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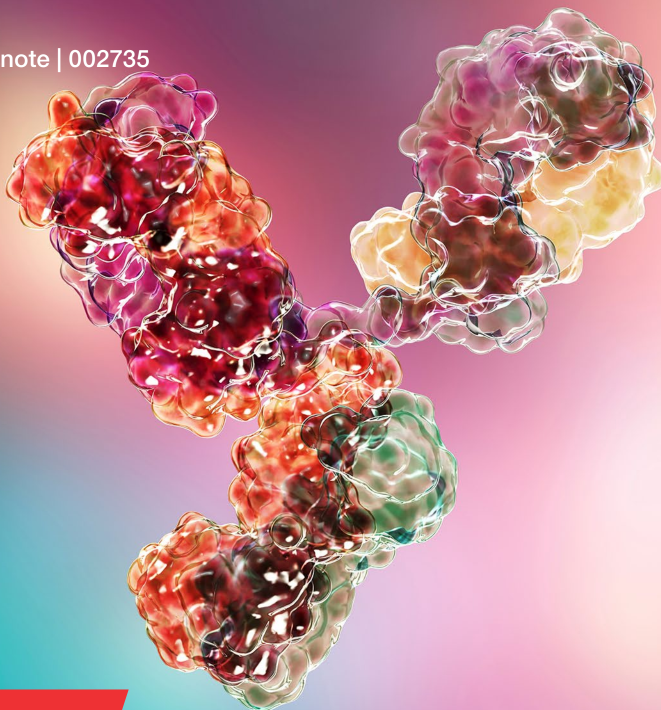

Biopharma

Performance comparison of reversed-phase C18 columns for peptide mapping of monoclonal antibodies

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Keywords

NIBRT, peptide mapping, reversed phase, MAM, monoclonal antibody, Hypersil GOLD Peptide column

Key benefits

- The Thermo Scientific™ Hypersil GOLD™ Peptide column provides excellent chromatographic performance related to peak shape, peak area, chromatographic resolution, and retention of hydrophobic as well as hydrophilic peptides.
- A wide range of modified and unmodified peptides in high and low abundance can be detected and quantified reliably and reproducibly.
- Highly reproducible run-to-run and lot-to-lot chromatographic results are obtained for mAb digest samples.

Goal

In this work the Hypersil GOLD Peptide reversed-phase C18 column was evaluated for biopharmaceutical peptide level characterization and to illustrate consistent lot-to-lot performance. Further analysis was done to compare the Hypersil GOLD Peptide column to cross vendor columns that are recommended for peptide mapping. Column performance was evaluated by investigating sequence coverage, post-translational modification separation, hydrophobic and hydrophilic peptide retention, and carry-over.

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Introduction

Peptide mapping analysis is routinely used in biotherapeutic characterization for the confirmation of the primary sequence and to investigate the presence and relative levels of post-translational modifications (PTMs) such as deamidation, oxidation, glycosylation, and more. Peptide mapping experiments are performed first by reduction and alkylation of the disulfide bonds followed by enzymatic digestion using proteases. The most commonly applied protease for monoclonal antibody (mAb) peptide mapping is trypsin, which cleaves specifically at the C-terminal of arginine (R) and lysine (K) residues.[1] Peptides are then separated by reversed-phase liquid chromatography, often using a C18 column, coupled to mass spectrometry. C18 columns recommended for peptide mapping differ in chemistry with various carbon load, particle size (µm), surface area (m^2/g), average pore size (Å), and pore volume (mL/g). Column characteristics can directly influence separation performance and, with a wide variety of columns available from various vendors, determining the appropriate column can often be expensive and time consuming.[2, 3]

mAbs undergo a variety of enzymatic and chemical modifications that can impact the overall stability, efficacy, and safety of the drug product. These are termed critical quality attributes (CQAs) and can range from impurity content, such as host cell proteins and residual DNA, to relative abundance of protein aggregates or fragments, to the presence of specific levels of PTMs on the protein backbone.[4] PTM analysis can be challenging due to often-subtle chemical differences between unmodified peptides and modified species, requiring efficient chromatographic separation.[5] Specific PTMs such as asparagine (N) deamidation can be particularly challenging in chromatography due to close elution of the deamidated species to the unmodified peptides, which requires high resolution in both chromatography and mass spectrometry to be able to distinguish a +0.98 Da mass shift that a deamidation event applies. Deamidation is typically triggered by exposure to environmental conditions, such as pH and temperature, and promotes antibody degradation making it a valuable CQA to separate and monitor.[6] In mAbs there exists a region known as the “PENNYK” motif, which is typically used for the evaluation of deamidation behavior due to the number of deamidation sites present within the peptide.

In this study, three lots of Hypersil GOLD Peptide columns were compared to four cross vendor columns. All columns investigated were of the same format (2.1 × 150 mm) with differences in particle and pore size, which ranged from 1.6 to 2.7 µm and 100 to 175 Å, respectively. Chromatographic conditions such as

