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HPLC analysis for *trans*-vitamin K_1 and dihydro-vitamin K_1 in margarines and margarine-like products using the C_{30} stationary phase

K.K. Cook, G.V. Mitchell, E. Grundel, J.I. Rader*

Office of Food Labeling, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 200 C St., S.W., Washington, DC 20204, USA

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Abstract

A C_{30} column successfully separated the *cis* and *trans* isomers of vitamin K_1 in margarines, reduced-fat margarine-like products and in their ingredient oils. We also measured the compound 2′3′-dihydro-vitamin K_1, a derivative formed during hydrogenation of oils containing vitamin K_1. We compared an enzymatic procedure, currently under AOAC collaborative study for milk and infant formulas, with a more direct extraction method in analyzing margarines and margarine-like products. Both methods have good precision and were applicable to the majority of products examined. Margarines or margarine-like products identifying liquid soybean oil, hydrogenated soybean oil or liquid canola oil as their primary ingredients contained about 50–160 μg vitamin K_1/100 g. Blends of sunflower and soybean oils contained < 50 μg vitamin K_1/100 g. Hardened or "stick" margarines contained more 2′3′-dihydro-vitamin K_1 than "soft" or "tub" products (122–285 μg/100 g vs 38–131 μg/100 g, respectively). Eight of 18 products (44%) contained 10% or more of the Reference Daily Intake for vitamin K_1 per serving. Higher-fat margarines contained more vitamin K_1 than their lower-fat counterparts.

Keywords: *trans*-Vitamin K_1; Dihydro-vitamin K_1; Margarines; Margarine-like products; C30 stationary phase

1. Introduction

1.1. Changes in US food labeling regulations

In 1995, the US Food and Drug Administration (FDA) (FDA, 1995) amended its regulations to establish Reference Daily Intakes (RDIs) for a number of nutrients, including vitamin K, for which label reference values were not included in federal regulations [Code of Federal Regulations (CFR), 1992 (101.9 (c) (7) (iv)]. Such reference values are necessary to permit the declaration of these nutrients in the nutritional labeling of foods and to permit nutrient content claims to be made for them. The agency also amended its regulations to make consideration of vitamin K mandatory in making a determination of whether a food is nutritionally inferior to a food for which it substitutes and that it resembles (FDA, 1995). These actions were intended to assist consumers in understanding the nutritional significance of foods in the context of a total daily diet.

1.2. Need for reliable data

Phylloquinone (vitamin K_1) is the primary dietary source of vitamin K (Booth & Suttie, 1998). Green leafy vegetables are generally considered to be the best dietary sources of vitamin K_1. The natural biologically active form of vitamin K_1 is the *trans* isomer while the inactive *cis* isomer is found predominantly in synthetic vitamin K_1, and may also arise as a result of exposure to light (Hwang, 1985; Indyk, 1988).

There is increasing need for more reliable data on the vitamin K content of foods, in part because of recent changes in US food labeling regulations and because changes in food formulations have led to the development of new lower fat products that may differ in vitamin K content from their full-fat counterparts.

To address the need for reliable data, a large collaborative study for the determination of vitamin K_1 in infant formula, unfortified liquid milk and milk powders,

* Corresponding author. Tel.: +1-202-205-5375; fax: +1-202-205-4594.

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using the enzymatic method of Indyk and Woollard (1997), is currently in progress and will be published in *JAOAC International*. This method uses reverse phase chromatography with fluorescence detection after hexane extraction of a lipase-digested sample.

The ability to separate *cis* and *trans* isomers of vitamin K_1 and to quantitate the *trans* isomer accurately is very important because of the dissimilar biological activities of these isomers. To date, the resolution of the *cis* and *trans* isomers of vitamin K_1 has only been achieved with normal phase HPLC (Dahl, 1987; Hwang, 1985). The polymeric C_{30} stationary phase was developed at the National Institutes of Standards and Technology (NIST) to resolve geometric isomers of carotenoids (Emenhiser, Sander, & Schwartz, 1995; Sander, Sharpless, Craft, & Wise, 1994). To date, its use in separation of isomers of vitamin K has not been reported.

1.3. Margarines and margarine-like products

Some edible oils also contain significant amounts of vitamin K_1 (e.g. soybean oil, canola oil) (Booth & Suttie, 1998). Such oils are major ingredients of many margarines, and their consumption contributes significantly to dietary vitamin K intake.

Prior to NLEA-related changes (Nutrition Labeling and Education Act, 1990) margarines were defined as products containing not less than 80% fat (Code of Federal Regulations, 1995). More recently, manufacturers provide margarine-like products with ''low-fat'', ''reduced-fat'' and other lower-fat claims to assist consumers in maintaining healthy dietary practices that include reductions in fat intake (FDA, 1993). There is little information available on the vitamin K content of these newer fat-modified margarine-like products. Additionally, many margarines and margarine-like products include a hydrogenated oil among their ingredients. Dihydro-vitamin K_1 is formed from vitamin K_1 during the hydrogenation process (Davidson, Booth, Dolnikowske, & Sadowski, 1996). This derivative has been detected in plasma following dietary intake of a hydrogenated vitamin K_1-rich vegetable oil (Booth, Davidson, Lichtenstein, & Sadowski, 1996a) and in infant formula (Indyk & Woollard, 1997). Its bioavailability is not known.

1.4. Purpose

The purpose of the present study was five-fold: (1) to determine whether the polymeric C_{30} column could resolve *cis* and *trans* isomers of vitamin K_1; (2) to extend the enzymatic method for vitamin K analysis to the analysis of margarine products; (3) to test a simpler method that utilizes only direct hexane extraction followed by filtration to extract vitamin K from margarines; (4) to measure dihydro-vitamin K_1 in margarines and margarine-like products, and (5) to extend the vitamin K food data base to the newer modified-fat margarine-like products.

