

## EVEREST™ Ultra High Resolution Reversed-Phase Columns and Media

Grace Vydac is proud to introduce its new EVEREST™ brand of HPLC columns. EVEREST columns are produced using a proprietary silica treatment with unique monomeric C18 bonding on 300 Å pore-size material. They have been demonstrated to provide high peak counts (indicative of high resolution) for tryptic digests of bovine serum albumin,  $\beta$ -lactoglobulin A, and fetuin under high sample loads. Compared to other leading commercial columns, EVEREST columns offer:

- high resolution
- unique selectivity for both hydrophilic and hydrophobic peptides
- excellent lot-to-lot reproducibility

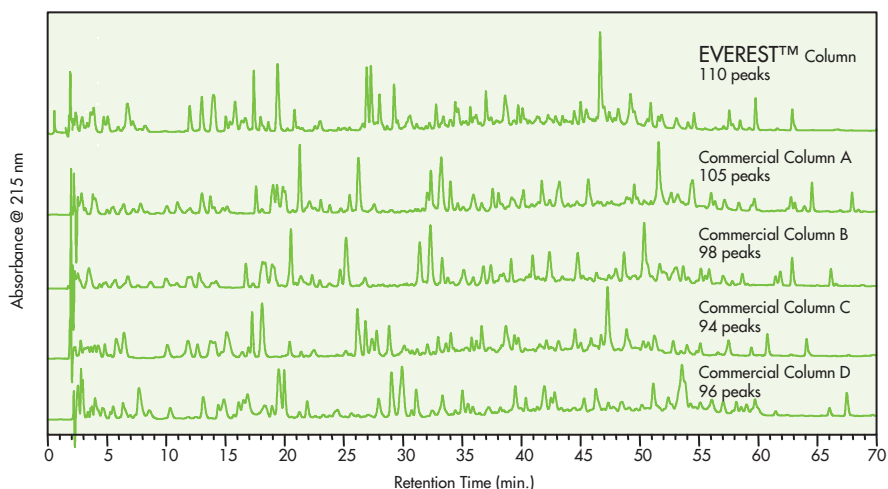
High peak counts for EVEREST columns are attributed to a novel silica treatment that provides improved bonding and peptide recoveries: hence the ability to detect trace level peptides. EVEREST columns are available for applications from nano/capillary LC/MS to preparative and process scale. They are especially beneficial for peptide mapping, purification of synthetic peptides, and high-peptide-load runs.

### Materials and Methods

#### Protein and Digest Preparation.

Insoluble trypsin attached to cross-linked beaded agarose (Sigma) was prepared and used following the manufacturer's guidelines. Briefly, the insoluble trypsin in the form of a suspension was gently mixed, and an aliquot was transferred to a graduated

### EVEREST Columns Outperform Many Commercial Columns for High Peptide Loads



**Figure 1.** Tryptic digest of bovine serum albumin on an EVEREST C18 column and four commercial C18 columns (all 300 Å pore size, 5  $\mu$ m particle size, 4.6 mm i.d. x 150 mm). Mobile Phase: A: 0.1% v/v TFA in water; B: 0.085% v/v TFA in acetonitrile. Gradient: 4% B for 5 min, 4-40% B in 75 min, 40-90% B in 10 min, 90% B for 10 min, equilibrate at 4% B for 20 min. Flow Rate: 1.0 mL/min. Detector: UV at 215 nm. Temperature: 22°C. Peptide load: 30  $\mu$ L of a 5.8  $\mu$ g/ $\mu$ L digest (174  $\mu$ g total peptide load). Peak numbers shown are the average of five replicate separations on each column.

microcentrifuge tube. The suspension was centrifuged (80 x g, 3 sec), and the supernatant discarded. The packed gel was resuspended and washed with 25 mM  $\text{NH}_4\text{HCO}_3$  (pH 7.8) a total of six times. The working trypsin suspension was prepared by adding enough buffer to create a 1:1 volume ratio of packed gel to 25 mM  $\text{NH}_4\text{HCO}_3$ . A 200  $\mu$ L volume of this suspension was added to 1000  $\mu$ L of each sample containing up to 6.25 mg of test protein in 25 mM  $\text{NH}_4\text{HCO}_3$  buffer. Test proteins included bovine serum albumin (BSA),  $\beta$ -lactoglobulin A (from bovine

