Testing ZORBAX GF-250 Performance Using Gel-Filtration Test Mixtures

Application

Biochemical

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Introduction

Gel-Filtration standards are used to define the performance and linear separation range of gel-filtration columns. A prior publication has already shown that the ZORBAX GF-250 column can readily separate the components of the Bio-Rad® gel-filtration standard covering the range between thyroglobulin (670 KDa) and Vitamin B_{12} (1.35 KDa) [1]. The plot is essentially reproduced below as Figure 1. The purpose of gel-filtration columns is to provide true separation of molecules by their molecular weight (MW) (or, more accurately, their volume when hydrated in solution). Sample analytes that are separated according to their MW fall close to a line in plots of log(MW) versus retention volume.

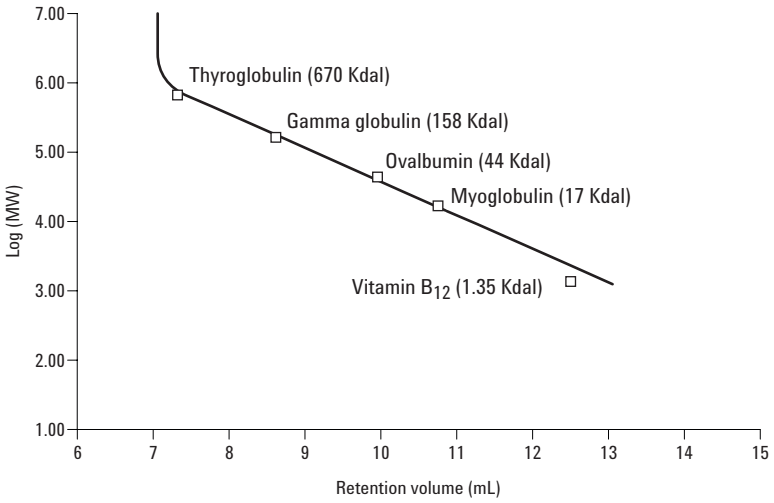

Figure 1. Retention volume versus log(MW) for the Bio-Rad standards separated on an Agilent ZORBAX GF-250 column.

Conditions

Column	**ZORBAX GF-250** (9.4 × 250 mm) p/n 884973-901
Mobile phase	200 mM Sodium phosphate, pH 7.0
Injection	5 µL
Temperature	Ambient
Detection	UV (254 nm)

Highlights

- High molecular weight (MW) proteins, differing at least two-fold in mass, are readily separated by ZORBAX GF-250 columns.
- Both the QA test mix and the Bio-Rad standards adequately test characteristics of the ZORBAX GF-250 columns.
- The physical strength of ZORBAX PSM particles facilitates excellent separations at 2 mL/min flow rates.

Results

This application note demonstrates the use of a different set of compounds (QA test mix) to characterize the ZORBAX GF-250 column and compares the results to the previously used Bio-Rad gel-filtration standard. Through this comparison, it will be shown that the Bio-Rad standards and QA test mix can be used to test performance of ZORBAX GF-250 columns. A particular test should be chosen and used to test the column when first received and anytime thereafter when the performance is in question. Figure 2 shows the two mixtures run on a ZORBAX GF-250 column under identical conditions at 1 mL/min.

