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Determination of vitamin K_1 in emulsified nutritional supplements by solid-phase extraction and high-performance liquid chromatography with postcolumn reduction on a platinum catalyst and fluorescece detection

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Abstract

Determination of small amounts of vitamin K_1 (0.8 μg/g) in nutritional supplements with high fat content (20 mg/g) was performed by solid-phase extraction and high-performance liquid chromatography (HPLC) with fluorescence detection after reduction on a platinum oxide catalyst. The concentration ratio of plant oils to vitamin K_1 (0.8 μg/g) was about 25 000:1. A sample solution was applied to a solid-phase extraction cartridge and vitamin K_1 was eluted with ethanol, followed by HPLC. The proposed method was simple, rapid (analysis time: ca. 12 min), sensitive [detection limit: ca. 0.1 pg per injection (100 μl) at a signal-to-noise ratio of 3:1], highly selective and reproducible [relative standard deviation: ca. 1.3% (n=5)]. The calibration graph of vitamin K_1 was linear in the range of 0–2 pg per injection (100 μl). Recovery of vitamin K_1 was over 90% by the standard addition method.

Keywords: Food analysis; Vitamins

1. Introduction

Solid-phase extraction (SPE) is a simple and rapid technique and has been applied to complex sample matrices for extraction of, among others, nutrients from foods and drugs and their metabolites from biological fluids [1]. It is important to optimize this step to ensure that the subsequent HPLC analysis is effective.

The trace amounts of vitamin K_1 (VK_1) in samples have been determined by high-performance liquid chromatography (HPLC) [2–6].

However, careful SPE conditions to optimize the retention and elution of trace amounts of VK_1 in samples high in fat, which forms an emulsion, have not been studied. It is necessary to remove the oily particles in samples with high fat content to optimize the retention of trace amounts of compounds before the SPE step, because large amounts of lipophilic species that are strongly retained on the reversed-phase SPE cartridge, such as Bond Elut C_{18}, reduce the capacity of the cartridge for these trace amounts of compounds.

The nutrient samples used here are in powdered form and contain 50 different components such as both water-soluble and fat-soluble vitamins, plant oils, amino acids, organic acids, sugar and minerals. The concentration of these compounds is over 10 000 times higher than that of VK_1. The concentration ratio of plant oils (20 mg/g) to VK_1 (0.8 μg/g) was about 25 000:1.

Usually, a hexane extraction method is used for

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the separation of oily particles in a sample, after the addition of an inorganic salt such as sodium chloride to the sample in a separatory funnel. However, this method takes a long time, because the sample often forms an emulsion, which may take as long as 1 h to break up. Furthermore it is not possible to separate the oily particles and fat-soluble VK_1 by the hexane extraction method. And it is also difficult to separate the two before SPE. It is important to separate the two on an SPE cartridge for the HPLC analysis.

In the present study, we report the feasibility of the complete separation of oils from VK_1 on the cartridges. This method allows the trace detection of VK_1 (800 ng/g) in emulsified nutritional supplements with high fat content (20 mg/g) by SPE and HPLC with fluorescence detection (FD) after reduction on a platinum oxide catalyst. This paper also deals with the periodic stability of VK_1 in diluted samples and eluates from the cartridge for the pre-analysis sample stabilization of VK_1.

2. Experimental

2.1. Reagents and materials

VK_1 used in this study was purchased from Wako (Osaka, Japan). All reagents were of analytical or HPLC grade. Nutritional supplements were commercially available. Lght-resistant brown volumetric flasks and glassware [7,8] were used. Bond Elut C_{18} cartridges (500 mg, 3 ml) were purchased from Varian (Harbor City, CA, USA).

2.2. Standard VK_1 preparation.

Standard VK_1 (40 mg) was freshly diluted with ethanol in a 50 ml brown volumetric flask prior to use. VK_1 in this stock solution (5 ml) was further diluted with ethanol in a second 50 ml brown volumetric flask. This VK_1 solution (5 ml) was again diluted with ethanol in a 50 ml brown volumetric flask. This procedure was repeated on the diluted solution and 2 ml of the resulting solution was diluted to 20 ml with ethanol containing a small amounts of water (ca. 350 mg, see Section 3.2) (to mimic the conditions of an extracted sample), which passes through a conditioned SPE cartridge, to yield a final concentration of 0.8 μg/100 ml.

