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Determination of Phylloquinone and Menaquinones in Food

Effect of Food Matrix on Circulating Vitamin K Concentrations

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Key Words

Phylloquinone · Menaquinone · Vitamin K · Food composition · Bioavailability · Anticoagulant, oral

Abstract

Fluctuations in international normalized ratio values are often ascribed to dietary changes in vitamin K intake. Here we present a database with vitamin K_1 and K_2 contents of a wide variety of food items. K_1 was mainly present in green vegetables and plant margarins, K_2 in meat, liver, butter, egg yolk, natto, cheese and curd cheese. To investigate the effect of the food matrix on vitamin K bioavailability, 6 healthy male volunteers consumed either a detergent-solubilized K_1 (3.5 μmol) or a meal consisting 400 g of spinach (3.5 μmol K_1) and 200 g of natto (3.1 μmol K_2). The absorption of pure K_1 was faster than that of food-bound K vitamins (serum peak values at 4 h vs. 6 h after ingestion). Moreover, circulating K_2 concentrations after the consumption of natto were about 10 times higher than those of K_1 after eating spinach. It is concluded that the contribution of K_2 vitamins (menaquinones) to the human vitamin K status is presently underestimated, and that their potential interference with oral anticoagulant treatment needs to be investigated.

Introduction

Vitamin K is an essential dietary micronutrient that facilitates the synthesis of specific blood coagulation factors and of proteins involved in bone metabolism and vascular biology [1, 2]. It serves as a cofactor for the membrane-bound microsomal enzyme γ-glutamylcarboxylase [3]. Dietary vitamin K is absorbed and transported in blood in its most stable form, i.e. as a quinone. Vitamin K occurs in two biologically active forms namely phylloquinone (also known as vitamin K_1)

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and the menaquinones (known by their group name vitamin K_2) [2–4]. All K vitamins have 2-methyl-1,4-naphthoquinone (also known as menadione) as a common ring structure, but differ from each other in the length and saturation degree of the polyisoprenoid side chain attached to the 3-position. Phylloquinone is produced by green plants, where it is tightly associated with the thylakoid membranes of the chloroplasts. It is a single compound containing 4 isoprenoid residues (one of which is unsaturated) in its aliphatic side chain. Menaquinones contain side chains of varying length; they are designated as MK-n where n denotes the number of isoprenoid residues, all of which are unsaturated. Long chain menaquinones (MK-7 through MK-10) are exclusively synthesized by bacteria [5, 6]. Menadione is often added to fortified animal food and must be converted in the liver into MK-4 before being active as a cofactor for γ-glutamylcarboxylase [7, 8]. In addition, a number of other tissues (notably pancreas, testis and vessel wall) are capable of converting phylloquinone into MK-4 [9, 10]. For these reasons animal products (meat, dairy, eggs) may contain relatively high concentrations of MK-4. It is well known that the bacterial flora in the colon produces large amounts of higher menaquinones (notably MK-10) [11], but since at the site of synthesis absorption seems to be unlikely, the question of whether and to which extent the intestinal flora contributes to the human vitamin K status is still unclear.

Warfarin and other 4-hydroxycoumarin derivatives are antagonists of vitamin K action and are effective antithrombotic agents (the so-called oral anticoagulants). They block the conversion of KO into K by inhibiting the enzyme KO reductase, thus hampering the recycling of vitamin K [12]. Under these conditions there is a 1:1 stoichiometric relation between KO formation and the number of Gla residues synthesized. It is known that 25% of the patients on oral anticoagulant treatment are not within their therapeutic range because of fluctuating international normalized ratio values [13]. Besides interfering drugs, age, poor compliance and concurrent diseases [14–18], unstable levels of anticoagulation are often ascribed to dietary influences, mainly fluctuating vitamin K intake [19–23].

