

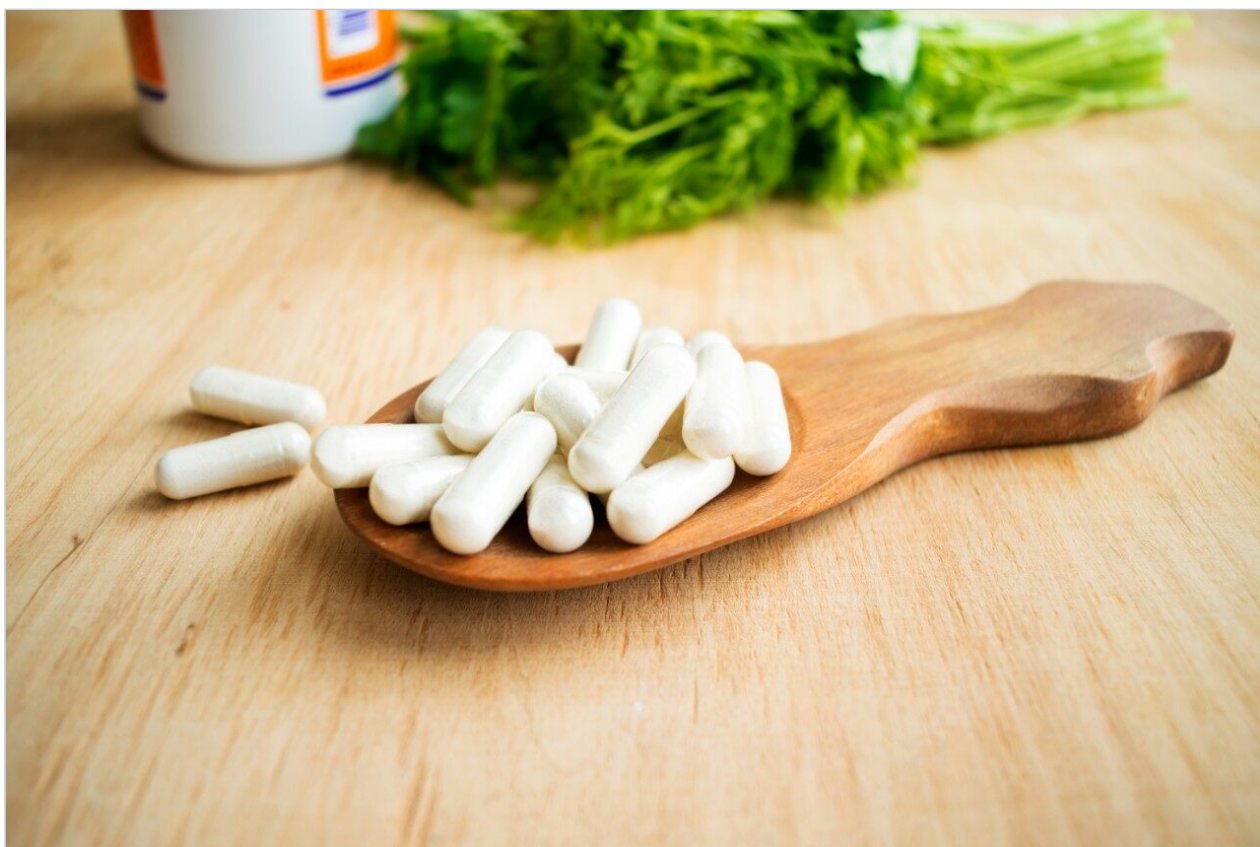
## Application Note

# Rapid Separation of Vitamin K<sub>1</sub> Isomers and Vitamin K<sub>2</sub> in Dietary Supplements Using UltraPerformance Convergence Chromatography with a C<sub>18</sub> Column

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## Abstract

In this application note presents the use of UltraPerformance Convergence Chromatography for a fast separation of vitamin K<sub>1</sub> *trans* and *cis* isomers and menatetrenone (MK-4), a common form of vitamin K<sub>2</sub>, on an ACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column.

