All You Wanted to Know about Method Development and Transfer, but Were Afraid to Ask

Stephanie Schuster[1], Thomas J. Waeghe[2], and Conner McHale[1]

[1]Advanced Materials Technology, Wilmington, DE 19810
[2]MAC-MOD Analytical, Chadds Ford, PA 19317

Method Transfer vs. Method Translation

- **Method transfer**
  - Move method from one column brand and particle size to another
  - Implement method in a different laboratory, different company or country
- **Method translation**
  - Move method from one particle size and/or column geometry to another with the same column brand
  - Move same column geometry and particle size to a different instrument brand (Δ delay volume, dispersion, etc.)
- **Typical Scenarios**
  - Transfer an HPLC method to a UHPLC column and system
    - e.g., TPP or SPP column to UHPLC SPP column
  - Translate a UHPLC method to an HPLC column and system
    - e.g., from R&D to QC
  - Direct implementation of an existing method
    - Only extracolumn volume, dispersion, delay volume and system max. pressure considerations

Questions to Ask
Method Transfer and Translation

- **Can the new instrument handle the pressure that the proposed new column will generate?**
- **Can you meet or exceed the original column's efficiency using the new instrument?**
- **Does the new instrument have low enough extracolumn dispersion to allow the required efficiency?**
- **Can the new instrument deliver the correct column temperature to match that of the original instrument?**
  - Does the instrument deliver the correct, accurate temperature?
  - How do the setpoint temperatures compare vs. actual temperatures for the instrument(s)?
- **To answer these questions, we need to be able to:**
  - Predict pressure
  - Predict efficiency
  - Measure extracolumn dispersion
  - Measure gradient dwell volume/delay volume

Important Method and Instrumental Parameters to Consider for Method Transfer and Translation

Isocratic Methods

- **Maximum Instrument Pressure**
  - Practical maximum operating pressure usually 75–80% of instrument maximum
- **Extracolumn volume**
  - Tubing
    - ID and Length
    - Homogeneous or heterogeneous IDs in sample flow path
  - Flow cell volume and path length
  - Injection volume
  - Injector type
    - Flow through needle vs. loop fill
- **Extracolumn dispersion**
  - Function of flow rate
  - Data Rate and Response Time
  - Instrument type
- **Column Heater Type and calibration**
  - Forced air, block/contact heater, heat tape wrap, etc.
  - Actual temperature vs. set point
- **Frictional Heating**
  - Effects on efficiency, peak width and selectivity

Gradient Methods

- **Same as for isocratic methods, except:**
  - Less impact on "efficiency" and peak capacity from precolumn tubing dispersion
- **Delay volume (aka dwell volume)**
  - High pressure mixing
    - Mixer volume
  - Low pressure mixing
  - Often a function of backpressure
    - $\propto$ column length
    - $\propto$ flow rate
    - $\propto$ 1/particle size, d_p

Pressure Estimation

To estimate pressure for a given column length and particle size, you need to know the following:

- **Flow rate (linear velocity)**
- **Column porosity (to calculate linear velocity)**
- **Column temperature**
- **Mobile phase viscosity as f(T)**
  - There are tables available for binary mixtures of ACN and MeOH with water
  - Tables for ternary mixtures (ACN, MeOH, water) or for binary mixtures of other solvents such as IPA, ethanol or THF with water are much harder to find.
- **Column Permeability (flow resistance parameter) is the most difficult to estimate**
- **If you have a column for a given product, you can estimate the permeability (flow resistance parameter) from the QC test conditions and reported pressure.**

Example

HALO 2 µm, 2.1 x 150 mm

- Mobile Phase A: ammonium formate, 10 mM, pH 3.7
- Mobile Phase B: CH_3CN
- Mobile phase composition: 50% B
- Flow Rate: 0.5 mL/min
- Temperature: 50 °C
- Viscosity, η: 0.51 cP
- Porosity: 0.506
- $V_M = \pi \times ID^2 \times L/(4 \times 1000) = 0.263$ mL
- $t_0 = 0.263/0.5 = 0.526$ min
- μ (mm/sec) = 150 mm/(0.526 x 60 sec/min) = 4.75 mm/sec

Φ Flow resistance parameter estimated at 600

$$\Delta P = \frac{\Phi \times \eta \times \mu \times L}{100 \times (d_p)^2}$$

$$\Delta P = \frac{600 \times 0.51 \times 4.75 \times 150}{100 \times 2.0^2} = 545 \text{ bar}$$

Efficiency Measurement or Theoretical Efficiency Estimation

- Theoretical plates, $N = L/(d_p \times h)$
- Column QC test report provides N and flow rate, but not dispersion of instrument used
- Conservative estimates of h for SPP particles
  - **2 µm**
    - 2.1 mm, 1.7
    - 3.0 mm, 1.6
  - **2.7 µm**
    - 2.1 mm, 1.7
    - 3.0 mm, 1.6
    - 4.6 mm, 1.4
  - **5 µm**
    - 2.1 mm, 1.7
    - 3.0 mm, 1.3
    - 4.6 mm, 1.3
- **TPP Particles**
  - **1.7 and 1.8 µm:** h ≈ 1.8–2.8
  - **3 µm:** h ≈ 2.2–2.3
  - **5 µm:** h ≈ 2.3–2.5
- Reduced plate height (*h*) varies with column diameter (4.6 < 3.0 < 2.1 mm ID)
- Easier to pack larger particles and larger ID columns to give higher N and lower *h* values

Some Examples

HALO 5 µm, 3 x 150 mm

- N ≈ 150 mm x 1000*/(1.3 x 4.6) ≈ 25,080

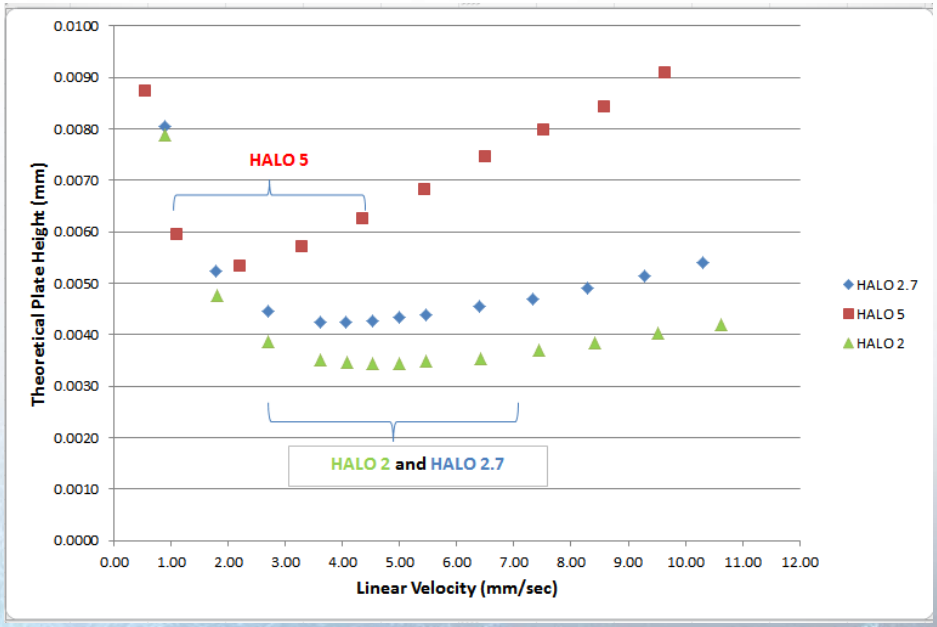
HALO 2 µm, 3 x 150 mm

N ≈ 150 mm x 1000*/(1.7 x 2) ≈ 44,120

HALO 2.7 µm, 4.6 x 250 mm

N ≈ 250 mm x 1000*/(1.4 x 2.7) ≈ 66,140!

