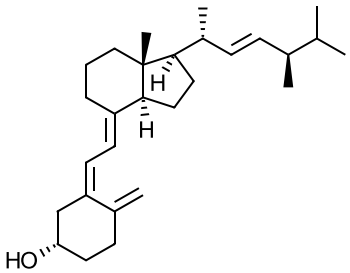
Separation of Vitamin D and Vitamin D Metabolites on FLARE C18 MM (Mixed Mode) HPLC Column

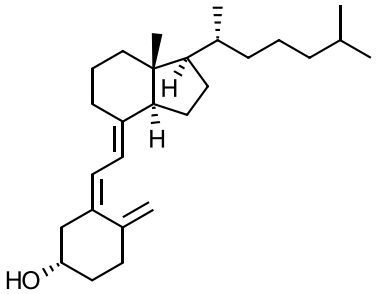
Introduction

In this technical note, the versatility of the diamond-based FLARE C18MM column is demonstrated in separating vitamin D_2 and D_3 and their derivatives. Additionally, a comparable separation on a silica-based C18 column is presented. Vitamin D is essential for adsorption of minerals in the intestines, for bone and muscular health as well as the prevention of chronic conditions such as autoimmune disorders, diabetes and heart disease. This essential vitamin is obtained from supplements and food such as meat and eggs and from exposure to sunlight. Through the analysis of vitamin D levels in human serum, current levels of vitamin D in an individual can be determined. When compared to standard levels, deficiencies can be accurately diagnosed and treated. The FLARE column, packed with 3.6µm superficially porous particles that have a unique stationary phase, allows for separation of closely related vitamin D compounds and their metabolites. The chromatographic resolution of these compounds is vital given that isomers of vitamin D are isobaric (same molecular weight) and MS detection is inadequate. The separations shown here are achievable on any traditional LC system and the solvents used are MS-compatible.

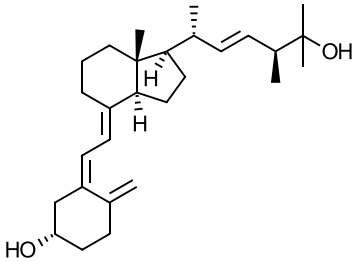
Vitamin D Chemical Structures



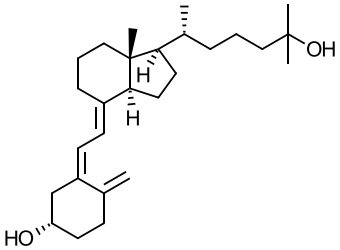
1. Vitamin D_2 (ergocalciferol)



2. Vitamin D_3 (cholecalciferol)



3. 25 (OH) vitamin D_2 (ercalcidiol)



4. 25 (OH) Vitamin D_3 (calcifediol)

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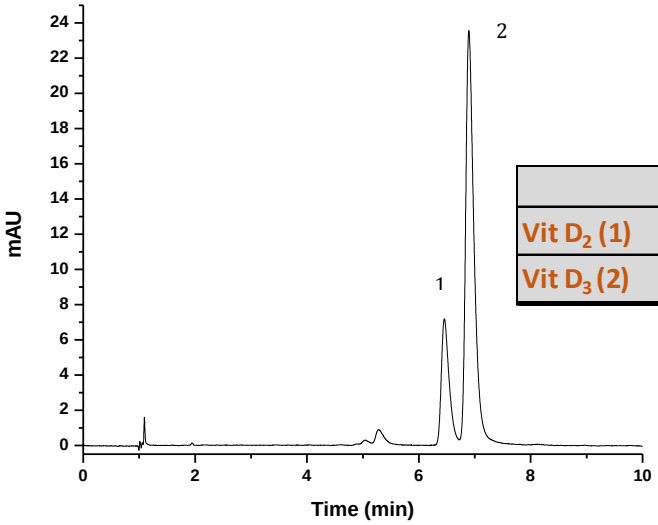
HPLC Conditions

Instrument: Agilent 1290
Column: FLARE C18MM 150 x 4.6mm, 3.6µm (SN: 15698.35-1); Competitor C18 fully porous silica C18, 150 x 4.6mm, 3.5µm
Mobile Phase: A: H_2O; B: ACN; A/B: 20/80
Flow Rate: 1.0 ml/min
Injection: 0.5 µl in ACN
Column Temperature: 35^0C
Elution Mode: Isocratic

Discussion

For the HPLC analysis, all tests were ran under isocratic conditions using a mobile phase containing water and ACN without any additives. The parameters are described in detail above. The analytes were dissolved in ACN to prepare test solutions of ca. 0.5mg/ml.