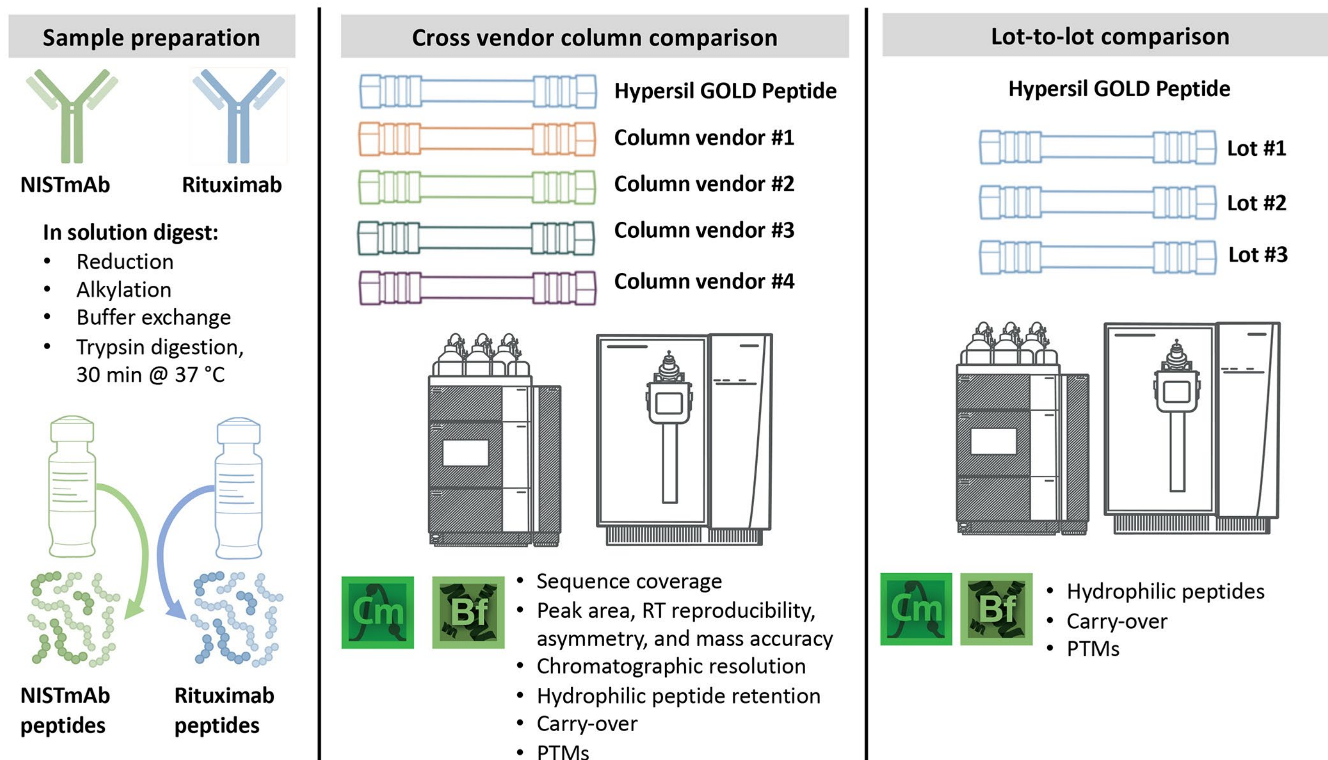

Figure 1. NISTmAb and rituximab were reduced, alkylated, and buffer exchanged before trypsin digestion. Using these samples, the Hypersil GOLD Peptide column was compared to four vendor columns. Each column was assessed based on sequence coverage, peak area, retention time reproducibility, peak asymmetry, mass accuracy, chromatographic resolution, hydrophilic peptide retention, peptide carry-over, and PTM abundance. Finally, three lots of the Hypersil GOLD Peptide column were compared by investigating hydrophilic peptides, peptide carry-over, and PTM relative abundance.

column temperature, mobile phase flow rate, and the amount of material loaded on column were kept consistent, although within the range recommended by each vendor. All columns were conditioned using the same procedure performing injection of BSA tryptic digest. NISTmAb digests were injected at the start and the end of the sequence as a system suitability test (SST) while six technical replicates of rituximab digests were investigated. For each column, chromatographic performance was evaluated by comparing the chromatographic profile and parameters such as sequence coverage, peak area, retention time precision (RT), asymmetry (A), chromatographic resolution, retention of both hydrophilic and hydrophobic peptides, and mass accuracy for confident identification. A specific focus was placed on peak resolution of the PENNYK peptide. For this investigation, NISTmAb digests were used due to a higher abundance of deamidation levels compared to rituximab, allowing for a more effective comparison of chromatographic resolution in this region. Finally, the relative abundances of relevant quality attributes, such as deamidation, oxidation, succinimide formation, and N- and C-terminal composition in rituximab, were examined.

Experimental

Recommended consumables

- NISTmAb Humanized IgG1κ Monoclonal Antibody (NIST™, RM 8671)
- MabThera™ (rituximab) chimeric IgG1 Monoclonal Antibody
- Thermo Scientific™ Pierce™ BSA Protein Digest, MS grade (P/N 88341)
- Thermo Scientific™ Water, UHPLC-MS grade (P/N W8-1)
- Thermo Scientific™ Acetonitrile, UHPLC-MS grade (P/N A956-1)
- Fisher Chemical™ Formic acid (FA) 99.0%, Optima™ LC-MS grade (P/N 10797488)
- Thermo Scientific Hypersil GOLD Peptide C18, 2.1 × 150 mm, 1.9 µm, 175 Å column (P/N 26002-152130)
- Column vendor 1 (2.1 × 150 mm, 2.7 µm, 120 Å)
- Column vendor 2 (2.1 × 150 mm, 1.7 µm, 130 Å)
- Column vendor 3 (2.1 × 150 mm, 1.7 µm, 130 Å)
- Column vendor 4 (2.1 × 150 mm, 1.6 µm, 100 Å)
- 8.0 M guanidine hydrochloride solution (Sigma-Aldrich, P/N G7294-100ML)
- Sodium hydroxide concentrate (Sigma, P/N 43617-1L)
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.9 (P/N 15567027)
- DL-Dithiothreitol (DTT) BioXtra ≥99% purity (Sigma-Aldrich, P/N D-5545)
- Sodium iodoacetate (IAC), BioUltra, >98% purity (Sigma-Aldrich, P/N I-9148)
- Thermo Scientific™ Pierce™ Trypsin-Protease, MS grade (P/N 90058)
- Bio-Spin™ P6-Desalting Spin Columns (BioRad, P/N 732-6227)
- Thermo Scientific™ SureSTART™ Screw vial kit (P/N 6AK92W)
- Thermo Scientific™ SureSTART™ 9 mm glass autosampler inserts, 400 µL (P/N 6PME04C1)
- Eppendorf™ Protein LoBind™ microcentrifuge tube 0.5 mL (P/N 022431064)
- Eppendorf™ Protein LoBind™ microcentrifuge tube 1.5 mL (P/N 022431081)

Sample handling equipment

- Thermo Scientific™ Vanquish™ Horizon UHPLC system (P/N 5400.0105) consisting of:
  - Thermo Scientific™ Vanquish™ System Base (P/N VF-S01-A-02)
  - Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
  - Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
  - Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
  - MS Connection Kit Vanquish (P/N 6720.0405)
- Thermo Scientific™ Orbitrap™ Exploris™ 240 mass spectrometer (P/N BRE725535)
- Fisherbrand™ accuSpin™ Micro 17 Microcentrifuge (P/N 13-100-675)
- Eppendorf™ ThermoMixer™ C (P/N 5382000023)

Software packages

- Thermo Scientific™ BioPharma Finder™ software, version 5.1
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.3.2

Preparation of BSA peptide standard

1 mL of 0.1% formic acid in UHPLC-MS grade water was added to the lyophilized BSA peptide standard. 200 µL was aliquoted into Thermo Scientific™ vials with a glass insert and frozen at -20 °C.

Reagent preparation

Solution 1: 7.0 M guanidine HCl, 100 mM Tris (pH 8.3)

87.5 mL of 8 M guanidine HCl and 10 mL of 1 M Tris-HCl pH 7.5 were added to a 100 mL volumetric flask and mixed by inversion. The pH was measured and adjusted to a pH of 8.3 with sodium hydroxide concentrate.

Solution 2: 500 mM DTT in solution 1

50 mg of DTT was weighed into a 1.5 mL Eppendorf tube and dissolved with 649 µL of solution 1.

