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**Methods of Analysis of Vitamin K: A Review**

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**ABSTRACT**

**Vitamin K1 (phylloquinone) and K2 (menaquinone) are available in nature and K3 (menadione) is a man-made vitamin. They perform many physiological functions in humans. Several analytical methods are available for the determination of vitamin K including spectrometric, fluorimetric, HPLC, electrochemical and microbiological methods. These methods have been applied to the assay of K vitamin in food, pharmaceuticals and biological fluids. The fluorimetric methods are more sensitive than the other methods. The details of these methods are reviewed in this article.**

**Keywords:** Phylloquinone, Menaquinone, Menadione, Spectrometric, Fluorimetric

**INTRODUCTION**

Dam, in 1935 [1] discovered the presence of an antihemorrhagic component [2] as a new fat-soluble vitamin and gave it the name of vitamin K (kaogulation; in German). It was isolated as yellow oil from alfalfa. The active principle of vitamin K is naphthoquinone nucleus and the parent compound of the vitamin K series is menadione (2-methyl-1,4-naphthoquinone) which is also known as man-made vitamin K or vitamin K3. Vitamin K1 was characterized as 2-methyl-3-phytyl-1,4-naphthoquinone [3] and is preferable called phylloquinone. Vitamin K2 (menaquinone) was isolated from alfalfa as a crystalline product. It has a side chain on the 3-position of naphthoquinone ring. Vitamin K2 has two subtypes i.e. MK-4 and MK-7 which are short-chain and long-chain menaquinones, respectively (Fig. 1). Most of the

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chemical and physical properties of different vitamin K forms are similar and this is due to the close structural relationship [4]. Menadione and menaquinones are solid while phylloquinone is liquid at room temperature. All forms of vitamin K are water insoluble and thermostable. They are sensitive to light and alkaline conditions but are stable in slight acidic and oxidizing conditions [5]. Green vegetables, cereals, red/white meat, eggs, some vegetable oils and liver are the good sources of vitamin K, particularly vitamin K1 and K2 [6, 7]. Menadione or vitamin K3 is found in supplements. Phylloquinone is widely distributed in blue-green algae and in higher plants and menaquinone is synthesized by bacteria and specific inhabitants of the human gut microflora. Its distribution is restricted as compared to phylloquinone [8]. Deficiency of vitamin K is uncommon in healthy people because of the widespread availability of the vitamin in foods. However, those taking anticoagulants [9] and those

with fat malabsorption are at risk of vitamin K deficiency [10].

The adequate intake (AI) level for vitamin K for healthy individuals is 2.5 µg/day for infants, 60 mcg/day for children and 120 µg/day and 90 µg/day for adult males and females respectively [11]. Vitamin K is well absorbed (70%) from small intestine [12]. Presence of fat in food enhances the absorption of this vitamin [32]. The bodily stores of vitamin are rapidly depleted [6]. Phylloquinone is excreted in urine and feces [14].

Vitamin K plays an important role in blood coagulation and helps stop bleeding. It is important for developing essential proteins for bones and blood [15]. It helps against the development of non-hodgkin lymphoma and high intake of this vitamin decreases the risk of hip fracture. Menadione is considered inferior to vitamin K1 for the treatment of drug-induced hypoprothrombinemia [16]. However, vitamin K3 clinically is an important chemotherapeutic agent [17, 18]. It is used in animal feeds and in fungicides [19]. It is an intermediate in the synthesis of vitamin K1 [20] and also for the treatment of osteoporosis [21].

Higher doses of phylloquinone and menaquinone have been found to be non-toxic, however, it is not the same in the case of menadione. Higher doses of vitamin K3 result in liver damage and hemolysis, especially in neonates. It is also responsible for causing hyperbilirubinemia and kernicterus in premature infants [17].

Adverse reactions of vitamin K include flushing, chest pain, weakness, peculiar sensation of taste, dyspnea, dizziness, sweating and brief hypotension [22,23]. Menadione ADRs include hypersensitivity or anaphylaxis and may include shocks and /or respiratory arrest [24].

Vitamin K antagonizes the effect of acenocoumarol, phenprocoumon, anisindione, diphenadione and phenindione [25]. Large doses of salicylates antagonize vitamin K [26].