*1000 µm/mm



Guiochon-Gritti Approach for Estimating Extracolumn Dispersion

$$\sigma^2_{obs} = \sigma^2_{ec} + \sigma^2_{col} = \sigma^2_{ec} + \left(\frac{V_0^2}{N_{theoretical}}\right)(1+k)^2$$

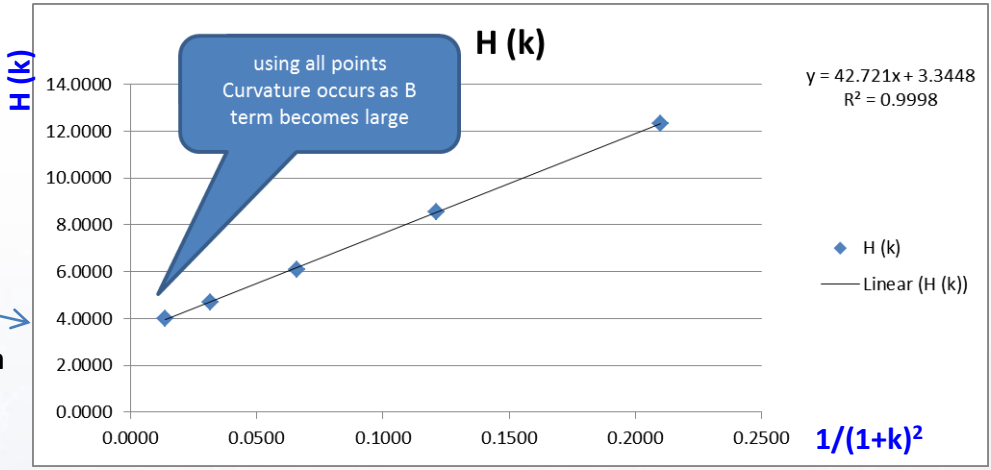
$$H_{obs}(k) = H_{theoretical} + L\left(\frac{\sigma^2_{ec}}{V_0^2}\right)\left(\frac{1}{(1+k)^2}\right)$$

$$Slope = L\left(\frac{\sigma^2_{ec}}{V_0^2}\right), \sigma^2_{ec} = \frac{V_0^2(mm^3)\times slope}{L\,(mm)}$$

1. **Chromatograph the mixture of homologs (plus uracil as t_0 marker) at the desired flow rate and linear velocity.**
2. **Obtain a performance report that shows plate count for each peak at half height**
3. **Plot the observed plate height in microns for each peak vs. $1/(1+k)^2$.**
4. **Note where the plot curves and include only those points from the first analyte forward.**
5. **Usually curvature occurs at or just before point for maximum plates vs. k is reached.**

Example for 2.1 x 100 mm, 2 µm SPP column
(0.5 µL injection, 0.4 mL/min with 50:50 CH3CN/water, 30 °C)

Analyte	Plates	RT	k	$1/(1+k)^2$	H (k)	h	% Max Plates
acetophenone	8118	1.024	1.18	0.2101	12.3183	6.1592	32%
propiophenone	11693	1.349	1.87	0.1210	8.5521	4.2761	45%
butyrophenone	16398	1.828	2.90	0.0659	6.0983	3.0492	64%
valerophenone	21408	2.632	4.61	0.0318	4.6712	2.3356	83%
hexanophenone	25054	4.000	7.52	0.0138	3.9914	1.9957	97%
heptanophenone	25738	6.295	12.41	0.0056	3.8853	1.9427	100%
octanophenone	24346	10.132	20.59	0.0021	4.1075	2.0537	95%



	L	100	mm
	V_0	187.7	µL
	V_0^2	35241.59	μL^2
	slope	42.7213	
	σ_{ec}^2	15.1	μL^2
$H_{intrinsic}$	intercept	3.34	µm
IBW	4 σ	15.5	µL
h		1.67	

H(k) = L x 1000/N(k)
$h = H(k)/d_p$

Accurate measurements of the true column efficiency and of the instrument band broadening contributions in the presence of a chromatographic column

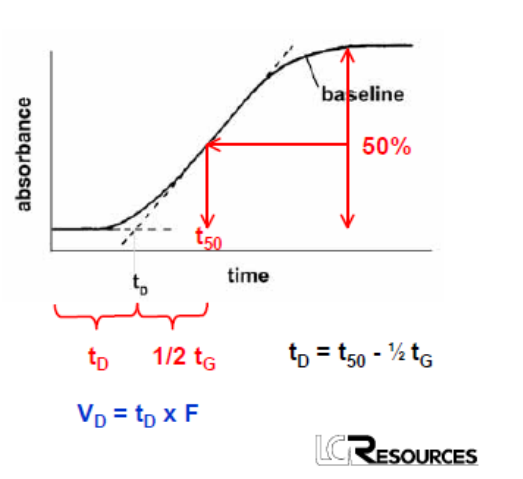
Journal of Chromatography A, 1327 (2014) 49– 56
Fabrice Gritti, Georges Guiochon

Excel calculator available on request from authors

Estimating Gradient Delay Volume (aka Dwell Volume)

Acetone Tracer Approach

- Install ZDV union in place of column
- A solvent: water
- B solvent: 0.1% (v/v) acetone in water
- Set a 0.5 or 1.0 min hold at start (0% B) to provide a flat portion initially
- Use a 10 min gradient time with hold for 5 min at %B final
- Flow Rates
  - 1 mL/min flow rate for 4.6 mm ID columns
  - 0.4 mL/min for 3 mm ID column
  - 0.2 or 0.25 mL/min for 2 mm ID columns



Note: If you use a 0.5 or 1.0 minute hold, remember to "back out" that portion of the calculated t_D and thus V_D

DryLab Software Approach

1. Sample: mixture of alkylphenones
2. Column: desired column
3. Flow rate: typical flow rate for column ID
4. Carry out 3 gradients (e.g., 5, 10 and 15 min) from 5 to 100% organic/water at the desired flow rate with column of interest.
5. Input 5 min and 10 min gradient data (RTs and PWs) into DryLab and vary dwell volume setting to obtain predicted RTs for 15 min run using those dwell volumes.
6. Find the delay volume setting that minimizes the error in RT for all peaks for predicted vs. actual 15 min run.
7. Estimate the dwell volume that minimizes the sum of the RT error differences by interpolation.
8. Input chromatograms into DryLab as CDF files or put retention times and peak widths into Excel table and paste into DryLab.
9. Note: a Microsoft Excel spreadsheet for carrying out the calculations is available from the authors based on the Reference 1 below.

