



A Media Platform That Allows Seamless Method Transfer Between UHPLC and Traditional HPLC Applications

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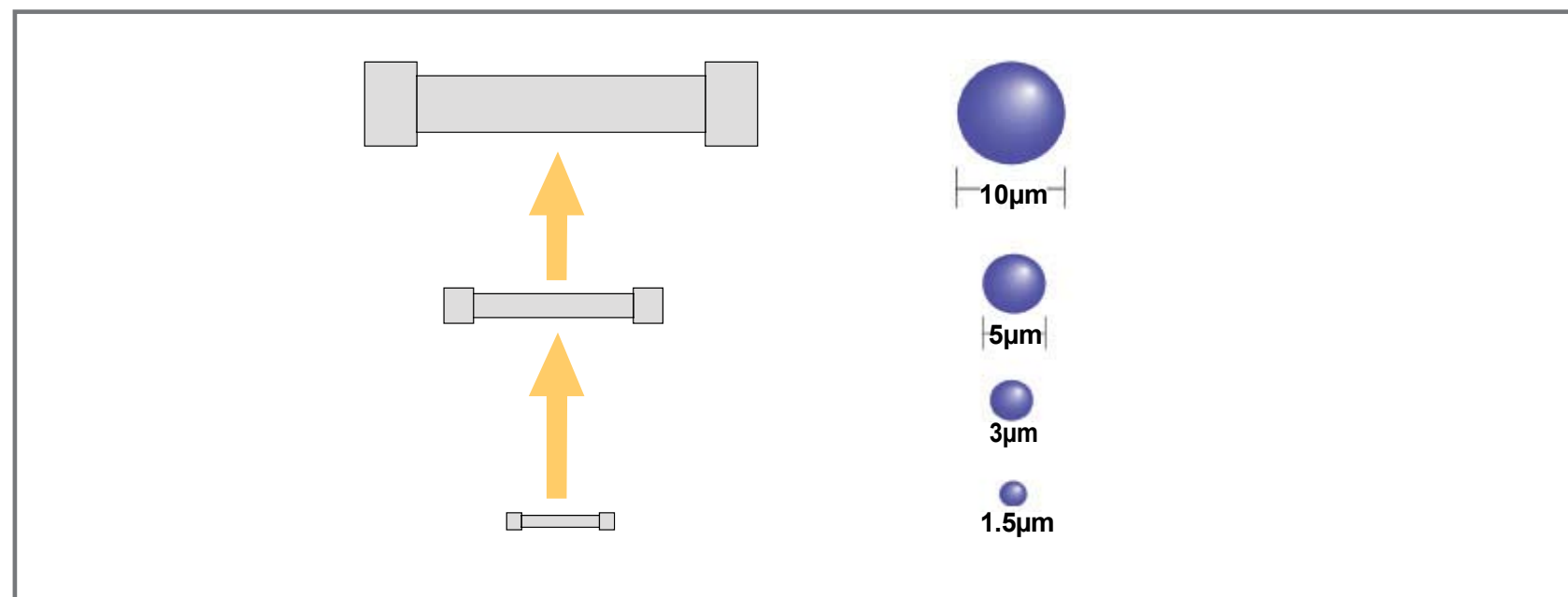
Abstract

The recent adoption of Fast LC systems within laboratory environment means there is a period of time when most labs have a variety of LC systems types – Traditional, UHPLC, and/or alternate Fast LC systems. Optimizing and transferring methods between systems has not been a simple and intuitive task. However, when the identical bonded phase is available in sub2, 3, 5, and 10µm particle sizes, it can be applied to appropriate formats to suit the system type. Here, we discuss the VisionHT™ column family which unifies LC technology platforms and improves laboratory efficiency. It offers a versatile range of phases, formats and particle sizes that proves ideal for separating everything from small polar molecules to larger protein/peptides.

Introduction

Method Transfer and Scalability Techniques

The VisionHT™ product line represents a Universal Column platform which allows the methodology to be developed in the classical 4.6 x 150mm 5µm format, and then transferred seamlessly to UHPLC style or alternate Fast LC system type. The opposite, of course, is also true – Develop/optimize methods on a Fast LC system and apply to the traditional LC system. A simple calculation to determine equivalent linear velocity is all that is necessary to seamlessly transfer methods between systems.



Practical Considerations When Transferring Methods Between Column Formats

Dead Volume

- Any volume after the injector can affect the separation by diffusing the sample. This decreases separation efficiency and affects separation.
- It is important to match the column type with the system type. Too short or narrow of a column on a large volume system will degrade separation performance.

Column Internal Diameter

Narrow ID (Use if sample limited or low concentration of target analyte)

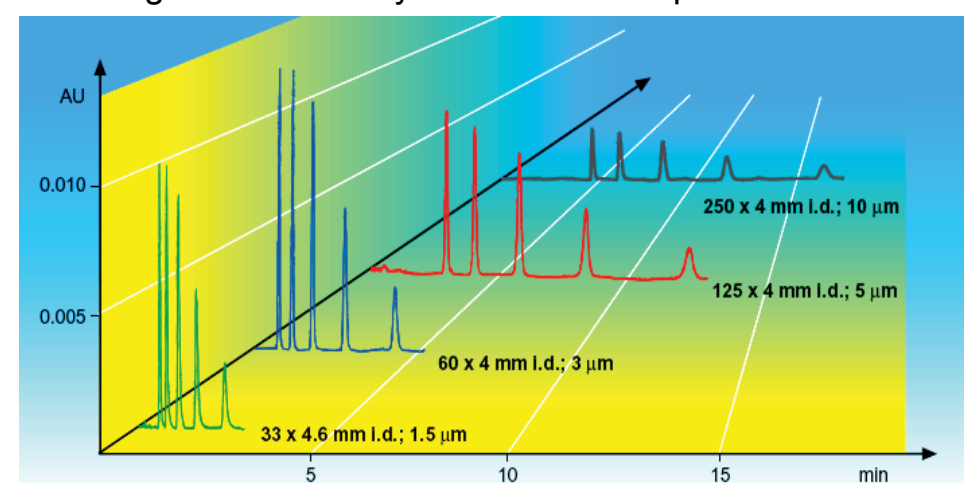
- Advantage: Increases sensitivity by keeping sample bands tighter as they move through the column
- Limitation: Lowers loading capacity

