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IMPLEMENTING 1.5 MILLIMETER INTERNAL DIAMETER COLUMNS AND A PORTABLE CAPILLARY COLUMN INSTRUMENT INTO MONOCLONAL ANTIBODY ANALYTICAL WORKFLOWS

by

Benjamin Patrick Libert

A Thesis

Submitted to the
Department of Chemistry and Biochemistry
College of Science and Mathematics
In partial fulfillment of the requirement
For the degree of
Master of Science in Pharmaceutical Sciences
at
Rowan University
December 7, 2023

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Dedication

This work is dedicated to my family.

Acknowledgements

I would like to thank Dr. Grinias for providing the wisdom, the inspiration and the freedom to experiment with prototype capillary LCs that made this work challenging and enjoyable. I thank Drs. Jonnalagadda and Caputo for their support throughout the program. I am grateful for meeting and working with the inspiring people of the Grinias Lab. A special thanks goes to Sam Foster for his vital contributions to this work. I would like to acknowledge Dr. Justin Godinho for helping catalyze my leap into graduate school. Many thanks go to Pat Sasso, Dr. Andrew Harron, and Dr. Taylor Harmon for a multitude of pleasant and enlightening discussions on mass spectrometry and life; their encouragement through the years was valuable to the completion of this work. Many thanks go to Dr. Mary Ellen McNally for her inspiration over the years. Many thanks to Dr. Mark Schure for his frequent improvised lectures on coding, chemistry, chromatography, and life. I would like to acknowledge Dr. Marc Goldfinger, Dr. Barry Boyes, Dr. Joe Destefano, Tim Langlois and the people at AMT for their generous support. Finally, my sincerest thanks go to my family and friends, because your love and support made this achievement possible. Thank you.

Abstract

Benjamin Patrick Libert
IMPLEMENTING 1.5 MILLIMETER INTERNAL DIAMETER COLUMNS AND A PORTABLE CAPILLARY COLUMN INSTRUMENT INTO MONOCLONAL ANTIBODY WORKFLOWS
2023 – 2024
James Grinias, Ph.D.
Master of Science in Pharmaceutical Sciences

Using 1.5 mm inner diameter (i.d.) columns to bridge the gap between routinely used 2.1 mm i.d. columns and capillary bore columns allows for a sequential but significant increase in performance without the need for specialized equipment associated with very low dispersion liquid chromatography (LC) systems. These 1.5 mm i.d. columns balance an increase in sensitivity compared to 2.1 mm i.d. columns (theoretically doubling the time-domain peak area in mass sensitive detectors for the same mass load), while mitigating the efficiency losses due to extra-column dispersion effects that are commonly observed with 1.0 mm i.d. columns. Here, the use of 1.5 mm i.d. columns was applied to LC/UV analysis of small molecules and LC/MS analysis of recombinant monoclonal antibodies. The 1.5 mm i.d. column exhibited a two-to-threefold improvement in analyte peak area signal for small molecules as well as for intact, subunit, and peptide levels of antibody analysis. Still, the utility of capillary columns for the analysis of recombinant monoclonal antibodies and other high molecular weight molecules has its benefits. Improvements in column performance and ruggedness along with instrumentation improvement would increase the use of capillary systems. One modern trend in capillary instrumentation is miniaturization of the equipment to such a small size that it is portable and can be used inside or outside of a laboratory. Examples of separations using such an instrument are shown.

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Chapter 1

Introduction & Methodology

Coupling liquid chromatography (LC) separations to electrospray ionization (ESI) sources for mass spectrometry (MS) detection without flow splitting requires columns with inner diameters (i.d.s) ≤ 2.1 mm [1]. In the regime of analytical-scale LC/MS separations, 1.0 mm i.d. columns are often used as an alternative to 2.1 mm i.d. These columns, due to their smaller i.d., require lower flow rates to achieve optimal linear velocity. Moreover, they generate higher sensitivity (more concentrated bands - *assuming identical mass load*) than can be achieved with larger diameters [2]. However, these columns often suffer from reduced chromatographic performance due to poorly packed beds [3,4] and the enhanced contribution of extra-column dispersion relative to intrinsically generated peak dispersion [5,6]. Recently, an intermediate column i.d., 1.5 mm, was shown to provide similar efficiency to 2.1 mm i.d. columns on currently available instrumentation [7]. As predicted theoretically, 1.5 mm i.d. columns exhibit optimal chromatographic performance (based on linear velocity) at half the flow rate of a 2.1 mm i.d. column. This has a twofold benefit in that it reduces solvent consumption and provides more favorable ESI conditions for desolvation and ionization for LC/MS analysis [8].

