SFC — a Powerful Tool for Polymethoxyflavone Isolation and Analysis

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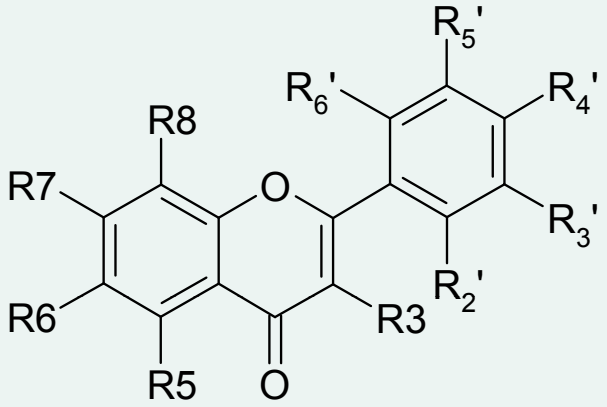
Introduction -- Polymethoxyflavones

- Polymethoxyflavones, or PMF, is a group of natural products that almost exclusively exist in citrus plants, especially in citrus peels.
- PMFs have a basic flavone skeleton with multiple substitutions of -methoxy groups.
- PMFs have received increasing attention for their health benefit, anti-inflammatory, anti-viral, etc.



R3 to R8 & R2' to R6' = H, or OMe

Polymethoxyflavone

Content

- Part I: Prep-SFC Isolation of polymethoxyflavones (PMFs) from citrus peel extract
- Part II: Analytical SFC method development for urinary metabolites of nobiletin
- Part III: Analytical method development for PMF product quality control

Part I.

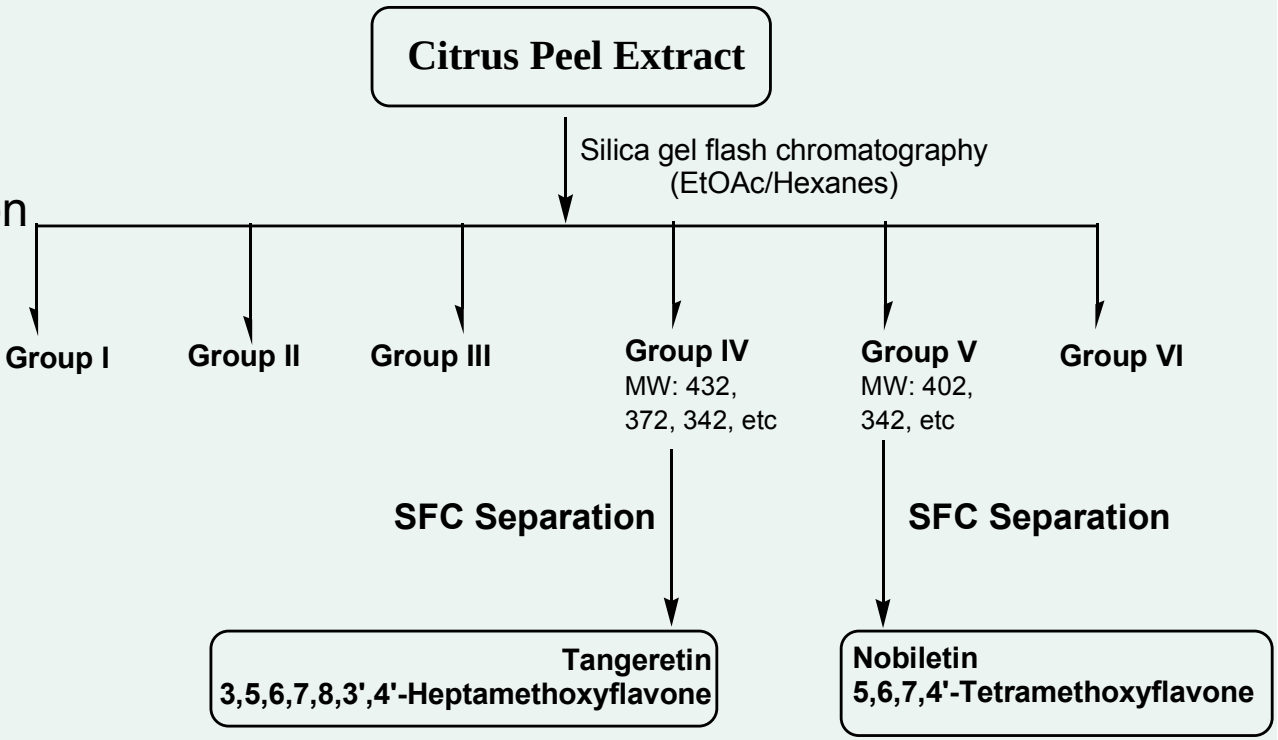
Prep-SFC Isolation of polymethoxyflavones from citrus peel extract

Isolation of PMF from Citrus Peels

- The major components in citrus peels include flavonoids (mainly PMFs), terpenoids, and other volatile compounds.
- Most of the time, PMF mixtures from citrus peel extracts were used *in vivo* study.
- The individual component of PMFs has been rarely used due to its limited availability and high cost.
- To better understand PMF efficacy and safety profile, individual PMF needs to be evaluated in *in vitro* and *in vivo* studies.

Isolation of PMFs from Citrus Peel Extract

- Normal phase flash chromatography for preliminary separation of crude citrus peel extracts.
- Collect six fractions based on polarity.
- Group IV and V are most of interests.
- Analytical method development found chiral SFC superior than other methods (LC, achiral SFC).