Solution 3: 500 mM IAC in solution 1

50 mg of IAC was weighed into a 1.5 mL Eppendorf tube and dissolved with 481 µL of solution 1. The solution was stored in the absence of light.

Solution 4: 50 mM DTT in solution 1

100 µL of solution 2 was added to 900 µL of solution 1 in a fresh 1.5 mL.

Solution 5: 50 mM Tris (pH 7.9)

10 mL of 1 M Tris-HCl pH 7.5 was added to a 200 mL volumetric flask and brought to a final volume of 200 mL using LC-MS grade water.

Solution 6: 10% Formic acid

1 mL of LC-MS grade formic acid was added to 9 mL of LC-MS grade water in a 15 mL tube.

Sample reduction and alkylation

100 µg of both NISTmAb and rituximab was diluted to 1 mg/mL with solution 1 to give a final volume of 100 µL. 2 µL of solution 2 was added to the diluted mAb sample and mixed by vortex. Reduction was carried out at room temperature for 30 minutes. 4 µL of solution 3 was added to each sample and mixed by vortex. Alkylation was carried out at room temperature for 20 minutes in darkness. Alkylation was quenched by adding 4 µL of solution 4 to each sample mixed by vortex.

Buffer exchange and tryptic digestion

BioSpin-6 columns were conditioned by centrifugation at 1,000 × *g* for 2 minutes. The flowthrough was discarded and 500 µL of solution 5 was added to the bed of the column and centrifuged at 1000 × *g* for 2 minutes. This step was repeated three times. 110 µL of the reduced and alkylated mAb sample was added to the bed of the column and the flowthrough was collected in a fresh 1.5 mL microcentrifuge tube by centrifugation at 1,000 × *g* for 4 minutes.

Pierce Trypsin protease was added to the samples at a 1:10 ratio and incubated at 37 °C for 30 minutes. 10% v/v formic acid was added at a 1:10 ratio to stop digestion. Samples were transferred to individual vials and frozen at -80 °C.

LC-MS conditions

Following MS system and mass calibration, columns were first conditioned by 10 injections of BSA before analysis of mAb samples using a Vanquish Horizon UHPLC system coupled to an Orbitrap Exploris 240 mass spectrometer. The LC parameters and gradient were kept consistent for all columns and are summarized in Table 1. MS parameters are summarized in Table 2.

Table 1. LC parameters and gradient applied for peptide mapping experiments

Parameter	Value	
Column temperature	50 °C	
Flow rate	0.25 mL/min	
Solvent A	H_20 + 0.1% formic acid	
Solvent B	ACN + 0.1% formic acid	
Gradient	Time (min)	% Solvent B
	0.0	1
	5.0	1
	6.0	10
	70.0	35
	72.0	90
	77.0	90
	79.0	1
	81.0	1
	83.5	10
	91.5	45
	93.0	90
	99.0	90
	101.0	1
	115.0	1
Injection volume	4 µL	
Needle wash	10% MeOH	
Seal rinse solution	10% MeOH	
Autosampler temperature	6 °C	
Thermostatting mode	Still Air	
Pre-heater	Not used	
Wash speed/time	30 µL/s for 10 s	

Table 2. MS parameters

Parameter	Value
Tune parameters	
Sheath gas flow rate	30 arbitrary units (au)
Auxiliary gas flow rate	10 au
Sweep gas flow rate	1 au
Spray voltage	Static at 3.5 kV
Ion transfer tube temperature	225 °C
Vaporizer temperature	200 °C
Full MS method parameters	
Polarity mode	Positive for the duration of the LC gradient to assess carry-over
Scan resolution	120,000
Scan range	*m/z* 200–2,000
RF lens	70%
Microscans	1
Normalized AGC target	300%
Maximum injection time	100 ms
Data-dependent MS^2 method parameters	
Scan resolution	15,000
Normalized AGC target	100%
Maximum injection time	250 ms
Isolation window	1.2 *m/z*
Normalized collision energy	27%
Dynamic exclusion duration	7 s
Mass tolerance	10 ppm

MS data processing

Peptide identification and product quality assessment was performed using BioPharma Finder software version 5.1, according to the parameters summarized in Table 3. Sequence coverage was assessed after applying some custom filters: only peptides with up to 1 missed cleavage were selected, while adducts, nonspecific, unknown modifications, and gas phase ions were excluded. A target peptide workbook was then created for rituximab sample containing a list of peptides to monitor and the most prominent quality attributes, including all the detected charge states. The target peptide workbook created within BioPharma Finder software was imported into Chromeleon CDS version 7.3.2, and the data were analyzed using the ICIS MS peak detection algorithm. Table 4 summarizes parameter settings applied for relative quantitation of most relevant quality attributes.

Table 3. BioPharma Finder software parameter settings for peptide mapping data analysis

Parameter	Value
Component detection	
Absolute MS signal threshold (counts)	2.0×10^4
Typical chromatographic peak width	0.3
Mass tolerance (ppm)	4.0
Maximum retention time shift (min)	0.5
Maximum mass (Da)	30,000
Identification	
Max peptide mass	7,000
Mass accuracy (ppm)	5
Min confidence	0.8
Max # modifications	1
Protease specificity	High
N-glycosylation	CHO
Modifications	
Static modifications	Carboxymethylation
Variable modifications	• N-term PyroGlu • C-term Lys • Deamidation (NQ) • Oxidation (MW) • Glycation (K) • Succinimide (ND)

Table 4. Chromeleon CDS parameter settings for target PQAs monitoring and quantitation

Parameter	Value
MS chromatogram settings	
Mass precision	5 decimal places
Mass tolerance (manually defined)	5 ppm
Smoothing	None
Composite scoring	
Pass score if at least	2 criteria passed
Fail score if less than	1 criterion passed
General MS criteria	
Isotopic dot product	≥0.9000
Mass accuracy	≤5.00 ppm
Peak apex alignment	≤0.50 min

Results and discussion

Beyond sample preparation and protein digestion, peptide separation is another critical aspect for a robust and reliable peptide mapping, with the goal of achieving complete protein sequence coverage and capturing as many product quality attributes (PQAs) as possible. There are multiple column types and formats commercially available to undertake this approach, and evaluation of chromatographic parameters is key to selecting the best column choice. The present study focuses on the comparison of the performance of the Hypersil GOLD Peptide column and four commercially available vendor columns with reversed-phase selectivity for peptide mapping applications. Monoclonal antibody-based samples were used to evaluate column's performance.

A commercial reference standard (NISTmAb) and a chimeric IgG1 molecule were digested with trypsin following the protocol described by Millán-Martín et al.[7] Trypsin digested samples were analyzed on a Vanquish Horizon UHPLC system coupled to a high resolution Orbitrap Exploris 240 MS detector using chromatographic conditions summarized in Tables 1 and 2. Figure 2 shows the comparison of overlaid total ion chromatograms (TIC) from six trypsin digested chimeric IgG1 samples obtained on five different C18 columns, where some monitored peptides are labeled with numbers. Selected peptides correspond to most hydrophilic and hydrophobic peptides, and peptides eluting along the chromatogram showing high and low intensity signals.

