The four pillars of protein characterization by hyphenated high resolution mass spectrometry
Part 1 of 4

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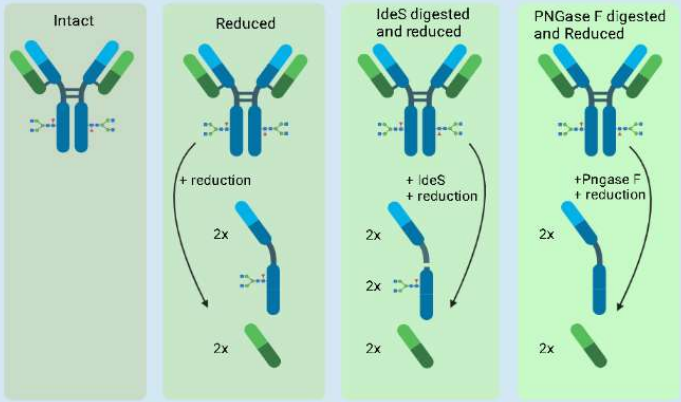
Intact & middle down ESI-MS

Introduction:
Intact analysis of proteins by LC-ESI-MS is commonly used to confirm the integrity of the intact protein by determination of the molecular weight of the intact molecule and other present size variants, e.g. the glycosylation profile.

To achieve a comprehensive data set, the intact molecule analysis is combined with additional middle down approaches (e.g. by applying enzymatic digestion with PNGase F or IdeS or by the application of a disulfide reduction step) which reduce complexity and result in an orthogonal view into the target molecule.

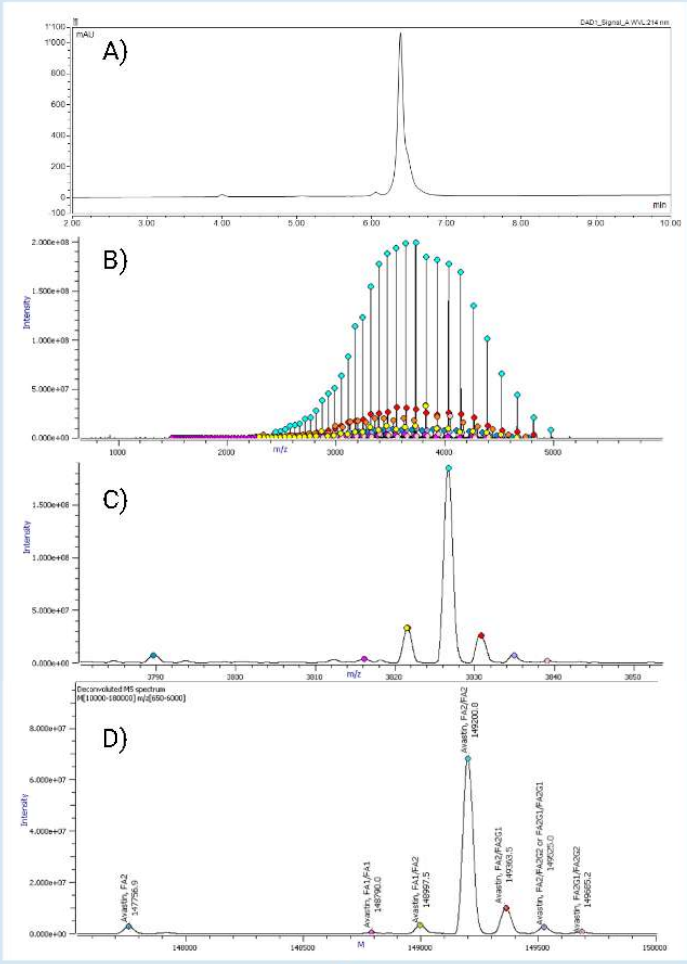
The intact analysis is well suited for the detection of post-translational modifications (PTMs) like lysine side chain glycation or backbone breaks (clippings). Other PTMs, as e.g. oxidations and glyocosylations, may be orthogonally confirmed by the peptide mapping approach.



