Separation of Isomers of Vitamin K1 Using Normal Phase HPLC

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Key Words
Phylloquinone, phytomenadione, phytonadione, vitamin K1, fat soluble vitamins, comparison of silica phases

Abstract
Vitamin K1 isomers and related compounds are separated using a silica HPLC column and a normal phase solvent system. The correct silica type and mobile phase equilibration are important considerations in order to achieve good resolution and retention time reproducibility.

Introduction

Vitamin K is believed to be an important factor in preventing a number of disease states and is essential in the synthesis of blood clotting agents. Consequently, there is obvious interest in monitoring this compound in foods, supplements, and humans. Vitamin K1, also known as phylloquinone or phytomenadione, is the dietary form of vitamin K and is an indicator of overall vitamin K status. The vitamin itself is consumed in the form of green leafy foods such as cabbages, onions, and broccoli. Where subjects are deficient in this vitamin, cholestasis will result and a synthetic oral supplement can be administered.

The British Pharmacopoeia (BP) and European Pharmacopoeia (EP) monographs include a normal-phase HPLC method for the determination of the *cis* and *trans* isomers and the *trans*-epoxy modification of vitamin K1. The method in this application note is derived from this methodology. This application note demonstrates the separation of these compounds using a normal phase approach and demonstrates that different silicas can be chromatographically very different.

Experimental Details

Consumables
Vitamin K1, containing both *cis* and *trans* isomers (from an accredited supplier)
trans-epoxy vitamin K1 EP reference (from an accredited supplier)
Fisher Scientific™ Heptane
Fisher Scientific Diisopropylether
Fisher Scientific Octanol

Thermo SCIENTIFIC

Sample Handling Equipment	Part Number
Thermo Scientific™ Finnpippettes™	4700850
Thermo Scientific™ Finntips™	940-370 (50 µL)
	9400-130 (200 µL)
	9401-110 (1000 µL)
Thermo Scientific 2 mL amber 8 mm vials and caps	60180-600

Separation Conditions		Part Number
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC	
Columns:	Thermo Scientific™ Hypersil™ Silica 5 µm, 250 × 4.6 mm	30005-254630
	Alternative silica column (Thermo Scientific™ Syncronis™ Silica 5 µm, 250 × 4.6 mm)	97005-254630
Mobile phase:	Heptane / diisopropylether / octanol (1000:3.3:0.67 v/v)	
Flow rate:	0.4 mL/min	
Run time:	30 min	
Column temperature:	Ambient (approx. 22 °C)	
UV detector wavelength:	254 nm	

Solutions

A vitamin K1 (*cis/trans*) stock solution was prepared by diluting 14 mg in 10 mL of mobile phase, then taking 50 µL of this and diluting to 500 µL to give a solution of 0.14 mg/mL.

A *trans*-epoxy vitamin K1 stock solution was prepared by taking a 10 mg ampoule and diluting to 1 mL in mobile phase. Then, 100 µL of this was diluted to 1 mL and 100 µL of the subsequent solution diluted to 225 µL to give a concentration of 0.4 mg/mL.

A test mixture of the *cis* and *trans* vitamin K1 and *trans*-epoxy vitamin K1 was prepared by mixing 500 µL of each of these solutions together. The final solution contained a combined concentration of the vitamin K1 of 70 µg/mL and the *trans*-epoxy vitamin K1 was 200 µg/mL.

Data Processing	
Software:	Thermo Scientific™ Chromeleon™ software version 7.1

Results

The separation of the *cis* and *trans* isomers of vitamin K1 and the *trans*-epoxy vitamin K1 was achieved on a Hypersil Silica column using a mobile phase of heptane with small amounts of polar modifiers (Figure 1). Sufficient resolution was achieved to allow for reliable quantification of each of the components (Table 1).

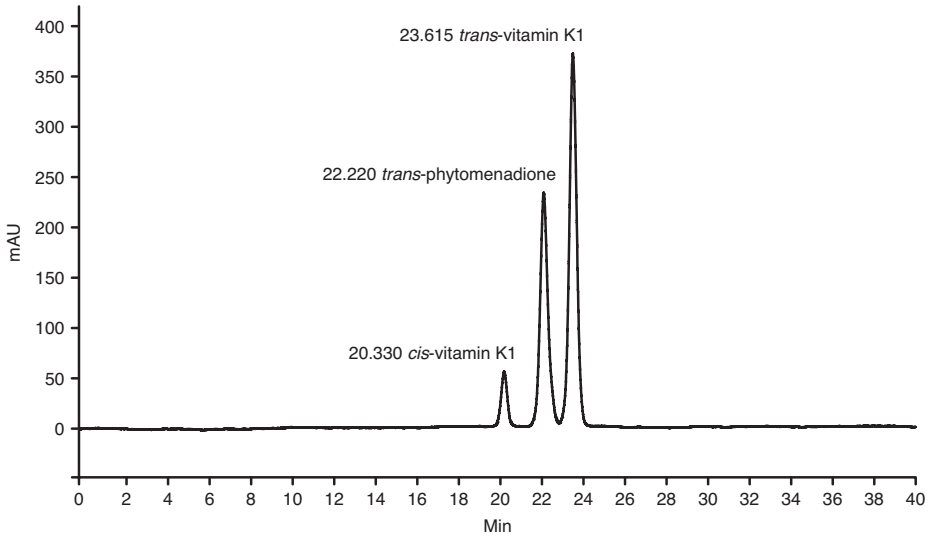

Figure 1: Chromatogram of isomers of vitamin K1 and *trans*-epoxy vitamin K1 on a Hypersil Silica column