In absolute amounts K_1 forms well over 80% of the total amount of vitamin K in the human diet, and most of our present knowledge on vitamin K concerns K_1. It is known, however, that the absorption from green vegetables is poor and that only 10–15% of the vitamin is bioavailable, whereas for K_2 vitamins this may be higher [24, 25]. Here we present a database on both dietary forms of vitamin K, phylloquinone and the menaquinones in a wide range of foods available on the Dutch market. Since the specimens selected formed a representative sample from the common Dutch foods the data presented here can be used in nutritional studies in The Netherlands. Furthermore, we compared the efficacy of absorption of phylloquinone and menaquinones as deduced from their serum profiles following oral ingestion.

Materials and Methods

Materials

Phylloquinone was obtained form Sigma (St. Louis, Mo., USA). The menaquinones (MK-4 through MK-10) and 2,3-dihydrophylloquinone were kind gifts from Hoffmann-La Roche (Basel, Switzerland). All common foods were obtained at local supermarkets. Konakion® (detergent-solubilized vitamin K_1 pharmaceutical product) was obtained from Hoffmann-La Roche. For the nutrition experiment we used creamed cooked spinach from Iglo Ola (Utrecht, The Netherlands), and natto, which was bought as a ready-to-use product at a local oriental store. Silica Sep-Pak cartridges were purchased from Millipore (Milford, Mass., USA). All other chemicals used were of the highest analytical grade.

Extraction of Food

The procedure for extraction and purification of vitamin K from beverages and dairy produce (except butter and cheese) was performed as described earlier [25] using 2,3-dihydrophylloquinone as an internal standard. Vegetables were bought as precooked deep-frozen products. Cooked vegetables and raw fruits were homogenized in a blender (Ultra Turrax; Janke & Kunkel, Staufen, Germany), and processed as described for cooked spinach [25]. Aliquots of 1 g of cheese, butter or margarine were extracted with 4 ml of 2-propanol, 20 ng internal standard (MK-6 for margarine, 2,3-dihydrophylloquinone for other products) and 2 ml of distilled water. The mixture was homogenized with a blender, warmed to a temperature of 60 °C and extracted with 8 ml of hexane. Raw meat and fish were cut into pieces, 1 g of which was supplemented with 2 ml of distilled water, 5 ng of internal standard (2,3-dihydrophylloquinone) and 4 ml of ethanol. Homogenization took place with a blender at room temperature, and 8 ml of hexane were used for extraction. Bread was dried and ground to powder in a mortar, 1-gram aliquots were supplemented with 5 ng internal standard (2,3-dihydrophylloquinone) and 4 ml of ethanol. After homogenization in a blender extraction took place with 8 ml of hexane. In all cases, the hexane phase was evaporated and redissolved in 2 ml of hexane. After prepurification over silica Sep-Pak cartridges the samples were ready to measure on reversed-phase HPLC. All samples were measured in duplicate.

Vitamin K Detection

Vitamin K was analyzed by HPLC using a C-18 reversed phase column and fluorometric detection after postcolumn electrochemical reduction as described previously [25]. Phylloquinone and the menaquinones were recorded in the same run. Because of the long retention times for the long-chain menaquinones the flow was increased from 0.5 to 1.0 ml/min at 11 min after injection. The interday variation was 6–8%.

Human Volunteer Study

A panel of 6 male volunteers took part in this protocol. Their mean age was 33.5 years, and their body mass index was 24.3 kg/m^2 (table 1). All participants were apparently healthy, and their serum lipid profiles were in the normal range. Neither medications nor vitamin supplements (other than the experimental supplements) were taken. The experimental protocol started at 8 a.m. after an overnight fast. At that time the participants received a breakfast containing either a diet low in vitamin K, a similar diet with additional detergent-solubilized phylloquinone, or a diet containing 400 g of spinach and 200 g of natto. All diets contained 30 g of fat. During the rest of the day participants were only allowed to have a lunch low in vitamin K (toast, marmalade, bananas, apples), and to drink orange juice and water ad libitum. After 6 p.m. and during the rest of the experiment only consumption of vitamin K-rich foods (spinach, broccoli, brussels sprouts, kale, natto and cheese) was prohibited. Blood samples were drawn by venipunctures at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 24, 48 and 72 h after start. Serum was prepared and 1-ml aliquots were kept frozen at – 80 °C until vitamin K determination. The study design was approved by the local Medical Ethics Committee, and informed consent was obtained from all subjects according to the institutional guidelines.