Plausible assignment	Expected mass [Da]	Detected mass [Da]	Delta Mass [ppm]	Relative MS intensity [%]
Avastin_LC_HC_HC_LC_K107-C214, FA2/FA2	137543.10	137542.96	-1.03	7.22
Avastin , FA2	147752.23	147756.95	31.91	2.95
Avastin , FA1/FA1	148791.18	148790.02	-7.78	0.67
Avastin , FA1/FA2	148994.38	148997.52	21.10	3.69
Avastin , FA2/FA2	149197.57	149200.75	21.32	71.42
Avastin , FA2/FA2G1	149359.71	149363.54	25.63	10.52
Avastin , FA2/FA2G2 or FA2G1/FA2G1	149521.85	149525.00	21.07	2.83
Avastin , FA2G1/FA2G2	149683.99	149685.20	8.11	0.71

Exemplary results of an intact evaluation of Avastin (Bevacizumab) is shown.

Exemplary results



A) LC-UV chromatogram of an intact measurement of Avastin

B) Summed MS spectrum of the whole peak group

C) Zoomed MS spectrum (showing the charge state z=39)

D) Deconvoluted MS spectrum (evaluated with Protein Metrics Intact)

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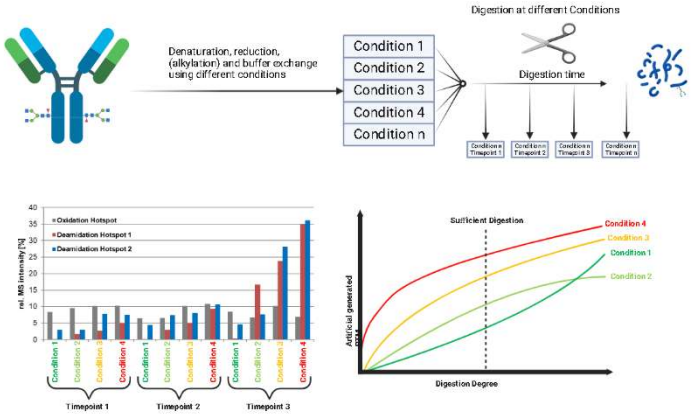
PepMap PTM (MAM)

Introduction:
The peptide mapping (PepMap) PTM approach (also known as multi attribute monitoring (MAM) or bottom up approach) is commonly used to confirm the protein backbone (amino acid sequence) and to identify site-specific potential post-translational modifications.

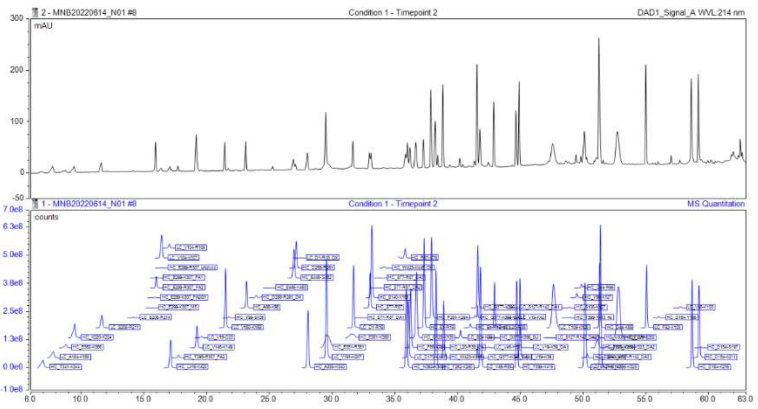
The protein is denatured and reduced followed by a proteolytic digestion with a suitable enzyme. Setting up a product-specific PepMap method typically starts with the application of an optimized generic platform method and screening of multiple digestion conditions. This is in order to determine the optimal method parameters for minimizing artificially generated modifications while maintaining suitable digestion to achieve a high amino acid sequence coverage.