### Benefits

- Fast and reliable separation of vitamin K<sub>1</sub> *trans* and *cis* isomers and MK-4 in less than three minutes.
- Separation is achieved on a C<sub>18</sub> column; no special C<sub>30</sub> column is needed.
- The use of carbon dioxide as the primary mobile phase minimizes organic solvent waste.

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## Introduction

Vitamin K<sub>1</sub> (phylloquinone) is an essential human nutrient produced in plants, especially green leafy vegetables. The vitamin K<sub>1</sub> in natural products exists mainly as the *trans* form, while the vitamin K<sub>1</sub> used in food supplementation is often synthetic K<sub>1</sub>, which may contain appreciable amounts of the *cis* form. The *trans*-vitamin K<sub>1</sub> is bioactive, while the *cis*-K<sub>1</sub> is not. It is highly desirable to separate the *trans*- and the *cis*-vitamin K<sub>1</sub> isomers to truly evaluate the nutritional value of the supplement ingredient. Available HPLC methods for the separation of vitamin K<sub>1</sub> isomers require C<sub>30</sub> columns. Their typical run time is about 20 minutes, and chlorinated solvents are used in some of the methods.<sup>1-3</sup>

UltraPerformance Convergence Chromatography (UPC<sup>2</sup>) is a separation technique that leverages the unique properties (i.e., low viscosity and high diffusivity) of compressed CO<sub>2</sub> at or near its supercritical state, as well as sub-2 micron particle packed columns to significantly improve the separation efficiency, speed, and selectivity.<sup>4</sup> This application note demonstrates a fast separation of vitamin K<sub>1</sub> *trans* and *cis* isomers and menatetrenone (MK-4), a common form of vitamin K<sub>2</sub>, by UPC<sup>2</sup> in less than three minutes on an ACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column. Figure 1 shows the structures of vitamin K<sub>1</sub> isomers and MK-4. Comparing to current LC-based vitamin K<sub>1</sub> *trans* and *cis* isomers analysis methods, this UPC<sup>2</sup> method is faster, simpler (no need to use a C<sub>30</sub> column), and it uses less organic solvent.