Larger ID (Use when needed to purify large sample amounts)

- Advantage: Increases sample loading capacity and over comes dead volume issues
- Limitation: Decreases sensitivity

Comparison of Speed, Sensitivity, and Resolution

Homologous Series – Alkyl Benzoates at equivalent flow rates



Stationary phase: 100Å ODS-2 FE;
Linear velocity: 1.8mm/s;
Eluent: ACN:H₂O = * 65:35, resp. ** 60:40;
Flow cell: 1.2µl/3mm with quick connector (100 x 0.1mm capillary);
Injection: 5µl benzoate test mixture / 1:100 dil. (methyl-, ethyl-, propyl-, butyl-, pentyl benzoate; 10–20mg/ml)

Speed, Resolution, and Sensitivity Data

| Col Length mm | Part Size µm | Analysis Time min | N/m Peak 5* 1/m | N/col 1/Col | Solv. use mL | Res 1&2 | Sensitivity Peak 1 AU | Pressure psig |
|---------------|--------------|-------------------|-----------------|-------------|--------------|---------|-----------------------|---------------|
| 250 | 10 | 18.1 | 36000 | 9000 | 10.9 | 4.8 | 0.004 | 200 |
| 125 | 5 | 9.4 | 75000 | 9375 | 5.6 | 5.2 | 0.008 | 500 |
| 60 | 3 | 4.8 | 120000 | 7200 | 2.9 | 4.6 | 0.013 | 1100 |
| 33 | 1.5 | 2.5 | 225000 | 7425 | 2 | 3.9 | 0.011 | 2500 |

Column i.d. 4mm
 Homologous Series - Alkyl benzoates at equivalent flow rates
 *pentyl benzoate

By reducing column length and particle size proportionally, overall analysis time is reduced and sensitivity is increased.

Physics of Method Transfer

When transferring your assay from one format to another there are some practical considerations that should be taken in account to maintain equivalent run conditions.

Column Length

$$L_2 = \frac{L_1 \times P_2}{P_1}$$

L = Length
P = Particle Size

$$45\text{mm} = \frac{150\text{mm} \times 1.5\mu\text{m}}{5\mu\text{m}}$$

Decreasing the particle size will increase the theoretical plate count of the column as well as the back pressure. By adjusting the column length we can maintain the separation.

Column Volume

$$F_2 = \frac{[D_2]^2 \times F_1}{[D_1]^2}$$

F = Flow
D = Diameter

$$0.208 = \frac{2.1^2 \times 1.0\text{mL/min}}{4.6^2}$$

The flow rate must be adjusted to maintain the linear velocity of the mobile phase traveling through the column which is essential to maintain peak efficiency and decrease associated backpressure.

Sample

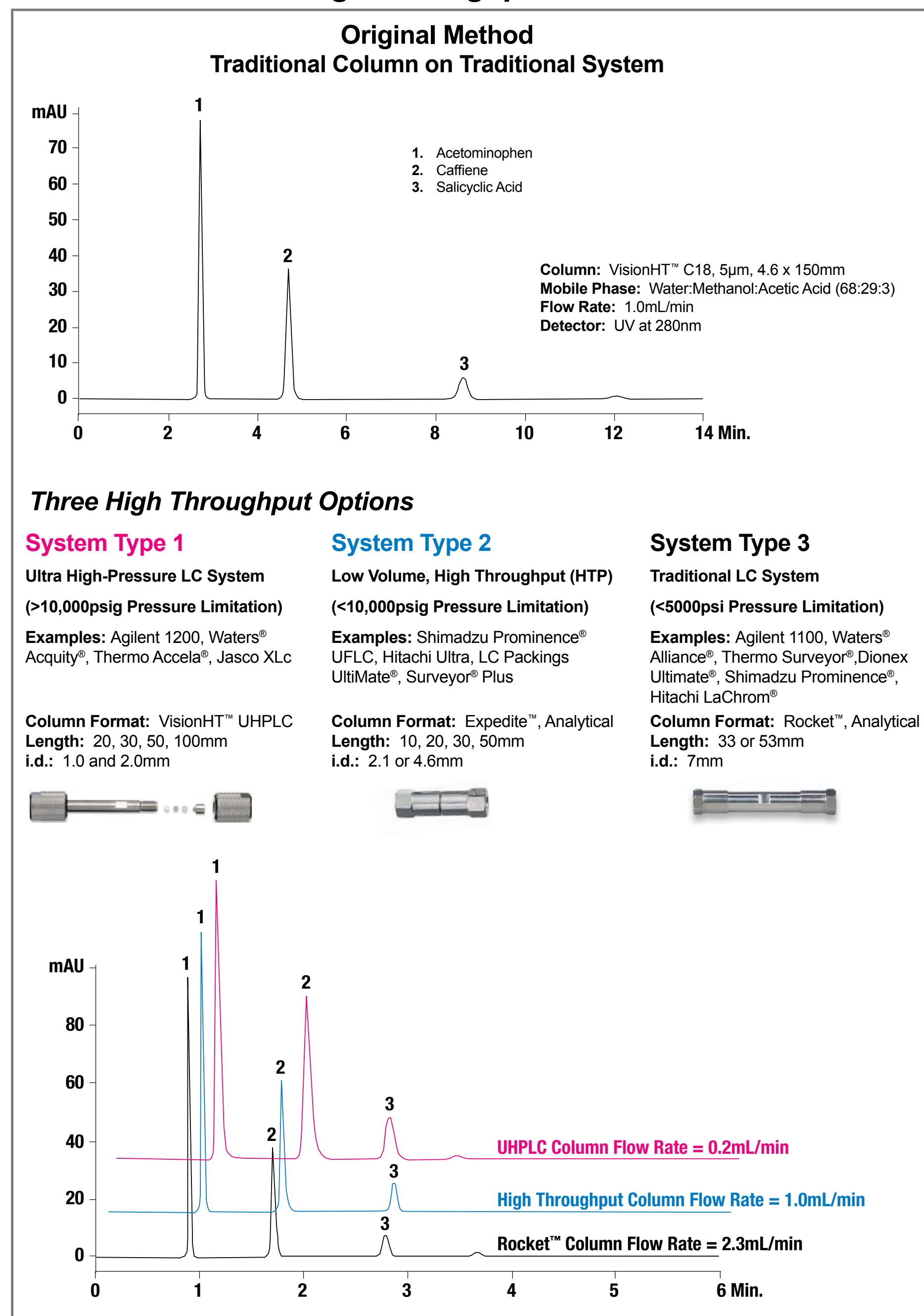
$$V_1 = V_2 \times \frac{D_2^2 \times L_2}{D_1^2 \times L_1}$$

