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Application of a simplified HPLC assay for the determination of phylloquinone (vitamin K_1) in animal and plant food items

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The lack of precise data in food composition tables demands intensive investigation of phylloquinone content in human food, for the purpose of nutritional intake assessment and support of anticoagulant drug therapy. The determination of small phylloquinone amounts requires sensitive and selective detection methods. Based on a RP-HPLC assay using post-column derivatization and fluorescence detection, and combined with a liquid–liquid sample clean-up, a rapid and routinely usable assay is presented. The application to foods is shown in the items of milk (0.36 ± 0.07 μg/100 g), other dairy products (yoghurt, 0.34 ± 0.04 μg/100 g), eggs (1.85 ± 0.99 μg/100 g), edible oils (from 0.97 to 112 μg/100 g), oatmeal (4.07 ± 0.35 μg/100 g), broccoli (195 ± 40 μg/100 g), cauliflower (12.0 ± 6.1 μg/100 g), carrots (5.94 ± 3.39 μg/100 g) and potatoes (1.62 ± 1.21 μg/100 g). The samples cover a range of foods, from those of fairly low to those of high phylloquinone content. High precision and wide application range both offer valuable instruments for food analysis and construction of current food composition and nutrition tables in the future. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

The role vitamin K plays as a cofactor in the post-translational synthesis of γ-carboxyglutamic acid in specific proteins in the liver is generally accepted. Most intensively investigated is the vitamin's importance for blood coagulation processes (Suttie, 1985, 1992). However, the occurrence of extrahepatic tissues containing vitamin K-dependent proteins has been observed (Olson & Suttie, 1977; Gallop *et al.*, 1980; Vermeer & Knapen, 1993).

For the further understanding of vitamin K's nutritional role, information about the concentration in foods is recommended to fill out current food composition tables (Booth *et al.*, 1993). These tables can serve as a basis for vitamin K-intake investigations.

Data concerning vitamin K content in recent tables (Olson, 1994) is based on indirect determination by curative bioassays done earlier (Matschiner & Doisy, 1966*a*,*b*) and then recalculated in an uncertain way. This situation has been overcome with the development of high-performance liquid chromatography (HPLC) as a physico-chemical method, which was first mainly used for the determination of phylloquinone in breast milk, cow's milk and infant formulas (Barnett *et al.*, 1980; Haroon *et al.*, 1982; Ball, 1988). The phylloquinone and menaquinones can now be measured directly, using spectrophotometric (UV), electrochemical or fluorescence detection, but the latter needs previous reduction of the quinone to its hydroquinone form, and in that case, reduction was carried out by wet-chemical post-column dosage of the reduction reagent (Sakano *et al.*, 1986), wet-chemical 'in-line', by post-column heating of the reduction reagent containing solvent (Lambert & De Leenheer, 1987; Lambert *et al.*, 1992), electrochemical (Langenberg *et al.*, 1986), photochemical (Indyk, 1988; Poulsen & Birks, 1989) or solid-phase procedures, either using platinum (Shino, 1988; Usui *et al.*, 1989) or zinc (Haroon *et al.*, 1987; Booth *et al.*, 1994). The reduction offers high sensitivity and selectivity, even with only small amounts present in the sample. Although HPLC facilitates the possibility of an easy-going analytical step, the whole analytical procedure remained cumbersome. Therefore, in the past, the main focus was on the improvement of sample clean-up. Nevertheless, assays using enzymatic hydrolysis (Barnett *et al.*, 1980; Zonta & Stancher, 1985), column chromatography (Haroon *et al.*, 1982; Hwang, 1985), thin-layer chromatography (Sakano *et al.*, 1986; Hiraishi, 1988), solid-phase extraction (Haroon & Hauschka, 1983; Booth *et al.*,

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1994), normal-phase HPLC (Shearer, 1986; Canfield *et al.*, 1991) or combinations of them (Haroon *et al.*, 1982; Shearer, 1986; Hirauchi *et al.*, 1989) were too laborious and time-consuming for routine purposes.

