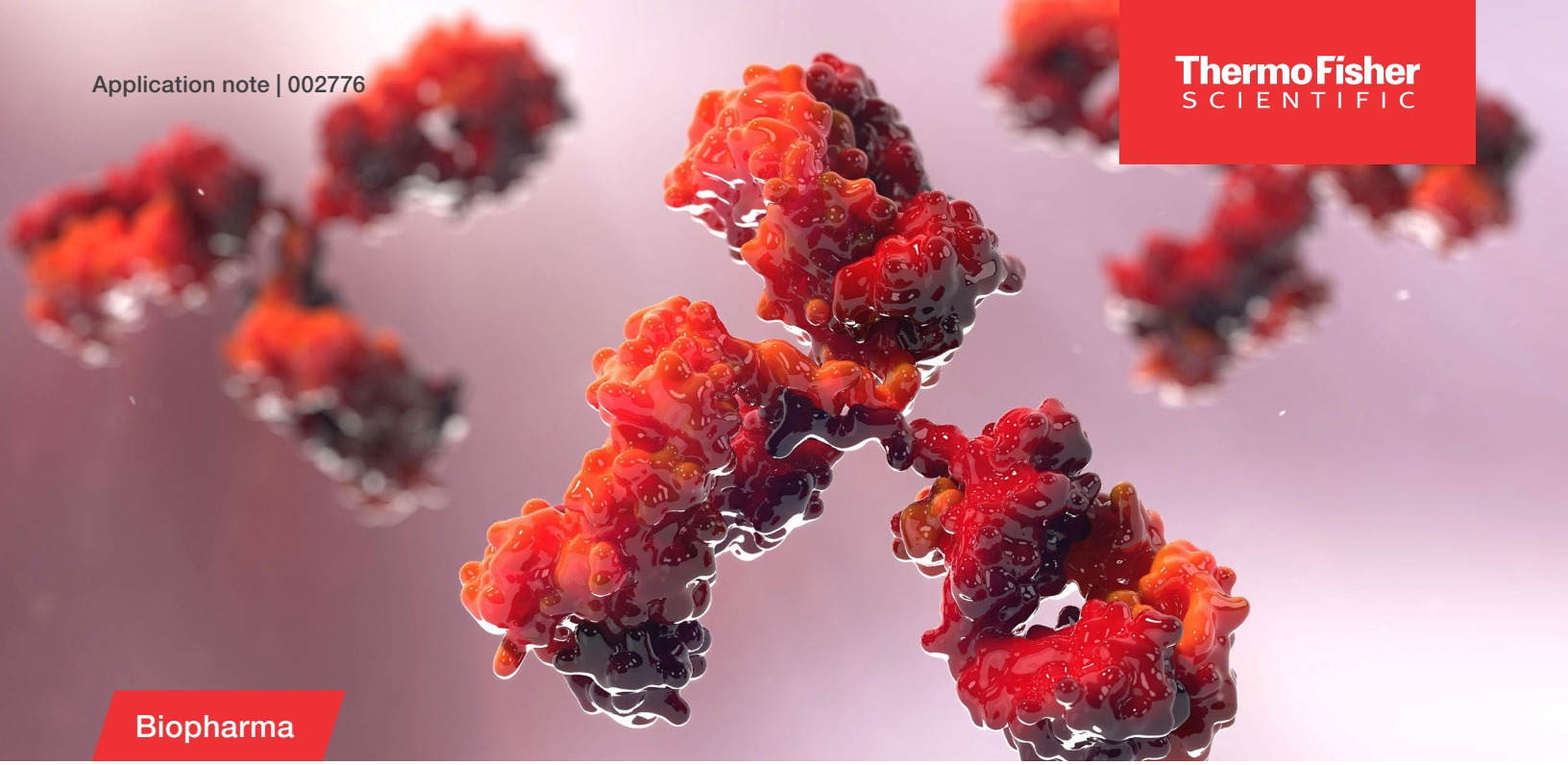
Application note | 002776



Biopharma

Hypersil GOLD Peptide column for reliable monitoring of glycopeptide variants in monoclonal antibodies by LC-UV-MS analysis

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Keywords

Peptide quantification, Hypersil GOLD Peptide column, LC column robustness, PTM, Vanquish Flex UHPLC, ISQ EM mass spectrometer, adalimumab, monoclonal antibody, mAb, SMART Digest Kit, trypsin

Application benefits

- Thermo Scientific™ Hypersil GOLD™ Peptide columns help achieve accurate and reproducible results when monitoring glycopeptide variants in mAb digest samples.
- Hypersil GOLD Peptide columns exhibit low column-to-column and lot-to-lot variability, resulting in consistent performance when analyzing glypeptides in mAb samples.