2.3. Sample preparation

The Bond Elut C_{18} cartridges were conditioned by washing with 5 ml of methanol and then with 10 ml of deionized water prior to use.

In a brown volumetric flask (100 ml) the nutrient (10 g) was dissolved in 5% (w/v) aqueous sodium sulfate solution containing 1 m*M* ethylenediaminetetraacetic acid disodium dihydrate (EDTA). This solution (1 ml) was applied to the conditioned Bond Elut C_{18} cartridge. The cartridge was washed with deionized water (10 ml) and then 10% aqueous ethanol solution (5 ml), followed by elution with ethanol (eluate volume; 10 ml). An aliquot (100 μl) was injected into the HPLC column.

2.4. Apparatus and conditions

A Model 655 A-12 high-performance liquid chromatograph (Hitachi, Tokyo, Japan), equipped with a Model F-1000 fluorescence detector (Hitachi), set at excitation wavelength 320 nm and emission wavelength 430 nm, was used. The samples were applied by a Rheodyne Model 7125 sample-loop injector with an effective volume of 100 μl. Separation took place on a 15×0.46 cm I.D. column of Inertsil ODS-2 (5 μm) (GL Sciences, Tokyo) with postcolumn reduction on a column of RC-10 platinum oxide catalyst (3×0.40 cm I.D., Irica, Kyoto, Japan), using methanol–ethanol (50:50) as the mobile phase. The flow-rate was 0.6 ml/min at 40°C.

3. Results and discussion

3.1. Spe (elution factors)

We reported in a previous paper [9] the determination of trace amounts of cyanocobalamin in foods by HPLC after SPE with Bond Elut C_{18} cartridges. It was found that three factors are significant for efficient retention and elution of cyanocobalamin: choice of eluent, eluate volume, and pH of the sample solutions. Consequently, these factors were

Table 1
Effect of eluents on the elution of VK_1. Standard VK_1 (80 ng) was injected into the cartridge followed by elution with each eluent (each eluate volume; 10 ml)

Eluent	Recovery (%)
Methanol	73.2
Ethanol	100
2-Propanol	52.5
Acetonitrile	73.4

also explored for the analysis of VK_1 in nutrients with high fat content.

3.1.1. Effect of eluent on elution of VK_1

Standard VK_1 (80 ng) was injected into the cartridge, followed by elution with four eluents (Table 1, methanol, ethanol, 2-propanol and acetonitrile). The optimal elution of VK_1 was provided by ethanol. Retention of VK_1 was tested by examining the cartridge effluent. We concluded that VK_1 was not detected in the aqueous unbonded fraction. VK_1 in aqueous solution was completely retained on the cartridge.

3.1.2. Effect of eluate volume on the elution of VK_1 from cartridge

The content (%) of VK_1 in successive 2 ml aliquots of eluate volume was determined, after standard VK_1 (80 ng) had been injected into the cartridge. VK_1 in each 2 ml fraction 1–5 was analyzed (Table 2). It was found that complete elution of VK_1 from the cartridge was obtained with ethanol (eluate volume 10 ml).

Table 2
Effect of ethanol volume on the elution of VK_1 from the cartridge. Standard VK_1 (80 ng) was injected into the cartridge followed by elution with successive 2 ml aliquots of ethanol. VK_1 in the fractions 1–5 (each 2 ml) was analyzed

Fraction (eluate volume 2 ml)	Recovery (%)
1	66.2
2	18.4
3	8.8
4	3.9
5	2.6

3.1.3. Effect of the pH of the sample solution on retention of VK_1

Standard VK_1 (80 ng) was diluted in 2 ml of 10 m*M* potassium phosphate buffer (pH 3 to 8.5) and was applied to the cartridge followed by elution with ethanol (eluate volume; 10 ml). VK_1 in each eluate was analyzed. Optimal recovery of VK_1 on the Bond Elut C_{18} cartridge was found over the sample pH range of 5.5–7.5 (Table 3). In other words, VK_1 retention is very sensitive to loading pH.

A summary of the optimized sample preparation method is thus: VK_1 (5 μg), diluted in 2 ml of 10 m*M* potassium phosphate buffer (pH 5.5 to 7.5) was applied to the conditioned Bond Elut C_{18} cartridge, followed by elution with ethanol (eluate volume, 10 ml).

