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APPLICATION NOTE 21879

Growth media effects on post-translational modifications investigated through peptide mapping LC-MS/MS analysis of anti-IL8 monoclonal antibody

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Keywords

NIBRT, biopharmaceuticals, biotherapeutics, comparability studies, CQAs, monoclonal antibodies (mAbs), IgG1, CHO DP12, anti-IL8, post-translational modifications (PTMs), peptide mapping, bottom-up, high throughput, Magnetic SMART Digest kit, KingFisher Duo Prime Purification System, Vanquish Flex Binary UHPLC System, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, Acclaim VANQUISH C18 column

Application benefits

- Speed and simplicity of data preparation leading to highly reproducible characterization results for therapeutic monoclonal antibodies (mAbs)
- Simple and rapid protein digestion for peptide mapping analysis of monoclonal antibodies (CHO-DP12) cultured in different types of media
- Automated magnetic bead technology allows excellent recovery of samples with high reproducibility and ease of use with less hands-on time
- High confidence in results with excellent quality data, excellent peptide coverage, and identification of post-translational modifications (PTMs) across all the samples

Goal

As the biopharmaceutical industry continues to expand, the demand for ancillary services such as cell culture media production and optimization is also increasing. Media used for cell cultivation and biopharmaceutical expression can affect cellular behavior with an associated potential influence in yield and quality of the expressed therapeutic protein. Media screening and optimization plays a key role in process development. The purpose of this study is to characterize proteins produced by Chinese hamster ovary (CHO) cell lines grown in different types of cell culture media to evaluate potential differences in their quality profile.

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Introduction

Optimization of cell culture media is an essential part of upstream process development during monoclonal antibody (mAb) production.[1] For large-scale biopharmaceutical production, fast growing, high producing, and robust cell lines are required.[2] Media development is essential to support these desired phenotypic characteristics of the production cell line by ensuring the provision of the required nutrients and elements. Chemically defined media (CDM) are currently used in several biotechnology processes for mAb manufacture. By definition, a CDM requires that all of the components are identified with exact concentrations known and that no materials of human or animal origin are used in medium formulation. Supplementation with recombinant growth factors and supplements such as plant hydrolysates may be required depending on the specific requirements of a particular cell line. Variability present in either the basal CDM or the supplements used to support the cell culture can affect the stability of a process, potentially affecting the yield or the quality of the produced biopharmaceutical. Therefore, a key challenge in media development and optimization is not only the evaluation of cell growth and titer, but also the evaluation of the resultant quality profile of the expressed biopharmaceutical and in particular, the PTMs present.

This application note presents the benefits of using the recently developed Thermo Scientific™ Magnetic SMART Digest™ kit to perform a peptide mapping based comparability study of an anti-IL8 IgG1 grown using different CDMs. We investigated the level of PTM changes across all the samples analyzed. The outlined method combines automated enzymatic digestion using the Magnetic SMART Digest kit on a Thermo Scientific™ KingFisher Duo Prime purification system, in combination with the high-resolution, accurate-mass (HRAM) capabilities of the Thermo Scientific™ Q Exactive™ Plus mass spectrometer. High-resolution chromatographic separation was achieved using the Thermo Scientific™ Acclaim™ VANQUISH™ C18 column on the Thermo Scientific™ Vanquish™ Flex UHPLC system. Thermo Scientific™ BioPharma Finder™ software version 3.0 was used for data processing and analysis of the experimental data.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ•cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical) (P/N 10505904)
- Acetonitrile, Optima™ LC/MS grade (Fisher Chemical) (P/N 10001334)
- Water with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 1011846)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Iodoacetamide, ≥99% (NMR), crystalline (IAA) (Sigma) (P/N I6125)
- KingFisher Deepwell, 96 well plate (P/N 95040450)
- KingFisher Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Equipment

- KingFisher Duo Prime purification system (P/N 5400110)
- Vanquish Flex Binary UHPLC system including:
  - Binary Pump F (P/N VF-P10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A)
  - System Base Vanquish Horizon (P/N VH-S01-A)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Sample preparation for monoclonal antibody purification