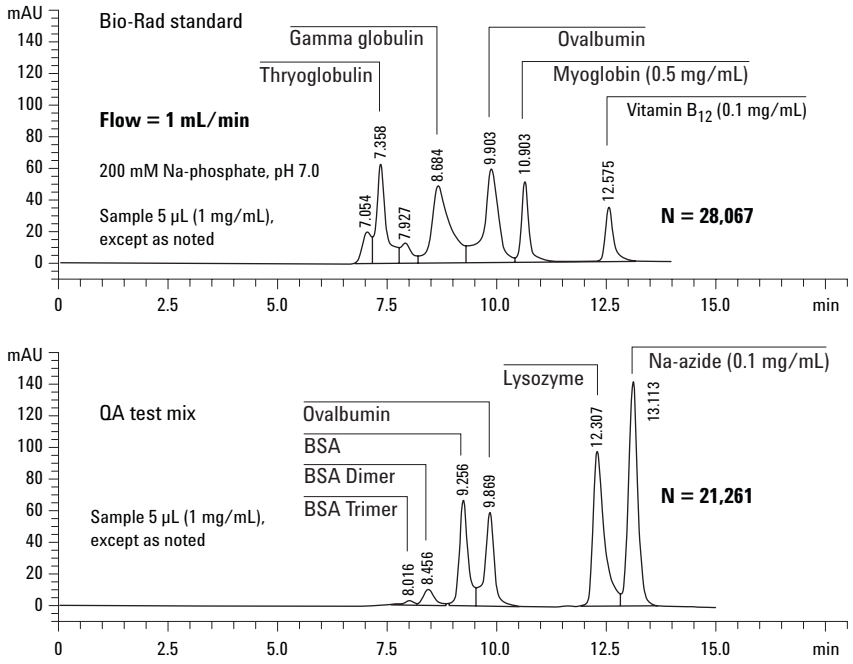

Figure 2. ZORBAX GF-250 gel-filtration chromatographic comparison of the QA test mix against the Bio-Rad standard. Conditions as in Figure 1.

A comparison of the chromatograms suggests that the molecules are eluting according to their MW (from large molecules to small molecules). Thyroglobulin, the largest protein by far, elutes as three peaks between 7 and 8 min. BSA (bovine serum albumin) also elutes early, BSA trimer first, followed by dimer and monomer. Lysozyme is a very basic protein that can be retained by interaction of its positive charge with negative charge on the packing surface. The elution position of lysozyme therefore can be used as a stringent test of any non-ideal behavior of the column packing. It is most impressive that the elution volumes for the Bio-Rad standard (Figure 2, top) match those obtained on a ZORBAX GF-250 column made 10 years earlier (Figure 1).

The small nonprotein molecules, vitamin B_{12} and sodium azide, elute last and are typically used to indicate the inclusion volume (t_o) and theoretical plate number (peak sharpness). Vitamin B_{12} is larger than azide and elutes somewhat earlier. In Figure 2, the lower plate number for azide (21K as opposed to 28K) is caused by the flow rate and will be discussed with Figure 4.

As mentioned for Figure 1, plots of log (MW) vs. retention volume indicate how closely sample compounds have been separated according to size for a particular column and set of conditions. The plot in Figure 3 shows how well the ZORBAX GF-250 column packing separates components of the QA test mix by their size.

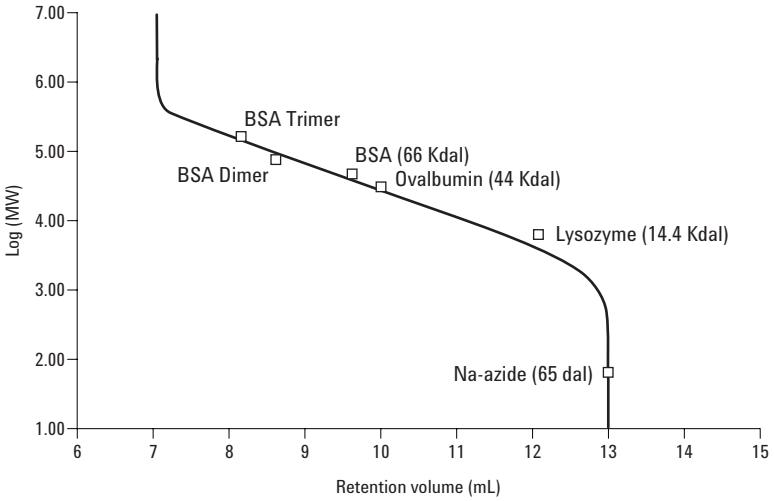

Figure 3. Retention volume versus log (MW) for the test mix components in Figure 2. Conditions as in Figure 1.

When found, deviation from ideal behavior (nonideal size exclusion) may be the result of ionic or hydrophobic interaction between the packing and the analyte. These effects are characterized for the ZORBAX GF-250 column material in some detail [2].

