Technical Overview

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Ultrafast Antibody Peptide Mapping with the Agilent 6545XT AdvanceBio LC/Q-TOF

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Introduction

This technical overview demonstrates the use of the Agilent 6545XT AdvanceBio LC/Q-TOF in the development of a rapid, online microdroplet digestion method. The described technique enables digestion times of less than 1 ms[1], a million-fold faster than those of conventional, in-solution digestion. Automated microdroplet digestions enable uninterrupted sample introduction using an Agilent 1290 Infinity II LC system, allowing for real-time peptide mapping. This approach achieved a digestion efficiency greater than 90% by evaluating the ion intensity change of the antibody before and after digestion.

To demonstrate the robustness of the method, peptide maps with high sequence coverage for in-solution-reduced NIST and bevacizumab mAbs were created using iterative MS/MS. Two critical antibody post-translational modifications (PTMs), asparagine deamidation and methionine oxidation, were characterized with MS/MS acquisition and quantified using a standard addition method, respectively.[2] Fast and reliable absolute antibody quantitation was also achieved.

Experimental

Instrumentation

The following instruments were used:

- 6545XT AdvanceBio LC/Q-TOF
- 1290 Infinity II LC system

Method

Online microdroplet digestion: A schematic of online microdroplet digestion of NIST mAb is presented in Figure 1A. Instruments were calibrated in high-resolution mode with a 4 GHz dynamic range and a standard mass range setting of *m/z* 50 to 3,200. Antibody and sequencing-grade trypsin were stored at 6 °C in the LC autosampler. These samples were serially aspirated, mixed in an injection loop, and injected into the flow path using an injector program as described by Gunawardena *et al.*[3] A 2-minute flow injection at 100 µL/min carried the mixture with the mobile phase solvent (5 mM ammonium bicarbonate buffer) into an Agilent Jet Stream source, where microdroplets were produced. For each injection, 1 µL of NIST mAb (1 µg/µL) and 1 µL of trypsin (0.1 µg/µL) were aspirated, giving a protein-to-enzyme ratio of 10:1 during microdroplet digestion. To enhance antibody sequence coverage depth, the protein and enzyme were mixed and injected multiple times, and previously selected precursor ions were dynamically excluded in each iteration.

Table 1. Chromatographic conditions.

Parameter	Value
UHPLC	Agilent 1290 Infinity II LC
Technique	Flow injection analysis
Column Oven Temperature	Ambient
Injection Volume	1 µL
Autosampler	5 ± 2 °C
Mobile Phase A	5 mM ammonium bicarbonate (ABC)
Gradient:	Time (min) Flow rate (mL/min) %A 0 0.3 100 0.1 0.3 100 0.2 0.025 100 1.9 0.025 100 2 0.3 100 3.5 0.3 100

Table 2. Injection program.

Function	Parameter
Draw	Draw 1.0 µL from reagent
Draw	Draw 1.0 µL from mAb
Mix	Mix 2 µL from air and repeat two times
Remote	Set remote line "Start" for 125 ms
Wait	Wait 0.1 min
Inject	Inject

Table 3. Optimized MS conditions using an Agilent 6545XT AdvanceBio LC-Q/TOF.

Parameter	Value
Drying Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer Gas	60 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	500 V
Ion Mode	AJS ESI positive
Fragmentor	130 V
Skimmer	65 V
MS Range	*m/z* 250–3,200
Acquisition Rate/Time	4 spectra/s

All other experimental details and methods are described by Xiao *et al.* in an open-access article.[4]

Results and discussion

Microdroplet digestion of reduced NIST mAb

To increase MS/MS identification depth and protein sequence coverage when analyzing the reduced NIST mAb, three iterative MS/MS analyses were carried out. The NIST mAb sample was reduced, alkylated, then desalted before microdroplet digestion, as described by Xiao *et al.*[4] When injected without trypsin, the reduced NIST mAb MS spectrum showed strong ion signals between *m/z* 800 and 2,400, related to charge state distributions of the light and heavy chain (Figure 1B). When trypsin and mAb were co-injected, the NIST mAb was digested online in microdroplets, and tryptic peptides were detected between *m/z* 300 and 1,300 region (Figure 1C). The MS spectrum also showed significantly decreased light- and heavy-chain ion signals relative to the undigested NIST mAb.

A sequence coverage of 53% was achieved through iterative MS/MS (Figure 1D), and most (84%) peptides identified through MS/MS had intensities above 5,000 counts (Figure 1E). An overall sequence coverage of 91% was possible through combining MS and MS/MS data.