Table 1. Characteristics of the subjects

	Mean	SEM
Age, years	33.5	2.57
Body mass index, kg/m^2	24.3	0.82
Triacylglycerol, mmol/l	0.87	0.14
Cholesterol, mmol/l	3.96	0.28
Vitamin K		
Phylloquinone, nmol/l	1.48	0.19
Menaquinones, nmol/l	n.d.	

Mean values ± SEM of 6 healthy male volunteers. n.d. = Not detectable.

Data Analysis

Serum vitamin K concentrations during 72 h after oral ingestion were recorded at indicated intervals. At each time point mean values ± SE for the 6 participants were calculated and plotted as a function of time. Blank values (no vitamin K ingested) were subtracted throughout the study.

Results

Vitamin K Content of Various Nutrients

For the determination of dietary phylloquinone and menaquinones we subdivided common foods into six categories: meat, fish, vegetables and fruits, dairy, oils and marga-

Table 2. Mean of K vitamins (μg/100 g or μg/100 ml) in various foods

Type of food	n	K_1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9
Meat								
Beef	7	0.6 (0.6–0.7)	1.1 (0.7–1.3)	–	–	–	–	–
Chicken breast	7	–	8.9 (6.4–11.3)	–	–	–	–	–
Chicken leg	7	–	8.5 (5.8–10.5)	–	–	–	–	–
Pork steak	7	0.3 (0.2–0.4)	2.1 (1.7–2.4)	–	–	0.5 (0.4–0.7)	1.1 (0.9–1.2)	–
Pork liver	7	0.2 (0.1–0.3)	0.3 (0.3–0.4)	–	–	–	–	–
Minced meat	7	2.4 (2.2–2.5)	6.7 (6.5–6.7)	–	–	–	–	–
Salami	7	2.3 (2.1–2.5)	9.0 (8.2–10.1)	–	–	–	–	–
Luncheon meat	7	3.9 (3.8–4.2)	7.7 (7.4–9.1)	–	–	–	–	–
Hare leg	7	4.8 (4.5–5.3)	0.1 (0.0–0.2)	–	–	–	–	–
Deer back	7	2.0 (1.9–2.2)	0.7 (0.6–0.7)	–	–	–	–	–
Goose leg	5	4.1 (3.5–4.8)	31.0 (28.2–33.1)	–	–	–	–	–
Goose liver paste	5	10.9 (9.3–12.1)	369 (317–419)	–	–	–	–	–
Duck breast	7	1.9 (1.7–2.2)	3.6 (3.3–3.9)					
Fish								
Prawn	7	0.1 (0.0–0.1)	–	–	–	–	–	–
Mackerel	7	2.2 (1.8–2.6)	0.4 (0.3–0.5)	–	–	–	–	–
Herring	7	0.1 (0.0–0.2)	–	–	–	–	–	–
Plaice	7	–	0.2 (0.1–0.3)	–	0.3 (0.2–0.3)	0.1 (0.0–0.1)	1.6 (1.3–1.8)	–
Eel	7	0.3 (0.2–0.5)	1.7 (1.4–2.1)	–	0.1 (0.0–0.2)	0.4 (0.2–0.6)	–	–
Salmon	7	0.1 (0.1–0.2)	0.5 (0.4–0.6)	–	–	–	–	–
Fruits and vegetables								
Kale	4	817 (752–881)	–	–	–	–	–	–
Spinach	6	387 (299–429)	–	–	–	–	–	–
Broccoli	5	156 (139–189)	–	–	–	–	–	–
Green peas	4	36.0 (31.2–39.4)	–	–	–	–	–	–
Sauerkraut	7	25.1 (23.8–27.5)	0.4 (0.3–0.5)	0.8 (0.6–1.0)	1.5 (1.4–1.6)	0.2 (0.1–0.3)	0.8 (0.6–0.9)	1.1 (0.9–1.3)
Natto	5	34.7 (31.2–36.7)	–	7.5 (7.1–7.8)	13.8 (12.7–14.8)	998 (882–1,034)	84.1 (78.3–89.8)	–
Banana	4	0.3 (0.2–0.4)	–	–	–	–	–	–
Apple	4	3.0 (2.7–3.4)	–	–	–	–	–	–
Orange	4	0.1 (0.1–0.2)	–					