2. Materials and methods

2.1. Margarines and oils

Eighteen margarines and one butter (in 453 g units) and four vegetable oils (in 473–946 g units) were purchased in local retail markets. The initial lots were purchased in October 1997, and the second lots were purchased in April 1998. The oils were those identified as major ingredients in the margarine products, i.e. liquid and partially hydrogenated soybean, corn, canola, and sunflower oil. The samples were stored refrigerated at 2–10°C and analyzed before their expiration dates. Table 1 lists the fat contents and major oil ingredients of the products analyzed.

2.2. HPLC system

The HPLC system consisted of a Perkin–Elmer LS-1 fluorescence detector (Perkin–Elmer Corporation, Norwalk, CT) combined with a Waters UK-6 injector and pumps controlled by Waters Maxima software (Waters Chromatography, Milford, MA, USA). The system

Table 1
Composition of margarines and margarine-like products

Product[a]	Fat (%)[b]	Form	Ingredients[c]
LS2	79	Stick	Liq and ph soybn
LS6	79	Tub	Liq soybn and ph soybn
LS1	79	Stick	Liq soybn, ph soybn, butter
CN2	79	Tub	Liq can, ph can
HS4	71	Tub	ph soybn, butrmlk
HS3	71	Stick	Veg oil blend (ph and liq soybn), butrmlk
HS6	71	Stick	Veg oil blend (ph soybn and liq soybn)
LS5	71	Tub	Liq soybn, liq can, butrmlk, h soybn, ph soybn
CR1	71	Stick	Liq crn, ph crn
SF1	71	Stick	Liq snflwr, h soybn, liq can, ph soybn
LS4	64	Squeeze	Liq soybn, ph cotneseed
HS5	64	Stick	Veg oil blend (ph and liq soybn)
HS1	57	Stick	ph and liq soybn
HS2	53	Stick	ph and liq soybn
LS3	50	Stick	Liq soybn and ph soybn
SF2	43	Tub	Liq snflwr, ph soybn, liq soybn, h soybn, liq can
CN1	14	Tub	Liq can, ph soy oil
CR2	0	Squeeze	Liq crn

[a] Oils are listed in their order of appearance in the ingredient lists of the products.

[b] Values for total fat (%) were calculated from label declarations of fat content per serving (g/srv).

[c] Abbreviations: Liq, liquid; soybn, soybean; h, hydrogenated; ph, partially hydrogenated; butrmlk, buttermilk; cotnseed, cottonseed; veg, vegetable; can, canola; crn, corn; snflwr, sunflower.

measures the fluorescence (λ_{ex} 243 nm, λ_{em} 430 nm) of vitamin K_1, menaquinones and dihydro-vitamin K_1 following post-column reduction with zinc to their corresponding hydroquinones (Haroon, Bacon, & Sadowske, 1987).

A Waters C_{18} insert guard column and a YMC C_{30} column (Wilmington, NC); 25×0.46 cm; 3 micron) followed by a Waters stainless steel guard column (20×4 mm id) which was open-packed with zinc powder was used. Periodic repacking of the zinc column was necessary for efficient reduction of the vitamin K compounds and removal of oxygen.

2.3. Standards and reagents

Vitamin K_1 standard (2-methyl-3-phytyl-1,4-naphthoquinone: 3-phytylmenadione) and vitamin K_2 (menatetrenone, MK4) were purchased from Sigma Chemical Company (St. Louis, MO, USA). 2′,3′-Dihydro-vitamin K_1 was generously provided by Hoffmann-La Roche (Basel, Switzerland).

Chemicals (e.g. hexane, methanol, ethanol, dichloromethane, acetonitrile, tetrahydrofuran, monobasic potassium phosphate, potassium carbonate, zinc chloride, anhydrous sodium acetate, and glacial acetic acid) were reagent grade or HPLC grade. Lipase (Type vii from *Candida rugosa*) was purchased from Sigma Chemical Company (St. Louis, MO). Zinc powder (200 mesh) was purchased from Alfa Aesar (Ward Hill, MA). The phosphate buffer (monobasic potassium phosphate) was adjusted to pH 8.0 with NaOH.

2.4. Preparation of standards

A stock solution of vitamin K_1 was prepared as follows: Approximately 150 mg of vitamin K_1 standard was weighed, dissolved in < 1 ml of hexane and diluted to 10 ml with methanol. These solvents were completely miscible in the proportions used. After thorough mixing, 100 μl was diluted to 25 ml with hexane and the vitamin K_1 concentration was determined spectrophotometrically at 248 nm ($E^{1\%}_{1cm}$ = 419, Merck). Two hundred and fifty μl of the stock solution was diluted to 250 ml with methanol to prepare an intermediate solution. Working standards containing from 0.54 to 53.6 ng/ml (*trans* + *cis*) vitamin K_1 were prepared from the intermediate solution.

The *cis* isomer of vitamin K_1 was isolated from a commercial standard using semi-preparative normal phase HPLC chromatography. It was then used to spike the vitamin K_1 standard in order to identify the *cis* isomer by correspondence of retention times.

2.5. Cis *and* trans *isomers*

The amounts of *cis* and *trans* isomers in the vitamin K_1 standard were calculated following column chromatography as follows: *trans* $K_1 = (Ht_{trans}/Ht_{trans} + _{cis}) \times Conc_{std}$, where Ht refers to the heights of the respective peaks.