V = Volume
D = Diameter
L = Length

$$0.69\mu\text{l} = 10 \times \frac{2.1^2 \times 50}{4.6^2 \times 150}$$

Decreases in column length and diameter lead to a decrease in column capacity. Decreasing the sample loading prevents column overload. Sample matrix effects will be more pronounced so that matching sample solvent to the mobile phase is critical.

Method Transfer to High Throughput Formats



The VisionHT™ column line represents a platform that allows the methodology to be developed in the classical 4.6 x 150mm 5μm format, and then transferred seamlessly to UHPLC style or alternate Fast LC system type. The opposite, of course, is also true – Develop/optimize methods on a Fast LC system and apply to the traditional LC system.

Grace® VisionHT™ Column Phase Specifications

Sub 2µm particles deliver speed and efficiency, but beyond that, having selectivity options can play a key role in separation success. Six VisionHT™ column high purity phases are available, each with unique separation benefits. C18-HL, with maximum bonded phase coverage, is ideal for complex hydrophobic samples. Use C18-B for basic compounds at neutral pH, which is often a requirement for mass spec work in the pharmaceutical industry. Reserve the C18 and C18-P for fastest analysis times. Both offer increased polar interactions to make neutral, non-polar compounds elute faster and retain polar compounds longer. The HILIC and Silica packings are normal phases that typically use near exclusive organic mobile phases; an advantage when seeking highest mass spec sensitivity.

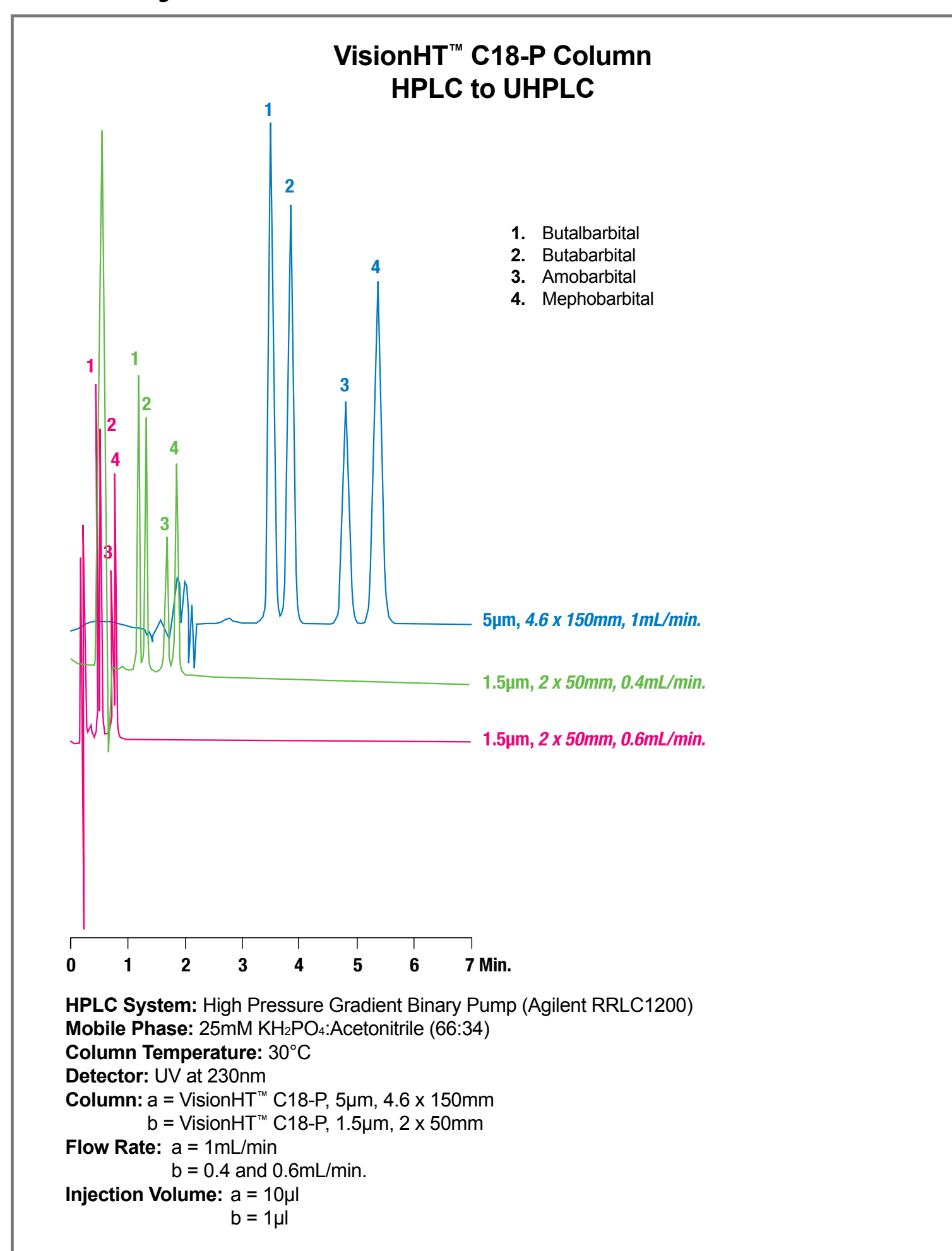
| Packing | Base Material | Particle Size | Carbon Load | Pore Size | Surface Area | Endcapped | pH Range* |
|---------|------------------|---------------|-------------|-----------|----------------------|-------------|-----------|
| C18-HL | Spherical Silica | 1.5,3,5,10µm | 10% | 120Å | 220m ² /g | Yes | 1–10 |
| C18-B | Spherical Silica | 1.5,3,5,10µm | 5% | 120Å | 220m ² /g | Proprietary | 1–10 |
| C18 | Spherical Silica | 1.5,3,5,10µm | 6% | 100Å | 200m ² /g | Yes | 1–10 |
| C18-P | Spherical Silica | 1.5,3,5,10µm | 5% | 100Å | 200m ² /g | No | 1–10 |
| HILIC | Spherical Silica | 1.5,3,5,10µm | NA | 120Å | 220m ² /g | No | 2–8 |
| Silica | Spherical Silica | 1.5,3,5,10µm | NA | 120Å | 220m ² /g | No | 2–8 |