CHO DP12 cells, clone#1934 ATCC™ CRL12445™, adapted to grow in suspension culture were used in this study. Three chemically defined media were evaluated for cultivation of the DP12 cells, referred to as Medium 1, 2, and 3. Each medium was supplemented with 4 mM L-glutamine and 200 nM methotrexate (MTX, Sigma-Aldrich). Cells were cultivated in 30 mL of media in 125 mL polycarbonate Erlenmeyer flasks with a starting density of 0.3×10^6 cells/mL. Incubator parameters were: 37 °C, 5% CO_2, and 80% humidity on a shaking platform of 125 rpm. Cultures were then transferred to 100 mL of media in 500 mL shaker flasks for production of larger quantities of mAb. The level of glucose was monitored during the cultivation and maintained at the level of 2 g/L by adding a glucose solution (Gibco). Cells were harvested after 14 days of culture by centrifugation, and the supernatants were clarified by sequential filtration. The samples of clarified media were passed through a HiTrap™ Protein A column, then washed with phosphate buffered saline (PBS) before elution of mAb from the Protein A column using 100 mM citric acid, pH 3.2. The mAb solutions were buffer exchanged in PBS, and the protein concentration was evaluated with a Nanodrop 2000 Spectrophotometer.

Protocol for sample preparation using a SMART Digest trypsin kit, magnetic bulk resin option (Magnetic SMART Digest)

Samples were prepared in triplicate and were diluted to 2 mg/mL in water. For each sample digest, sample and buffers were added to each lane of a KingFisher Deepwell 96 well plate as outlined in Table 1. Bead "wash buffer" was prepared by diluting SMART Digest buffer 1:4 (v/v) in water. Bead buffer was neat SMART Digest buffer. Digestion was performed using the Kingfisher Duo Prime purification system with Thermo Scientific™ BindIt™ software (version 4.0), using the protocol outlined in Table 2. Samples were incubated for 45 minutes at 70 °C on medium mixing speed (to prevent sedimentation of beads), with post-digestion cooling carried out to 10 °C. Following digestion, disulfide bonds were reduced with 10 mM DTT for 20 minutes at room temperature and subsequently alkylated with 3 mM IAA in darkness for 15 minutes at room temperature (for DTT and IAA final concentrations are listed). All samples were diluted to 0.1% formic acid (FA) in water and 3 µg were loaded on column for all runs.

Table 1. KingFisher Duo Prime plate layout utilized for sample preparation. Reagents and associated volumes placed in each well are outlined.

Lane	Content	Volume Applied to Each Well (µL)
A	SMART Digest buffer	150
	Sample (2 mg/mL)	50
B	Tip Comb	
C	Empty	
D	Magnetic SMART Beads	15
	Bead Buffer (SMART Digest buffer)	100
E	Bead Wash Buffer (SMART Digest buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

Table 2. Protocol for automated peptide mapping using a KingFisher Duo Prime system

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	45 min Medium Mix	3 count, 15 s	During digestion: 70 °C Post-digestion: 10 °C	A
Release Beads	Yes, Fast	–	–	–	F

LC conditions

Column:	Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.2 µm, 2.1 × 250 mm
Mobile Phase A:	0.1% formic acid aqueous solution
Mobile Phase B:	0.1% formic acid solution in acetonitrile
Flow Rate:	0.3 mL/min
Column Temperature:	25 °C (Still air mode)
Autosampler Temp:	5 °C
Injection Volume:	12 µL
Injection Wash Solvent:	MeOH:H_2O, 10:90 (v/v)
Needle Wash:	Enabled pre-injection
Gradient:	Table 3 for details

Table 3. Mobile phase gradient for UHPLC separation of peptides

Time (min)	Flow (mL/min)	% Mobile Phase B	Curve
0.00	0.300	2.0	5
45.0	0.300	40.0	5
46.0	0.300	80.0	5
50.0	0.300	80.0	5
50.5	0.300	2.0	5
65.0	0.300	2.0	5

MS conditions

Detailed MS method parameters are shown in Tables 4 and 5.