2.6. Dihydro-vitamin K_1 and vitamin K_2

Concentrations of working standards were 12 to 119 ng/ml for 2′,3′-dihydro-vitamin K_1 and 0.57 to 56.8 ng/ml for vitamin K_2 (MK4). Both the dihydro-vitamin K_1 and vitamin K_2 were characterized spectrophotometrically. The purity of each compound was > 90%. Because of uncertainties with respect to the appropriate extinction coefficient, however, the concentration of dihydro-vitamin K_1 was calculated on a weight basis.

2.7. Preparation of samples

Stick margarines and the butter product were composited as 453 g units (each representing a single lot) by mixing in a food processor. We composited multiple sticks of the products because vitamin K_1 content was found to vary within individual sticks. "Tub" and "squeeze" margarine products and oils were stirred or shaken to ensure homogeneous sampling. Compositing, sampling and analysis were performed under yellow lights to prevent degradation of vitamin K_1. Portions of 0.2 to 0.5 g (5 g for fat-free products) of each homogenized sample were weighed in triplicate for each extraction procedure. Each sample underwent an enzymatic extraction and a direct extraction procedure for vitamin K.

2.8. Enzymatic digestion and extraction

The samples were weighed into 38×200 mm round bottom screw cap centrifuge tubes. Five ml of warm (< 40°C) water was added to each tube and the method of Indyk and Woollard (1997) was followed. Briefly, 5.0 ml of phosphate buffer was added, followed by 1.0 g lipase. The tubes were stoppered and the contents vortexed, then shaken for 7 min. Samples were incubated for 120 min at 37°C in a sonicator bath with shaking at 20 min intervals. Following cooling to room temperature, 10 ml of reagent alcohol [methanol: absolute ethanol (v/v), 5:95] was added, the tubes inverted, and 1 g of potassium carbonate was added. The contents of the sample tubes were mixed. Hexane (30 ml) was added, the tubes were shaken for 7 min, followed by centrifugation at 1000 rev per min (180×*g*) for 10 min. At this stage, extracts were held at < 4°C under nitrogen for 24 h if necessary. For estimation of vitamin K_1 at very low levels, hexane was removed under nitrogen from a 5 ml portion of the extract and the residue was dissolved in 1.0 ml methanol.

2.9. Direct extraction

Samples were weighed into a 50 ml tube and 20 ml of hexane added. Samples were homogenized for 30 s at a

speed of approximately 25,000 rev/min in a Polytron (Brinkmann Instruments, Inc., Westbury, NY) and filtered through an 0.45 micron Autovial glass microfiber filter. A volume of extract containing vitamin K within the range of determination was transferred to a small test tube and the hexane evaporated under nitrogen.

2.10. HPLC determination

We used the mobile phase and conditions described by Indyk and Woollard (1997) (i.e. dichloromethane:methanol, 100 + 900 v/v containing zinc chloride). However, we diluted the buffer to 1 liter with methanol, and then added 5 ml of this buffer to the mobile phase. This resulted in concentrations of 50 micromolar $ZnCl_2$, 25 micromolar anhydrous sodium acetate and 25 micromolar glacial acetic acid. Slight modifications were needed to resolve interfering peaks in canola oil and canola oil-containing products and these were achieved either by using acetonitrile in place of dichloromethane, or methanol at a reduced flow rate (0.8 ml/min).

The residues from each extraction were stored at −80°C until HPLC analysis. Prior to HPLC analysis, the residue from the direct extraction was dissolved in tetrahydrofuran:methanol (1:1)(v/v). The residue resulting from the enzymatic digestion and extraction procedure was completely soluble in methanol.

2.11. Recovery studies

A reduced-fat margarine (Product HS3, Table 1), spiked and unspiked, was assayed in triplicate following extraction by both methods. The spike consisted of an amount of vitamin K_1 standard (containing *cis* and *trans* isomers) approximating the vitamin K_1 content in the margarine-like product. Recovery of *trans*-vitamin K_1 was calculated as follows: vitamin K1 recovered, %: $(C_s\text{-}C_u)/(C_a)\times 100$ where C_s = concentration of *trans*-vitamin K_1 measured in the spiked test portion; C_u = concentration of *trans*-vitamin K_1 measured in the unspiked test portion (average of triplicate determinations); and C_a = amount of *trans*-vitamin K_1 added per gram of the spiked test portion.

2.12. Analysis of NIST reference material 1846

NIST Standard Reference Material (SRM) 1846 (NIST, 1996) is a spray-dried milk-based infant formula powder that is intended primarily for use in validating methods for determining proximates, minerals and certain vitamins in infant formula and similar matrices (Sharpless et al., 1997). Replicate analyses were performed from each of three packets of SRM 1846 with both the enzymatic digestion and direct extraction procedures. The information value for vitamin K_1 in this reference material is 1 mg/kg (NIST, 1996).

2.13. Statistical analysis

The significance of differences between values obtained by the direct extraction method and the enzymatic method was determined by two-tailed Student *t*-tests (Datamost, Salt Lake City, UT). Differences with $p \leqslant 0.05$ are considered statistically significant.

3. Results and discussion

3.1. Vitamin K isomer separation

To date, the geometric isomers *cis* and *trans*-vitamin K_1 have been separated only by methods that used normal phase chromatography with the less-sensitive, less-specific UV detection. We are unaware of any reverse phase HPLC methods that separate these isomers.

We report here that the C_{30} column separates *cis* and *trans* isomers of vitamin K (Figs. 1 and 2). Not only was confirmation based on identical retention times of standards for *cis* and *trans* vitamin K_1 and dihydro-vitamin K_1, but we also observed the loss of peak response from chromatograms when the zinc column was removed. This column has been used previously to significantly improve the separation of carotenoid isomers (Emenhiser et al., 1995) and tocopherol isomers (Strohschein, Pursch, Lubda, & Albert, 1998).