Excel calculator available on request from authors

1. **LC-GC Magazine, 1990, Vol. 8, Number 7, 524-537**
   "Reproducibility Problems in Gradient Elution Caused by Differing Equipment.
2. **J Chromatogr A. 2014 Nov 21; 1369: 73–82.**
   "Measure Your Gradient": A New Way to Measure Gradients in High Performance Liquid Chromatography by Mass Spectrometric or Absorbance Detection

Instrumentation Configurations for Dispersion and Delay Volume

- **Agilent 1200 Low Dispersion Configuration**
  - Binary pump, mixer removed, pulse dampener bypassed, 600 bar max.
  - All sample flow path tubing 0.127 mm ID
  - Automatic delay volume reduction (ADVR)
  - Micro flow cell, 2 µL, path length 3 mm
  - Data rate: various 10 Hz/80 Hz
  - Response time: 0.5 sec/0.025 sec
- **Agilent 1100 Low Dispersion Configuration**
  - Quaternary pump, low pressure mixing, 400 bar max.
  - All sample flow path tubing 0.127 mm ID
  - 3 µL TCC heat exchanger
  - Semi-micro flow cell (5 µL, heat exchanger bypassed, path length 6 mm)
  - Data rate: fastest setting 13.7 Hz
  - Response time: 0.0625 sec
- **Agilent 1100 Standard Configuration**
  - Quaternary pump, low pressure mixing, 400 bar max.
  - All sample flow path tubing 0.178 mm ID
  - 3 µL TCC heat exchanger
  - Standard flow cell (14 µL, path length 10 mm)
  - Data Rate: fastest setting 13.7 Hz
  - Response time: 0.0625 sec

Column Geometries for all Dispersion and Delay Volume Experiments

- 3 x 50 mm, HALO 2 µm
- 3 x 50 mm, HALO 2.7 µm
- 3 x 50 mm, HALO 5 µm

3 Flow Rates

- 0.43 mL/min
- 0.64 mL/min (not for delay volume expts)
- 0.75 mL/min

Dwell Volume Estimates

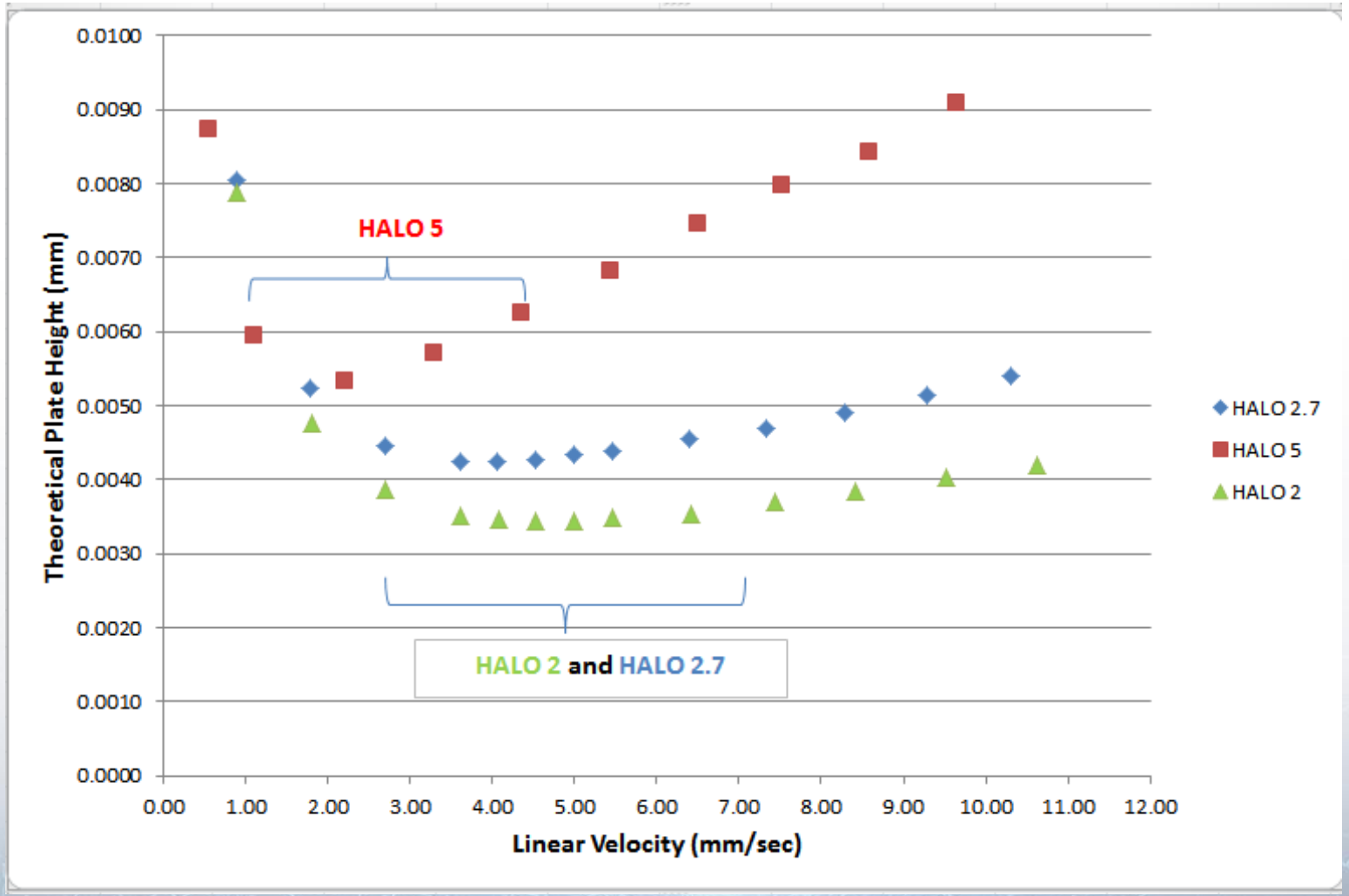
Agilent 1100 optimized			
Flow Rate	HALO 2 DryLab	HALO 5 DryLab	Step Gradient
0.43	1.02	1.01	1.00
0.75	1.04	1.04	1.08

Agilent 1100 Standard Configuration			
Flow Rate	HALO 2 DryLab	HALO 5 DryLab	Step Gradient
0.43	1.10	1.10	
0.75	1.12	1.03	

Nexera			
Flow Rate	HALO 2 DryLab	HALO 5 DryLab	Step Gradient
0.43		0.44	
0.75		0.45	

Van Deemter Plots for HALO 2, HALO 2.7 and HALO 5

Optimum linear velocity ranges vary by particle size



Efficiency and Dispersion Results for HALO 2, 2.7 and 5 µm, 3 x 50 mm Columns Using Agilent 1100 and 1200 Instruments

Agilent 1200
(0.127 mm ID tubing and 2 µL flow cell)

