Determination of Phylloquinone and Menaquinones in Animal Products with Fluorescence Detection after Postcolumn Reduction with Metallic Zinc

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A high-performance liquid chromatographic (HPLC) method for the determination of phylloquinone and menaquinones in foods of animal origin is described. The K vitamers were quantified with a fluorescence detector after postcolumn reduction with metallic zinc using $K_{1(25)}$ as an internal standard. Extraction was done either with 2-propanol−hexane (meat and fish products) or with acid hydrolysis method (dairy products). Prior to quantification, sample extracts were purified by semipreparative HPLC; in addition, the fats of cheese and rainbow trout samples were removed with lipase hydrolysis. By this method the phylloquinone and menaquinones (MK-4 to MK-10) present in a few representative samples of different animal food groups were determined. HPLC-MS was used to confirm the identification of K vitamers. Long-chain menaquinones were found from bovine and pig livers as well as from various cheeses. The total vitamin K contents calculated as the sum of quantified K vitamers were in general low (mean content 10−100 ng/g); the highest amount was analyzed in chicken meat (600 ng/g).

Keywords: *Menaquinone; phylloquinone; vitamin K; HPLC; animal products*

INTRODUCTION

Vitamin K exists naturally as either vitamin K_1 (phylloquinone), which is synthesized by plants, or as vitamin K_2 (menaquinones). Menaquinones (MK-*n*) are of microbial origin and classified on the basis of the number of isoprene units (2−15) in their side chains. Menadione (vitamin K_3) is a synthetic form of vitamin K, which is generally used in commercial animal feeds. Its vitamin K activity results from its in vivo conversion to MK-4 in different animals (Dialameh et al., 1971; Udagawa et al., 1993). Vitamin K is a cofactor in the posttranslational synthesis of γ-carboxyglutamic acid in several proteins, which have been found for example in bone. These findings have expanded the physiological role of vitamin K; earlier it was believed that vitamin K affects only blood coagulation (Shearer, 1995). Thus vitamin K has been under active research during recent years, and reliable data on vitamin K contents in foods are needed.

Phylloquinone is in general recognized as the main dietary form of vitamin K, and green vegetables and plant oils are regarded to be its best sources (Booth et al., 1996; Koivu et al., 1997; Piironen et al., 1997). Although there is now a significant and growing database for phylloquinone in plant foods, reliable data on phylloquinone and menaquinones in animal products do not exist. On the basis of the few studies done, it is assumed that the menaquinone contents are low except in some fermented soybean products and cheese (Shino 1988; Shearer et al., 1996). However, the role of animal food as a source of vitamin K has to be evaluated further. It has been suggested that bioavailability of phylloquinone from vegetables may be poor (Gijsbers et al., 1996) and on the other hand that intestinal synthesis of menaquinones is not sufficient but dietary vitamin K is also needed (Vermeer et al., 1995). Therefore more information is needed on the concentrations of vitamin K in animal food.

Due to the low concentrations of menaquinones in biological samples, their detection has remained a great challenge. Although reverse-phase high-performance liquid chromatography (HPLC) with electrochemical (EC) or fluorometric detection after postcolumn reduction provides enough sensitivity and specificity for phylloquinone analysis, much progress has to be made in analytical methods before reliable data on menaquinones in animal products are produced. In addition, their reliable identification is difficult and has to be confirmed at least with two detection systems. A lot of effort is also required for the extraction and purification steps because of the complexity of animal food matrices.

Here we describe a HPLC method for determining phylloquinone and menaquinones (MK-4 to MK-10) in animal products. The main purpose of the present study was to develop a separation and quantification method for menaquinones and validate an efficient extraction and purification system for foods of animal origin. The identification of menaquinones was confirmed with HPLC-MS. The method developed was applied in analyzing the presence of phylloquinone and different menaquinones in a few animal products to get information for further studies. The samples were selected to represent basic food groups, fish, meat, and dairy products, to give an overview of the distribution of vitamin K among animal products. The selected samples were regarded to be important sources of animal food in the Finnish diet.

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MATERIALS AND METHODS

Sampling. A few examples from meat, fish, and dairy products (Table 1) were selected for analysis; they were purchased from 10 retail stores representing the four major food chains in the Helsinki area in 1998. There were 8−10 subsamples weighing 0.2−1.0 kg of each food item, and one pooled sample was prepared to present each sample type.

The samples were determined according to how they are consumed; i.e., only edible parts were analyzed. Each subsample was homogenized, and identical amounts (usually 150 g) of each were added to the pool. The pooled samples were mixed and vacuum-packed as 50 g portions in plastic bags and stored at −20 °C in the dark until analyzed (generally 1−3 weeks). All work was performed under subdued light. The vitamin K contents (phylloquinone and menaquinones 4−10) of each sample were determined in triplicate. In addition, the moisture of all samples was determined by drying at 100 ± 2 °C to a constant weight (AOAC 952.08, modified; AOAC, 1990). The AOAC method was also used to determine the fat contents of the meat and fish samples (AOAC 948.15; AOAC, 1990). The fat contents of dairy products (Table 1) are from the specifications of the producer.

