

Phylloquinone (Vitamin K₁) in Cereal Products

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ABSTRACT

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The significance of cereals as dietary sources of phylloquinone (vitamin K₁) was estimated by analyzing the phylloquinone content in some representatives of milling and bakery products commercially available in Finland. For extraction of phylloquinone, two procedures were compared. Routine determinations were made by isopropanol-hexane extraction. After purification of the extracts with semipreparative straight-phase HPLC, phylloquinone was quantified by reverse-phase HPLC with dual-electrode electrochemical (EC) detection. Menaquinone-4 (MK-4) was used as an internal standard. The phylloquinone content of some fat-containing bakery products were, however, quantified by the external standard method. The

highest phylloquinone content was found in doughnuts (8.5 µg/100 g), while the phylloquinone content of other bakery products varied at 3–5 µg/100 g. Among milling products, the best source of phylloquinone was rye meal (5.9 µg/100 g). The phylloquinone content of the other milling products, except whole wheat flour (4.0 µg/100 g), was very low (<3 µg/100 g). Due to this low phylloquinone content, cereal products cannot be regarded as significant sources of vitamin K, despite their overall significance in diets. The contribution to the average daily dietary intake of vitamin K in Finland was estimated to be ≈6 µg, which is 7–10% of the recommended daily intake.

Vitamin K has been historically identified for its role in blood coagulation (Shearer 1995), in which it functions as an essential cofactor in the posttranslational synthesis of prothrombin and other clotting proteins. It is currently known that vitamin K dependent proteins also include proteins affecting bone metabolism (Shearer 1995). The significance of vitamin K has thus expanded.

It was earlier believed that primary deficiency of vitamin K is uncommon (Suttie 1992). Deficiency may, however, be more prevalent, as new sensitive tests based on detecting undercarboxylation of vitamin K dependent proteins have indicated (Vermeer et al 1995). It has also been shown that bioavailability of vitamin K can vary greatly depending on the sources of the vitamin (Gijssbers et al 1996). On the other hand, dietary phylloquinone (vitamin K₁) impairs the efficiency of anticoagulant drugs. All these new findings have raised questions about adequacy of the current daily Recommended Dietary Allowance (RDA) (Food and Nutrition Board 1989) and the requirement of more reliable data on the vitamin K₁ content of different foods.

The phylloquinone content of cereal products has been examined in only a few studies (Booth et al 1993, 1995). Furthermore, previous data are available for only small number of products (e.g., rye has rarely been examined). Based on limited previous data, cereal products are generally regarded as poor sources of phylloquinone (Booth et al 1993, 1995). The consumption of cereal products is, however, fairly heavy in many countries. Therefore, more information is needed on the concentration of phylloquinone in various cereal products to estimate the dietary intake of this vitamin. These data are also needed in planning diets for people undergoing anticoagulant therapy.

In a recent article, we described an HPLC method for determining phylloquinone in oils and margarines (Piironen et al 1997). The aim of this study was to develop an efficient extraction procedure for milling and bakery products. The extraction procedure developed was applied to analyzing the presence of phylloquinone by HPLC in the main milling products and also in some bakery products available in Finland.

MATERIALS AND METHODS

Sampling

Samples of selected milling and bakery products (Table I) were purchased from 10 retail stores representing the four major food chains in the Helsinki area during the winter of 1997. There were eight to 10 subsamples (0.2–1.0 kg) of each food item, and one pooled sample was prepared representing each sample type. Flours did not require pretreatment, while subsamples of bakery products were diced. Equal weights (usually 100 g) of each subsample were added to the pool. The pooled samples were mixed and divided into portions of ≈100 g, vacuum-packed in plastic bags, and stored at –20°C in the dark until analyzed, usually for one to three weeks.

Sample Preparation

All work was performed under subdued light conditions. The phylloquinone content of the samples was analyzed in triplicate. Rye meal was used as a reference sample, which was analyzed in duplicate in every second sample series. In addition, a blank without the internal standard (menaquinone-4 [MK-4]), was analyzed for every sample item. Phylloquinone and MK-4 were purchased from Sigma (St. Louis, MO). Standard stock and working solutions were used as previously described, and all reagents were also the same as in our earlier study (Piironen et al 1997).