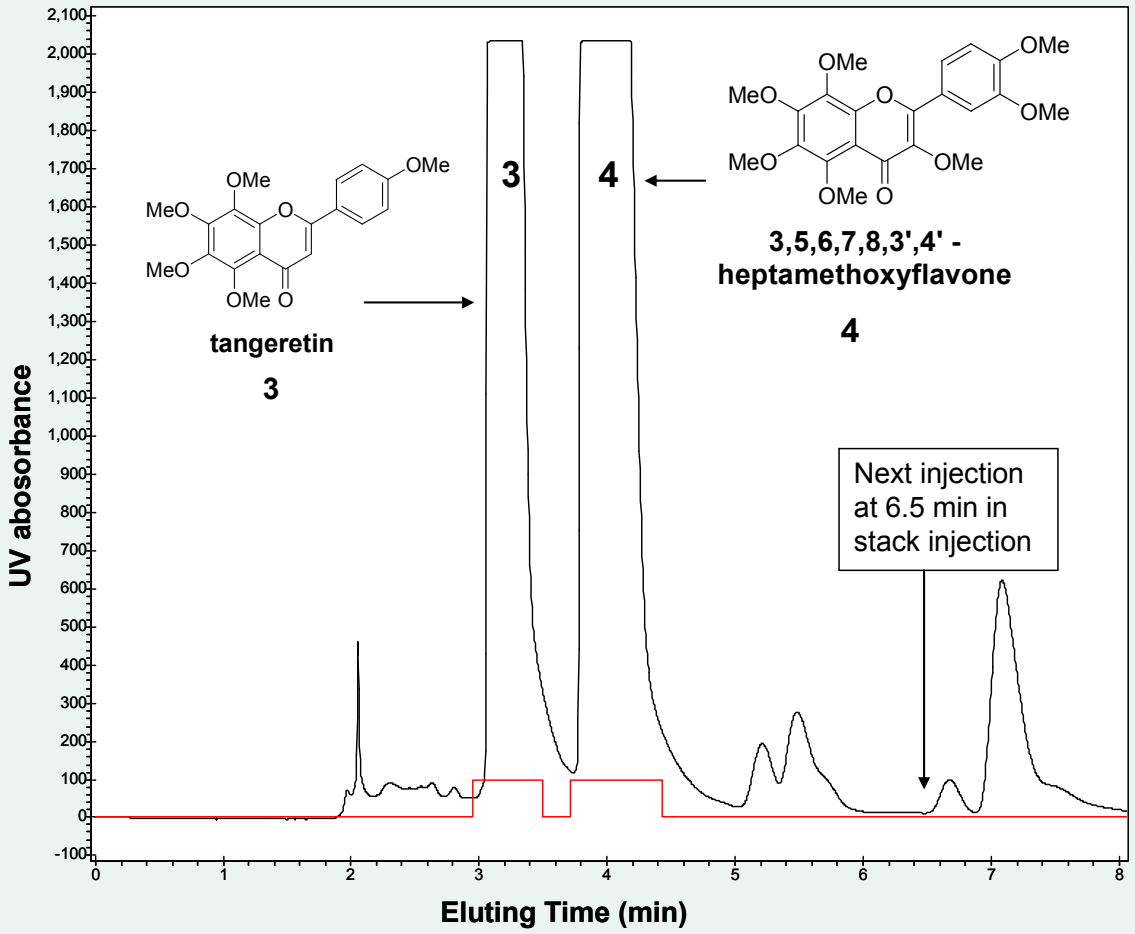


Prep Chiral SFC Conditions

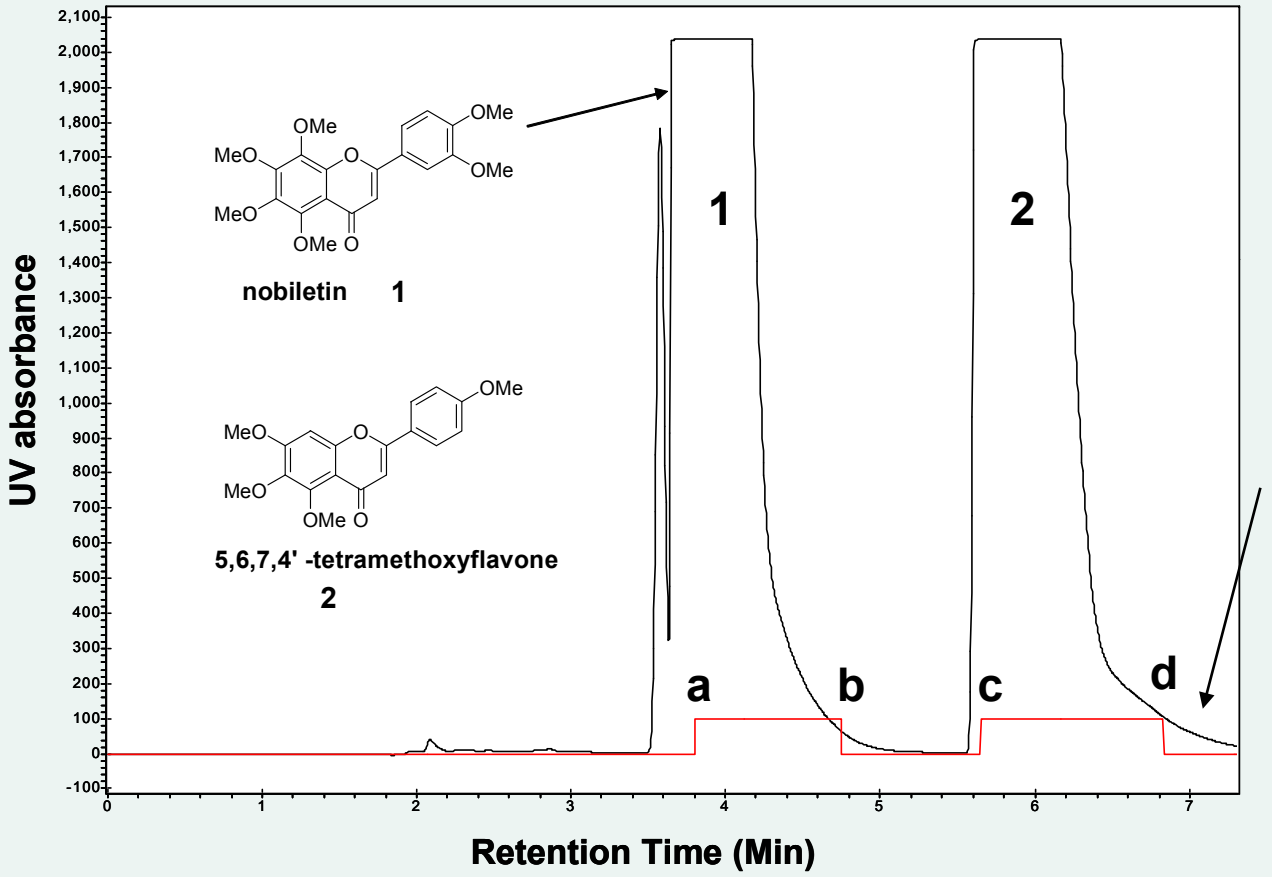
- Berger MultiGram II Prep-SFC
- Column: Daicel Chiralpak AD 250 x 30 mm, 10 µm
- Column temperature: 30 ºC
- CO_2 Pressure: 100 bar
- Co-solvent: methanol 45%
- Flow rate: 70 mL/min
- UV: 220 nm
- Injection: Stack Injections with 0.8 mL per injection

J. Chromatogr. B, 846 (2007) 291-297

Chiral SFC Separation of Group IV

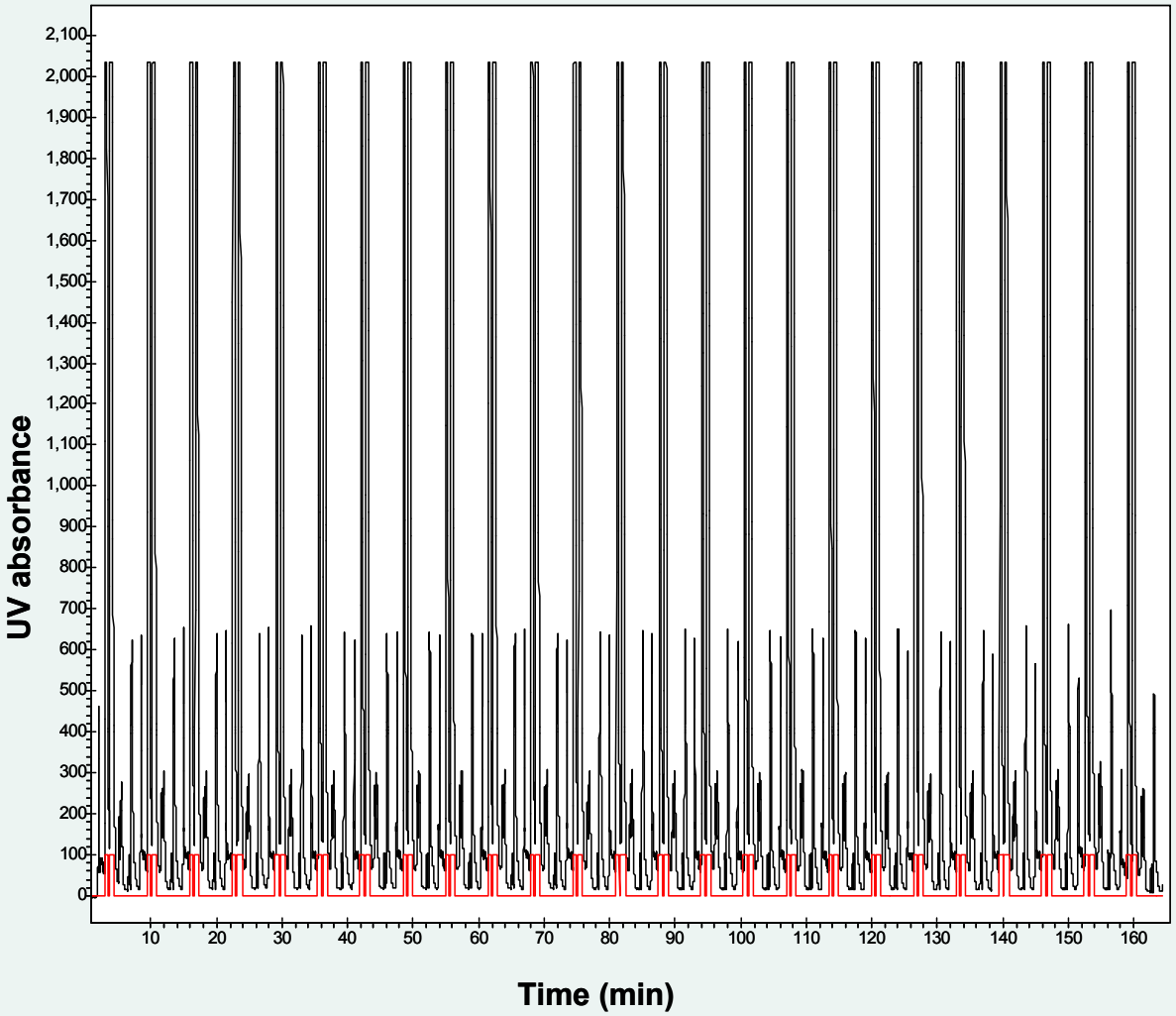


Chiral SFC Separation of Group V



Cycle time: 7 min

Stack Injections of Group V



25 stack injections,

80 mg of crude Group V per injection

SFC Isolation--Summary

Method	Preparative Chiral SFC			
Column	Daicel Chiralpak AD, 30 x 250 mm, 10 μm			
Co-Solvent	45% Methanol			
Groups	Group IV		Group V	
Amount loaded	2.0 g		3.3 g	
Isolated PMF	Tangeretin	3,5,6,7,8,3′,4′-Heptamethoxyflavone	Nobiletin	5,6,7,8,4′-Tetramethoxyflavone
Isolated amount	0.72 g	0.73 g	1.68 g	1.30 g
Purity by HPLC-UV/MS	> 99	> 99	> 99	> 99
Time per injection cycle	6.5 min		7 min	
Total run time	163 min		175 min	

Fun Calculations

Total pure PMF obtained on prep-SFC:

0.72 g + 0.73 g + 1.68 g + 1.30 g = 4.43 g

Price for pure PMF:

$300,000 per gram*

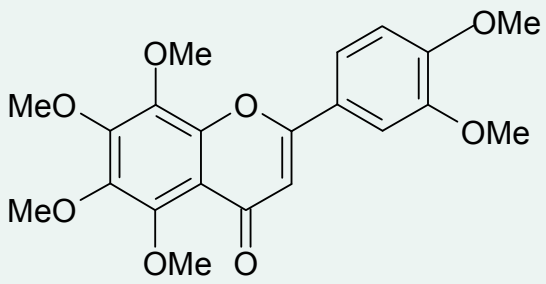
Become a millionaire in one day:

4.43 g x $300,000/g = $1.3 m

* *Journal of Agricultural and Food Chemistry*, 2006, *54*, 4176

Part II.