*Choice of buffer is critical at pH >8

Small Molecule Method Transfer

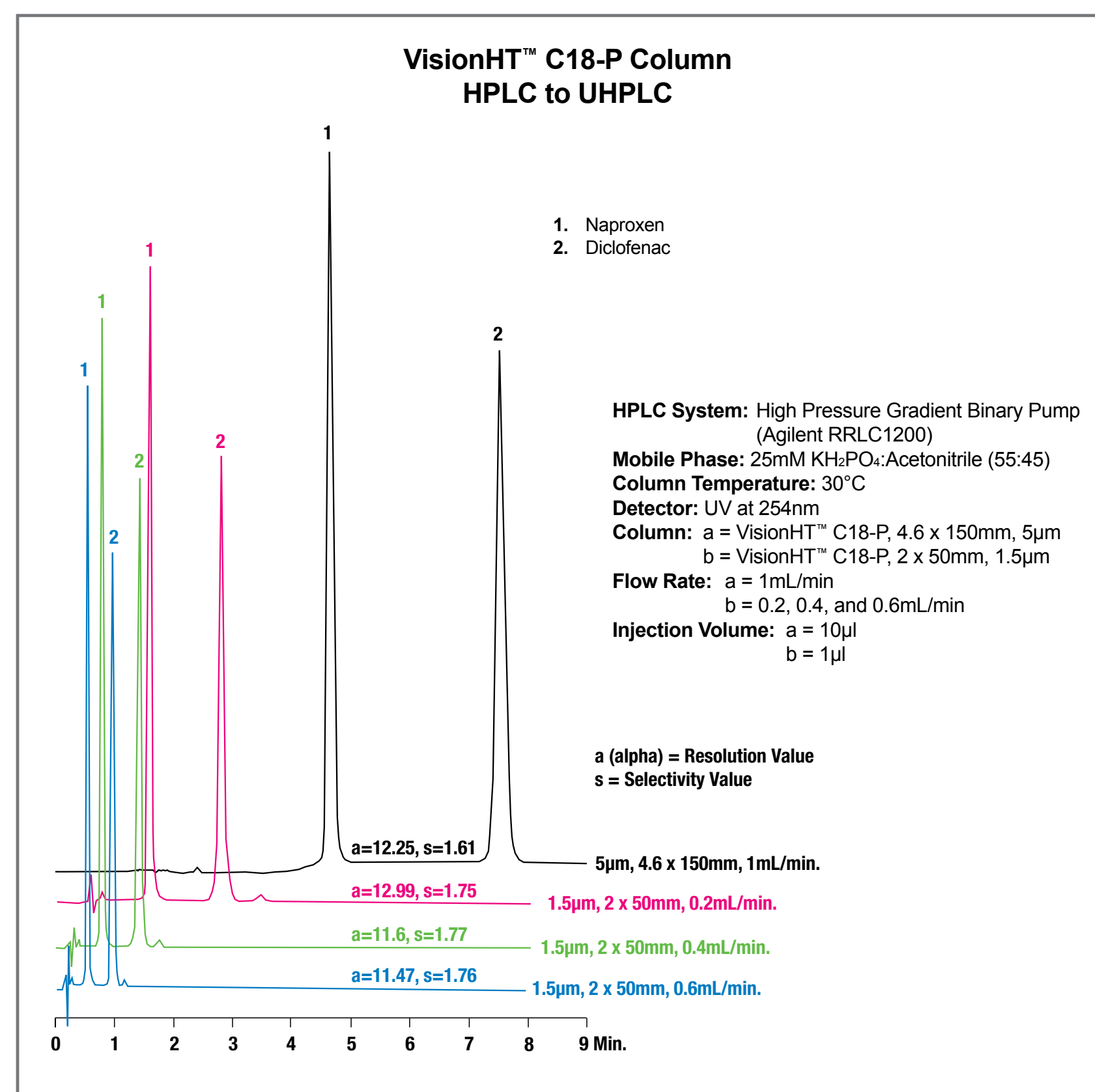
VisionHT™ media can be used in multiple column formats for use on a variety of systems. Here we demonstrate transferring of methods from one format to another in order to improve productivity.