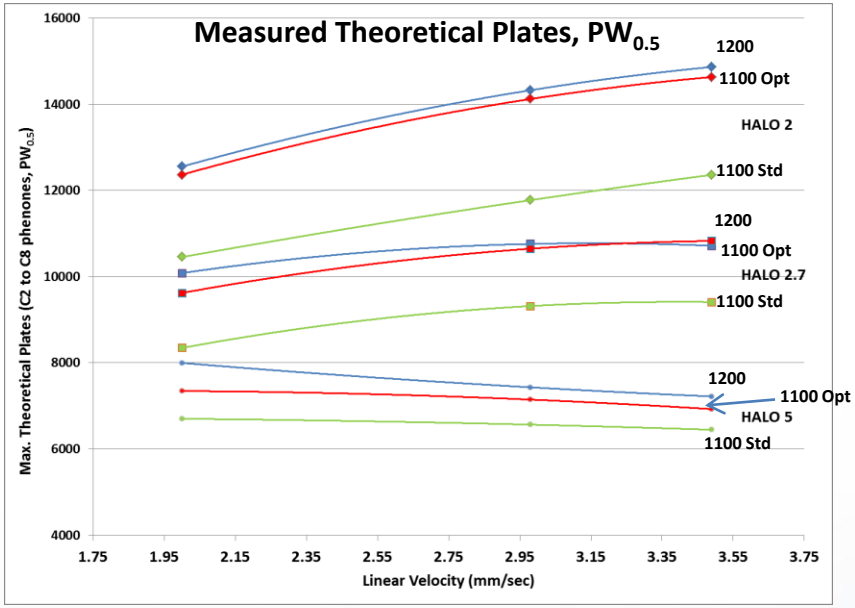
	HALO 2		HALO 2.7		HALO 5	
Flow Rate	Average N	σ^2	Average N	σ^2	Average N	σ^2
0.43	12554	7.0	10083	4.7	7997	5.8
0.64	14327	7.7	10760	5.5	7431	6.8
0.75	14867	7.9	10717	5.7	7220	5.7

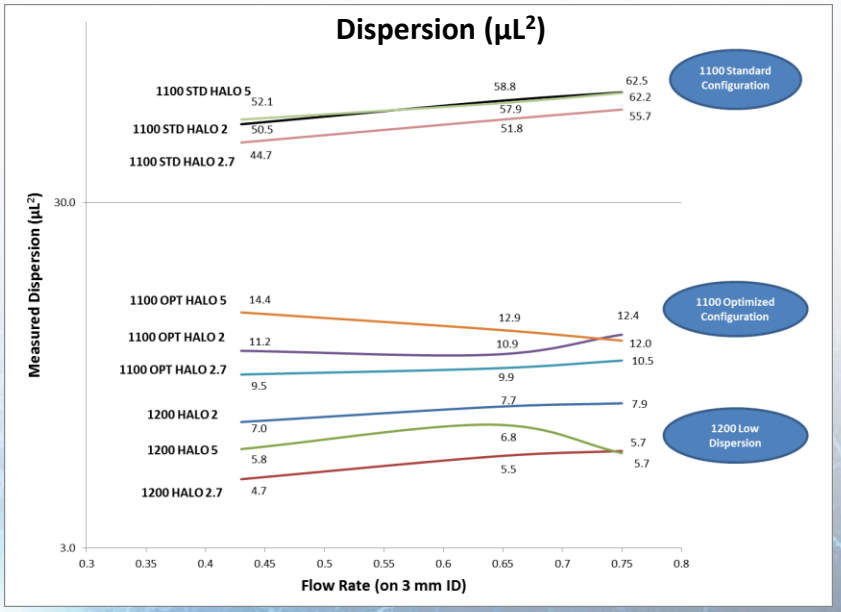
Agilent 1100 Optimized
(0.127 mm ID tubing and bypassed semi-micro flow cell)

	HALO 2		HALO 2.7		HALO 5	
Flow Rate	Average N	σ^2	Average N	σ^2	Average N	σ^2
0.43	12367	11.2	9621	9.5	7345	14.4
0.64	14123	10.9	10649	9.9	7146	12.9
0.75	14634	12.4	10829	10.5	6926	12.0

Agilent 1100 Standard Configuration
(14 µL Flow Cell and 0.17 mm ID tubing)

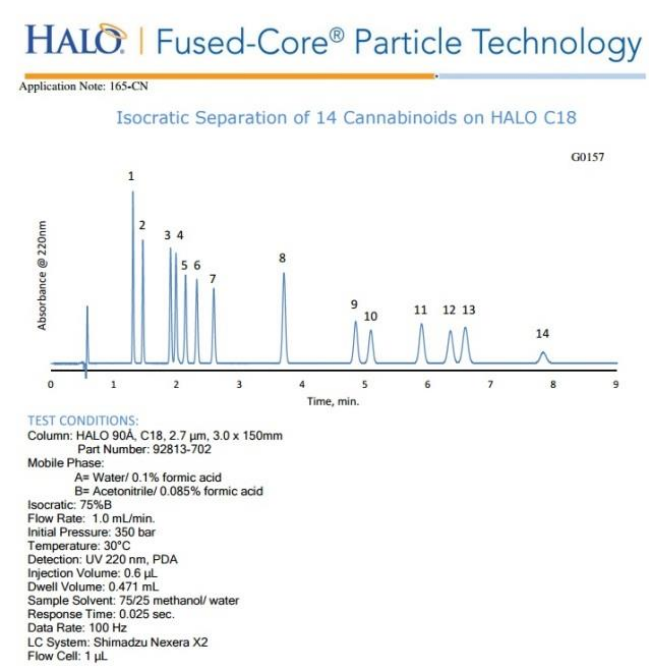
	HALO 2		HALO 2.7		HALO 5	
Flow Rate	Average N	σ^2	Average N	σ^2	Average N	σ^2
0.43	10454	50.5	8345	44.7	6701	52.1
0.64	11776	58.8	9318	51.8	6565	57.9
0.75	12363	62.5	9410	55.7	6447	62.2





Isocratic Separation: Cannabinoids

- 3 x 150 mm, 2.7 µm HALO C18
- 75:25 ACN/water 0.1% HCOOH
- 1 mL/min (4.67 mm/sec)
- 30 °C
- 0.6 µL injection
- Pressure: 350 bar
- Instrument: Shimadzu Nexera



3 x 150 mm, HALO 5

- Adjust flow rate to 0.6 mL/min due to lower optimum µ for HALO 5 (2.8 mm/sec)
- V_{inj} same at 1 µL
- Pressure will be much lower