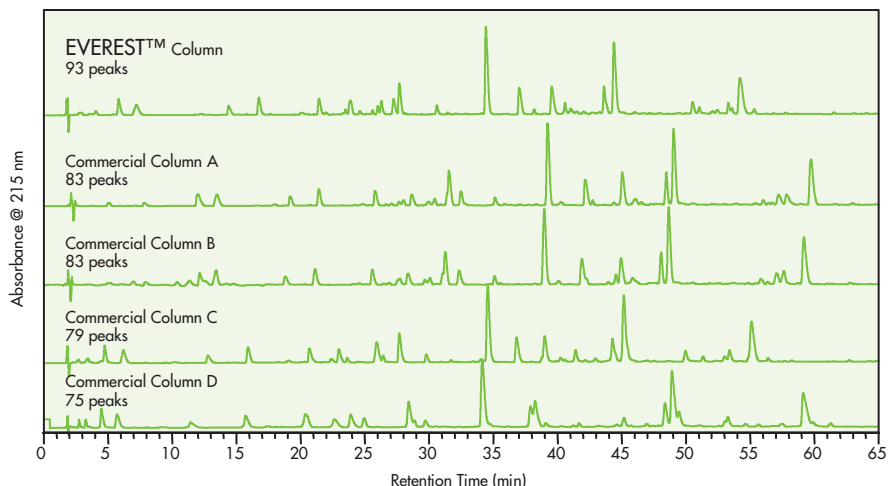
milk), fetuin (a glycoprotein from fetal calf serum), and cytochrome c (from bovine heart), all from Sigma. Incubations were performed at 37°C for 15 h, with mixing on a rotary shaker. Following incubation, samples were centrifuged (80 x g, 3 sec) to pellet insoluble trypsin. The supernatant was transferred to a new microcentrifuge tube and centrifuged at higher g-force (16,000 x g, 10 min). The pellet was discarded and sufficient aqueous 1% v/v trifluoroacetic acid (TFA) added to the supernatant to bring the final concentration to 0.09% v/v TFA.

**Instrumentation, Equipment, and Integration Parameters.** A Dionex HPLC system equipped with a P580A LPG quaternary gradient pump, an integrated degasser, a UVD 170S variable wavelength detector, an ASI-100 autosampler, and a CHROMELEON® (version 6.00) data system was used for the analyses. Samples consisting of 25 to 30  $\mu\text{L}$  of the trypsin digest were injected onto an EVEREST reversed-phase C18 HPLC column (300  $\text{\AA}$  pore size, 5  $\mu\text{m}$  particle size, 4.6 mm i.d. x 150 mm). Other commercially available columns of the same hardware dimensions were also tested. All columns had monomeric C18 bonding on silica with pore and particle sizes of the same classification (i.e., 300  $\text{\AA}$ , 5  $\mu\text{m}$ ). Mobile phases and gradient conditions are described in the figure legends. Peak counts for a given trypsin digest sample were the average of three to five individual injections, and were obtained using the CHROMELEON software set with the following parameters: inhibit integration until 2.4 minutes; valley-to-valley on; rider threshold 10%, minimum area 0.25 mAU-min; minimum width 0.1 min; inhibit integration after 70 min for BSA,  $\beta$ -lactoglobulin A, and cytochrome c; inhibit integration after 80 min for fetuin.