Table 4. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Thermo Scientific™ Ion Max source with HESI-II probe
Sheath Gas Pressure	40 psi
Auxiliary Gas Flow	10 arbitrary units
Probe Heater Temperature	400 °C
Source Voltage	3.8 kV
Capillary Temperature	320 °C
S-lens RF Voltage	50 V

Table 5. MS method parameters utilized for peptide mapping analysis

General	Setting	MS^2 Parameters	Setting
Run Time	0 to 65 min	Resolution Settings	17,500
Polarity	Positive	AGC Target Value	1.0×10^5
Full MS Parameters	**Setting**	Isolation Width	2.0 *m/z*
Full MS Mass Range	200–2,000 *m/z*	Signal Threshold	1.0×10^4
Resolution Settings	70,000	Normalized Collision Energy (HCD)	28
AGC Target Value	3.0×10^6	Top-N MS^2	5
Max Injection Time	100 ms	Max Injection Time	200 ms
Default Charge State	2	Fixed First Mass	–
SID	0 eV	Dynamic Exclusion	7.0 s
Microscans	1	Loop Count	5

Data processing and software

Thermo Scientific™ Xcalibur™ software version 4.0.27.13 (Cat. No. OPTON-30487) was used for data acquisition and analysis. For data processing, Thermo Scientific™ Biopharma Finder™ software version 3.0 was applied.

Detailed parameter settings are shown in Table 6. Rstudio software (Version 1.0.143) was used to produce Pearson correlation scatter plot, Box plot, and Venn diagrams from http://www.rstudio.org [3] (Figure 3).

Table 6. Biopharma Finder 3.0 software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS Signal Threshold	1 × 10^6 counts
Typical Chromatographic Peak Width	0.3 min
Relative MS Signal Threshold (% base peak)	1.00
Relative Analog Threshold (% of highest peak)	1.00
Width of Gaussian Filter (Represented as 1/n of chromatographic peak width)	3
Minimum Valley to be Considered as Two Chromatographic Peaks	80.0%
Minimum MS Peak Width	1.20 Da
Maximum MS Peak Width	4.20 Da
Mass Tolerance (ppm for high-res or Da for low-res)	4.00
Maximum Retention Time Shift	1.69 min
Maximum Mass	30,000 Da
Mass Centroiding Cutoff (% from base)	15
Identification	**Setting**
Maximum Peptide Mass	7,000
Mass Accuracy	5 ppm
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	1
Unspecified Modification	-58 to +162 Da
N-Glycosylation	CHO
Protease Specificity	High
Static Modifications	**Setting**
Side Chain	Carbamidomethylation
Variable Modifications	**Setting**
N Terminal	Gln→Pyro Glu
C Terminal	Loss of lysine
Side Chain	Deamidation (N) Deamidation (Q) Glycation (K) Oxidation (MW) Isomerization (D)

Results and discussion

Peptide mapping analysis was carried out by using the Magnetic SMART Digest kit in combination with the KingFisher Duo Prime system, which simplifies the sample preparation and reduces preparation time and user errors, providing significant improvements in reproducibility and robustness between samples analyzed.

The chromatograms (Figure 1A) show three biological replicates overlaid of anti-IL8 IgG1 monoclonal antibody produced in the different cell culture media and digested with the Magnetic SMART Digest trypsin kit. Excellent reproducibility between each biological replicate was observed between all the different media analyzed. In-depth analysis of single ion chromatogram (SIC) and MS/MS spectra for selected peptide “SSTLTLSK” from 181aa to 189aa of anti-IL8 IgG1 light chain shows the same elution time at 22.09 min present across all samples analyzed with the same MS/MS fragmentation (Figure 1B).

A visual comparison of the peptide maps across IgG1 samples derived from different media shows a good similarity between Media 1 and Media 2, while differences in the fingerprint were observed in Media 3, potentially due to a different array of PTMs.