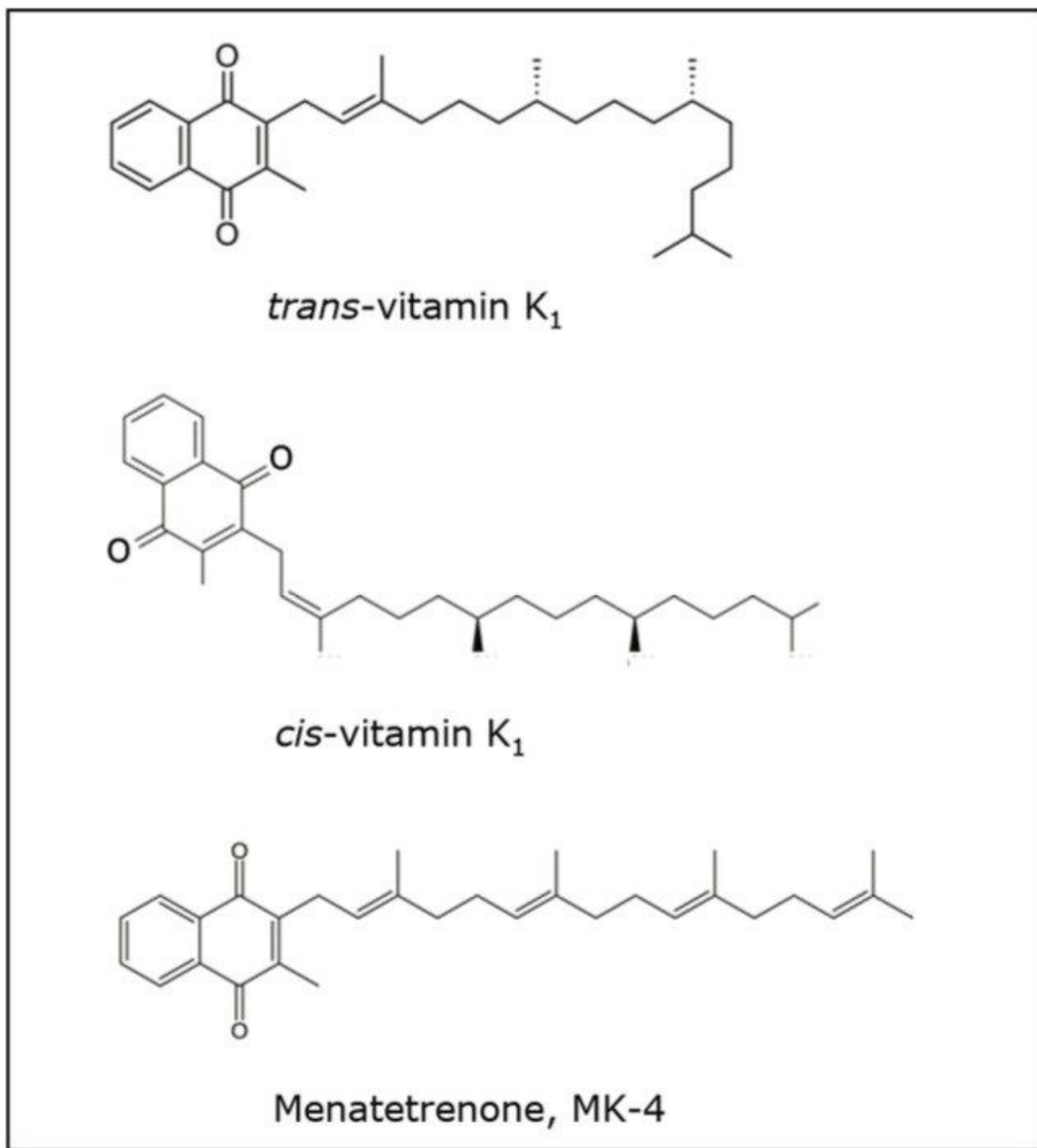


Figure 1. Structures of *trans*- and *cis*-vitamin K<sub>1</sub> and menatetrenone.

Experimental

## Sample preparation

Sample preparation Vitamin K<sub>1</sub> (Sigma-Aldrich) and MK-4 (Sigma-Aldrich) were weighed and dissolved in iso-octane (ReagentPlus, Sigma-Aldrich) to obtain a stock solution at 1 mg/mL. Intermediate and working standard solutions were obtained by serial dilution of the stock solution with iso-octane. Vitamin K<sub>1</sub> supplement tablets were purchased from a local store and were ground into a powder and extracted with iso-octane. The supernatant was filtered with a 0.45-µm PTFE syringe filter and diluted before injection.

## Conditions

### UPC<sub>2</sub> conditions

System	ACQUITY UPC <sup>2</sup> with ACQUITY UPC <sup>2</sup> PDA Detector
Software	Empower 3
Detection	UV at 243 nm (compensation reference 400 to 500 nm, res. 6 nm)
Column	ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB 3.0 x 100 mm, 1.8 µm
Column temp.	50 °C
Sample temp.	10 °C
Injection volume	20 µL (Full loop)
Flow rate	3.00 mL/min
Mobile phase A	Compressed CO <sub>2</sub>
Mobile phase B	Acetonitrile/methanol mixture (50/50 v/v)
Run time	4 min

## UPC<sup>2</sup> conditions

ABPR pressure	1500 psi
Gradient	0.5% B for 2 min, ramp to 20% B in 1.5 min, hold at 20% B for 0.5 min

