Eliminate your Application and Chromatography Challenges

LC Application Scientist Session

Information Contributed by USA HPLC Applications Scientist Team

Speaker: Lorena Lopez
LC Application Scientist
Lexington, MA

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DE:7419791667
This Session will cover

Troubleshooting your Method, Application / Chromatography
• Sample prep considerations
• Troubleshooting by following the LC flow. Mobile Phase and System Hygiene
• Step through Method Setup to highlight parameters that are critical but often overlooked or misunderstood

Optimizing your Application / Method Transfer considerations
• What needs to be considered when implementing App notes and transferring method between systems
• Delay volume, column void volumes
• Capillary selection and connections
• Column considerations (dimension and particle size)

Advancing your Application / Chromatography
• How to choose the appropriate LC system for the application that is going to be run.
• Where to find resources or info
Sources of Error Generated During Chromatographic Analysis

- Sample preparation (30%)
- Operator (19%)
- Columns (11%)
- Contamination (4%)
- Sample introduction (6%)
- Chromatography (7%)
- Integration (6%)
- Instrument (8%)
- Calibration (9%)

Data taken from Agilent Technologies survey
## What is Method Transfer?

<table>
<thead>
<tr>
<th>Different Aspects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>From particle size to particle size (HPLC to UHPLC)</strong></td>
</tr>
<tr>
<td>- often associated with a change of instruments due to constraints in max. pressure, extra-column band broadening etc.. Some fine-tuning of method might be required due to frictional heating effects</td>
</tr>
<tr>
<td>- available tools: Agilent Method translator, Third Party Method development SW (S-Matrix, ACD Labs, ChromSword, etc.)</td>
</tr>
<tr>
<td><strong>From one eluent type or phase chemistry to an other</strong></td>
</tr>
<tr>
<td>- Method development</td>
</tr>
<tr>
<td>- available tools: Method development SW (ACD Labs, ChromSword, S-Matrix etc.)</td>
</tr>
<tr>
<td><strong>From column dimension to column dimension (e.g. 4.6 to 2.1 mm i.d., 50 mm to 100 mm)</strong></td>
</tr>
<tr>
<td>- recalculate flow rates, recalculate gradient times, adjust connection capillaries and flow cells, due to delay volume results may vary, method might need revalidation</td>
</tr>
<tr>
<td>- available tools: Method Translator</td>
</tr>
<tr>
<td><strong>From Instrument to Instrument</strong></td>
</tr>
<tr>
<td>- with method/instrument change: Isocratic Hold / Pre-injection, Vol. Modification</td>
</tr>
<tr>
<td>- available tools w/o method or instrument change: none until ISET</td>
</tr>
</tbody>
</table>
Method Transfer Considerations & Application Optimization

Method transfer — What went wrong?

Legacy method

Method transfer — What went wrong?
Design Differences (U)HPLC Systems

- Delay volumes (gradient formation)
- Power range (flow x pressure)
- Extra column volume
- Temperature
- Data rates
- Sensitivity

Problem:
Instrument to Instrument Method Transferability
- Not possible
- Requires Re-Development
- Requires Re-Validation

Resulting in huge additional cost factor
Instrument to Instrument Method Transferability
- Important Parameters

- **Pump**
  - Delay volume
  - Gradient mixing behavior
  - Pressure x flow rate

- **Sampler**
  - Delay volume
  - Extra column volume
  - Injection volume

- **Column Thermostat**
  - Temperature profile
  - Extra column volume

- **Detector**
  - Data rate
  - Extra column volume
  - Path-length

- **Retention Time**
- **Resolution**
- **Sensitivity**
Comparison of Gradient Delay Volume (Dwell Volume)

1290 Infinity II Flexible Pump (Quaternary)
- Integrated Degasser
- 4 solvent channels with concurrent mixing of all 4 channels
- Lower in price, typically, than binary pump

1290 Infinity II High Speed Pump (Binary)
- Integrated Degasser
- 4 solvent channels available, mixing of 2 channels possible
- Better performance concept is widely accepted
- Greater control over dwell volume vs. Quaternary pump

*Incl. Degasser for both pumps
**with solvent selection valve
1290 Family Delay Volume Profiles

Blue – Binary with V35 mixer
Red – Quat with no mixer
Green – Quat w/ V380 mixer

0.5ml/min
A: 50% MeOH/water
B: 50% MeOH with 500mm AmmOAc
Step from 0% B to 20% B at 10 min.

