Optimization of the Rapid HPLC Analysis of Monoclonal Intact Antibodies, Light Chains, and Heavy Chains Using ZORBAX Poroshell Columns

Technical Overview

Biochemical

Cliff Woodward and Robert D. Ricker

Highlights

- High velocity gradient separations using ZORBAX Poroshell technology result in high-resolution analysis of intact antibodies, heavy chains, and light chains, with short run times and exceptional peak shape and peak efficiences.
- Method development is significantly more rapid when using ZORBAX Poroshell HPLC columns – reductions of up to 90% in analysis time are easily achieved.
- ZORBAX Poroshell 300SB columns are available in a variety of internal diameters (0.3, 0.5, 1.0, and 2.1-mm id) and bonded phases (300SB-C3, 300SB-C8, 300SB-C18, and 300Extend-C18) for optimal sensitivity, resolution, and speed.
- ZORBAX Poroshell resolution is maximized by using an HPLC instrument optimized for low-volume peaks of very narrow width.

Challenges to Monoclonal Antibody Analyses

Antibodies represent a class of proteins that are the key to immunological systems. They bind to an antigen (protein, glycoprotein, DNA, etc.) with a high degree of specificity, making them extremely valuable for use in diagnostics, general research, and for therapeutics. Structurally, IgG, a commonly studied class of antibodies, is comprised of two heavy chains (50 kDa each) and two light chains (25 kDa each) attached through disulfide bridges. Commonly glycosylated, their carboxy-terminal domains are conserved; but their amino-terminal domains are variable in amino-acid sequence, resulting in the degree of antibody specificity and diversity.

A variety of enzymes and chemical treatments are available to help in elucidation of antibody structure. These may involve (but are certainly not limited to) cleavage of the disulfide bonds to separate an antibody into free heavy and light chains, the removal of carbohydrate moieties, and the removal of a C-terminal lysine. Other useful modifications are achieved by cleavage of whole antibodies with papain or pepsin. Use of these antibody treatments in combination with HPLC (for separation, isolation, and analysis) is an invaluable tool for understanding and verifying antibody structure and function.

Traditionally, intact antibodies and their fragments are analyzed with limited success using wide-pore, totally-porous particle, reversed-phase HPLC. Because of their large size (150 kDa for the 4-protein subchains) and limited structural diversity, analysis times are often unacceptably long and antibody peaks can elute as broad bands thereby compromising resolution. In contrast, high-efficiency ZORBAX Poroshell HPLC columns easily separate antibodies and their fragments in mere minutes and with high efficiency – eluting large molecules similar in structure as distinct, narrow bands.

The following discussion describes how the unique design of the ZORBAX Poroshell particle is key to achieving these remarkable results. Two appendixes were added at the end of this document to facilitate the optimal use of ZORBAX Poroshell technology. Appendix A addresses how column dimensions, gradient time, and flow rate are interrelated. These parameters can then be used to transfer methods from other columns to Poroshell columns of different dimensions. This is especially useful since Poroshell columns typically operate at flow rates 5 to 10 times faster than those used on a typical column of the same diameter. This is why the term "high velocity" was coined – the mobile phase travels at a very high velocity down the column. However, this does not necessarily mean high flow rate since velocity is the flow rate relative to the column diameter. The equation in Appendix A can be used to maintain an elution pattern, increase resolution, or decrease analysis time. Appendix B discusses some of the key instrument parameters that should be considered for obtaining the maximum power of Poroshell technology. Some of the more important factors include using reduced extra-column volume, using a small-volume flow cell, and using a very rapid response setting on the detector.

The ZORBAX Poroshell Design Maintains Sharp Peaks while Dramatically Reducing Analysis Time of Antibodies and Other Large Biomolecules

ZORBAX Poroshell particles consist of a solid, silica core covered by a superficially porous surface, that allows large molecules to quickly diffuse in and out of the thin, porous layer (see Figure 1). Pores in the thin, outer layers measure 300 Å in diameter and are maximally bonded with sterically protected ligands (300SB-C18, 300SB-C8, or 300SB-C3) for maximum column life at low pH, (for example, with trifluoroacetic acid (TFA) and formic acid mobile phase modifiers). A 300Extend-C18 bonding provides extended high pH stability for additional method flexibility. During the separation, large molecules rapidly diffuse in and out of the thin porous shell – allowing for the use of higher flow rates and correspondingly short gradient times – with large molecules like antibodies eluting in seconds as easily resolved, narrow bands.