Table 2 (continued)

Type of food	n	K_1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9
Dairy produce								
Whole milk	6	0.5 (0.4–0.6)	0.8 (0.7–0.9)	0.1 (0.0–0.1)	–	–	–	–
Skimmed milk	6	–	–	–	–	–	–	–
Buttermilk	6	–	0.2 (0.2–0.3)	0.1 (0.1–0.2)	0.1 (0.0–0.2)	0.1 (0.1–0.3)	0.6 (0.5–0.6)	1.4 (1.2–1.6)
Whole yoghurt	6	0.4 (0.3–0.5)	0.6 (0.5–0.7)	0.1 (0.0–0.2)	–	–	0.2 (0.2–0.3)	–
Skimmed yoghurt	6	–	–	–	–	–	0.1 (0.0–0.2)	–
Whipping cream	6	5.1 (4.9–5.5)	5.4 (5.2–5.6)	–	–	–	–	–
Chocolate	6	6.6 (6.4–6.7)	1.5 (1.4–1.6)	–	–	–	–	–
Hard cheeses	15	10.4 (9.4–12.1)	4.7 (4.2–6.6)	1.5 (1.3–1.7)	0.8 (0.6–1.0)	1.3 (1.1–1.5)	16.9 (14.9–18.2)	51.1 (45.3–54.9)
Soft cheeses	15	2.6 (2.4–2.9)	3.7 (3.3–3.9)	0.3 (0.2–0.4)	0.5 (0.6–0.7)	1.0 (0.9–1.1)	11.4 (10.7–12.2)	39.6 (35.1–42.7)
Curd cheese	12	0.3 (0.2–0.4)	0.4 (0.3–0.6)	0.1 (0.0–0.2)	0.2 (0.1–0.3)	0.3 (0.2–0.5)	5.1 (4.8–5.4)	18.7 (18.1–19.2)
Egg yolk	8	2.1 (1.9–2.3)	31.4 (29.1–33.5)	–	0.7 (0.6–0.8)	–	–	–
Egg albumen	8	–	0.9 (0.8–1.0)	–	–	–	–	–
Oils and margarines								
Margarine	6	93.2 (85.6–98.3)	–	–	–	–	–	–
Butter	6	14.9 (13.2–15.9)	15.0 (13.5–15.9)	–	–	–	–	–
Corn oil	6	2.9 (2.7–3.1)	–	–	–	–	–	–
Sunflower oil	6	5.7 (5.5–5.9)	–	–	–	–	–	–
Olive oil	6	53.7 (49.9–57.2)	–	–	–	–	–	–
Bread								
Rue bread	6	0.7 (0.5–0.9)	–	–	–	–	–	–
Wheaten bread	6	1.1 (1.0–1.2)	–	–	–	–	–	–
Sourdough bread	6	1.0 (0.9–1.1)	–	–	–	–	–	–
Buckwheat bread	6	3.0 (2.8–3.4)	–	–	–	1.1 (1.0–1.2)	–	–
Beverages								
Tea	4	0.3 (0.2–0.4)	–	–	–	–	–	–
Coffee	4	–	–	–	–	–	–	–
Orange juice	4	–	–	–	–	–	–	–

All samples were assessed in duplicate. Values are mean values. Highest and lowest values are given in parentheses. Foods were bought from shops in and around Maastricht. MK-10 was not detectable in any of the foods. N = Number of different samples tested; – = not detectable.