Figure 1 shows the analysis of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). The compound structures were shown earlier. A baseline separation with Rs of 1.73 can be observed for these closely related compounds using a 150 x 4.6 mm FLARE C18MM column. The separation run time is less than 10 mins.



	Rt (mins)	Rs	α	N
Vit D_2 (1)	6.453			10830
Vit D_3 (2)	6.891	1.73	1.09	11417

Figure 1: Separation of vitamin D_2 (1) and vitamin D_3 (2) on **FLARE C18MM column**

For comparison, the same separation was performed on a leading silica-based C18 column. Under identical separation conditions, the best resolution obtained was 0.83- since Rs is less than 1.5 this will make quantitation difficult, likely resulting in erroneous analytical results. Moreover, the retention time was over 17 minutes, almost three times as on the FLARE column.

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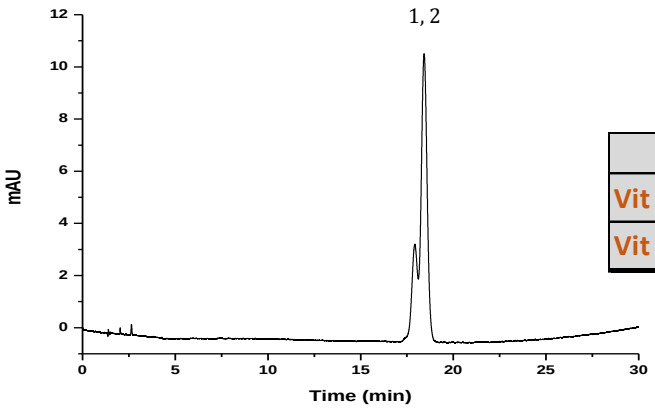
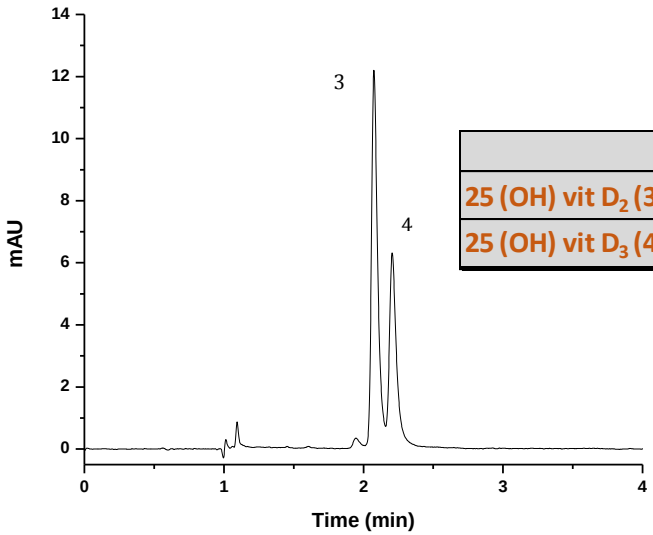


	Rt (mins)	Rs
Vit D_2 (1)	17.925	
Vit D_3 (2)	18.426	0.83

Figure 2: Separation of vitamin D_2 and vitamin D_3 on a **leading silica C18 column**

In the human body, vitamins D_2 and D_3 are converted by the liver into various compounds which are more abundant and can be monitored to determine vitamin D levels. The amount of these compounds in human serum also indicates how well the body is metabolizing the vitamin. There are various metabolic products on the market, but among the most important are 25 (OH) Vitamin D_2 (ercalcidiol) and 25 (OH) vitamin D_3 (calcifediol). In Figure 3 below, a baseline separation of these two metabolites is accomplished in less than 6 minutes.