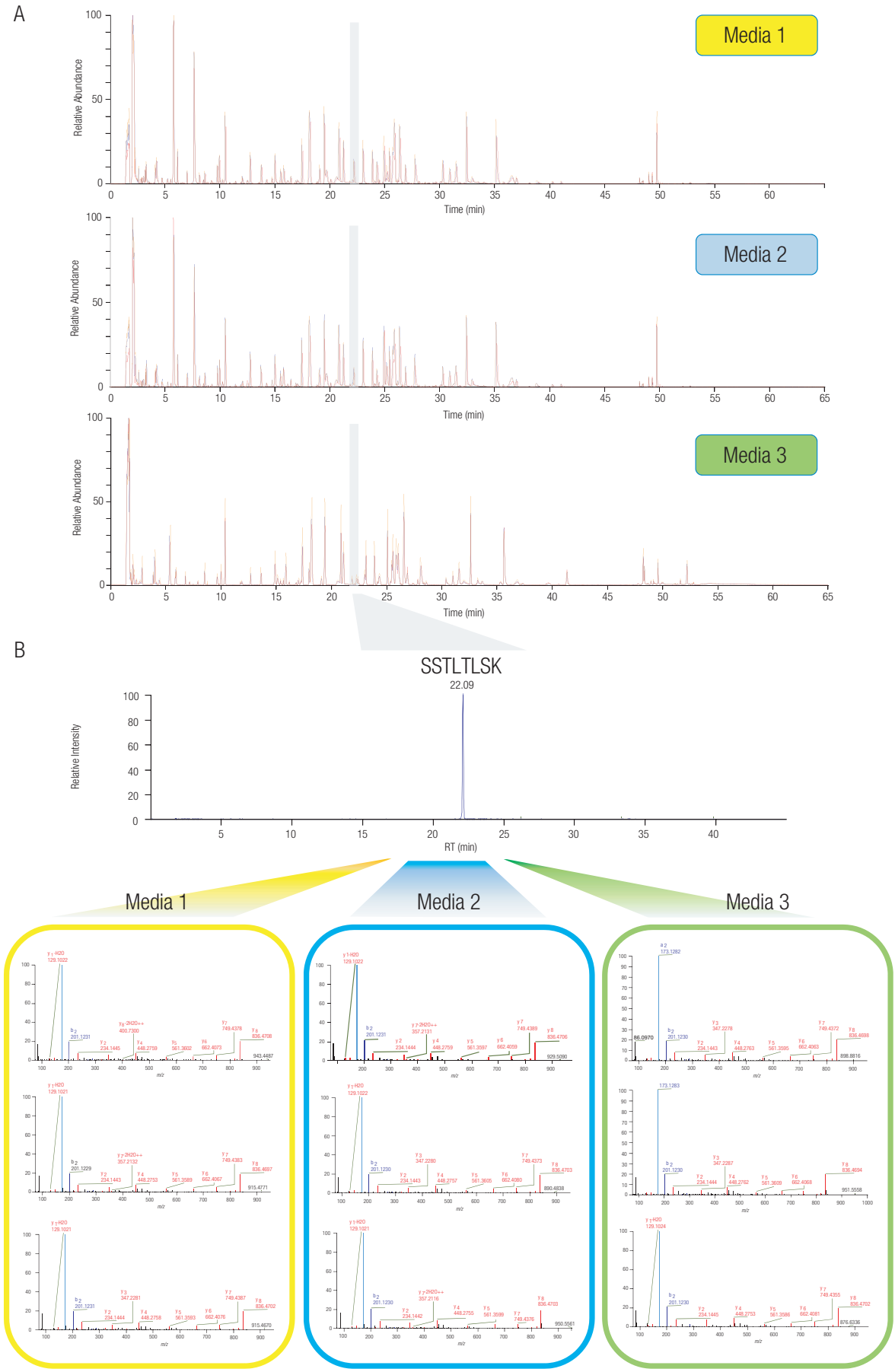

Figure 1. (A) Base peak chromatograms (BPCs) obtained from peptide mapping experiments of anti-IL8 IgG1 from CHO DP12 cell line growing in three different culture conditions, after Magnetic SMART digestion with the KingFisher Duo Prime system. (B) Representative SIC of peptide "SSTLTLSK" and MS/MS spectra obtained for each replicate.

To investigate any difference across the samples analyzed, we examined each LC-MS/MS analysis evaluating the count of detected MS peaks, their relative abundance (RA), peak MS area, sequence coverage (Table 7), and the array of PTMs (Table 8). One hundred percent of sequence coverage was achieved for both the light and heavy chain of the anti-IL8 monoclonal antibody in all the samples analyzed. Further, the total number of detected MS peaks in the samples only varied between 202 and 224 for light chain and 451 and 523 for heavy chain. We observed an increased number of MS peaks detected in Medium 3 for both heavy and light chains.

