

UHPLC Method Development Options for a Vitamin D2 and D3 Separation

Application Note

Food

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Abstract

Vitamin D2 and D3 are partially separated by an isocratic LC method with a C18 column and methanol/acetonitrile mobile phase. Several optimization parameters were examined to increase resolution (R_s) including column selectivity and column length. A 2.1 mm \times 150 mm Agilent ZORBAX StableBond-C18 provided the best resolution. Further fine tuning included faster flow rates and mobile phase strength resulting in short analysis times of 3 to 1.5 minutes with R_s of 1.6 to 1.4. The performance of a 3.0 mm internal diameter (id) column was also compared to that of the 2.1 mm id column demonstrating higher sample loading before any loss of resolution in the closely spaced peaks.

Introduction

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 (milk and cheese)).

Analyzing Vitamin D in foods via LC/UV is popular because it is robust and inexpensive; however the food usually requires sample preparation such as saponification and extraction, which is complicated and time consuming [1]. Fortified foods include yogurt, cheese and milk, but orange juice and breakfast cereals are now often fortified. These newly fortified low-fat foods do not need saponification but are likely to still need time-consuming sample preparation. Reducing separation time while maintaining resolution of D2 and D3 is beneficial to the sample preparation-analytical separation workflow.



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Experimental

See individual figures for LC method details in addition to the following:

Standards: A standard stock solution of cholecalciferol (762 ppm) and ergocalciferol (1000 ppm) was made in a glycerin/water solution and diluted 1:20 with 50/50 acetonitrile/water. All samples were syringe filtered (0.2 μm) into autosampler vials for analysis.

LC: Agilent 1290 Infinity with G4212A DAD (diode array detector)

RRHD Columns for High Pressure Use:

Description	Size (mm)	Particle Size (μm)	Eclipse Plus C18	SB-C18	SB-C8
Solvent Saver HD, 1200 bar	3.0 \times 100	1.8	959758-302	858700-302	
Solvent Saver HD, 1200 bar	3.0 \times 50	1.8	959757-302	857700-302	
Narrow Bore RRHD, 1200 bar	2.1 \times 150	1.8	959759-902	859700-902	859700-906
Narrow Bore RRHD, 1200 bar	2.1 \times 100	1.8	959758-902	858700-902	858700-906
Narrow Bore RRHD, 1200 bar	2.1 \times 50	1.8	959757-902	857700-902	857700-906

Detection: Signal = 280,4 nm, Reference = 360,100 nm UV

Temperature: 25 $^{\circ}\text{C}$, maintained by the thermally controlled column compartment

Results and Discussion

A previously published fat soluble vitamin method demonstrated the different column selectivity (α) between Agilent ZORBAX Eclipse XDB-C18 and Agilent ZORBAX StableBond-

C18 (SB-C18) for vitamins D2 and D3 [2]. A new and improved ZORBAX stationary phase, Eclipse Plus C18 has superior silica manufacturing techniques and bonding technology over previous ZORBAX phases. Eclipse Plus C18 had not been previously evaluated for D2 and D3 selectivity and so was compared to SB-C18 (Figure 1).