Menadione is degraded in the presences of light and is also destroyed by reducing agents and alkali solutions. It forms complexes with electron donors such as caffeine, nicotinamide and salicylic acid and these complexes have a stabilizing effect on its photodegradation [27].

## ANALYTICAL METHODS

The major problem in the analysis of vitamin K arises due to its light sensitivity and low concentration within complex matrices. Therefore, the entire sample prepared should be protected from light by using amber glassware. Many methods have been used for the analysis of vitamin K analogues, phylloquinone, menaquinone and menadione. Some of these methods are mentioned below.

## SPECTROMETRIC METHODS

### *UV spectrometric methods*

Phylloquinone (vitamin K1) has been determined by UV spectrometry at 249 nm using 420 as a value of A (1 %, 1 cm) at this wavelength [28]. Spectrometric methods have been utilized for the determination of vitamin K3 alone [29] and also in the presence of its decomposition products [30]. A method has been proposed for the analysis of vitamin K3 which is simple, rapid, selective and sensitive. The method is based on the reaction of menadione with piperidine and malononitrile and measurement of absorbance at 510 and 580 nm, respectively. There are no interferences from vitamin K1 and K2 or any of the pharmaceutical excipients and the average recovery is 98% [31]. Hassan et al. developed a spectrometric method for the determination of menadione. The method is based on the condensation reaction of vitamin K3 with acetylacetone and diethylmalonate in the presences of ammonia with an accuracy of  $\pm 0.2\%$ . In this method the reaction of vitamin with alkalies has also been evaluated [32]. Menadione has also been determined by the formation of its 2, 4-dinitrophenylhydrazone and measurement of absorbance at 635 nm [33]. Menadione and menadione sodium bisulphate have been determined

by their reaction with ethyl cyanoacetate and ammonium hydroxide in ethanol to give a stable blue colour. The colour is increased at 570 nm for menadione and at 582 nm for menadione sodium bisulphate. The colour reaction obeys Beer's Law [34]. Some other colorimetric methods for the determination of menadione have also been reported [35, 36].

#### *Fluorimetric methods*

A fluorimetric detection of phylloquinone in plasma has been performed. Using conventional chromatographic extraction vitamin K1 was quantified and detected fluorimetrically after post-column reduction to hydroquinone with zinc metal. The mean concentration of vitamin K1 was 0.55 µg/L and the lower limit of detection was about 0.05 µg/L [37]. Menadione exhibit fluorescence in the region of 407 nm when complexed to β-cyclodextrin in an aqueous medium. The measurements are performed at pH 6.2 by adding 0.1 per mole citrate buffer and  $6.4 \times 10^{-3}$  M of β-cyclodextrin. The calibration graph is linear within the range of 0.1–2.0 mg/L with the repeatability of 2.2%. The method can be successfully applied to the determination of the vitamin in pharmaceutical products [38].

### **CHROMATOGRAPHIC METHODS**

#### *HPLC methods*

A ionization (ESI) method for the assay of phylloquinone (vitamin K1) has been proposed. The atomic pressure chemical ionization (APCI) method has been used with LC/MS for the determination of vitamin K1. The linearity was found up to 5.4 µg/L and the recovery was above 98% [39]. This method is more sensitive, simple and reliable than the electrospray ionization (ESI). Phylloquinone has been determined in plasma specimens with the help of LC-APCI/MS method. The method is sensitive enough for the simultaneous determination of both labeled and unlabelled vitamin K1. The minimum detectable concentrations of labeled and unlabelled phylloquinone were 0.08 and 0.05 pmol/injection and the RSD was found to be 6.6% and 96.2%