## Results and Discussion:

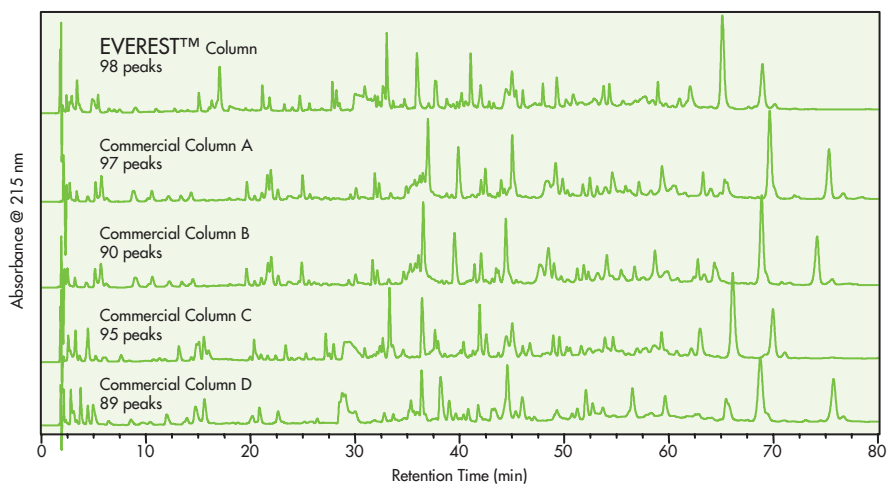
**Peak Performance: BSA,  $\beta$ -lactoglobulin, and Fetuin Tryptic Digest.** Separation of a tryptic digest of BSA on an EVEREST column and four commercial columns is presented in Figure 1. All five columns provide different selectivity and resolving power. Repeat injections of a 174  $\mu\text{g}$  peptide load were performed on all columns. At this high peptide load, excellent peak count (110 peaks) is observed for the EVEREST column, with 15% better resolution than commercial column D.

### EVEREST Column vs. Commercial C18 for Digest of $\beta$ -Lactoglobulin A, High Loading



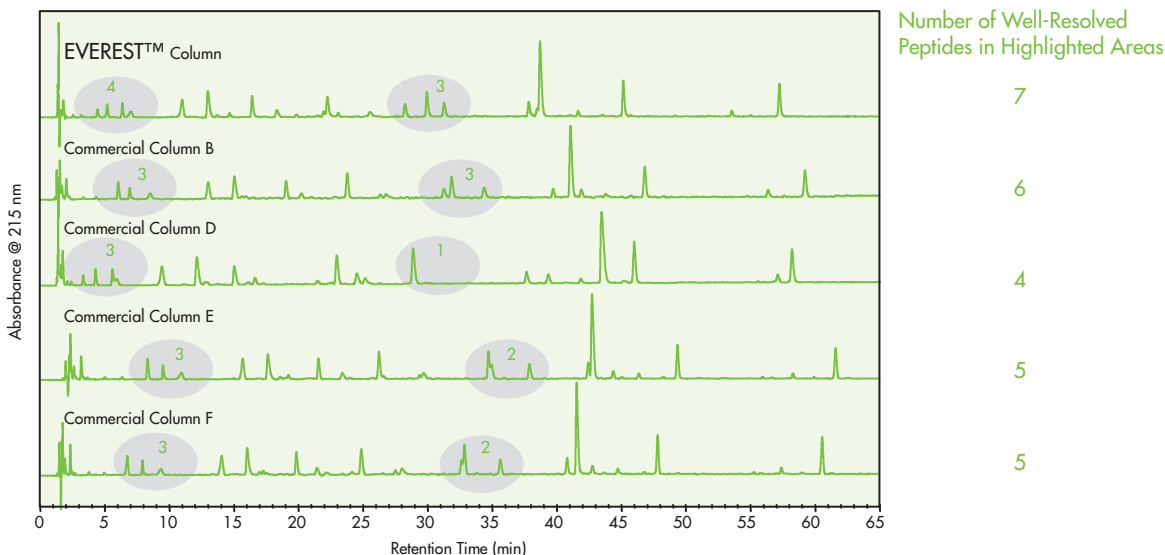
**Figure 2.** Comparison of tryptic digest of  $\beta$ -lactoglobulin A on an EVEREST C18 column and four different commercial C18 columns (all 300  $\text{\AA}$  pore size, 5  $\mu\text{m}$  particle size, 4.6 mm i.d. x 150 mm). Solvent composition and run conditions as in Figure 1. Peptide load: 30  $\mu\text{L}$  of a 6.25  $\mu\text{g}/\mu\text{L}$  digest (188  $\mu\text{g}$  total peptide load). Peak numbers shown are the average of three replicate separations on each column.