Standards. Phylloquinone, 2',3'-dihydrovitamin K_1, $K_{1(25)}$ [2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-naphthalenedione] and menaquinones were either purchased (Sigma Chemical Co., St. Louis, MO) or received as gifts (Hoffman-La Roche and Co., Basel, Switzerland, and Eisai Co., Tokyo, Japan). Standard stock and working solutions were used as previously described (Piironen et al., 1997). The concentrations of the phylloquinone, $K_{1(25)}$, and MK-4 solutions were confirmed by analyzing them spectrochemically at 249 nm ($E^{1\%}_{1cm} = 419$, Merck).

$K_{1(25)}$, a synthetic analogue of phylloquinone (produced by the substitution of a 25-carbon side chain to menadione), was used as an internal standard. In earlier studies (Koivu et al., 1997,1998; Piironen et al., 1997) we have used MK-4 as the internal standard. Since it is not suitable for animal products containing MK-4 naturally, we tested two alternatives: $K_{1(25)}$ and dihydrovitamin K_1. Two reasons supported the selection of $K_{1(25)}$ for animal food analysis; first, other components had not the same elution characteristics, whereas dihydrovitamin K_1 had the same retention time as MK-6. Second, we have not found any evidence to support the existence of $K_{1(25)}$ in foods. For each item a blank sample without the internal standard ($K_{1(25)}$) was analyzed first to confirm the validity of its use.

Preparation of Meat and Fish Samples. For the extraction of phylloquinone and menaquinones from meat and fish products two methods were compared: extraction with 2-propanol−hexane and with chloroform−methanol. Bovine liver was used as a test material. The efficiency of each method was estimated by monitoring the contents of K_1, MK-4, MK-8, and MK-9, as well as the recovery of $K_{1(25)}$, and the repeatability of the results**.** The reliability of the methods was further confirmed by comparing the ratios of both phylloquinone and menaquinones with $K_{1(25)}$ in the first and second extractions. Differences between methods were determined using the paired *t* test.

For the routine determinations of meat and fish products, extracting with 2-propanol−hexane was chosen. The extraction procedure is described in our previous paper (Koivu et al., 1997). Briefly, the method involves the digestion of a sample (3 g) with the internal standard (50−400 ng) and 10 mL of 2-propanol in a boiling water bath for 5 min. After cooling, 10 mL of 2-propanol is added and the sample is homogenized for 2 min. *n*-Hexane (10 mL) is added to the mixture twice, and it is homogenized for 2 min after each addition. This is followed by adding 10 mL of water and shaking rapidly. Finally the extract is centrifuged at 1500*g* for 5 min after which 10 mL of a *n*-hexane phase is evaporated to dryness and redissolved in 1 mL of hexane. Prior to purification with semipreparative HPLC, the sample is filtered through a membrane filter (Puradisk 25 TF 0.45 mμ, Whatman, Ann Arbor, MI). The fat of rainbow trout was, however, removed with lipase hydrolysis (as related below) before HPLC purification. When evaluating

Table 1. Vitamin K Contents of Animal Products

food item	dry matter (%)	% fat/ fresh wt	vitamin K content in foods,[a] ng/g							
			phylloquinone	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	sum
fishes											
rainbow trout, cultivated	32.8	12.4	5.6 ± 0.31	31 ± 2.4	0.9 ± 0.19	nd	2.0 ± 0.58	nd	nd	nd	40
pike-perch	21.4	0.9	1.3 ± 0.20	1.9 ± 0.25	0.49 ± 0.044	0.52 ± 0.008	4.9 ± 1.28	nd	nd	nd	9
Baltic herring	22.9	6.4	11.5 ± 0.17	2.07 ± 0.019	UK	nd	nd	nd	nd	nd	14
meat products											
pig liver[c]	27.7	4.0	12 ± 2.7	10.8 ± 1.44	nd	nd	16 ± 2.7	25 ± 5.2	6 ± 1.8	8 ± 2.9	78
bovine liver	29.5	3.6	58 ± 4.1	6.8 ± 1.03	nd[f]	9.44 ± 0.118	25.6 ± 0.59	13.8 ± 0.55	9.8 ± 0.70	14 ± 1.7	138
beef meat, roast	26.5	3.2	6.7 ± 0.53	28 ± 2.5	1.2 ± 0.29	nd	1.17 ± 0.131	4.0 ± 0.53	nd	nd	41
pork meat, chop	29.9	7.1	nd	31 ± 4.6	nd	nd	1.2 ± 0.35	nd	nd	nd	32
chicken meat, leg and thigh	30.5	11.2	nd	600 ± 82	nd	nd	nd	nd	nd	nd	600
dairy products											
soured whole milk		2.5	2.17 ± 0.050	5.7 ± 0.18	2.93 ± 0.019	1.7 ± 0.29	4.1 ± 0.40	20.1 ± 1.27	47 ± 1.6	nd	84
yogurt, plain		2.5	2.10 ± 0.042	3.6 ± 0.28	1.01 ± 0.061	nd	nd	nd	nd	nd	7
cheese, Edam type[c]	63.2	30	19.1 ± 1.31	33 ± 2.5	10.5 ± 0.71	5.6 ± 0.52	12.6 ± 0.82	105 ± 7.5	300 ± 26	8.6 ± 1.27	494
cheese, Emmental type[b,d]	63.4	30	25.8 ± 0.61	52.3 ± 1.00	nd[f]	traces[g]	traces[g]	nd[f]	nd[f]	nd	78
cheese, Emmental type[e]	56.9	24	30 ± 2.9	61 ± 5.1	nd[f]	traces[g]	nd	nd[f]	nd[f]	nd	91