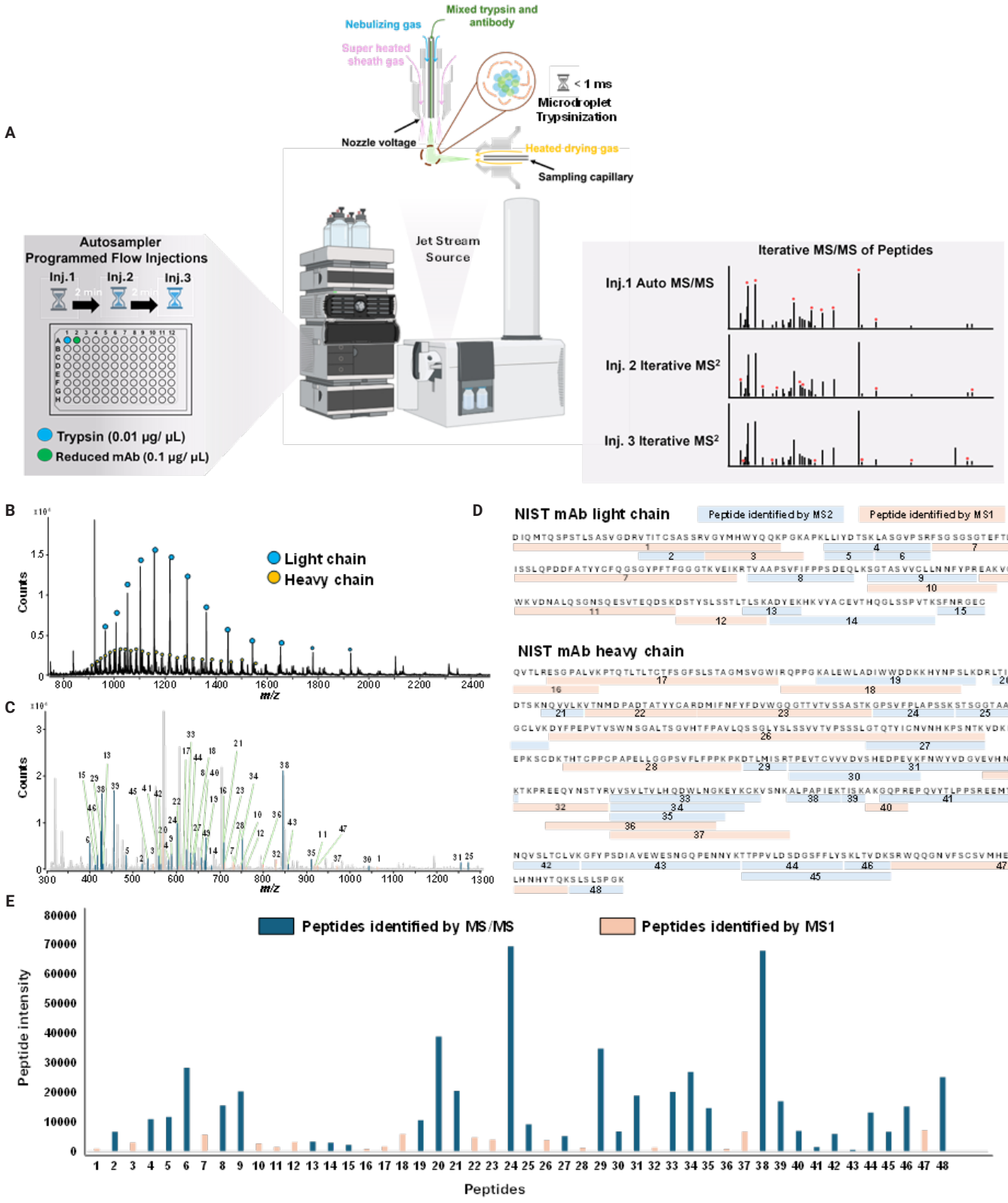

Figure 1. (A) Scheme illustrating microdroplet digestion of antibody with trypsin, performed with automated flow injection and iterative MS/MS using an Agilent 1290 Infinity II LC system coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF. MS spectra of (B) reduced NIST mAb before microdroplet digestion and (C) reduced mAb after microdroplet digestion. (D) Peptide map for NIST mAb showing data from both MS and MS/MS analyses. (E) Chart showing the peak intensities for the identified peptides.