LC/MS has found utility in the characterization of monoclonal antibody (mAb) critical quality attributes (CQAs). For example, post-translational modifications that arise from upstream and downstream processing of mAbs [9,10] must be rigorously controlled to ensure mAb products are produced in a safe and robust manner [11].[1]

[1] Portions of this chapter were previously published as B.P. Libert, J.M. Godinho, S.W. Foster, J.P. Grinias, B.E. Boyes, Implementing 1.5 mm internal diameter columns into analytical workflows. J. Chromatogr. A 1676 (2022) 463207. They are reprinted here with permission from Elsevier.

Apart from using bottom-up LC/MS for mAb therapeutic process control, bottom-up approaches have been used to monitor the clearance of mAbs in animal and human serum via surrogate peptides. The pharmacokinetic study of mAbs and their surrogate peptides that are present in pre-clinical and clinical serum samples provides guidance on how to dose mAbs efficiently and safely. However, the surrogate peptide monitoring approach continues to experience significant challenges, including low analyte sensitivity, which can negatively affect the lower limit of quantitation [12-14].

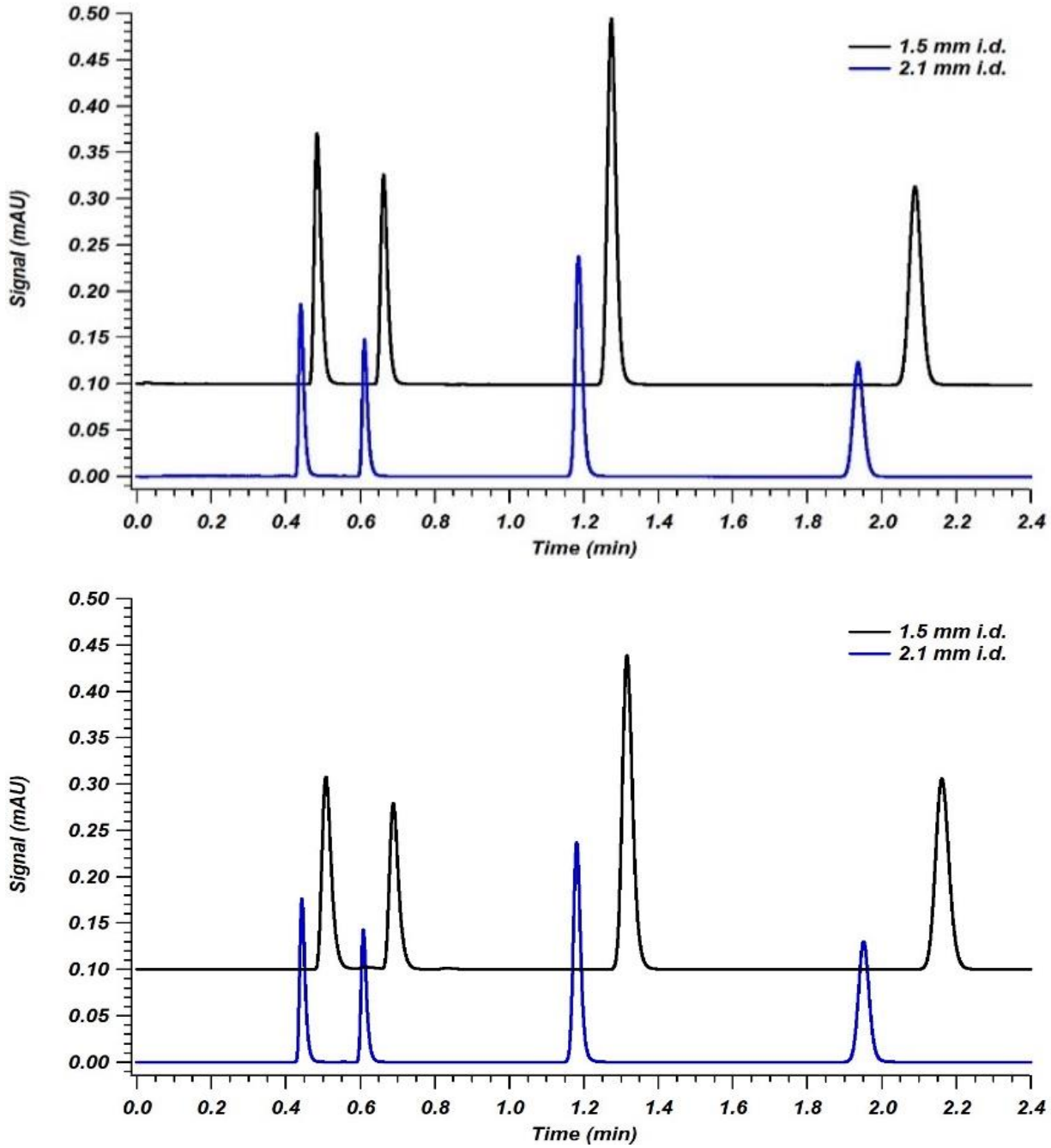
Analyte signal inherently increases with higher concentration in absorbance detection [8]. Thus, with use of a 1.5 mm i.d. column in which linear velocity is maintained at approximately half the flow rate of that with a 2.1 mm i.d. column, peaks of similar width will contain twice the concentration of analyte in the smaller diameter column if the same analyte mass is loaded onto column. This enhancement in sensitivity was explored in this study on two separate UHPLC instrument platforms. 1.5 and 2.1 mm i.d. columns were then compared by reverse phase LC/MS separations, demonstrating that an increase in MS area counts is achieved at the intact mAb, mAb subunit, and peptide levels when using the smaller diameter column under identical sample load conditions, as MS signal using ESI also increases with higher analyte concentration [15]. This enables bottom-up and top-down mAb LC/MS monitoring approaches with higher sensitivity while requiring no change in the LC/MS instrument configuration.