Assessment of sequence coverage

Initially, sequence coverage was evaluated using BioPharma Finder software through a peptide mapping analysis experiment based on MS/MS data. Results showed full or almost full protein sequence coverage for all the columns tested. As an example, the sequence coverage map is shown for rituximab heavy chain for each of the tested columns (Figure 3). All the columns tested provided similar results in terms of sequence coverage with some differences in terms of low intensity peptide EPQVYTLPPSR which could not be detected for vendor columns 2, 3, and 4, thus resulting in a sequence coverage of 97.6% for the heavy chain, while 98.6% was attained for the light chain for all the columns, where missing peptide EAK could only be detected by full MS in all cases.

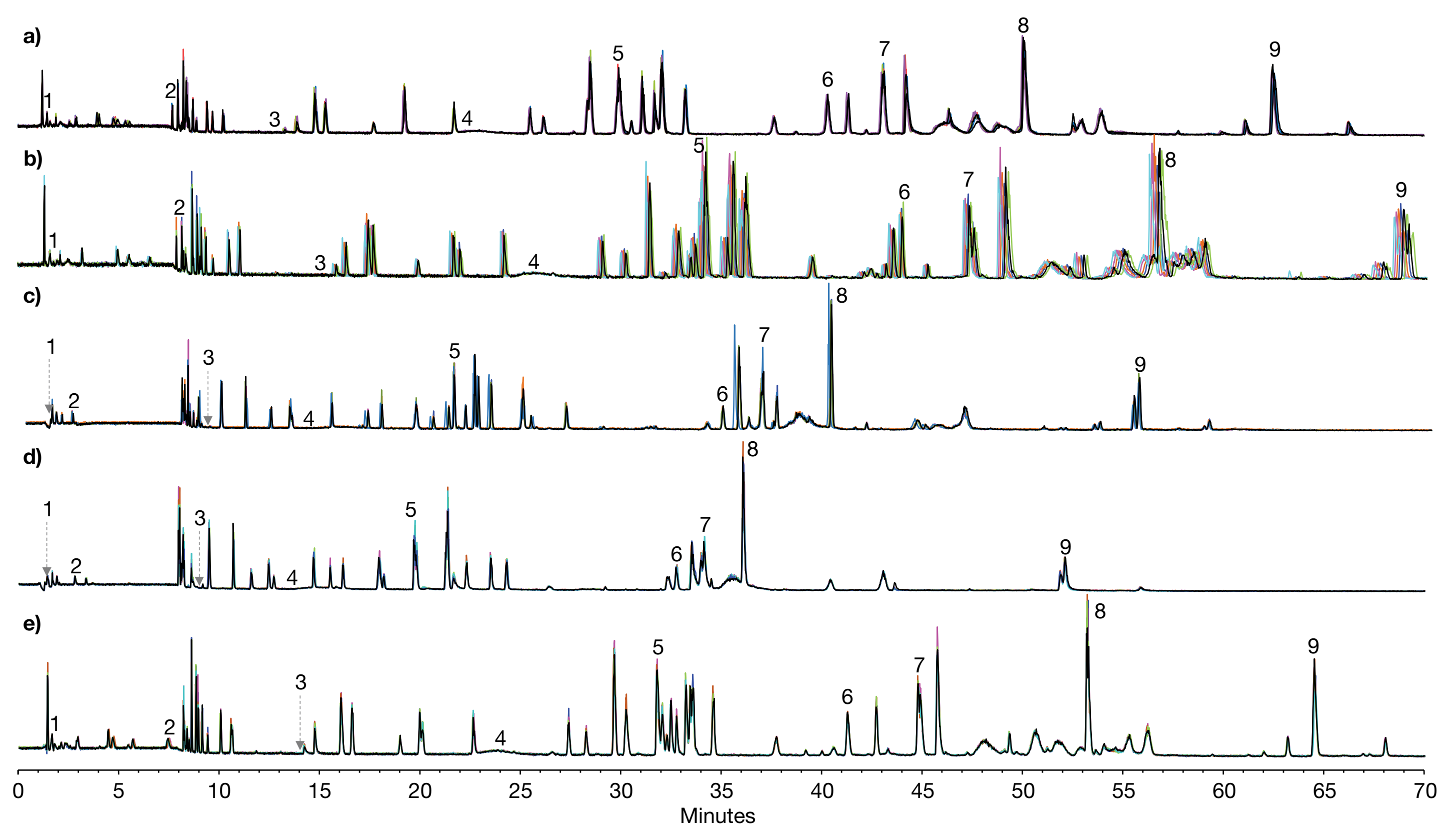

Figure 2. Total ion chromatograms (TIC) obtained from chimeric IgG1 (rituximab) digest sample (n = 6), separated via a 70 min gradient on five different C18 columns: (a) column vendor 1; (b) column vendor 2; (c) column vendor 3; (d) column vendor 4; (e) Hypersil GOLD Peptide column, lot #2. Monitored peptides are numbered from 1 to 9 on all the tested columns: 1. VSNK, 2. ADYEK, 3. SLSLSPG[Lys], 4. QVQLQQPGAELVKPGASVK, 5. FNWYVDGVEVHNAK, 6. GFYPSDIAVEWESNGQPENNYK, 7. GLEWIGAIYPGNGDTSYNQK, 8. VVSVLTVLHQDWLNGK, 9. DYFPEPVTVSWNSGALTS_QTYICNVNHKPSNTK.