3.2. Total vitamin K_1 in NIST SRM 1846

Results of determination of *cis* and *trans* isomers and total vitamin K_1 following enzymatic digestion of SRM 1846 are shown in Table 2. Total vitamin K_1 (mean ± SD, 10 determinations) was found to be 948 ng/g (850 ± 60 ng/g *trans*-vitamin K_1 + 98 ± 20 *cis*-vitamin K_1 = 948 ng/g). This result is in excellent agreement with the informational mass fraction value of 1 mg vitamin K_1/kg (1000 ng/g) for this Standard Reference Material.

The results of analysis of SRM 1846, a vitamin K_1-fortified product and the only reference material currently available with an information value for total vitamin K_1 content, demonstrate the accuracy of the enzymatic method as applied to infant formula. The data also confirms complete resolution of *cis* and *trans* vitamin K_1 isomers present in fortified infant formula and represents the first demonstration that the C_{30} column can unambiguously estimate the bioactive *trans* isomer of this vitamin.

We also used the direct extraction procedure to analyze vitamin K in SRM 1846. Results were significantly lower than those obtained with the enzymatic digestion and extraction procedure (data not shown). The lower recovery using the direct extraction may have been due to the inability of the hexane to completely dissolve the

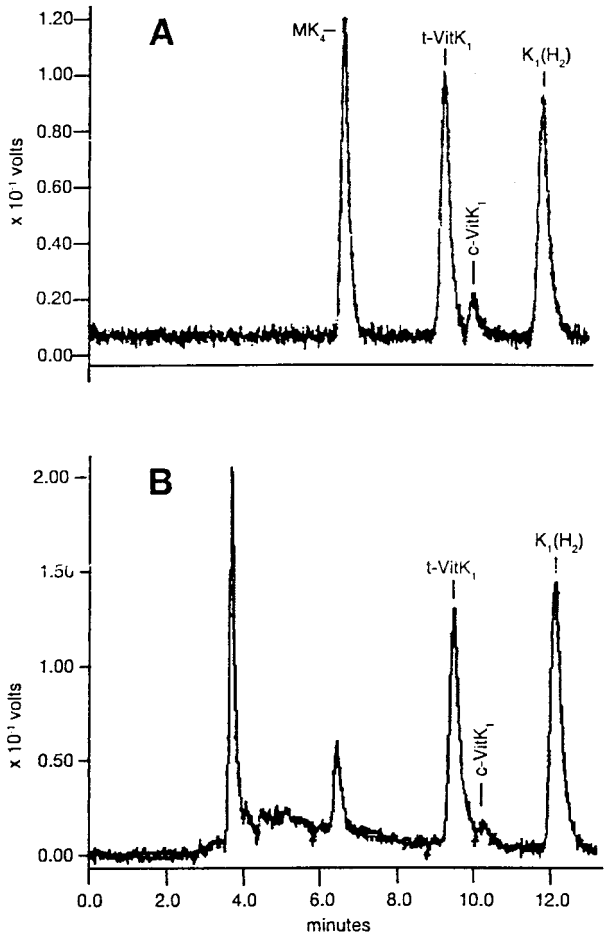

Fig. 1. Separation of vitamin K compounds by reverse-phase HPLC on a YMC C_{30} column. Panel A shows the separation of a mixture of pure standards of vitamin K_2 (MK_4), *cis*- and *trans*-vitamin K_1, and dihydro-vitamin K_1 [$K_1(H_2)$]. Panel B shows the separation of *cis*- and *trans*-vitamin K_1 and dihydro-vitamin K_1 extracted from a soybean oil spread (product HS3, Table 1). The mobile phase consisted of a mixture of dichloromethane and methanol (100 ml dichloromethane +900 ml methanol) and 5 ml of a methanolic solution of 10 mM zinc chloride, 5 mM anhydrous sodium acetate and 5 mM glacial acetic acid; flow rate 1.5 ml/min.

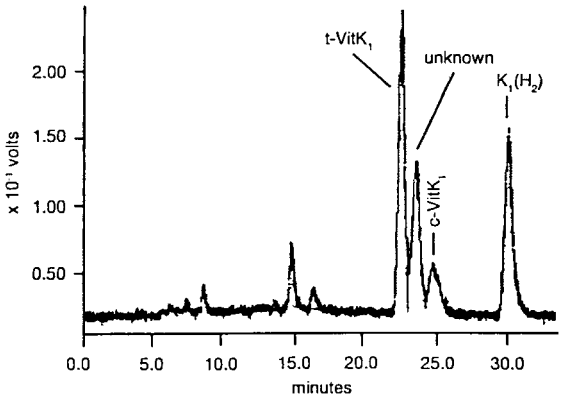

Fig. 2. Separation of vitamin K compounds by reverse-phase HPLC on a YMC C_{30} column. The panel shows the separation of *cis*- and *trans*-vitamin K_1, an unknown peak, and dihydro-vitamin K_1 [$K_1(H_2)$] extracted from a canola oil margarine (product CN2, Table 1). The mobile phase consisted of 0.5% dichloromethane, 99.5% methanol, and 5 ml of a methanolic solution of 10 mM zinc chloride, 5 mM anhydrous sodium acetate and 5 mM glacial acetic acid; flow rate 0.8 ml/min.

