Fat-Soluble Vitamins Analysis on an Agilent ZORBAX Eclipse PAH Polymeric C18 Bonded Column

Application Note

Food

Authors

Rongjie FU
Agilent Technologies (Shanghai) Co. Ltd.
412 Ying Lun Road
Waigaoqiao Free Trade Zone
Shanghai 200131
P.R. China

Jianzhong LI and Ying Wang
Agilent Technology Inc.
P.R. China

Abstract

Fat-soluble vitamins are highly lipophilic molecules which are analyzed by normal phase HPLC or reversed phase HPLC with a methanol/acetonitrile mobile phase. A new reversed phase HPLC method was developed for the Agilent ZORBAX Eclipse PAH column for vitamins A, D2, D3 and E. This polymeric C18 phase column provides baseline separation of vitamins D2 and D3, with a short analysis time of only three minutes. The methanol and water mobile phase made this compatible with LC/MS/MS with electrospray ionization.

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Introduction

Vitamins are essential nutrients that are necessary in small amounts for various functions in the human body. Vitamins are divided into two groups: water-soluble (such as B-complex and C vitamins) and fat-soluble (such as vitamins A, D, E and K). Fat-soluble vitamins perform many functions in the body. Vitamin A plays an important role in helping the eyes adjust to light changes, bone growth, tooth development, reproduction, cell division and gene expression. Vitamin D helps the body to use calcium and phosphorous, and it increases the amount of calcium absorbed from the small intestine to help form and maintain bones. Children especially need adequate amounts of vitamin D to develop strong bones and healthy teeth. Vitamin E acts as an antioxidant, protecting vitamins A and C, red blood cells and essential fatty acids from destruction. [1] Though fat-soluble vitamins are beneficial for human health, they have a toxic effect if taken at very high levels. Therefore, the quantity of fat-soluble vitamins in fortified foods must be controlled.

HPLC methods are usually used to analyze for these vitamins. Vitamins A and D are often analyzed by normal phase LC as described in the USP method for vitamin A and D. [2] Vitamin D has different forms; vitamins D2 and D3 are the most important compounds. They have similar structures that make them difficult to separate by HPLC. Recent research showed vitamin D2 is much less effective than vitamin D3 in humans. [3] Therefore, most foods fortified with Vitamin D use cholecalciferol (vitamin D3). Some analytical methods for Vitamin D in food use ergocalciferol (Vitamin D2) as an internal standard (for example, The Association of Official Analytical Chemists International (AOACI) method 2002.05: cholecalciferol (vitamin D3) in selected foods, such as milk and cheese). Therefore, it is important that any method used is capable of accurately separating vitamins D2 and D3. [4]

This application note describes a method for simultaneously analyzing vitamins A, D2, D3 and E on a polymeric bonding C18 phase. This C18 phase gives the selectivity necessary for polynuclear aromatic hydrocarbons PAH's, some of which separate on the basis of shape. The selectivity of this phase compared to that of typical monomeric bonding C18 phases makes it ideal for separating vitamins D2 and D3, using the same principle of shape selectivity but with a shorter analysis time. The simple mobile phase of methanol and water is also ideal for LC/MS with electrospray ionization (ESI).

Experimental

HPLC conditions:

Instrument:	Agilent 1200SL series LC system with binary pump
Column:	Agilent ZORBAX Rapid Resolution HT Eclipse PAH, 4.6 mm × 50 mm, 1.8 µm
Temperature:	40 °C
Mobile phase:	92% methanol/8% water
Flow rate:	2 ml/min (0.6 ml/min for MS detector)
Wavelength:	325 nm for VA/280 nm for VD and VE
Injection:	2 µL
Standards:	Vitamin A, ergocalciferol (vitamin D2), cholecalciferol (vitamin D3) and Vitamin E in methanol

Mass conditions:

Mass instrument:	Agilent 6460 triple quadrupole LC/MS system
Ion source:	ESI
Polarity:	Postive/Negative
Source temp:	325 °C
Gas flow:	5 L/min
Nebulizer:	55 psi
Sheath gas temp:	400 °C
Sheath gas flow:	12 L/min
Nozzle voltage:	Negative 1000 V; Positive 0 V
Ion spray voltage:	4000 V
Resolution:	Q1 (unit) Q3 (unit)
Scan mode:	Multiple Reaction Monitoring (MRM)