Goal

Demonstrating the benefits of the Hypersil GOLD Peptide C18 column in LC-UV-SQMS analysis of tryptic mAb digest samples

Introduction

Therapeutic monoclonal antibodies (mAbs) are recombinant proteins produced by highly complex ectopic expression in cell culture and used in biotherapeutics. They are susceptible to various chemical and enzymatic modifications during expression, purification, and long-term storage.[1,2]

During the development phase, a detailed characterization of the mAb is conducted to verify its correct sequence and to determine modifications in its chemical structure that could constitute potential quality attributes. For this purpose, high-resolution accurate mass (HRAM) mass spectrometry and MS/MS analysis are applied to localize and quantify these post-translational modifications (PTMs), such as glycopeptides, but also stress-induced modifications on the peptide level. Once characterized, monitoring can be transferred to a comprehensive MS1-only approach.

Monitoring of PTMs requires careful consideration of several key factors, including reproducibility, sensitivity, precision, and throughput. Hypersil GOLD Peptide columns are specifically designed to provide outstanding performance and exceptional durability, making them the ideal choice for your peptide monitoring needs. Thermo Scientific™ columns are chromatographically tested during quality control (QC) in the manufacturing process to ensure column-to-column and lot-to-lot reproduciblity. In particular, Hypersil GOLD Peptide columns with 1.9 µm particle size deliver sharp symmetric peaks with higher efficiency, ensuring greater sensitivity and LOQs.

In this study, a liquid chromatography method using the Hypersil GOLD Peptide column along with ultraviolet (UV) detection coupled to a single quadrupole mass spectrometer (SQMS) was used to monitor glycopeptide variants for the IgG I class monoclonal antibody adalimumab. To perform monitoring on the peptide level, the protein must be digested into peptides. For this purpose, the Thermo Scientific™ SMART Digest™ Trypsin Kit in combination with a reducing agent was used. The endoprotease trypsin cleaves the peptide bonds at the C-terminal side of the amino acids arginine (R) and lysine (K) except for -Arg-Pro- and -Lys-Pro- bonds which are normally resistant to proteolysis. This results in an antibody-specific set of peptides which is ready for subsequent analysis by LC-UV-SQMS.

The data obtained in this study demonstrate the excellent column-to-column and lot-to-lot consistency of the Hypersil GOLD Peptide column, which is important for the use in routine QC analysis.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm at 25 °C, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification (P/N 50136149)
- Acetonitrile, Optima™ LC/MS grade, Fisher Scientific™ (P/N A955)
- Methanol, Optima™ LC/MS grade, Fisher Scientific™ (P/N A456)
- Formic acid, Optima™ LC/MS grade, Fisher Chemical™ (P/N A117)
- Thermo Scientific™ SMART Digest™ Trypsin Kit (Non-Magnetic, low pH) (P/N 60109-101-LPH)
- Thermo Scientific™ Bond-Breaker™ TCEP Solution, Neutral pH (P/N 77720)
- Adalimumab, 9.7 mg/mL, purchased from a reputable vendor

Sample handling

- Fisherbrand™ Mini Centrifuge (P/N 12-006-901)
- Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ Digital Heating Shaking Drybath (P/N 88880028)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 100–1,000 µL (P/N 4641100N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 10–100 µL (P/N 4641070N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 1–10 µL (P/N 4641030N)
- Fisherbrand™ Locking-Lid Microcentrifuge Tubes with Polypropylene Snap-Cap™ (P/N 11976955)
- Thermo Scientific™ SureSTART™ 2 mL Polypropylene Snap Top Microvials for <2 mL Samples, Level 1 Everyday Analysis (P/N 6ERV11-03PPCF)
- Thermo Scientific™ SureSTART™ 11 mm Snap Caps, Level 2 High-throughput Applications (P/N 6ARC11ST1)

Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:

- System Base Vanquish Horizon/Flex (P/N VF-S01-A-02)
- Vanquish Binary Pump F (P/N VF-P10-A-01)
- Vanquish Split Sampler FT (P/N VF-A10-A-02)
- Vanquish Column Compartment H (P/N VH-C10-A-03)
- Vanquish Variable Wavelength Detector F (P/N VF-D40-A)

Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer (P/N ISQEM-ESI)

Sample preparation

An adalimumab sample was digested in triplicate together with a control sample containing water only.