Monitoring deamidation of NIST mAb

Deamidation is a common antibody post-translational modification where amide groups in asparagine (Asn; N) or glutamine (Gln; Q) residues are converted to carboxylic acids. This process can impact antibody stability and pharmacokinetics, and so monitoring deamidation is critical during antibody production. The MS/MS analysis of NIST mAb identified five Asp-containing peptides (Figure 1D), which were used to develop a method for monitoring deamidation.

NIST mAb was artificially deamidated in 1 M Tris buffer (pH 8) for 5 or 7 days. A control sample involved incubating NIST mAb in deionized water (neutral pH) for the same period. Target MS/MS was performed on the Asp-containing peptide ions during online microdroplet digestion for deamidation monitoring.

The fragments of the +3 ion of an Asp-containing peptide (with a sequence GFYPSDIAVEWESNGQPENNYK and *m/z* 848.71) were used to evaluate deamidation, and included y_1–y_{11} ions except y_9 (Figure 2A). The y_6 and y_{10} fragments were chosen for monitoring deamidation levels at certain asparagine residues. The y_6 ion showed identical isotopic distributions under and deamidation and control conditions after 5 days of incubation, suggesting minimal deamidation at the N392 and N393 residues (Figure 2B). By contrast, the y_{10}^{+1} isotopic peak showed a significant increase after deamidation (Figure 2C).

Figure 2D shows that, based on the y_6 ion, deamidation is minimal at N392 and N393 after 5 days' incubation in both the control and in Tris buffer (5% and 7%, respectively). However, as concerns the y_{10} ion, deamidation was 15% and 39% for antibody incubated in deionized water and Tris buffer, respectively. These results indicate that N387 (NG motif) is more likely to be deamidated than N392 and N393 (NN motif). With 7 days of incubation, deamidation increased to approximately 11% in the case of quantification based on the y_6 ion, and to 45% in the case of y_{10}-related quantification (Figure 2E).

Further peptides (TPEVTCVVVDVSHEDPEVKFNWYV-DGVEVHNAK, VVSVLTVLHQDWLNGKEYK, NQVVLK, and SGTASVVCLLNNFYPR) were also studied. Results indicate that Asn is more likely to be deamidated when followed by smaller residues and is less vulnerable to deamidation when followed by larger residues.