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## Results and Discussion

Vitamin K<sub>1</sub> *cis* and *trans* isomers and MK-4 were baseline separated in less than three minutes by UPC<sup>2</sup> using a single UPC<sup>2</sup> HSS C<sub>18</sub> SB Column (3.0 x 100 mm, 1.8 μm). The *cis* form eluted first, followed by the *trans* form, then the MK-4, as shown in Figure 2. The USP resolution between the critical pair, the *cis*- and the *trans*-K<sub>1</sub>, was 1.7 (Table 1). In the gradient program, the initial two-minute isocratic elution at 0.5% B was necessary for the baseline separation of the *cis*- and the *trans*-vitamin K<sub>1</sub>. Precise control of the mobile phase B delivery volume at 0.5% is critical for the critical pair separation. The ACQUITY UPC<sup>2</sup> System is the only SFC system on the market that can provide this level of precision control. Following the isocratic hold, a generic gradient from 0.5% to 20% B was used in the study. This gradient range could be modified in applications depending on the retention of the actual vitamin K<sub>2</sub> homologues of interest. MK-4 was included in this study because it is a common form of vitamin K<sub>2</sub>, and it is structurally the closest vitamin K<sub>2</sub> to K<sub>1</sub>. Other forms of vitamin K<sub>2</sub>, such as MK-7, have longer side chains, and tend to be retained longer at column. They can therefore be easily separated from vitamin K<sub>1</sub>. The total run time was four minutes, which was at least five times faster than the typical run time for HPLC methods using C<sub>30</sub> columns. The organic solvent consumption was less than 1 mL per injection, which is only a fraction of the typical 15 to 30 mL of solvent used in LC methods.

