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Monitoring oxidation in recombinant monoclonal antibodies at subunit level through two-dimensional liquid chromatography coupled with mass spectrometry

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Introduction

Oxidation, induced by reactive oxygen species, is a prevalent chemical modification in monoclonal antibodies (mAbs), affecting amino acid residues such as cysteine (C), methionine (M), tryptophan (W), tyrosine (Y), lysine (K), and others (Gupta *et al.,* 2022). Each residue's oxidation uniquely influences the protein's structure and function. Methionine is highly prone to oxidation in mAbs, typically resulting in the formation of a methionine sulfoxide product with increased polarity. These methionine residues are found in both the antigen-binding (Fab) and effector activity (Fc) domains of a mAb, with those situated on the surface being particularly susceptible to oxidation (Hageman *et al.,* 2019). The increasing popularity of two-dimensional liquid chromatography (2D-LC) has led to a significant rise in the integration of two complementary chromatographic techniques, coupled with mass spectrometry (MS), aiming to provide comprehensive insights into mAb post-translational modifications (PTMs) (Sarin et al., 2022). The proposed 2D WCX-RP-MS workflow enables the analysis of mAbs at the subunit level, providing an automated analytical platform for characterizing and identifying oxidized regions within both the Fc and Fab domains.

Experimental

An online 2D-LC workflow was performed on a 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, Germany) equipped with one autosampler (G7167B), two binary pumps (G7120A), two multicolumn compartments (G7116B), two DAD detector (G7115A). Two dimensions were interconnected through one 2-Position/8 -Port valve (G1170A) with one ASM valve and two 6-position/14-port multiple heart cutting (MHC) valves. Each 6-position/14-port valve is installed with six 40 µL stainless steel loops.

The WCX chromatography was performed in the first dimension using an Agilent BioMab, NP5, PEEK column (2.1 X 250 mm, 5 µm) and the WCX peaks were selected through heart-cut mode which was analyzed in the second dimension by RPC chromatography using an Agilent Zorbax 300SB-C8 RP column (2.1 x 150 mm, 5µm). The peaks of 1D were transferred to 2D using a typical heart-cut method. When peak 1 was being analysed in 2D, the other peak fraction was parked in the 40ul MHC loop. Further, all peaks were analysed sequentially. The 2D-LC system was coupled to 6545XT AdvanceBio LC/Q-TOF system (Agilent Technologies, USA) equipped with Dual Agilent JetStream electrospray ionization (AJS-ESI) source.

The 2D-LC and MS data were acquired in OpenLAB CDS Chemstation 10.0 and MassHunter software 10.0 (Agilent Technologies), respectively. Data analysis was performed in BioConfirm software version 10.0 (Agilent Technologies).