[a] Mean ± SD ($n = 3$). [b] $n = 8$. [c] $n = 4$. [d] Aged over 180 days. [e] Aged over 90 days. [f] Found by fluorescence detection, but HPLC-MS did not confirm identification. [g] Found by HPLC-MS, but under quantification limit of fluorescence detector. nd = Not detected. UK = Unknown whether MK-5 was present. sum = Summarized amount of phylloquinone and MK-4 to MK-10 (ng/g).

the efficiency of the extraction method, the residue of the first extraction was reextracted with hexane (20 mL). The mixture was homogenized for 1 min and centrifuged for 5 min, and evaporation, dissolving, and filtering were performed as in the first extraction.

In the second method chloroform−methanol was used as the extraction solvent. Extraction was done as in our previous paper (Koivu et al., 1997). The internal standard (50−400 ng) and 10 mL of 2-propanol were first added to a homogeneous, weighed (3 g) sample. The tube was immersed in a boiling water bath for 5 min; after cooling, 10 mL of 2-propanol was added and the sample homogenized for 2 min. The mixture was centrifuged at 2000*g* for 5 min; after phase separation the upper layer was collected in a flask. A 20 mL aliquot of chloroform−methanol (1:1) was added to the residue and the mixture homogenized for 2 min and filtered into the flask. The combined extract was filtered and evaporated to dryness using a rotavapor. Ethanol (10 mL) was added and the evaporation repeated. The residue was dissolved in *n*-hexane and handled as described for the extraction with 2-propanol−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 h, after which the sample was filtered, evaporated, and redissolved in the same manner as in the first extraction.

Purification of sample extracts by semipreparative straight-phase HPLC was performed as described by Piironen et al. (1997); only the collection time was modified. The vitamers (cis and trans isomers of phylloquinone, $K_{1(25)}$, and MK-4 to MK-10) were separated with a (Porasil (10 μm, 300 × 3.9 Millipore Corp., Milford, MA) column, in which the mobile phase was n-hexane containing 1% diethyl ether with a flow rate of 1.5 mL/min and injection volume of 500 mL. The collection time was begun 1.5 min prior to elution of the *cis*-phylloquinone and ended 2 min after the elution of MK−4; $K_{1(25)}$, *trans*-phylloquinone, and other menaquinones eluted between these two forms. The collected fraction was evaporated and redissolved in 0.25 mL of the mobile phase used in analytical HPLC.

Preparation of Dairy Product Samples. When searching for methods for routine analysis of phylloquinone and menaquinones from dairy products, development work was focused on extraction and purification steps using cheese (Emmental type, aged over 90 days) as the test material. First two extracting methods were compared: extraction with 2-propanol−hexane and acid hydrolysis method, in which K vitamers were extracted with ethers after HCl digestion. Because of the high fat content of cheese, another purification step was added before purification by semipreparative HPLC. Two methods, enzymatic digestion with lipase and liquid-phase reductive extraction, were tested for this purpose. During the development process, the vitamin K contents, the recovery of $K_{1(25)}$, and repeatability of results were monitored, and the differences between methods were determined using the paired *t* test as in the case of meat products.

Acid hydrolysis method (modification of the AOAC method, 1990) was selected for routine analysis of vitamin K from dairy products. In this method the internal standard (50−400 ng) and 10 mL of H_2O were added to the sample (3 g) in a Mojonnier flask. After adding 10 mL of HCl (37%), the contents of the flasks were mixed and then they were immersed in a boiling water bath. Two digestion times (10 and 20 min) were compared. After cooling, diethyl ether (25 mL) was added and the mixture shaken for 1 min, after which petroleum ether (25 mL) was added and the mixture shaken again. The diethyl ether−petroleum ether phase was separated, and the extraction was repeated twice with 15 mL of the solvents. The combined extract was evaporated using a rotavapor and purified with lipase hydrolysis and further by semipreparative HPLC.

The second extraction method tested for dairy products was extraction with 2-propanol−hexane with a 5 min digestion time. The extraction procedure was the same as in the case of meat products. After extraction the residue was dissolved in hexane and handled as after the acid hydrolysis method.

