Utilizing Gradient Manipulation and 1.8 µm LC Columns for High Resolution Analysis of Complex Natural Products

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Introduction

The gradient slope is the chief parameter modified for optimizing RP gradient separations of complex mixtures such as natural product extracts. Other method parameters that maximize resolution in gradient methods such as column length, particle size, and flow rate are sometimes ignored due to limitations of the LC system including the column. Licorice Root extract and other natural products will be separated using UHPLC instruments which provide the flexibility to better use a wider range of LC parameters including column length, temperature, and flow rate for optimum resolution. The UHPLC instruments will be used with 1.8 µm columns to evaluate how column length (efficiency), flow rate, and other method parameters in addition to gradient slope, can be optimized to produce narrower peaks, higher peak capacity, and the best resolution for complex samples requiring gradients.

Peak Capacity

$$P_c = 1 + \frac{t_g}{(1/n)\,\Sigma_1^n w}$$

t_g = gradient run time
n = number of peaks measured for width
$\Sigma^n w$ = sum of peaks widths

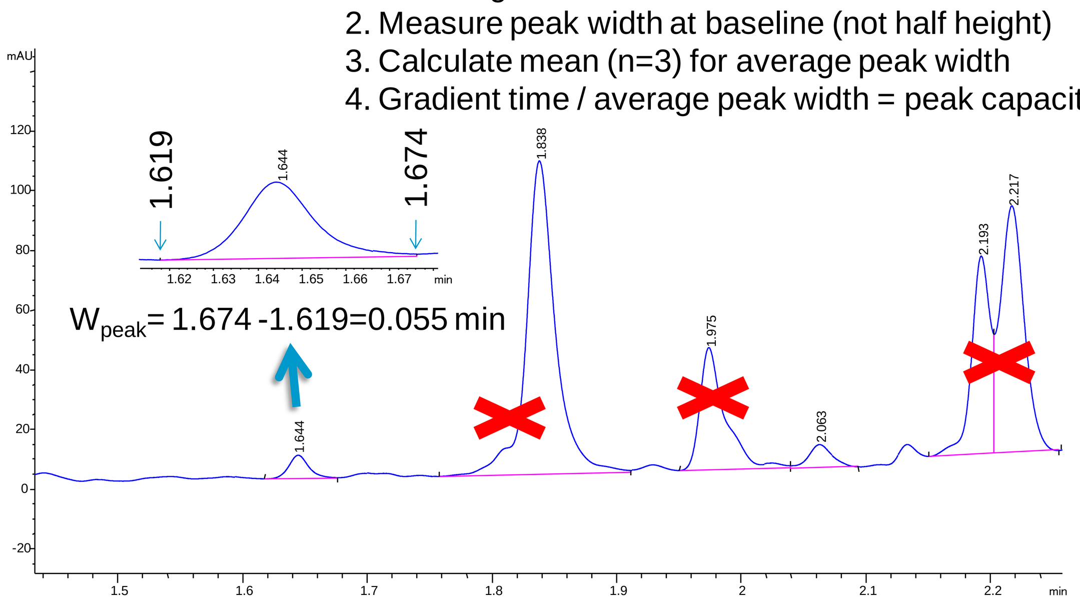
•Assume peaks elute across entire gradient

Neue, U.D., "Theory of peak capacity in gradient elution" J. Chromatogr. A 1079 (2005) 153-161

Peak capacity is sometimes used to measure column performance for gradient analyses. The higher the number of peaks across the gradient, the better the column performance.

Peak Width

1. Choose 3 peaks, avoid co-eluting peaks:
   - Early gradient eluter
   - Middle gradient eluter
   - Late gradient eluter
2. Measure peak width at baseline (not half height)
3. Calculate mean (n=3) for average peak width
4. Gradient time / average peak width = peak capacity



Gradient Manipulations:

1. Varied Column Length, Fixed Gradient Time
2. Varied Gradient Time, Fixed Column Length
3. Varied Flow Rate, Fixed Gradient Time
4. Varied Flow Rate, Scaled Gradient Time
5. Elevated Temperature

Column length, gradient time and flow rate have a direct effect on gradient retention (k*). Manipulating k* is useful to change resolution.

$$k^* = \frac{t_g F}{V_{column\ void}}$$

t_g = gradient run time
F = flow rate
$V_{column\ void} = t_0$ F *(void time x flow rate)*

Temperature also influences resolution by changing the selectivity (α).