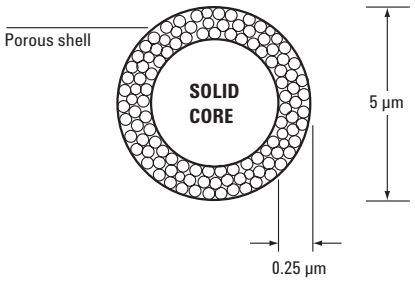
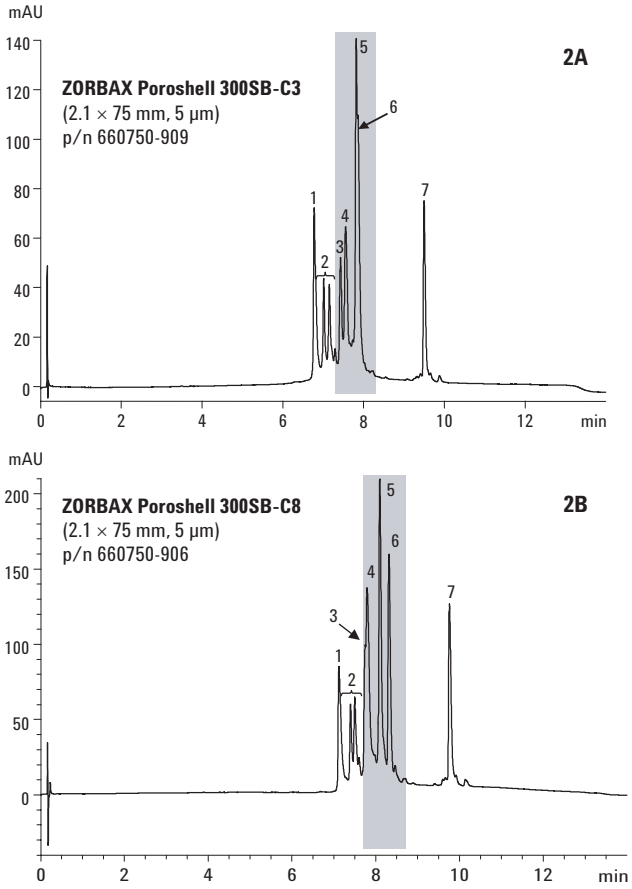


Figure 1. Representation of a 5-µm superficially porous ZORBAX Poroshell particle.

Poroshell 300SB-C3 and 300SB-C8 Separate Intact Monoclonal Antibodies

In Figure 2A, ZORBAX Poroshell columns were used for rapid method development and analysis of seven closely-related, intact, monoclonal IgG antibodies. The seven antibodies are very similar in molecular weight and are quite large (approximately 150 kDa). All seven antibodies are known to be glycosylated, further complicating the analysis. In the first separation, a rapid multisegment gradient using an optimized Agilent 1100 system and a ZORBAX Poroshell 300SB-C3 column completely resolves all but antibodies 5 and 6 within the total run time of 14 minutes.

A change of bonded phase type is often the quickest way to change method selectivity or band spacing. Consistent with this thinking, the analysis was rerun with identical conditions using a ZORBAX Poroshell 300SB-C8 (see Figure 2B). This second run was successful at separating peaks 5 and 6, but resulted in loss of separation between peaks 3 and 4. Note that the second antibody elutes as a doublet in both approaches. With no additional method development, two types of Poroshell columns may be utilized to separate all seven antibodies in as little as 28 min. Running two methods sequentially also serves to provide duplicate quantitation data that may be helpful to the analyst.



Conditions

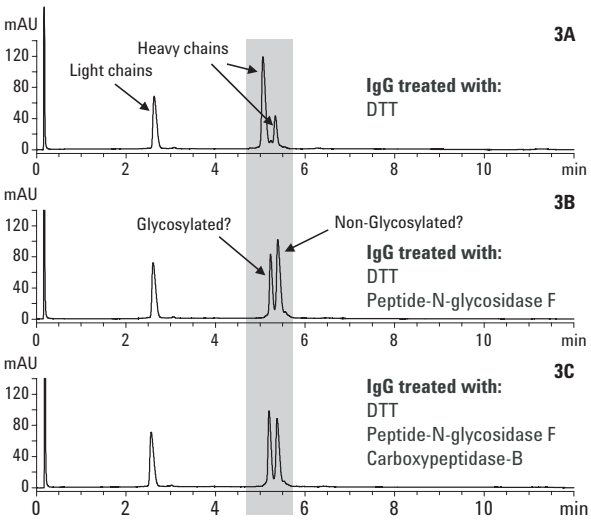
Mobile phase	A = H_2O-ACN (90:10) B = H_2O-ACN (10:90) Both A and B contain 0.1% TFA and 3 mL/L of PEG 300.
Temperature	70 °C
Flow rate	1.0 mL/min
Detection	UV (210 nm)
Multisegment timetable	
Time (min)	% Solvent B
0.00	19
12.00	41
12.10	19
14.00	19