The pH value in the nutrient sample solution was about 6 ± 0.3. Thus, the sample can be applied to the SPE cartridge without further pH adjustment (a benefit in routine sample preparation).

A large volume (10 ml) of eluate is needed to elute VK_1, because VK_1 is very strongly adsorbed on a C_{18} cartridge (500 mg).

3.1.4. Effect of sodium sulfate concentration on recovery of VK_1

A second purpose of this work was to determine the effect of sodium sulfate concentration on recovery of VK_1.

The nutrient often forms an emulsion. Usually, inorganic salt such as sodium chloride is employed to break up the emulsion. Therefore, the effect of sodium sulfate concentration on the recovery of VK_1 from the SPE was examined. It was found that

Table 3
Effect of pH value in sample solution on the retention of VK_1. Standard VK_1 (80 ng) diluted in 2 ml of each 50 m*M* potassium phosphate buffer (pH 3–9) was applied to the cartridge followed by the elution with ethanol (each eluate volume 10 ml)

Loading pH	Recovery (%)
3.0	85.1
4.5	89.5
5.5	100
6.0	100
6.8	100
7.5	100
9.0	98.2

Table 4
Effect of sodium sulfate concentration on Recovery (%) of VK_1 in real sample solution

Sodium sulfate concentration (%, w/v)	Recovery (%)
0	9.1
5	100
10	100
15	100

suitable sodium sulfate concentrations were above 1% (Table 4). To ensure a rugged method, we adopted a 5% (w/v) sodium sulfate aqueous solution.

3.1.5. Effect of EDTA on recovery of VK_1

VK_1 is known to be unstable in the presence of metal ions. A third purpose of this work was to determine the effect of EDTA on the recovery of VK_1.

This was examined for the sample preparation step. The stability of VK_1 is evident from Table 5. It was found that VK_1 in the sample solution diluted with 5% aqueous sodium sulfate containing 1 m*M* EDTA was stable for 5 h at 25°C. However, VK_1 in a sample solution diluted in 5% aqueous sodium sulfate without 1 m*M* EDTA was stable for only 0.5 h and then decreased over time. After 5 h, the content of VK_1 had decreased to only.

From the above, it is clear that the addition of 1 m*M* EDTA to samples is effective for the stabilization of VK_1. This is probably because the EDTA chelates metal ions, thus preventing them from oxidizing the analyte.

3.2. Chromatography

Firstly, we focused on the effect of dilvents on the sharpness of the VK_1 peak on the chromatogram.

When VK_1 was diluted with water-free ethanol, a broad VK_1 peak was obtained. On the other hand, when VK_1 was diluted with ethanol containing a trace amounts of water, which was obtained after that eluent (ethanol) had been passed through the conditioned cartridge, a sharp VK_1 peak was obtained.

Table 5
Effect of addition of 1 m*M* EDTA on the recovery (%) of VK_1 in real sample solution

EDTA	Recovery (%)				
	0	1	2	3	5 (h)
Addition	100	100	100	100	100
No addition	100	85.4	67.7	35.3	15.5

The broad peak may be due to the fact that the separation was carried out immediately after injection without complete concentration of VK_1 on the top of the analytical column, owing to the large injection volume (100 μl). The sharp peak may be caused by sample enrichment at the top of the column. Whatever the cause, it is fortuitous that the optimum conditions coincide with those that result from SPE. About 350 mg of water ($n=5$) is retained on a conditioned cartridge. Thus, it was necessary to prepare both the standard and sample VK_1 in the same way. In the present study, 100 μl of ethanol solution was injected into the analytical column. If the injection volume was smaller (20 μl), a sharp VK_1 peak was obtained, irrespective of the water content of the dissolving agents.

VK_1 and idebenone in biological samples, which have quinone-like molecule structures, were analyzed by HPLC with FD and electrochemical detection offer reduction on a platinum oxide catalyst [3,10].

VK_1 in nutrient samples with high fat content treated with a Bond Elut C_{18} cartridge can be obtained by HPLC with FD on an Inertsil ODS-2 column with postcolumn platinum oxide reduction (Fig. 1). The analysis time was about 12 min.