We investigated in depth the global level of peptides across all the samples by selecting the most abundant peptides quantified at both MS1 and MS2 levels. The results were analyzed using differential cluster analysis combined with scatter plots and Person correlation to measure the linear correlation between samples across all the media (Figure 2A).[3] Statistically, the strongest linear relationship is indicated by a correlation coefficient of -1 or 1. Overall, we observed a higher and significant correlation between all the biological replicates associated to Medium 1 and 2, between 0.99 and 1, while Medium 3 showed slightly less correlation from 0.79 to 0.84. To address the reduction of linear coefficient in Medium 3 we plotted $\log_2$ scale of whole peptides signal abundance (both MS1 and MS2 level) as a function of the amount of sample digested. Boxplot distribution showed a similar amount of sample has been processed and each media reported an overall similar distribution (Figure 2B). We noticed the median value in Medium 3 is slightly increased compared with Medium 1 and 2. This is confirmed by the presence of 39 unique peptides in Medium 3 as obtained by Venn diagram (Figure 2C). To characterize any other significant differences between the samples we investigated the PTMs profiles.

Table 7. MS features identified across the different cell medium with the average total number of MS peaks found

Proteins	Sample	Replicates	Number of MS Peaks	Sequence Coverage
Light chain				
	Media 1	1	202	100%
		2	202	100%
		3	208	100%
	Media 2	1	208	100%
		2	216	100%
		3	215	100%
	Media 3	1	222	100%
		2	224	100%
		3	223	100%
Heavy chain				
	Media 1	1	456	100%
		2	473	100%
		3	485	100%
	Media 2	1	451	100%
		2	493	100%
		3	493	100%
	Media 3	1	522	100%
		2	513	100%
		3	523	100%

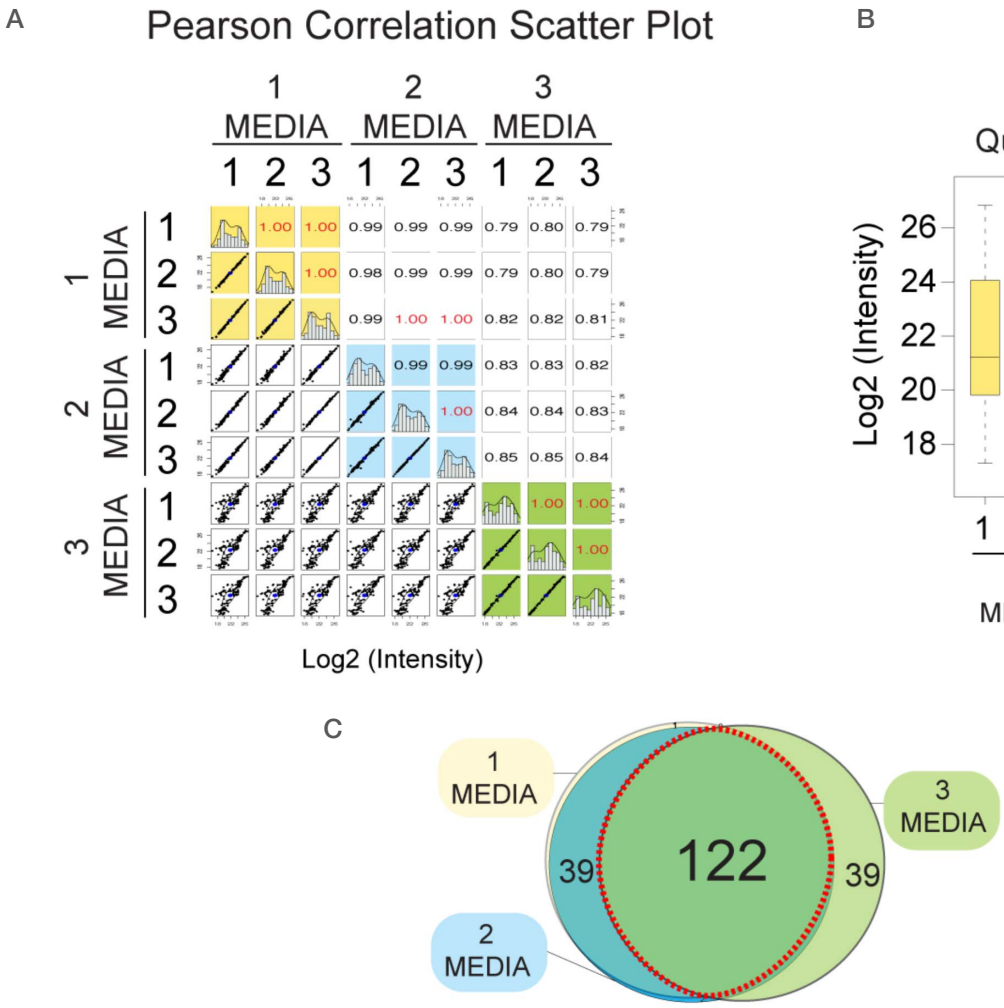

Figure 2. Multi-comparison quantitative analysis of unique peptides present in all CDM. (A) Scatter plot cluster combined with Pearson correlation coefficient; (B) Boxplot analysis; (C) Venn Diagram. Yellow, blue, and green correspond to Medium 1, 2, and 3.