Figure 2. Antibody separations using ZORBAX Poroshell 300SB columns C3 (2A), and C8 (2B).

Separation of Antibody Heavy Chains

As one begins to study the more detailed structure/function of antibodies, chemical or enzymatic techniques may be used to obtain free antibody heavy chains. ZORBAX Poroshell columns are very effective in providing high-velocity, high-resolution separations of antibody heavy chains (50 kDa). The chromatograms in Figure 3 show a rapid reversed-phase analysis optimized for the separation of antibody heavy-chain forms with peaks eluting in less than 6 minutes.

Figure 3A shows the IgG antibody sample separated on ZORBAX Poroshell 300SB-C8, after treatment with DTT to break disulfide bonds. The two IgG heavy-chain forms, elute as two distinct peaks, that differ in either structure or mass. Subsequent treatment of the sample with peptide-N-glycosidase F results in removal of carbohydrate moieties from the protein chains (Figure 3B). There is a change in the peak ratios as well as some indication of change in retention. However, two peaks are still present, indicating that the heavy chains were most likely not simply different carbohydrate modifications of a single underlying protein. In Figure 3C, the sample was further modified by treatment with carboxypeptidase B to remove C-terminal lysines, if present, from the protein chains. Additional changes in the peak ratio and retention occur, but two peaks remain. The second peak could result from the presence of unusual modifications, different primary sequence, or differences in folding. To gain additional information about the heavy chain forms present in this sample, chromatographic analysis lends itself to



Conditions

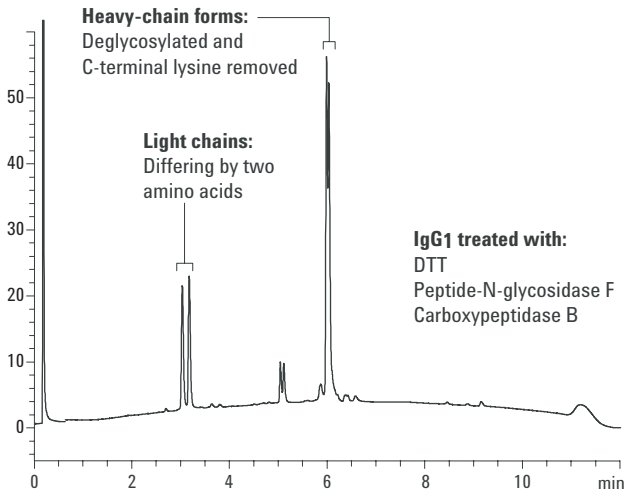
Column	**ZORBAX Poroshell 300SB-C8** (2.1 × 75 mm, 5 µm) p/n 660750-906
Mobile phase	A = H_2O-ACN (90:10) B = H_2O-ACN (10:90) Both A and B contain 0.1% TFA and 3 mL/L of PEG 300.
Temperature	70 °C
Flow rate	1.0 mL/min
Detection	UV (210 nm)
Gradient timetable	
Time (min)	% Solvent B
0.00	25.0
10.00	40.0
10.10	25.0
12.00	25.0

Figure 3. Chromatographic comparison of antibody IgG after different chemical treatments.

the isolation of chromatographic peaks for downstream analysis. While the light chains are present in this sample and may be seen eluting slightly later than 2.5 minutes, the separation was not optimized for their analysis.

Separation of Antibody-Light Chains

Modification of the gradient conditions allows the rapid, high-resolution separation of antibody-light chains (25 kDa) using a ZORBAX Poroshell 300SB-C8 column (Figure 4). This sample was treated with DTT, peptide-N-glycosidase F, and carboxypeptidase B before chromatographic separation. While resolution of the heavy chains is reduced in this gradient run, the gradient profile was optimized so that one may distinguish between light chains known to differ by only two amino acids (peaks eluting just after 3 min). This rapid, high-resolution analysis can be used to quantitate the chain forms or isolate antibody chains for downstream use.