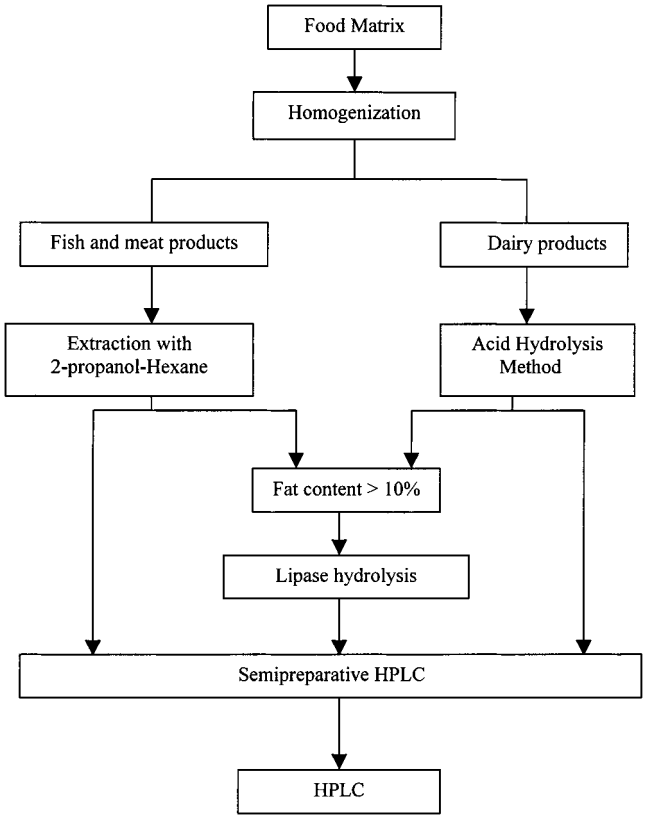

Figure 1. Flowchart for the analytical scheme of vitamin K determination in different food matrices.

In routine determinations of cheese and rainbow trout samples enzymatic removal of fat was used as the first purification step for the extracts. In this method (a modification of the method of Indyk and Woollard (1997)) 2 g of lipase powder (type VII from *Candida cylindracea*, L1754, Sigma) and 40 mL of phosphate buffer (0.8 M, pH 8.0) were added to the dry extraction residue. The tubes were shacked and then incubated at 37 °C for 120 min in a shaking water bath. After cooling to room temperature, 20 mL of ethanol was added and the contents of the tubes were mixed. This was followed by reextraction of K vitamers; 10 mL of hexane was added to the tubes twice and homogenized for 2 min after each addition. After phase separation the hexane layer was transferred to a flask and evaporated using a rotavapor. The residue was redissolved in hexane, filtered, and purified further by semipreparative HPLC as described earlier.

Liquid-phase reductive extraction (Booth et al., 1994) was tested as an alternative purification method. The extraction residue was redissolved in 4 mL of hexane, after which 8 mL of reductive extraction mixture (containing 10 g/L $ZnCl_2$, 30 mL/L acetic acid, and 970 mL/L acetonitrile) and approximately 50 mg of zinc metal (particle size < 45 μm, Merck, Darmstadt, Germany) were added. The tubes were vortexed (5 min) to reduce K vitamers to their acetonitrile−soluble hydroquinones, centrifuged (1000g, 2 min), and the upper layer containing the contaminating lipids was removed. A 4 mL aliquot of H_20 and 8 mL of *n*-hexane were added to the residue to oxidize the hydroquinones to their hexane-soluble quinones before repetition of vortexing and centrifugation; after which the upper *n*-hexane layer containing K vitamers was removed and evaporated, and the residue was handled in the same way as after lipase hydrolysis.

Summary of Sample Preparation. The chosen methods used in routine analysis of animal food items are summarized in Figure 1. The K vitamers were extracted from meat and fish products with 2-propanol−hexane using 5 min digestion time, whereas the acid hydrolysis method with 10 min digestion time was used for the extraction of dairy products. In the case of food items with low fat content (<10%) the sample

Table 2. Extraction of Vitamin K from Bovine Liver with 2-Propanol−Hexane (Method A) and with Chloroform−Methanol (Method B)

method	N	phylloquinone[a] ng/g	MK-4[a] ng/g	MK-8[a] ng/g	MK-9[a] ng/g	recovery of $K_{1(25)}$[a] %
A[b]	11	54 ± 7.7	6.1 ± 1.06	24 ± 4.9	11 ± 6.8	99 + 19.0
B[b]	11	56 ± 5.0	5.8 ± 0.81	21 ± 4.7	9 ± 4.4	80 ± 16

[a] Mean ± SD. [b] Digestion time 5 min.

extracts were purified with semipreparative HPLC. Samples with high fat content, all cheeses and rainbow trout, were first purified with lipase treatment after extraction and further by semipreparative HPLC.