	Rt (mins)	Rs	α	N
25 (OH) vit D_2 (3)	2.075			11773
25 (OH) vit D_3 (4)	2.205	1.6	1.23	10307

Figure 3: Separation of vitamin D_2 and vitamin D_3 metabolites, 25 (OH) vitamin D_2 and 25 (OH) vitamin D_3, on **FLARE C18MM column**

To improve the utility of this work, in future versions of this technical note, the following iterations will be considered:

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1. Investigation of other vitamin D metabolites, including 3-*epi*-25-Hydroxyvitamin D_3 and 1,25 diOH vitamin D_2 and D_3.
2. Analysis of vitamin D in serum and/or blood matrices

Conclusion

The Diamond Analytics FLARE core-shell column is effective in analyzing vitamin D and its metabolites. The added advantage of this column is its ability to operate under a wide range of solvent, temperature and pH conditions. The unique selectivity of the FLARE column allows for separation, identification and quantitation of isomeric forms of vitamin D. The method presented above is MS-compatible and can be used on any HPLC system.

References

1. Hymoller, Lone; Jensen, Soren Krogh. Vitamin D analysis in plasma by high performance liquid chromatography (HPLC) with C30 reversed phase column and UV detection – Easy and acetonitrile-free. J. CHrom A, 2011, 1218(14), 1835-1841
2. Lensmeyer, Gary L; Wiebe, Donald A.,; Binkley, Neil; Drezner, Marc K. HPLC Method for 25-Hydroxyvitamin D Measurement: Comparison with Contemporary Assays. Clinical Chemistry, 2006, 52(6), 1120-1126

About Diamond Analytics

Diamond Analytics, a US Synthetic company, expands the existing range of analytical capabilities in separation science by providing diamond-based solutions that allow for the exploration of novel chemistries. The company is a pioneer in the creation of non-silica diamond core-shell particles that are chemically modified for use in High Performance Liquid Chromatography (HPLC).

As a result of breakthroughs in diamond technology, Diamond Analytics' HPLC columns offer expanded pH range capability (1-13), elevated temperature ranges (up to 100 °C), increased longevity, novel selectivity and uncompromising efficiency—leading to a longer, more productive life and increased total cost savings. All Diamond Analytics columns are LC-MS compatible and can operate from 0% to 100% organic/aqueous mobile phases. Particle manufacturing uses 99.999% pure diamonds to minimize secondary interactions with metals.

The parent company, US Synthetic, was founded in 1978. The Diamond Analytics division was created in 2005 with over 50 years of combined experience in chromatography.

Column Phases

FLARE C18 MM (Mixed-Mode)
Operates primarily in a reversed-phase and anion exchange mode, depending on the pH of the mobile phase. At low to medium pH, the column is a good option for analysis of neutral compounds, acids and anionic compounds. At high pH, the column is also a good option for analysis of bases with good peak shape.

FLARE C18+
Exhibits a permanent positive charge regardless of the pH of the mobile phase. It is a good option for the analysis of polar molecules (including carbohydrates, sugars and steroids) and anionic species. It also works for hydrophobic compounds where low retention is desired.

FLARE HILIC
Has a hydrophilic amino-diol surface and is able to retain polar molecules at all pH conditions. Works well for the analysis of sugars, nuclotides, and polar APIs.

FLARE Wide Pore (WP) C18
Works primarily in reversed phase mode due to the column's protein interactions with C18 ligands. Optimal pore size and premeability allows for retention of both small and large proteins (up to 500kDa). Has been effectively used in analyzing mAbs, ADCs, insulin, hemoglonin, etc.

Column Dimensions

ID/Length (mm)	20	30	50	75	100	150
2.1	x	x	x	x	x	x
4.6	x	x	x	x	x	x

Other custom dimensions are also available from capillary to prep

For more information or to order a column,

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