### EVEREST Column vs. Commercial C18 for Digest of Fetuin, High Loading



**Figure 3.** Comparison of tryptic digest of fetuin (a glycoprotein) on an EVEREST C18 column and four different commercial C18 columns (all 300  $\text{\AA}$  pore size, 5  $\mu\text{m}$  particle size, 4.6 mm i.d. x 150 mm). Solvent composition and run conditions as in Figure 1. Peptide load: 30  $\mu\text{L}$  of a 6  $\mu\text{g}/\mu\text{L}$  digest (180  $\mu\text{g}$  total peptide load). Peak numbers shown are the average of three replicate separations on each column.

## Selectivity of EVEREST C18 Column and Others for Tryptic Digest of Cytochrome C



**Figure 4.** Comparison of tryptic digest of cytochrome c on an EVEREST column and four different commercial columns (all C18, 300 Å pore size, 5 µm particle size, 4.6 mm i.d. x 150 mm). Mobile Phase: A: 0.1% v/v TFA in water; B: 0.085% v/v TFA in acetonitrile. Gradient: 4-40% B in 75 min, 40-90% B in 10 min, 90% B for 10 min, equilibrate at 4% B for 15 min. Flow Rate: 1.5 mL/min. Detector: UV at 215 nm. Temperature: 22°C. Peptide load: 25 µL of a 1.7 µg/µL digest (42 µg). The EVEREST column offers unique selectivity (see encircled regions) for both hydrophilic and hydrophobic peptides. In this case with the cytochrome c digest, various peptides are better (baseline) resolved on the EVEREST column.

The peak counts obtained for the BSA digest surpass the theoretical cleavages. There are 77 theoretical trypsin cleavage sites, based on the Swiss Protein data bank. This “discrepancy” is the result of the integration parameters being set such that the data system will be able to detect a large number of “trace level” peaks. With these particular parameters, the laboratory-determined peak count data will deviate from the theoretical peak count, likely due to cleavage from very small amounts of contaminating proteases (in commercially available enzyme preparations, or from bacterial contamination). Furthermore, the commercially available protein standards usually have some impurities. The obvious interest in detecting trace level peaks is to obtain a measure of resolution. It is also clear that at high peptide loading, the UV

spectrophotometer can detect “trace level” peaks.

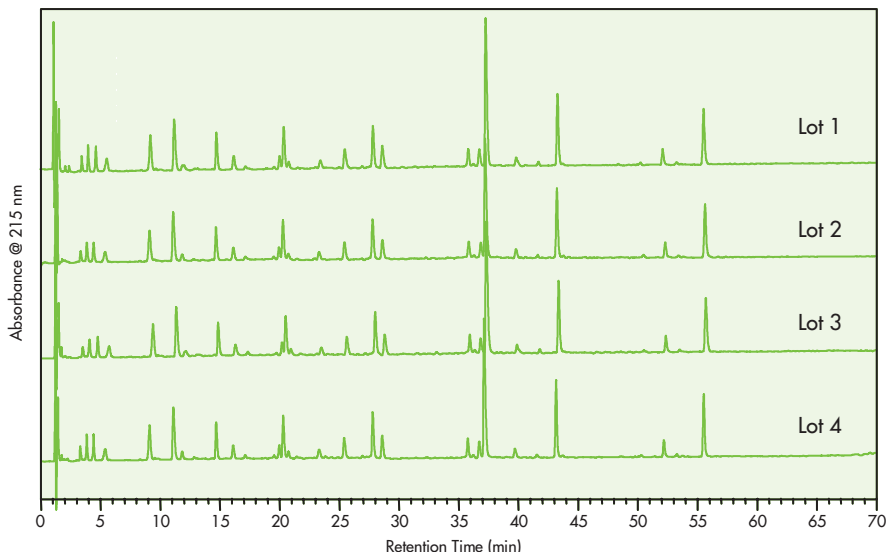
Chromatograms for the tryptic digests of β-lactoglobulin and fetuin on an EVEREST column and four commercial columns are presented in Figures 2 and 3. As with the BSA tryptic digest, the EVEREST column is able to provide high resolution separations for the β-lactoglobulin and fetuin digests with 93 and 98 peaks detected, respectively. The EVEREST column exhibits 12% higher peak count than commercial columns A and B based on the β-lactoglobulin tryptic digests at high peptide load; and, it is 24% better than commercial column D for the same sample.

