

High Resolution Liquid Chromatography with Commercial HPLC Systems and Ascentis® Express HPLC Columns:

Increasing Resolution by Column Coupling

William Campbell, Wendy Roe, Wayne K. Way
 wayne.way@sial.com

In this study, high resolution HPLC with efficiencies greater than 100,000 plates/column was achieved under moderate conditions. Specifically, Ascentis Express columns were coupled and used on a standard Agilent® 1200 HPLC system. Both isocratic and gradient examples are illustrated.

Introduction

High resolution liquid chromatography is important in many areas including pharmaceutical product development, natural product chemistry, and synthetic peptide mapping, just to name a few. A common need is LC methods that provide optimum assurance to the purity of peaks. For instance, in stress studies of active pharmaceutical ingredients (API), the ability to unambiguously quantitate and subsequently identify and potentially purify degradants of the API are paramount to the determination of the efficacy and safety of the pharmaceutical product. Modern hyphenated techniques such as LC-MS and LC-NMR often rely on the purity of a chromatographic peak for structural identification and confirmation.

To date, reducing the particle size of the packing in HPLC columns has been the strategy of many column manufacturers to provide higher resolution columns. Smaller particles have higher efficiencies and therefore can provide higher resolution. Unfortunately, column back pressure increases at a greater rate than efficiency as one decreases particle size. Therefore high resolution LC with small particles (sub 2 µm) is difficult even with modern LC systems. For this reason, a particle with high efficiencies yet low backpressure would be a more suitable candidate for high resolution LC.

Ascentis Express columns provide a breakthrough in high resolution LC performance. Based on Fused-Core™ particle technology, Ascentis Express provides the high efficiency based benefits of sub-2 µm particles but at much lower backpressure. Due to the high efficiencies at low back pressures, Ascentis Express can provide high resolution chromatography that was previously unattainable on commercial LC systems.

The Fused-Core particle consists of a 1.7 µm solid core and a 0.5 µm porous shell. A major benefit of the Fused-Core particle is the small diffusion path (0.5 µm) compared to conventional fully porous particles. The shorter diffusion path reduces axial dispersion of solutes and minimizes peak

broadening. In fact, Ascentis Express columns are able to achieve efficiencies of 240,000 N/m, which is similar to that obtained with sub-2 µm particle columns, even though the backpressures are only 50% of that achieved under similar conditions with sub-2 µm particles. This means that Ascentis Express can turn almost any LC system into a high resolution workhorse for your lab.

Column coupling in HPLC is gaining interest since LC systems are being designed to withstand column back pressures of up to 15,000 psi. Column coupling is a simple and practical way to increase resolution by simply increasing column length. Because Ascentis Express HPLC columns provide higher efficiencies at any pressure compared to 3 µm and sub-2 µm particles, the coupling of Ascentis Express columns enables significantly higher resolution than any other column on any commercial HPLC system.

In this study, coupled Ascentis Express C18 columns were used on a standard Agilent 1200 HPLC system. Efficiencies greater than 100,000 plates/column are demonstrated in the isocratic separation of benzene and D₆-benzene. Ascentis Express column coupling is further applied to the analysis of a synthetic hydrophilic peptide to separate the target peptide from its deletion side products.

Separation of Benzene and Deuterobenzene

Figure 1 shows the efficiency obtained by coupling Ascentis Express columns together. Due to space limits in the column heater, three 15 cm x 4.6 mm columns and one 10 cm x 4.6 mm column were used to obtain a total column length of 55 cm.

Figure 2 shows a plot of efficiency as a function of column length. The linearity indicates that efficiency is not sacrificed due to coupling hardware. It should be noted that efficiencies of greater than 100,000 were achieved under isocratic conditions with a modest back pressure of 7,000 psi.

Analysis of Synthetic Hydrophilic Peptide

Amino acid deletion products frequently result during the preparation of synthetic peptides. Since deletion may happen at any stage of the synthesis, the deletion products are diverse yet very similar to the target peptide. This process provides a unique and difficult separation challenge. Following is a study carried out on a hydrophilic synthetic peptide. The 12-mer peptide is composed of cysteine, proline, lysine, serine, phenylalanine and aspartic acid residues. Any one of the amino acids may be deleted at any position, thus a large number of possible deletion

Figure 1. Separation of Benzene from Deuterated Benzene Using an Ascentis Express C18, 55 cm x 4.6 mm I.D.

column: Ascentis Express C18, 55 cm x 4.6 mm I.D.
mobile phase: 55:45, acetonitrile:water
flow rate: 1.0 mL/min.
temp.: 50 °C
det.: 254 nm
injection: 10 µL

2. Acetophenone = 120,045 plates
3. D₆ Benzene = 101,852 plates
4. Benzene = 104,463 plates
5. Toluene = 101,281 plates
Pressure: = 480 bar (7,000 psi)