Figure 1: Agilent 2D-LC 1290 Infinity II UHPLC system

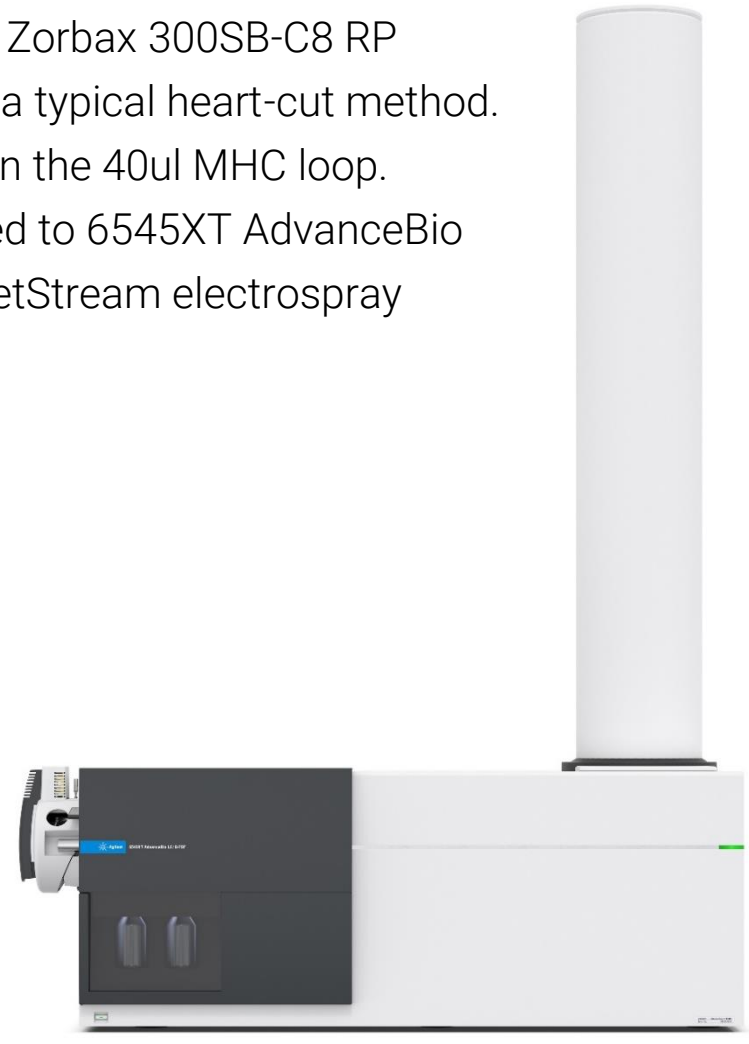

Figure 2: Agilent 6545XT AdvanceBio LC/Q-TOF system

Results and Discussion

Weak-cation exchange (WCX) column in the 1D resolves the Fab, Fc subunits and their oxidized variants at their respective positions. When the peaks from 1D are passed on to the 2D RPC column using a heart-cut approach, further enhancement of separation is obtained. The mass spectrometer identifies each 2D peak with a charge state of +10 to +30 and an *m/z* range of 1000-5000. For the control sample of mAb A, five peaks obtained in the 1D WCX column are selected and passed on to the 2D RPC column for further analysis. Figure 4A (red) shows the profiles obtained in the 1D WCX, and Figures 4B-4F (red) depict the corresponding peaks obtained in the 2D RPC method. Deconvoluted masses for the 2D WCX-RPC peaks are summarized in Table 1.

For the oxidized mAb A analysis, an identical workflow to the control sample was chosen and analyzed in the 2D-LC system (Figures 4A-4F, blue). A total of four peaks resolved in the 1D WCX column were heart-cut and sent to the 2D RPC column for further analysis. The profile obtained from the oxidized sample is overlaid with the control sample profile (Figure 4A) to depict the peak changes between the two samples. The 1D WCX oxidation is quantifiable and ranges from 2.7% to 15.1 % from 2 hours to 8 hours, but such quantification is challenging for the 1D RPC method due to partially resolves the Fab oxidized peaks and shows no difference in the Fc oxidation profile. On the other hand, the 2D-LC WCX-RP-MS method identifies 10 oxidation sites at Fab (+KTH), Fab (-KTH), and Fc fragments, including methionine and tryptophan residues.