	Retention Time on Hypersil Silica		Resolution on Different Silica Types	
Compound	**RT/min**	**% RSD (n=3)**	**Hypersil**	**Syncronis**
cis-vitamin K1	20.46	0.6	-	-
trans-epoxy vitamin K1	22.40	0.8	3.07	3.51
trans-vitamin K1	23.82	0.9	2.08	0.60

Table 1: Resolution of components

The Importance of Equilibration

Equilibration on silica columns can take longer than the usual ten column volumes of mobile phase used with reversed phase packing materials. In this case, stable retention of components was achieved after approximately 2 hours of flushing with the mobile phase at 0.4 mL/min, equating to approximately twenty column volumes. In an experiment where the column had been allowed to rest overnight in the mobile phase and then equilibrated for only 30 minutes, equating to approximately 5 column volumes, the elution order of the *trans*-epoxy vitamin K1 and *trans*-vitamin K1 reversed (Figure 2). Different methods will require different times to achieve chromatographic stability and should always be evaluated; this is particularly important for normal phase methods.

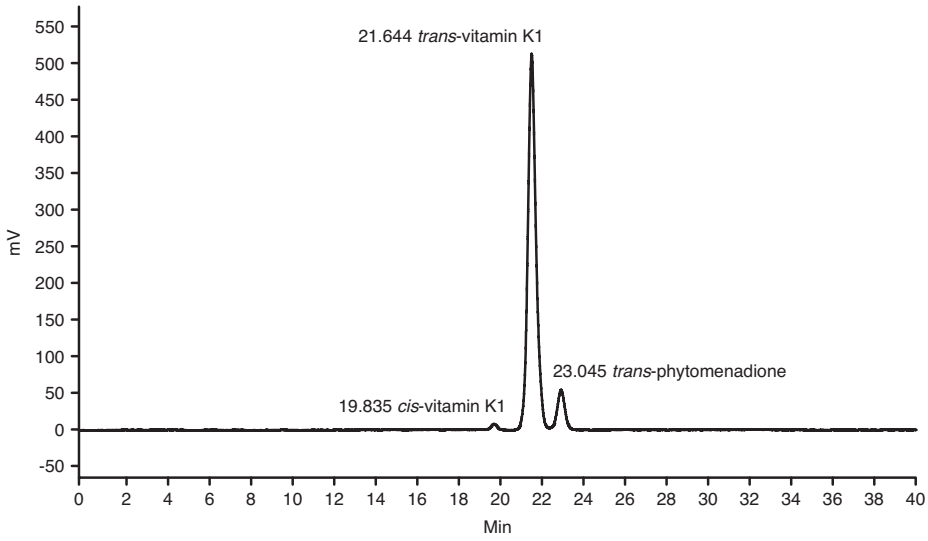

Figure 2: Chromatogram of components after a reduced equilibration time of 30 min on a Hypersil Silica column

The Effect of Different Silicas

The Hypersil Silica column was replaced with a column packed with a different silica of the same particle size, length, and diameter and evaluated using the same method (Figure 3). In this case, retention of all components was greater and, while the elution order remained the same, the resolution between the *trans*-epoxy vitamin K1 and the *trans*-vitamin K1 was insufficient to quantify these components (Table 1). Clearly, the physical and chemical properties of the silica, such as surface area, hydrogen bonding, and steric effects were responsible for these chromatographic differences. It should never be assumed that one column packing can be substituted for another and expect it to provide the same selectivity.

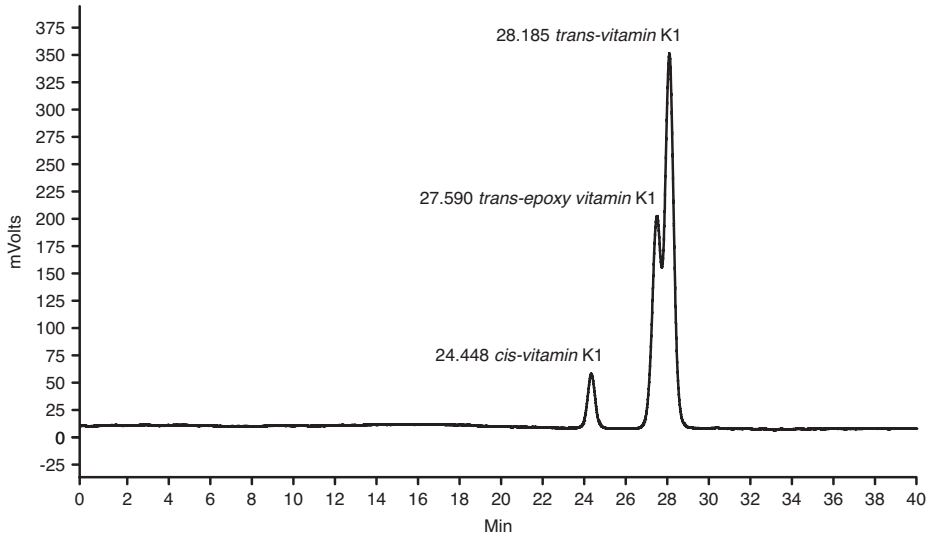

Figure 3: Chromatogram of isomers of vitamin K1 on a high surface area column

Conclusion

- Hypersil Silica can separate *cis* and *trans* isomers of vitamin K1 as well as the *trans*-epoxy form.
- Different silicas can demonstrate different chromatographic selectivities due to manufacturing and subsequent physical and chemical differences.
- Good column equilibration is critical to achieving reproducible retention times and selectivity and should always be evaluated for a method. Normal phase separations typically require longer equilibration than those using reversed phase methods.

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