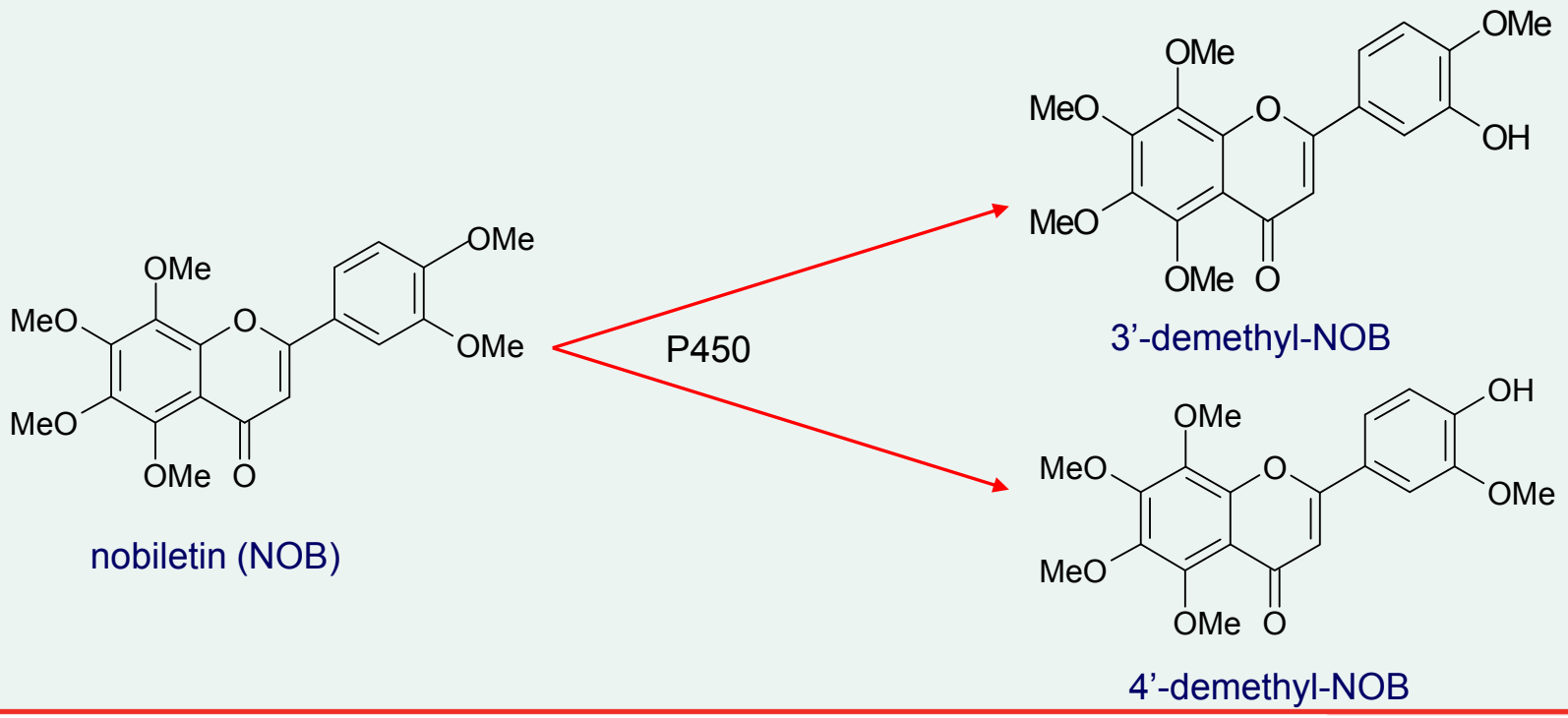
Analytical SFC method development for mice urinary metabolites of nobiletin



Nobiletin

Nobiletin Metabolites

- Nobiletin (NOB) is currently recognized as a promising anti-inflammatory and anti-tumor agent.
- Nobiletin is demethylated by hepatic p450 enzyme yielding two major hydroxylated metabolites: 3'-demethyl-NOB and 4'-demethyl-NOB.

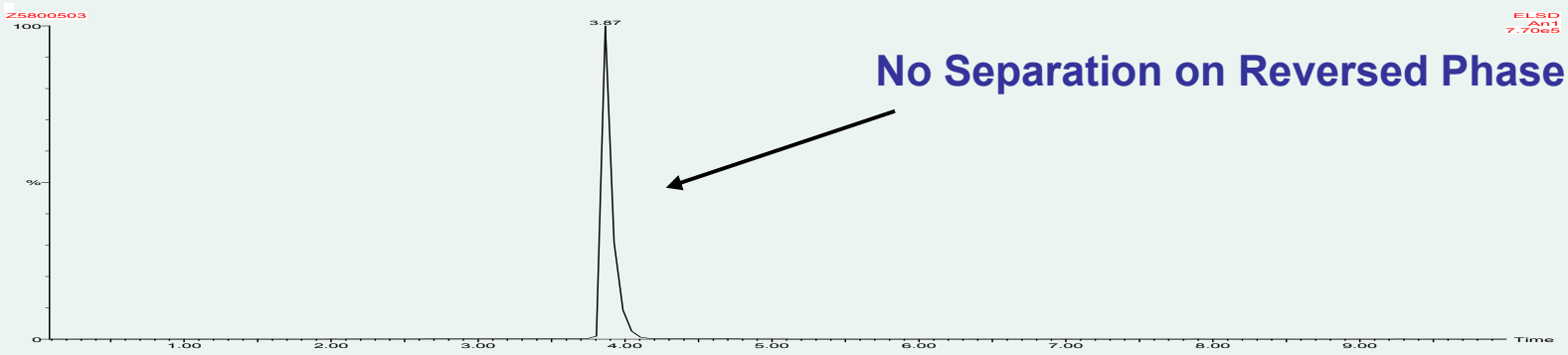


nobiletin (NOB)