Method Transfer of Barbiturates for Fast LC Analysis

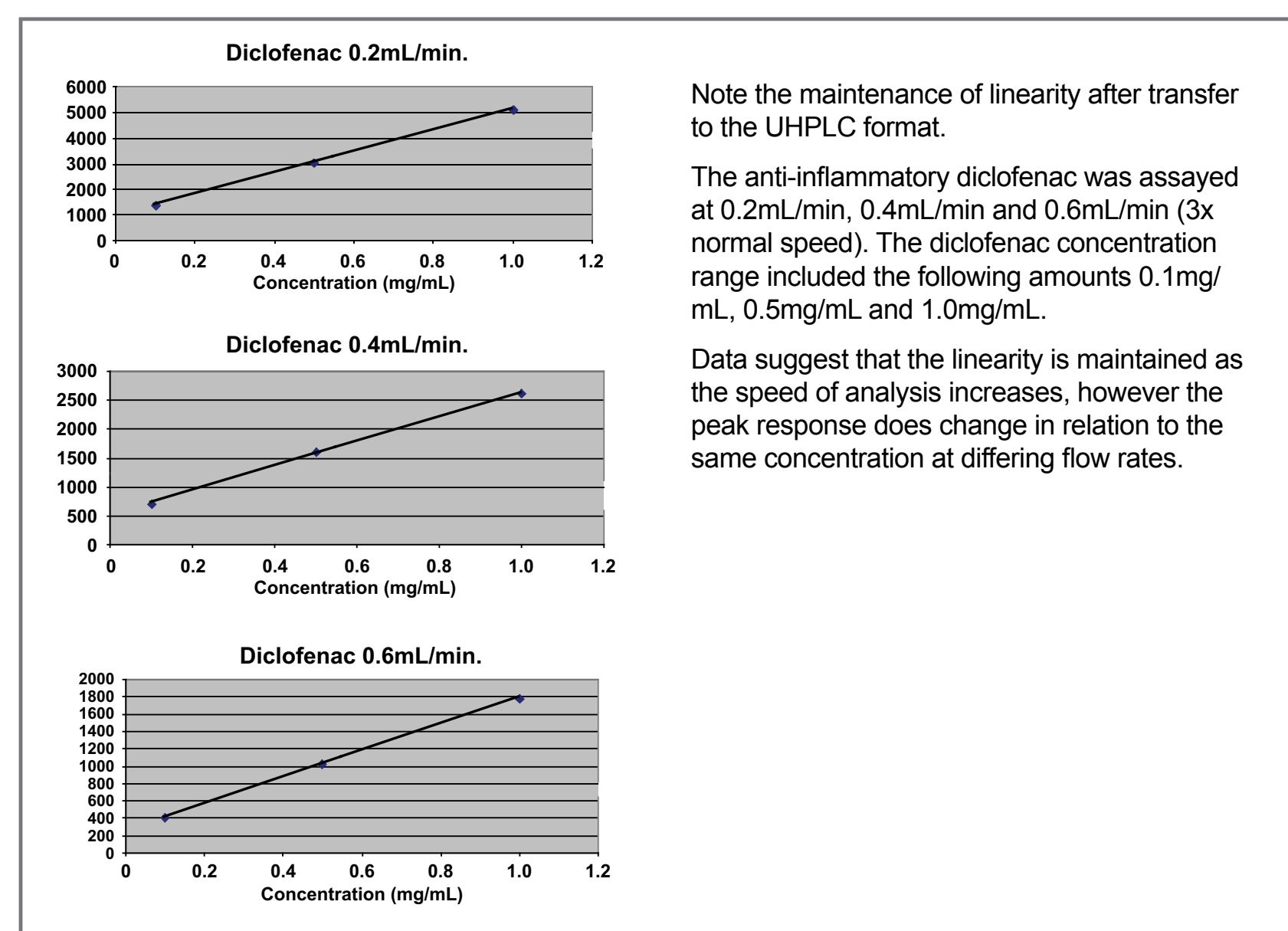


Practical example of transferring a method established on an analytical format column (5µm, 4.6 x 150mm) to a fast UHPLC format (1.5µm, 2 x 50mm) for either degradant/impurity monitoring, or for finish product QC.

Method Transfer of Anti-inflammatory Drugs for Fast LC Analysis



Linearity of Anti-inflammatory After Transfer to UHPLC Format



Results

When compared to traditional 4.6mm HPLC columns the following benefits can be achieved while still maintaining excellent linearity.