3 x 50 mm, HALO 2

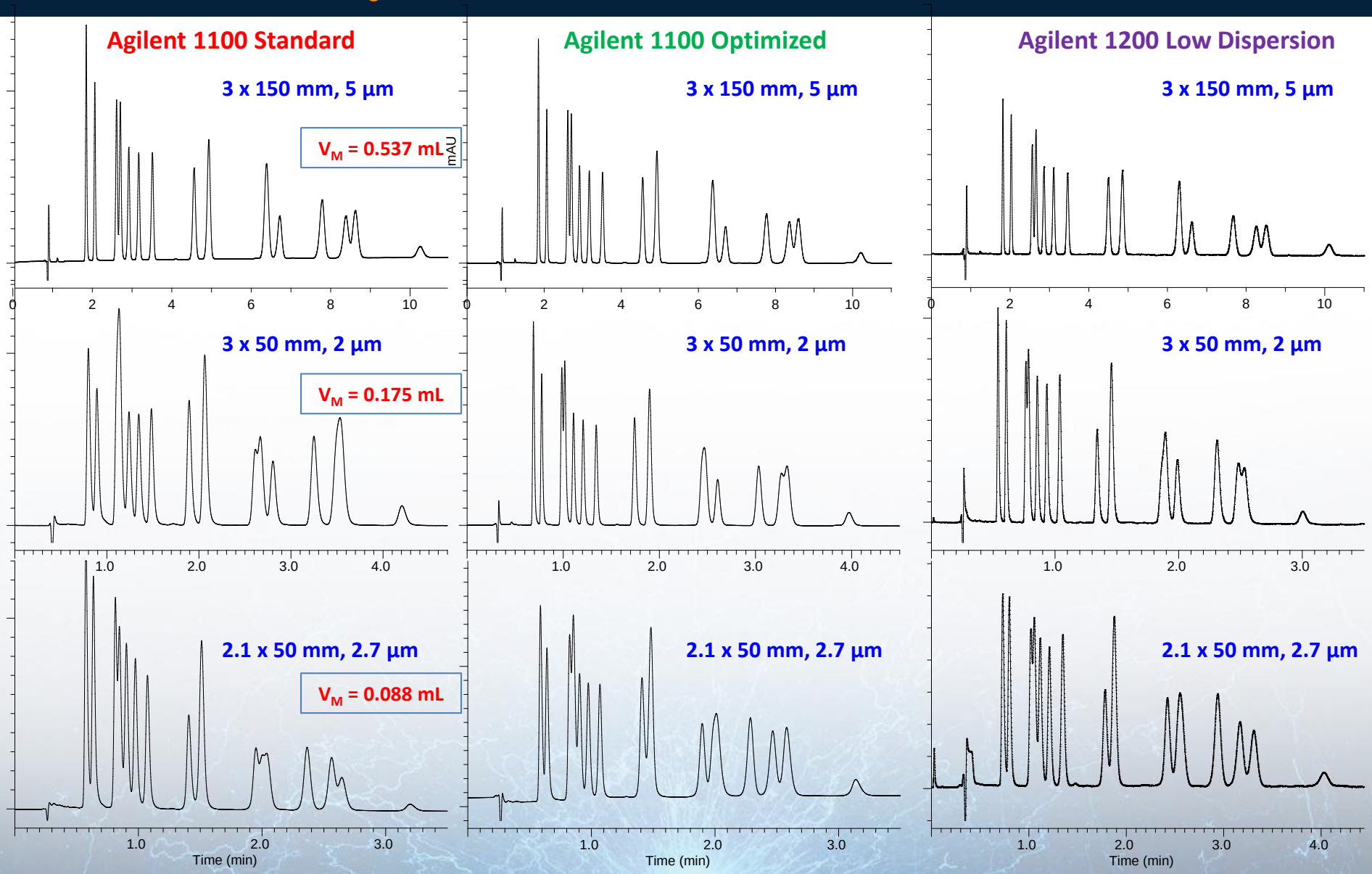
- Flow rate same at 0.6 mL/min (2.8 mm/sec)
- V_{inj} reduce to 0.5 µL
- Pressure will be 350 x (1/3) x $(2.7/2)^2$ ~ 210 bar

2.1 x 50 mm, HALO 2.7

- Flow rate to 0.294 mL/min (2.8 mm/sec)
- V_{inj} reduce to 0.3 µL
- Pressure will be 350 x (1/3) ~ 150 bar

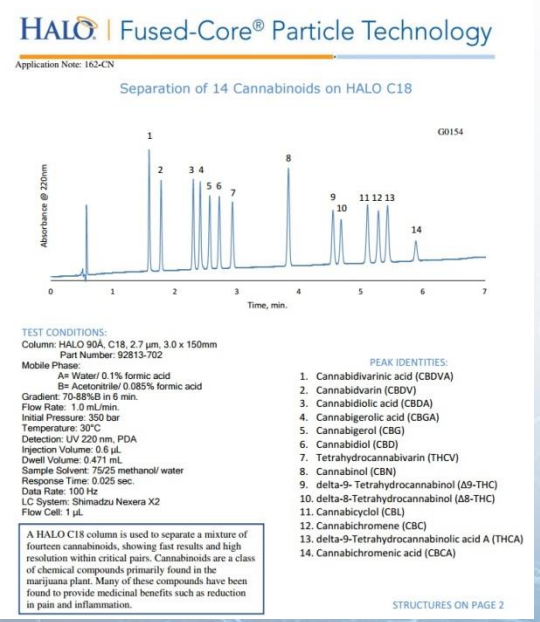
Cannabinoids: Isocratic Separations

75:25 CH_3CN/water with 0.1% HCOOH, 30 °C at 2.8 mm/sec



Gradient Separation: Cannabinoids

- 3 x 150 mm, 2.7 µm HALO C18
- Gradient from 70 to 88% in 6 min
- 1 mL/min (4.67 mm/sec)
- 30 °C
- 0.6 µL injection
- Starting Pressure: 350 bar
- Instrument: Shimadzu Nexera



HALO® | Fused-Core® Particle Technology

Application Note: 162-CN

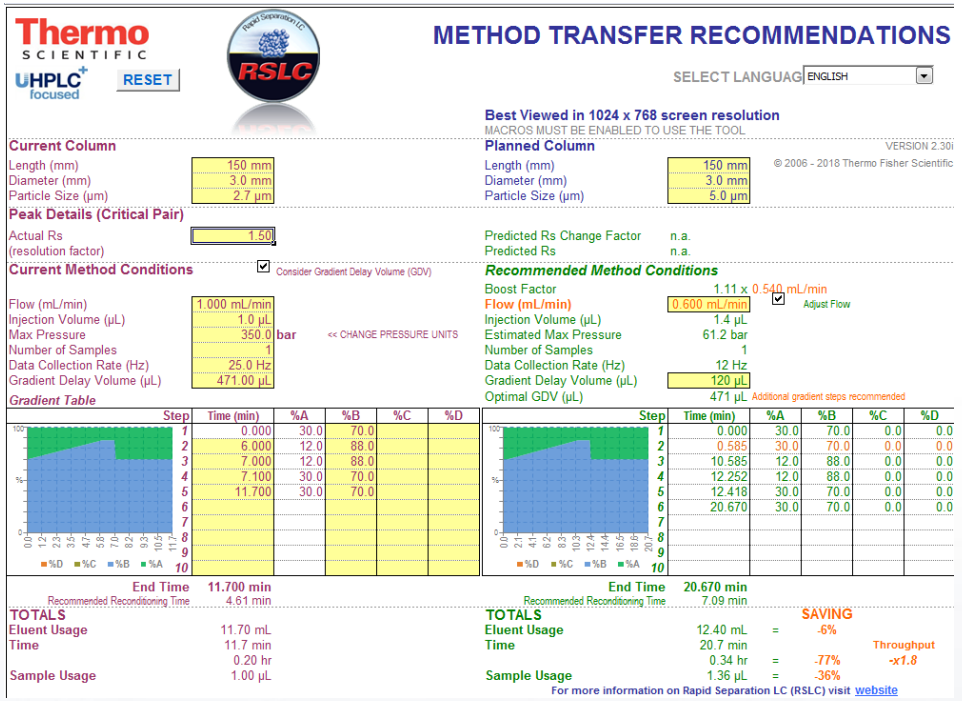
Separation of 14 Cannabinoids on HALO C18

TEST CONDITIONS:
Column: HALO 90Å, C18, 2.7 µm, 3.0 x 150mm
Part Number: 92813-702
Mobile Phase:
A= Water/ 0.1% formic acid
B= Acetonitrile/ 0.085% formic acid
Gradient: 70-88%B in 6 min.
Flow Rate: 1.0 mL/min.
Initial Pressure: 350 bar
Temperature: 30°C
Detection: UV 220 nm, PDA
Injection Volume: 0.6 µL
Dwell Volume: 0.471 mL
Sample Solvent: 75/25 methanol/ water
Response Time: 0.025 sec.
Data Rate: 100 Hz
LC System: Shimadzu Nexera X2
Flow Cell: 1 µL

A HALO C18 column is used to separate a mixture of fourteen cannabinoids, showing fast results and high resolution within critical pairs. Cannabinoids are a class of chemical compounds primarily found in the marijuana plant. Many of these compounds have been found to provide medicinal benefits such as reduction in pain and inflammation.