The method presented here offers an easier and more rapid sample preparation, without any semi-preparative chromatographic steps. A liquid–liquid extraction, as employed by Cham *et al.* (1989), was used as the purification step. Depending on the food matrix, slight adaptations of the extraction solvent were made. The determination of phylloquinone follows a fluorescence detection method combined with post-column reduction, utilizing zinc metal as reported by Haroon *et al.* (1987) and evaluated for food analysis by Booth *et al.* (1994).

MATERIALS AND METHODS

Reagents

Methanol, 2-propanol, ethanol, *n*-hexane, dichloromethane, all of HPLC quality (Riedel-de Haën, Seelze, Germany); vitamin K_1 (phylloquinone), biochemical reagent (Merck, Darmstadt, Germany); 2′,3′-dihydrophylloquinone (contributed by Dr P. Hofmann, Hoffmann-LaRoche, Basle, Switzerland); zinc powder, analytical grade, particle size < 60 μm (Merck, Darmstadt, Germany); zinc chloride, sodium acetate (anhydrous) and acetic acid (99.8%), all analytical grade (Riedel-de Haën, Seelze, Germany); bidistilled water.

Apparatus other than HPLC

Laboratory blender (HO 3, Bühler, Tübingen, Germany); laboratory grinder (Janke and Kunkel, Staufen, Germany); screw-capped tubes of borosilicate glass (16×100 mm, Pyrex), of which the caps contained Teflon inlets; rotary evaporator (Heidolph, Kelheim, Germany); luminescence spectrometer LS50 (Perkin-Elmer, Überlingen, Germany).

Sampling

Because of phylloquinone's sensitivity to light irradiation, all procedures were carried out under subdued daylight. All food items, such as milk and other dairy products, edible oils, oatmeal, carrots, potatoes, cauliflower and broccoli, were purchased randomly from the local retail market. The milk and yoghurt samples (3.6% fat) were from different lots, the eggs were from different shops. The non-edible portions were removed from the eggs and the vegetables. Eggs were homogenized directly and 0.1 g was taken for extraction. Carrots and potatoes were homogenized with added water, deep-frozen and thawed to break down the cells. Two grammes of the suspension was extracted. Broccoli and cauliflower were homogenized without water after the heads were divided and opposite eighths homogenized together. Two grammes of the chopped material was extracted. Oatmeal was crushed and 0.1 g was extracted. Milk and other dairy products, in portions of 0.1 g, were extracted directly. The edible oils required no special preparation, so they could be dissolved in *n*-hexane directly, in appropriate amounts (approximately 25–150 mg in 50 ml). Aliquots of the solutions were taken for the following clean-up step. The materials and their corresponding extraction solvents are shown in detail in Table 1.

Extraction and clean-up

For all samples, appropriate amounts of the internal standard (2′,3′-dihydrophylloquinone dissolved in ethanol) were added prior to extraction (Table 1). The samples were extracted in glass-tubes. After mixing vigorously for at least 2 min, the samples were centrifuged for 5 min at 3000 rev/min. When the extraction

Table 1. Food samples and corresponding extraction solvents and added amounts of internal standard

Sample	Approximate sample amount (g)	Solvent[a]	Volume (ml)	Internal standard (ng added)
Egg	0.1[b]	B	7	1
Whole milk (3.6% fat)	0.1[b]	B	7	1
Yoghurt (3.6% fat)	0.1[b]	B	7	1
Cheese (Emmentaler)	0.1	A	8	5
Oatmeal	0.1	A	8	5
Carrot	2.0[c]	B	6	1
Potato	2.0[c]	B	6	1
Broccoli	2.0	A	200[d]	5[e]
Cauliflower	2.0	A	200	5[e]
Edible oils	⩽0.15	C	50	1[f]

[a]A, dichloromethane–methanol (2:1, v/v); B, 2-propanol–*n*-hexane (3:1, v/v); C, *n*-hexane.
[b]1 ml distilled water added for better separation of phases.
[c]Aqueous suspension (sample/water ratio approx. 1/3).
[d]Required further dilution.
[e]Added to 1 ml extract of solvent A.
[f]Added to 4 ml hexane solution.

solvent contained hexane, the upper hexane layer was taken for the following clean-up step. In the case of the solvent other than hexane, an aliquot was evaporated, the residue redissolved in hexane and then followed by purification, as described below. Equal volumes of a mixture of methanol–water (9:1, v/v) were added to the hexane extracts, mixed vigorously for 2 min and centrifuged for 5 min at 3000 rev/min. The upper hexane layer was removed and evaporated to dryness at 40°C and simultaneously under vacuum for 15 min. After dissolving the residue in 150 μl of the eluent, it was injected into the HPLC.