Compound name	Precursor ion	Product ion	Fragmentor	Collision Energy	Polarity
Vitamin A	269.2	213.2	110	7	Positive
Vitamin D2	397.2	379.4	120	4	Positive
Vitamin D3	385.3	367.3	120	5	Positive
Vitamin E	429.3	163.1	150	25	Negative

Delta EMV: 200 V

Results and Discussion

Vitamins A, D2 and D3 are usually separated with normal phase columns due to the highly lipophilic properties of these molecules. The methods developed on reversed phase columns generally use methanol/acetonitrile as the mobile phase, but mostly do not baseline resolve Vitamins D2 and D3. A previous application was developed for fat-soluble vitamins on Agilent ZORBAX Eclipse XDB C18 and Agilent ZORBAX StableBond SB-C18 columns, but with lower resolution of vitamins D2 and D3 [5]. A new application compared the separation of vitamins D2 and D3 with Stablebond C18 bonded phase to the same separation with Eclipse Plus C18 bonded phase. The SB-C18 may resolve the D vitamins better because of the accessible silanols on the surface of the stationary phase. However, the longer column increases the resolution but also increases the analysis time. The flow rate can be raised to decrease the time. The method was developed as a UHPLC method for high pressure exceeding 600 bar [4].

The results of this study determined that a fat-soluble vitamins analysis on an Eclipse PAH column provides better resolution and shorter analysis time compared to Eclipse Plus C18. Vitamins D2 and D3 were baseline separated in 3 minutes (Figure 1). The polymeric bonding C18 phase made it easy for D2 and D3 to resolve completely. The pressure was only 268 bar with a 2 mL/min flow rate, which can be performed on any LC instrument.