Table 2
Measurements of *cis*- and *trans*-vitamin K_1 (ng/g) in NIST SRM 1846 Infant Formula power after enzymatic digestion[a]

	Vitamin k_1		
Experiment	*trans*[b]	(*cis*)[c]	Total[d]
1	928	(75)	1003
	934	(103)	1037
	946	(146)	1092
2	794	(91)	885
	830	(86)	916
	809	(95)	904
3	826	(106)	932
	810	(87)	897
	802	(87)	889
	826	(106)	932
Mean	850 ± 60	(98 ± 20)	948

[a] Each value represents the result of a single determination.
[b] The amount of *trans*-vitamin K_1 in the standard was determined as described in the text.
[c] Values for the *cis* isomer are shown in parentheses.
[d] The informational mass fraction value for total vitamin K_1 in NIST SRM 1846 Infant Formula, determined by enzymatic digestion followed by reverse-phase liquid chromatography with UV absorbance detection (Sharpless et al., 1997) is 1000 ng/g.

200 μm beadlet capsules used to fortify with fat-soluble vitamins. These encapsulated vitamins were included in the vitamin premix of the infant formula powder (NIST, 1996).

3.3. *Recovery of* trans *vitamin* K_1

Vitamin K_1 was added to a margarine-like product (Product HS-3, Table 1), extracted by the two procedures and analyzed as described above. Recoveries of *trans* vitamin K_1 following use of the enzymatic extraction ranged from 82 to 100%; those following the direct extraction procedure were 93 to 99% (Table 3). Despite its numerous steps, the enzymatic procedure provides comparable recoveries to those of the direct method.

3.4. *Analysis of margarines and margarine-like products*

3.4.1. *Comparison of extraction methods*

We used both extraction procedures to specifically determine *trans*-vitamin K_1 and dihydro-vitamin K_1 in a number of margarines and margarine-like products (Tables 4 and 5). Only a few of the products showed significant differences in results obtained by the two methods. The average of values obtained by the two methods provided an estimate of *trans* vitamin K_1 in samples of the first lot.

During analysis for vitamin K in foods containing hydrogenated oils, Booth, Pennington, and Sadowski (1996b) observed a chromatographic peak later identified as 2′,3′-dihydro-vitamin K_1, a product of the hydrogenation of phylloquinone rich vegetable oils (Davidson et al., 1996). Dihydro-vitamin K_1 has been measured in the plasma following dietary intake of the compound in a stick margarine (Booth, Davidson et al., 1996a) and has also been reported in infant formula (Indyk & Woollard, 1997). The biological activity of this compound has not been determined.

Dihydro-vitamin K_1 contents determined following both methods were generally found to be equivalent. For two products, however, the results obtained by the two extractions were significantly different (LS2 and HS2).

Margarines in the US are not fortified with vitamin K_1. In 1993, a provisional table of vitamin K_1 content of foods listed the median content of vitamin K_1 for margarines as 51 with a range of 4 to 97 μg/100 g (Booth, Sadowiski, Weihrauch & Ferland, 1993). Piironen, Kolvu, Tammisalo, and Mattila (1997) reported that in margarines from retail markets in Finland, vitamin K_1 concentrations were generally correlated with fat content.

In the present study, we found that "tub" or soft margarine products contained higher levels of vitamin K_1 than "stick" margarine products containing similar levels of fat and similar oil sources. These findings are comparable to those of Piironen et al. (1997).

Table 3
Recovery of *trans*-vitamin K_1 (ng/g) from a spiked margarine-like product

Method	*trans*-Vitamin K_1[a]			
	Added	Endogenous[b]	Analyzed[c]	Recovered[d]
Enzymatic	402	464	793	82
	415	464	840	90
	358	464	822	100
Direct	321	471	789	99
	288	471	740	93
	303	471	764	97

[a] Vitamin K_1 standard was added to 0.3 to 0.4 g portions of sample HS-3 (Table 1), a reduced fat, soybean oil margarine-like product containing 71% fat.

[b] Values for the unspiked ("endogenous") determinations are means of triplicate analyses using the enzymatic digestion or direct extraction methods.

[c] Each value for the spiked samples ("analyzed") represents the results of a single determination.

[d] Determination of the amount of *trans*-vitamin K_1 in the standard and calculation of % recovery are described in the text.

Table 4
Measurement of *trans*-viamin K_1 (μg/100 g) in margarines and margarine-like products following enzymatic digestion or direct extraction[a]

Product[b]			*trans*-Vitamin K_1			
	Form	Fat (%)	Enzymatic	Direct	p[c]	Mean value[d]
LS6	Tub	79	163.2 (8.4)	158.7 (10.0)	NS[f]	161
LS1	Stick	79	94.5 (7.6)	86.8 (0.6)	NS	90.2
CN2	Tub	79	80 (7.0)	78.4 (12.0)	NS	79
LS2	Stick	79	72.2 (1.6)	63.0 (1.3)	$\leqslant 0.014$	67.6
LS5	Tub	71	118.0 (2.9)	114.1 (2.3)	$\leqslant 0.033$	116
HS4	Tub	71	110.6 (6.4)	108.5 (8.6)	NS	110
HS3	Stick	71	56.9 (8.8)	56.6 (2.0)	NS	56.8
HS6	Stick	71	55.5	–	–	–
SF1	Stick	71	26.2 (1.5)	23.4 (0.5)	NS	24.8
CR1[e]	Stick	71	2.7 (0.2)	–	–	–
LS4	Squeeze	64	129.9 (4.2)	138.7 (5.8)	NS	134.3
HS5	Stick	64	66			
HS1	Stick	57	53.9 (4.4)	52.7 (5.1)	NS	53.3
HS2	Stick	53	35.0 (1.3)	30.3 (0.4)	$\leqslant 0.025$	32.6
LS3	Stick	50	54.2 (2.2)	52.8 (1.6)	NS	53.5
SF2	Tub	43	15.5 (0.9)	14.2 (0.6)	NS	14.8
CN1	Tub	14	14.3	–	–	–
CR2	Squeeze	0 0.33		0.24	NS	0.285

[a] Values are means (standard deviations) of three determinations each for enzymatic digestion or direct extraction procedures, with the exceptions of products HS5, HS6 and CN1, which are results of single determinations.

