, 1% fat, nonfat
Yogurt	16	Full fat and nonfat
Greek yogurt	16	Full fat and nonfat
Kefirs	4	Low fat
Cream	5	Heavy, light, half/half
Processed cheese	9	American cheese
Fresh cheese	12	Goat, Feta, Ricotta, Cotija, cottage and mozzarella cheeses
	12	Mozzarella part skim
	8	Reduced-fat cottage cheese
Blue cheese	10	Gorgonzola and blue cheeses
Soft cheeses	14	Brie, camembert, crème fraiche, limburger, mascarpone
Semi-soft cheeses	10	Montery jack, Havarti, fontina, gouda, Swiss and cream cheeses
Hard cheeses	12	Cheddar and parmesan
	10	Reduced-fat cheddar

Table 2. Vitamin K content of regular yogurt and Greek yogurt varies by fat content.

Vitamin K (μg/100g)	Yogurt		Greek yogurt	
	Full fat n=9	Fat free n=7	Full fat n=6	Fat free n=10
PK	0.4±0.1*	ND	0.3±0.1*	ND
MK4	0.7±0.3	ND	0.8±0.1*	ND
MK5	ND	ND	ND	ND
MK6	ND	ND	ND	ND
MK7	ND	ND	ND	ND
MK8	ND	ND	ND	ND
MK9	13.2±4.8*	ND	14.8±2.2*	ND
MK10	1.6±0.6*	ND	1.8±0.6*	ND
MK11	8.4±0.8*	ND	8.7±0.8*	ND
MK12	ND	ND	ND	ND
MK13	ND	ND	ND	ND
Total	26.3±6.4*	ND	28.2±2.7*	ND
Fat content (%)	4.6±0.5*	0.0	4.0±0.2*	0.0

1. Values are mean ± SEM.
2. ND: non-detectable. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 μg/100g).
3. * Significant difference between full fat and fat free within yogurt group at $P < 0.05$.
4. Total: Sum of PK and MK4 to MK13

Table 3. Vitamin K content of dairy products comparing fat and fat free or reduced fat products.

Dairy products	n	PK	Menaquinones										Total
			MK4	MK5	MK6	MK7	MK8	MK9	MK10	MK11	MK12	MK13	
Cottage cheese													
4% fat	6	0.3±0.1	0.3+0.1	0.5±0.2	0.5±0.1	0.6±0.2	2.5±0.7*	8.0±1.4*	0.4±0.2	39.1±3.0*	ND	ND	52.7±3.4*
Reduced-fat	8	ND	ND	ND	ND	ND	0.8±0.4	2.3±0.6	0.3±0.2	5.0±1.6	ND	ND	10.3±1.4
Cheddar cheese													
Full fat	12	2.4±0.1*	9.5±0.4*	0.4±0.1	0.9±0.2*	0.8±0.2	5.6±0.8	175±12.1*	42.9±7.8*	42.2±3.5*	1.3±0.1*	ND	281±11.9*
Reduced-fat	10	0.5±0.1	1.8+0.5	ND	ND	0.7±0.1	4.0±0.7	22.6±4.2	1.5±0.7	16.3±3.7	ND	ND	49.0±7.9
Cream													
Heavy	2	2.4±0.1	9.3±0.8	ND	ND	ND	ND	442±30.2	85.2±10.9	44.3±9.4	2.6±0.1	ND	587±27.8
Light	1	1.2	5.3	ND	ND	ND	ND	103†	13.0†	24.5	1.0	ND	149†
Half/half	2	0.8±0.1†	2.3±0.4†	ND	ND	ND	ND	40.4±17.3†	4.5±2.5†	35.5±11.3	ND	ND	85.1±3.8†