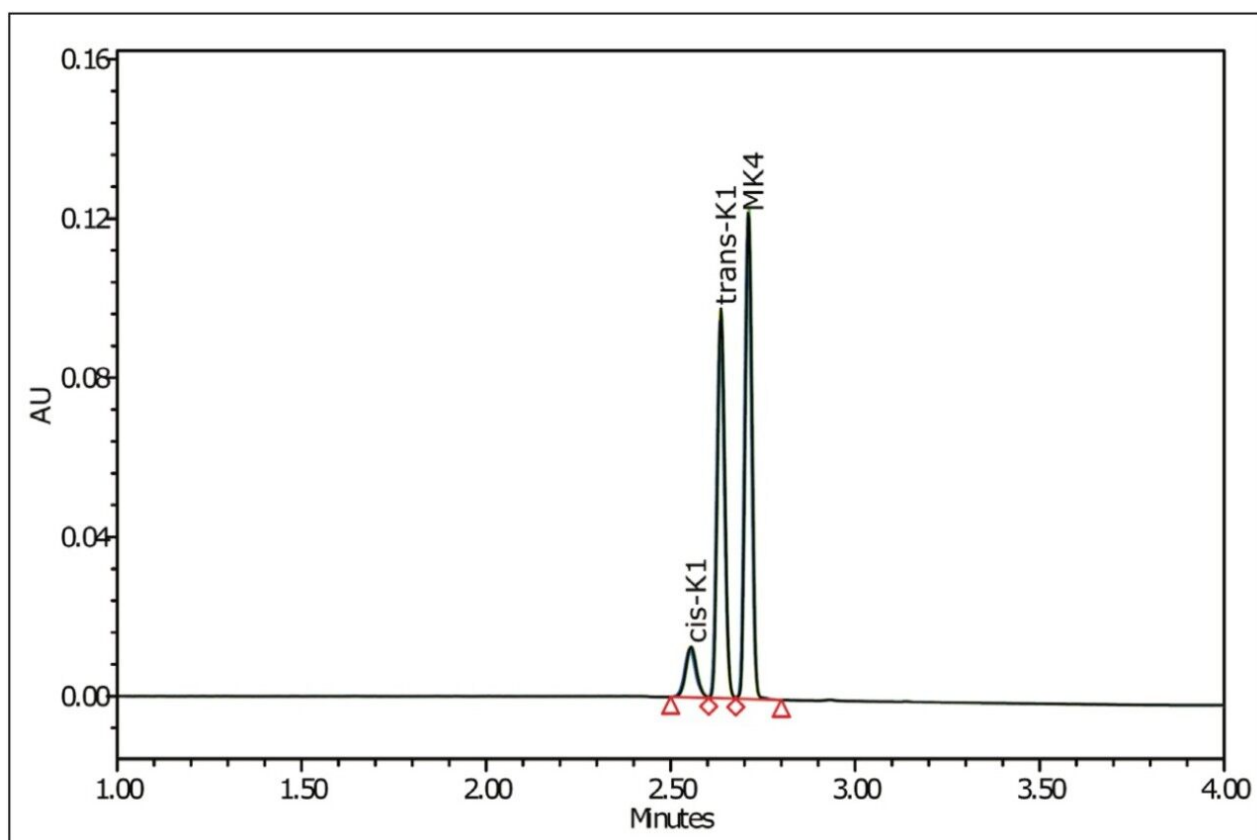


Figure 2. Chromatogram overlay of vitamin K<sub>1</sub> isomers and MK-4 standard mixture (n=10).

	RT (min)	RTRSD	Peak area RSD	Resolution	Resolution RSD
<i>cis</i> -vitamin K <sub>1</sub>	2.553	0.08%	0.6%	–	–
<i>trans</i> -vitamin K <sub>1</sub>	2.636	0.05%	0.2%	1.7	1.1%
MK-4	2.710	0.05%	0.2%	2.0	0.9%

Table 1. Results of replicate analysis of vitamin K standard mixture (n=10).

Ten replicate analyses of a standard mixture demonstrated excellent repeatability (Table 1). The limits of quantitation (LOQ), estimated at a signal-to-noise ratio at 10, were 0.06, 0.06, and 0.04 µg/mL for the *cis*-vitamin K<sub>1</sub>, the *trans*-vitamin K<sub>1</sub> and the MK-4, respectively (Table 2). Excellent linearity ( $R^2 > 0.998$ ) was obtained for these compounds (Table 2). Analysis of a commercial vitamin K supplement product also showed excellent repeatability and resolution (Figure3). In this product, the *cis*-K<sub>1</sub> was found to account for 11.2% of the total vitamin K<sub>1</sub> (Table 3).

Parameters	<i>cis</i> -vitamin K <sub>1</sub>	<i>trans</i> -vitamin K <sub>1</sub>	MK-4
Range (µg/mL)	0.03 to 1.5	0.02 to 8.5	0.02 to 10
Regression (R <sup>2</sup> )	0.9980	0.9997	0.9999
Slopes (mV sec mL/µg)	17.7	16.3	16.0
LOQ (µg/mL)	0.06	0.06	0.04

Table 2. LOQ and linearity.