[b] Oils in ingredient lists on the product labels are listed in Table 1.

[c] The significance of differences between values obtained by the two methods was determined by two-tailed Student t-tests. Pairs of values with $p \leqslant 0.05$ are considered significantly different.

[d] For each product, a mean value (far right column, above) was calculated as the average of the means obtained by the enzymatic digestion and direct extraction procedures.

[e] During analysis of product CR1 following direct extaction, a small peak was observed in the *trans*-vitamin K_1 region but the software could not integrate it.

[f] NS, not significant.

Table 5
Measurement of 2′,3′-dihydrovitamin K_1 (μg/100 g) in margarine and margarine-like products following enzymatic digestion or direct extration[a]

Product[b]			Dihydrovitamin K_1			
	Form	Fat (%)	Enzymatic	Direct	p[c]	Mean value[d]
LS2	Stick	79	244.8 (6.1)	224.3 (3.6)	⩽0.05	234.6
LS1	Stick	79	168.0 (11.0)	161.8 (1.2)	NS[f]	164.9
CN2	Tub	79	133.4 (10.0)	127.7 (13.0)	NS	130.6
LS6	Tub	79	87.4 (9.8)	77.9 (2.5)	NS	82.7
HS6	Stick	71	189	–		
HS3	Stick	71	170.9 (17.0)	177.6 (5.1)	NS	174.3
HS4	Tub	71	137.2 (2.8)	143 (17.0)	NS	140.3
SF1	Stick	71	143.8 (4.8)	132.2 (2.7)	NS	138.0
LS5	Tub	71	42.2 (0.4)	41.2 (6.7)	NS	41.7
CR1[e]	Stick	71	8.3 (1.6)	–	–	8.3
HS5	Stick	64	168	–		
LS4	Squeese	64	ND	ND	–	–
HS1	Stick	57	163.2 (9.9)	166.7 (16.0)	NS	165.0
HS2	Stick	53	191.1 (1.5)	172.4 (3.4)	⩽0.01	181.8
LS3	Stick	50	124.3 (2.6)	119.0 (5.2)	NS	121.7
SF2	Tub	43	38.8 (1.7)	37.1 (0.8)	NS	38.0
CN1	Tub	14	20.0	–		–
CR2	Squeeze	0	ND	ND		–

[a] Values are means (standard deviations) of three determinations each for enzymatic or direct extractions, with the exceptions of products HS6, HS5 and CN1, which are results of single determinations.

[b] Oils in ingredient lists on the product labels are listed in Table 1.

[c] The significance of differences between values obtained by the two methods was determined by two-taied Student t-tests. Pairs of values with $p \leqslant 0.05$ are considered significantly different.

[d] For each product, a mean value (far right column, above) was calculated as the average of the means obtained by the enzymatic and direct extractions.

[e] During analysis of product CR1 following direct extraction, a small peak was observed in the 2′,3′-dihydrovitamin K_1 region but the software could not integrate it.

[f] Abbreviations: ND, not detected; NS, not significant.

Dihydro-vitamin K_1 was present in higher amounts in the ''stick'' margarines than in the softer ''tub'' margarines. Dihydro-vitamin K_1 was not detected in a ''squeeze'' margarine-like product. The highest reported amount of dihydro-vitamin K_1 in soybean oil stick margarines is 132 μg/100 g (Booth et al., 1996b). We found 244 μg/100 g dihydro-vitamin K_1 in a soybean oil ''stick'' margarine (product LS2). These differences may be due to differences in methodology, product composition, or as yet unrecognized geographic, seasonal or processing factors.

3.4.2. Butter and oils

The results of triplicate analyses of vitamin K_1 in butter and in oils using the two extraction methods are shown in Table 6. The oils were chosen because they were the major oil sources identified in the ingredient lists of the margarines and margarine-like products studied (Table 1). Analytical precision for the direct extraction method is generally superior to that of the enzymatic method, demonstrating one advantage of the simpler procedure. Determination of vitamin K_1 in canola oil and canola oil margarines by either extraction method required separating a peak that co-eluted with vitamin K_1 under the usual HPLC conditions. The

Table 6
Analysis of vitamin K_1 (μg/100 g) in vegetable oils and butter using two extraction procedures[a]

Method	Vitamin K_1			
	Enzymatic		Direct	
	trans	*cis*	*trans*	cis
Oils				
Soybean	114.2 (7.9)	20.4 (1.1)	102.5 (3.6)	17.1 (0.8)
Canola	107.9 (15.9)	9.0 (7.1)	97.7 (8.3)	10.4 (1.2)
Sunflower[b]	1.9 (0.3)	0.8	1.7 (0.35)	–
Corn	1.25 (0.3)	0.8	0.95 (0.1)	0.4
Butter				
Butter	8.9 (1.9)	ND[c]	6.3 (1.1)	ND

[a] Values are means (standard deviations) of three determinations each for the enzymatic or direct extractions. The ingredient lists of these products did not identify any hydrogenated components. Dihydro-vitamin K_1 was not observed in any of these products.

[b] During analysis of sunflower oil following direct extraction, a small peak was observed in the *cis*-vitamin K_1 region, but the software could not integrate it.

[c] ND, not detected.