- Maintain resolution as assay length decreases
- Maintain selectivity as assay length decreases
- Reduce analysis time by greater than 87%
- Reduce solvent consumption by greater than 89%
- Decrease sample loading and requirements by 90%

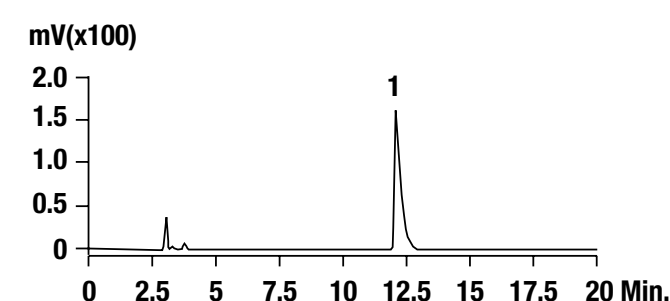
Method Transfer From HPLC to UHPLC for Melamine

Milk, infant formula, and other dairy products were recently found contaminated with melamine. After 2007, and after more recent melamine contamination outbreaks, there is an urgent need for analytical methods that can identify and quantify melamine in food. Current melamine methods involve LC-MS and GC-MS. GC-MS requires derivatization, and LC-MS methods generally use gradient conditions that require column clean up and re-equilibration.

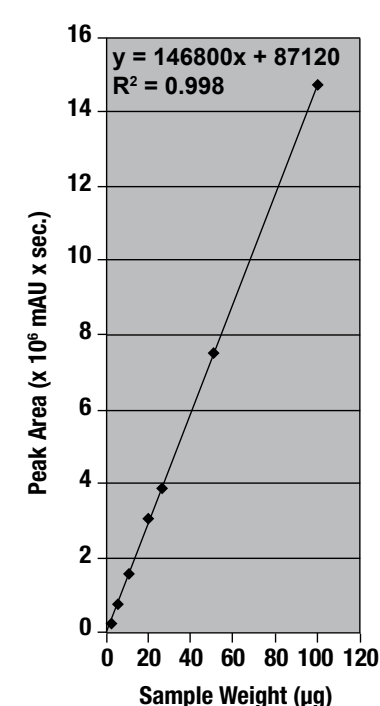
Grace has developed MS-compatible LC methods for melamine using an HILIC media platform that can be applied to both traditional HPLC as well as UHPLC systems. Melamine was analyzed with a standard HPLC system using a 5µm particle HILIC phase packed into a 250 x 4.6mm column. The 1.5µm version of this phase was then packed into a high throughput format conducive to UHPLC and fast LC systems. Both methods deliver excellent linearity and use isocratic elution for fast analysis without the need for re-equilibration.

HPLC Method for Melamine

This HPLC analytical method for melamine fulfills the FDA requirements using an HILIC column and an ionizable mobile phase compatible with mass spec. Low UV detections offers excellent linearity between 40ng and 100µg.

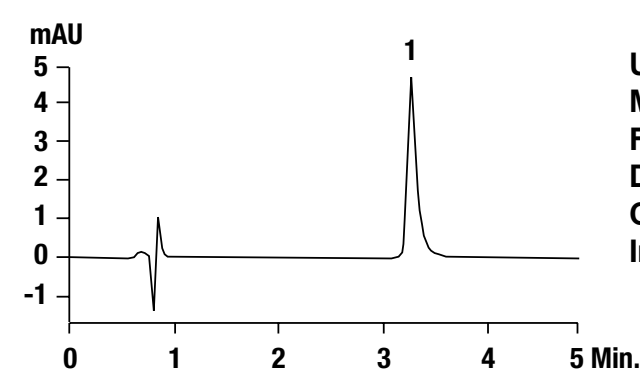


HPLC Column: VisionHT™ HILIC, 5µm, 4.6 x 250mm (Part No. 86466)
Mobile Phase: Acetonitrile:10mM Ammonium Acetate in Water (95:5)
Flow Rate: 1mL/min
Detection: UV at 240nm
Column Temperature: 30°C
Injection: 40µg/mL x 20µL



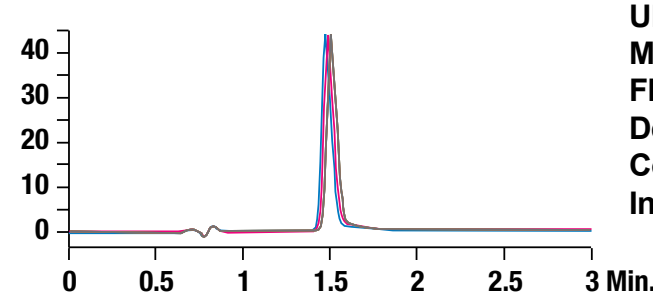
UHPLC Method for Melamine

Compared to the conventional HPLC method, the UHPLC method is 4 times faster. With the use of 1.5µm particles, optimal linear velocities extend over a wider range. Therefore, it is possible to maintain efficiency and resolution while running samples at faster flow rates.

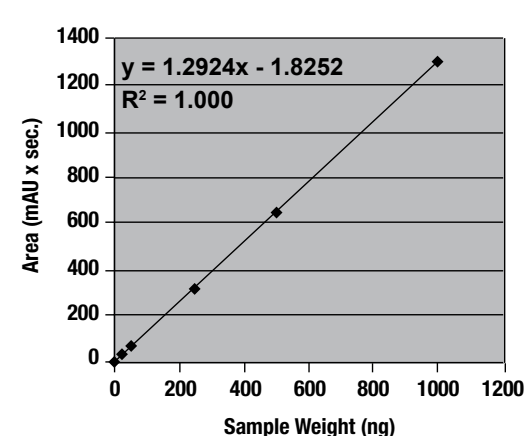


UHPLC Column: VisionHT™ HILIC, 1.5µm, 2 x 50mm (Part No. 5141919)
Mobile Phase: Acetonitrile:10mM Ammonia Acetate in Water (95:5)
Flow Rate: 0.2mL/min
Detection: UV at 240nm
Column Temperature: 30°C
Injection: 50µg/mL x 0.5µL