respectively [40]. A method has been developed by Paroni et al. for the determination of vitamin K1 in plasma by HPLC with fluorescence detection. The method is simple with high throughput [41]. A precise and accurate method has been proposed for the analysis of vitamin K1 in multivitamin preparations by RP-HPLC technique. The measurements were made at the wavelength of 248 nm [42]. In serum, phylloquinone has been analysed by solid-phase extraction and narrow-bore HPLC method with multichannel UV detection. This sensitive method uses 2.1 mm (i.d.) column with a non-aqueous eluent at 248 nm with a recovery of about 76% [43]. For the determination of phylloquinone in foods, an HPLC method has been developed using fluorescence detection. Inter- and intra- precision of the assay lies within 6.6–13.6%. By using this method the endogenous dihydrophylloquinone has also been identified in the presences of hydrogenated oils [44]. A RP-HPLC method has been proposed for the analysis of menaquinone mixtures of microbial origin. The method has been found to be easy and sensitive [45]. Research has been conducted for the analysis of menaquinone and demethylmenaquinone mixtures from bacteria by RP-HPLC [46]. This technique has also been utilized for separation of the menaquinone mixtures of microbial origin on the bases of the chain length and the degree of hydrogenation of side-chain [47]. For the pharmacokinetic study of menaquinone-4 in dogs, a simple and sensitive method has been established using HPLC technique with fluorescence detection [48].

Schneiderman et al. have proposed a method for the determination of menadione in animal feed using RP-HPLC with reductive electrochemical detection at a silver electrode at -0.75 V vs. calomel electrode. The minimum detected quantity of vitamin K3 was 125 pg. The method showed 90.5% recovery and the RSD of 2.2% [49]. Another HPLC method for the determination of menadione in animal feed with fluorimetric detection using a post-column zinc reducer at 325 nm excitation and 425 nm emission wavelength has been reported. Menadione was extracted with methanol and with the help of sodium

carbonate was converted to the oil-soluble menadione. Average recoveries were greater than 90% and the average RSD was 5.5% [50]. A group of worker has performed RP-HPLC for the determination of vitamin K3 with a dual-electrode amperometric detector. The detection was found to be more sensitive than the UV detection. The linear response was within 35 ng to 15 µg with the detection limit of 15 ng [51]. HPLC conditions for the assay of K vitamins are given in Table 2.

### ELECTROCHEMICAL METHODS

A planar glassy-carbon electrode has been used to study the reduction of phyloquinone by cyclic voltametry. The reaction was found to be quasi-reversible with adsorption. The normal range of concentration was determined as 0.08–1.24 ng/ml and the mean value was 0.41 ng/ml. The endogenous levels of phyloquinone have been determined by liquid chromatography with electrochemical detection [52].

A potentiometric method has been developed for the determination of vitamin K3. The dissociation constant and stability constants were determined at 298 K and at 0.1 M ionic strength [53]. An electrochemical method for the assay of menadione is based on the appearance of a well defined polarographic catalytic wave with a peak potential of -0.95 V in 0.2 mol/L HOAc-NaOAc (pH 4.7) buffer solution containing KIO<sub>3</sub>. The sensitivity of the method was 10 times higher than in the absence of KIO<sub>3</sub> [54]. Two methods i.e. direct and indirect have been developed for the determination of menadione in pharmaceutical products. Vitamin K3 is oxidized in the presences of sulfite. The method is simple and fast and the detection limit was found to be 7×10<sup>-7</sup>M [55].

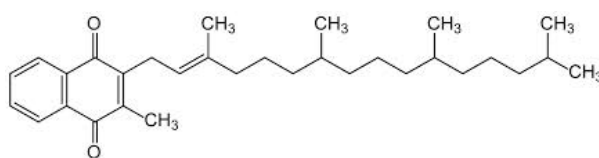
### MICROBIOLOGICAL METHODS

A microbiological method is based on the fact that staphylococci, micrococci and the strains intermediate in guanine+cytosine content contains menaquinone categorized as 'normal' and 'hydrogenated'.

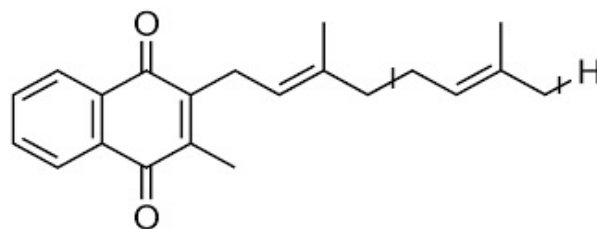
Occurrence of vitamin K2 in these strains has been determined in the range of 31.0–61.4% and 66.3–73.3% for normal and hydrogenated manauquinone, respectively [56]. *Bacillus subtilis* synthesizes vitamin K2 and the synthesis increases with the number of cells. This property has been utilized for the determination of menaquinone in bacterial cells and is secreted in the form of a soluble complex with a specific acidic binding factor. The factor is purified by DEAE ion-exchange chromatography and examined by polyacrylamide disc gel electrophoresis [57].