For the extraction of phylloquinone from milling and bakery products, two methods were tested: extraction with isopropanol-hexane and with chloroform-methanol. Rye meal and rye bread were used as test materials. During the developing process, the phylloquinone content, recovery of MK-4, and repeatability of the results were monitored. The ratio of phylloquinone to MK-4 in the first and second extractions of rye meal were compared to further confirm the reliability of the methods. In addition, simplicity of the extraction procedure was considered when selecting the method. The differences between the results of the two methods were determined with the paired *t* test.

For routine determinations of milling and bakery products, extraction with isopropanol-hexane with 10 min of digestion time was chosen. The extraction procedure used was partly based on that of Langenberg et al (1986). In this method, a 2.5-g sample was weighed into a centrifuge tube and MK-4 (40–365 ng) was added. After adding 15 mL of isopropanol, the sample was digested in a boiling waterbath. Two digestion times (5 and 10 min) were compared for the rye meal. After cooling, 10 mL of isopropanol was added and the sample was homogenized with a mixer (Ultra-Thurax T25, Janke and Kunkel, Germany) for 2 min. *n*-Hexane (10 mL)

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was added to the mixture twice; the mixture was homogenized for 2 min after each addition. This was followed by adding 10 mL of water and shaking rapidly. The mixture was centrifuged at $1,500 \times g$ for 5 min (Sorval RC2-B, Ivan Sorval Inc., Norwalk, CT), after which an appropriate amount (5–10 mL) of the *n*-hexane phase was evaporated to dryness and the residue redissolved in 1 mL of *n*-hexane. Prior to purification with semipreparative HPLC, the sample extract was run through a membrane filter (Puradisc 25 TF 0.45 μ m, Whatman, Ann Arbor, MI). The residue of the first extraction was reextracted with *n*-hexane (10 mL) followed by homogenization (1 min) and centrifugation (5 min). Evaporation, dissolving, and filtration were performed as in the first extraction. The second extraction was performed to further confirm the efficiency of the extraction procedure. For some bakery products (Danish pastry, Karelian pie, doughnuts, and sweet wheat bread) the same method without added MK-4 was used.

In the second method, extraction was performed using chloroform-methanol (1:1) as a solvent. MK-4 (40–365 ng) and 15 mL of isopropanol were first added to a homogeneous, weighed sample (2.5 g). The tube was kept in the boiling waterbath for 10 min. After cooling, 10 mL of isopropanol was added and the sample was homogenized with the mixer for 2 min. The mixture was centrifuged at $2,000 \times g$ for 5 min. After phase separation, the upper layer was collected in a flask. The extraction was repeated with 20 mL of chloroform-methanol (1:1). The extract was rehomogenized for 2 min and centrifuged for 5 min. The supernatant was added to the flask and the combined extract was evaporated to dryness using a rotavapor. Ethanol (10 mL) was added and the evaporation repeated. The residue was dissolved in *n*-hexane and treated as described for the extraction with isopropanol-hexane. In this case, the second extraction was performed with 40 mL of chloroform-methanol. The mixture was shaken with a magnetic stirrer for 1 hr, after which the sample was centrifuged, evaporated, and redissolved in the same manner as in the first extraction.

TABLE I
Phylloquinone Content^a of Cereals and Bakery Products

Sample	Phylloquinone (μ g/100 g)	CV% ^b
Milling products		
Barley meal	2.2 \pm 0.08	3.9
Oats, rolled	2.1 \pm 0.14	6.9
Rice, polished	0.16 \pm 0.01	6.1
Rye meal ^c	5.9 \pm 0.37	6.3
Wheat flour, all purpose (\approx 0.5% ash)	1.0 \pm 0.14	15
Whole wheat flour	2.6 \pm 0.14	5.3
Wheat germ	4.0 \pm 0.11	2.7
Bakery products		
Danish pastry ^d	5.1 \pm 0.65	13
Doughnuts, raised ^d	8.2 \pm 1.56	19
Karelia pie ^d	4.0 \pm 0.90	22
Sweet wheat bread ^d	3.1 \pm 0.07	2.4
French rolls	1.8 \pm 0.04	2.1
Rye bread	4.3 \pm 0.14	3.2
Yeast bread	3.0 \pm 0.05	1.8

^a Mean \pm standard deviation ($n = 3$).