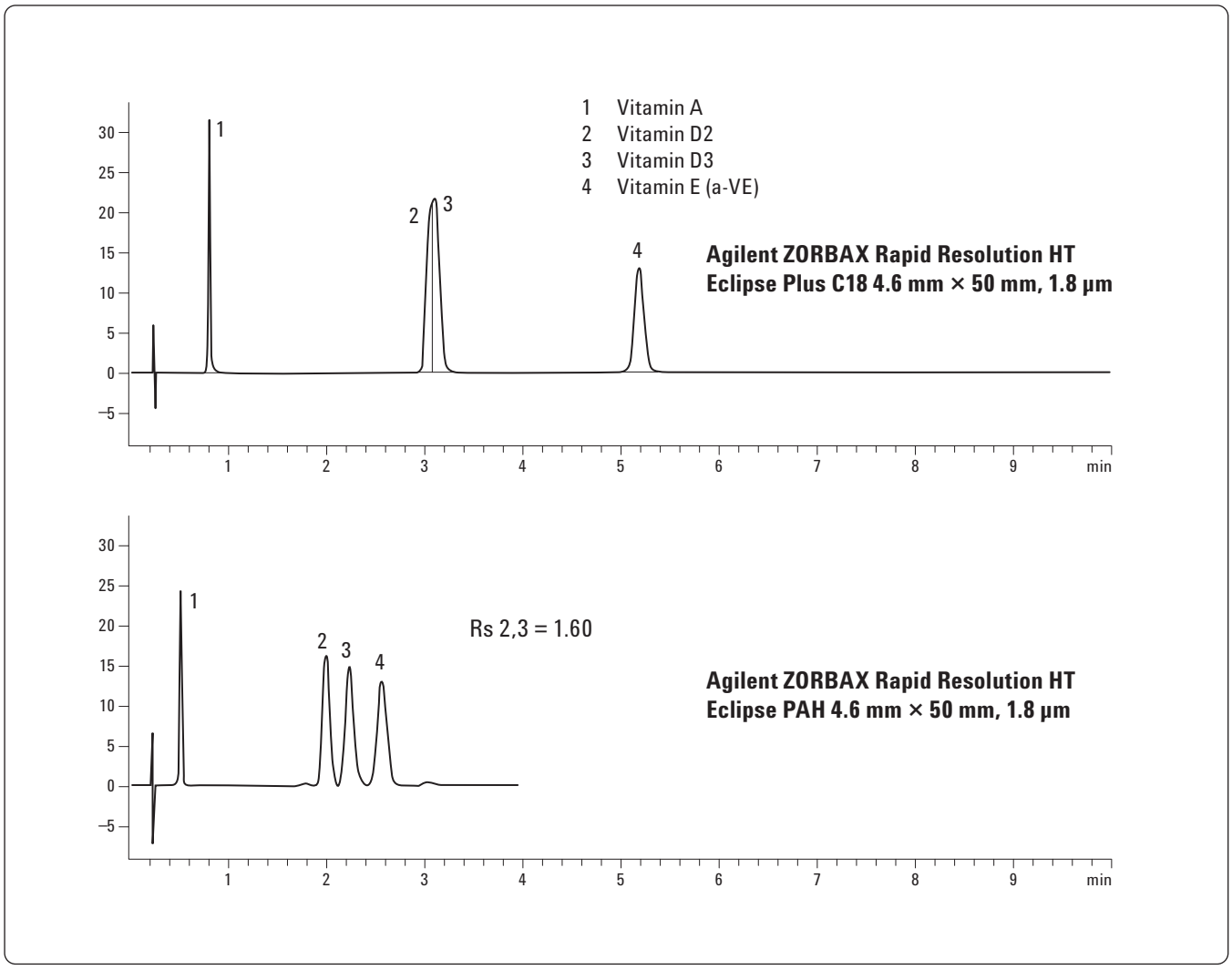

Figure 1. Chromatograms for the separation of four fat-soluble vitamins on different Agilent ZORBAX C18 columns.

Figures 2 and 3 show the total ion chromatogram (TIC) and multiple reaction monitoring (MRM) of four vitamins on the ZORBAX Eclipse PAH column. All four compounds were well resolved with no ion depression, which made it possible to get very high MS sensitivity.

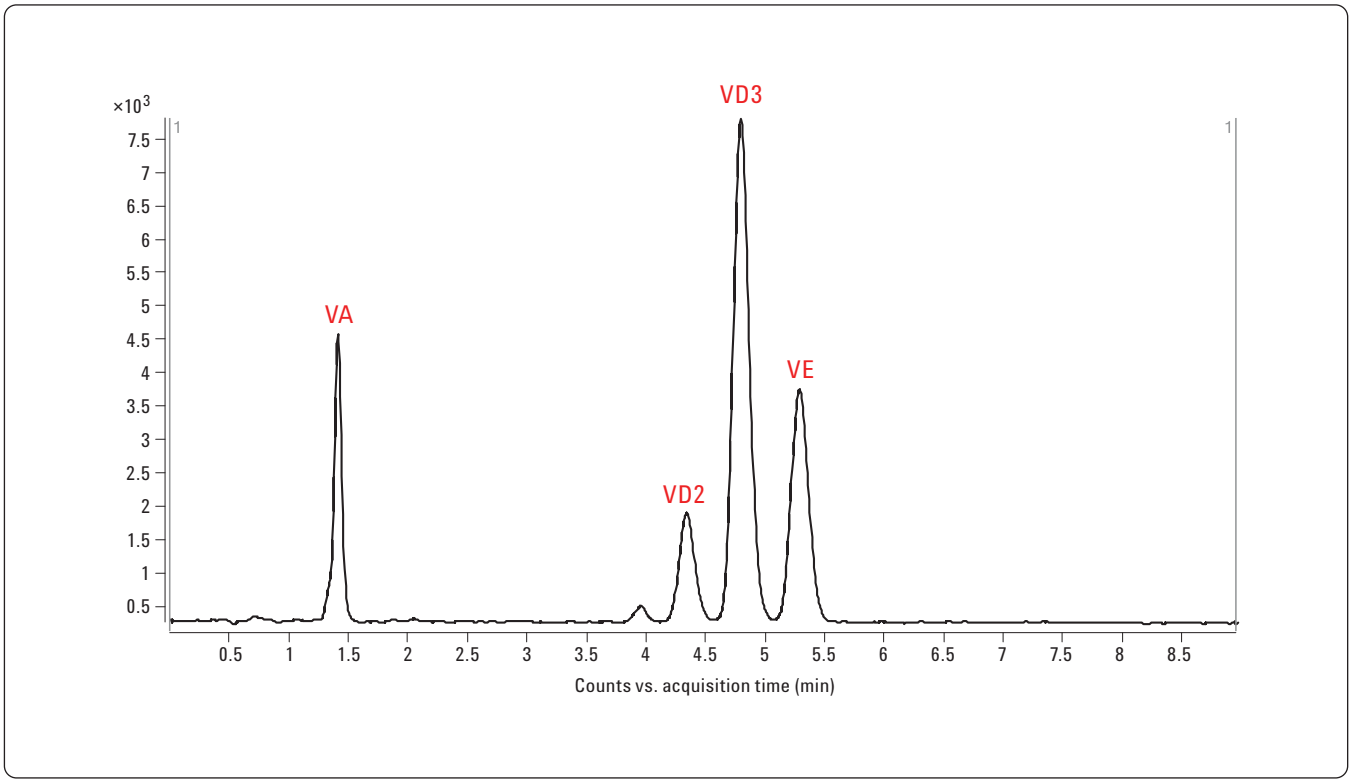

Figure 2. TIC of four fat-soluble vitamins on an Agilent ZORBAX Eclipse PAH column.