Table 7
trans-vitamin K_1 (μg/100 g) and 2′,3′-dihydro-vitamin K_1 (μg/100 g) in two lots of margarine products

Product[a]			*trans*-vitamin K_1		Dihydrovitamin K_1	
	Form	Fat (%)	Lot 1[b]	Lot 2[c]	Lot 1[b]	Lot 2[c]
LS6	Tub	79	161	143	83	96
LS1	Stick	79	90	55	165	36.9
CN2	Tub	79	79	43.4	131	106
LS2	Stick	79	68	22.9	235	141
LS5	Tub	71	116	82	41.7	34.5
HS4	Tub	71	110	47	140	142
HS3	Stick	71	57	63	174	221
HS6	Stick	71	55.5	42.7	189	191
SF1	Stick	71	24.8	3.3	138	25.7
CR1	Stick	71	2.7	4.6	8.3	16.1
LS4	Squeeze	64	134	115	ND[d]	ND
HS5	Stick	64	66	–	168	–
HS1	Stick	57	53	22.1	165	123
HS2	Stick	53	32.6	23.5	182	95
LS3	Stick	50	54	37.8	122	141
SF2	Tub	43	14.8	15.9	38.0	29.1
CN1	Tub	14	14.3	13.6	20.0	28.3
CR2	Squeeze	0	0.29	0.3	ND	ND

[a] Oils in ingredient lists on the product labels are listed in Table 1.
[b] Values for Lot 1 are means of the results obtained by the enzymatic and direct extraction methods (Tables 4 and 5).
[c] Values for Lot 2 are the results of single determinations.
[d] ND, not detected.

resolution of this peak (Fig. 2) was improved using a reduced flow rate (e.g. 0.8 ml/min) and methanol(100%) or complete with a mobile phase of acetonitrile in methanol(12.5, 87.5%, v/v). Both eluents included the required $ZnCl_2$ buffer solution. This unidentified compound peak, like vitamin K compound peaks, is not detected following removal of the zinc column.

The total vitamin K_1 content of the oils analyzed (Table 6) falls within the ranges reported in the literature. Concentrations reported for vitamin K_1 in major edible oils are (μg/100 g): soybean, 103-333 (Ferland and Sadowski, 1992; Moussa, De–Passe, Lompret, Harte, & Girardet, 1994; Piironen et al., 1997; Zonta & Stancher, 1985); rapeseed or canola, 125–348 (Gao & Ackman, 1995; Piironen et al.); corn, 1.63–4.18 (Ferland & Sadowski); and sunflower, 6.49–11.77 (Ferland & Sadowski; Piironen et al.). Our results for corn oil are slightly below the range stated above, but may be within the analytical error of measurement. The amount of vitamin K_1 found in butter agrees with results of Booth, Sadowski, and Pennington (1995) and Piironen et al. who reported levels of 6–8 μg/100 g for butter. Concentrations of vitamin K_1 in the oils were lower than total vitamin K_1 and dihydro-vitamin K_1 in the derived margarine products. The storage of the oils in clear plastic bottles on shelves in retail markets may have

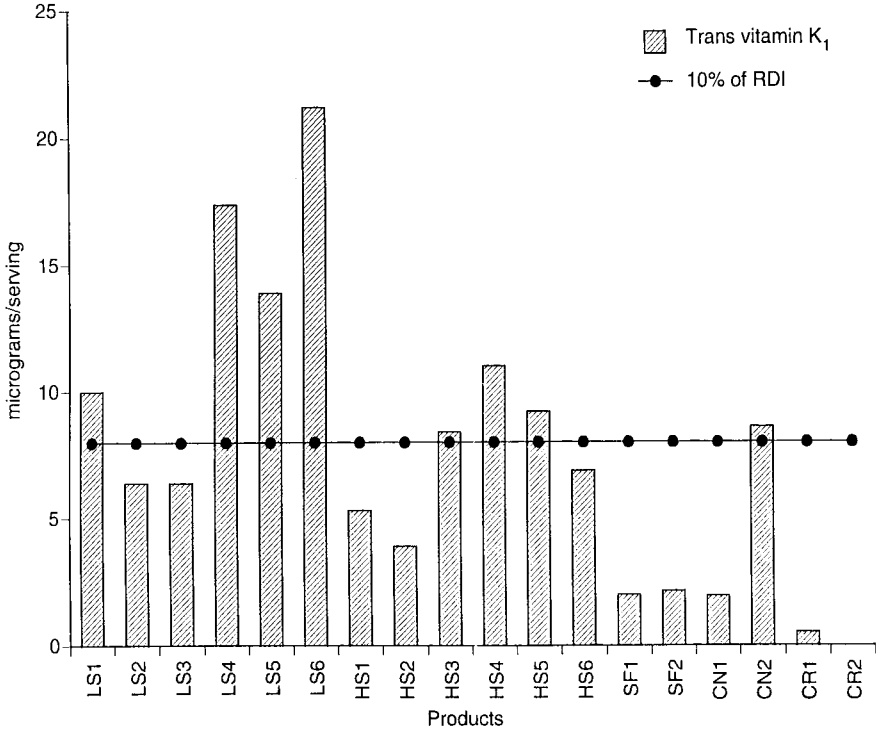

Fig. 3. Relationship between vitamin K_1 content of margarines and Reference Daily Intake (RDI). Concentrations of vitamin K_1 in margarines are expressed as micrograms/serving. Values for trans-vitamin K_1 are means of results from analysis of two lots of the products (Table 7). Oil ingredients identified on the product labels are listed in Table 1. The bold line is drawn at 8 micrograms vitamin K_1/serving (i.e. 10% of the Reference Daily Intake (RDI) of 80 μg).