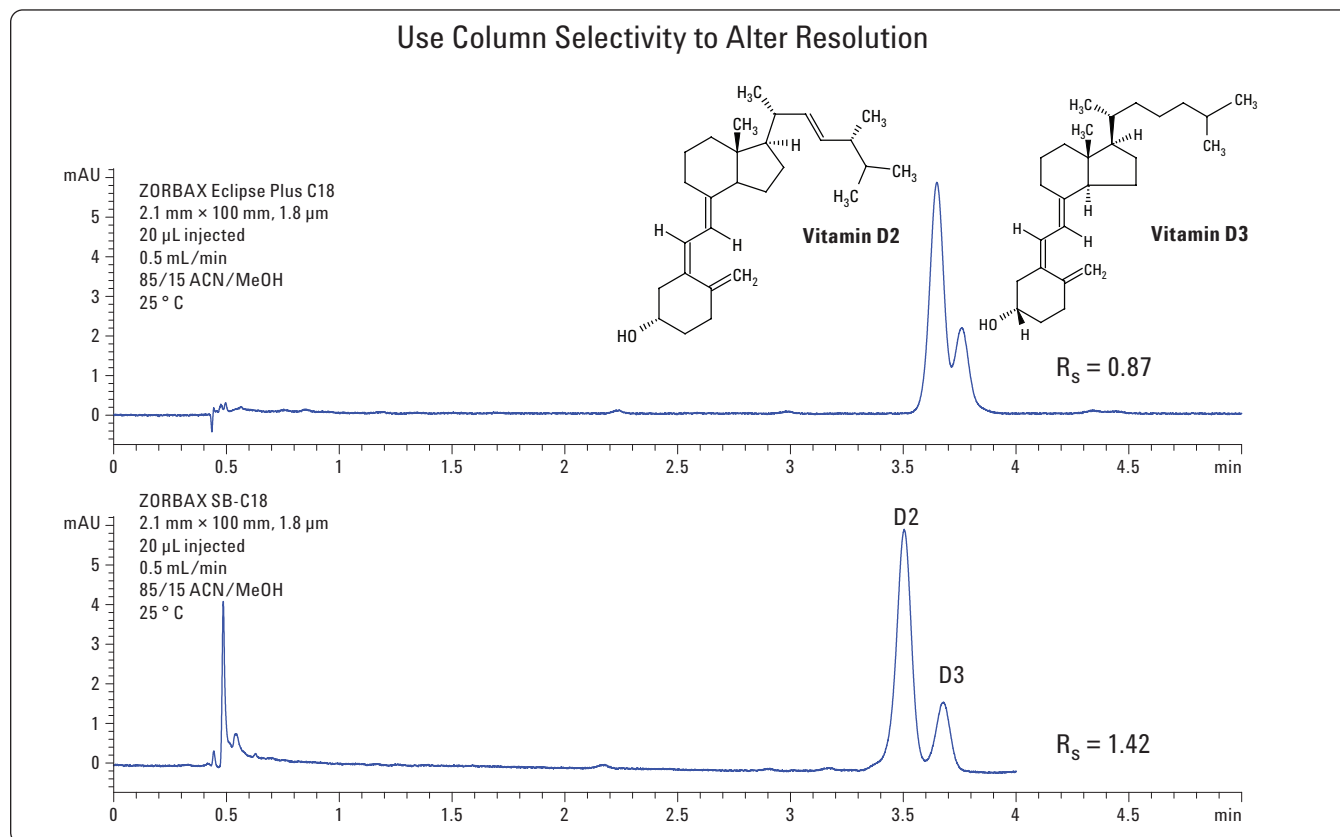


Figure 1. Vitamin D2 and D3 on distinct C18 columns.

The similar molecular structures of D2 and D3 result in similar retention, making them challenging to resolve. The SB-C18 resolved the D vitamins better compared to ZORBAX Eclipse Plus C18, under the same conditions. SB-C18 may resolve the D vitamins better because of the accessible silanols on the surface of the stationary phase. Silanols are endcapped on the Eclipse Plus phase to minimize secondary interactions of polar analytes, and allow longer column lifetime at higher pH. Accessible silanols found on SB-C18 are advantageous here, providing more selectivity to improve resolution. Column (stationary phase) substitution is an easy and fast method development technique for changing selectivity and consequently changing resolution.

Replacing a shorter column with a longer one is another easy and fast method development technique for changing resolution by increasing efficiency (N) (Figure 2). The 150 mm column increased resolution but also increased analysis time proportionally to the column length.