Experimental

The UHPLC: Agilent 1290 Infinity

- Column Temperature: 26°C (1.6 µL TCC heat exchanger)
- DAD: 280, 4 Ref =off, 60 µm flow cell
- Peak Width >0.003 min (0.062 s response time) (80 Hz)

Complex Natural Products

Sample Preparation

Licorice Root *(Glycyrrhiza giabra)*

Goldenseal Root *(Hydratis canadensis)*

Echinacea Root *(Echinacea purpurea and angustifolia)*

Three liquid herbal supplements were procured from a local vendor and diluted 1:100 in de-ionized water, then filtered (0.2 µm regenerated cellulose) into autosampler vials for analysis.

The Mobile and Stationary Phase

Stationary Phase: ZORBAX RRHD Eclipse Plus C18

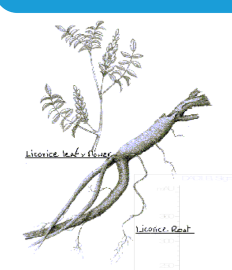
2.1 x 50mm, 1.8 µm PN 959757-902
2.1 x 100 mm 1.8 µm PN 959758-902
2.1 x 150 mm 1.8 µm PN 959759-902

RRHD (Rapid Resolution High Definition) columns contain 1.8 µm ZORBAX particles and are designed to operate at pressures up to 1200 bar for instruments such as the Agilent 1290 Infinity UHPLC.

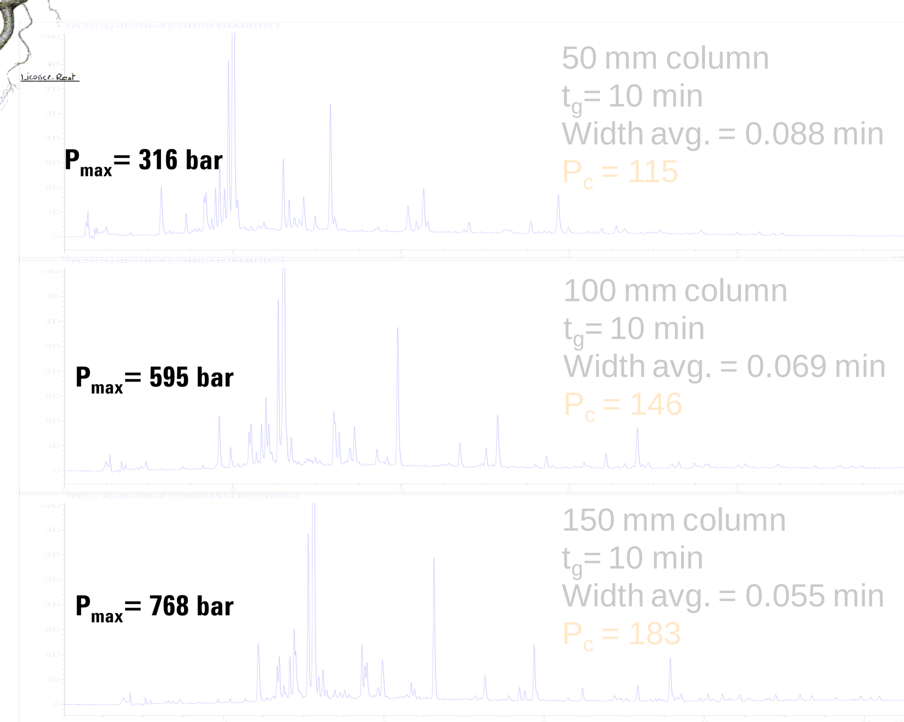
Mobile phase A: Water with 0.1% formic acid (v/v)
Mobile phase B: Acetonitrile with 0.1% formic acid (v/v)