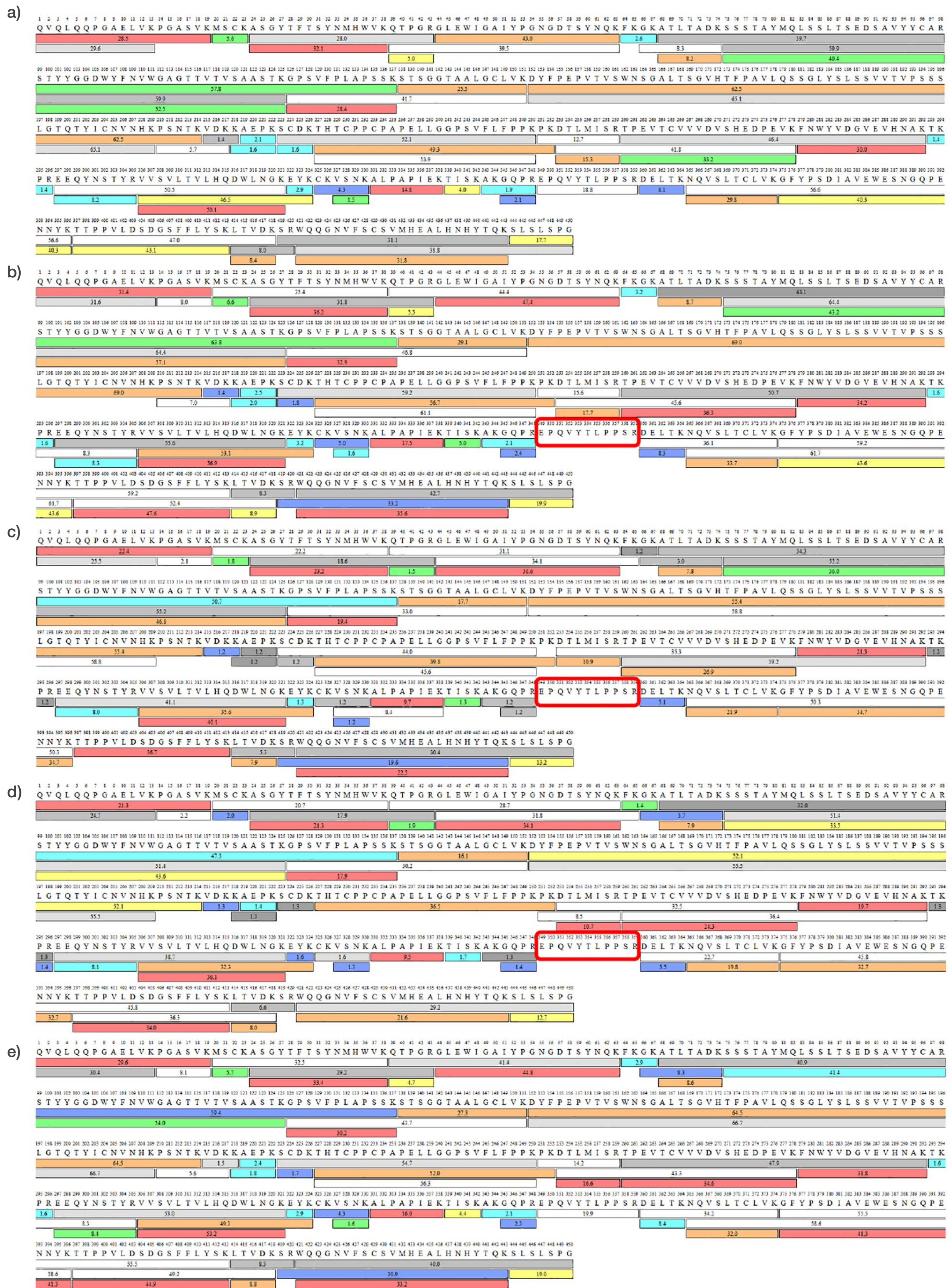

Figure 3. Sequence coverage map of rituximab heavy chain obtained using in-solution tryptic digestion on five different C18 columns where the missing peptide is highlighted with a red box: (a) column vendor 1; (b) column vendor 2; (c) column vendor 3; (d) column vendor 4; (e) Hypersil GOLD Peptide column, lot #2. The colored bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the peptide recovery in the MS1 scan: red >50%, orange >20%, and yellow >10% represent good recovery. Green, >5%, light blue >2%, and cyan >1% represent fair recovery, and grey-white scale shows poorly recovered peptides.

Assessment of peak area, RT reproducibility, peak asymmetry, and mass accuracy

In-depth data comparison across the five selected columns was performed on the basis of several parameters (Table 5) including reproducibility of the peak area (peak area %CV ≤ 10), reproducibility of the retention time (RT %CV ≤ 2), peak asymmetry, and mass accuracy (≤ 5 ppm).

Table 5 (part 1). Evaluation of chromatographic performance for the rituximab peptide mapping analysis. Values outside the established acceptance criteria are highlighted in red.

Rituximab peptides (n=6)	Peak area (Avg.)	%CV (Area)	Avg. RT (min)	%CV (RT)	Max RT shift (min)	Asymmetry
Column vendor 1 (150 × 2.1 mm, 2.7 µm, 120 Å)						
1. VSNK	4.50E+08	10.49%	1.44	0.55%	0.02	1.04
2. ADYEK	8.14E+08	6.95%	7.67	0.15%	0.03	1.25
3. SLSLSPG[Lys]	3.12E+07	2.73%	12.92	0.20%	0.05	0.93
4. QVQLQQPGAELVKPGASVK	1.12E+07	5.72%	22.28	0.14%	0.08	1.08
5. FNWYVDGVEVHNAK	9.10E+09	3.72%	29.93	0.11%	0.08	1.65
6. GFYPSDIAVEWESNGQPENNYK	3.68E+09	3.28%	40.30	0.04%	0.03	1.15
7. GLEWIGAIYPGNGDTSYNQK	7.66E+09	3.42%	43.01	0.07%	0.07	1.50
8. VVSVLTVLHQDWLNGK	1.94E+10	5.05%	50.04	0.07%	0.09	1.98
9. DYFPEPVTV_NVNHKPSNTK	2.78E+09	9.04%	62.47	0.12%	0.19	1.35
Column vendor 2 (150 × 2.1 mm, 1.7 µm, 130 Å)						
1. VSNK	4.33E+08	8.45%	1.61	0.54%	0.02	1.11
2. ADYEK	7.92E+08	11.29%	7.88	0.05%	0.01	1.78
3. SLSLSPG[Lys]	3.05E+07	3.52%	15.28	0.47%	0.20	0.95
4. QVQLQQPGAELVKPGASVK	1.18E+07	6.31%	25.49	0.41%	0.28	1.01
5. FNWYVDGVEVHNAK	9.61E+09	4.11%	34.02	0.45%	0.41	2.44
6. GFYPSDIAVEWESNGQPENNYK	3.78E+09	3.90%	43.43	0.24%	0.29	1.33
7. GLEWIGAIYPGNGDTSYNQK	7.19E+09	5.10%	47.17	0.26%	0.33	1.79
8. VVSVLTVLHQDWLNGK	1.99E+10	4.56%	56.52	0.42%	0.61	2.92
9. DYFPEPVTV_NVNHKPSNTK	2.67E+09	7.94%	68.68	0.36%	0.64	1.64
Column vendor 3 (150 × 2.1 mm, 1.7 µm, 130 Å)						
1. VSNK	6.03E+07	5.62%	1.22	0.44%	0.02	1.21
2. ADYEK	6.77E+08	14.09%	2.30	1.09%	0.06	1.36
3. SLSLSPG[Lys]	2.53E+07	5.09%	8.95	0.19%	0.04	0.97
4. QVQLQQPGAELVKPGASVK	6.68E+06	5.22%	14.38	0.37%	0.15	1.14
5. FNWYVDGVEVHNAK	6.87E+09	6.17%	21.31	0.08%	0.05	1.19
6. GFYPSDIAVEWESNGQPENNYK	2.53E+09	1.00%	34.70	0.05%	0.05	1.04
7. GLEWIGAIYPGNGDTSYNQK	5.07E+09	3.96%	36.60	0.07%	0.07	1.20
8. VVSVLTVLHQDWLNGK	1.50E+10	6.35%	40.06	0.12%	0.13	1.26
9. DYFPEPVTV_NVNHKPSNTK	2.51E+09	8.96%	55.41	0.04%	0.06	1.12
Column vendor 4 (150 × 2.1 mm, 1.6 µm, 100 Å)						
1. VSNK	6.11E+07	9.05%	1.29	0.55%	0.02	1.13
2. ADYEK	7.84E+08	3.40%	2.78	0.30%	0.02	1.67
3. SLSLSPG[Lys]	3.65E+07	3.97%	8.85	0.10%	0.02	1.10
4. QVQLQQPGAELVKPGASVK	8.28E+06	5.77%	13.52	0.13%	0.05	1.15
5. FNWYVDGVEVHNAK	7.67E+09	6.90%	19.69	0.17%	0.07	1.91
6. GFYPSDIAVEWESNGQPENNYK	2.63E+09	4.14%	32.74	0.06%	0.05	1.13
7. GLEWIGAIYPGNGDTSYNQK	5.13E+09	4.21%	34.11	0.04%	0.04	1.34
8. VVSVLTVLHQDWLNGK	1.37E+10	3.52%	36.05	0.06%	0.05	1.98
9. DYFPEPVTV_NVNHKPSNTK	2.12E+09	7.90%	52.07	0.05%	0.06	2.01