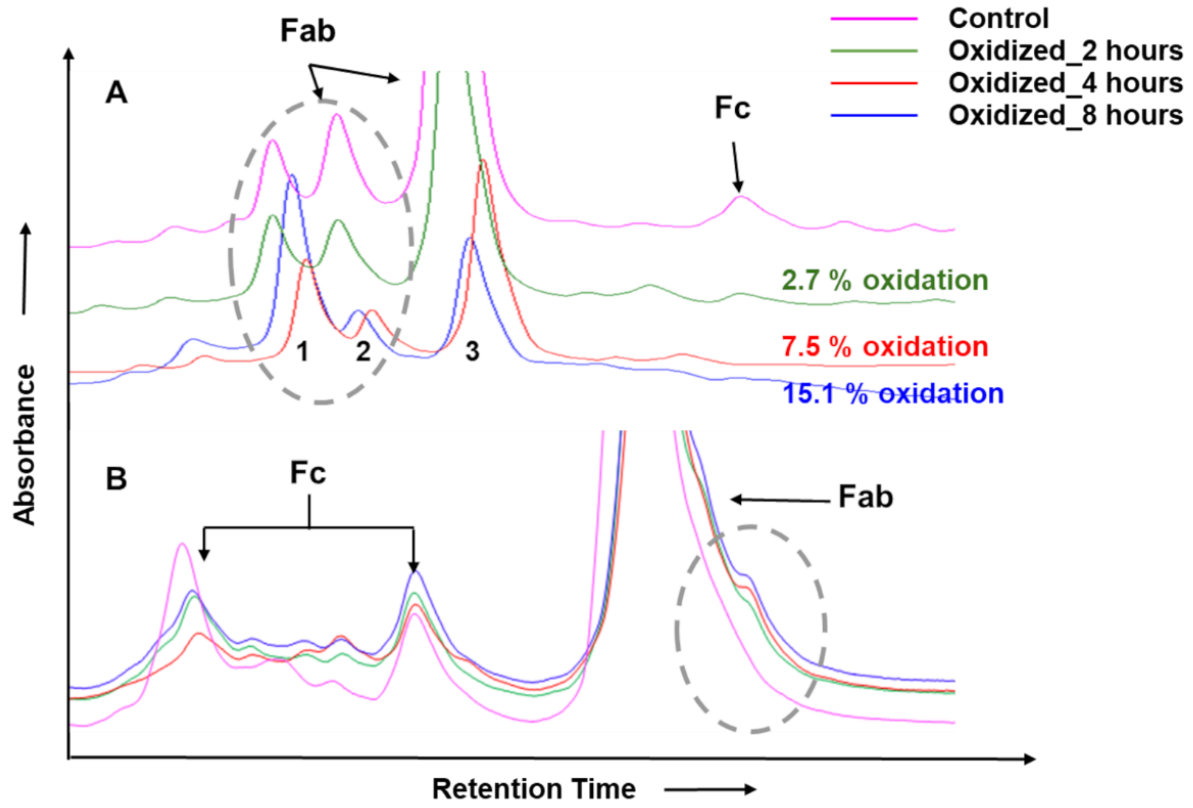

Figure 3 (A): Subunit WCX analysis of control and oxidized-digested mAb A samples; (B): Subunit RPC analysis of control and oxidized-digested mAb A samples