Gradient: 10 to 100% B
Flow: 0.4 mL/min. unless otherwise indicated

Results

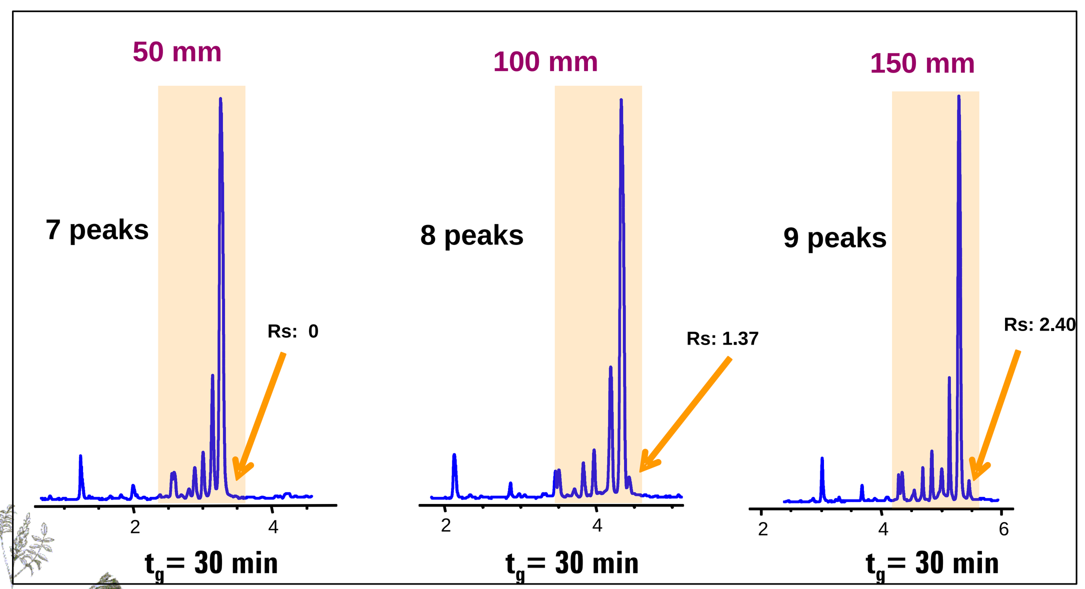


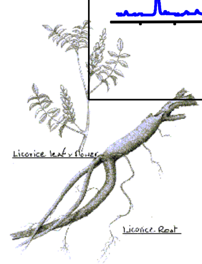
Effect of Column Length on P_c
Licorice Root



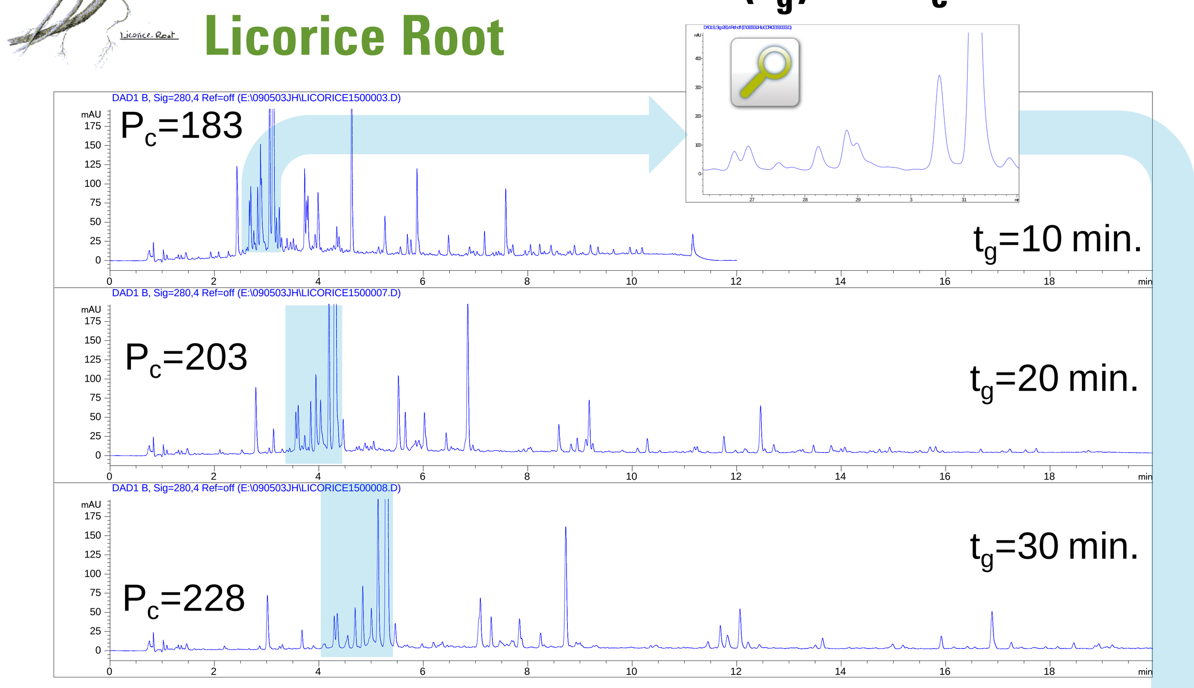
Gradient time primarily determines analysis time. Higher efficiency of longer columns produces narrower peaks, resulting in increased peak capacity.
The higher pressure from the longer columns is well within the operating range of the RRHD columns and Agilent 1290 Infinity UHPLC.