Analytical HPLC. The phylloquinone and menaquinone contents were quantified with reverse-phase HPLC with a fluorescence detector after postcolumn reduction with metallic zinc. The chromatographic apparatus, except detector, was the same as in our previous paper (Piironen et al., 1997); fluorescence was monitored using a Waters 470 fluorescence detector (Waters Associates, Milford, MA) with excitation and emission wavelengths set at 238 and 425 nm.

The analytical column was a Vydac 201 TP54 column (5 μm, 250 × 4.6 mm; The Separation Group, Hesperia, CA). Fluorescent derivatives of K vitamers were produced online using a postcolumn chemical reactor (2.1 × 50 mm) packed with zinc metal (particle size < 45 μm, Merck). The reactor was placed between the chromatography column and the detector. In addition, an oxygen-scrubber consisting of a stainless steel column (4 × 125 mm) packed with 5% platinum-on-alumina (Merck) was connected between the pump and the injector. The mobile phase consists of 83% methanol and 17% ethanol containing 10 mM $ZnCl_2$, 5 mM sodium acetate, and 5 mM acetic acid. Separation of phylloquinone and menaquinones was achieved by isocratic elution starting with a flow rate 0.8 mL/min. After 8 min the flow rate was increased to 1.5 mL/min in 4 min; the 1.5 mL/min flow rate was maintained for 28 min before returning back to the initial conditions. The injection volume was 50 μL. The vitamin K contents of the samples were quantified by the internal standard method based on the peak areas, in which the response factor was determined daily at three concentration levels.

Method Validation. Identification of K vitamers was based on their retention times in the analytical HPLC. The identification and quantification of most of the samples (bovine and pig livers, chicken meat, soured whole milk, and all cheese samples) were confirmed by HPLC-MS analysis, in which the separation of phylloquinone and menaquinones was based on the analytical HPLC procedure used with the fluorescence detector. The postcolumn chemical reactor and oxygen scrubber were omitted from the HPLC system. The mobile phase consisted of only methanol (83%) and ethanol (17%), and the flow rate was 0.8 mL/min. The mass spectrometric detection was carried out with an ion-trap mass spectrometer (Esquire-LC, Bruker Daltonik, Bremen, Germany) using positive ion atmospheric pressure chemical ionization (APCI). Selected ion monitoring for protonated K vitamers was performed by measuring the intensity of mass:charge ratios of 445, 451, 513, 521, 581, 649, 717, 785, and 853 for $[MK\text{-}4 + H]^+$, $[K_1 + H]^+$, $[MK\text{-}5 + H]^+$, $[K_{1(25)} + H]^+$, $[MK\text{-}6 + H]^+$, $[MK\text{-}7 + H]^+$, $[MK\text{-}8 + H]^+$, $[MK\text{-}9 + H]^+$, and $[MK\text{-}10 + H]^+$, respectively. The temperature and flow rate of the drying gas (nitrogen) was set at 5 l/min and 350 °C, the APCI interface temperature being 400 °C. The pressure of the nebulizer gas (nitrogen) was 50 psi. The voltage of the corona discharge needle was +1.8 kV. The beginning of the chromatographic track including MK-4, MK-5, and phylloquinone was monitored with the trap drive value of 41 and with capillary exit and skimmer voltages of 122 and 44 V, while higher mass analytes (MK-6 to MK-10) were detected using the trap drive value of 51 and with capillary exit and skimmer voltages of 110 and 36 V. The scan range used was 150−900 m/z. Quantification was made with the internal standard method.

The linearity ranges of the standard curves for phylloquinone, $K_{1(25)}$, and menaquinones, as well as their detection and quantification limits were determined. The accuracy of the method was monitored by testing the recoveries of phylloquinone, MK-4, MK-8, and MK-9 calculated by the internal standard method for two materials (bovine liver and Emmental type cheese). In addition, the recoveries of phylloquinone, MK-4, MK-8, and MK-9 calculated by the external standard method were compared with that of the internal standard ($K_{1(25)}$). The daily variations in detector response and the retention times of the analytical HPLC were monitored with standard injections after every third sample. In the preparative HPLC standards (mixture containing K_1, $K_{1(25)}$, MK-4, MK-8, and MK-9) were injected to confirm the retention times for the collection. Reproducibility of the method was tested by analyzing triplicate samples.

RESULTS AND DISCUSSION

Analytical Method. We have previously described an HPLC method for the determination of phylloquinone in oils and margarines, vegetables, and cereal products (Koivu et al., 1997; 1998; Piironen et al., 1997). In the present study the method was modified for determining the presence of phylloquinone and menaquinones in animal products. Only a few studies, in which menaquinones in foods have been determined, exist; therefore special attention was focused on the detection and separation of the K vitamers. Our previous studies used electrochemical (EC) detection; in this study the method was changed to fluorescence detection, because efficient separation of different K vitamers was not possible in a reasonable time with an EC detector. With a fluorescence detector both gradient elution and the changing of the flow rate during analysis are possible, so it offers more alternatives for achieving better and more practical separation of different vitamin K forms. In addition, the fluorescence detector was more sensitive. Development work was also focused on extraction and purification of the samples. Two different procedures were selected for routine analysis; one for meat and fish products and another for dairy products (Figure 1).