3'-demethyl-NOB

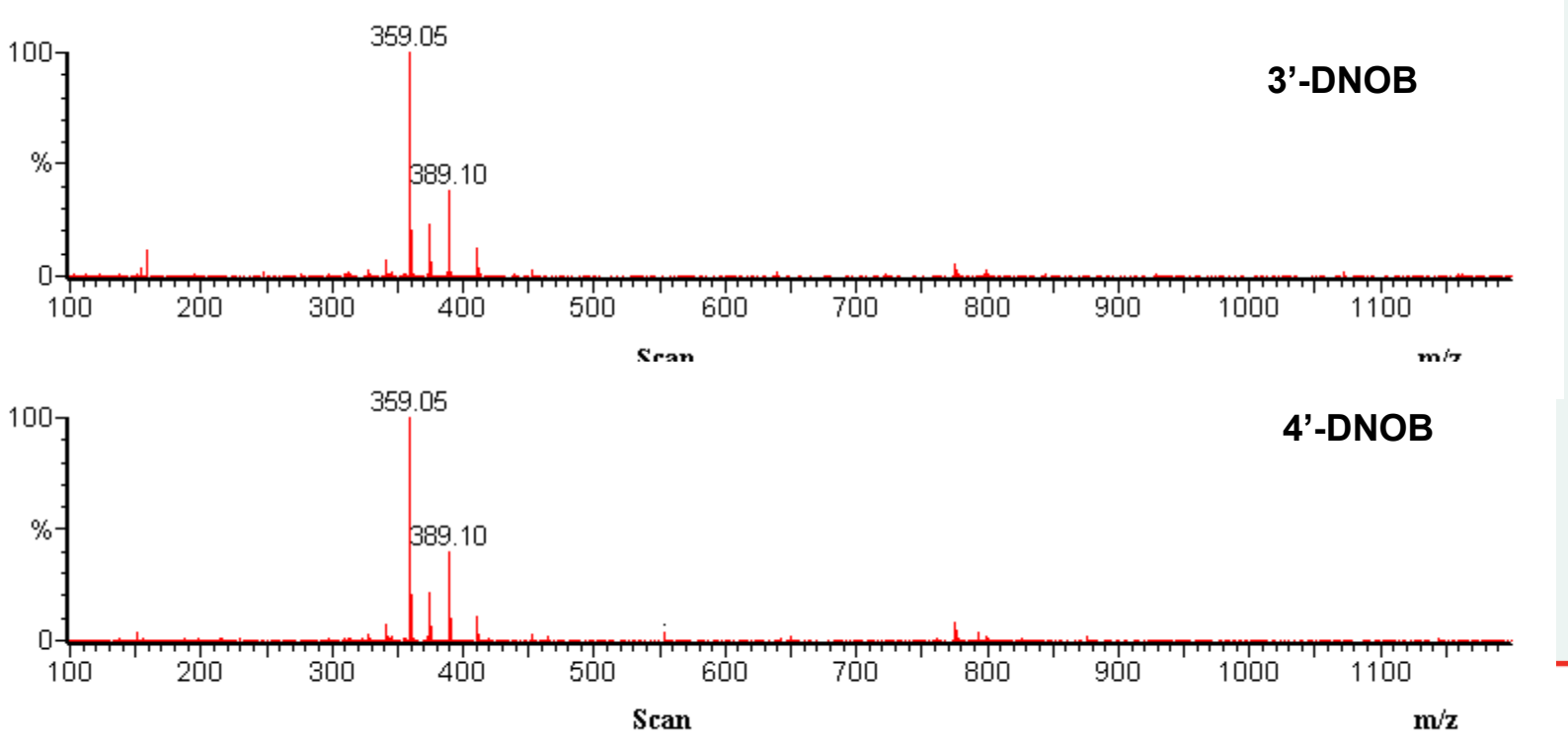
4'-demethyl-NOB

Analytical Challenges of 3'-DNOB and 4'-DNOB



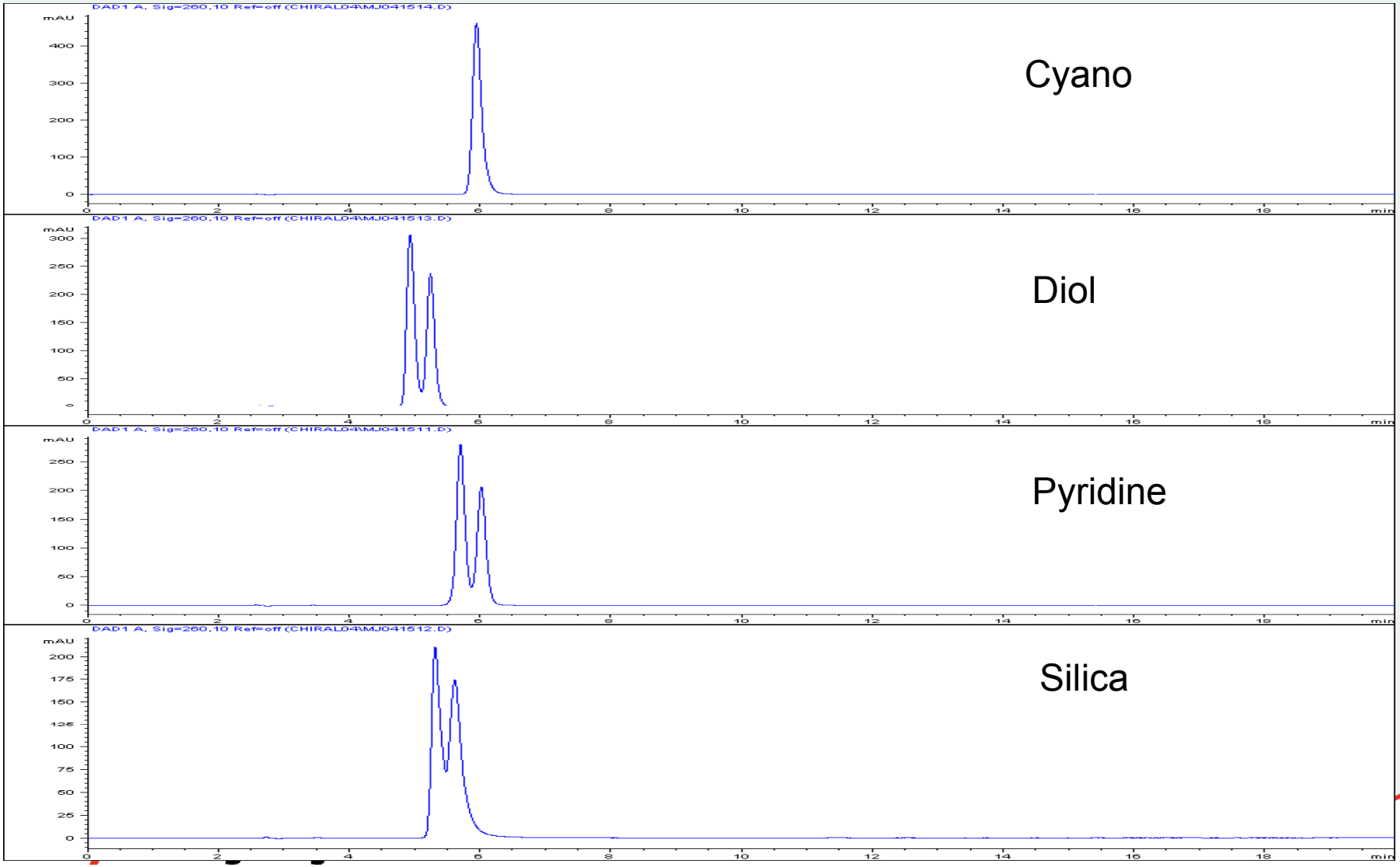
No Separation on Reversed Phase LC

Mass Spectrum:



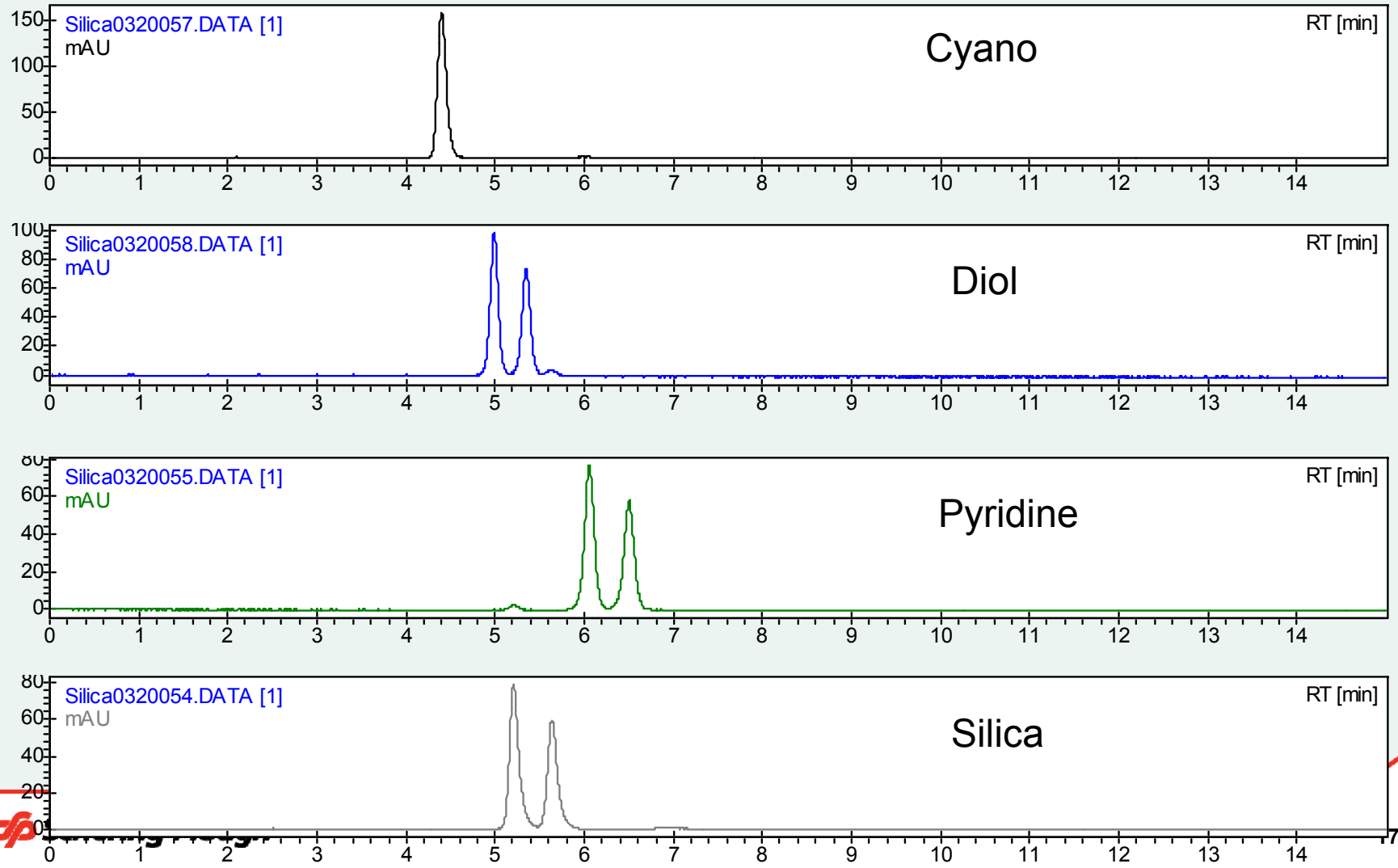
Normal Phase LC Separation of 3'-DNOB and 4'-DNOB on Achiral Columns