^b Coefficient of variation.

^c $n = 12$.

^d Calculated by external standard method.

Purification with Semipreparative Straight-Phase HPLC

Purification of the sample extracts with semipreparative HPLC was performed using the HPLC apparatus described by Piironen et al (1997). The vitamers (*cis* and *trans* isomers of phylloquinone and MK-4) were separated with a μ Porasil column (5 μ m, 300 \times 3.9 mm; Millipore Corp., Milford, MA). The mobile phase was *n*-hexane containing 1% diethyl ether with a flow rate of 1.5 mL/min and injection volume of 300–500 μ L. Collection began 2 min before elution of the *cis* isomer of phylloquinone and ended 1.5 min after the elution of MK-4. The collected fraction was evaporated and redissolved in 0.5 mL of the mobile phase used in analytical HPLC.

Analytical HPLC

The phylloquinone content of the samples was quantified by reverse-phase HPLC with a dual-electrode electrochemical (EC) detector. The method was based on that of Hart et al (1985) and the apparatus used was the same as described by Piironen et al (1997). The analytical column was a Vydac 201 TP54 column (5 μ m, 250 \times 4.6 mm) (The Separation Group, Hesperia, CA) and the mobile phase consisted of 96% methanol and 0.05M Na acetate buffer (pH 3) at a flow rate of 1.0 mL/min; the injection volume was 30 μ L. The detector was operated in the redox mode in which the upstream electrode (-1.1 V) reduced the vitamin K compounds and the downstream electrode (0 V) reoxidized the compounds. The phylloquinone content of samples was quantified by the internal standard method, based on the peak areas in which the response factor was determined twice during the research at three concentration levels (0.2–0.9 ng/injection) and daily at one concentration level (0.7 ng/injection). Blank tests were done for every sample to confirm reliable quantification. Danish pastry, Karelian pie, doughnuts, and sweet wheat bread showed a peak eluting at the retention time of MK-4. Depending on the type and amount of fat added, these products may contain endogenous MK-4, even in amounts similar to phylloquinone; therefore the phylloquinone content was quantified by the external standard method.

Method Validation

The accuracy of the method chosen was tested by determining the recoveries of phylloquinone and MK-4 in rye meal and rye bread and phylloquinone added to Danish pastry. Repeatability of the determinations was monitored by analyzing a reference sample in every second sample series. Daily variations in detector response and the retention times in analytical HPLC were monitored with standard injections after every third sample. In preparative HPLC, standards were injected to confirm the retention times for collection. To avoid the carry-through effect, the mobile phase was injected after every standard injection in both HPLC systems.

RESULTS AND DISCUSSION

Analytical Method

We have previously described an HPLC method for the determination of phylloquinone in oils and margarines (Piironen et al 1997). The method presented here for the determination of phylloquinone in cereal and bakery products is a modification of this earlier method. The major modification relates to the extraction procedure, which was modified suitable for cereals. The purifica-

TABLE II
Extraction of Phylloquinone by Two Different Procedures^a

Sample	Isopropanol-Hexane			Chloroform-Methanol		
	<i>n</i>	Phylloquinone (μ g/100 g)	Recovery of MK-4 ^b (%)	<i>n</i>	Phylloquinone (μ g/100 g)	Recovery of MK-4 ^b (%)
Rye meal	11	6.1 \pm 0.38	78 \pm 8.6	6	6.1 \pm 0.21	83 \pm 16
Rye bread	4	4.6 \pm 0.17	97 \pm 4.8	4	4.3 \pm 0.15	93 \pm 10

^a Mean \pm standard deviation.

^b Menaquinone-4 used as an internal standard.

tion by semipreparative HPLC and quantification by analytical HPLC were done as in our earlier study.