Conditions

Column	**ZORBAX Poroshell 300SB-C8** (2.1 × 75 mm, 5 µm) p/n 6600750-906
Mobile phase	A = H_2O-ACN (90:10) B = H_2O-ACN (10:90) Both A and B contain 0.1% TFA and 3 mL/L of PEG 300.
Temperature	70 °C
Flow rate	1.0 mL/min
Detection	UV (210 nm)

Gradient timetable	
Time (min)	% Solvent B
0.00	20.0
10.00	50.0
10.10	20.0
12.00	20.0

Figure 4. Chromatogram showing clear separation between heavy and light antibody chains.

Conclusion

The structure of ZORBAX Poroshell particles allows very efficient separation and short analysis times for intact monoclonal antibodies, their heavy chains and light chains, facilitating high-resolution, high-throughput analyses. ZORBAX Poroshell 300SB HPLC columns enhance peak shape and recovery through simplified interaction with the bonded phase. Use of different Poroshell bonded phases, including 300SB-C18, 300SB-C8, and 300SB-C3, can be crucial to successful separation of challenging bio-samples, especially if these proteins are very large and heterogeneous.

IgG samples may be analyzed directly or after treatment with various chemicals and enzymes such as DTT (for separation of heavy and light chains), peptide N glycosidase F (a carbohydrate cleaving enzyme), or carboxypeptidase B (for removal of C-terminal lysines) followed by chromatographic separation. The resulting peaks give information about structure and function. Thus, Poroshell high-resolution analysis can be used to quantitate whole antibodies, chains, and various chain conformers; follow enzymatic reactions and modifications, or to isolate antibodies or antibody chains for downstream use.

The speed that ZORBAX Poroshell technology contributes to rapid development of these separation methods should not be overlooked. Method development can account for a considerable amount of the time spent in achieving an analysis with the resolution seen here. This is especially true for protein separations that would typically be 1-hour long. In short, the 15-min chromatographic runtimes achieved here using Poroshell technology speed up method development four-fold. This gives you additional time to increase the resolution of the separation.

Appendix A

Increased Flow Rate and Decreased Column Length Leads to a Decrease in Gradient Retention

Since flow rate and gradient time are linked by the gradient relationship below; a five-fold increase in flow rate can be paired with a five-fold decrease in gradient time without affecting gradient retention (k).

$$\text{Retention (k)} = \frac{t_G \times F}{C \times V_m}$$

Where:

t_G = Gradient time in minutes

F = Flow rate in mL/min

V_m = Volume of the packed column in mL

C = A constant (provided that the change in organic concentration during the gradient are unchanged from run to run)

Gradient retention is analagous to isocratic retention, where larger values of k translate into more retention and typically more resolution, with an expected increase in peak width and decrease in peak height. More importantly in this case, if gradient retention is kept constant from one chromatographic run to another, then the peak elution pattern for the separation will not change. Combining this formula with the understanding that superficially porous (Poroshell) particles may be used at high linear velocities, one can choose gradient and flow rate conditions that optimize use of Poroshell technology.

When large molecules such as antibodies are separated on porous particles, increasing HPLC flow rate results in broad peaks due to poor mass transfer. As described in Figure 1, the Poroshell design restricts the protein diffusion path length, so that flow rates can be increased to reduce run time, while retaining sharp, symmetrical peaks. Since the 1.0-mL/min flow rate used in the antibody applications is five-times higher than that typically selected for a 2.1-mm diameter column (that is, 0.2 mL/min), gradient time (t_G) can be reduced five-fold.

Similarly, since most Poroshell columns are half the length (and volume, V_m) of a standard 150-mm column, gradient time can be reduced another two-fold – ten-fold overall, when combined with a five-fold increase in flow. For example, a gradient time of 30 minutes in a 4.6 × 150-mm column could be reduced to 3 minutes on a 2.1 × 75-mm Poroshell column without changing the relative retention or resolution of the separation. This is an impressive order-of-magnitude reduction in analysis time!

Gradient Retention is Maintained when Product of Gradient Time and Flow Rate is Constant

Additionally, the four chromatograms in Figure A1 were obtained by use of the formula above to maintain the same relative gradient retention on a Poroshell 300SB-C18 column. The gradient time, as shown in the chromatogram, was adjusted with the change in flow rate so that k remained constant and the peptide/protein sample gave the same elution pattern when aligned.

