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Multi-Attribute Monitoring of Antibody Variants through Hydrophobic and Cation-Exchange Chromatography Coupled with Native Mass Spectrometry

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Introduction

Two-dimensional liquid chromatography (2D-LC) is a recent innovative analytical technique that is a step towards multidimensional chromatography capable of resolving mAb heterogeneities. Recently, an advanced 2D-LC hardware system has been successfully developed, where the eluents from the first dimension (1D) are selectively or completely transferred to the column in the second dimension (2D). Such a novel workflow has overcome the burden of the offline approach, subsequently reducing sample loss and time of analysis (Camperi et al., 2021) . The development of 2D-LC methods coupled with MS has further led to a plethora of ways to separate variants in proteomics effectively including advancements in the MAM analytical workflow (Wu et al., 2022). This study presents a 2D-LC separation system with hydrophobic interaction chromatography (HIC) in the first dimension and weak cation exchange chromatography (WCX) in the second dimension. The method utilizes only volatile salts and is based on the orthogonality between the two chromatographic modes to provide additional separation, characterization and identification of mAb variants.

Experimental

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

Experimental

The monoclonal antibody mAb A was resolved by HIC using an AdvanceBio HIC column (4.6 X 100 mm, 3.5 µm, Agilent Technologies) in the first dimension and WCX chromatography was performed using an Agilent Bio WCX column (NP5, 4.6 x 250 mm, 5µm, Agilent Technologies) in the second dimension on a 1290 Infinity II UHPLC system (Agilent Technologies, Waldbronn, Germany). For native MS analysis, the 2D-LC system was coupled to 6545XT AdvanceBio LC/Q-TOF system (Agilent Technologies, USA) equipped with Dual Agilent JetStream electrospray ionization (Dual AJS-ESI) source. The MS parameters were drying gas at 350 °C, drying gas flow 12 L/min, nebulizer pressure 35 psig, sheath gas temperature at 300 °C, sheath gas flow at 12 L/min, nozzle voltage 5500 V, and Fragmentor voltage 200 V. MS data was collected in profile mode at a rate of 1 spectrum per sec with a 2500-20000 *m/z* range. Data was acquired using MassHunter Workstation LC/MS Data Acquisition 10.0 software (Agilent Technologies).