40/60 hexane/ethanol, 1.0 mL/min



SFC Separation of 3'-DNOB and 4'-DNOB on Normal Phase Achiral Columns

20% MeOH as modifier, 2.0 mL/min, CO_2 100 bar, 30ºC



LC vs SFC: Comparison of Selectivity and Resolution on Same Achiral Columns

	Selectivity (α)		Resolution (R_S)	
	LC	SFC	LC	SFC
Cyano	1.00	1.00	0	0
Diol	1.06	1.07	1.11	1.99
Pyridine	1.05	1.07	1.10	2.16
Silica	1.05	1.18	1.06	2.20

SFC > LC

$$Rs = \frac{\sqrt{N}}{4} \times \left(\frac{\alpha - 1}{\alpha}\right) \times \left(\frac{1 + k'}{k'}\right)$$

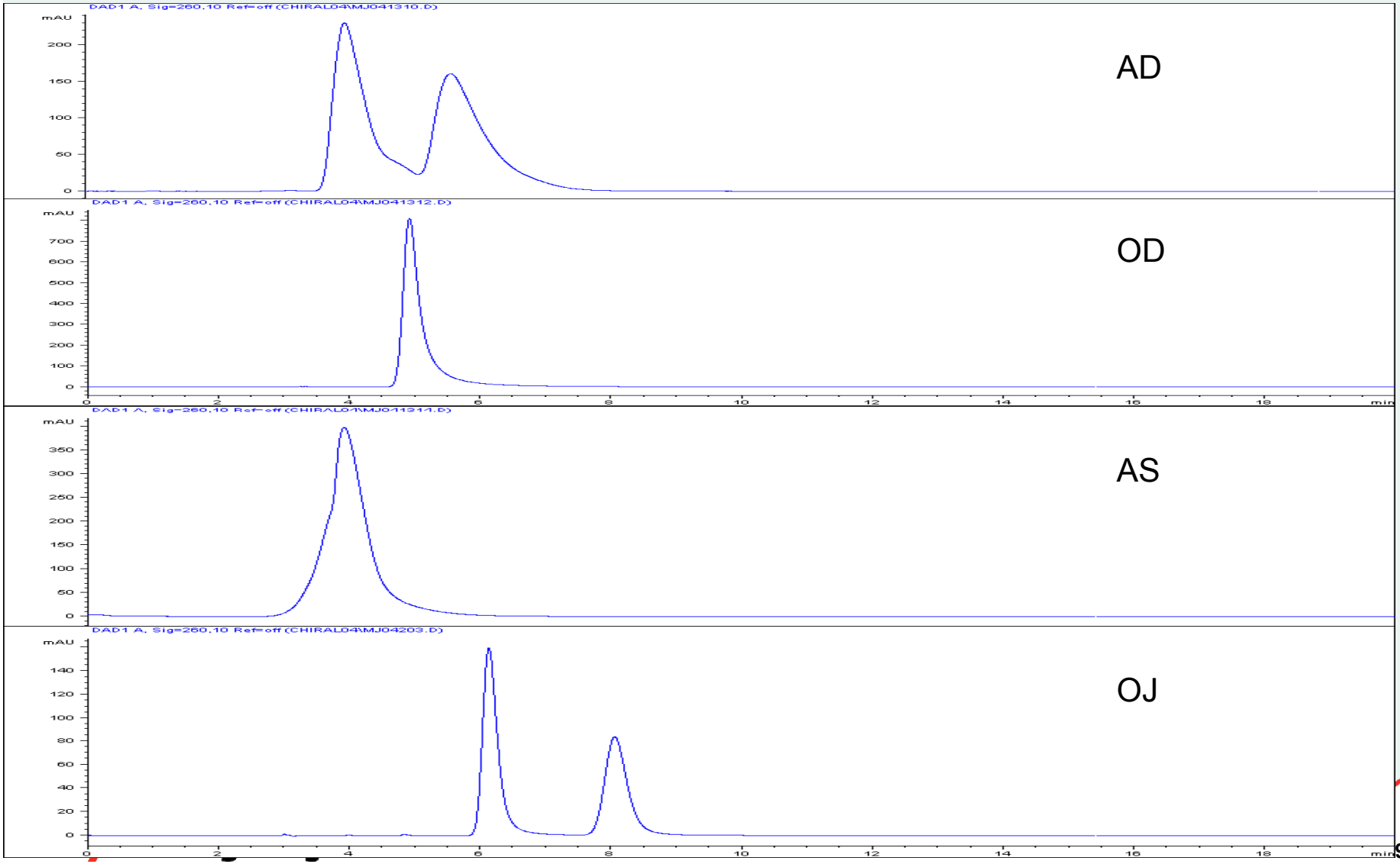
LC vs. SFC

Similar selectivity

Higher resolution!!