Prior to the sample preparation, the PCR tubes from the SMART Digest Kit were briefly centrifuged to spin down the beads. 150 µL of the SMART Digest low pH buffer (Kit component 2), 20 µL adalimumab, 30 µL water, and 2 µL TCEP were added to each tube. The control sample was prepared in the same way by using water instead of adalimumab sample.

After mixing gently, the samples in the SMART Digest PCR tubes were transferred to 0.5 mL tubes. The digestion was performed according to the SMART Digest Kit User Manual. Afterwards, the samples were cooled for 1 min in an ice bath and 2 µL formic acid were added to stop the digestion. After briefly mixing the samples on a vortexer, they were centrifuged for 2 min to spin down the beads. Subsequently, 100 µL of each sample were carefully transferred and pooled into one HPLC vial to obtain one single sample for the study.

The sample and the control were kept at 6 °C and analyzed within seven days.

Chromatographic conditions

Table 1. Chromatographic conditions

Parameter	Value
Column	Hypersil GOLD Peptide 150 × 2.1 mm, 1.9 µm (P/N 26002-152130)
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Acetonitrile + 0.1% formic acid
Gradient	Time (min) %B 0.0 2.0 0.5 2.0 1.0 9.0 22.0 35.0 23.0 90.0 26.0 90.0 27.0 2.0 45.0 2.0
Flow rate	0.25 mL/min
Column temp.	50 °C (with active pre-heater at 50 °C, forced-air mode, fan speed 5)
Autosampler temp.	6 °C
Needle wash solution	10/90 (v/v) acetonitrile/water + 0.1% formic acid
Needle wash mode	Before draw
Injection volume	5 µL
UV wavelength	214 nm
Data collection rate, response time	20.0 Hz, 0.20 s

MS settings

Table 2. Ion source and scan settings for the mass spectrometer

Parameter	Value
Ionization mode	HESI
Polarity (Spray voltage)	Positive (+3,000 V)
Method type	Component mode
Resulting total scan time	0.500 s
SIM scan width	1.00 amu
Lowest dwell time	0.046 s
CID voltage	20 V
Vaporizer temperature	144 °C
Ion transfer tube temperature	300 °C
Gas flow pressure	• Sheath gas: 32.3 psig • Auxilliary gas: 3.6 psig • Sweep gas: 0.5 psig

Chromatography Data System

The Thermo Scientific™ Chromeleon™ 7.3.1 CDS was used for data acquisition and processing.

Results and discussion

Adalimumab sample was separated with replicate injections on three different lots of Hypersil GOLD Peptide columns. Chromatograms of five consecutive injections are shown in Figure 1. Three peptides were selected to demonstrate column lot-to-lot reproducibility of retention time, peak width (50%), and absolute peak area based on UV signal. The selection covers the entire elution window from early to late eluting peptides. Excellent results were obtained with relative standard deviation (%RSD) for retention time ≤0.6, peak width (50%) ≤3.9, and peak area ≤6.2 as illustrated in Figure 2.