When the two different extraction methods for meat products were tested, no statistically significant differences were observed in the extraction efficiency between 2-propanol−hexane and chloroform−methanol (Table 2). The analyzed concentrations of phylloquinone as well as of MK-4, MK-8, and MK-9 in bovine liver were similar with both methods. The recovery of $K_{1(25)}$ was, however, significantly better ($p < 0.02$) in 2-propanol−hexane extraction. On the other hand, the ratios of both phylloquinone and menaquinones to $K_{1(25)}$ were similar in the first and second extractions in both methods, indicating that the extractability of endogenous K vitamers was similar to that of the added standard. In addition, extraction with 2-propanol−hexane was shown to be reproducible and easy to perform. When considering all the reasons mentioned above, extraction with 2-propanol−hexane was chosen for routine vitamin K analysis of meat and fish products.

Both tested extraction methods, the acid hydrolysis method and extraction with 2-propanol−hexane, resulted in quite similar phylloquinone, MK-4, and MK-8

Table 3. Extraction of Vitamin K from Cheese (Emmental Type) with 2-Propanol−Hexane (A) and by Acid Hydrolysis Method with 20 (B) and 10 min (C) Digestion

method	N	phylloquinone[a] ng/g	MK-4[a] ng/g	MK-8[a] ng/g	MK-9[a] ng/g	recovery of $K_{1(25)}$[a] %
A	6	28.9 ± 0.83	59 ± 3.9	2.8 ± 0.52	5 ± 2.1	76 ± 15.5
B	6	28 ± 2.4	57 ± 7.3	2.9 ± 1.21	8.1 ± 1.33	77 ± 13.4
C	8	25.8 ± 0.61	52.3 ± 1.00	3.2 ± 0.88	12 ± 2.9	86 ± 6.5

[a] Mean ± SD. Sample extracts were purified with lipase hydrolysis and semipreparative HPLC.

contents in cheese (Emmental type) and the recovery of $K_{1(25)}$ (Table 3). However, with the acid hydrolysis method the MK-9 content was significantly higher ($p < 0.02$). It was shown that a shortened digestion time (from 20 to 10 min) in the acid hydrolysis method significantly increased the MK-9 content of cheese ($p < 0.01$) and also slightly increased the MK-8 content. Furthermore, the recovery of $K_{1(25)}$ was better after a shorter digestion time, which indicated that the long digestion in HCl may destroy vitamin K. In addition, the most repeatable results were obtained after this method. Therefore in routine analysis of dairy products extraction was done with ethers after 10 min digestion in HCl.

Nonpolar components such as triacylglycerols had to be removed from the sample extracts, because the residues have to be dissolved in a mobile phase consisting of methanol and ethanol. When the fat content of the sample was low (<10%), that was most effectively and easily done with the semipreparative HPLC. In other studies (Hirauchi et al., 1989a; Usui et al., 1989; Udagawa et al., 1993) solid-phase extraction has been used for purification in menaquinone analysis. In preliminary studies both silica and C18 cartridges were tested for purification of bovine liver. Because of marked differences in the polarity of various vitamin K forms, effective removal of nonpolar components with the C18 cartridge was not possible without losses in the recoveries of long-chain menaquinones. Due to the complexity of the sample matrix, the purification capacity of silica cartridges alone was not enough, although also large cartridges (up to 2 g) were tested. Efficient purification of the bovine liver extract was achieved by using silica solid-phase extraction and semipreparative HPLC purification together. Solid-phase extraction was, however, omitted during the development of the analyzing method, because its repeatability was poor and purification by semipreparative HPLC alone was found to be effective enough.

HPLC purification also adequately removed other possible interfering components when the separation capacity of the column was maintained by washing it regularly. The *cis* and *trans* isomers of phylloquinone and $K_{1(25)}$ were eluted from the column approximately in 5.8, 6.8, and 6.3 min, respectively. Different menaquinones eluted after these forms in reverse order to their polarity; MK-4 eluted last in approximately 8.6 min.

However, it was observed that purification with semipreparative HPLC was not effective enough for cheese and rainbow trout samples because of their high fat content (>10%). Instead of this, satisfactory purification was obtained using semipreparative HPLC after lipase hydrolysis. The suitability of lipase hydrolysis to vitamin K analysis was confirmed by the moderate recovery of $K_{1(25)}$ (77 ± 14.0%, $n = 12$), whereas recovery was poor (36 ± 5.8%, $n = 11$) with the liquid-phase reductive extraction method. For routine determinations of animal products with high fat content lipase hydrolysis followed by semipreparative HPLC was selected for the purification method.