Normal Phase LC Separation of 3'-DNOB and 4'-DNOB on Chiral Columns

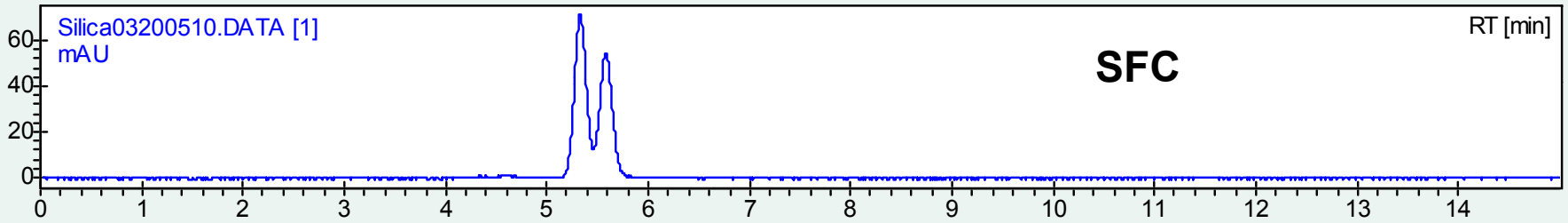
40/60 hexane/ethanol, 1.0 mL/min

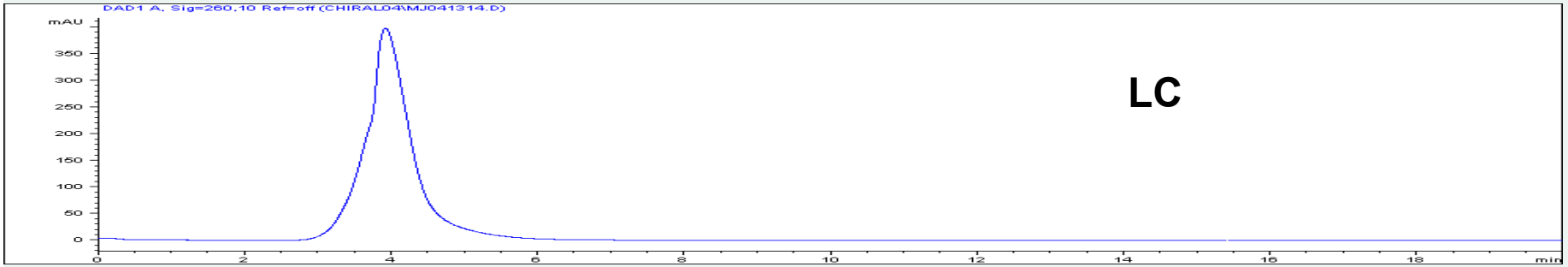


Comparative SFC Separation of 3'-DNOB and 4'-DNOB on Chiral Column

20% MeOH as modifier, 2.0 mL/min, CO_2 100 bar, 30ºC

Column: Chiralpak AS

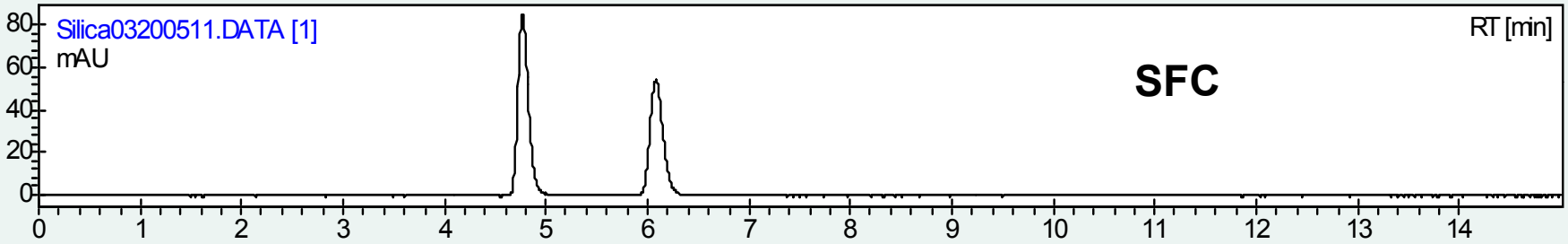


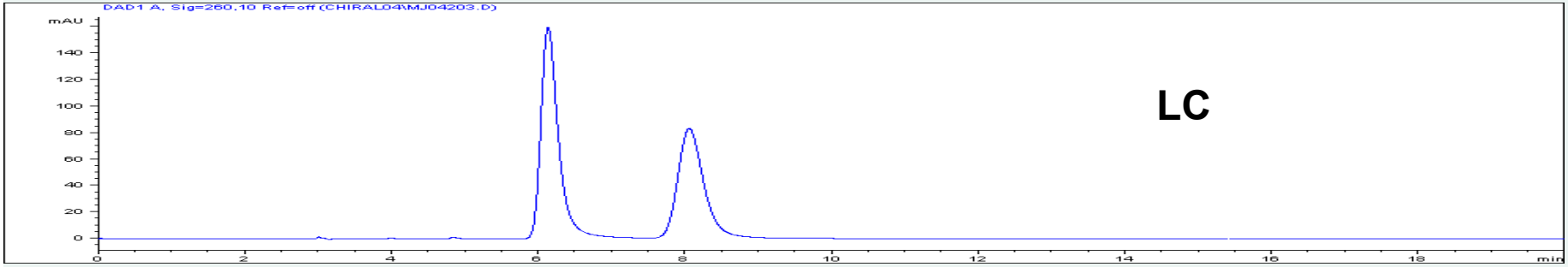


Comparative SFC Separation of 3'-DNOB and 4'-DNOB on Chiral Column

20% MeOH as modifier, 2.0 mL/min, CO_2 100 bar, 30ºC

Column: Chiralcel OJ

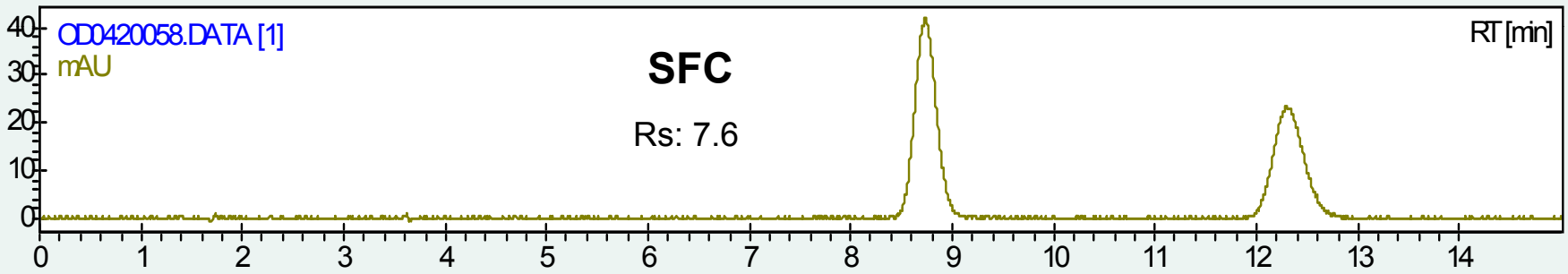


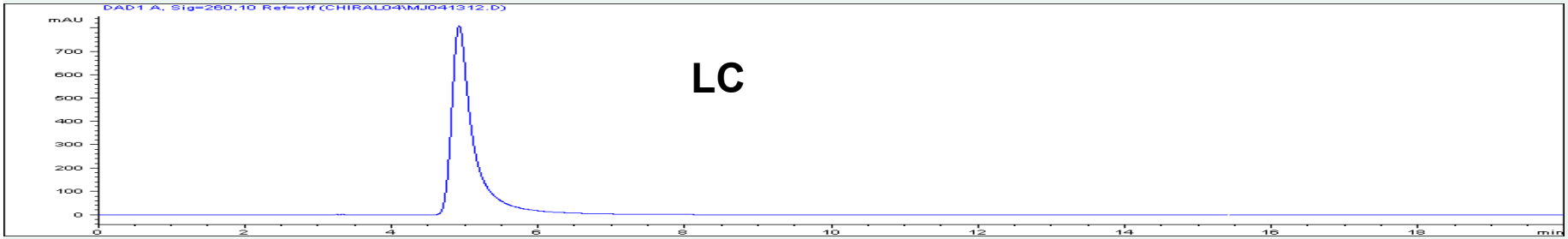


Comparative SFC Separation of 3'-DNOB and 4'-DNOB on Chiral Column

20% MeOH as modifier, 2.0 mL/min, CO_2 100 bar, 30ºC

Column: Chiralcel OD

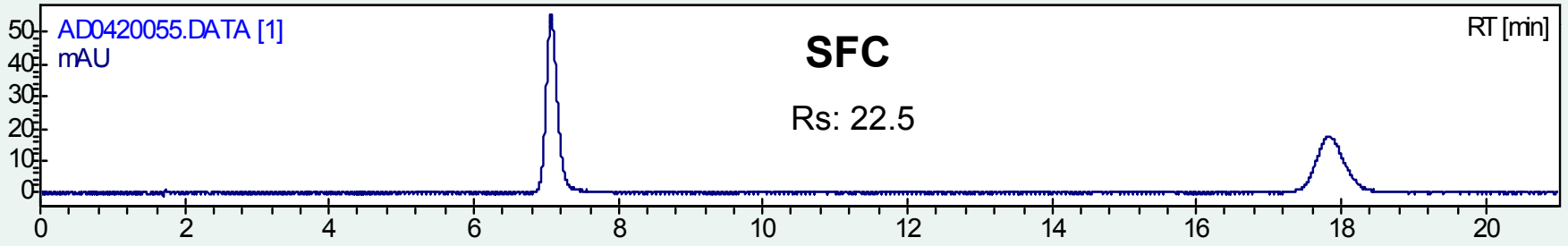


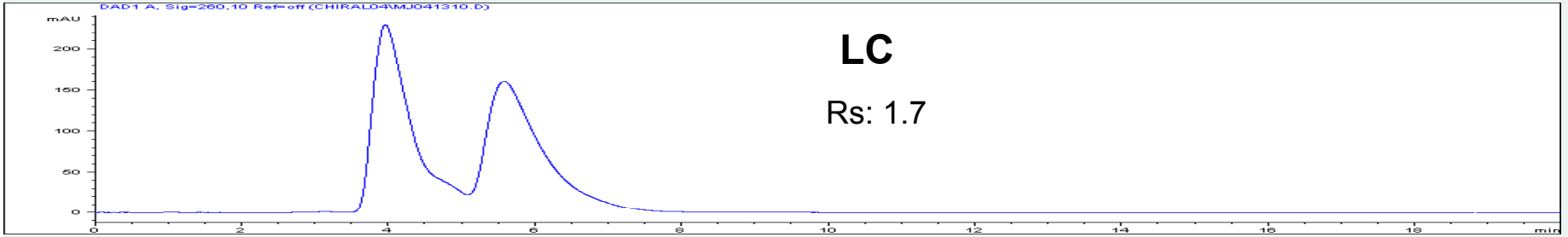


Comparative SFC Separation of 3'-DNOB and 4'-DNOB on Chiral Column

20% MeOH as modifier, 2.0 mL/min, CO_2 100 bar, 30ºC

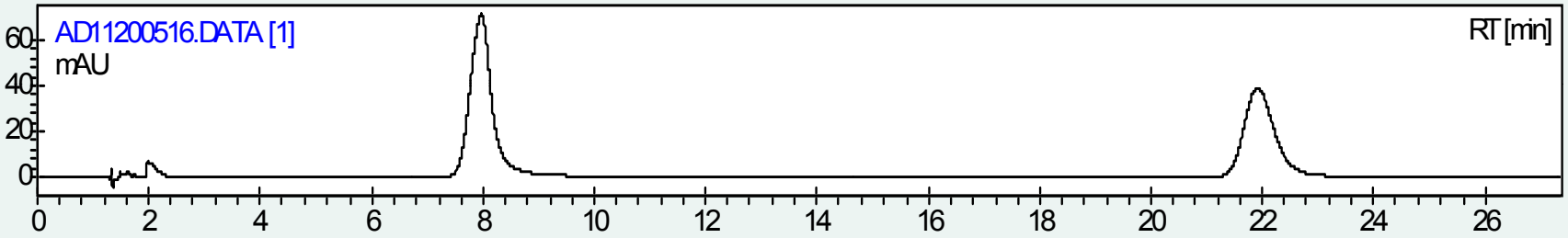
Column: Chiralpak AD



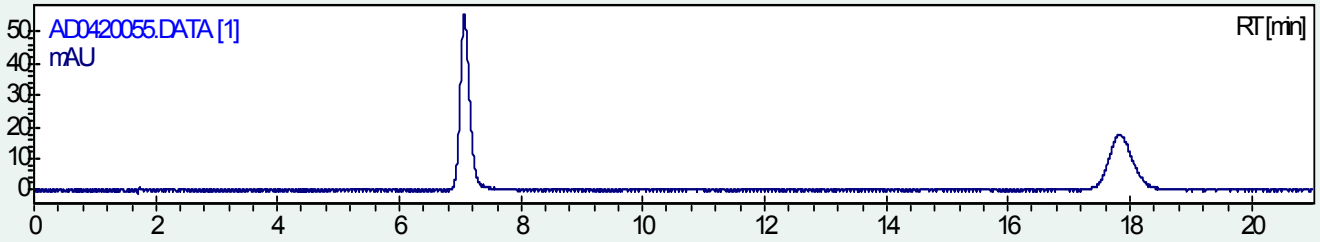


Effect of the Modifier

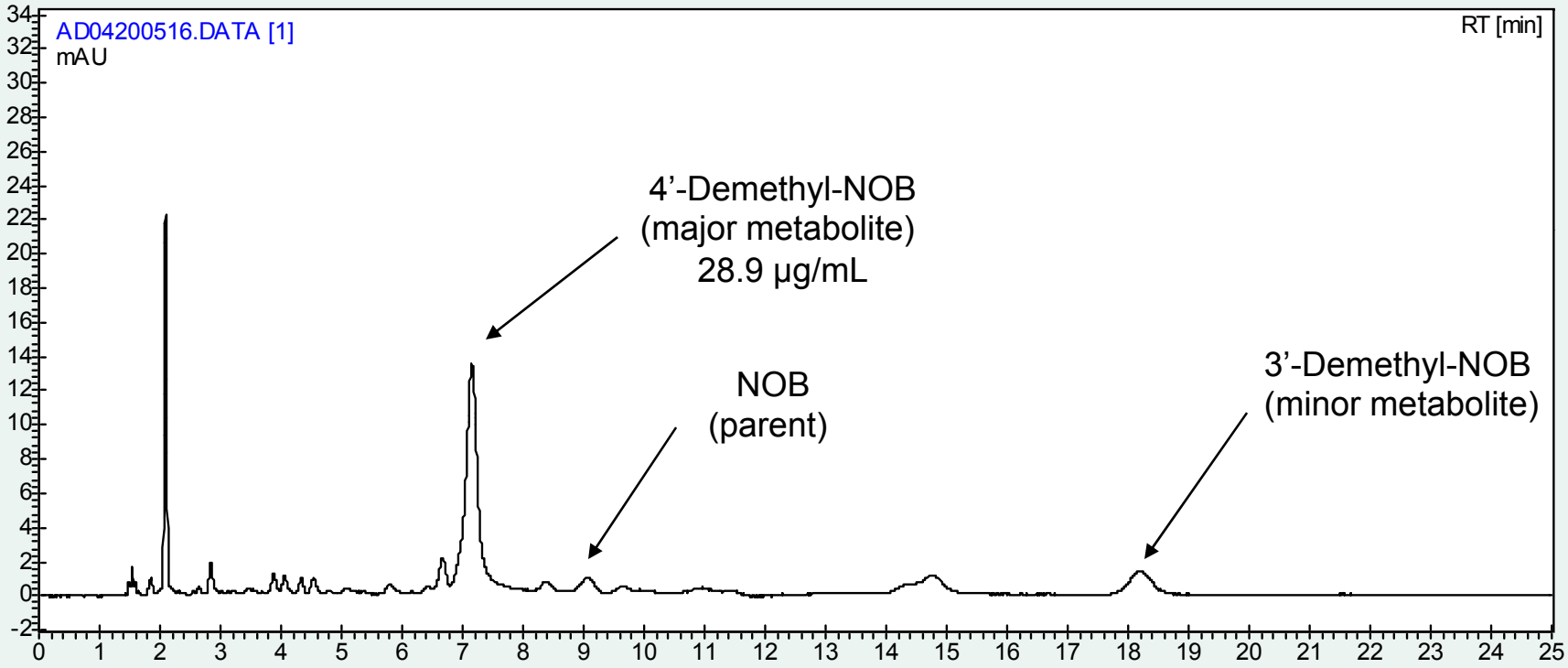
SFC: AD, 20% ethanol



SFC: AD, 20% methanol



Identification of NOB Hydroxylated Metabolites In Mice Urine



SFC with AD column, 20% MeOH as modifier, 2.0 mL/min, CO_2 100 bar, 30ºC, 5µL injection of urine sample.

Biomed. Chromatogr., 20 (2006) 1206-1215