System includes G4226A Autosampler, in Mainpass Position, TCC with 65um (i.d.) PEEK restrictor (operating pressure for these tests 350 bar) and G4212B DAD with std 10mm 1ul MaxLight flow cell
1290 Delay Volume – 10-90% Transition

Transition Volume Calculations

Quat with mixer V380

(~12.85-11.21) x 500 ul/min = 820 ul

Quat without mixer

(~11.58-10.92) x 500 ul/min = 330 ul

Binary with V35 mixer (standard)

(10.58-10.32) x 500 ul/min = 130 ul
Method Transfer to UHPLC instruments
Impact of delay volume and mixing behavior...

- Programmed gradient step
- 1290 Infinity II High Speed Pump
- 1260 Infinity Binary Pump

What happens with a programmed gradient?

- Gradient slope/Mixing behavior
- Delay volume

1200 Series
1290 Infinity II

Total delay volume of the system (sum of capillaries, mixer, cells, valves..)
Chromatographic Test Result; Different Delay Volumes

Instrument with smaller dwell volume

Instrument with larger dwell volume
Example -- How to Calculate Delay Volume

Time(50%) – Time(step) x Flowrate
= dwell volume
(4.04-2.00) min. x 500ul/min
= 1020ul
## Summary of Available Data

### Delay Volume Summary for Agilent Infinity 1290 Systems

<table>
<thead>
<tr>
<th>Pump</th>
<th>Configuration</th>
<th>Sampler</th>
<th>Liftoff</th>
<th>Midpoint</th>
<th>Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1290 bin @ 350 Bar</td>
<td>35ul JetWeaver</td>
<td>G4226A</td>
<td>125</td>
<td>225</td>
<td>130</td>
</tr>
<tr>
<td>1290 quat @ 350 Bar</td>
<td>no mixer</td>
<td>G4226A</td>
<td>375</td>
<td>570</td>
<td>330</td>
</tr>
<tr>
<td>1290 quat @ 350 Bar</td>
<td>380ul JetWeaver</td>
<td>G4226A</td>
<td>450</td>
<td>820</td>
<td>820</td>
</tr>
<tr>
<td>For Reference…</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1260 bin @ 350 Bar</td>
<td>as shipped</td>
<td>G1367E</td>
<td>720</td>
<td>1105</td>
<td>545</td>
</tr>
</tbody>
</table>
## Summary of Available Data

### Delay Volume Summary for Binary 1260 Systems

<table>
<thead>
<tr>
<th>Pump</th>
<th>Configuration</th>
<th>Sampler</th>
<th>Liftoff</th>
<th>Midpoint</th>
<th>Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1260 bin @ 350 Bar</td>
<td>as shipped</td>
<td>G1367E</td>
<td>720</td>
<td>1105</td>
<td>545</td>
</tr>
<tr>
<td>1260 bin @ 350 Bar</td>
<td>minus damper</td>
<td>G1367E</td>
<td>635</td>
<td>955</td>
<td>460</td>
</tr>
<tr>
<td>1260 bin @ 350 Bar</td>
<td>minus damper with purge valve in channel A</td>
<td>G1367E</td>
<td>550</td>
<td>835</td>
<td>425</td>
</tr>
<tr>
<td>1260 bin @ 350 Bar</td>
<td>minus mixer/damper with purge valve in channel A</td>
<td>G1367E</td>
<td>270</td>
<td>375</td>
<td>175</td>
</tr>
<tr>
<td>1260 bin @ 350 Bar</td>
<td>minus mixer/damper with purge valve in channel A</td>
<td>G1367E with bypass (ADVR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>For Reference</td>
<td>1290 bin @ 350 Bar</td>
<td>35ul JetWeaver</td>
<td>G4226A</td>
<td>125</td>
<td>225</td>
</tr>
</tbody>
</table>
One-Click LC System Emulation

Intelligent System Emulation Technology (ISET)

- Emulates other (U)HPLC instruments – by a simple mouse click
- Runs existing (U)HPLC methods – without modifying method or system
- Delivers same retention times and peak resolution – for infinitely better method transfer
Intelligent System Emulation Technology (ISET)

How does it work?

Same gradient conditions by
1) moving the gradient
2) emulating the gradient mixing behavior

- Programmed gradient
- 1290 gradient
- 1200 gradient
Intelligent System Emulation Technology (ISET)

1200 Quat

1290 Binary no ISET

1290 Binary with ISET
Can you tell which is which?
You can run the fastest UHPLC methods, and still run your legacy methods
Lowest dispersion for highest resolution

Agilent A-Line Quick Connect UHPLC column fittings for truly dead-volume-free fluidic connections

- Tool-free connection up to 1300 bar
- Spring loaded design for easy and truly zero-dead volume connection
- Removable and reusable for all column types

System dispersion

- “Dispersion is the sample bandspreading or dilution which occurs in connecting tubing, sample valves, flow cells and in column end-fittings.”