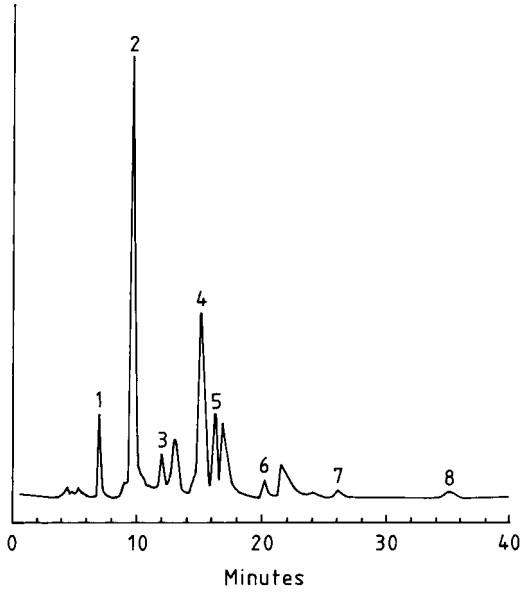

Figure 2. Analytical HPLC chromatogram of bovine liver. Peaks: (1) MK-4; (2) K_1; (3) MK-6; (4) $K_{1(25)}$; (5) MK-7; (6) MK-8; (7) MK-9; (8) MK-10.

In the analytical HPLC method a good separation was achieved using MeOH:EtOH (83:17) containing 10 mM $ZnCl_2$, 5 mM sodium acetate, and 5 mM acetic acid as a mobile phase. The chromatogram of the bovine liver is shown as an example (Figure 2).

Under the conditions described, phylloquinone eluted in approximately 9.6 min, $K_{1(25)}$ in 15.1 min, and menaquinones (MK-4 to MK-10) in 6.9, 8.9, 12.0, 16.2, 20.3, 26.1, and 35.2 min, respectively. Minor day-to-day variations appeared in retention times, but the within-run variation was insignificant.

In previous studies (Haroon et al., 1986, 1987) MeOH:CH_2Cl_2 has been used as a mobile phase when detecting vitamin K with fluorescence detection after reduction with metallic zinc. We, however, observed that the baseline disturbances could be diminished by replacing CH_2Cl_2 with EtOH, which resulted in increased selectivity of fluorescence detection. Earlier Isshiki et al. (1987) had used MeOH:EtOH (60:40) as a mobile phase in vitamin K analysis with EC detection; in our chromatographic system better peak separation was achieved by increasing the proportion of MeOH (from 60 to 83%) in the mobile phase. On the other hand, elution of long-chain menaquinones (>MK-8) became slower and their peaks broader. This effect was partly diminished by increasing the flow rate after 8 min. Despite this change quenching of fluorescence was considerable in the case of long-chain menaquinones. We tested also gradient elution in which the proportion of MeOH in the mobile phase was decreased during analysis, but no good baseline was achieved. In the development of the detection system the sensitivity was further increased

Table 4. Recoveries of Phylloquinone, MK-4, MK-8, MK-9, and the Internal Standard ($K_{1(25)}$) in Bovine Liver and Cheese (Emmental Type)

food item	N	recovery,[a] %				
		phylloquinone	MK-4	MK-8	MK-9	$K_{1(25)}$
bovine liver						
IS	9	92 ± 12.3	92 ± 10.9	74 ± 5.8	70 ± 11.3	
ES	7	85 ± 12.4	71 ± 3.7	56 ± 6.6	53 ± 6.2	70 ± 14.3
cheese						
IS	6	99 ± 13.5	90 ± 19	60 ± 20		
ES	6	90 ± 22	80 ± 18	51 ± 14.6		73 ± 7.0

[a] Mean ± SD. − = Not analyzed. IS = Calculated by the internal standard method. ES = Calculated by the external standard method.

with changing wavelengths of the detector: emission from 244 to 238 nm and extinction from 418 to 425 nm (the originals from the study of Haroon et al. (1986)). In addition, the fluorescence response of the K vitamers was increased by enlarging the zinc column. The reactor has to be refilled once a month to maintain its operation capacity as acceptable.

The detection limits of standard compounds, defined as a signal twice the height of the noise level, were 25 pg for phylloquinone, MK-4, and MK-5; 60 pg for MK-7 and $K_{1(25)}$; 155 pg for MK-8 and MK-9; and 400 pg for MK-10. The quantification limits for phylloquinone and MK-4 were 0.5 ng/g in meat and fish samples and 1 ng/g in dairy products; long-chain menaquinones (>MK-6) could be quantified in levels above 1 ng/g in meat and fish samples and above 2 ng/g in dairy products. The detector response was linear for different vitamin K forms in the tested range of 0.1−15 ng per injection (coefficient of correlation, 0.9991). The variation in the detector response in analytical HPLC was tested daily. The coefficient of within-day variation (CV) for the peak areas of phylloquinone was 2.6%, for the internal standard ($K_{1(25)}$) 2.4%, and for MK-4 and MK-8 4.0 and 3.6%, respectively (n > 3/day, 36 days). The day-to-day CV were 9.0, 13.9, 12.2, and 18.1% (n = 36), respectively.