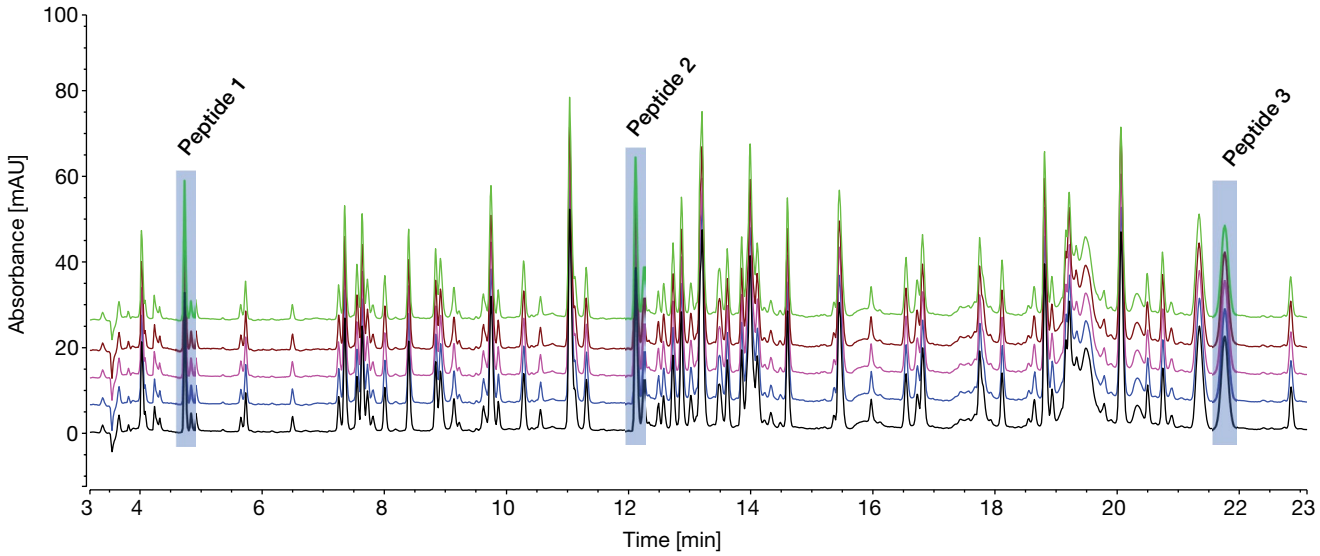

Figure 1. UV chromatograms of five consecutive injections of adalimumab tryptic digest sample with detection wavelength of 214 nm

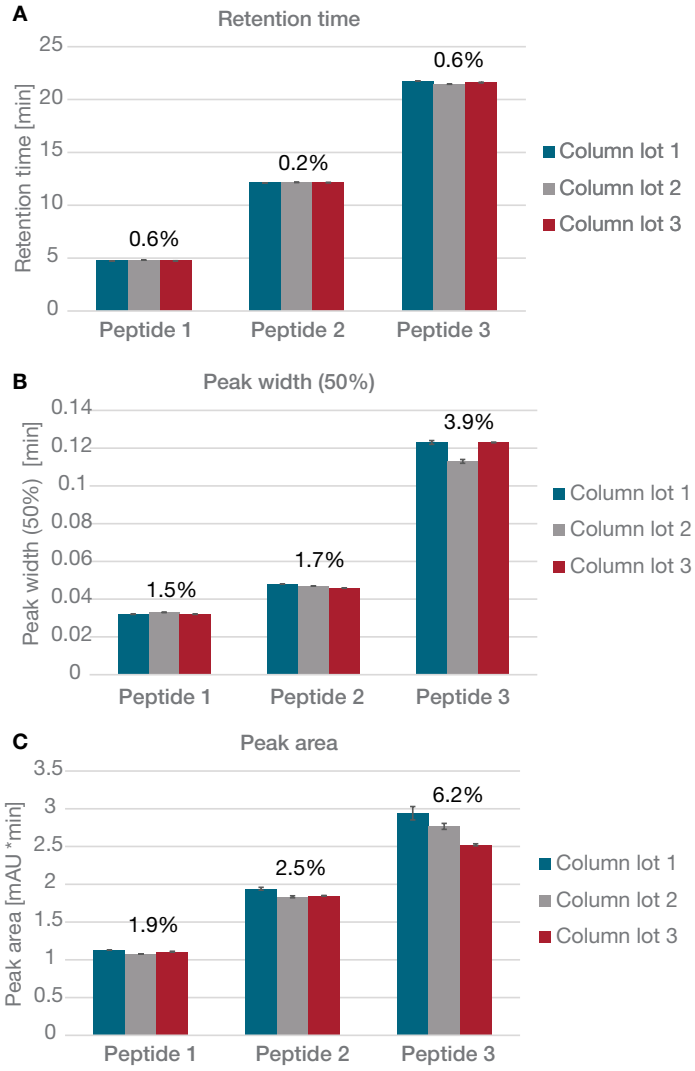

Figure 2. Column lot-to-lot reproducibility for (A) retention time, (B) peak width (50%), and (C) absolute peak area with error bars indicating the standard deviation for five consecutive injections for each column of each lot. The numbers above the bars represent %RSD values obtained when comparing the three column lots.

In addition, reproducibility was assessed for relative abundance of glycopeptide variants in the adalimumab sample based on SQMS signal. Relative abundance of the glycopeptides was evaluated by summing the peak areas of each variant, A2G0F, A2G1F, and A2G2F, and dividing by the total sum of the peak areas of all variants. Robust quantification of glycopeptides can be achieved with %RSD ≤5.2 (Figure 3).