Both tested extraction methods, isopropanol-hexane and chloroform-methanol, resulted in quite similar phylloquinone results for rye meal and the recoveries of MK-4 for both sample matrices (Table II). Extraction with isopropanol-hexane, however, resulted in significantly ($P < 0.01$) higher phylloquinone content for rye bread than extraction with the chloroform-methanol. The ratio of phylloquinone to MK-4 was similar in the first and the second extractions regardless of the extraction solvents used, which was an indication of the similar extractability of endogenous phylloquinone and the added standard (MK-4). Since extraction with isopropanol-hexane was at least as efficient as extraction with chloroform-methanol and also more simple to perform, it was chosen for routine determinations. Both digestion times tested (5 and 10 min) gave the same phylloquinone content for rye meal (6.1 $\mu\text{g}/100\text{ g}$) when extracting with isopropanol-hexane. However, the recovery of MK-4 was slightly better with the digestion time chosen (10 min) ($78 \pm 8.6\%$ compared with $69 \pm 11.5\%$). More repeatable results were obtained after 10 min of digestion. Coefficient of variation (CV) between phylloquinone analyses was 3.2% ($n = 11$) compared with 6.1% ($n = 5$).

The good recoveries of phylloquinone calculated by the internal standard method were an indication of the accuracy of the method. For rye meal the recovery was $96 \pm 3.0\%$ (CV = 3.2%, $n = 5$), and for rye bread $92 \pm 1.6\%$ (CV = 1.7%, $n = 3$). The recovery tests also confirmed the similar behavior of phylloquinone and MK-4 during analysis. The recoveries of phylloquinone and MK-4 (calculated by the external standard method) added to rye meal ($n = 5$) were $85 \pm 12\%$ (CV = 14%) and $86 \pm 13\%$ (CV = 15%), and $91 \pm 19\%$ (CV = 20%) and $98 \pm 15\%$ (CV = 15%) when added to rye bread ($n = 3$). The recovery (calculated by the external standard method) of phylloquinone added to Danish pastry was $95 \pm 5\%$ (CV = 5.6%, $n = 2$).

In analytical HPLC, good separation of phylloquinone and MK-4 from the other components of the sample was obtained. No interfering peaks eluted at the retention time of MK-4 in the samples containing no added fat. The chromatograms of whole wheat flour are shown as illustrations (Fig. 1). Phylloquinone eluted in ≈ 9.9 min and MK-4 in 6.5 min. The day-to-day variations ($n = 18$) in the retention times were 1.1 and 0.8%, respectively, while the within-day variation was insignificant. The retention times in the semipreparative HPLC averaged 7.1 min for the *cis* isomer of vitamin K_1 and 8.3 min for the *trans* isomer; the retention time of MK-4 was 11.3 min.

The variation in EC detector response in analytical HPLC was tested daily. The within-day CV for the peak areas of phylloquinone was 2.8% and for the internal standard (MK-4) CV = 2.5% ($n > 3$ per day, 18 days). The day-to-day CV = 6.7 and 8.2% ($n = 18$), respectively. The response factor for phylloquinone, using MK-4 as an internal standard, was 0.967 ± 0.012 (day-to-day CV = 2.2%, $n = 18$). The response factor was also determined twice at three different concentration levels. Between these two determinations CV = 2% and between different levels CV = 1.7%. The reliability of the results was further confirmed by the good day-to-day repeatability observed in analyses of the reference sample. In the phylloquinone content of rye meal CV = 5.8% (six days). Normally, when analyzing samples in triplicate CV < 10%. It was greater for samples where phylloquinone content was quantified by the external standard method (CV = 10–22%).

Phylloquinone Content of Milling and Bakery Products

The phylloquinone content of the samples analyzed were at most moderate (Table I). Among the bakery products analyzed, the highest phylloquinone content was found in raised doughnuts (8.2 $\mu\text{g}/100\text{ g}$). The phylloquinone content of other bakery products containing added fat was, in general, 3–5 $\mu\text{g}/100\text{ g}$, whereas French rolls contained phylloquinone in considerably lower

amounts (1.8 $\mu\text{g}/100\text{ g}$). Rye meal was the best source of phylloquinone (5.9 $\mu\text{g}/100\text{ g}$) among the milling products analyzed. The phylloquinone content of the other milling products, except whole wheat flour (4.0 $\mu\text{g}/100\text{ g}$), was very low (<3 $\mu\text{g}/100\text{ g}$).