Table 5 (part 2). Evaluation of chromatographic performance for the rituximab peptide mapping analysis. Values outside the established acceptance criteria are highlighted in red.

Rituximab peptides (n=6)	Peak area (Avg.)	%CV (Area)	Avg. RT (min)	%CV (RT)	Max RT shift (min)	Asymmetry
Hypersil GOLD Peptide (150 × 2.1 mm, 1.9 µm, 175 Å)						
1. VSNK	5.21E+08	4.71%	1.64	0.53%	0.02	1.01
2. ADYEK	1.06E+09	2.12%	7.43	0.60%	0.12	1.24
3. SLSLSPG[Lys]	3.96E+07	1.45%	14.35	0.07%	0.03	0.92
4. QVQLQQPGAELVKPGASVK	1.33E+07	4.48%	23.65	0.03%	0.01	1.05
5. FNWYVDGVEVHNAK	1.08E+10	5.29%	31.76	0.06%	0.04	1.76
6. GFYPSDIAVEWESNGQPENNYK	3.56E+09	3.52%	41.24	0.03%	0.03	1.22
7. GLEWIGAIYPGNGDTSYNQK	6.87E+09	5.26%	44.76	0.02%	0.02	1.53
8. VVSVLTVLHQDWLNGK	1.93E+10	3.37%	53.19	0.04%	0.05	1.54
9. DYFPEPVTV_NVNHKPSNTK	2.99E+09	9.30%	64.48	0.02%	0.05	1.24

Results showed excellent peak area (%CV ≤ 10) and retention time reproducibility (%CV ≤ 2) for all columns, with some peptides showing higher values when using column vendor 3 (i.e. ADYEK, peak area %CV of 14.09) but in all cases still passing the evaluation criteria set for a %CV < 15. Retention time shifts (≤0.3 min acceptance criteria) were noticeably high for column vendor 2, with values over 0.3 min for peptides 7, 8, and 9, while observed values for the other column tested were <0.2 in all cases for all the measured peptides. Peak asymmetry is a measure of peak fronting or tailing, and it also helps to evaluate column quality. For ideal peaks, the asymmetry (A) should be equal to 1. According to the United States Pharmacopeia (USP)[8] and revised chapter of the European Pharmacopoeia (Ph. Eur.)[9] the default A range is extended from 0.8 to 1.8. For the evaluated peptides, the Hypersil GOLD Peptide column showed the best asymmetry values—close to 1 for the majority of the peaks and, in all cases, within the acceptance range. Column vendor 2 showed the highest asymmetry values and column vendor 1 also showed high values (A > 1.8) for some peptides (i.e., peptide 5 and peptide 8, respectively). It is also important to highlight that column vendor 1 showed big peak asymmetry (peak tailing) when evaluating BSA digest (data not shown), which prevented measurement of some deamidated species (i.e, peptide HLVDEPQNLIK), which are monitored during the system performance evaluation test as previously described.[10] Mass accuracies were monitored for all the measured peptide signals and all detected charge states, and observed values were within ±5 ppm in all cases.

Assessment of chromatographic resolution

Chromatographic resolution was evaluated next as this parameter helps to assess the separation capability of the column according to the Ph. Eur formula.[9] A specific focus was placed on the ability to separate the various deamidated forms of the PENNYK peptide as it is considered a well-known deamidation hot spot of the mAb Fc region. Deamidation can diminish the activity and stability of an antibody, thus, identification of asparagine (N) sites that are prone to deamidation is critical for the development of therapeutic mAbs.[11] Deamidation of the PENNYK peptide was investigated for NISTmAb reference standard, as it showed higher abundances compared to rituximab sample. Figure 4 shows the extracted ion chromatogram for this peptide obtained for the five tested columns. The main peak labeled as peak 1 corresponds to the unmodified form of the peptide, while peak 2 and peak 3 represent two deamidated species eluting before and after the main peak. As an example, corresponding mass spectra is indicated for Figure 4e, where observed signals are those for charge state +3 (z = 3). Results showed that vendor 3 (Figure 4c) and vendor 4 (Figure 4d) columns could not provide the chromatographic resolution needed for assessing deamidation on the PENNYK peptide, while vendor 1 (Figure 4a), vendor 2 (Figure 4b), and Hypersil GOLD Peptide (Figure 4e) columns provided good resolution, with values between 1.4 and 1.9 for peak 2 and between 2.0 and 4.3 for peak 3.