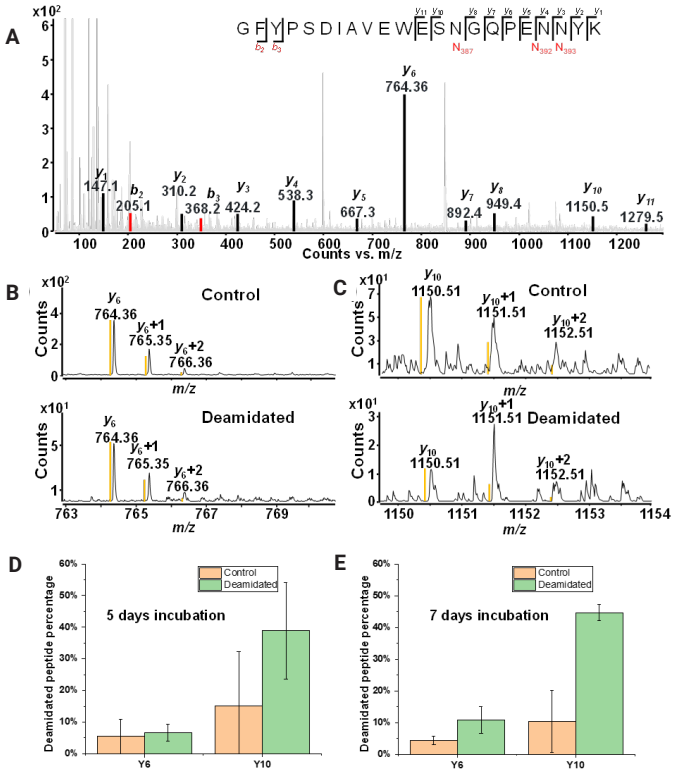

Figure 2. (A) MS/MS spectrum of the +3 ion of the GFYPSDIAVEWESNGQPENNYK peptide (*m/z* 848.71). Zoomed-in MS spectra showing (B) the y_6 fragment ion and (C) the y_{10} fragment ion of *m/z* 848.71 produced through online microdroplet digestion of NIST mAb incubated in deionized water (control) versus in 1 M Tris buffer (pH 8, deamidation condition) for 5 days. These spectra indicate minimal deamidation on N392 and N393. Theoretical isotopic distribution patterns of the fragment ions are shown in yellow for comparison. The y_{10} fragment ion of deamidated GFYPSDIAVEWESNGQPENNYK shows a higher y_{10} + 1 peak intensity compared to the y_{10} monoisotopic peak, indicating deamidation to N387. The deamidation percentages of GFYPSDIAVEWESNGQPENNYK were calculated for NIST mAb incubated in deionized water and in Tris buffer for (D) 5 days and (E) 7 days, based on quantitative analysis of the y_6 and y_{10} fragment ions.

Quantification of methionine oxidation

Methionine oxidation quantification is a critical quality attribute (CQA) for antibodies, particularly because the DTLMISR peptide is located near the neonatal Fc receptor binding interface, which is highly exposed to solvent and therefore very sensitive to oxidation.[5]

Antibody oxidation quantification is generally performed through LC/MS/MS peptide mapping, enabling reliable measurement of oxidation at each methionine residue.[6] However, online microdroplet digestion accelerates analysis by eliminating in-solution digestion, and minimizes oxidation products that can appear during sample preparation.

Figure 3A shows that microdroplet digestion of hydrogen peroxide-stressed NIST mAb produced both the DTLMISR peptide ion (+2, *m/z* 418.22) and the oxidized variant DTLM(O)ISR (+2, *m/z* 426.22). Methionine oxidation is indicated by the 16 Da mass shift between these two ions. Targeted MS/MS was then performed on *m/z* 418.22 and *m/z* 426.22. The y_4, y_5, and y_6 ions showed a 16 Da increase in the oxidized form, further indicating methionine oxidation (Figures 3B and 3C).

In direct comparison of oxidation levels, online microdroplet digestion showed lower oxidation (4.7%) than in-solution digestion (6.1%). Control injections of the DTLMISR peptide revealed minimal in-source oxidation, as the oxidized DTLM(O)ISR ion was found to be less than 1% of the intact peptide. This suggests that most oxidation occurs before microdroplet digestion.

The relative quantification of DTLMISR oxidation was performed by online microdroplet antibody digestion under different stress conditions. The peak intensities of *m/z* 418.22 and *m/z* 426.22 were compared using an equation described by Xiao *et al.*[4] and the percentages of methionine oxidation for stressed and non-stressed NIST mAb were calculated. Figure 3E shows the methionine oxidation percentages for stressed compared to non-stressed NIST mAb, using either MS data (i.e., the intensities of +2 ions of DTLMISR and DTLM(O)ISR) or MS/MS data (i.e., y_5 fragment ions from collision-induced dissociation of +2 ions of DTLMISR and DTLM(O)ISR). However, for relative quantification results to be valid, oxidized and non-oxidized peptides must show similar ionization efficiencies, which is not always the case.