1.1 Analytical Scale Small Molecule Analysis

A recent study comparing the use of 1.5 and 2.1 mm i.d. columns described a small decrease in peak MS signal for analyses using the 1.5 mm i.d. column when the injected sample volumes were scaled to account for the smaller internal diameter compared to the 2.1 mm i.d. column [7]. This observation was attributed to the slightly wider peaks observed on 1.5 mm i.d. columns, which arise due to the larger impact of extra-column dispersion when using an identical instrument for both column types. The focus of this study is to compare the measured signal between these two column types when injecting identical sample volumes. In an initial test of a simple mixture with absorbance detection, by swapping the column and decreasing the flow rate by half (to maintain similar linear velocity) the analysis yielded a two-fold increase in area counts on two separate instruments (**Figure 1**).

Figure 1

Comparison of Small Molecule Isocratic Separations on the 1.5 and 2.1 mm i.d. Columns



Note: Shimadzu Nexera X2 UHPLC (top) and Thermo Vanquish Horizon (bottom).

This increase arises from the same sample mass being contained within a smaller diluent volume (lower flow rate and lower inter- and intraparticle volumes normalized for column length). Thus, the sample is less diluted on the 1.5 mm i.d. column, leading to higher concentrations and consequently higher absorbance peak area signal.

1.2 Sample Preparation and Instrument Methods

1.2.1 Absorbance Detection of Small Molecule Test Mixture

Uracil, phenol, 1-chloro-4-nitrobenzene, and naphthalene were obtained from MilliporeSigma (St. Louis, MO) for use in a small molecule test mixture. Comparison of detector signal using absorbance detection between the 1.5 mm i.d. and 2.1 mm i.d. columns was performed under isocratic conditions of 60:40 v/v acetonitrile:water. Analysis was performed on two distinct UHPLC platforms: Nexera X2 UHPLC (Shimadzu, Columbia, MD) and Vanquish Horizon UHPLC (Thermo Fisher Scientific, Germering, Germany). The 1.5 mm i.d. columns were operated at 0.2 mL/min and the 2.1 mm i.d. columns were operated at 0.4 mL/min. For both columns on both instruments, the column oven was set to 30ºC and 0.5 µL of the sample mixture was injected.