PEAK IDENTITIES:
1. Cannabidivarinic acid (CBDVA)
2. Cannabidvarin (CBDV)
3. Cannabidiolic acid (CBDA)
4. Cannabigerolic acid (CBGA)
5. Cannabigerol (CBG)
6. Cannabidiol (CBD)
7. Tetrahydrocannabivarin (THCV)
8. Cannabinol (CBN)
9. delta-9- Tetrahydrocannabinol (Δ9-THC)
10. delta-8-Tetrahydrocannabinol (Δ8-THC)
11. Cannabicyclol (CBL)
12. Cannabichromene (CBC)
13. delta-9-Tetrahydrocannabinolic acid A (THCA)
14. Cannabichromenic acid (CBCA)

STRUCTURES ON PAGE 2



Thermo SCIENTIFIC
UHPLC+ focused
RESET

RSLC

METHOD TRANSFER RECOMMENDATIONS

SELECT LANGUAG ENGLISH

Best Viewed in 1024 x 768 screen resolution
MACROS MUST BE ENABLED TO USE THE TOOL

VERSION 2.30i
© 2006 - 2018 Thermo Fisher Scientific

Current Column		Planned Column	
Length (mm)	150 mm	Length (mm)	150 mm
Diameter (mm)	3.0 mm	Diameter (mm)	3.0 mm
Particle Size (µm)	2.7 µm	Particle Size (µm)	5.0 µm

Peak Details (Critical Pair)

Actual Rs (resolution factor)	1.50	Predicted Rs Change Factor	n.a.
		Predicted Rs	n.a.

Current Method Conditions		Recommended Method Conditions	
	Consider Gradient Delay Volume (GDV)	Boost Factor	1.11 x 0.540 mL/min
Flow (mL/min)	1.000 mL/min	Flow (mL/min)	0.600 mL/min Adjust Flow
Injection Volume (µL)	1.0 µL	Injection Volume (µL)	1.4 µL
Max Pressure	350.0 bar << CHANGE PRESSURE UNITS	Estimated Max Pressure	61.2 bar
Number of Samples	1	Number of Samples	1
Data Collection Rate (Hz)	25.0 Hz	Data Collection Rate (Hz)	12 Hz
Gradient Delay Volume (µL)	471.00 µL	Gradient Delay Volume (µL)	120 µL
		Optimal GDV (µL)	471 µL Additional gradient steps recommended

Gradient Table

Step	Time (min)	%A	%B	%C	%D
1	0.000	30.0	70.0		
2	6.000	12.0	88.0		
3	7.000	12.0	88.0		
4	7.100	30.0	70.0		
5	11.700	30.0	70.0		
6					
7					
8					
9					
10					

%D %C %B %A

End Time 11.700 min
Recommended Reconditioning Time 4.61 min

TOTALS	
Eluent Usage	11.70 mL
Time	11.7 min
	0.20 hr
Sample Usage	1.00 µL

Step	Time (min)	%A	%B	%C	%D
1	0.000	30.0	70.0	0.0	0.0
2	0.585	30.0	70.0	0.0	0.0
3	10.585	12.0	88.0	0.0	0.0
4	12.252	12.0	88.0	0.0	0.0
5	12.418	30.0	70.0	0.0	0.0
6	20.670	30.0	70.0	0.0	0.0
7					
8					
9					
10					

%D %C %B %A

End Time 20.670 min
Recommended Reconditioning Time 7.09 min

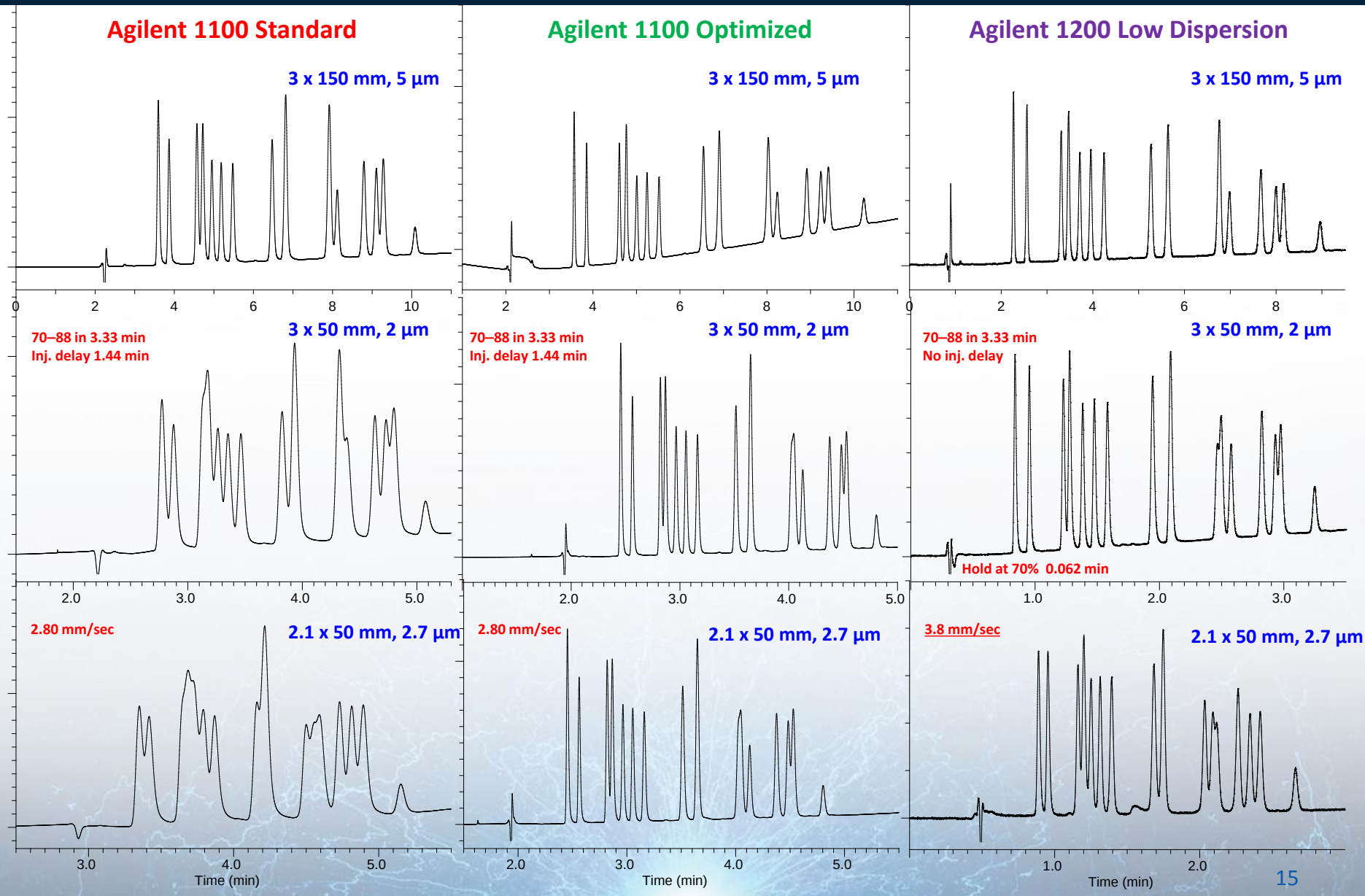
TOTALS			SAVING	
Eluent Usage	12.40 mL	=	-6%	
Time	20.7 min			Throughput
	0.34 hr	=	-77%	-x1.8
Sample Usage	1.36 µL	=	-36%	