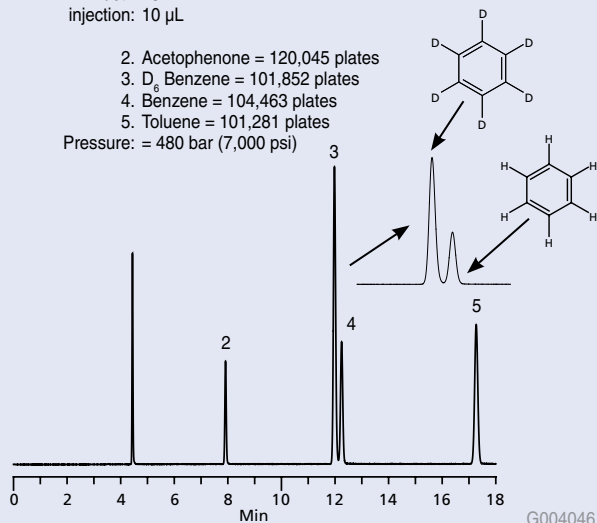
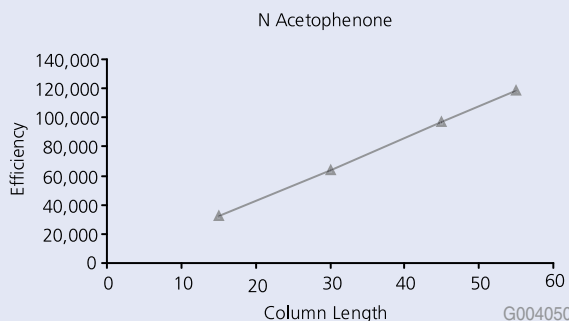


Figure 2. Efficiency as a Function of Column Length

Efficiency is Linear with Respect to Column Length
Indicating no Loss Due to Column Coupling



impurities may exist in the end product. Separation and identification of these side products is important.

Figure 3 demonstrates the effect of Ascentis Express column coupling on this separation. The column length was extended to 30 cm and compared to the 15 cm. The gradient rate was adjusted to account for the added column length. Comparison of the data shows the enhanced resolution obtained for several of the deletion products.

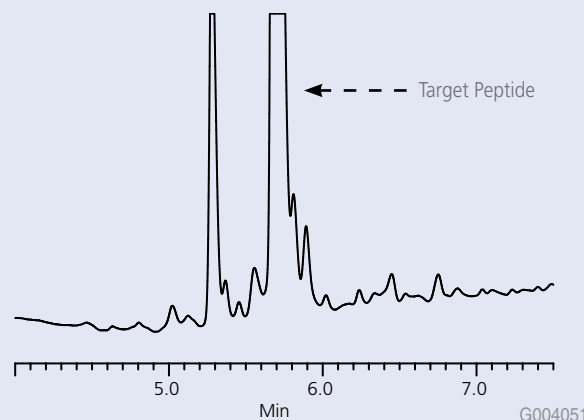
Conclusion

This study illustrates the potential for high resolution LC using Ascentis Express HPLC columns under moderate conditions with commercial instrumentation. Dramatic improvements in resolving power beyond that shown in this study are possible with elevated temperature and ultra-high pressure instrumentation.

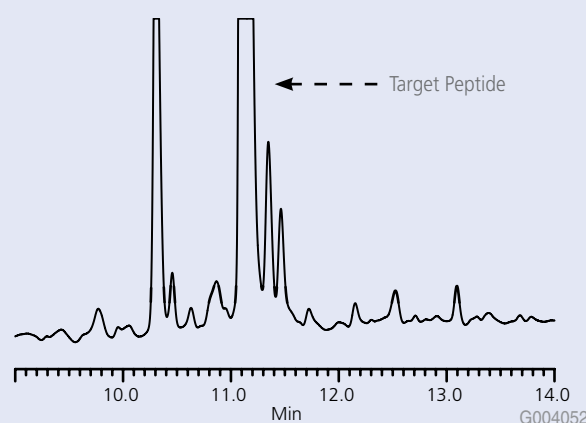
Figure 3. Gradient Elution of a Synthetic Peptide and its Deletion Products: Comparison of an Ascentis Express C18 at 15 and 30 cm Column Lengths

column: Ascentis Express C18
mobile phase A: 10% acetonitrile
mobile phase B: 100% acetonitrile / Both 0.1% TFA
flow rate: 1.0 mL/min.
temp.: 35 °C
det.: 210 nm
injection: 10 µL injection (0.1 mg/mL total peptide)

Ascentis Express C18 (15 cm x 4.6 mm I.D.)
Gradient: 0% B – 20% B (10 min.)



Ascentis Express C18 (30 cm x 4.6 mm I.D.)
Gradient: 0% B – 20% B (20 min.)



+ Featured Products

Column Dimensions	C18	C8
Ascentis Express	Cat. No.	Cat. No.
3 cm x 2.1 mm I.D.	53802-U	53839-U
5 cm x 2.1 mm I.D.	53822-U	53831-U
7.5 cm x 2.1 mm I.D.	53804-U	53843-U
10 cm x 2.1 mm I.D.	53823-U	53832-U
15 cm x 2.1 mm I.D.	53825-U	53834-U
3 cm x 3.0 mm I.D.	53805-U	53844-U
5 cm x 3.0 mm I.D.	53811-U	53848-U
7.5 cm x 3.0 mm I.D.	53812-U	53849-U
10 cm x 3.0 mm I.D.	53814-U	53852-U
15 cm x 3.0 mm I.D.	53816-U	53853-U
3 cm x 4.6 mm I.D.	53818-U	53857-U
5 cm x 4.6 mm I.D.	53826-U	53836-U
7.5 cm x 4.6 mm I.D.	53819-U	53858-U
10 cm x 4.6 mm I.D.	53827-U	53837-U
15 cm x 4.6 mm I.D.	53829-U	53838-U