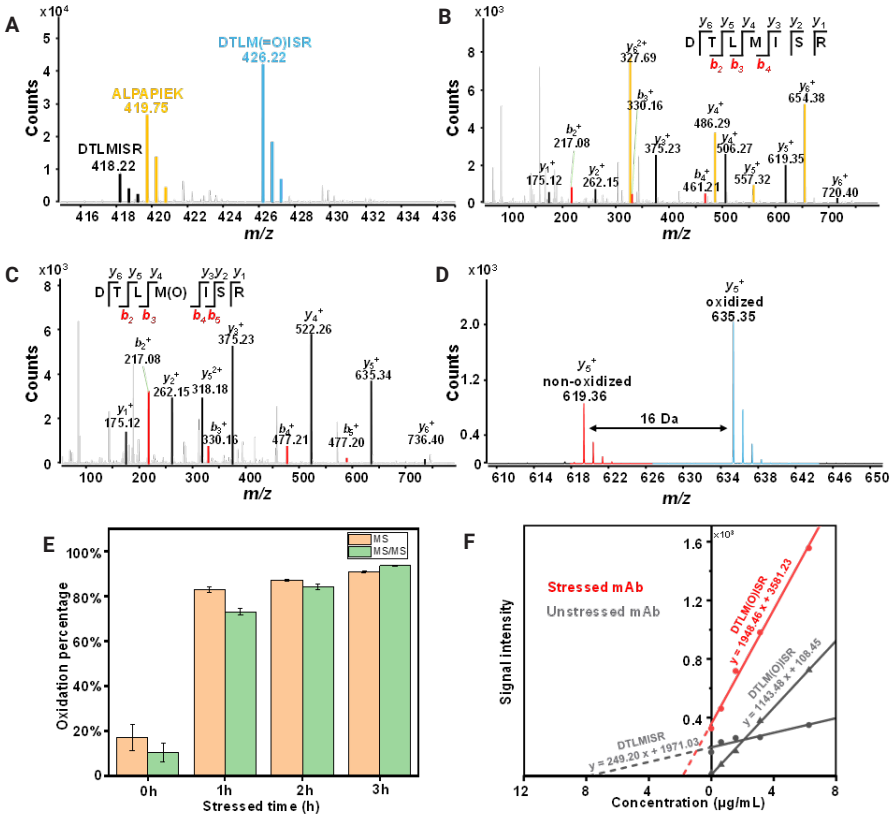

Figure 3. (A) Zoomed-in MS spectrum of microdroplet-digested NIST mAb stressed by hydrogen peroxide, showing ions of DTLMISR and its oxidation product DTLM(O)ISR. (B) Target MS/MS spectrum of the +2 ion of peptide DTLMISR (*m/z* 418.22); y and b ions are marked in black and red, respectively. The yellow peaks are fragment ions from +2 ion of ALPAPIEK. which was co-fragmented with DTLMISR. (C) Target MS/MS spectrum of the +2 ion of DTLM(O)ISR (*m/z* 426.22); identified y and b ions were labeled in black and red, respectively. (D) Overlapped MS/MS spectrum showing two y_5 fragment ions (differing by 16 Da) generated from collision-induced dissociation (CID) from +2 ions of DTLMISR (*m/z* 418.22) and DTLM(O)ISR (*m/z* 426.22). (E) Bar graph showing the methionine oxidation percentages for stressed versus non-stressed NIST mAb under different stress conditions using either MS data (i.e., the intensities of +2 ions of DTLMISR and DTLM(O)ISR) and MS/MS data (i.e., y_5 fragment ions from CID of +2 ions of DTLMISR and DTLM(O)ISR). (F) Absolute quantification of DTLMISR and the oxidized DTLM(O)ISR peptide with a standard-addition method.

For more accurate measurement of oxidation, absolute quantification of the DTLMISR peptide and the oxidized DTLM(O)ISR peptide was carried out using a standard-addition method. Hydrogen-peroxide-stressed or non-stressed NIST mAbs were spiked with synthetic DTLM(O) ISR, followed by online microdroplet digestion. Non-stressed NIST mAb was spiked with synthetic DTLMISR. Figure 3F shows that the oxidized peptide DTLM(O)ISR produced a significantly higher slope than the non-oxidized DTLMISR, which indicates greater ionization efficiency for DTLM(O)ISR. This finding enabled correction of the relative quantification results by accounting for ionization efficiency differences.

Absolute antibody quantification

In the biopharmaceutical industry, the current standard technique for antibody quantification is bottom-up LC/MS.[7] However, this approach can be hindered by challenges related to surrogate peptide selection, sample loss during preparation, and PTMs that negatively affect peptide yield and quantification. By contrast, microdroplet digestion simplifies sample preparation, minimizes sample loss, and reduces issues related to PTMs. Absolute antibody quantification is made possible with an online microdroplet digestion method that involves spiking the target antibody with a heavy isotope-labeled antibody internal standard.

A target light bevacizumab mAb was spiked with a heavy isotope-labeled bevacizumab mAb in a 1:1 mass ratio. Ten pairs of heavy and light peptides were identified after microdroplet digestion of the mixed antibodies, each with peak heights close to a 1:1 ratio (Figure 4A). As an example, the signal of the proteotypic peptide ALPAPIEK was successfully detected. There was a mass shift of 8 Da between ALPAPIEK-light (*m/z* 838.50) and ALPAPIEK-heavy (*m/z* 846.51) due to the [$^{13}C_6$, $^{15}N_2$]-Lys label (Figure 4B).

In online microdroplet digestion of light and heavy bevacizumab mAb, the MS1 peak heights for ALPAPIEK-heavy and for ALPAPIEK-light differed by only 3.3%. Targeted MS/MS was performed on both ions, resulting in the y_4 fragment ions at *m/z* 486.29 (light) and *m/z* 494.30 (heavy, Figure 4C). Online microdroplet digestion was then carried out for quantification: Heavy and light bevacizumab were mixed at increasing ratios. A 2:1 heavy:light mixture produced a corresponding 2:1 peak-intensity ratio for ALPAPIEK (Figure 4D), and the same 2:1 ratio of heavy/light y_4 product ions (Figure 4E). These results demonstrate the reliability of microdroplet digestion for use in quantitation.