For more information on Rapid Separation LC (RSLC) visit website

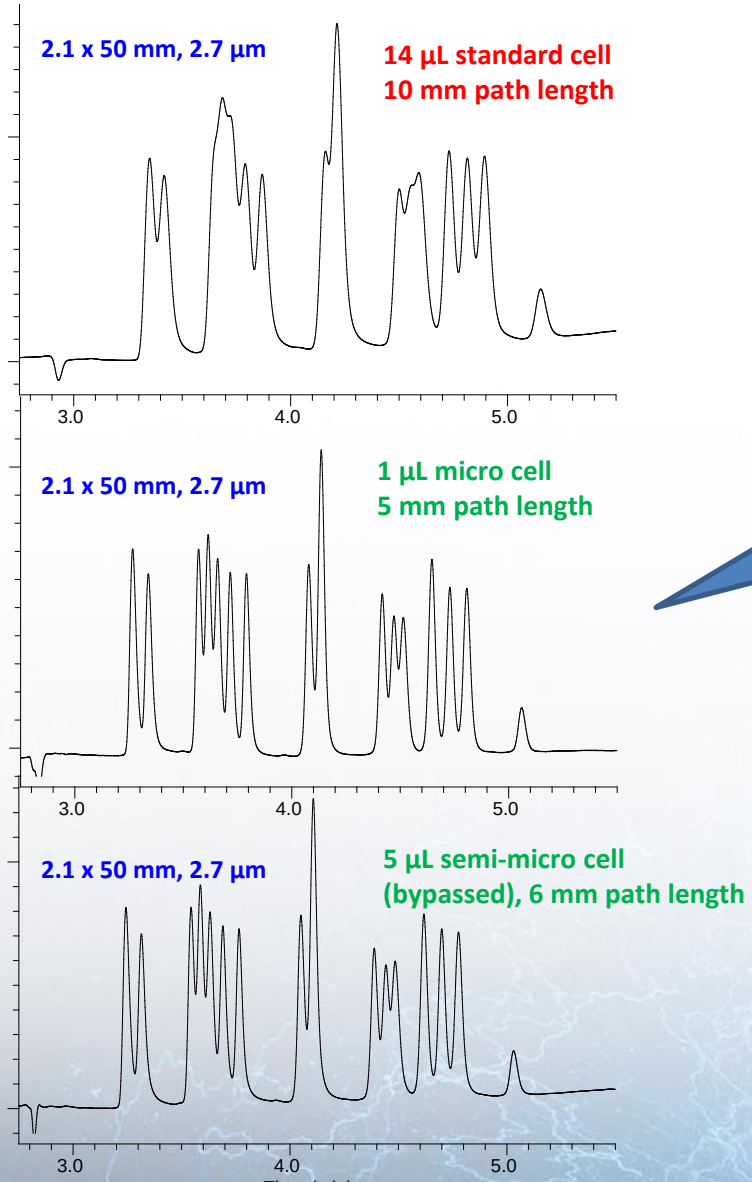
Input delay volume for “new” instrument.
Flow rate
Used calculated injection delay as needed for 3 x 50 and 2.1 x 50 mm columns.

Cannabinoids: Gradient Separations

70 to 88% CH_3CN/water (0.1% HCOOH)

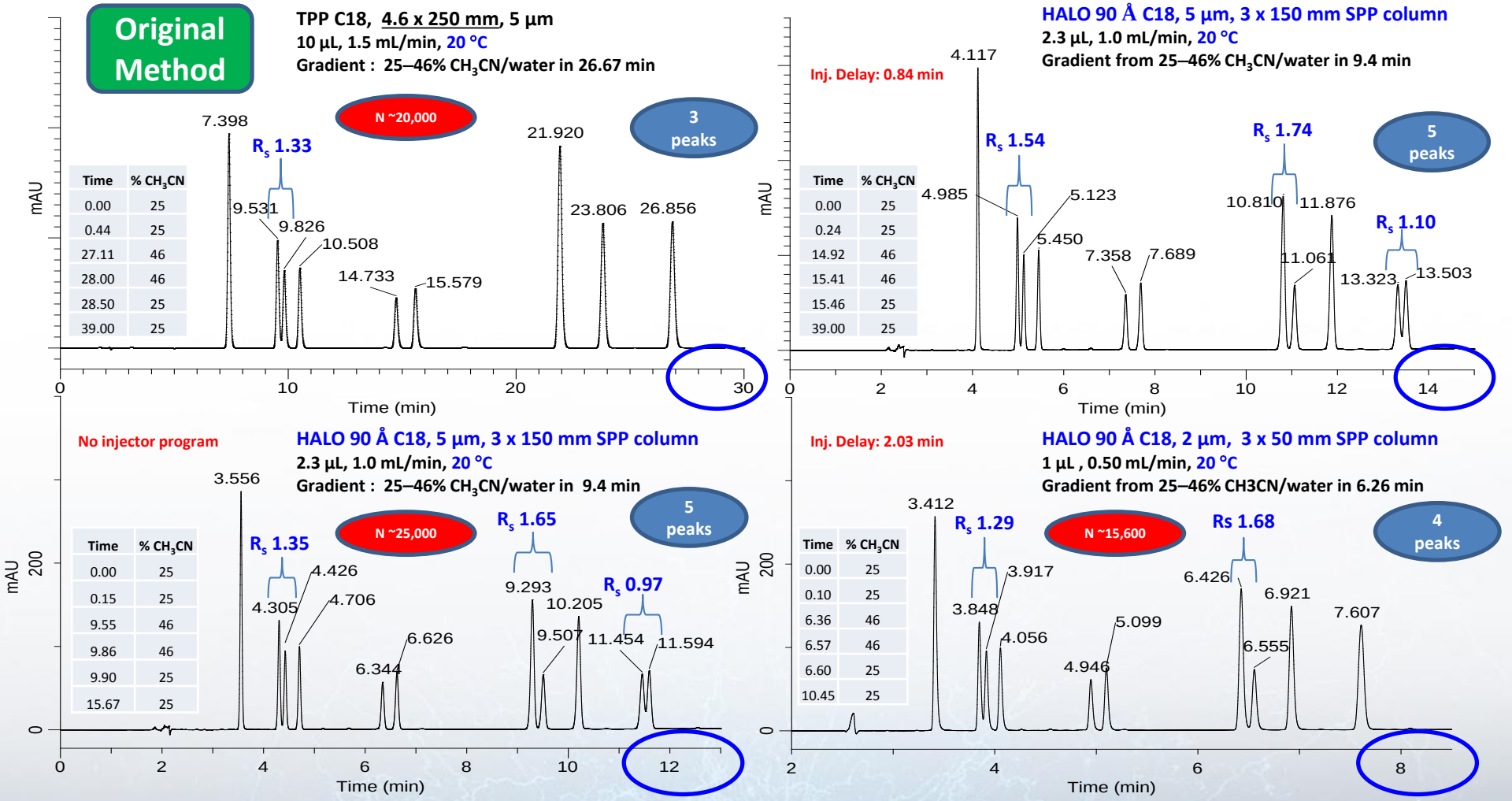


Example Translation from 3 x150 mm HALO 2.7 to 2.1 x 50 mm, HALO 2.7 on Agilent 1100 configuration (standard , micro, semi-micro flow cells)



