SFC Analytical Method Development for Vitamin D3 and Related Compounds

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Introduction

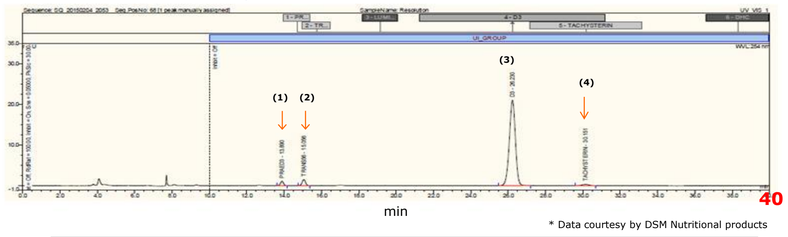
By utilizing the advantages of Supercritical Fluid Chromatography (SFC) such as high permeability and diffusibility, we could generally achieve higher resolution by SFC analysis in a shorter run time than by HPLC. Thus a combination of achiral columns and SFC is one of the effective strategies for reducing analysis cycle time of natural products such as fat-soluble vitamins and terpenoids.

It has currently been recognized that some normal-phase HPLC methods to assay Vitamin D3 and three related compounds (Pre-Cholecalciferol, 5,6-*trans*-Cholecalciferol, Tachysterol$_3$) with various impurities in nutritional products are insufficient because analysis times of them are slightly longer (40 minutes；shown below) and integration of them into a single method is difficult.

In this research, we tried to develop a robust and efficient SFC method to solve these problems.

LC Method Condition

Column : Hypersil SILICA (3 µm) 250 x 4.6 mmi.d.
Mobile phase : n-Hexane (including 1-Octanol, 1-pentanol and acetonitrile)
Detection : UV at 254 nm
Flow rate : 0.8 mL/min



* Data courtesy by DSM Nutritional products

Those compounds are referred to on chromatograms by the numbers indicated.
(1) Pre-cholecalciferol (3) Cholecalciferol (Vitamin D3)
(2) 5,6-*trans*-Cholecalciferol (4) Tachysterol$_3$

Guidelines for the SFC Method

- Single method which can be applied to all samples.
- Analysis time to be under 10 minutes.
- Resolution (Rs) to be not less than 1.5 (between Pre-cholecalciferol and 5,6-*trans*-Cholecalciferol).
- Tailing factor to be between 0.8 and 1.5.
- Repeatability of the method to be within RSD 1.0% for six consecutive injections.
- Highly robust, as wide linearity range as HPLC method and accurate method.

Samples[*1]

Number	Compound	Concentration (if not specified)
1	Cholecalciferol	5 µg/mL
2	Concentrated cholecalciferol powder, thermally stressed[*2]	25 µg/mL
3	Animal nutrition powder form (Rovimix® AD3 1000/200)	25 µg/mL
4	Water-miscible form	25 µg/mL
5	Oily form	25 µg/mL

[*1] Supplied by DSM Nutritional Products
[*2] Intentionally prepared to assign cholecalciferol related compounds. Not commercially available.

1. Method Scouting (Outline of Method Scouting)

[Column]
① YMC-Triart Diol
② YMC-Triart PFP
③ YMC-Triart C18
④ YMC-Pack SIL
⑤ YMC-Pack CN
5 µm, 150X4.6 mmI.D.