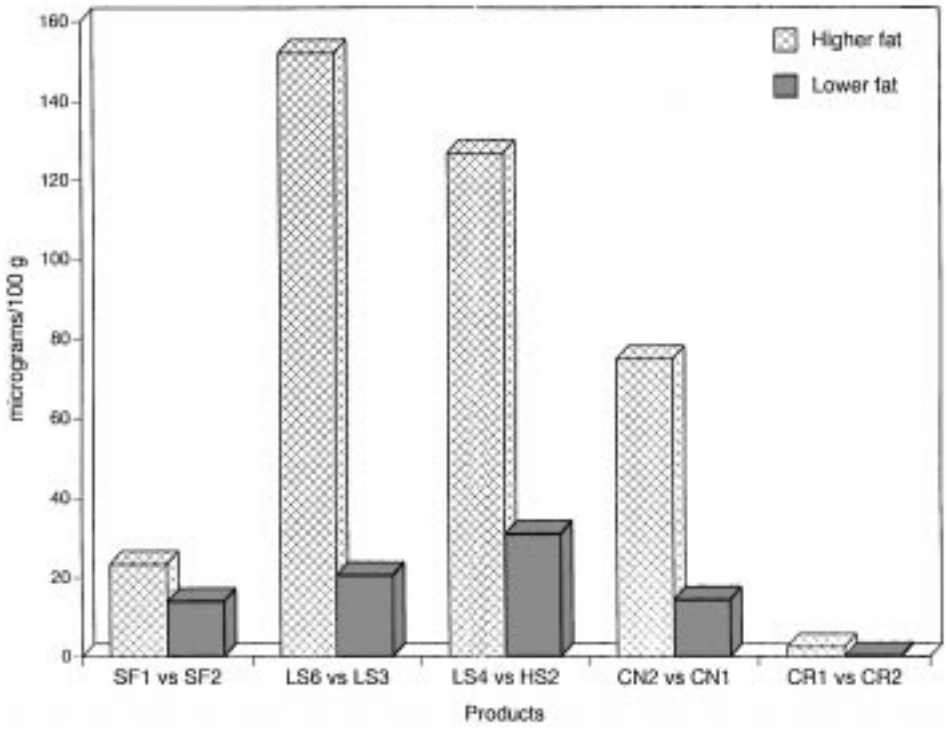

Fig. 4. Relationship between vitamin K_1 content and fat content for several margarines. Comparison of concentrations of vitamin K_1 (micrograms/100 g) in five margarines with those in lower-fat products of similar oil composition. Oil ingredients identified on the product labels are listed in Table 1.

contributed to loss of vitamin K_1 through photo-degradation (Hwang, 1985; Indyk, 1988).

3.4.3. Cis *and* trans *isomers*

We found *cis*- and *trans*-vitamin K_1 in the majority of products we analyzed. *Cis*-vitamin K_1 usually made up 10% or less of the vitamin K_1 present and was not quantified in most cases due to the small size of the *cis*-peak. The *cis*-vitamin K_1 peak could be reliably quantitated in soybean oil and canola oil. The cis component contributed approximately 17% to the total amount of vitamin K_1 in soybean oil. Hwang (1985) reported that exposure of a standard solution of vitamin K_1 to light resulted in increases in the *cis* isomer and decreases in the *trans* isomer, followed by overall loss of total vitamin K_1. While we did not study the effect of light exposure during retail storage, such exposure may increase the relative proportion of *cis* isomer concurrent with photo-degradation of total vitamin K_1.

3.4.4. Lot-to-lot variation

We analyzed vitamin K independently in two lots of each product (Table 7). Although the second lot was tested only once, some products showed consistency in vitamin K content between lots, while others showed significant lot-to-lot variation. Some factors that may affect vitamin K_1 content between lots are degree of hydrogenation of oil ingredients, differences in the ratios of liquid and hydrogenated soybean oils to other ingredient oils, quality of the oil, storage conditions, possible seasonal variations and the changes in oils used.

Fig. 3 shows the relationship between concentrations of vitamin K_1 in margarine products, expressed as micrograms/serving (average of two lots), and the Reference Daily Intake [RDI; CFR §101.9 (c) (8) (iv)] of 8 μg/serving. A product containing 10% of the RDI of a nutrient is considered to be a "good source" of that nutrient. About 45% of the products analyzed in this study provide 10% or more of the RDI.

We also compared the vitamin K_1 content of five margarines with concentrations of vitamin K_1 in lower-fat products of similar oil compositions and/or that were produced by the same manufacturer (Fig. 4). The lower fat products contained significantly lower amounts of vitamin K. Such products contribute little or no vitamin K to the diet.

4. Conclusions

To date, the method of Indyk and Woollard (1997), which offers significant improvements over the current AOAC (1996) official method 992.27 and other methods requiring solid-phase clean-up techniques, has not been extended to matrices other than infant formula, unfortified liquid milk and milk powders. Data from our study now support the use of a direct extraction and demonstrate the utility of the enzymatic digestion procedure for other food matrices such as margarines

and margarine-like products. The C_{30} column successfully resolves the *cis* and *trans* isomers of vitamin K_1 in foods. This had not been possible using the conventional C_{18} columns and therefore, use of the C_{30} column extends RP-HPLC techniques to specific estimation of the bioactive form of vitamin K present in foods.

Dihydro-vitamin K_1, frequently in high proportions relative to total vitamin K_1, was found in all products listing a hydrogenated oil among their ingredients. Stick margarines contain higher amounts of the dihydro derivative than tub margarines or squeeze margarine-like products. Lower fat margarine-like products contained significantly lower amounts of vitamin K and contribute little or no vitamin K to the diet.

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