9 injections in parallel shows good reproducibility



UHPLC Column: VisionHT™ HILIC, 1.5µm, 2 x 50mm (Part No. 5141919)
Mobile Phase: Acetonitrile:Water(20mM Ammonium Formate) (90:10)
Flow Rate: 0.2mL/min
Detection: UV at 240nm
Column Temperature: 30°C
Injection: 50µg/mL x 0.1µL

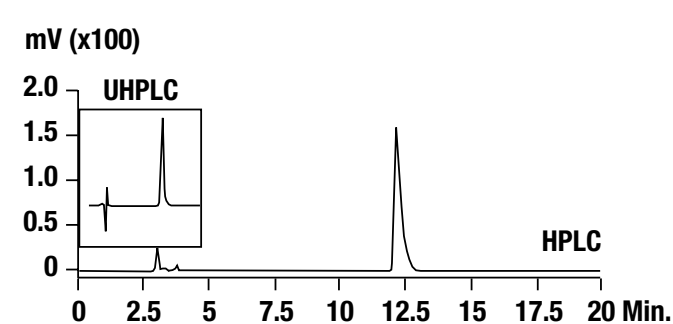


| Conc. | Inj. (µL) | Weight (ng) | Peak Area |
|---------|-----------|-------------|-----------|
| 50µg/mL | 0.1 | 5 | 7.3 |
| 50µg/mL | 0.5 | 25 | 32 |
| 50µg/mL | 1 | 50 | 63 |
| 50µg/mL | 5 | 250 | 318 |
| 50µg/mL | 10 | 500 | 641 |
| 50µg/mL | 20 | 1000 | 1293 |

This method exhibits excellent linear response between 5ng and 1000ng for accurate quantitation.

HPLC Methods Transfer to UHPLC

Compared to a conventional HPLC method, the UHPLC method is 4 times faster.



| | HILIC Column | Flow Rate | Time (min) | Conc. | Inj. Range | Loading Range |
|-------|--------------------------------------|-----------|------------|---------|------------|---------------|
| HPLC | Alltima™ HP HILIC, 5µm, 4.6 x 250mmL | 1.0mL/min | 12.090 | 40µg/mL | 1-100µL | 40ng-4mg |
| UHPLC | VisionHT™ HILIC, 1.5µm, 2.0 x 50mmL | 0.2mL/min | 3.259 | 50µg/mL | 0.1-20µL | 5ng-100ng |

Improve Resolution of Complex Protein Digest Samples

By utilizing different column dimensions, improvements in resolution can be balanced against desired speed. Here we compared complex protein digests with competitive sub-2 μ m columns and investigated resolution improvements with changes in column dimensions.

Sample Analysis Conditions

HPLC System: High Pressure Gradient Binary Pump (Agilent RRLC1200)

Mobile Phase A: 0.1% TFA in Water

Mobile Phase B: 0.085% TFA in Acetonitrile

Gradient (min., %B): (0, 4), (2, 4), (40, 50), (45, 90), (55, 90)

Flow Rate: 0.2mL/min.