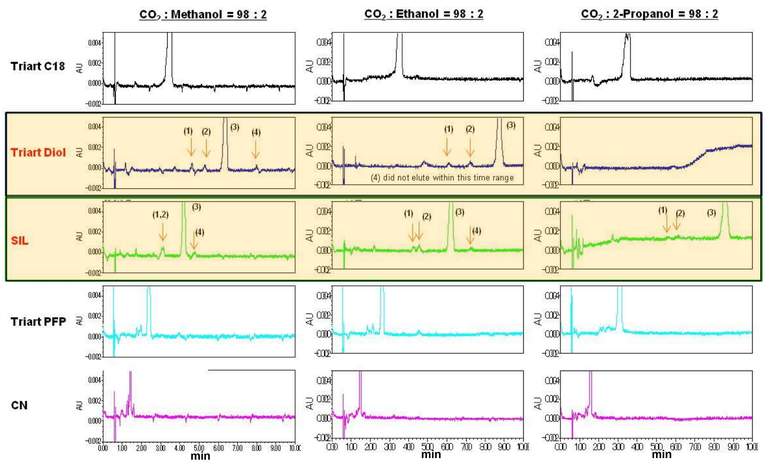
×

[Mobile phase]
❶ A) CO_2 B) methanol
❷ A) CO_2 B) ethanol
❸ A) CO_2 B) 2-propanol
2 %B

Flow rate : 3.0 mL/min
Temperature : 35 ℃
Detection : UV at 254 nm
Back pressure : 2000 psi
Sample : Sample #2
Injection : 10 µL
System : Waters UPC^2

1-1. Packing Material Selection

- Triart Diol and SIL showed good separation.



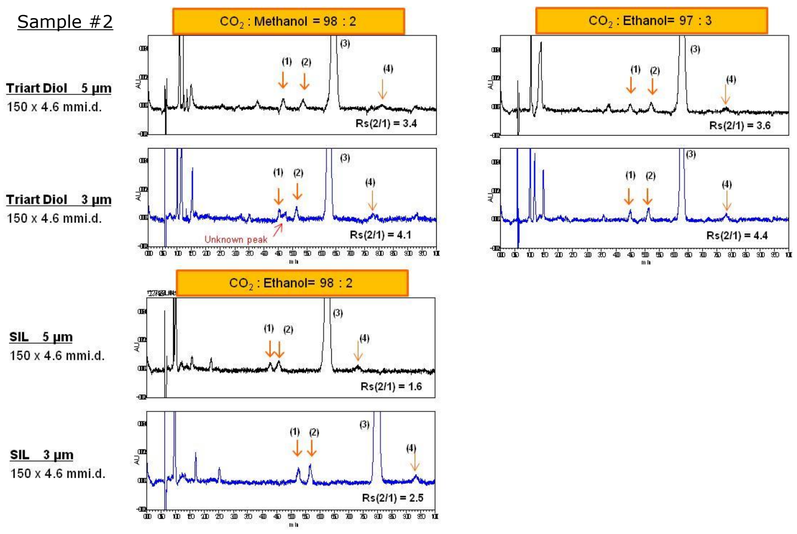
1-2. Modifier Selection

- Methanol or ethanol is the candidate to obtain good separation. (detected chromatograms above)

2. Optimization

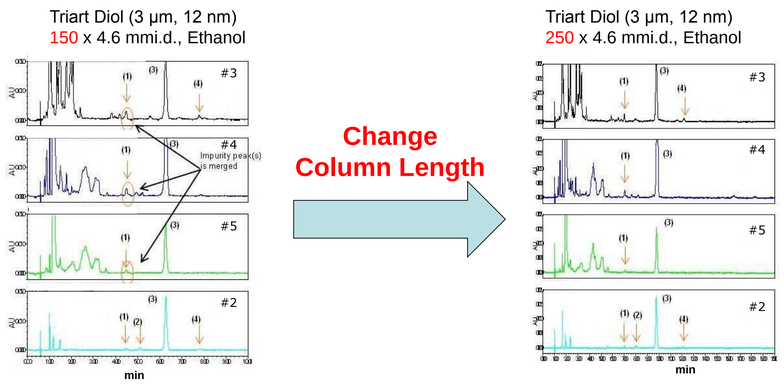
2-1. Particle Size (5 µm → 3 µm)

- 3 µm is the better option to obtain more efficient method.
- SIL showed unknown retention difference between two particles. Column history or slight difference in gel parameters might be the cause. Therefore SIL is not recommended from the standpoint of method robustness.