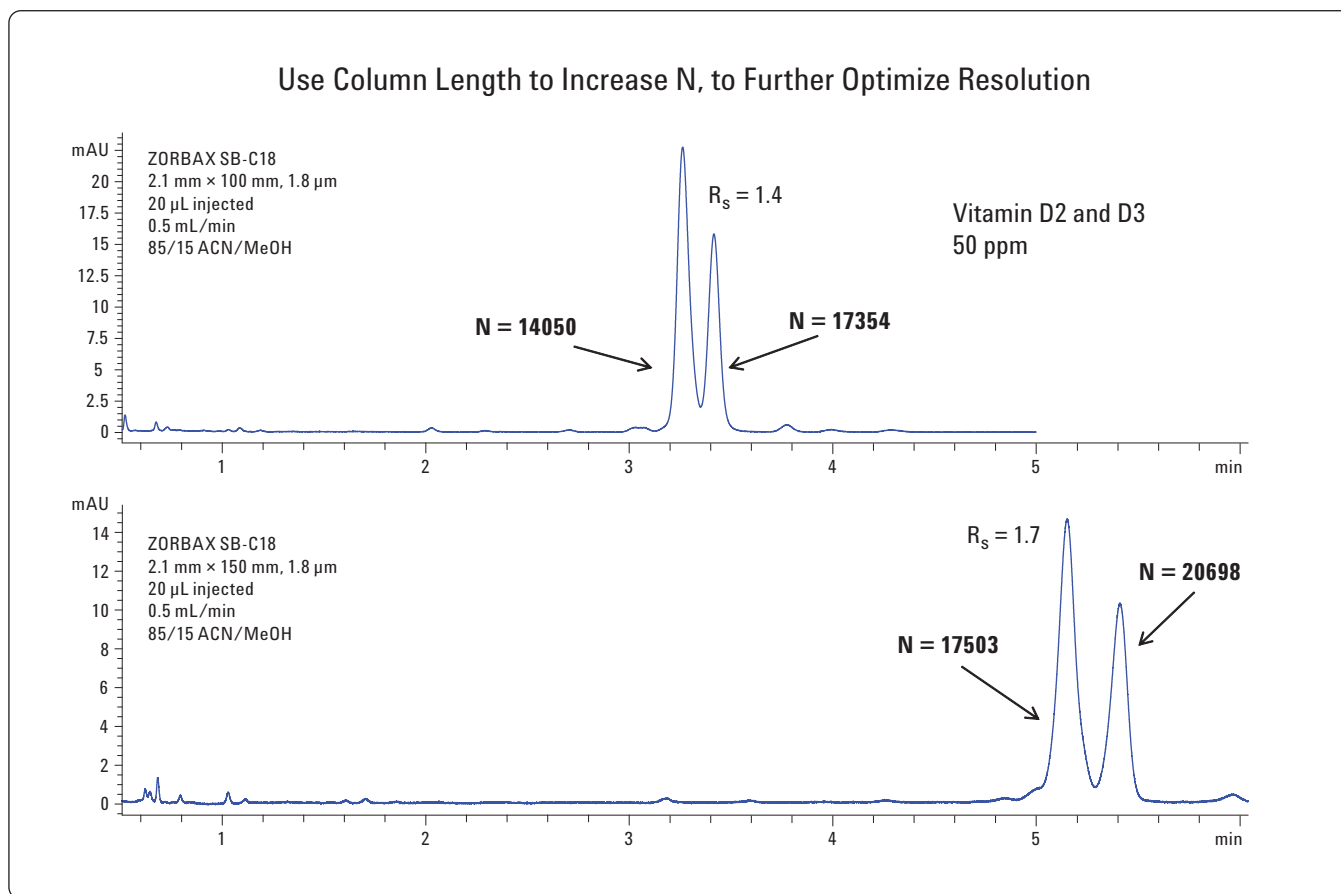


Figure 2. Comparison of a 100 mm to a 150 mm RRHD column.

The added analysis time can be reduced by increasing the flow rate. Figure 3 shows the vitamin D separation on a 150 mm column at three different flow rates (with a slightly different mobile phase composition). Retention was reduced proportionally to flow rate. Increasing the flow rate from 1 to 1.5 reduced peak retention by one-third. Developing the method with sub-two micron particles, 100 and 150 mm column lengths and high flow rates upgraded the HPLC method to a UHPLC method.

