



# Separating Vitamin D<sub>2</sub> and D<sub>3</sub> their 25-OH Metabolites and C-3 Epimers



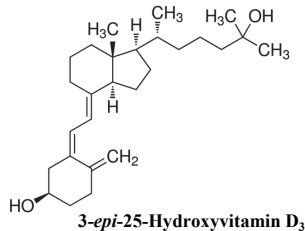
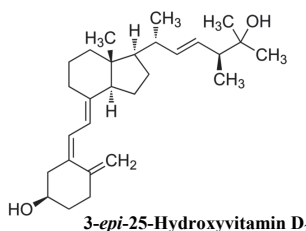
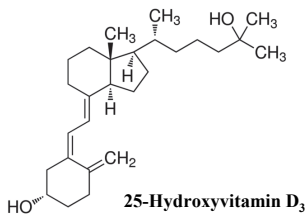
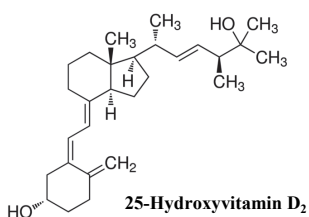
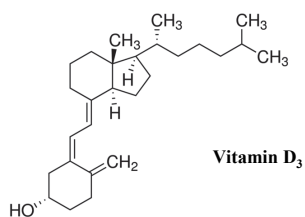
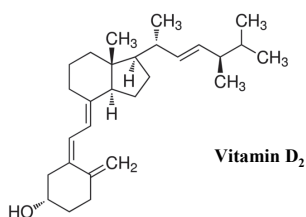
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## Introduction

The accuracy of current vitamin D measurements by immunoassays and LCMS have been questioned due to the overlapping LC peaks with identical m/z values epimers. To solve this problem, we have developed a new HPLC method to achieve baseline separation of vitamin D<sub>2</sub>/D<sub>3</sub>, their 25-OH metabolites and C3-epimers in one single run. A novel core-shell type reversed-phase HPLC column with Cholesterol as the functional group (Cosmocore Cholester) is used in this study. The Cosmocore Cholester column has similar hydrophobicity as regular C18 columns, so it is used in the same exact way. The baseline separation is so complete that it can be used for quantification by UV detector alone at low concentrations. Gradient condition can be employed to further separate vitamin D<sub>2</sub> and vitamin D<sub>3</sub> and their four metabolites/epimers, all in one LCMS (or UV) run.

## Vitamin D Structures

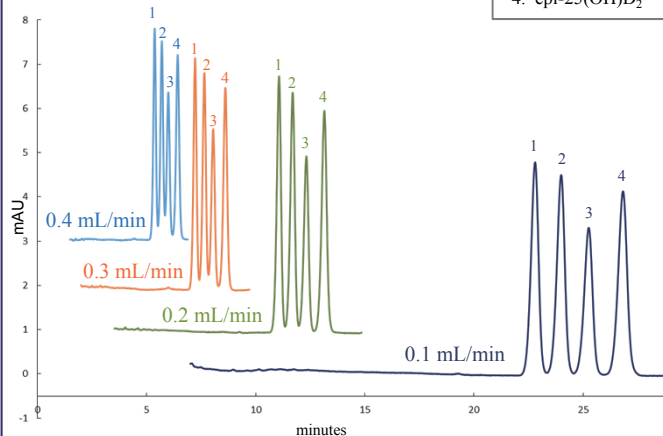


## Vitamin D Metabolite and Epimer Separation – Flow Rate Comparison

Column: COSMOCORE Cholester  
Column size: 2.1x150mm, 2.6 μm core-shell particles  
Flow rate: 0.4 mL/min  
Temperature: 50 °C  
Detection: UV 265 nm  
Isocratic: 20A:80B A: 0.1% formic acid in H<sub>2</sub>O B: methanol

### Samples

1. 25(OH)D<sub>3</sub>
2. *epi*-25(OH)D<sub>3</sub>
3. 25(OH)D<sub>2</sub>
4. *epi*-25(OH)D<sub>2</sub>

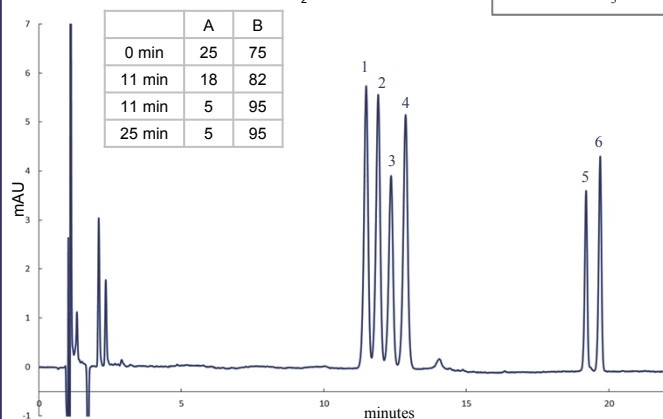


## Vitamin D, Metabolite, and Epimer Separation

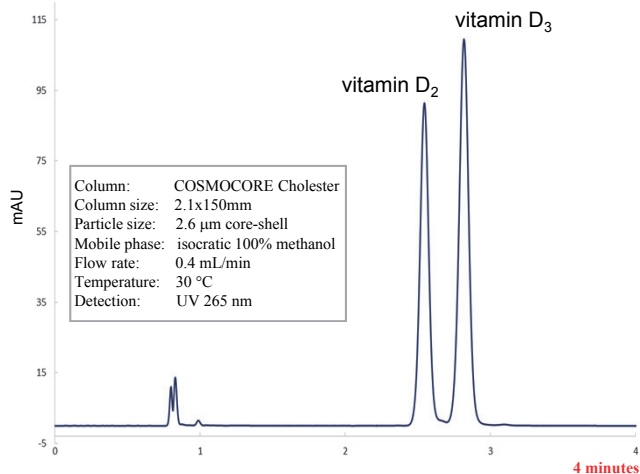
Column: COSMOCORE Cholester  
Column size: 2.1x150mm, 2.6 μm core-shell particles  
Flow rate: 0.3 mL/min  
Temperature: 50 °C  
Detection: UV 265 nm  
Data: blank subtraction performed  
Gradient: A: 0.1% formic acid in H<sub>2</sub>O B: methanol

### Samples

1. 25(OH)D<sub>3</sub>
2. *epi*-25(OH)D<sub>3</sub>
3. 25(OH)D<sub>2</sub>
4. *epi*-25(OH)D<sub>2</sub>
5. vitamin D<sub>2</sub>
6. vitamin D<sub>3</sub>



## Vitamin D Separation



## Conclusion

- Vitamin D<sub>2</sub> and D<sub>3</sub> isocratic separation under 3 minutes using 100% MeOH
- 25(OH) Vitamin D<sub>2</sub> and D<sub>3</sub> metabolites and C-3 epimers can be baseline separated under isocratic condition at low concentration
- All six vitamin D and associated metabolites were separated in a single HPLC gradient run.