All conditions held constant on Agilent 1100 in standard configuration except flow cell (compare vs. bottom left preceding slide)

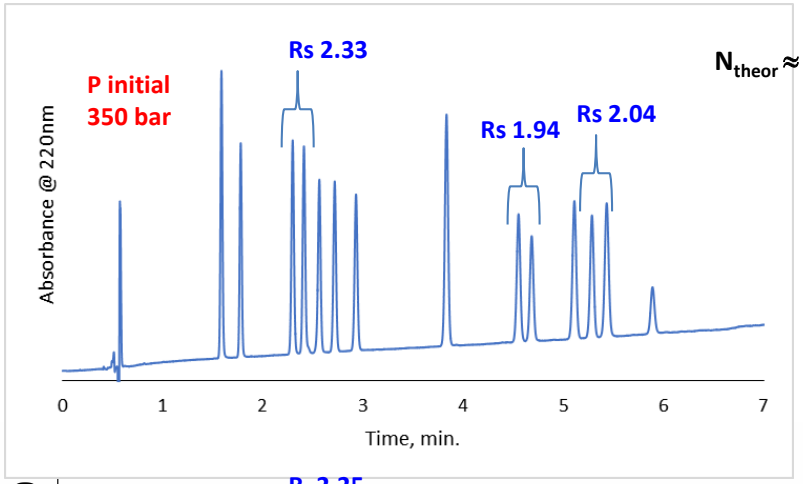
Transfer of 11-Steroid Separation from 4.6 x 250 mm, 5 µm TPP to 3 x 150 mm, 5 µm SPP and 3 x 50 mm, 2 µm SPP

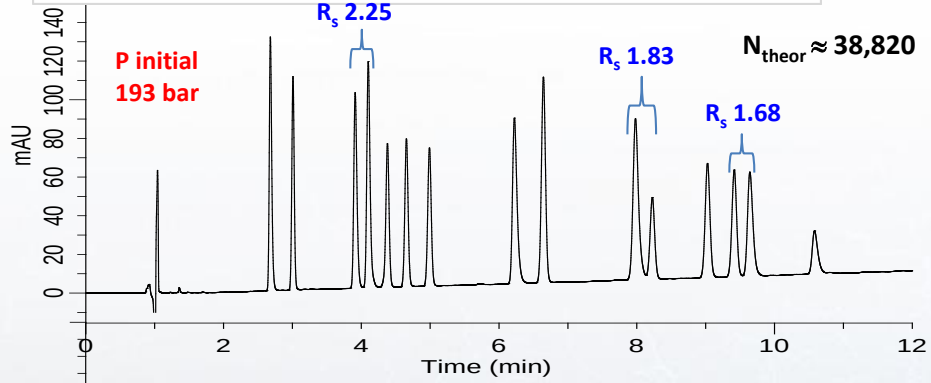


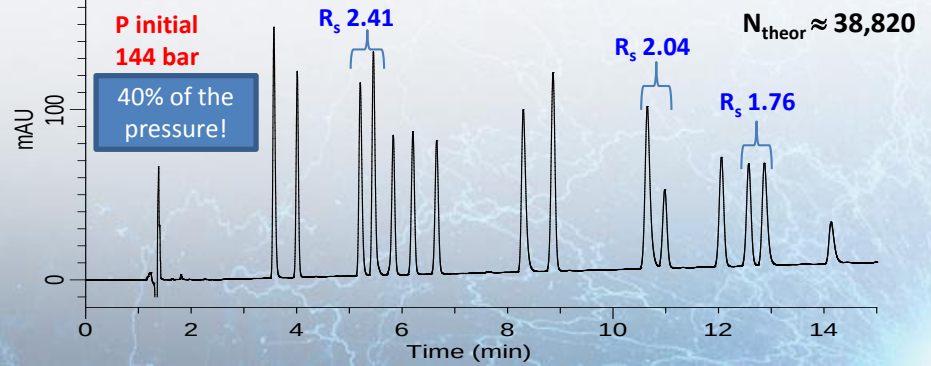
Analyte Elution order on HALO 5: (1) estriol, (2) prednisolone , (3) hydrocortisone, (4) cortisone, (5) dexamethasone, (6) corticosterone, (7) 17-β-estradiol, (8) 17-α-estradiol, (9) estrone, (10) epi-testosterone, (11) cortisone acetate

NOTE: Separation was transferred from a method on 4.6 x 150 mm, 3 µm TPP column to 4.6 x 250 mm, 5 µm TPP column

Cannabinoids: Gradient Translation from 3 x 150 mm, 2.7 µm HALO C18 to 4.6 x 250 mm, 5 µm HALO C18







Shimadzu Nexera, Delay volume, 0.47 mL
HALO 90 Å C18, 2.7 µm, 3 x 150 mm
Flow rate, 1.0 mL/min; 30 °C
Gradient: 70 to 88% ACN/water (0.1% HCOOH) in 6 min
Inj. Vol.: 1 µL
Linear velocity: 4.66 mm/sec

Instrument	Dimensions	Flow Rate	d_p (µm)	N_{theor}	V_M	µ (mm/sec)	P_c	Limiting Rs
Nexera	3 x 150	1.00	2.7	39700	0.537	4.66	125	1.94
Agilent 1100 Optimized	4.6 x250	2.00	5	38820	2.10	3.96	126	1.68
Agilent 1100 Optimized	4.6 x 250	1.50	5	38820	2.10	2.97	136	1.76

Agilent 1100 Optimized, Delay volume, 1.02 mL
HALO 90 Å C18, 5 µm, 4.6 x 250 mm
Flow rate, 2.0 mL/min; 30 °C
Gradient: 70 to 88% ACN/water (0.1% HCOOH) in 11.76 min
Inj. Vol.: 4 µL
Linear velocity: 3.96 mm/sec

Time	%B
0.00	70
0.41	70
12.17	88
14.13	88
14.32	70
20.60	70

Agilent 1100 Optimized, Delay volume, 1.02 mL
HALO 90 Å C18, 5 µm, 4.6 x 250 mm
Flow rate, 1.5 mL/min; 30 °C
Gradient: 70 to 88% ACN/water (0.1% HCOOH) in 15.67min
Inj. Vol.: 4 µL
Linear velocity: 2.97 mm/sec

Time	%B
0.00	70
0.55	70
16.22	88
18.84	88
19.10	70
27.47	70

Summary and Conclusions

- Described the key parameters to be measured and assessed for the columns and instruments
- Knowledge of the gradient delay volume, instrument dispersion and other instrument parameters, along with column theoretical and actual performance under prescribed conditions is important.
- Method translation can be done quite readily if proper measurements and calculations are made beforehand.
- Transfer between different column brands (even with the same stationary phase type (C18, phenyl, cyano, etc.)
  - always subject to selectivity changes and may require separation re-development and optimization ("adequatization").
- The web site www.hplccolumns.org with the Hydrophobic Subtraction Model of Lloyd R. Snyder, John Dolan and Peter Carr is strongly recommended for identifying alternative, "equivalent" columns.

HALO®