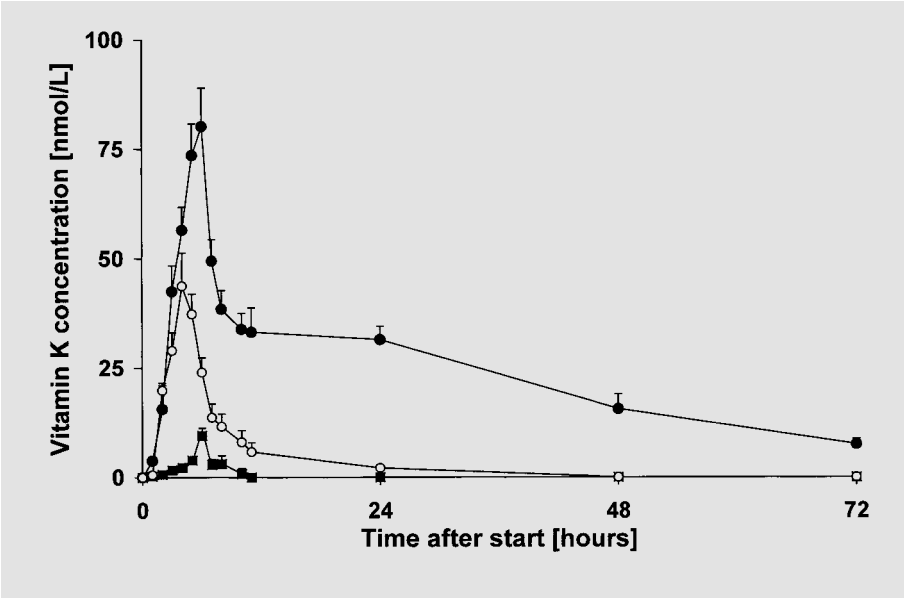

Fig. 1. Serum vitamin K following the oral intake of either Konakion or a meal containing spinach and natto. The ingested Konakion contained 3.5 μ*M* K_1, the mixed meal contained 3.5 μ*M* of K_1 and 3.1 μ*M* of MK-7. Points represent mean values from 6 volunteers, error bars represent SEM. ○ = K_1 after Konakion; ■ = K_1 after mixed meal; ● = MK-7 after mixed meal.

rines, bread, and beverages. At least three to six different samples or brands were obtained in various local supermarkets, and mean values for each product are given in table 2 together with their ranges for each product. High amounts of K_1 were found in green leafy vegetables, broccoli, sauerkraut and margarines based on plant oils. Meat, fish, dairy produce and eggs contained both K_1 and MK-4 with relatively high MK-4 concentrations in goose meat and liver, butter and egg yolk. Long-chain menaquinones were mainly found in curd cheese, hard (Dutch) and soft (French) cheeses, probably derived from the bacterial starter fermentation. Very rich in menaquinones was the Japanese food natto, which consists of fermented soy beans. No substantial differences were found between free-range products (eggs, chicken, meat) and those from factory farms. The fact that fermented beverages like beer and wines did not contain detectable amounts of menaquinones is probably due to the fact that moulds do not synthesize menaquinones [26].