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

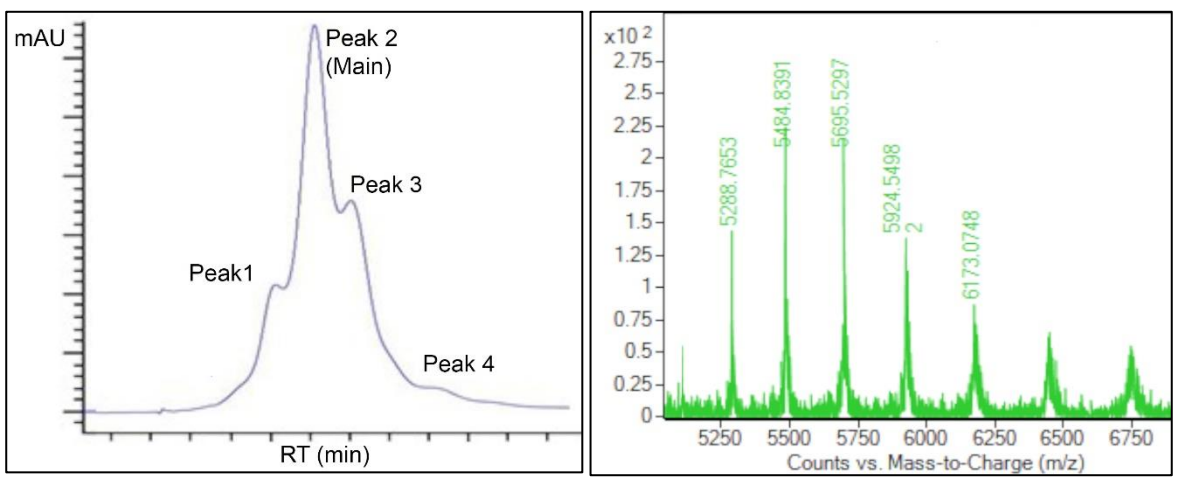
Results and Discussion

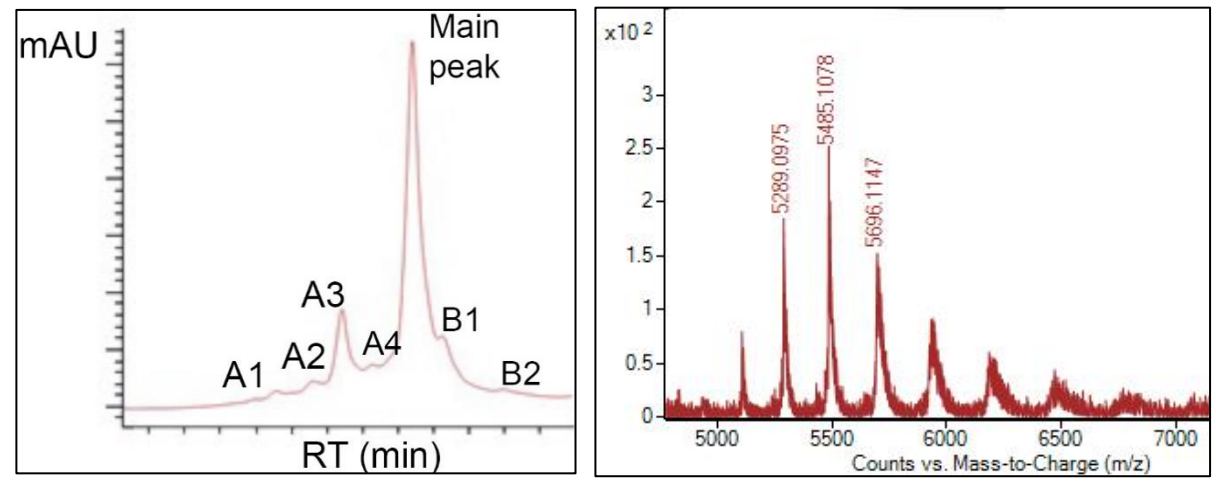
Native HIC-MS

Hydrophobic interaction chromatography (HIC) resolves complex proteins in their native structures on the basis of their hydrophobicity with reducing salt gradient conditions containing less or no organic solvent modifiers. In recent years, HIC has been coupled with mass spectrometry (MS) using volatile buffer salts to characterise hydrophobic variants in their intact native state (Chen et al., 2016). Wei et al., 2019 reported a HIC-MS method for the separation and analysis of oxidized monoclonal antibody isoforms. In the present study, an online 2D-LC workflow of ammonium acetate buffer condition-HIC coupled with MS was employed to separate and analyse hydrophobic variants of mAb A. The mAb A elutes into one main peak and three minor peaks. The HIC peaks of mAb A contained a charge state envelope in the range of 5000-7000 *m/z* and the distribution of charge states range from +21 to +29 which indicates that mAb A has been ionized in the native state (Figure 3).

Native WCX-MS

Weak cation exchange chromatography (WCX) is a nondenaturing analytical technique extensively applied to separate and isolate charge variants of mAbs. Recent advances in the MS-compatible volatile mobile phases allow a direct coupling of WCX chromatography to MS, which potentially enables the characterization of charge variants with low abundance in the native state. In the present study, the native WCX-MS method was used to analyze charge variants of mAb A, which resulted in adequate resolution of acidic and basic peaks. The charge state envelope had a range of +23 to +29 and a *m/z* range of 5000–7000, depicting ionization in the native state of mAb A. A total of seven highly resolved peaks were obtained for mAb A, including four acidic peaks (A1-A4), two basic peaks (B1-B2) and a main peak (M1) (Figure 4).