**Table 1:** Physicochemical properties of vitamin K

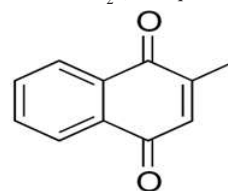
	Phylloquinone	Menaquinone	Menadione
Empirical formula	C <sub>31</sub> H <sub>46</sub> O <sub>2</sub>	C <sub>46</sub> H <sub>64</sub> O <sub>2</sub>	C <sub>11</sub> H <sub>8</sub> O <sub>2</sub>
Molar mass	450.70	648.99	172.18
Crystalline form	Pale yellow crystals	Light yellow microcrystal	Bright yellow crystals
Odor			Very faint acrid odor
Boiling point	140–145 °C	200 °C	
Melting point	-20 °C	54 °C	105–107 °C
Solubility, mg/100 ml			
Water	Insoluble	Insoluble	Insoluble
Absolute ethanol	Sparingly soluble	Sparingly soluble	1.6 mg
Acetone	Soluble		
Chloroform	Soluble		
Benzene			10 g
Absorption maxima (nm)	242, 248, 260, 269, 325	243, 248, 261, 270	245, 251, 333



Vitamin K<sub>1</sub> (phylloquinone)



Vitamin K<sub>2</sub> (menaquinone)



Vitamin K<sub>3</sub> (menadione)

**Figure 1:** Chemical structures of vitamin K1, K2 and K3.

**Table 2:** HPLC conditions for the assay of K vitamins

Material	Technique	Column	Mobile phase	Flow rate ml/min	Detection nm	Conc. Range µg/ml	Reference
<b>Vitamin K<sub>1</sub> : Phytoquinone</b>							
Phytoquinone in nutritional products	HPLC	C 30	Acetonitrile: methanol (50:50, v/v)	0.5			58
K <sub>1</sub> and D <sub>3</sub> in rat plasma	HPLC-UV	Xterra RP 18	Methanol: water (93:7, v/v)	0.5	239		59
Vitamin K <sub>1</sub>	HPLC	Luna C18	Methanol	1.2	248	25	42
K <sub>1</sub> and K <sub>3</sub> in cosmetics	HPLC	C 18	50% acetonitrile: methanol (75:25, v/v)		333		60
K <sub>1</sub> in vegetable juices	HPLC	C 18	Methanol: ethanol (95: 5, v/v)		$\lambda_{ex}$ 240 $\lambda_{em}$ 430		61
K1 in oils	HPLC	Supelco C18	Methanol-acetonitrile-water (88:10:2, v/v)		270		62
Phylloquinone in milk and infant formulas	HPLC	C18	Methanol-2-propanol-ethyl-acetate-water (450:350:145:135, v/v)		269, 277		63
Phylloquinone in vegetables	HPLC	LiChrosorb RP-8	Methanol		247	50	64
Phylloquinone and menaquinone-n in foods	HPLC	Nucleosil C 18	Methanol-ethanol-water (1:2:0.06, v/v)		FL 254 / 430	0.1 ng/ml	65
Vitamin K <sub>1</sub> in green vegetables	RP-HPLC	Hypersil ODS	Dicloromethane-methanol (20:80, v/v) + 10 mM ZnCl <sub>2</sub> + 5 mM CH <sub>3</sub> COOH and 5mM NaAc		248/418		66
<b>Vitamin K<sub>2</sub> : Menaquinones</b>							
Menaquinone (MK-7) and phylloquinone in human milk	HPLC	Radial Pak C 18	Ethanol-hexane-water (90:6:3) + 25 mM TBAP		EC -0.6 V/ + 0.2 V		67
<b>Vitamin K<sub>3</sub> : Menadione</b>							
Menadione, retinyl acetate, cholecalciferol, $\alpha$ tocopherol, $\alpha$ tocopherol acetate	HPLC	C 30	95% methanol, 0.55% aqueous solution and 5% DI water				68
Menadione in feed	HPLC	Atlantis dC 18	Methanol:water (98:2, v/v)		230, 265		69

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