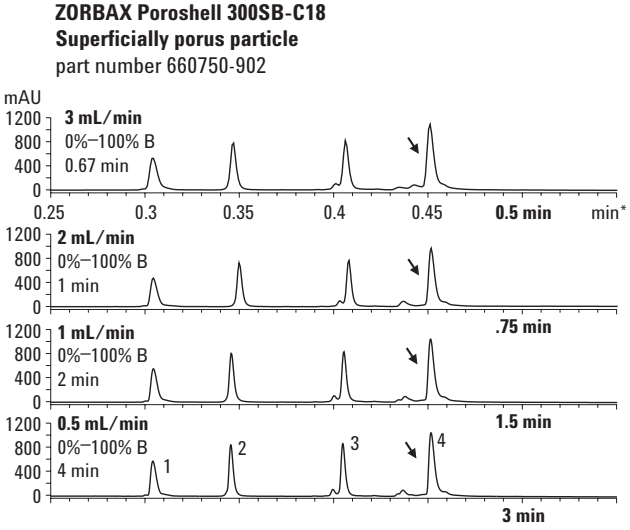


Figure A1. Comparison of chromatographic separations on Poroshell at different flow rates with adjusted gradient times.

Rapid Scouting Runs for Method Development

The additional benefit of high-speed method development when using Poroshell columns should not be overlooked. A series of method-development scouting runs may be made in one-fifth or one-tenth the time allowing for additional optimization or freeing the investigator for data evaluation and other pressing work.

Appendix B

Maximize Poroshell Resolution with an Optimized HPLC Instrument Configuration

With standard Poroshell column diameters of 2.1, 1.0, and 0.5 mm, the volume of eluting sample peaks is quite small, making it important to know if your current instrument configuration can contribute to peak broadening. For example, for low-volume peak widths, the response time (peak-width setting) of the detector and the extra-column-volume (injection size, tubing diameter, and flow-cell volume), if not properly selected, could increase and distort Poroshell sample peak width. Evaluating these instrument parameters before starting method development will ensure you maximize the resolving power of your Poroshell separations.

Below four peptides and proteins, neurotensin, ribonuclease A, lysozyme and myoglobin are separated on either a 2.1-mm or 1.0-mm id Poroshell 300SB-C18 column using an Agilent 1100 HPLC instrument to evaluate the effects of instrument settings and configurations on Poroshell peak efficiency. The Agilent 1100 HPLC System includes a Well-Plate Sampler with Automatic Delay Volume Reduction and a Binary Pump with Piston Stroke set to 20 µL and mixing column removed. Operating temperature is 70 °C and detection is at 215 nm.

Peak-Width Setting

With increased column efficiency and decreasing column diameter, the sample peak volume becomes surprisingly small. For example, a peak eluting from a 2.1-mm id column has one-fifth the volume of the same peak eluting from a 4.6-mm id column and peaks eluting from a 1.0-mm id column have peak volumes of one-twentieth that obtained on the standard 4.6-mm id column. In fact, peaks eluting from a 2.1-mm id Poroshell column can have peak widths on the order of several seconds or even a fraction of a second. Therefore, if the peak-width setting of the detector is not set low enough, peak widths will widen and resolution between closely eluting bands can be lost. In fact, data developed at Agilent Technologies shows that peak widths increase from 18% to 51% for the separation of four proteins using a 2.1 × 75-mm Poroshell 300SB-C18 column with a diode array detector (DAD) peak-width setting of 0.2 min compared with a DAD peak-width setting of <0.01 min. Peak widths averaged 6 seconds (see Table B1).

Table B1. Effect of Detector Response-Time Setting on the Peak Widths of a Peptide-Protein Sample Mixture Analyzed on ZORBAX Poroshell

Peak	% Increase in peak width
1	18
2	35
3	51
4	35

Values for percent increase in peak width are calculated using ratio of peak widths at half height for peaks with DAD peak-width settings of 0.20 min and <0.01 min. For experimental details see Agilent Technical Overview 5988-9998EN [1].

Extra-Column-Volume (ECV)

ECV is a function of the volume of HPLC system components between the injector and the detector, which includes the injection tubing, and detector volume but excludes the column itself. Low-volume peaks are particularly susceptible to broadening in the instrument extra-column volume of your system and should be evaluated on a regular basis.