Recoveries of the added standards in bovine liver and cheese are summarized in Table 4. The good recoveries of phylloquinone and MK-4 calculated by the internal standard method for both tested samples (85−99%) were an indication of the accuracy of the methods for various animal food matrices. The recoveries of MK-8 and MK-9 were lower (53−74%), but acceptable. Similar recoveries of the internal standard ($K_{1(25)}$) and phylloquinone as well as MK-4, MK-8, and MK-9, calculated by the external standard method, indicated the similarity of their behavior during extraction, purification, and analysis. In routine determinations the recovery of $K_{1(25)}$ was usually 65−90%. The low CV (<15%) between triplicate analyses indicated the good reproducibility of the method. For some sample items, a greater CV (15−30%) was acceptable, because the phylloquinone or menaquinone content analyzed was low (<5 ng/g).

We used HPLC-MS to verify the identification of phylloquinone and menaquinones in the selected samples. The selected ion chromatograms of all samples tested confirmed the identification of most of the K vitamers. However, MK-5 in bovine liver and MK-5, MK-8, and MK-9 in Emmental type cheeses found by fluorescence detector were not detected with HPLC-MS. The detected K vitamers were quantified from HPLC-MS in the same levels as they were determined from the fluorescence detector. In the case of Emmental cheeses the presence of MK-6 and MK-7 (only in cheese aged over 90 days) were confirmed with the MS analysis, although their concentrations were below the quantification limit of the fluorescence detector. Because HPLC-MS showed that there maybe some interfering compounds eluting with the menaquinones, the fluorescence data have to be confirmed with another detection system to avoid overestimation of vitamin K contents in animal products.

Vitamin K in Animal Products. The aim of this study was to establish the presence of different menaquinones in animal products. The phylloquinone and menaquinone contents of the analyzed samples are summarized in Table 1; as expected, the summarized amounts of K vitamers in fish, meat, and dairy products (10−100 ng/g) were very low in general. Even the highest vitamin K contents found in this study; 600 ng/g for chicken meat and 494 ng/g for Edam type cheese are only moderate when compared to the phylloquinone contents in green vegetables (>1000 ng/g; Koivu et al. (1997)) or to the menaquinone content in the fermented soybean product, natto (12700 ng/g; Shino (1988)).

Among the fish samples analyzed the highest vitamin K content was found in cultivated fish: rainbow trout, mainly in the form of MK-4 (31 ng/g). This probably originates in animal feed containing menadione, which is converted to MK-4 in tissues (Dialameh et al., 1971; Udagawa et al., 1993), because earlier salmon has been reported to contain only 15 ng/g of MK-4 (Hirauchi et al., 1989b). Lean fishes, Baltic herring and pike-perch, contained only low amounts of phylloquinone, MK-4, and MK-7. No long-chain menaquinones (>MK-8) were found in any fish.

In agreement with the study of Hirauchi et al. (1989a), we found long-chain menaquinones among meat samples only in the liver. The sum of different forms was 2-fold in bovine liver compared to pig liver. Hirauchi et al. (1989a) found MK-11 to MK-14 to be the dominant vitamin K forms in bovine liver; in this study, however, these forms were not analyzed at all. As in other studies (Hirauchi et al., 1989a,b; Schurgers et al., 1999) MK-4 was the dominant vitamin K form in different meats (chicken, beef, pork); its concentration was considerably high only in chicken (600 ng/g). This finding supports the assumption of Will et al. (1992) about the conversion of phylloquinone to MK-4 in chicks, although MK-4 can also originate in menadione in animal feed.

Among dairy products various menaquinones were found in different cheeses and soured whole milk. This is in agreement with earlier studies (Hirauchi et al., 1989b; Shearer et al., 1996; Schurgers et al., 1999) in which cheese is observed to be one of the few sources of menaquinones. In this study the cheese type was observed to affect greatly both the distribution and levels of K vitamers; the total amount was higher in Edam type cheese than in Emmental type cheeses due to its higher MK-8 and MK-9 concentrations. Both

Emmental type cheeses contained significant amounts of only MK-4 and phylloquinone regardless of aging time. As in previous studies (Hirauchi et al., 1989b; Schurgers et al., 1999) yogurt contained only very low amounts of phylloquinone and MK-4.

Vitamin K contents reported here for fish, meat, and dairy products are in general in accordance with the previous data (Hirauchi et al., 1989a,b; Shearer et al., 1996; Schurgers et al., 1999). Exact comparison of the results was not possible because sample information was often insufficient; especially in the case of cheese, differences between cheese types seem to be significant. On the other hand, in the case of vegetables a significant variation in phylloquinone content is observed (Ferland and Sadowski, 1992; Koivu et al., 1997); thus some natural variation also in vitamin K contents of animal products is expectable. Moreover, validation of the previously used extraction methods is not always documented. In addition, our study showed that HPLC with fluorescence detector alone is not enough for unambiguous detection of K vitamers. In conclusion, both phylloquinone and menaquinone contents of animal products are low; thus they probably play at most only a moderate role in vitamin K nutrition.

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