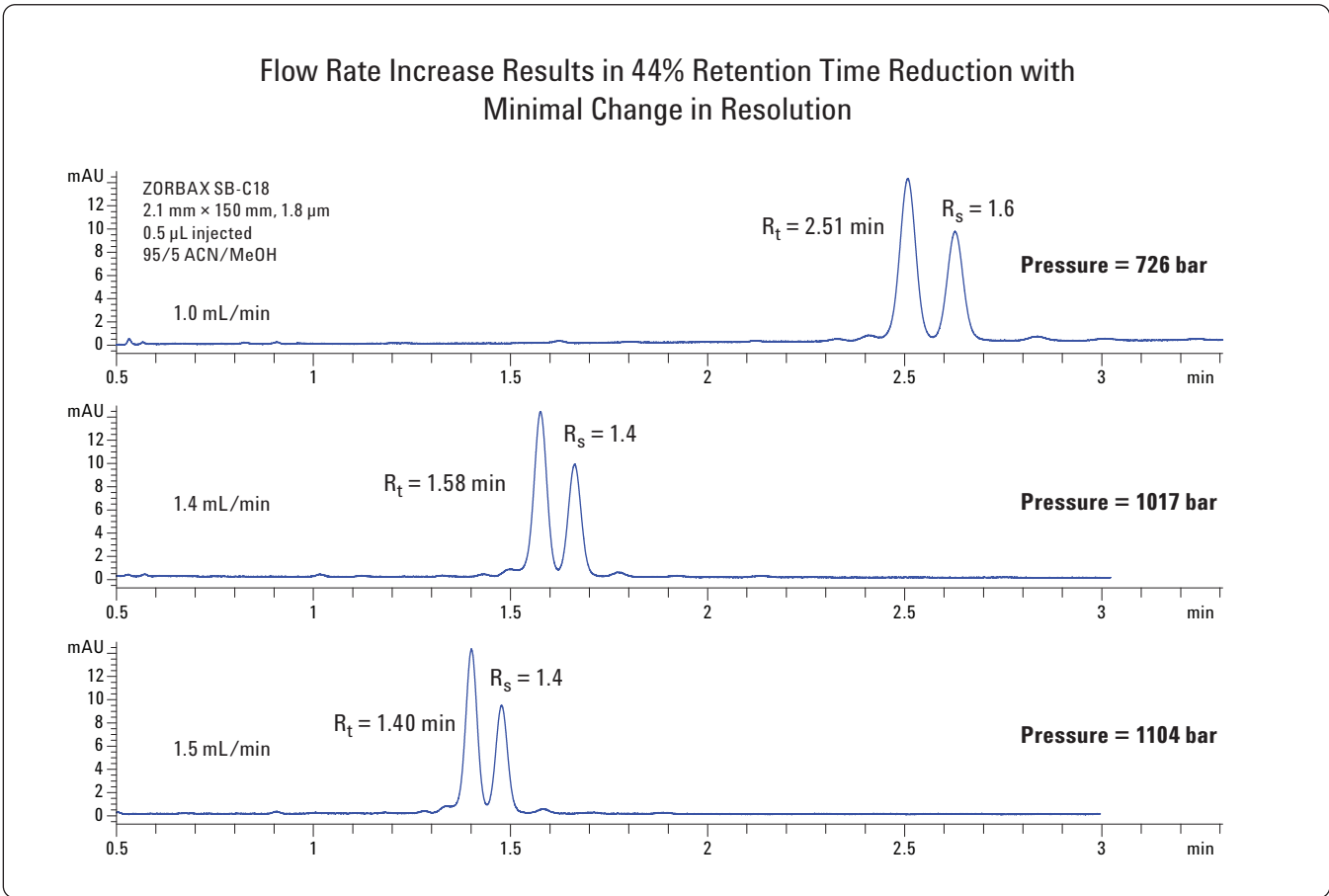


Figure 3. Comparison of flow rate on resolution of Vitamin D2 and D3.

3.0 mm id Solvent Saver Columns

Scaling or transferring a method from a 4.6 mm id to a 3.0 mm id column is advantageous because the flow rate is reduced proportionally to the ratio of the squares of the column diameters ($F_2 = F_1 (id_{col2} / id_{col1})^2$), thus reducing solvent usage by half. It can also be advantageous to scale from 2.1 mm id narrow bore to 3.0 mm id columns. Solvent Saver columns can handle more sample than smaller 2.1 mm columns. If enough sample is available, such as typical food analyses, it may be desirable to inject large volumes to detect

minor peaks that would otherwise be below the quantification limit if a smaller injection amount was used.

Sample overload however may occur with large injections. Injection volume or injection mass overload can cause peak broadening. Broadening of closely spaced peaks results in decreased resolution. Figure 4 compares different injection volumes on a 2.1 mm and 3.0 mm id column. The loss of resolution as sample load increases is more evident on the smaller diameter column.