The limit of detection according to Fig. 1. was ca. 0.1 pg per injection (100 μl) at a signal-to-noise ratio 3:1. It was necessary to inject 100 μl of sample solution for trace amount determination, because the SPE method developed actually dilutes the VK_1. The on-column focusing of VK_1 by the ethanol/water injection conditions improves detection. A similar technique has previously been used [9,11,12].

3.3. Determination of VK_1

The calibration graph for VK_1 was constructed by plotting the peak height of VK_1 against the amount of VK_1 added. Satisfactory linearity was obtained in the range of 0–2 pg on column ($y=4.9354x-0.091$, y=peak height, x=amount of VK_1 in pg).

Table 6 shows analytical data for VK_1 in nutrition-

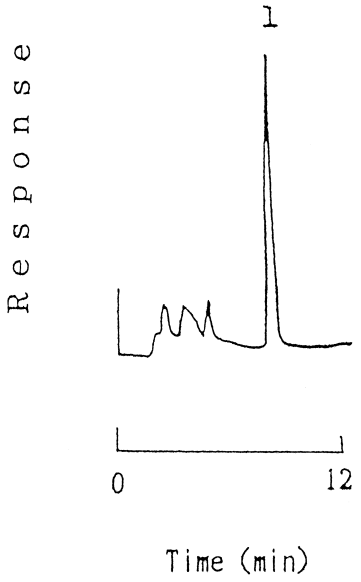

Fig. 1. Chromatogram of VK_1 in the SPE eluate of an emulsified nutritional supplement by HPLC after reduction on a platinum catalyst and FD after SPE. HPLC out on a 15×0.46 cm I.D. column of Inertsil ODS-2 (5 μm) with RC-10 postcolumn reduction (3×0.40 cm I.D.). Mobile phase, methanol–ethanol (50:50); flow-rate, 0.6 ml/min at 40°C. Peak 1=VK_1 (0.81 ng injected).

al supplements with high fat content. There was good agreement with small RSD (1.3–3.1%) in VK_1 value in nutritional supplements between the concentration indicated according to the label and that found by this method, irrespective of the indicated ratio between plant oil and VK_1) was over 25 000:1.

A known amount of VK_1 was added to the nutrient and overall recoveries were estimated by the standard addition method, as shown in Table 7. The recovery of VK_1 was above 90%. The relative standard deviation (RSD) was 1.3% (n=5) without addition of VK_1.

3.4. Stability of VK_1

Finally, we examined the stability of VK_1 in the nutrient. The VK_1 concentrations in both the nutrient sample solutions and ethanol eluates obtained from SPE, stored at both 5°C and 25°C for 24 h were studied to determine how long VK_1 in each sample was stable.

VK_1 in the ethanol eluates.was stable for 24 h under both conditions. On the other hand, in the nutrient sample solutions VK_1 was stable for 5 h

Table 6
Analytical data for VK_1 in emulsified nutritional supplements

Sample	Ratio plant oil/VK_1	Concentration VK_1 (μg per 100 g)	Concentration determined (μg per 100 g)	RSD (n=5) %
A	25 000	80	81	1.3
B	166 666	23	21	3.1
C	156 520	56	54	2.8

Table 7
Recoveries of VK_1 added to emulsified nutritional supplement fat count

Added (μg per 100 g)	Found (μg per 100 g)	Recovery (μg per 100 g)	Recovery (%)
0	81[a]	–	–
40	118	37	92.5
80	157	76	95.0
160	234	153	95.6

[a] RSD: 1.3% (n=5).

under both conditions. When the nutrient sample was stored for 24 h at either 5°C or 25°C, the VK_1 content (%) was about 42.9 and 43.9%, respectively. Thus, VK_1 in the eluate obtained after SPE was quite stable.

4. Conclusion

A simple, rapid and highly selective analytical method for VK_1 in the nutrient samples with high fat content has been developed, based on SPE and HPLC on an Inertsil ODS-2 column with postcolumn reduction on a platinum oxide catalyst and FD (excitation wavelength 320 nm, emission wavelength 430 nm). It was demonstrated that it is feasible to obtain complete retention and isolation on the cartridge of trace amounts of VK_1 in nutrients containing large amounts of fat. The effects of the pH of the sample solution, nature of the eluent and of eluate volume on the retention on and elution from Bond Elut C_{18} cartridge were examined.

The stability of VK_1 in both the nutrient sample solution and eluate obtained after SPE was also examined by the proposed method.

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