Increasing P_c Increases Rs of Licorice Root

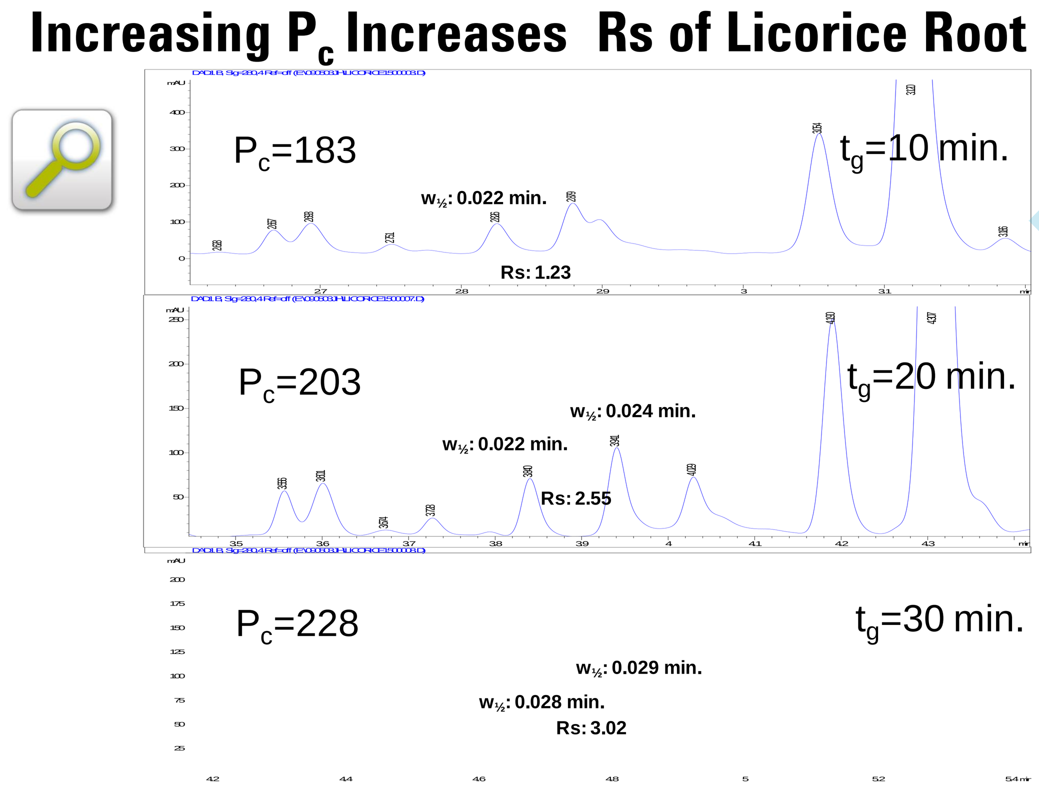




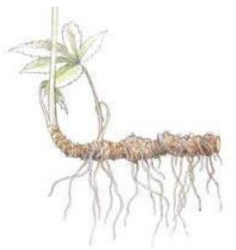
Effect of Gradient Time (t_g) on P_c
Licorice Root