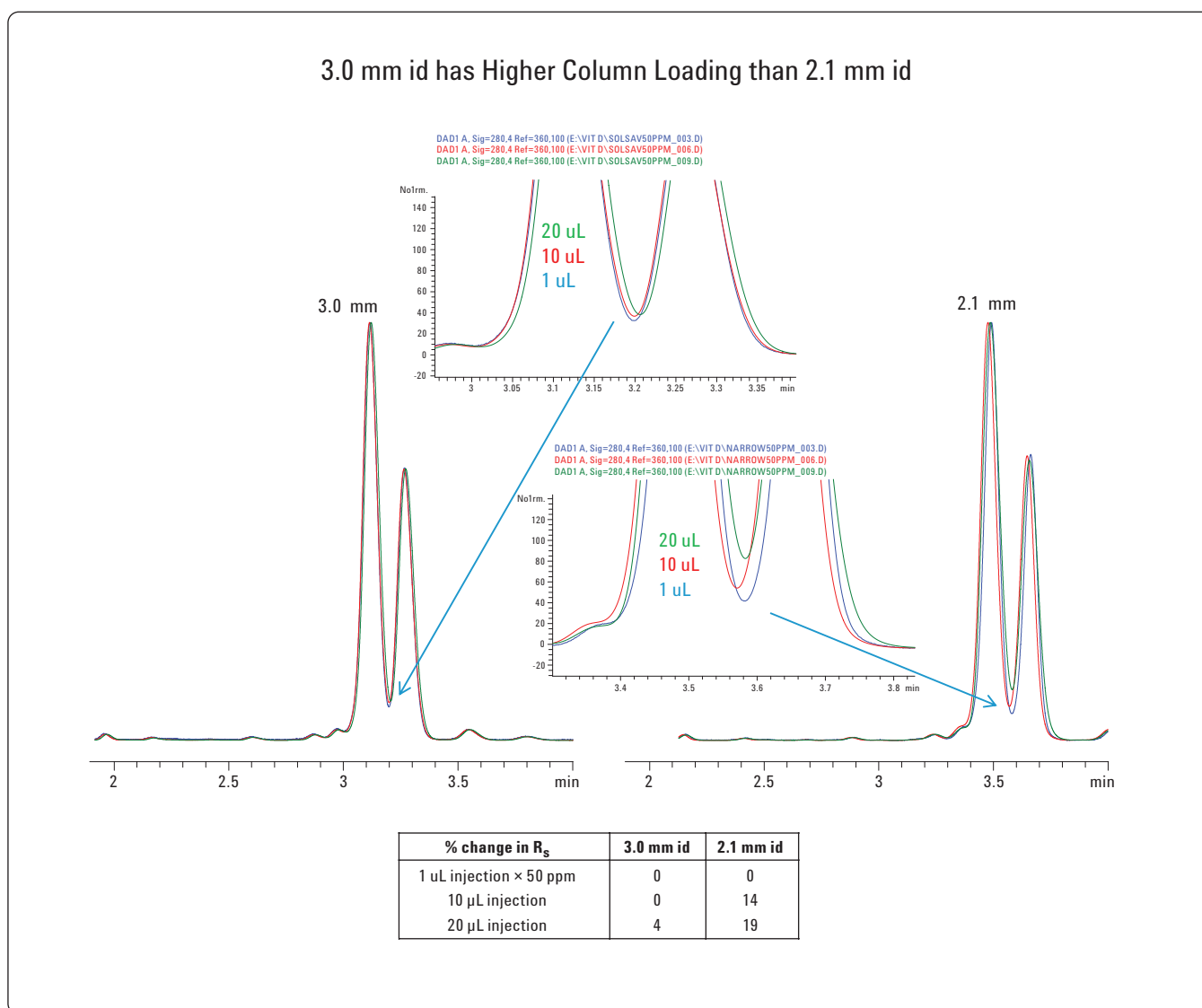


Figure 4. Comparison of injection amount on resolution between a 3.0 mm and a 2.1 mm id RRHD column.

Conclusion

Many method parameters can be fine tuned to improve a separation. Substituting columns is an easy and fast method development tool. Stationary phase or column selectivity can immediately alter resolution while keeping the mobile phase constant; even different C18 phases can have different selectivity. Using a longer column immediately adds efficiency (theoretical plates, N) and therefore increases resolution. Longer analysis times associated with longer columns can be countered by faster flow rates. RRHD columns and the Agilent 1290 Infinity LC are designed to operate up to 1200 bar to accommodate sub-two micron particle packing in 100 and 150 mm columns, with faster flow rates. This is popularly known as a UHPLC method. A vitamin D2 and D3 UHPLC method was developed from a HPLC method by substituting RRHD columns and increasing flow rates.

References

1. W. Craig Byrdwell et al, "Analyzing Vitamin D in Foods and Supplements: Methodologic Challenges," *American Journal of Clinical Nutrition*, 88(2)554S-557S, 2008
2. R. Ricker, "Comparison of Non-Endcapped Sterically Protected Silanes to Fully Endcapped Dimethylsilane", Hewlett Packard Lab Insights, FD15, 1998

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