The resulting peptides are LC-UV MS/MSMS analyzed, applying state-of-the-art UHPLC-Orbitrap technology. After the identification of modified and unmodified peptides, a relative quantification is applied, e.g. determining the relative % peak area of an oxidized peptide vs. the corresponding unmodified peptide.



The scheme above shows exemplarily how the applied screening conditions result in a different degree of artificially induced oxidation and deamidation. Suitable PepMap conditions are identified by this approach, which should be applied subsequently for test sample analysis.

Exemplary results



Top panel: LC-UV chromatogram (black). Bottom Panel: Extracted ion chromatogram used for MS identification (blue).

Subunit	Peptide Name	Position	Modification	MS peak area in %
Light Chain (LC)	LC_D1-R18_OX	M4	Oxidation	0.31
	LC_S127-R142_DA	N137, N138	Deamidation	0.16
	LC_V146-K149_OX	W148	Oxidation	1.53
Heavy Chain (HC)	HC_E1-R19_pE	E1	Pyroglutamate	4.33
	HC_L20-R38_OX	M34 (W36)	Oxidation	0.80
	HC_G44-K65_DSU	D63	Succinimide from Asp	0.31
	HC_S77-R87_OX	M83	Oxidation	0.52
	HC_D255-R261_OX	M258	Oxidation	6.86
	HC_F281-K294_OX	W283	Oxidation	0.51
	HC_E299-R307	N303	Unglycosylated	2.68
	HC_E299-R307_FA2	N303	N-Glycan: FA2	79.32
	HC_E299-R307_A2	N303	N-Glycan: A2	1.42
	HC_E299-R307_FA2G1	N303	N-Glycan: FA2G1	7.43
	HC_E299-R307_FA1	N303	N-Glycan: FA1	8.47
	HC_E299-R307_M5	N303	N-Glycan: M5	0.68
	HC_V308-K323_DA	N321	Deamidation	0.96
	HC_V329-K332_DA	N331	Deamidation	3.09
	HC_E362-K366_OX	M364	Oxidation	8.09
	HC_N367-K376_DA	N367	Deamidation	0.39
	HC_G377-K398_NSU	N390, N395, N396	Succinimide from Asn	1.78
	HC_G377-K398_DA	N390, N395, N396	Deamidation	5.19
	HC_T399-K415_DSU	D405, D407	Succinimide from Asp	0.60
	HC_T399-K415_ID	D405, D407	Isoaspartate	0.09
	HC_W423-K445_OX	(W423) M434	Oxidation	2.14
	HC_S446-G452	G452	Absence of C-terminal Lys	98.19

Exemplary results of a PepMap PTM evaluation of Avastin (Bevacizumab) is shown.

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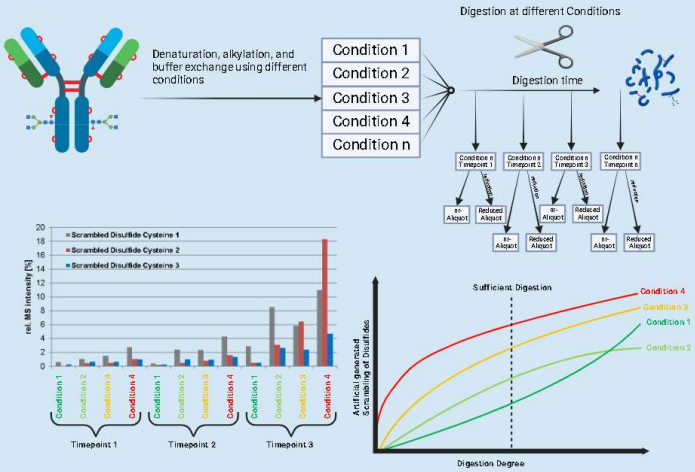
Disulfide PepMap