HPLC

Apparatus

High Precision Pump Model 300B; Gynkotek ODS Hypersil, 250 mm×4.6 mm i.d., 5 μm; guard-column, Gynkotek ODS Hypersil, 20 mm×4.6 mm i.d., 5 μm (all from Gynkotek, Germering, Germany); reduction-column, 20×4.0 mm i.d. (Bischoff, Leonberg, Germany), dry filled with zinc powder; injection device, Rheodyne (Cotati, USA); column-heating, cosytherm (Labortechnik Barkey, Bielefeld, Germany) and U3 (Julabo, Seelbach, Germany); Fluorescence Spectrophotometer F-1050; Chromato-Integrator D-2500 (both from Merck, Darmstadt, Germany).

Conditions

The mobile phase contained 100 ml of dichloromethane and 900 ml methanol. This solvent was then combined with 5 ml of a methanolic solution, containing 1.37 g of zinc chloride, 0.41 g of sodium acetate and 0.30 g of acetic acid. The flow rate was 1.0 ml/min, and the three columns were heated at 40°C. The injection loop volume was 100 μl. Detection was carried out with an excitation wavelength of 243 nm and an emission wavelength of 430 nm (confirmed by scanning a solution of the 1,4-dihydrophylloquinone). The concentrations were calculated by peak height ratios, using a linear regression curve from standard solutions containing 0.13, 0.5, 2.0 and 10 ng phylloquinone per injection (100 μl) and each containing 1.0 ng 2′,3′-dihydrophylloquinone as an internal standard. If necessary, dilution steps were taken into account. Figure 1 summarizes the complete analytical procedure.

Test of method performance

To obtain the precision for this method, the same food items were analysed in replicate. For broccoli and cauliflower, aliquots from the first extraction were tested several times. For recovery experiments, known amounts of phylloquinone were added to the sample prior to extraction. The recovery was obtained by substraction of the phylloquinone concentration in the non-treated sample. Peak purity for all items was tested and confirmed by using several excitation wavelengths (243, 272 and 330 nm) and comparing the peak ratios to those obtained from standard solutions. Every food item was tested without the addition of the internal standard to confirm the absence of 2′,3′-dihydrophylloquinone in these materials, because Booth *et al.* (1994) reported the occurrence of the internal standard in bread samples. None of the investigated foods showed any signal interfering with the internal standard.

RESULTS AND DISCUSSION

Phylloquinone contents in foods

Characteristic chromatograms for some food items are shown in Fig. 2. The phylloquinone peak and that of the internal standard are well separated from matrix signals. Table 2 gives an overview of the phylloquinone content obtained from 13 food samples. It can be seen that the presented method works as well with samples of low phylloquinone content, such as eggs, milk and other dairy products, carrots, potatoes and several edible oils, as it does with food items that are rich in phylloquinone, such as broccoli or pumpkin seed oil. Consequently, a wide range of food matrices is represented. The results are in good accordance with those reported by Booth *et al.* (1994), who found 0.30 μg/100 g phylloquinone for milk, or those of Ferland & Sadowski

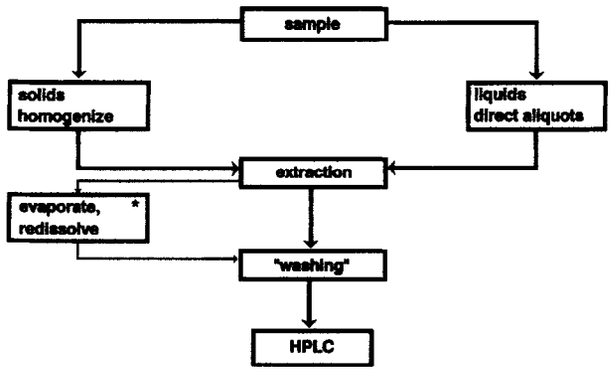

Fig. 1. Analytical scheme for the phylloquinone assay. *Only for solid samples where hexane was not the first extraction solvent.