Bioavailability of K Vitamins from Food

To examine the blank values (serum vitamin K at low vitamin K intake) 6 male volunteers received a vitamin K-poor breakfast with blood sampling (up to 72 h) as indicated. These blank values (data not shown) were subtracted from those obtained after controlled vitamin K intake. Based on the analyses summarized in table 2 we have prepared meals consisting of 400 g cooked spinach (equivalent to 3.5 μmol of K_1), 200 g natto (3.1 μmol of MK-7), supplemented with corn oil to a total fat content of 30 g. Postprandial

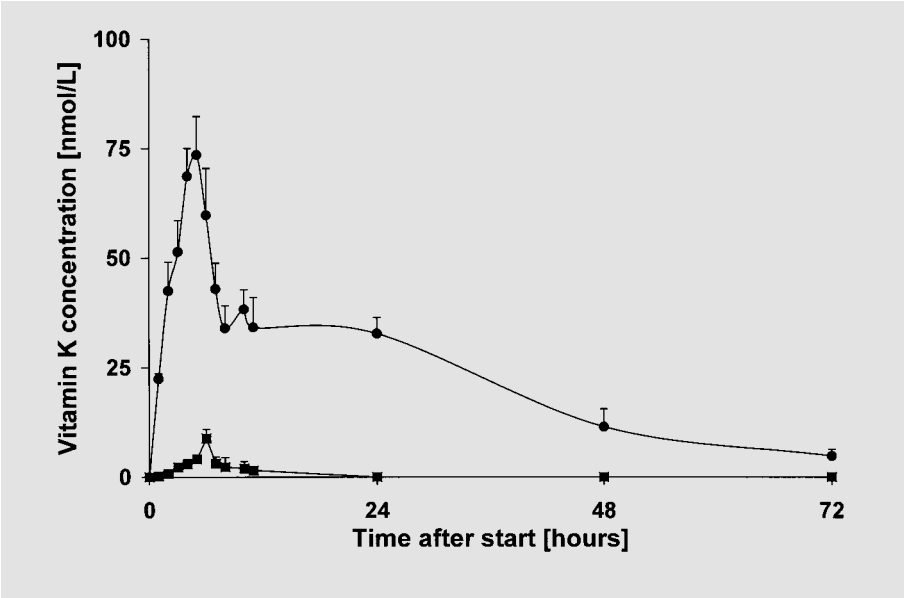

Fig. 2. Serum vitamin K_1 and MK-7 following the separate intake of either spinach (3.5 μ*M* K_1) or natto (3.1 μ*M* MK-7). Points represent mean values from 6 volunteers, error bars represent SEM. ■ = K_1; ● = MK-7.

serum vitamin K concentrations are given in figure 1. One week later the volunteers received a vitamin K-poor breakfast supplemented with 3.5 μmol of Konakion. Peak values for serum vitamin K (both K_1 and MK-7) were found at 6 h following the meal, and at 4 h after intake of the pure compound. The very poor absorption from green vegetables becomes clear by comparing the difference between the curves for K_1 pure compound and the similar amount of K_1 from spinach. Remarkably, MK-7 from natto was absorbed extremely well with peak values even higher than those for detergent-solubilized K_1. After having reached their peak levels a rapid disappearance of both K_1 and MK-7 was observed, but MK-7 showed complex pharmacokinetics, with slow disappearance during the second part of the curve, while it remained detectable for at least 72 h. The half-life times for both K_1 and MK-7 between 6 and 8 h postprandially were about 1.5 h, whereas during the later phases of MK-7 disappearance the half-life time was about 50 h. To exclude mutual interference of absorption (e.g. by competition for the same binding protein), the above experiment was repeated in a design in which spinach and natto were given in two separate meals with a 1-week interval. The serum curves are shown in figure 2 and are comparable to those obtained after the combined meal.

The above absorption curves were repeated for other foods: broccoli as source for K_1 and curd cheese and egg yolk as sources for higher menaquinones (MK-8 and MK-9) and MK-4, respectively [Schurgers, unpubl. data]. In all cases it was found that K_1 absorption from vegetables was very poor (5–10% without concomitant fat intake and 10–15% if tak-

en together with 30 g fat), whereas menaquinone absorption from dairy produce and natto was much better, probably almost complete.