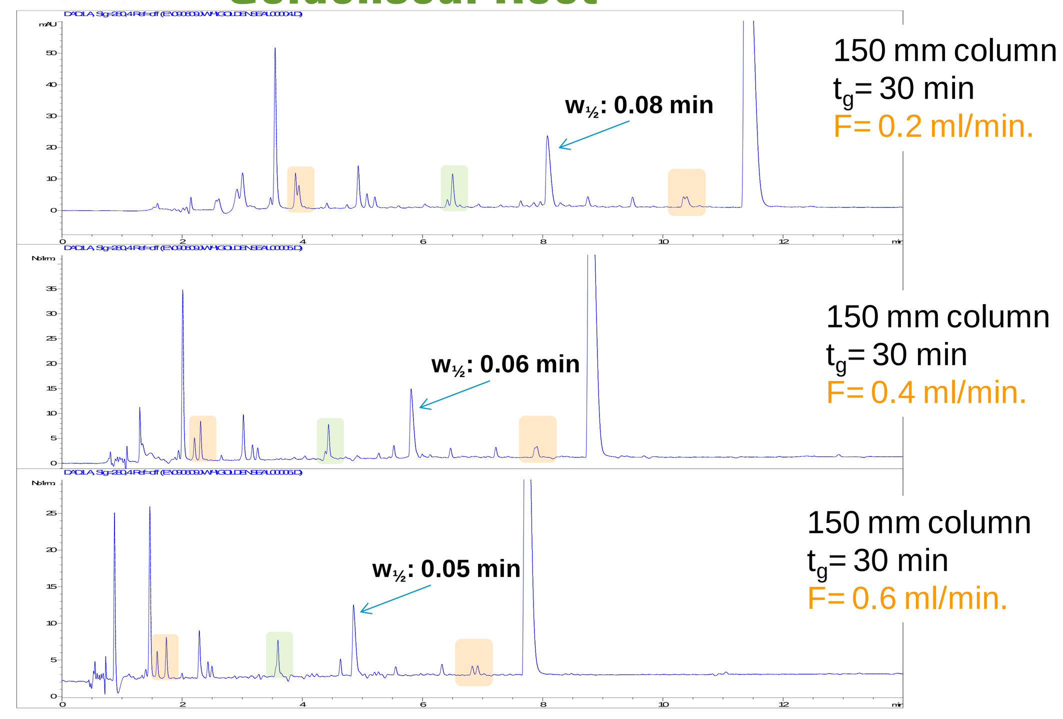
Increasing P_c Increases Rs of Licorice Root



Changing the gradient time from 10 to 30 min. increased peak capacity by 20%. In the top chromatogram this does not appear to be a significant increase in column performance, but a magnified view of closely eluting peaks in the 2 to 5 min. range shows the resolution factor improving significantly, despite wider peaks.