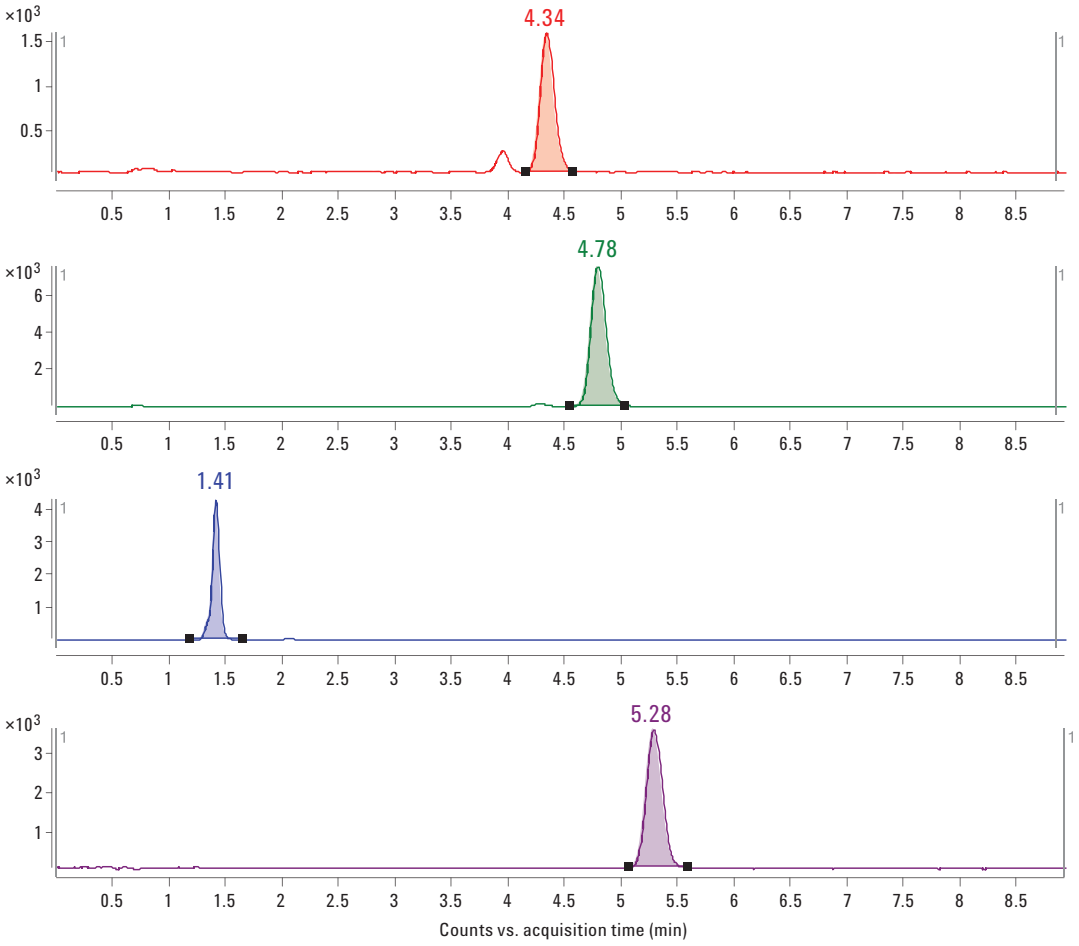

Figure 3. MRM of four fat-soluble vitamins on Agilent ZORBAX Eclipse PAH column.

Conclusion

The unique selectivity of a polymerically bonded C18 column, such as the Agilent ZORBAX Eclipse PAH column makes it possible to separate compounds with very similar structures like vitamins D2 and D3. The method developed in this application note resolved four fat-soluble vitamins simultaneously, in 3 minutes, and is compatible with the MS detector. The method can be run on any LC instrument and is suitable for the analysis of fat-soluble vitamins in foods.

References

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Printed in the USA
February 15, 2010
5990-5342EN

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