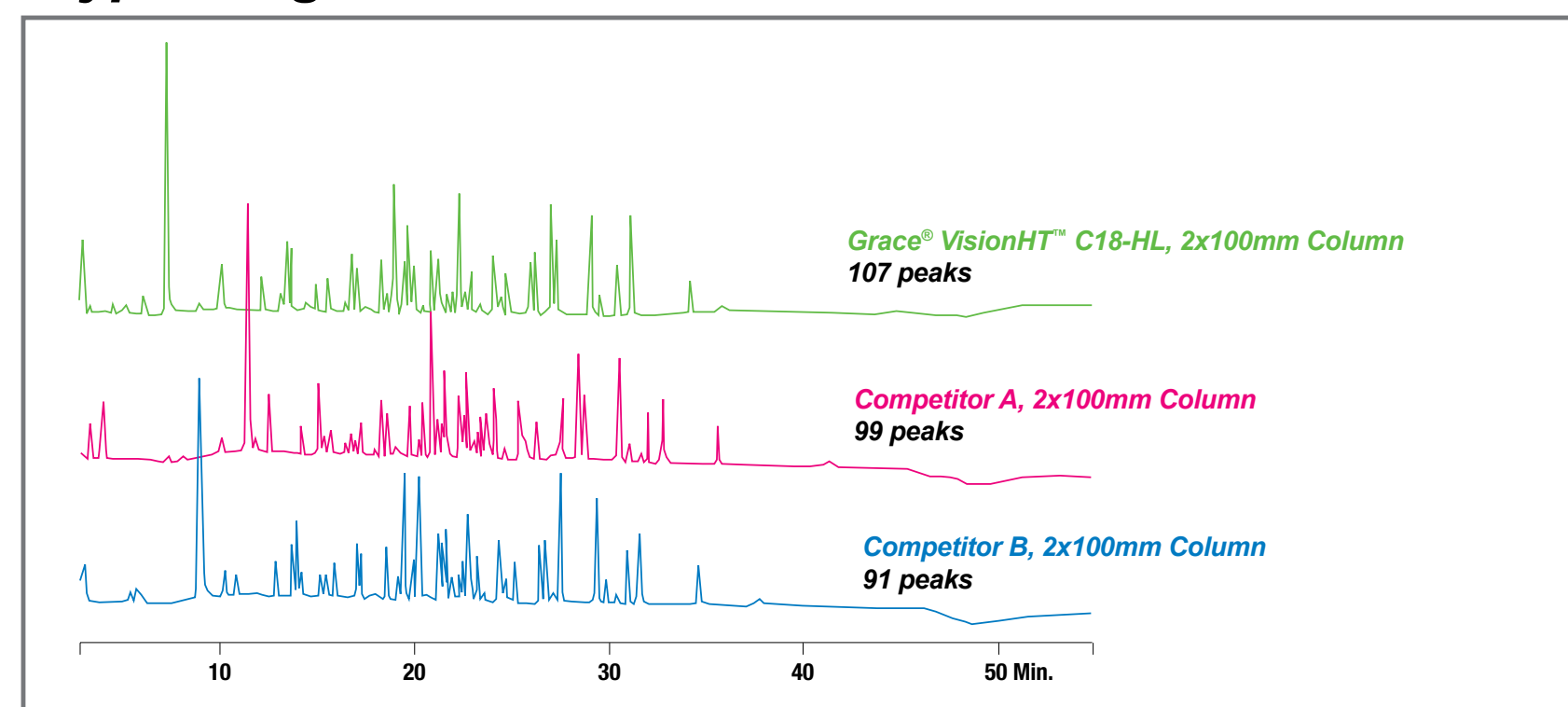
Column Temperature: 30°C

Detector: UV at 215nm

Injection Volume: 5 μ L of a 0.55 mg/mL sample

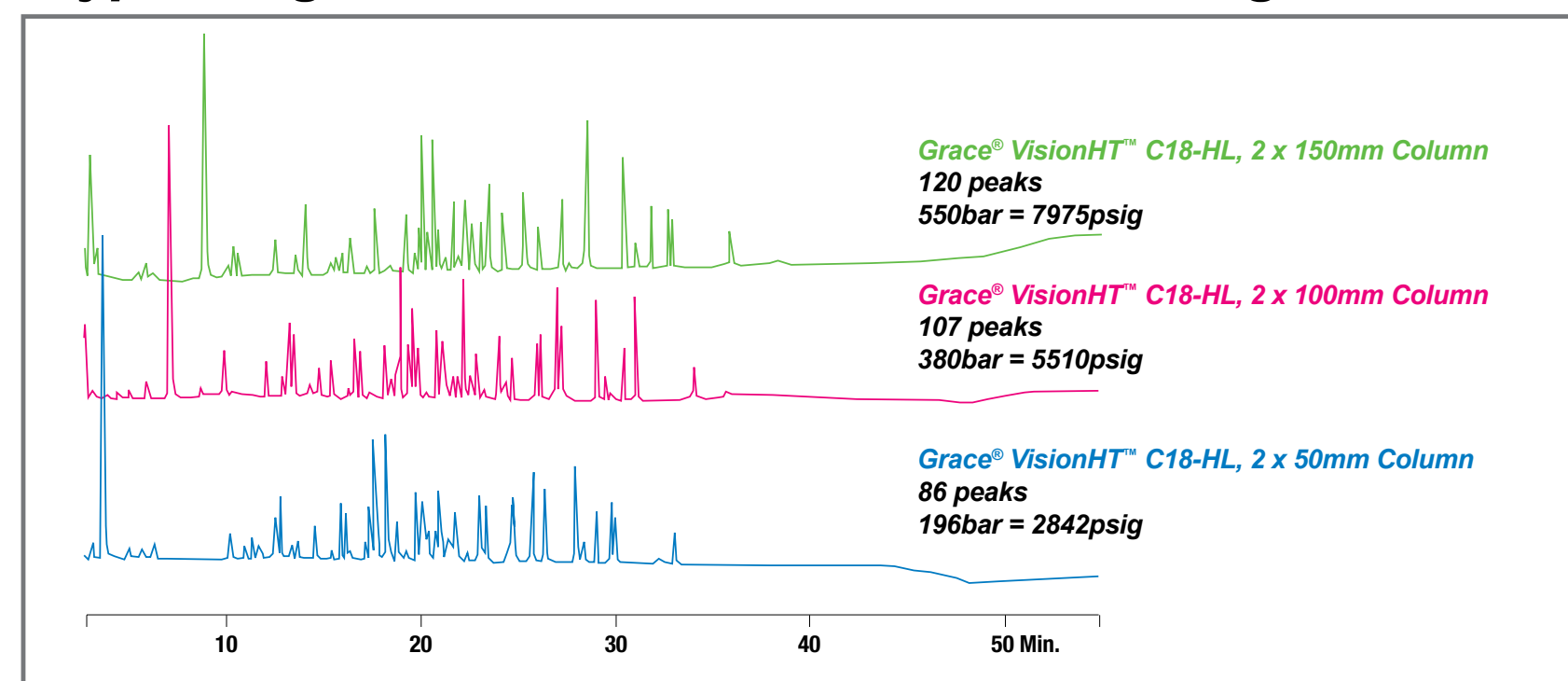
Note: Peak integration starts at 2.5 minutes

Grace® VisionHT™ Column vs. Other UHPLC Columns: Tryptic Digest of BSA



Excellent resolution for tryptic digest sample run on a VisionHT™ column compared to competitor columns.

Tryptic Digest of BSA: Effect of Column Length



Increased resolution and number of peaks with longer column length.

Conclusions

Optimizing and transferring methods between systems can be made simple when the identical bonded phase is available in 1.5, 3, 5, and 10 μ m particle sizes. With a single media platform, methods can be applied to appropriate formats to suit the system type. VisionHT™ HPLC columns range from UHPLC compatible to microbore through analytical and preparative. The columns are manufactured under a highly controlled process including silica production, bonding, and column packing and QC.

VisionHT™ columns offer the following benefits:

- Seamless media offering in a variety of column formats, ranging from fast HPLC to preparative. Enabling quick and easy method development and transfer.
- Wide range of phases for improved selectivity and method optimization.
- Applicability to small molecule and large molecule applications.

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