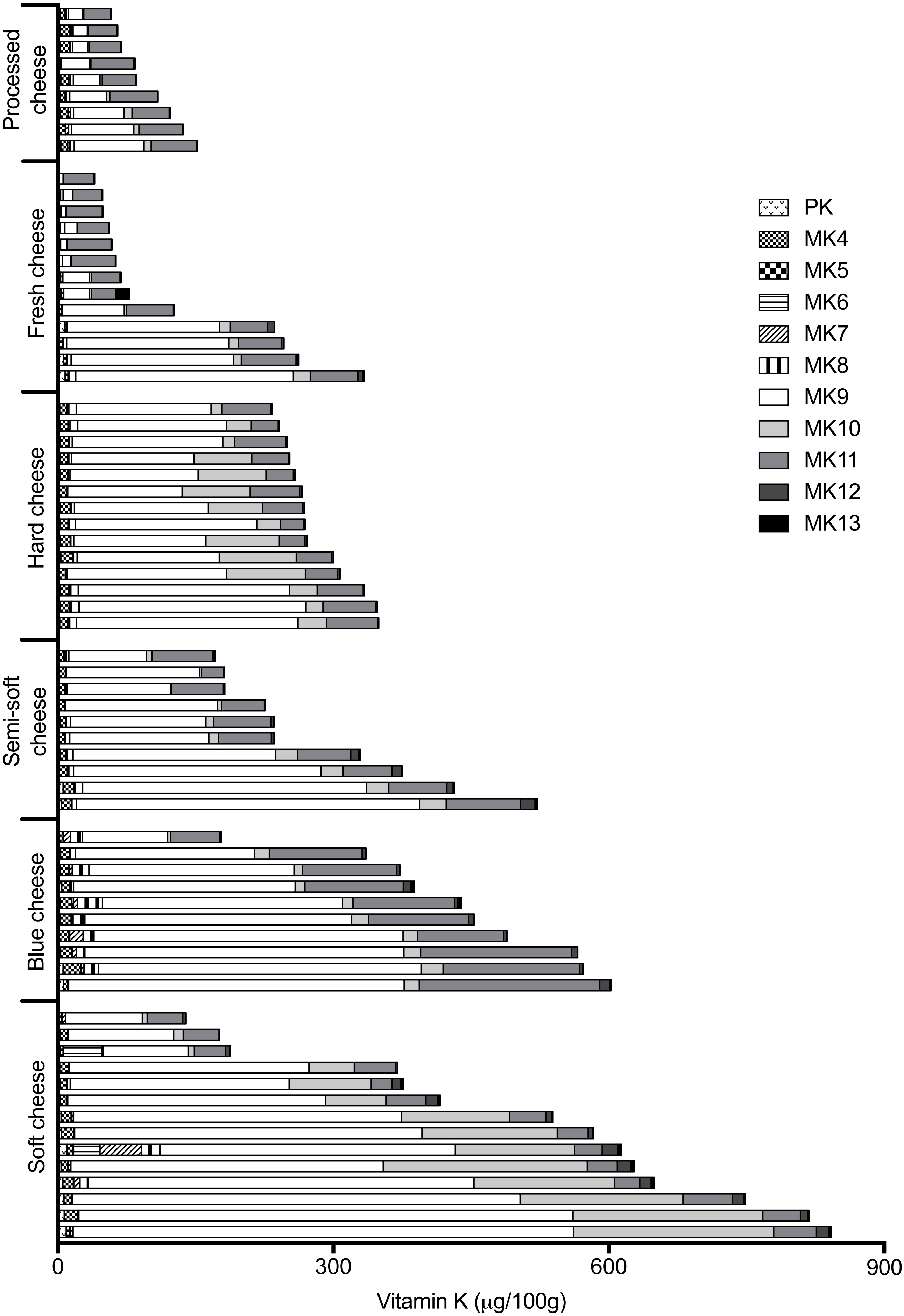
1. Values are mean ± SEM. ND: non-detectable. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 μg/100g).
2. Total: Sum of PK and MK4 to MK13.
3. *Significant difference between full fat and fat free/reduced fat within each dairy product category. Significance at *P* < 0.05.
4. † Significance determined by general linear model, with heavy cream as the reference group. Significance at *P* < 0.05.