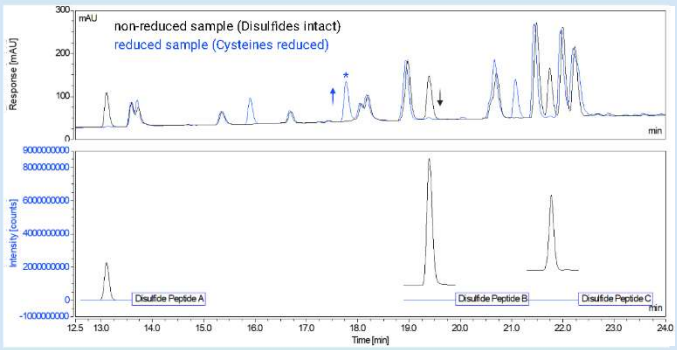
Introduction:
The disulfide PepMap approach is used to confirm the disulfide linkages in a protein. In the first step, the free thiols of the protein are blocked by alkylation, followed by a proteolytic digestion under non-reducing conditions. The disulfide linked di-peptides are analyzed by LC-MS/MSMS, identified and relatively compared (relative % peak area of expected linked disulfide vs. scrambled disulfide).

Further this approach is well suited for relative quantification of PTMs that are only present under non-reduced conditions, as e.g. trisulfides. For method setup a similar condition screening approach as described for the PepMap PTM is applied to minimize artificially induced disulfide scrambling.



The scheme above shows exemplarily how the digestion condition affects the amount of scrambled disulfide.

Exemplary results



Top panel: LC-UV chromatogram. Bottom Panel: Extracted ion chromatogram.
Both figures show the non-reduced (black) and reduced (blue) preparation. Disulfide peptide peaks are disappearing after reduction (black arrow). Reduced cysteine carrying peptide peaks are appearing (blue arrow).

	Relative MS peak area [%]			
Cysteine	Expected	Scrambled	Trisulfide	Thioether
LC_C1	99.8	0.2	-	-
LC_C2	98.9	1.1	-	-
LC_C3	97.9	2.1	-	-
LC_C4	99.7	0.3	-	-
LC_C5	98.6	0.8	0.3	0.3
HC_C1	100.0	-	-	-
HC_C2	100.0	-	-	-
HC_C3	98.5	0.9	0.2	0.4
HC_C4	99.3	0.7	-	-
HC_C5	100.0	-	-	-
HC_C6-9 (Hinge)	*	*	*	*
HC_C10	100.0	-	-	-
HC_C11	99.9	0.1	-	-
HC_C12	99.9	0.1	-	-
HC_C13	99.5	0.5	-	-

Isoform *	Rel. MS peak area [%]
IgG2-A	70.2
IgG2-A/B	10.5
IgG2-B	19.3

Exemplary results of a disulfide PepMap evaluation of an IgG2 is shown including the relative quantification of the IgG2 isoforms (IgG2-A, IgG2-A/B and IgG2-B).

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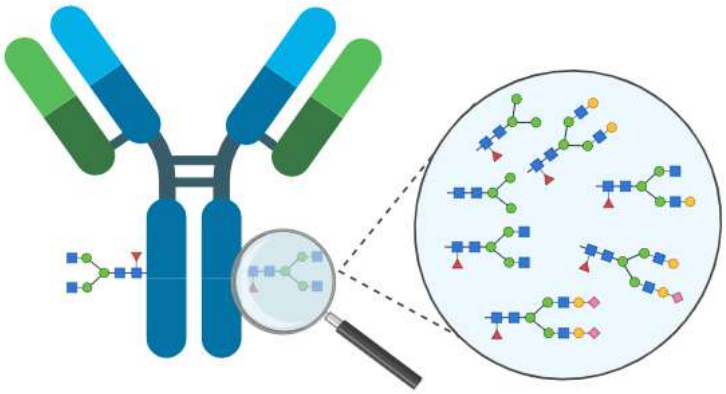
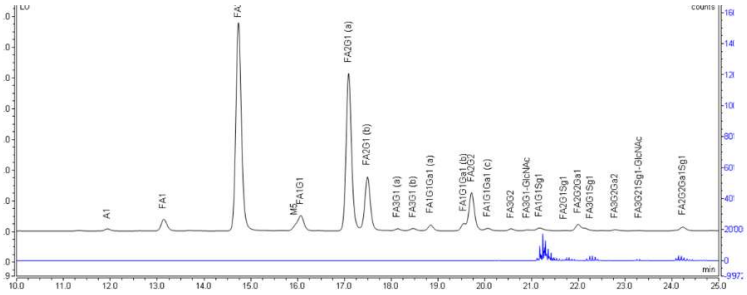
N-glycosylation analysis