Relative abundance levels of each PTM modification across all the samples were identified and exhibited low standard deviations across triplicate sample preparation (<2%), highlighting the remarkably robust, high reproducibility of this workflow (Figure 3 and Table 8). A tilde (~) before the modification indicates the modification was found on the tryptic peptide but could not be localized to a specific amino acid with MS/MS spectra. We noticed a reduction of deamidation levels on residues N84, N320, and N394 present in Medium 3. Similarly, a reduction of oxidation level for residue M433 was observed, while oxidation for M257 was higher with Medium 2. The most remarkable differences are found on the *N*-glycosylation levels. Indeed, for both mono- and bi-antennary *N*-glycan structures, non-galactosylated species show higher values in Medium 1 (13.14% and 67.70%, respectively), their abundancies decrease in Medium 2 (10.24% and 52.94%, respectively) but are still higher than in Medium 3 (8.07% and 46.57%, respectively). The opposite trend is shown for the mono- and bi-antennary *N*-glycan structures bearing one or two terminal galactoses. The difference in *N*-glycan profiles is probably caused by the different levels of nutrients present in the three media tested. These differences however can have an influence on the produced IgG1 effector function.[4] Overall, while the three media seem to have different consequences on *N*-glycan profiles, the total abundance of other detected modifications (deamidation and oxidation) establishes a good similarity between Media 1 and 2, while Medium 3 shows reduced levels of these PTMs (respectively ~4% for deamidation and ~2% for oxidation, Figure 3B).

Table 8. Summary of PTMs identified and quantified by LC-MS/MS analysis of the tryptic digest of anti-IL8 IgG1 grown in three different media. MS data were processing using BioPharma Finder 3.0 software.

	Media 1		Media 2		Media 3		Sequence
Modification	Relative Abundance (n=3)	RSD (n=3)	Relative Abundance (n=3)	RSD (n=3)	Relative Abundance (n=3)	RSD (n=3)	
Q316+Deamidation	2.00	0.15	2.18	0.03	1.75	0.02	TVLHQDWLNGK
N84+Deamidation	0.92	0.06	0.96	0.04	0.46	0.01	NTAYLQMNSLR
N291+Deamidation	0.51	0.04	0.55	0.02	0.38	0.01	FNWYVDGVEVHNAK
N320+Deamidation	2.84	0.11	2.89	0.08	1.46	0.19	VVSVLTVLHQDWLNGK
N366+Deamidation	1.47	0.10	1.67	0.08	1.13	0.06	NQVSLTCLVK
~N394+Deamidation	2.86	0.69	2.44	0.27	1.01	0.14	GFYPSDIAVEWESNGQPENNYK
M83+Oxidation	0.52	0.16	0.49	0.09	0.47	0.06	NTAYLQMNSLR
M257+Oxidation	3.72	0.12	5.01	0.05	3.80	0.15	DTLMISR
M433+Oxidation	3.94	0.20	3.49	0.26	2.10	0.07	WQQGNVFSCSVMHEALHNHYTQK
D285+Isomerization	1.58	0.10	1.77	0.02	1.63	0.02	FNWYVDGVEVHNAK
N302+A1G0F	13.14	0.32	10.24	1.33	8.07	0.20	EEQYNSTYR
N302+A1G1F	1.28	0.07	2.59	0.10	2.71	0.05	EEQYNSTYR
N302+A2G0F	67.70	0.12	52.94	0.90	46.57	0.21	EEQYNSTYR
N302+A2G1F	15.90	0.33	28.74	1.04	40.29	0.16	EEQYNSTYR
N302+A2G2F	1.98	0.07	5.62	0.19	6.88	0.21	EEQYNSTYR
D406+Isomerization	0.57	0.05	0.66	0.05	0.58	0.02	TTPPVLDSDGSFFLYSK