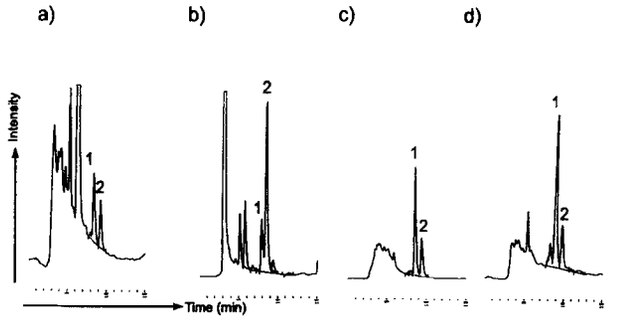

Fig. 2. Chromatograms of some food samples (phylloquinone concentrations are given in parentheses). (a) Egg (1.30 μg/100 g); (b) carrot (3.28 μg/100 g); (c) cauliflower (14.8 μg/100 g); and (d) pumpkin seed oil (113 μg/100 g). (1) Phylloquinone and (2) 2′,3′-dihydrophylloquinone (internal standard).

Table 2. Phylloquinone content of several foods and precision and recovery data

Food sample	Items[a] $n=$	Phylloquinone content (μg/100 g)			Within-run CV (%)	Items $n=$	Recovery (%)
		Mean ± SD	Min.	Max.			
Egg	11	1.85 ± 0.99	0.48	3.51	4.8	10	101
Whole milk	7	0.36 ± 0.07	0.30	0.50	4.4	10	99
Yoghurt	4	0.34 ± 0.04	0.20	0.36	9.8	8	98
Cheese (Emmentaler)	5	2.39 ± 0.08	2.29	2.47	8.3	9	n.d.
Oatmeal	4	4.07 ± 0.35	3.72	4.53	4.9	5	n.d.
Carrot	6	5.94 ± 3.39	3.12	11.8	4.7	10	98
Potato	5	1.62 ± 1.21	0.49	3.41	6.5	10	101
Broccoli	7	195 ± 40	137	247	7.6[b]	8	97
Cauliflower	4	12.0 ± 6.1	6.4	19.3	3.0[b]	9	n.d.
Sunflower oil	1	0.97	—	—	8.4	5	n.d.
Corn oil	1	1.63	—	—	5.2	5	n.d.
Olive oil	1	16.5	—	—	7.9	5	95
Pumpkin seed oil	1	112	—	—	1.7	5	n.d.

[a]Between two and five analyses per item.
[b]Except the extraction step.
n.d., not determined.

(1992*b*) or Langenberg *et al.* (1986), reporting 178 μg/100 g or 205 μg/100 g phylloquinone for broccoli. For edible oils, we see the same tendency as do Ferland & Sadowski (1992*a*), who showed that corn and sunflower oil are poor sources (2.91 μg/100 g, 9.03 μg/100 g) and olive oil is a medium source (55.5 μg/100 g) of phylloquinone. The fact that they are not really in the same range as our results could be caused by storage degradation of the phylloquinone from being on the shelf (Ferland & Sadowski, 1992*a*). Except for the pumpkin seed oil, all vegetable oils were presented in clear glass bottles, which did not protect from light exposure. Pumpkin seed oil, a speciality of Styria in Austria, contains a high phylloquinone concentration of 113 μg/100 g.

Method performance

The investigations in linearity, precision, and recovery led to satisfying results, as summarized in Table 2. It should be emphasized that the coefficient of variation did not exceed 10% for any matrix and there were no remarkable losses over the whole analytical process.

CONCLUSION

After a long period of laborious and time-consuming methods for the determination of vitamin K_1, the demand for assays of more simplicity and rapidity has become indispensable. HPLC assays have facilitated the analyst's work, but sample preparation has remained cumbersome. In our work, we demonstrate that the determination of phylloquinone will no longer be a question for specialized laboratories, but will offer the feasability of phylloquinone analyses to laboratories provided with widely available equipment and inexpensive chemicals.

In the future the offered data concerning the phylloquinone content of foods will be increasingly trustworthy. This will enable scientists to draw up current food composition and nutrition tables for the purpose of more intense nutrient intake studies.

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