Part III.

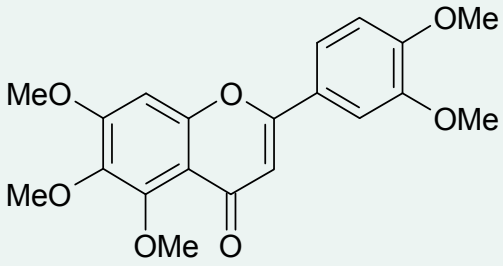
Analytical method development for PMF product quality control

Quantitative Analysis of Major PMFs in Citrus Peel Extracts

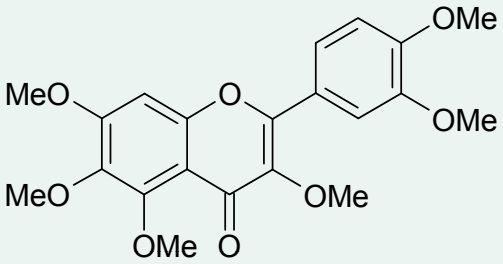
- Most *in vitro* and *in vivo* PMF studies using citrus peel extracts (CPE) instead of individual PMFs
- Little is known about the correlation between the bioactivity and the individual PMF content in the CPE
- CPE from various genus or using different extraction process will change PMF content
- Need a quantitative method to simultaneously analyze individual PMF in CPE

J. Sep. Sci., 31 (2008) 30-37

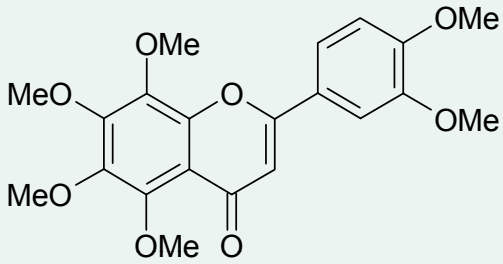
Six Major PMFs in the Citrus Genus



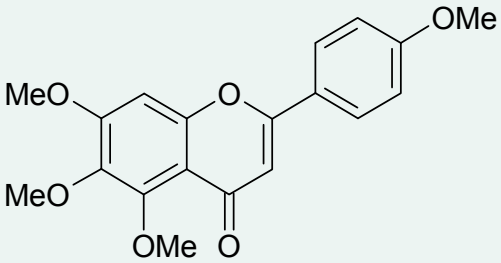
Sinensetin



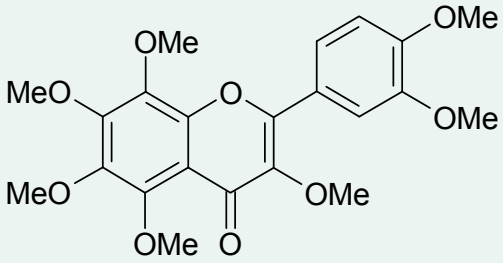
3,5,6,7,3',4'-Hexamethoxyflavone



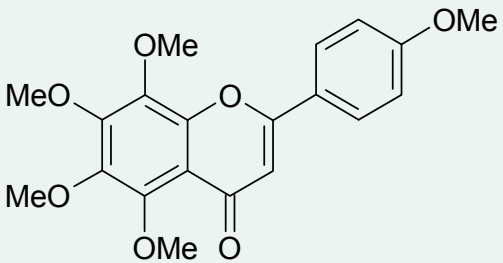
Nobiletin



5,6,7,4'-Tetramethoxyflavone



3,5,6,7,8,3',4'-Heptamethoxyflavone

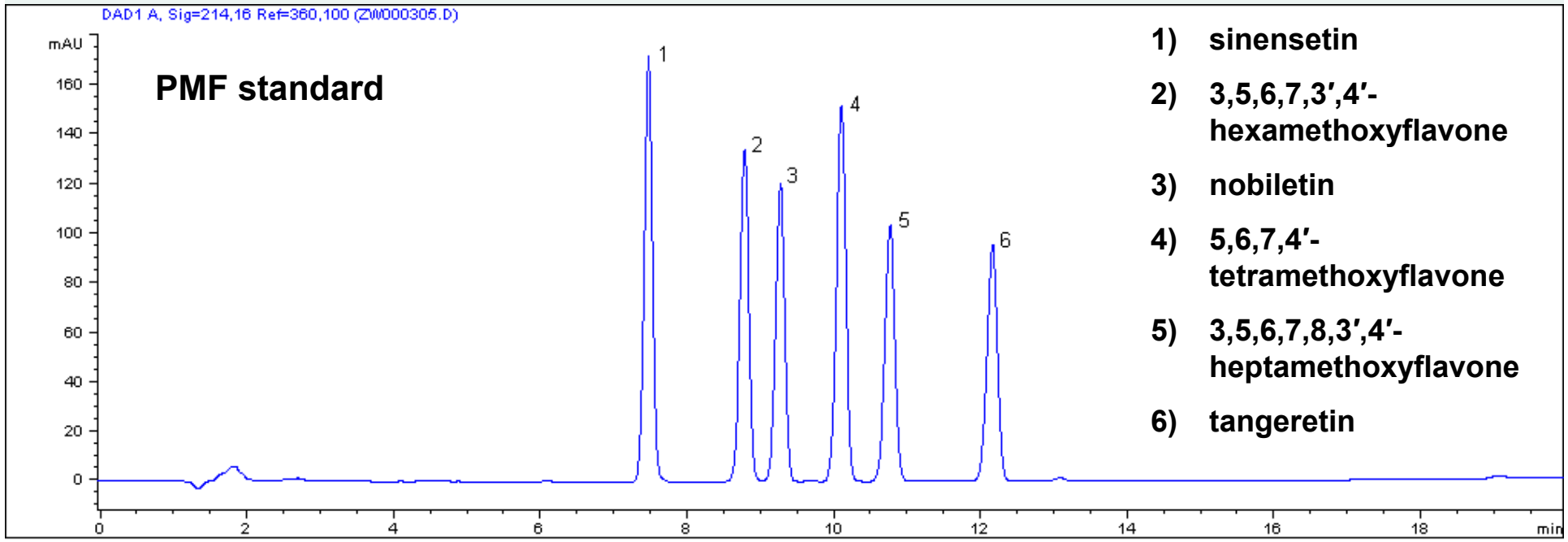


Tangeretin

Exploration of Various Separation Approaches

- Normal phase LC
  - Evaluated achiral and chiral columns
  - Poor separations
- Analytical SFC
  - Evaluated achiral and chiral columns
  - Improved separation using chiral column (AD)
  - Instrument availability concern in QC lab
- Reversed phase LC
  - Evaluated achiral and chiral columns
  - Best separation using RP-AmideC16
  - Method to be optimized and validated