- Capillaries (Inner diameter, length)

Peak height: Loss of sensitivity
Peak width: Loss of resolution
# Tubing Pressure and Internal Volume

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Black</th>
<th>Red</th>
<th>Green</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tubing Description</strong></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>i.d. (mm)</td>
<td>0.075</td>
<td>0.127</td>
<td>0.178</td>
<td>0.254</td>
</tr>
<tr>
<td>Total Length (mm)</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td>1.000</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Solvent Name</td>
<td>ACN/wa</td>
<td>ACN/wa</td>
<td>ACN/wa</td>
<td>ACN/wa</td>
</tr>
<tr>
<td>Viscosity (cP)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Internal Volume (ul)</td>
<td>2.209</td>
<td>6.334</td>
<td>12.4</td>
<td>25.3</td>
</tr>
<tr>
<td>Expected pressure (bar)</td>
<td>129.1</td>
<td>15.7</td>
<td>4.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

500mm – typical sample flow path with UV detn. LC/MS -- near 1000mm
## Tubing Dimensions and System Dispersion

<table>
<thead>
<tr>
<th></th>
<th>HPLC 1260</th>
<th>UHPLC 1290</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column to Detector</strong></td>
<td>18-30cm 0.17 18-30cm 0.12</td>
<td>18-30cm 0.12 25cm 0.075</td>
</tr>
<tr>
<td><strong>TCC outlet to column</strong></td>
<td>10cm 0.17 10cm 0.12</td>
<td>10cm 0.12 10cm 0.075</td>
</tr>
<tr>
<td><strong>ALS to TCC inlet</strong></td>
<td>18-30cm 0.17 18-30cm 0.12</td>
<td>18-30cm 0.12 45cm 0.075</td>
</tr>
<tr>
<td><strong>Needle Seat</strong></td>
<td>Green 0.17 - 2.3uL Red 0.12 - 1.7uL</td>
<td>Red 0.12 - 1.7uL Black 0.075 - 0.9uL</td>
</tr>
<tr>
<td><strong>Pump to ALS</strong></td>
<td>20-40cm 0.17 20-40cm 0.12</td>
<td>20-40cm 0.12 20cm 0.075</td>
</tr>
</tbody>
</table>

Optimize Dispersion for Small Columns
2.1 x 50 mm Poroshell 120 1.9µm

Figure 1A. The performance of an Agilent InfinityLab Poroshell 1.9 µm column is improved when LC system volume is reduced by using smaller internal diameter capillaries and a smaller volume detector flow cell.

Figure 1B. The performance of an Agilent InfinityLab Poroshell 1.9 µm column is improved when LC system volume is reduced by using smaller internal diameter capillaries and a smaller volume detector flow cell.
Optimizing System Dispersion on the Agilent 1290 Infinity II LC

Isocratic separation of dimethyl phthalate, diethyl phthalate, biphenyl, and o-terphenyl

App Note: 5991-5984EN
Increased Pressure Requirements (column particle size)

<table>
<thead>
<tr>
<th>(d_p) (µm)</th>
<th>(\Delta P) (bar)</th>
<th>(N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>14.5</td>
<td>25,000</td>
</tr>
<tr>
<td>3.0</td>
<td>66.9</td>
<td>41,000</td>
</tr>
<tr>
<td>1.5</td>
<td>531</td>
<td>83,000</td>
</tr>
<tr>
<td>1.0</td>
<td>1800</td>
<td>125,000</td>
</tr>
<tr>
<td>0.75</td>
<td>4270</td>
<td>166,000</td>
</tr>
<tr>
<td>0.50</td>
<td>14400</td>
<td>250,000</td>
</tr>
</tbody>
</table>

For a 30 cm column, 5.0 to 0.50 µm particle size reduction:

- \(N\) (theoretical plates) increases 10-fold
- \(\Delta P\) (change in pressure) increases 1000-fold
## Column Length and Particle Size

<table>
<thead>
<tr>
<th>Column Length (mm)</th>
<th>Column Efficiency N(5 µm)</th>
<th>Column Efficiency N(3.5 µm)</th>
<th>Column Efficiency N(1.8 µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>12,500</td>
<td>21,000</td>
<td>35,000</td>
</tr>
<tr>
<td>100</td>
<td>8,500</td>
<td>14,000</td>
<td>23,250</td>
</tr>
<tr>
<td>75</td>
<td>6,000</td>
<td>10,500</td>
<td>17,500</td>
</tr>
<tr>
<td>50</td>
<td>4,200</td>
<td>7,000</td>
<td>12,000</td>
</tr>
<tr>
<td>30</td>
<td>N.A.</td>
<td>4,200</td>
<td>6,500</td>
</tr>
<tr>
<td>15</td>
<td>N.A.</td>
<td>2,100</td>
<td>2,500</td>
</tr>
</tbody>
</table>

* Reduction in analysis time compared to 150 mm column; all columns 4.6-mm i.d.