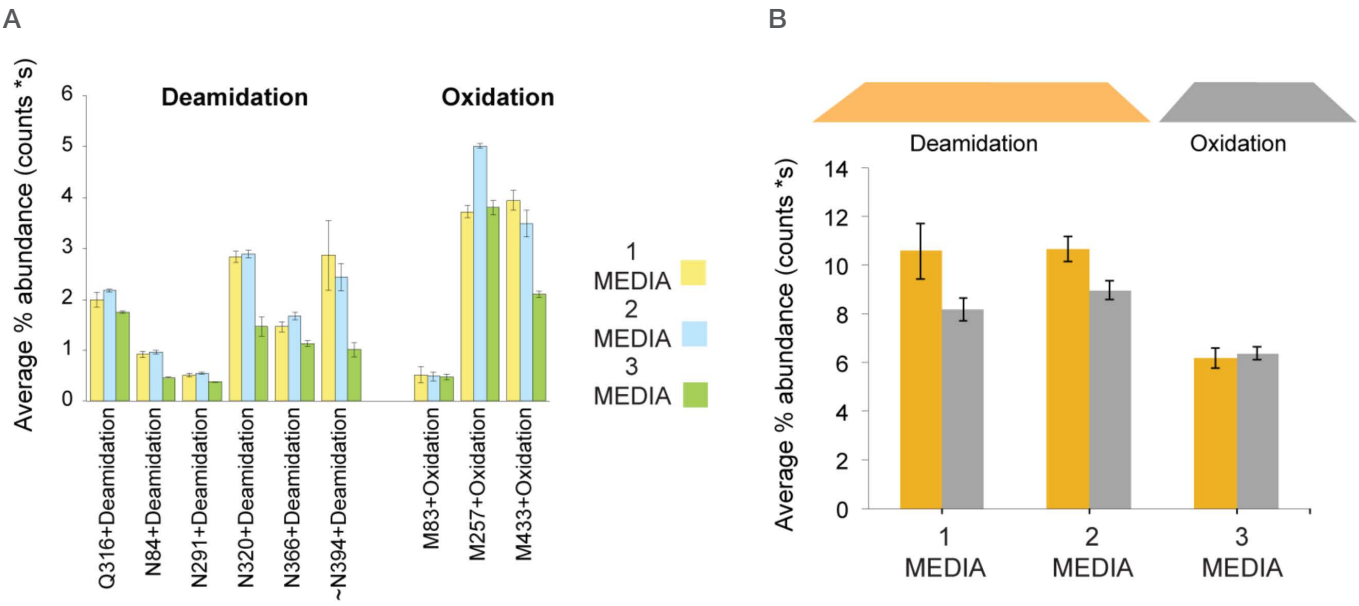

Figure 3. (A) Average relative abundance (n =3) of identified deamidation and oxidation for anti-IL8 IgG1; (B) Global relative abundance (n =3) of the selected modifications across different growth media used

Conclusions

- The Magnetic SMART Digest kit provides simple and rapid protein digestion for peptide mapping analysis and PTM investigations, achieved in less than 2 hours as shown using the anti-IL8 monoclonal antibody.

- Analysis of IgG1 produced in three different chemically defined media gave excellent quality data with high confidence in the results using this workflow. Excellent sequence coverage (~100%) and low levels of sample preparation-induced PTMs were observed with the Magnetic SMART Digest kit, enabling confident evaluation of produced mAbs in early stages of product development.

- The most striking differences in PTMs are found on the *N*-glycosylation levels, probably caused by the different levels of nutrients present in the three media.

- Deamidation and oxidation are significantly reduced in anti-IL8 IgG1 antibody purified in Medium 3.

- The KingFisher Duo Prime system offers an automated option for biotherapeutic digestion for up to 12 samples at a time. In combination with Magnetic SMART Digest kits, it simplifies the process, reduces sample handling, the time needed for sample preparation, and increases sample preparation reproducibility and confident transfer of methods between laboratories enabling high-throughput analysis with reduced method development time.

- The Q Exactive Plus system has been proven to deliver excellent mass accuracy and highly sensitive MS results for protein identification and detailed peptide mapping. BioPharma Finder 3.0 software can provide automatic data processing, peptide sequence matching, and protein sequence coverage mapping accurately and with high confidence.[5]

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