Furthermore, column-to-column reproducibility of the relative abundance of glycopeptides was examined. For this purpose, the digest was analyzed on three columns from two different lots. As can be seen in Figure 4, %RSD is excellent with ≤3.0%.

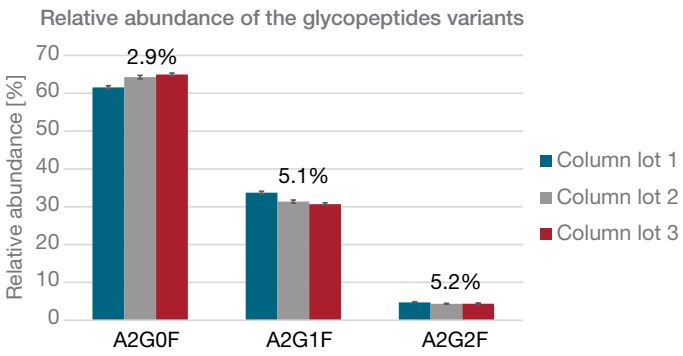

Figure 3. Column lot-to-lot reproducibility of relative abundance for glycopeptide variants with error bars indicating the standard deviation for five consecutive injections for each column of each lot. The numbers above the bars represent %RSD values obtained when comparing the three column lots.

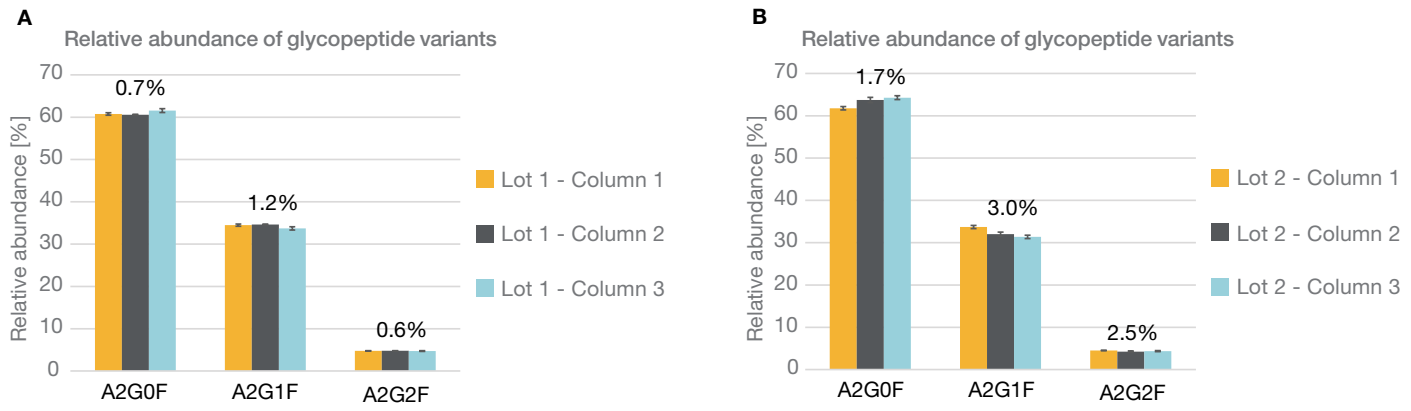

Figure 4. Column-to-column comparison for three columns each from two different lots with error bars indicating the standard deviation for five consecutive injections for each column. The numbers above the bars represent %RSD values obtained when comparing the three columns from the same lot.

Conclusion

In this study, we have evaluated the performance of the Hypersil GOLD Peptide column for peptide monitoring analysis. The obtained results demonstrate the following:

- Excellent column lot-to-lot reproducibility with %RSD RT ≤0.6,%RSD peak width (50%) ≤3.9, and %RSD peak area ≤6.2 for peptides across the entire elution window
- Consistent column-to-column results for the quantification of glycopeptides with %RSD of relative abundance ≤5.2 and ≤3.0, respectively

The results demonstrate the exceptional capabilities of the Hypersil GOLD Peptide column for LC-UV-SQMS monitoring of post-translational modifications in monoclonal antibodies.

References

1. Gucinski, A.C.; Boyne, M.T. Evaluation of intact mass spectrometry for the quantitative analysis of protein therapeutics. *Analytical Chemistry,* **2012,** *84*(18), 8045–8051.
2. Jefferis, R. Posttranslational modifications and the immunogenicity of biotherapeutics. *Journal of Immunology Research,* **2016,** 1–15.

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