Discussion

In this paper we describe the phylloquinone and menaquinone content of various foods available on the Dutch market. All K vitamins were quantified in the same run after a slight modification of our previously reported procedure [25]. It was confirmed that phylloquinone is mainly present in green vegetables, margarins and some plant oils such as olive oil. Since these data are similar to those reported by others [27–29] we have focussed on the menaquinones in food. MK-4 was present in nearly all animal products (meat, dairy produce, eggs), but the fact that there were no substantial differences between game (hare, deer), free-range animals and those from factory farms suggests that conversion of menadione from fortified animal food (used at factory farms) does not contribute substantially to the total tissue MK-4 stores. Rather, it seems that the major part of MK-4 in animal products originates from conversion of K_1 as was also reported to occur in rats [10]. Relatively high concentrations of long-chain menaquinones were found in all cheeses. As was suggested by Shearer [26], they probably originate from bacteria present in the starter cultures used to induce fermentation. On the basis of food frequency questionnaires and the data in table 2 it has been calculated that phylloquinone forms almost 90% of the total dietary vitamin K intake in the Dutch population, whereas menaquinones account for less than 12% [6]. Phylloquinone, however, is tightly bound to the thylakoid membranes of plant chloroplasts, and the efficacy of its liberation therefrom in the digestive tract is poor [24, 25]. This was confirmed in an experiment in which we compared the serum concentration vitamin K profiles after ingestion of similar amounts of K_1 from spinach and from a detergent-solubilized pharmaceutical product. To compare the efficacy of absorption of phylloquinone and menaquinone we have chosen a design in which K_1 was obtained from spinach and MK-7 from natto. In this way the molar concentrations of both K vitamers could be kept similar. As is shown in figure 1, the postprandial serum concentrations of MK-7 were much higher than those of K_1, with a peak height difference of more than 10-fold. Both absorption peaks occurred 2 h later than that for the detergent-solubilized product. From the curves obtained, it may be concluded that the contribution of MK-7 from natto to the total bioavailable pool of vitamin K is much higher than estimated on the basis of intake. Menaquinones from other sources (cheeses, egg yolk) were absorbed with comparable efficacy as was MK-7 [Schurgers, unpubl. data], suggesting that the contribution of menaquinones to the total human vitamin K status is much higher than generally assumed, and may equal that of K_1.

Another remarkable difference between K_1 and menaquinones was that the former had a disappearance curve with an apparent half-life time of 1.5 h, whereas the long chain menaquinones (not MK-4) had more complex disappearance curves with a very long half-life time. Rapid clearance is consistent with the previously reported uptake and transport of K vitamins in chylomicrons, from where they are cleared by the liver during the first 8 postprandial hours. The very long half-life times of the higher menaquinones suggest that these vitamers (and not K_1 and MK-4) are redistributed by the liver and set free in the circulation in low and high density lipoproteins. It is well known that LDL may be present in the

circulation for several days. The long residence times of higher menaquinones in the circulation implies that they are available for extrahepatic tissue uptake for much longer periods than is phylloquinone. Both because of their high postprandial serum concentration and their slow clearance, the importance of higher menaquinones for extrahepatic tissues such as bone and arterial vessel wall may be underestimated if only dietary intake is regarded. Since vitamin K-dependent proteins have been reported to be involved in the regulation of calcium deposition in bone [30] and in the prevention of arterial calcification [31], intake of higher menaquinones may be important for functions of vitamin K not related with blood coagulation.

The high efficacy of menaquinone absorption may also have consequences for subjects on oral anticoagulant treatment. In attempts to identify potential causes of unstable anticoagulation, menaquinone intake has been ignored thus far. Our data demonstrate that this is not justified. Their efficient absorption combined with long serum and tissue half-life times [32] suggests that menaquinones from curd and cheese may accumulate at repeated intake and are a potential cause of disturbance of anticoagulant therapy. This is even more so for subjects consuming natto. Although in general natto is not eaten by Caucasians, dietary habits may survive after migration of subjects from Asiatic countries so that hematologists in western countries may be confronted with this unsuspected source of highly bioavailable vitamin K.

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