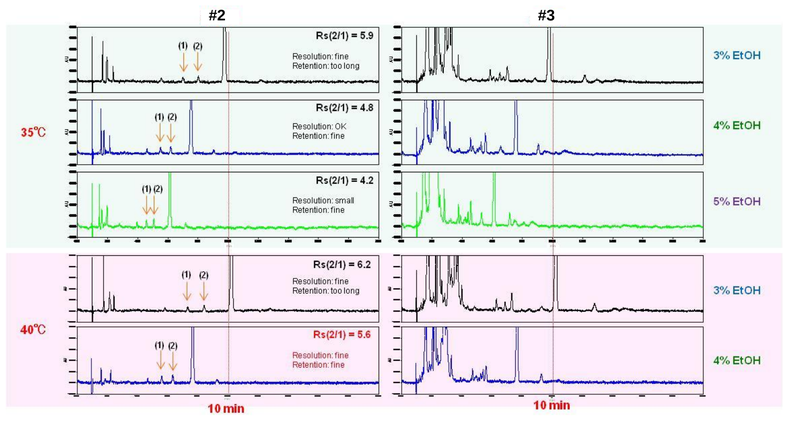
2-2. Column Size (150X4.6 mmi.d. → 250X4.6 mmi.d.)

- Pre-cholecalciferol and impurity were well separated on Sample #3, 4, and 5 using 250 mm length column.



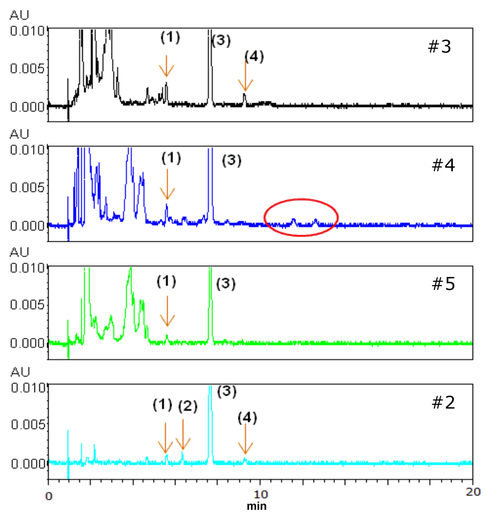
2-3. Column Temperature and Modifier ratio

- Column temperature of 40℃ gave slightly longer retention than 35℃, but resolution was better on 40℃.
- By setting the modifier ratio at 4-5%, total analysis time of < 10 min is achieved.
- 4% Ethanol + 40℃ is determined as the optimal conditions.



3. Analysis of nutritional product

- Separation of major components and impurities was achieved.
- Total analysis time was within 10 min. (Only except for #4; impurity peak eluted (marked in circle) at around 12 min.)



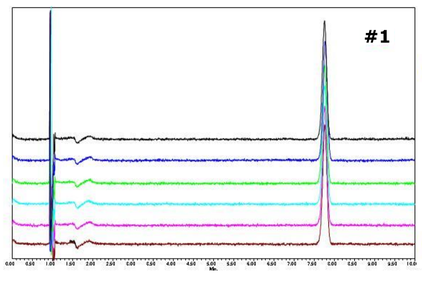
#2	Tf	Rs
(1)	0.973	
(2)	0.949	3.879
(3)	0.947	6.873
(4)	0.764	8.034

4. System Validation

4-1. Injection Reproducibility

- RSD ≤ 1.0 for all parameters is achieved on Cholecalciferol.

Overlay of six consecutive injections



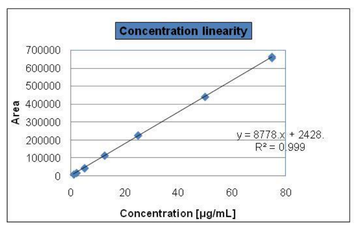
Summary of six consecutive injections

Cholecalciferol			
n	tR(min)	Area	Height
1	7.756	125697	16137
2	7.769	125745	16173
3	7.755	127199	16137
4	7.767	126166	16160
5	7.761	125980	16242
6	7.762	126667	16327
AVG.	7.762	126242	16196
STD.	0.006	585.65	74.919
RSD.	0.073	0.464	0.463

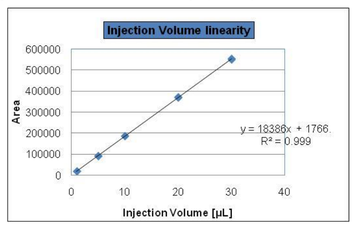
4-2. Linearity

- Linearity of both concentration and injection volume is confirmed.