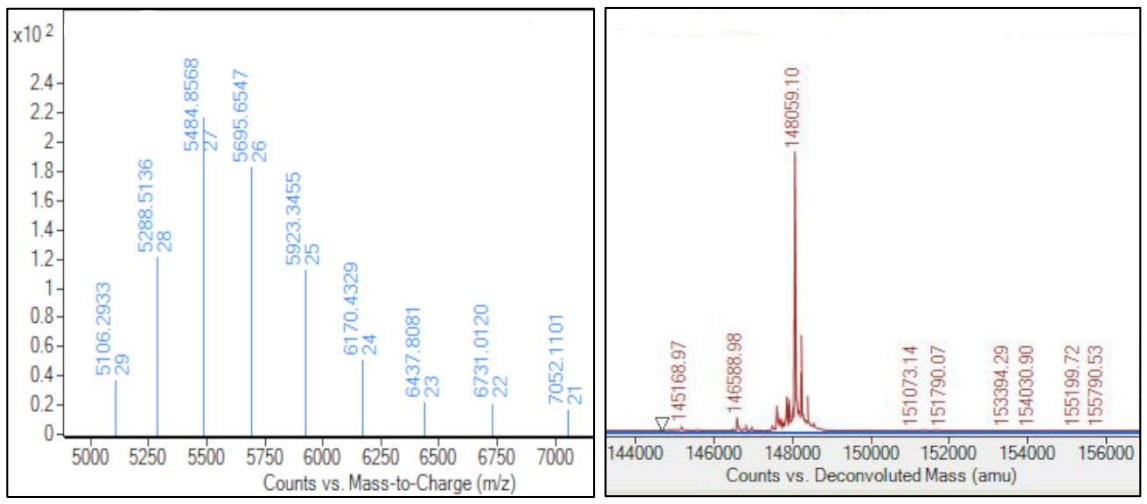


Figure 3: Native HIC-MS analysis of mAb A: (A) HIC base peak chromatogram of mAb A under the UV; (B) Charge-state pattern of mAb A which confirm ionization under native form; (C) Isotopic resolution of native mAb A; (D) Deconvoluted spectrum of mAb A.

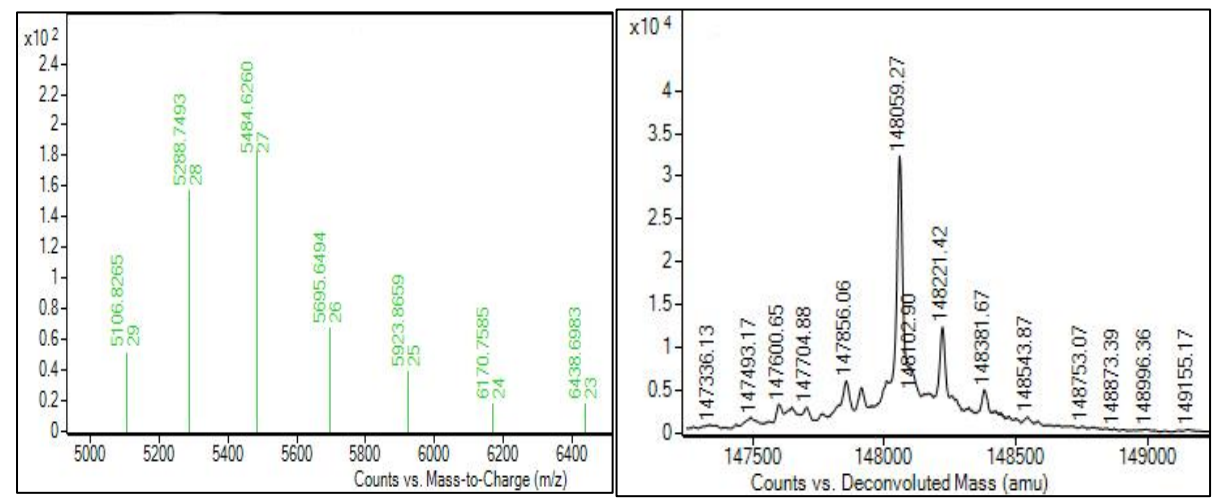


Figure 4: Native WCX-MS analysis of mAb A: (A) WCX base peak chromatogram of mAb A under the UV; (B) Charge-state pattern of mAb A which confirm ionization under native form; (C) Isotopic resolution of native mAb A; (D) Deconvoluted spectrum of mAb A.

Results and Discussion

2D-HIC-WCX

As a stand-alone chromatography, HIC resolves mAb variants based on their hydrophobicity, whereas IEC resolves them based on the overall charge of the protein variants. The 2D-LC technique provides a platform for the analysis of a sample through two chromatographies simultaneously. In the present study, an online 2D-LC workflow coupled with mass spectrometry was performed to analyze hydrophobic variants in the first dimension using the HIC column and charge variants in the second dimension using the WCX column. The HIC in the first dimension resolved the hydrophobic variants of mAb A into four prominent peaks. A heart-cut approach using the ChemStation C.01.07 software was utilized to cut each resolved peak from HIC (as shown in Figure 5) for collection and transfer to the second dimension WCX to separate the charge variants. The main peak of mAb A in the HIC showed improved selectivity in the second dimension with the separation into acidic variants, basic variants, and a major peak. The pre-peaks and post-peaks of mAb A in the HIC were further analyzed in the second dimension, which showed low resolution and eluted as single peaks. The low resolution may be attributed to the low intensity of pre and post-peaks as compared to the intensity of the main peak in the first dimension.