A calibration curve was then generated by correlating the ALPAPIEK heavy-to-light peak-intensity ratio with the injected amount of labeled antibody. The resulting curve showed excellent linearity (R^2 = 0.99) with 1/x weighting. Based on the calibration curve, when y = 1, the amount of the target light antibody is 12.58 ng, which was close to the theoretical injection amount (12.5 ng), demonstrating high accuracy.

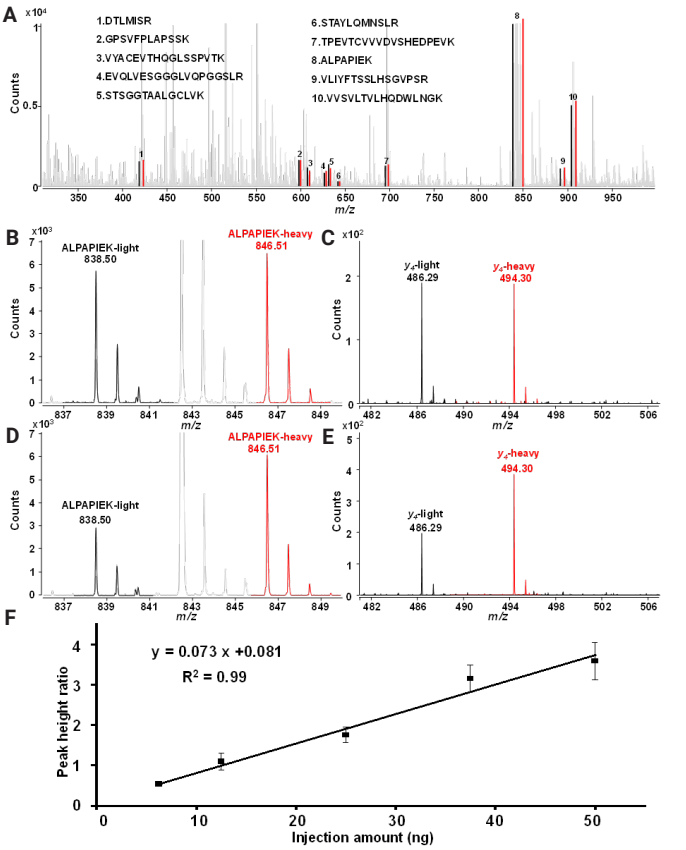

Figure 4. (A) MS spectrum showing the results of online microdroplet digestion of a mixture of 1:1 light- and heavy-isotope-labeled bevacizumab mAbs; 10 pairs of light (black) and heavy (red) peptide ions were identified. (B) MS spectrum of heavy and light ALPAPIEK peptide ion (light: *m/z* 838.50; heavy: *m/z* 846.51) generated by online microdroplet digestion of 1:1 ratio mixed heavy and light bevacizumab mAbs. (C) MS spectrum of y_4 ions produced by target MS/MS of *m/z* 838.50 and *m/z* 846.51 from 1:1 ratio mixed heavy and light bevacizumab mAbs. (D) MS spectrum of heavy and light ALPAPIEK peptide ions generated by online microdroplet digestion of 2:1 ratio mixed heavy and light bevacizumab mAbs. (E) MS spectrum of y_4 ions produced by target MS/MS of *m/z* 838.50 and *m/z* 846.51 from 2:1 ratio mixed heavy and light bevacizumab mAbs. (F) Calibration curve of the peak height ratio of heavy y_4 over light y_4 of ALPAPIEK peptide ion against the injection amount of heavy bevacizumab mAb.

Conclusion

An ultrafast and automated antibody characterization and quantitation method was developed using online microdroplet trypsin digestion coupled with MS/MS analysis on an Agilent 6545XT AdvanceBio LC/Q-TOF. This approach achieved digestion within milliseconds and sample injection cycle times of ~2.5 minutes. The method provided high sequence coverage and enabled the sensitive detection and accurate quantification of PTMs such as asparagine deamidation (by relative quantitation using MS/MS fragment ions) and methionine oxidation (by absolute quantitation via standard additional method). The method also allowed for absolute quantification of antibodies through spiking of isotopically labeled antibody standards.

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