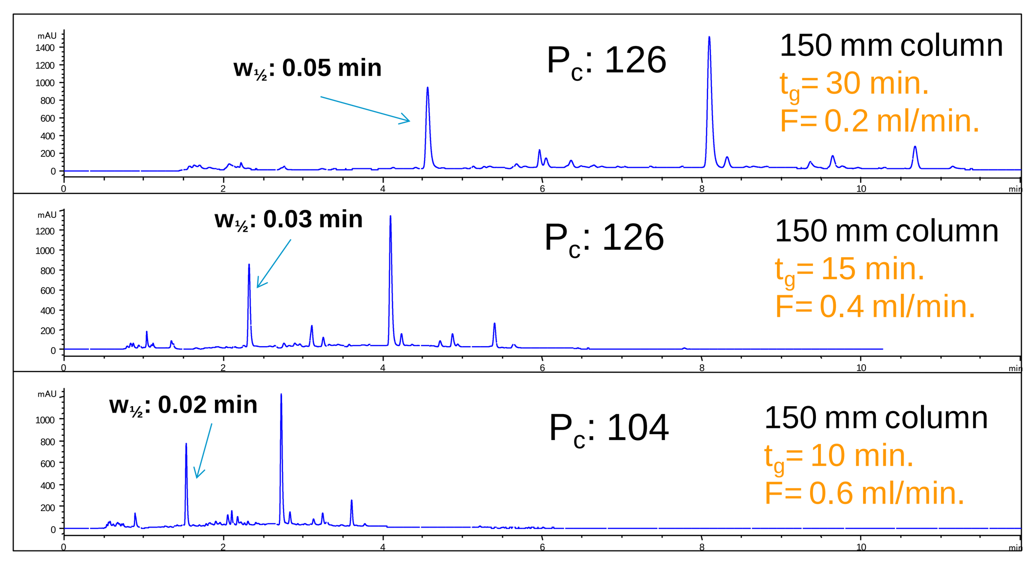


Effect of Flow Rate on P_c
Goldenseal Root



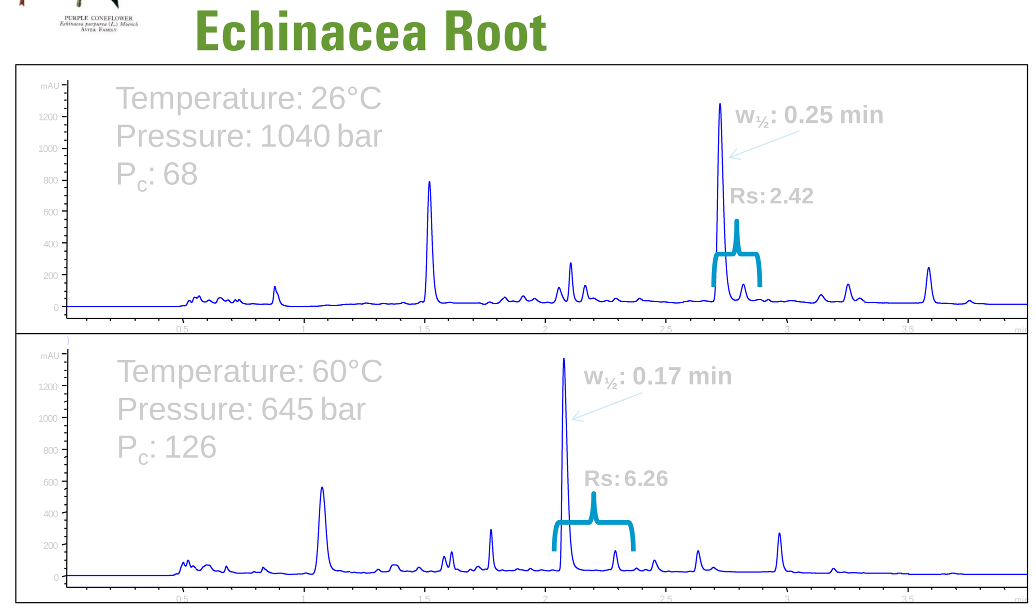
Increasing flow rate while keeping the gradient time fixed also reduces peak width, and increases peak capacity. It also changes selectivity and has an effect on resolution. In this goldenseal example, resolution improved for the critical pairs shaded orange, but resolution worsened for the green shaded pair. Retention time is also reduced.

Effect of Flow Rate: Scaled Gradient
Echinacea Root



Tripling the flow rate requires reducing the gradient time by one-third to proportionally scale the gradient. Peak capacity was reduced slightly, but resolution was maintained and analysis time was reduced threefold.

Effect of Temperature
Echinacea Root



Elevated temperature reduces peak width, and therefore increases peak capacity. Temperature can also alter selectivity so may or may not improve the separation. In this echinacea separation elevated temperature did improve resolution. Elevated temperature reduces mobile phase viscosity, lowering system pressure. This shortens analysis time and can be advantageous for more viscous solvents such as methanol.

Conclusion

Four variables that can improve peak capacity and resolution in 1.8 µm gradient methods are:

- Longer Columns
- Longer Gradient Times
- Higher Flow Rates
- Temperature

Agilent RRHD columns and the 1290 Infinity UHPLC are designed for higher system pressure, so these four variables can be better utilized for gradient method development of complex samples.

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