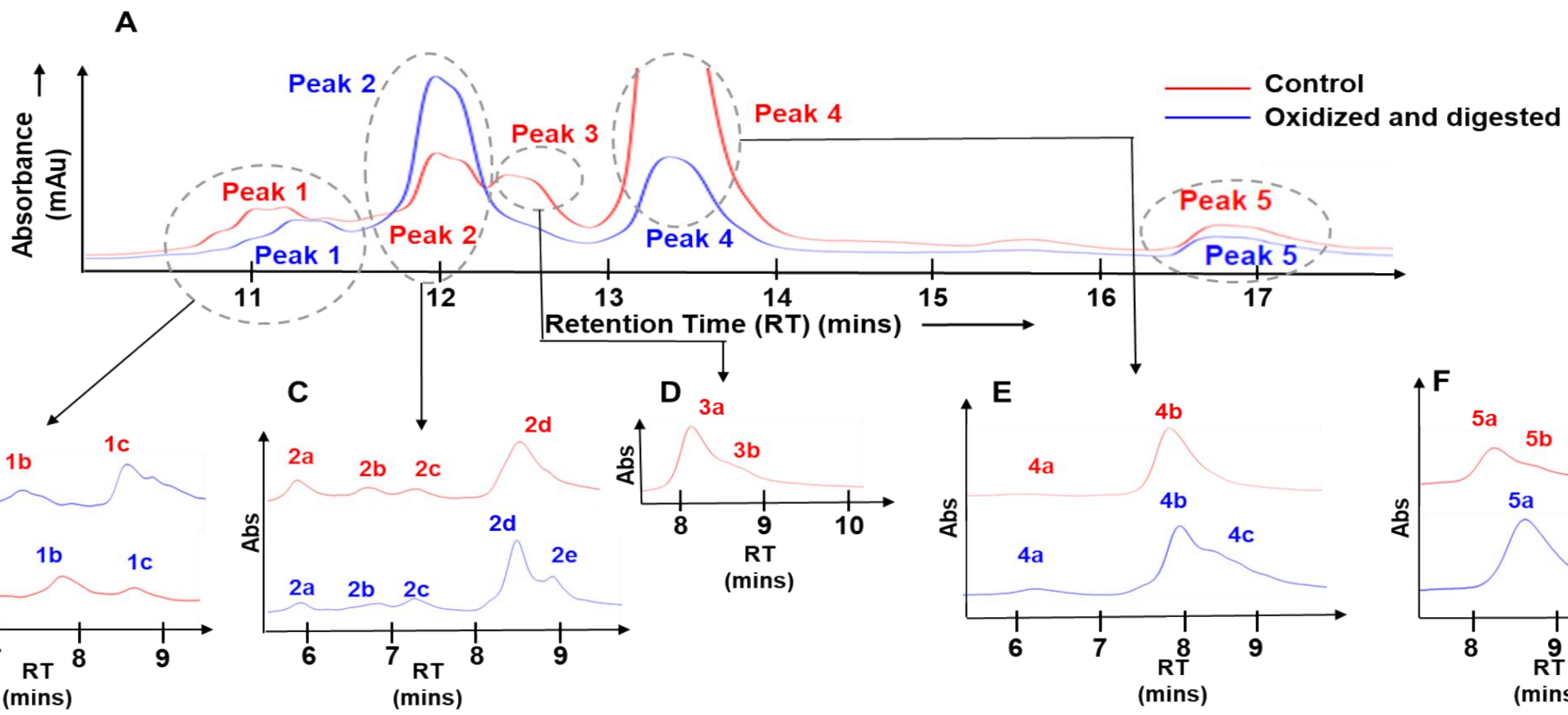

Figure 4 (A): D1 WCX analysis of control and oxidized-digested mAb A in the 2D-LC setup; (B-F): D2 RPC analysis of control and oxidized-digested mAb A samples in the 2D-LC setup

Results and Discussion

Table 1 Summarized deconvoluted masses of WCX-RP-MS subunit analysis of mAb A

Control			Oxidized			Mass Difference between control & oxidized (Da)
D^1_WCX	D^2_RPC	Mass (Da)	D^1_WCX	D^2_RPC	Mass (Da)	
Peak 1	1a	25516.9	Peak 1	1a	23883.3	-1633.6
	1b	10492.7		1b	10492.5	-0.2
	1c	10355.6		1c	10355.6	0.0
Peak 2 (Fab -KTH)	2a	25516.8	Peak 2 (Fab -KTH)	2a	25564.9	48.0
	2b	10566.0		2b	12122.6	1556.6
	2c	23440.7		2c	23440.7	0.0
	2d	47272.9		2d	47272.4	-0.5
Peak 3 (Fab -KTH)	3a	23440.8		2e	23834.7	NA
	3b	47272.5				
Peak 4 (Fab +KTH)	4a	23440.8	Peak 4 (Fab +KTH)	4a	23440.8	0.0
	4b	47638.8		4b	47638.6	-0.2
				4c	47687.3	48.5
Peak 5 (Fc)	5a	52934.5	Peak 5 (Fc)	5a	52999.7	65.3
	5b	13612.9		5b	23664.86	10051.94

Table 2 Summarized Oxidation site of mAb A after tryptic digestion under reduced condition **(A/C – Light Chain, B/D – Heavy Chain)**

Sequence	Location	Pred mods
TVLHQDWLNGK	B (310-320)/D (310-320)	Oxidation (W) 7
ASQDVNTAVAWY	A (25-36)/C (25-36)	Oxidation (W) 11
WGGDGFYAMDYWGQGTLVTVSSASTK	B (99-124)/D (99-124)	Oxidation (W) 1
AMDYWGQGTLVTVSSASTK	B (106-124)/D (106-124)	Oxidation (W) 5
SVMHEALHNHYTQK	B (429-442)/D (429-442)	Oxidation (M) 3
DTLMISR	B (252-258)/D (252-258)	Oxidation (M) 4
NTAYLQMN	B (77-84)/D (77-84)	Oxidation (M) 7
DIQMTQSPSSLSASVGDR	A (1-18)/C (1-18)	Oxidation (M) 4
AMDYWGQGTLVTVSSASTK	B (106-124)/D (106-124)	Oxidation (M) 2
EEMTKNQVSLTC	B (359-370)/D (359-370)	Oxidation (M) 3

Conclusions

The oxidized peaks not resolved by 1D methods are successfully resolved by the D2 RPC column with additional HC-LC information. It should be highlighted that the proposed method completely uses MS compatible buffers and is a faster method to identify mAb modification and their correlation in different mAb fragments compared to the tedious bottom-up approach. A general mAb bottom-up analysis takes up to 24-48 hours with the risk of sample loss, degradation, and induced modifications. In the present study, sample preparation took only 4-10 hours (depending on oxidation incubation), and sample analysis took only 3 hours. Hence, 7-13 hours is enough for the subunit analysis with a low risk of sample loss and degradation. The present study focuses on oxidation as the chemical modification of interest, but the workflow can be extended to other critical charge modifications easily resolved by the WCX column.

References

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