The QA test mix was run on the ZORBAX GF-250 column at 1 and 2 mL/min. See Figure 4. This was done for several reasons: first, to demonstrate the excellent performance of ZORBAX GF-250 particles at higher pressures; second, to point out the improvement in theoretical plates achieved for azide at higher flow rates; third, to reproduce and demonstrate the same conditions under which ZORBAX GF-250 batch materials are tested (lower chromatogram).

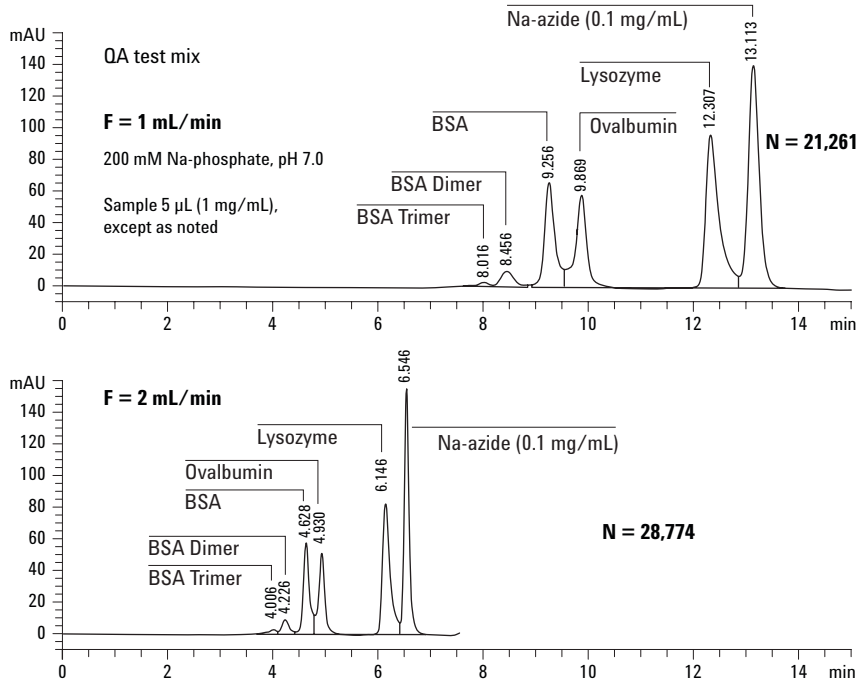

Figure 4. Comparison of chromatographic performance of the ZORBAX GF-250 column in the separation of the QA test mix at two flow rates.

Because of their extreme rigidity, ZORBAX GF-250 particles may be used at higher-than-usual flow rates, achieving very rapid gel-filtration separations. Sodium azide is a very small molecule with a high diffusion rate. At flow rates of 1 mL/min, it has time to broaden as it moves down the column. At 2 mL/min, azide plates jump to >28,000 (similar to the plate number found for vitamin B_{12} in Figure 2). Note that proteins are large molecules with much lower diffusion rates. They will elute with somewhat lower plate values as flow rate is increased.

Finally, this is the test performed on every batch of ZORBAX GF-250 packing material. All gel-filtration columns should be tested upon receipt to verify performance. This result may then be used to verify performance of the column any time it is in question. Both sodium azide (at 2 mL/min) and vitamin B_{12} (at 1 mL/min) elute with similar plate values. This should be used as an indicator of: bed integrity. While columns received may have very high plate values, the minimum specification set by the manufacturer may be much lower. Check with the manufacturer or in the column test sheet for the actual acceptable value. Finally, the elution time and separation between sample components within the test mix should be used to test the column for chemical integrity.

References

1. R. D. Ricker, "Calibration of a ZORBAX GF-250 Column Using Bio-Rad Gel-Filtration Standards," Agilent Technologies publication 5988-6323EN www.agilent.com/chem
2. R. D. Ricker, L. A. Sandoval, J. D. Justice, and F. O. Geiser, (1995) Multivariate visualization in the size-exclusion chromatography and pattern recognition of biological samples. *J. Chromatogr. A,* **691,** 67-79.

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