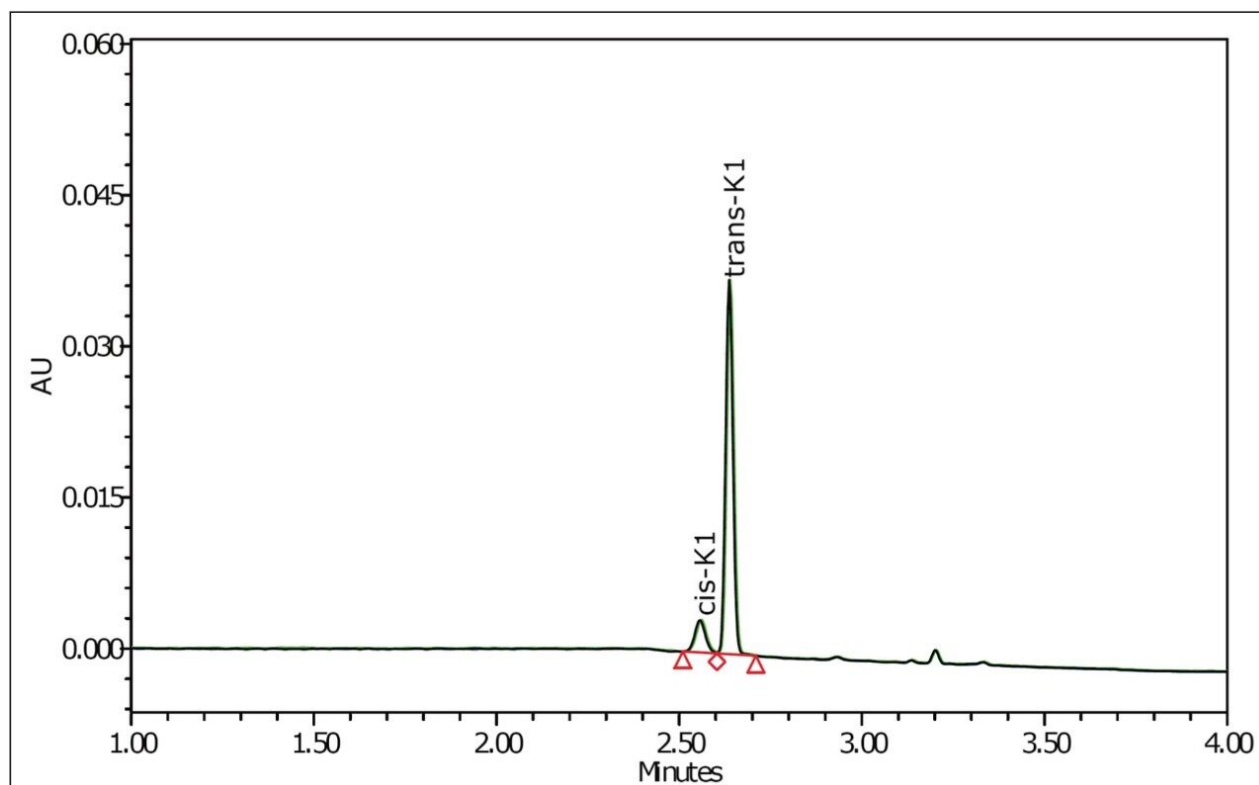


Figure 3. Chromatogram overlay of replicate analysis of vitamin K tablet (n=3).

	RT		Conc.		% of total K <sub>1</sub> Conc.
	Mean (Min)	RSD (%)	Mean (µg/mL)	RSD (%)	
<i>cis</i> -vitamin K <sub>1</sub>	2.558	0.09	0.38	2.1	11.2
<i>trans</i> -vitamin K <sub>1</sub>	2.638	0.06	3.20	0.3	88.8

Table 3. Results of replicate analysis of vitamin K supplement tablet (n=3).

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## Conclusion

UPC<sup>2</sup> Technology enables a rapid separation of the *cis*- and the *trans*-vitamin K<sub>1</sub> isomers and MK-4 on an ACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column in less than three minutes. The analysis time is at least five times faster than the current available HPLC methods, and no special C<sub>30</sub> column is needed. This UPC<sup>2</sup> method has excellent separation selectivity, resolution, sensitivity, repeatability, and it uses much less solvent than HPLC methods. UPC<sup>2</sup> can potentially be used by food ingredient testing labs for routine vitamin K analysis with significant increases in throughput and decreases in operating cost.

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## References

1. AOAC Official Method 999.15 Vitamin K in milk and infant formulas liquid chromatographic method. *AOAC International*. 2005.
2. Woollard DC, Indyk HE, Fong BY, Cook KK. Determination of vitamin K<sub>1</sub> isomers in foods by liquid chromatography with C<sub>30</sub> bonded-phase column. *J AOAC International* 85(3):682-691. 2002
3. Huang B, Zheng F, Fu S, Yao J, Tao B, Ren Y. UPLC-ESI-MS/MS for determining *trans*- and *cis*-vitamin K<sub>1</sub> in infant formulas: method and applications. *Eur Food Res Technol*;235(5):873-879. Nov. 2012.
4. Aubin A. Analysis of fat-soluble vitamin capsules using UltraPerformance Convergence Chromatography. Waters Application Note No. 720004394en. June, 2012.

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## Featured Products

ACQUITY UPC<sup>2</sup> System <<https://www.waters.com/134658367>>

ACQUITY UPLC PDA Detector <<https://www.waters.com/514225>>

Empower 3 Chromatography Data Software <<https://www.waters.com/513188>>

Available for purchase online



Viridis HSS C18 SB Column, 100Å, 1.8 µm, 3 mm X 100 mm, 1/pkg <  
<https://www.waters.com/waters/partDetail.htm?partNumber=186006623>>

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