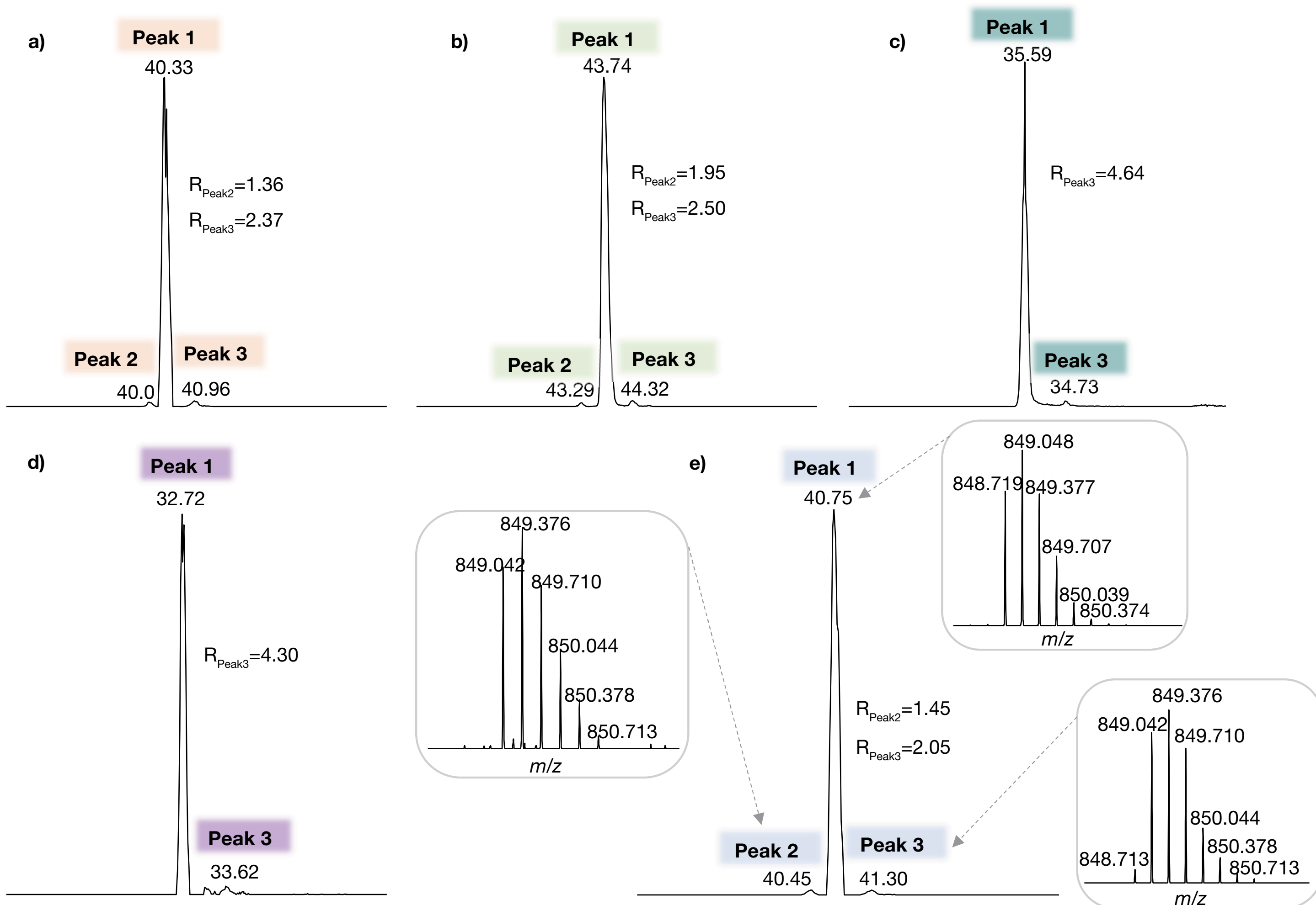

Figure 4. Extracted ion chromatograms for the Fc peptide GFYPSDIAVEWESNGQPENNYK obtained for all the tested columns from the sum of all detected charge states and 5 ppm of mass tolerance: (a) column vendor 1; (b) column vendor 2; (c) column vendor 3; (d) column vendor 4; (e) Hypersil GOLD Peptide column, lot #2. Retention times for all identified peaks are indicated. Peak 1: GFYPSDIAVEWESNGQPENNYK; Peak 2: GFYPSDIAVEWESN[Deam]GQPENNYK; and Peak 3: GFYPSDIAVEWESNGQPEN[Deam]NYK.

Assessment of hydrophilic peptide retention

A recent study indicates that the deamidation on VSNK peptide has a functional impact on antibody-dependent cell-mediated cytotoxicity (ADCC) and Fc receptor binding for IgG1 mAbs.[12] In peptide mapping, the main limitation is that deamidation of VSNK is often missed because this hydrophilic peptide is eluted near the void volume of most reversed-phase (RP) columns after Lys-C or trypsin digestion.[13] The next step focused on the evaluation of the most hydrophilic peptides detected for rituximab tryptic digest. Table 6 shows the average retention times obtained for each of the columns tested. Vendor 3 and vendor 4 columns show absent resolution for measured hydrophilic peptides, where most of them eluted at 1.2 min and 1.3 min, respectively. Additionally, important information is missing when using vendor 3 column as the deamidated peptide VSNK cannot be detected. Vendor 2 and the Hypersil GOLD Peptide columns showed better chromatographic resolution for early eluting peptides, and in general both columns provided a better distribution of the peptides along the full chromatographic gradient as previously shown in Figure 2 and Table 5.

Assessment of carry-over

Evaluation of carry-over was next performed by calculating relative abundances of hydrophobic peptides signals for two consecutive blank injections analyzed after six injections of rituximab tryptic digests. Table 7 summarizes the observed retention times and calculated relative abundances for the selected peptides, highlighting the low carry-over effect observed for all the columns tested. The heavy chain peptide D152-K214 shows the highest carry-over effect for the Hypersil GOLD Peptide column, but in all cases is below 7% in terms of relative abundance compared to the signals observed for rituximab drug product.[14] It is worth mentioning that peptide D152-K214 showed good asymmetry values for all the tested columns, in all cases between 1.0 and 1.4. It is not the most hydrophobic detected peptide, which suggests that possibly other interaction mechanisms may be happening.

Assessment of PTM quantitation

A key aspect to demonstrate a robust and reliable peptide mapping analysis relies on the quantitation of the most relevant PTMs. For this reason, PTM levels were evaluated for rituximab samples for each of the columns tested using a targeted approach through Chromeleon CDS, including deamidation, succinimide formation, oxidation, and N- terminus pyroglutamate (PyroGlu) formation and C-terminal lysine (Lys) content. Excellent reproducibility was observed in most cases for intermediate precision on each of the competitor columns tested (Table 8).

For repeatability analysis, mean % relative abundance and %CV of six replicate injections of rituximab samples were calculated for each of the competitor columns tested. For intermediate precision analysis, mean % relative abundance and %CV of six pooled injections from each Hypersil GOLD Peptide column lot were calculated. We observed less than 10% CV for repeatability analysis and intermediate precision. Only very low abundant modification, such as the PENNYK peptide deamidation, showed %CV values slightly over 10%. Observed results are in accordance with previous studies[10] and published acceptance criteria during biotherapeutics analytical development[15].