Method Optimization and Validation

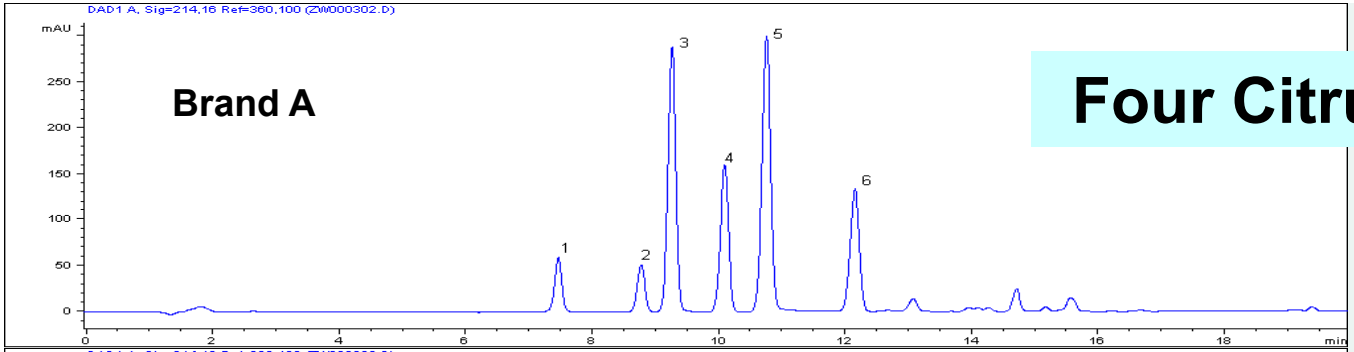


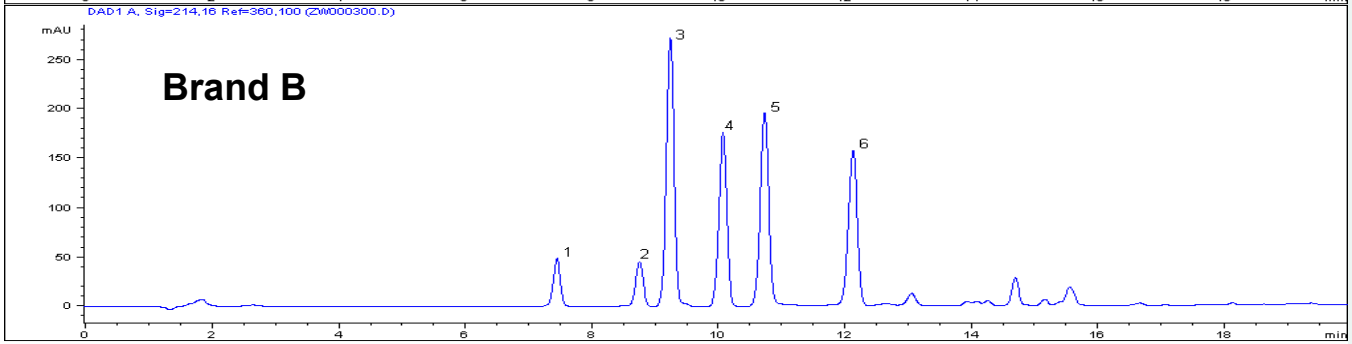
Chromatographic conditions:

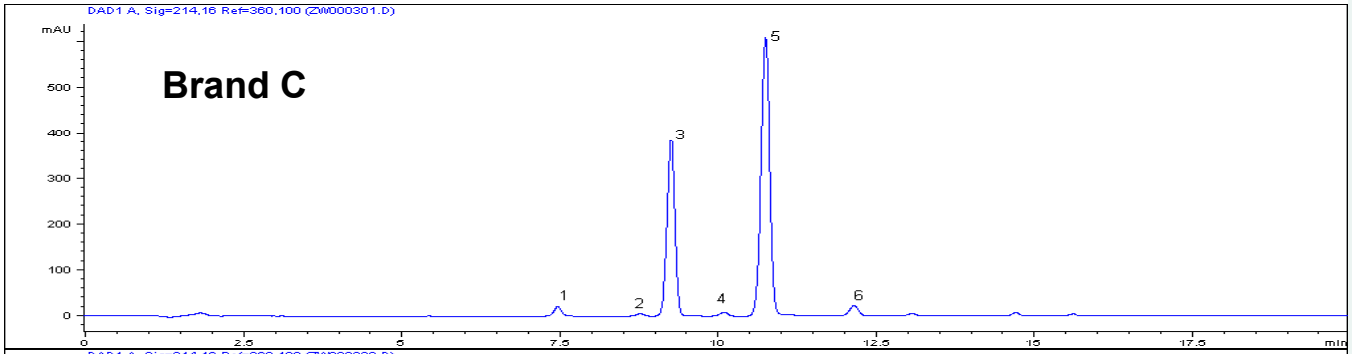
RP-AmideC16 column, water/MeCN gradient elution, 35 ˚C, 5 µL injection, 214 nm

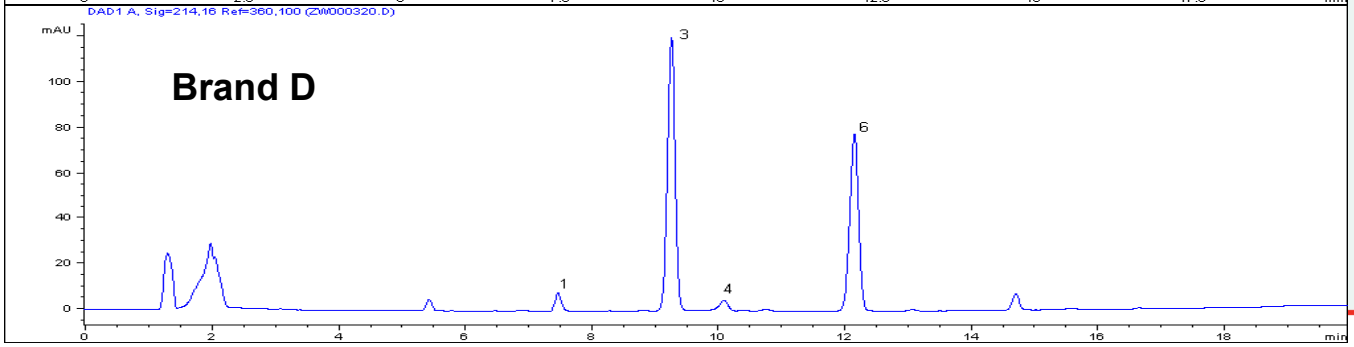
Method was further validated in linearity /range, accuracy /precision, LOD/LOQ

Four Citrus Peel Extracts









1) sinensetin
2) 3,5,6,7,3′,4′-hexamethoxyflavone
3) nobiletin
4) 5,6,7,4′-tetramethoxyflavone
5) 3,5,6,7,8,3′,4′-heptamethoxyflavone
6) tangeretin

Analysis of PMFs in Four Citrus Peel Extracts

Analyte	Weight percentage (%)			
	Brand A	Brand B	Brand C	Brand D
sinensetin	3.9	3.2	1.4	1.0
hexamethoxyflavone	4.1	3.7	0.4	-
nobiletin	26.1	24.4	33.2	10.5
tetramethoxyflavone	11.4	12.5	0.6	0.4
heptamethoxyflavone	31.2	20.7	62.3	-
tangeretin	15.4	18.1	2.8	8.2
Total % of PMFs	**92.1**	**82.6**	**100.7**	**20.1**

J. Sep. Sci., 31 (2008) 30-37

Summary

- Prep SFC is a powerful tool to isolate the PMFs from citrus peel extracts.
- Analytical SFC using chiral column provided superior separation of two urinary metabolites (regio-isomers) of nobiletin.
- For this cases, the separation mechanism of chiral SFC is different to that of chiral LC for the same chiral stationary phases.
- SFC has its niche in separation science. It is the analyst's job to pick up the right tool base on the purpose of the task.

Acknowledgment

WellGen

Dr. Shiming Li

Hoffmann-La Roche

Ted Lambros

Margaret Jonca