Introduction:
The gold standard for N-glycosylation analysis is the release of protein-attached N-glycans by digestion with PNGase F followed by a fluorescence labeling. Labeled N-glycans are analyzed by hydrophilic interaction chromatography (HILIC) based UHPLC-Fluorescence-MS/MSMS.

Hereby the fluorescence read out for relative % peak area determination of the N-glycan species typically results in a higher sensitivity compared to the PepMap PTM (MAM) approach.

Coupling high-resolution MS hyphenated to the fluorescence detection is used for initial identification of the N-glycans and for peak characterization.

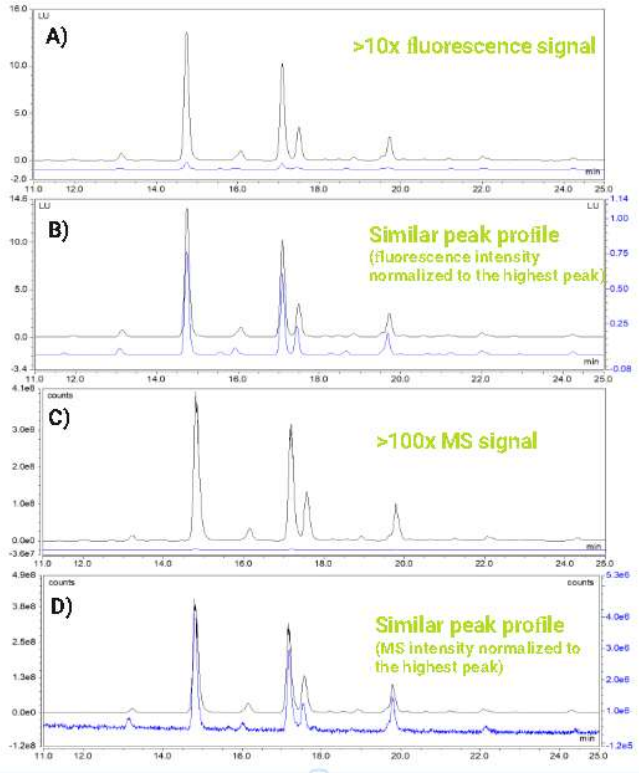




Overlay of RapiFluor labeled N-glycan analysis:

Black trace: HILIC-fluorescence chromatogram
Blue Trace: MS/MS marker ion extraction for N-glycolylneuraminic acid (NGNA). This marker ion can be used to confirm the presence of NGNA containing N-glycans.

Label comparison



The fluorescence label used for the last decade was 2-AA or 2-AB. Recent developments resulted in improved, commercially available fluorescence labels which enable more efficient sample preparation workflows and result in higher quality data due to increased fluorescence and MS/MSMS signal response (e.g. Waters RapiFluor).

A) and B) LC-fluorescence chromatogram comparison of RapiFluor (black trace) and 2-AB (blue trace)
C) and D): Total ion current (TIC) chromatogram comparing RapiFluor (black trace) with 2-AB (blue trace).
The additional MS signal intensity allows for MS2 confirmation of marker ions - even for minor species.