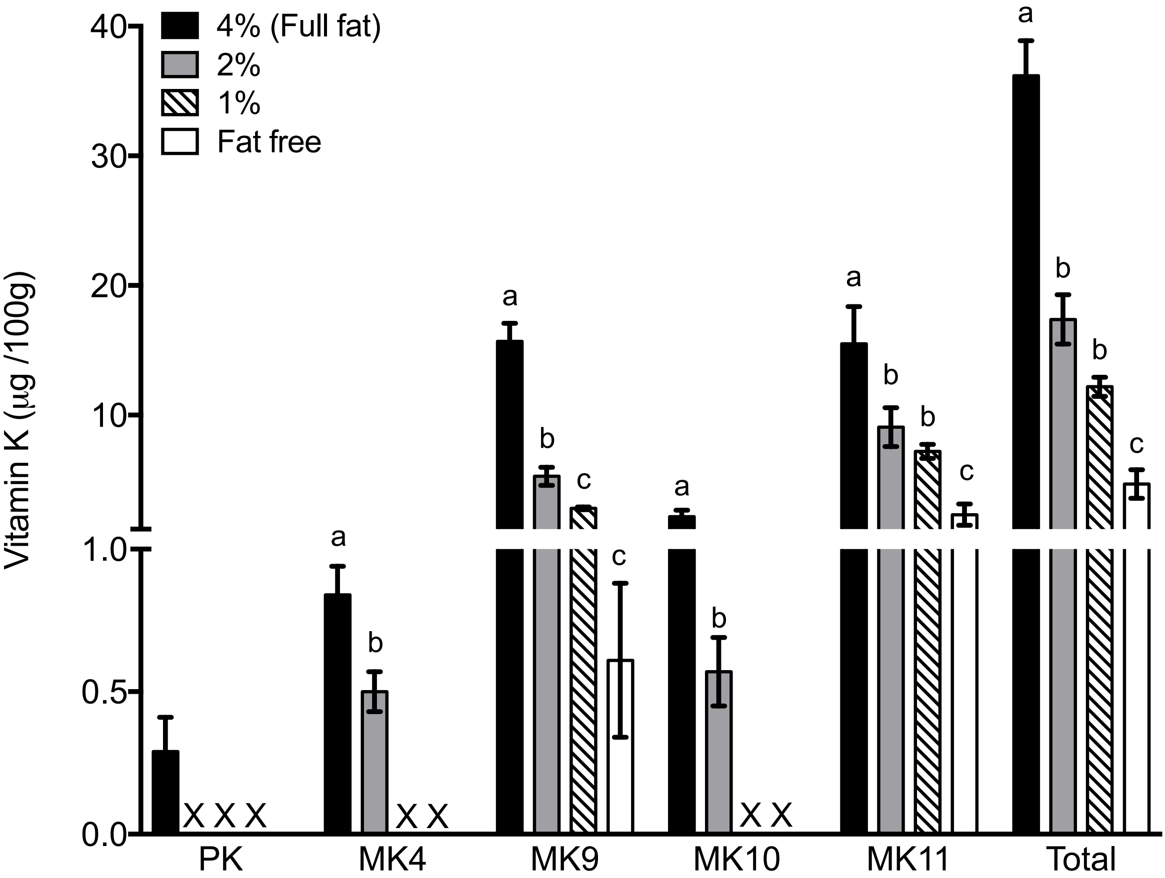
Figure legends

Figure 1. Vitamin K content of different cheeses.

Figure 2. Vitamin K concentrations of full fat (4%), 2%, 1% and fat free milk.

Values are mean ± SEM. X: non-detectable. MK5-8 and MK12-13 were not detected in any milk samples. Total: Sum of PK and MK4 to MK13. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 µg/100g). $^{a-c}$ means with different letters are significantly different ($p < 0.05$). Total vitamin K indicates the sum of PK and all MK forms.


Processed cheese
Fresh cheese
Hard cheese
Semi-soft cheese
Blue cheese
Soft cheese
PK
MK4
MK5
MK6
MK7
MK8
MK9
MK10
MK11
MK12
MK13
0
300
600
900
Vitamin K (μg/100g)


4% (Full fat)
2%
1%
Fat free
Vitamin K (µg /100g)
40
30
20
10
1.0
0.5
0.0
PK
MK4
MK9
MK10
MK11
Total
a
b
c
X X X
X X

Supplemental Table 1 Vitamin K content of cheese products (μg/100g).

Cheeses	n	PK	Menaquinones										Total
			MK4	MK5	MK6	MK7	MK8	MK9	MK10	MK11	MK12	MK13	
Blue cheese	10	3.2±0.5	10.3±1.3	0.6±0.2	ND	4.0±1.3	11.6±2.5	271±27.2	14.3±1.8	119±12.8	5.0±0.9	1.3±0.5	440±40.7
Fresh cheese	13	2.3±0.8	1.3±0.4	ND	ND	0.8±0.2	2.3±0.6	71.4±23.5	4.5±1.6	41.7±28.0	1.6±0.6	1.7±1.1	128.4±28.4
Soft cheese	14	4.5±0.7	8.1±1.0	ND	5.3±3.5	4.7±3.1	2.6±1.5	319±40.8	114±20.9	37.7±2.2	8.7±1.4	1.9±0.4	506±62.5
Semi-soft cheese	10	2.4±0.4	7.1±0.7	ND	ND	0.6±0.1	3.8±0.8	198±29.4	13.3±3.4	56.9±4.6	5.1±1.6	1.0±0.3	289±38.1
Processed cheese	9	2.5±0.2	7.0±10	ND	ND	1.2±0.3	2.8±0.4	38.6±7.7	3.7±1.0	40.8±2.8	ND	ND	98.1±11.2
Hard cheese	14	2.3±0.1	9.1±0.5	ND	ND	0.8±0.2	4.8±0.7	172±5.4	48.3±7.7	42.5±3.2	1.5±0.1	ND	282±5.0
Mozzarella part skim	12	1.4±0.1	1.4±0.3	ND	ND	0.5±0.1	ND	51.8±7.5	7.7±2.5	37.9±5.3	0.9±1.1	ND	106±10.6

Values are mean ± SEM. ND: non-detectable. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 μg/100g). Total: Sum of PK and MK4 to MK13.