Table 6. Evaluation of retention time behavior for most hydrophilic peptides detected for rituximab tryptic digest (n = 6)

Peptide	Vendor 1	Vendor 2	Vendor 3	Vendor 4	Hypersil GOLD Peptide Lot #1	Hypersil GOLD Peptide Lot #2	Hypersil GOLD Peptide Lot #3	Hypersil GOLD Peptide lot-to-lot
	Avg. RT (min)							Stdev
TKPR	1.41	1.57	1.19	1.29	1.50	1.58	1.55	0.04
VSNK	1.44	1.61	1.22	1.29	1.56	1.64	1.61	0.04
SCDK	1.59	1.78	1.23	1.29	1.67	1.75	1.72	0.04
KAEPK	1.61	1.95	1.22	1.29	1.69	1.78	1.76	0.05
VSN[Deam]K	1.85	2.08	-	1.30	1.95	2.04	2.02	0.05
AKGQPR	1.88	2.10	1.22	1.29	2.01	2.10	2.08	0.05
GQPR	2.09	1.95	1.22	1.29	1.69	1.78	1.76	0.05
AEPK	2.10	2.49	1.22	1.44	2.26	2.37	2.36	0.06

Table 7. Evaluation of carry-over: relative abundances of hydrophobic peptide signals for blank injections after six injections of rituximab tryptic digests and retention time for the monitored peptide (highlighted in blue). The relative abundance has been calculated against the average peak area of the six rituximab injections.

	HC: V306-K321	LC: S126-R141	HC: S99-K137	HC: D152-K214	HC: D152-K209
Vendor 1	50.11 min	53.95 min	57.77 min	62.54 min	65.05 min
Blank_1	0.01	0.00	1.86	0.06	0.06
Blank_2	0.00	0.00	0.33	0.00	0.00
Vendor 2	56.85 min	59.11 min	63.74 min	69.01 min	---
Blank_1	0.01	0.00	2.68	0.08	---
Blank_2	0.00	0.00	0.10	0.01	---
Vendor 3	40.10 min	46.76 min	50.70 min	55.44 min	58.80 min
Blank_1	0.01	0.00	0.75	0.09	0.00
Blank_2	0.00	0.00	0.05	0.01	0.00
Vendor 4	36.06 min	43.03 min	47.32 min	52.10min	55.53 min
Blank_1	0.01	0.00	0.05	0.01	0.00
Blank_2	0.00	0.00	0.00	0.00	0.00
Hypersil GOLD Peptide Lot #1	52.46 min	55.51 min	58.76 min	63.70 min	66.04 min
Blank_1	0.02	0.01	4.19	0.30	0.00
Blank_2	0.01	0.00	1.09	0.09	0.00
Hypersil GOLD Peptide Lot #2	53.17 min	56.18 min	59.40 min	64.48 min	66.74 min
Blank_1	0.01	0.00	4.48	0.19	0.00
Blank_2	0.00	0.00	1.32	0.06	0.00
Hypersil GOLD Peptide Lot #3	52.79 min	55.78 min	58.96 min	63.88 min	66.17 min
Blank_1	0.01	0.00	6.86	0.21	0.00
Blank_2	0.00	0.00	2.32	0.05	0.00
Hypersil GOLD Peptide lot-to-lot	stdev	stdev	stdev	stdev	stdev
Blank_1	0.01	0.01	1.47	0.06	0.00
Blank_2	0.01	0.00	0.65	0.02	0.00

Table 8. Summary of PTMs evaluation for rituximab drug product. Values represent % relative abundances for the monitored quality attributes based on six replicates for each of the vendor columns and eighteen replicates for Hypersil GOLD Peptide column considering three column lots were evaluated. % CV values are shown in parenthesis for the replicate injections.

Peptide	Description	Vendor 1 (n = 6)	Vendor 2 (n = 6)	Vendor 3 (n =6)	Vendor 4 (n = 6)	Hypersil GOLD Peptide (n = 18)
GLEWIGAIYPG**N**GDTSYNQK	Deamidation	0.95 (7.2%)	0.34 (10.5%)	0.87 (4.3%)	0.90 (5.4%)	0.39 (6.8%)
GLEWIGAIYPG**N**GDTSYNQK	Succinimide	0.77 (4.4%)	0.94 (6.5%)	0.84 (4.1%)	0.90 (7.8%)	0.72 (4.4%)
VVSVLTVLHQDWL**N**GK	Succinimide	0.72 (1.7%)	0.63 (5.4%)	0.65 (8.4%)	0.69 (2.1%)	0.80 (5.3%)
GFYPSDIAVEWES**N**GQPE**N**NYK	Deamidation	0.03 (5.7%)	0.02 (6.4%)	0.04 (5.5%)	0.04 (5.5%)	0.03 (10.2%)
GFYPSDIAVEWES**N**GQPE**N**NYK	Succinimide	0.71 (5.7%)	0.68 (6.4%)	0.70 (5.5%)	0.75 (2.8%)	0.70 (6.5%)
WQQGNVFSCSV**M**HEALHNHYTQK	Oxidation	0.15 (17.8%)	0.17 (5.2%)	0.19 (10.3%)	---	0.19 (3.0%)
DTL**M**ISR	Oxidation	2.05 (5.1%)	2.06 (6.1%)	2.31 (19.1%)	4.40 (10.3%)	2.49 (5.3%)
QVQLQQPGAELVKPGASVK	PyroGlu	99.86 (0.0%)	99.85 (0.0%)	99.88 (0.0%)	99.87 (0.0%)	99.88 (0.0%)
QIVLSQSPAILSASPGEK	PyroGlu	98.06 (0.0%)	97.15 (0.3%)	96.42 (0.2%)	95.72 (0.3%)	97.97 (0.3%)
SLSLSP**G**	Lys	2.73 (6.1%)	2.72 (5.0%)	2.93 (6.2%)	3.47 (4.5%)	2.97 (2.6%)

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Conclusions

This study demonstrates the benefits of the Hypersil GOLD Peptide column for peptide level biopharma characterization based on analysis of a comprehensive dataset comprising rituximab and NISTmAb digest samples.

- The Hypersil GOLD Peptide column produces peaks with good asymmetry values, provides strong retention time reproducibility, retained, and separated hydrophilic peptides, and has very good chromatographic resolution values for deamidated species in the PENNYK region.
- The Hypersil GOLD Peptide column facilitates the detection of a wide range of CQAs with a strong level of reproducibility recorded across three different column lots. This low lot-to-lot variability provides confidence in analysis in particular where necessitated in a QC environment.
- The Hypersil GOLD Peptide column performs comparably if not better than several cross vendor columns with a high level of retention time precision, sequence coverage and good peak asymmetry values.

The benefits of the Hypersil GOLD Peptide column described here enable this column to uniquely meet the demands of peptide level analysis where consistent lot-to-lot performance is required.

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