Concentration linearity (Sample: #1)



Injection volume linearity (Sample: #1 = 25 µg/mL)

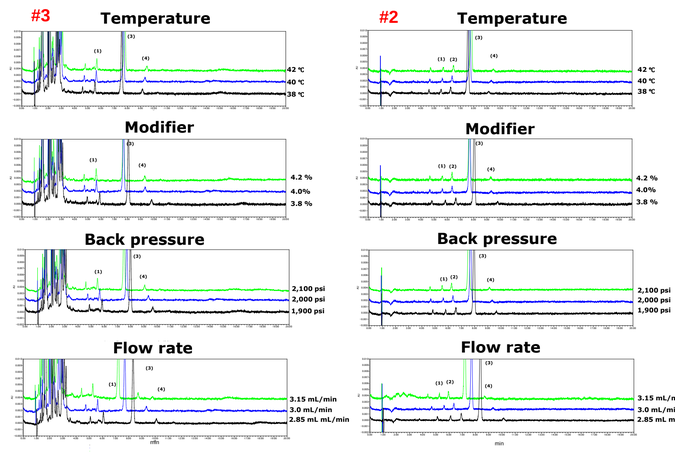


4-3. Robustness

- Cholecalciferol concentrations under each condition were consistent and within acceptable RSD.
- It is concluded that the method developed has sufficient robustness.

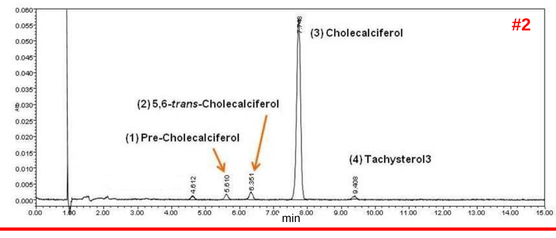
Impact of each parameter below was estimated

- Sample: #3, #2
- Parameters changed
  - Temperature : 38℃ & 42℃ (n=2)
  - Modifier : 3.8% & 4.2% (n=2)
  - Backpressure : 1,900 psi & 2,100 psi (n=2)
  - Flow rate : 2.85 mL/min & 3.15 mL/min (n=2)



Developed SFC Method Condition

Column : Triart Diol (3 µm, 12 nm) 250 x 4.6 mmi.d.
Mobile phase : CO_2/ Ethanol (96/4)
Flow rate : 3.0 mL/min
Temperature : 40 ℃
Detection : UV at 254 nm
Back pressure : 2,000 psi (as BPR)
System : Waters UPC^2



Cross-Reference of Guidelines and Final Results

Item	Requirements	Proposed method
Total run time	Less than 10 min	Less than 10 min. except for #4 (Impurities in #4 will elute at around 12min)
Resolution	Rs>1.5 (as Pre-cholecalciferol and 5,6-trans-Cholecalciferol)	Achieved
Tailing factor	0.8 < TF <1.5	Achieved
Injection reproducibility	RSD: less than 1.0% for six consecutive injections	RSD of Cholecalciferol is less than 1% for all parameters (tR, Area, and Height).
Method robustness	As robust as possible	High separation reproducibility is achieved between two lots.
Robustness	Adequate robustness	Method robustness is confirmed. Quantitative result is not affected by method parameters and separation is consistent between column lots
Linearity	Linearity	Linearity is secured between 0.01 µg and 0.75 µg loading

Conclusion

We have developed a robust and efficient SFC analysis method for Vitamin D3 and three related compounds. The established method can separate Vitamin D3, its related compounds, and impurities in the nutritional products. Analysis time was decreased to ten minutes with keeping good peek separation. Furthermore, analysis reproducibility and linearity offered reliability which is commonly required as a quantitative analysis method.

Acknowledgement

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