### Analysis Time*

- **Pressure**
  - Analysis Time: -33%
  - Peak Volume: -50%
  - Solvent Usage: -67%

- **Efficiency (N)**
  - Analysis Time: -80%
  - Solvent Usage: -90%
### Predicting Column Pressure and Performance

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Media</th>
<th>ZORBAX</th>
<th>Poroshell 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.d. (mm)</td>
<td></td>
<td>2.100</td>
<td>3.000</td>
</tr>
<tr>
<td>Length (mm)</td>
<td></td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Particle Size (uM)</td>
<td></td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Column void fraction</td>
<td></td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td>Permeability (PF)</td>
<td></td>
<td>900</td>
<td>900</td>
</tr>
<tr>
<td>Reduced plate height value</td>
<td></td>
<td>2.30</td>
<td>2.30</td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td></td>
<td>0.5000</td>
<td>1.000</td>
</tr>
<tr>
<td>Linear Velocity (mm/sec)</td>
<td></td>
<td>4.01</td>
<td>3.93</td>
</tr>
<tr>
<td>Viscosity (cP) 25C ACN/Water</td>
<td></td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Column Volume (ml)</td>
<td></td>
<td>0.104</td>
<td>0.212</td>
</tr>
<tr>
<td>Expected pressure (bar)</td>
<td></td>
<td>401.0</td>
<td>393.0</td>
</tr>
<tr>
<td>Estimated Efficiency** (Neue)</td>
<td></td>
<td>12077</td>
<td>12077</td>
</tr>
</tbody>
</table>

**Estimates do not consider system dispersion effects. Actual results will vary.
Yield estimates – 1.8um particles 2.1-4.6mm i.d.
Multi-Detector Connections Bandspreading

[Graph showing UV connected before FLD and FLD connected to column directly, 50cm red PEEK]
Use The Right Tool for the Job

We might get this to work, but the results may not be what we needed
(U)HPLC Systems

- Each component of the system has settable values, however not all settable values can be achieved.

- Before using or purchasing a system, the user must be certain that the desired parameters can be run accurately and reproducibly.

- Instrument specifications should be obtained from the appropriate manuals.

- From these specifications, it can be determined if that component may be used.
Pump Parameters

- Low flow rate (<0.5 ml/min) start to move to binary pump due to lower delay volumes
- Shallow gradient (<1%B/min) requires binary pump due to lower delay volume and more precise changes

Note flow rate 0.2 ml/min

Note gradient change (%B/min)
Shorter (<150mm) and narrower columns (<3mm) may have lower injection volumes. Make sure the sampler precision and accuracy are correct for low volumes.

More sensitive detectors (or higher salt concentrations in the buffers) may require additional wash steps for both the needle and needle seat.
As columns become shorter peaks elute more quickly requiring detector data rates to increase.
Resources — Primers

5990-7595EN
The LC Handbook
Guide to LC Columns and Method Development

5991-2359EN
Two Dimensional Liquid Chromatography

5990-3777EN
High Performance Capillary Electrophoresis

5991-5509EN
Supercritical Fluid Chromatography

5989-6639EN
Principles in Preparative HPLC

5991-3326EN
Sample Preparation Fundamentals for Chromatography

5980-1397EN
Fundamentals of UV-visible Spectroscopy
Resources for Support

- Collection of LC resources: https://community.agilent.com/docs/DOC-1852-lc-insights-to-go#jive_content_id_LC_Troubleshooting
- Agilent support resources: https://community.agilent.com/community/resources
- Agilent University: http://www.agilent.com/crosslab/university
- Agilent resource center: http://www.agilent.com/chem/agilentresources
- InfinityLab Supplies Catalog (5991-8031EN)
- Your local FSE and Specialists
- Youtube – Agilent Channel

- Sales and support phone assistance (US and Canada): 1-800-227-9770 Phone Tree Navigation Assistance

gc-column-support@agilent.com
lc-column-support@agilent.com
spp-support@agilent.com
spectro-supplies-support@agilent.com
Thanks for your attention!
Universal Low Dispersion Fittings

Quick Connect

- Spring pushes capillary constantly towards receiving port
- Guaranteed zero dead volume

Quick Turn

- Hand-tighten the blue nut until feeling the first resistance
- Depress the lever, 1300 bar tight!

Agilent application note 5991-5525EN