For this particular comparative study of column performance, we have tested 150 mm length columns. However, for real-world applications, if one desires the best resolution for enzymatic digests, it is recommended that a longer 250 mm column or a shallower gradient be used. (Reference: Grace Vydac Handbook, 3rd ed.)

### Selectivity

An excellent approach to evaluating selectivity of different columns is to chromatograph a tryptic digest of cytochrome c. Among the columns tested, the EVEREST column offers unique selectivity which allows the best separation of a group of hydrophilic and hydrophobic peptides (Figure 4).

### Tryptic Digest of Cytochrome C on EVEREST C18 Columns



**Figure 5.** Tryptic digest of cytochrome c showing lot-to-lot reproducibility of EVEREST C18 columns (300 Å pore size, 5 µm particle size, 4.6 mm i.d. x 150 mm). Solvent composition and run conditions as in Figure 4. Peptide load: 25 µL of a 1.7 µg/µL digest (42 µg). Excellent lot-to-lot reproducibility ensures confidence in the column validation process.

	Lot 1	Lot 2	Lot 3	Lot 4		
Major Peak #	RT <sup>a</sup> (min)	RT <sup>a</sup> (min)	RT <sup>a</sup> (min)	RT <sup>a</sup> (min)	AVE ± 1 SD (min)	RSD <sup>b</sup> (%)
1	9.4	9.1	9.1	9.2	9.2 ± 0.13	1.41
2	11.4	11.1	11.1	11.2	11.2 ± 0.12	1.07
3	14.8	14.7	14.7	14.7	14.7 ± 0.08	0.54
4	20.5	20.3	20.3	20.3	20.4 ± 0.10	0.49
5	25.6	25.4	25.4	25.4	25.5 ± 0.10	0.39
6	28.0	27.8	27.8	27.8	27.8 ± 0.10	0.36
7	28.8	28.6	28.6	28.6	28.6 ± 0.10	0.35
8	37.3	37.2	37.1	37.2	37.2 ± 0.08	0.22
9	43.4	43.2	43.2	43.3	43.3 ± 0.09	0.21
10	55.7	55.6	55.5	55.5	55.6 ± 0.09	0.16

<sup>a</sup> RT = retention time <sup>b</sup> RSD = relative standard deviation

**Table 1.** Tryptic digest of cytochrome c showing lot-to-lot reproducibility of EVEREST C18 columns. Ten of the major peaks were selected for analysis.

### Lot-to-Lot Reproducibility

Robust polypeptide assays (e.g., of biotechnology-derived therapeutic products) require HPLC columns to be reproducible in selectivity and resolution from lot-to-lot (Ref.: AN9703). The separation of peptide fragments from an enzymatic digest is an effective test of lot-to-lot reproducibility for reversed-phase columns. A tryptic digest of cytochrome c was chromatographed on four different lots of EVEREST C18 columns (Figure 5). The excellent reproducibility, especially as shown by the statistical data presented in Table 1, results from rigorous controls in Grace Vydac's manufacturing process.

The Separations Group began producing VYDAC® 300 Å synthetic silica from purified organic silicates almost three decades ago. Today, as a part of W. R. Grace & Co.-Conn., there have been great strides in the development of new silica technologies. The outstanding performance of Grace Vydac's EVEREST columns may be attributed to a novel, proprietary silica treatment and a unique bonding chemistry.

### References

1. The Grace Vydac Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC, 3rd Edition
2. Grace Vydac Application Note # AN9703. "Developing a Robust Reversed-Phase Method for the Analysis of Polypeptides."

To place an order, call (800) 347-6378, fax (508) 485-5736 your Grace/Vydac distributor.  
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