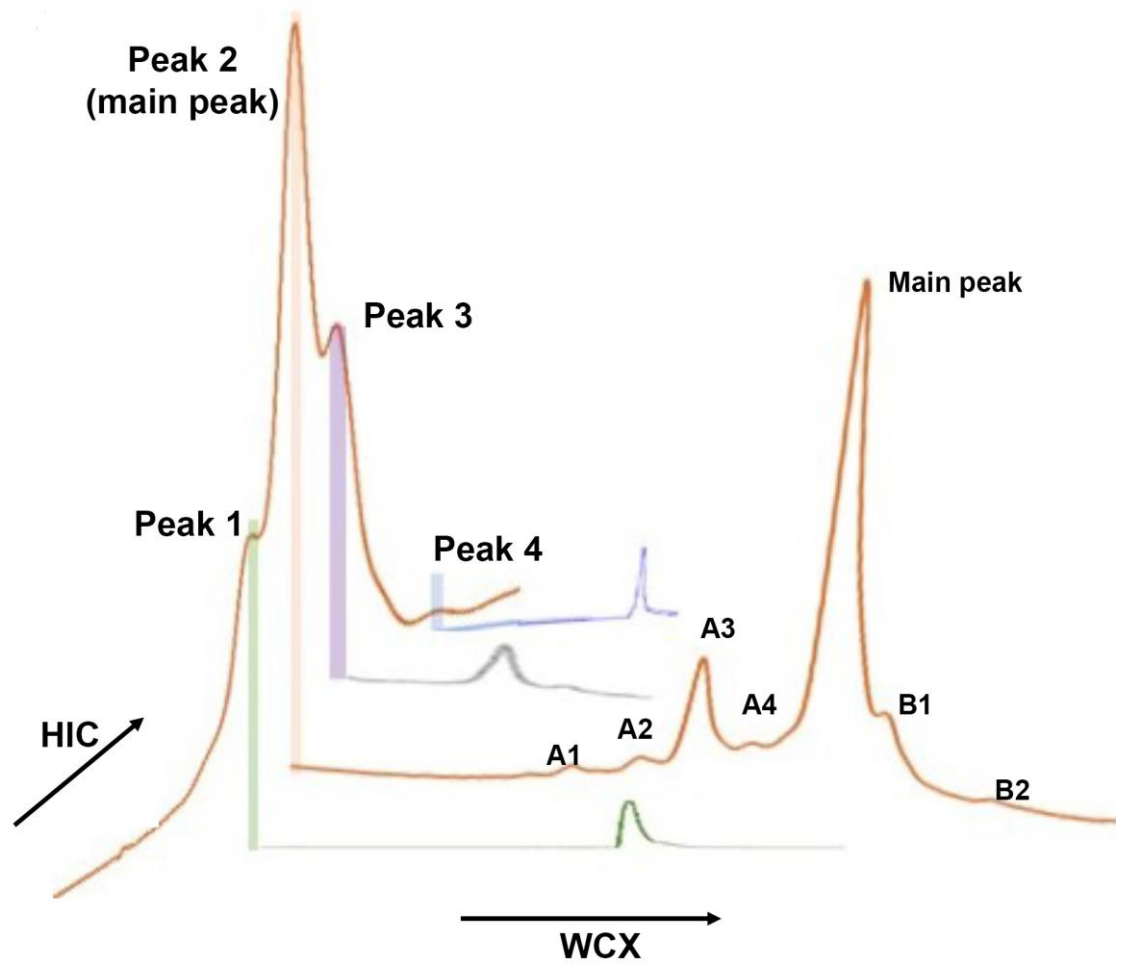


Figure 5: 2D HIC-WCX base peak chromatogram of mAb A under the UV; each HIC peak was transferred to the WCX column through a heart-cut 2D-LC method.

Conclusions

The present study demonstrated an efficient 2D-LC/MS work flow which characterized all abundant oxidative and deamidated species through first dimension HIC chromatography and further, HIC peaks were resolved into acidic and basic variants by the second dimension WCX chromatography which were characterized and analysed by employing online native-mass spectrometry.

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