In previous studies, the phylloquinone content of only a small number of milling and bakery products was investigated. Booth et al (1995) reported that rye bread contained 3.0 $\mu\text{g}/100\text{ g}$ of phylloquinone and various wheat breads contained 1.9–3.5 $\mu\text{g}/100\text{ g}$. These figures are in agreement with our results and also with that of Weihrauch and Chatra (1993) for an assortment of breads (3 $\mu\text{g}/100\text{ g}$), rice (1 $\mu\text{g}/100\text{ g}$), and wheat flour (0.6 $\mu\text{g}/100\text{ g}$). Our result with wheat flour is slightly higher (1.0 $\mu\text{g}/100\text{ g}$), whereas in rice, we determined only 0.16 $\mu\text{g}/100\text{ g}$ of phylloquinone. The phylloquinone content we obtained for barley meal (2.2 $\mu\text{g}/100\text{ g}$) is twofold when compared with the result of Sakano et al (1993) (1 $\mu\text{g}/100\text{ g}$). However, variations in milling practices make exact comparisons difficult. Furthermore, Ferland and Sadowski (1992) showed that the soil and climate considerably affect the phylloquinone content of green vegetables. It is very probable that growing conditions of cereals used as raw material may be another factor causing variation in phylloquinone content of milling products. For bakery products, the type and amount of added fat and also the processing technique may affect the phylloquinone content.

Despite great consumption (64 kg/year per person) of cereals and bakery products in Finland, they cannot be regarded as significant sources of dietary phylloquinone. Based on the average per capita consumption in 1990 (Statistics Finland 1993) and the present results, the average intake of vitamin K from this food group was estimated to be 6 $\mu\text{g}/\text{day}$, which is only 7–10% of the current RDA (Food and Nutrition Board 1989).

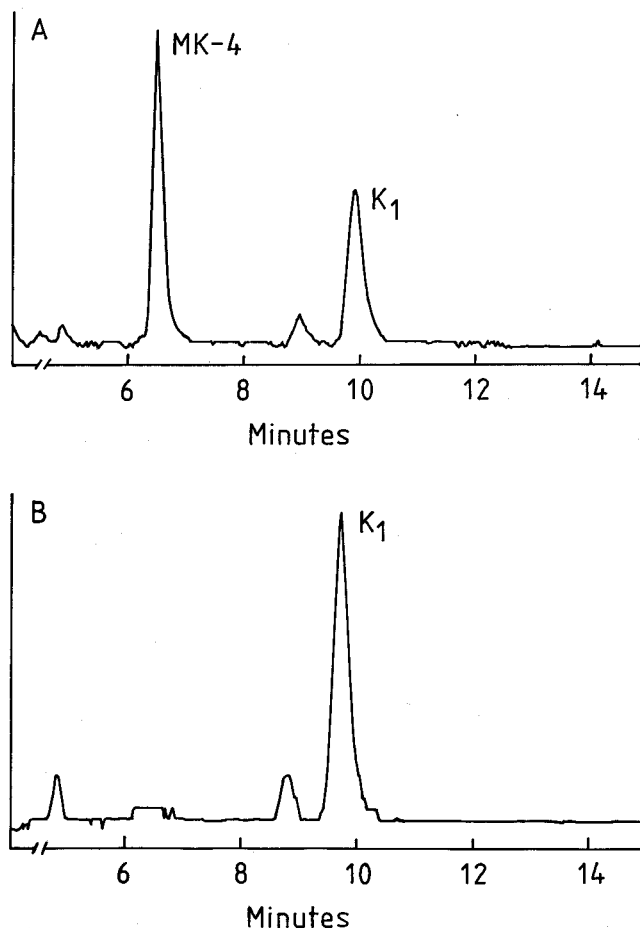


Fig 1. Analytical HPLC chromatograms of a whole wheat flour sample with (A) and without (B) MK-4.

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