Instrument Tubing

Care must be taken when using 1-mm id Poroshell columns. Specifically, it is recommended that you plumb your system with 0.12-mm id tubing instead of 0.17-mm id tubing. Use of the larger diameter tubing has shown to increase peak width up to 33% and increase tailing by up to 58%. (See Table B2).

Table B2. Effect of Column-Connector Tubing on the Peak Shape of a Peptide-Protein Sample Mixture Analyzed on ZORBAX Poroshell

Peak	% Change in peak width	% Change in symmetry value
1	+29	–12
2	+25	+58
3	+33	+33
4	+28	+49

Values for percent change in peak widths are calculated as the ratio of peak width at half height for peaks generated from both 0.12- and 0.17-mm id tubing systems. Percent change in symmetry values are derived in a similar way using pseudomoment analysis as reported in the Agilent ChemStation documentation. For experimental details see Agilent Technical Overview 5988-9998EN.

Detector Flow Cell

Proper selection of a dectector cell is key to maintaining narrow, symmetrical, peak widths when using high-efficiency, narrow-bore columns. Comparing the peak widths for the same set of proteins eluting from the 1.0 × 75-mm Poroshell 300SB-C18 column, when using a 1.7-µL or a 13-µL detector flow cell, shows that peak widths are up to 96% wider when using the larger volume flow cell (See Table B3).

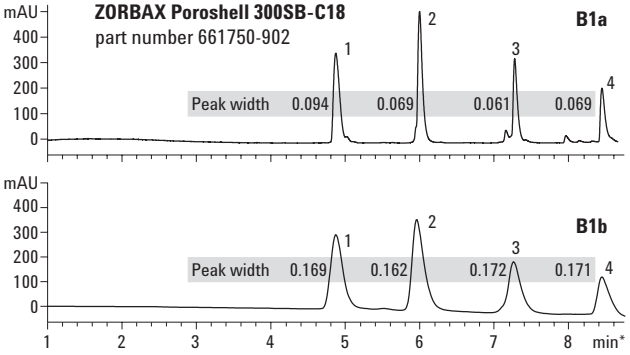
Table B3. Effect of Detector Cell Volume on the Peak Shape of a Peptide-Protein Sample Mixture Analyzed on ZORBAX Poroshell

Peak	% Change in peak width
1	+36
2	+74
3	+96
4	+75

Values for percent change in peak widths are calculated using ratio of peak width at half height for peaks generated using 1.7 and 13-µL cell volumes. For experimental details see Agilent Technical Overview 5988-9998EN.

Optimized Instrument Configuration

To emphasize the importance of evaluating your instrument for separations conducted on a 1.0 × 75-mm Poroshell 300SB, results on an Agilent 1100 optimized system configuration that includes a <0.01-min detector peak-width setting, 0.12-mm id tubing, and a 1.7-µL flow cell are compared with a non-optimized Agilent 1100 system having a 0.20-min detector peak-width setting, 0.17-mm id tubing and a 13-µL flow cell. Data in Figure B1 and Table B4 show that peak widths are improved by 80% to 180% – up to a three-fold improvement when operating with the optimized Agilent 1100 instrument configuration.



Conditions

Column	**ZORBAX Poroshell 300SB-C18** (1.0 × 75 mm, 5 µm) p/n 660750-902 A. Optimized system B. Non-optimized system
Mobile phase	A = 0.1% TFA in H_2O B = 0.07% TFA Acetonitrile
Gradient	5% to 60% B in 8.8 min
Flow rate	113 µL/min

*Peak widths of neurotensin, ribonuclease A, lysozyme, and myoglobin are noticebly narrower and more finely detailed when separated using the optimized Agilent 1100 instrument configuration.

Figure B1. Protein separations as a function of LC system volume. The upper chromatogram (B1a) used the optimized configuration.

Table B4. Combined Effects of Peak-Width Setting, Tubing Volume, and Detector Cell Volume on Peak Width

Peak	% Change with peak width
1	+79
2	+135
3	+182
4	+148

Values for percent change in peak width are calculated using ratio of peak width at half height for peaks using the optimized and non-optimized instrument configurations. For more experimental details see Technical Overview 5988-9998EN.

Reference

1. Cliff Woodward and Robert Ricker, “Optimization of Agilent 1100 HP LC System for Superior Results with ZORBAX Poroshell Columns,” Agilent Technologies, publication 5988-9998EN www.agilent.com/chem

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The authors, Cliff Woodward and Robert Ricker, are Application Biochemists based at Agilent Technologies, Wilmington, Delaware.

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