1.2.2 Intact mAb Sample Preparation

100 mM ammonium bicarbonate pH 8.0 (Sigma, St. Louis, MO) was prepared in deionized water obtained from an in-house source (also used for all other aqueous solutions in this study). The concentration of intact trastuzumab drug product (21mg/mL injectable formulation (Herceptin, Roche), from a pharmaceutical supplier) was confirmed

spectrophotometrically (ε_{280nm} = 225,000 M^{-1} cm^{-1}). The sample was buffer exchanged into the ammonium bicarbonate using a Microcon Ultracel PL-30s centrifugal filter (MilliporeSigma, Burlington, MA) spun at 10,000 rpm until most of the drug product excipients were eliminated (3-4 replicates at 5 min each) and then checked for concentration spectrophotometrically. The buffer exchanged sample was then analyzed by LC/MS with a total on-column mAb mass of 3 µg.

1.2.3 Reduced mAb Sample Preparation

Trastuzumab drug product was denatured with 6 M guanidine hydrochloride (Thermo Fisher Scientific, Waltham, MA) in 100 mM Tris HCl at pH 7.8 (Sigma, St Louis, MO) and reduced with 12.5 mM dithiothreitol (DTT, Thermo Fisher Scientific, Waltham, MA) for 45 min at 60°C. The sample was alkylated with 20 mM iodoacetamide (Thermo Fisher Scientific, Rockford, IL) in the dark for 30 min at room temperature. Excess iodoacetamide was quenched with 20 mM DTT. The solution was diluted with 100 mM Tris pH 7.8 to 1.5 M guanidine HCl. The reduced and alkylated mAb was buffer exchanged into 0.1% TFA (Thermo Fisher Scientific, Rockford, IL) using a 5,000 MWCO Vivaspin 2 centrifuge filter (Sartorius, Bohemia, NY) spun at 4,200 rpm. For LC/MS analysis, 2 µg were injected on-column.

1.2.4 Enzymatic Digestion

Trastuzumab was buffer exchanged into 100mM Tris HCl pH 7.8 using a Microcon Ultracel PL-30 centrifugal filter. FabRICATOR (Genovis, Cambridge, MA) was combined with trastuzumab according to the supplier's directions (post-exchange), then incubated for

30 minutes at 37°C. The sample was diluted into 0.1%TFA and then analyzed by LC/MS with a total on-column IdeS-digested mAb mass of 2 µg.

Trypsin Gold MS grade (Promega, Madison, WI) was combined with reduced/alkylated trastuzumab according to the supplier's directions and incubated at 37°C for 16 h. The tryptic digest was acidified with 0.5% formic acid prior to LC/MS analysis of 0.3 µg of on-column digest mass.

1.2.5 LC/MS Analysis Conditions

Separations for the intact, reduced, and IdeS-digested samples were performed with a Shimadzu Nexera X2 UHPLC system (Columbia, MD) equipped with a Halo 2.7 µm 1000 Å diphenyl column (Advanced Materials Technology, Wilmington, DE). The column temperature was maintained at 60 °C and the flow rate was set at either 0.4 mL/min (2.1 ×150 mm) or 0.2 mL/min (1.5 ×150 mm). Mobile phase A was composed of 0.1% difluoroacetic acid, DFA, (Sigma, St. Louis, MO) in water and mobile phase B was composed of 0.1% DFA in acetonitrile:n-propanol (1:1, both LC/MS grade from Sigma, St. Louis, MO). DFA was selected as the mobile phase additive based on its balance between chromatographic efficiency and enhanced ionization compared to formic acid and trifluoroacetic acid, respectively [16]. For the 1.5 mm i.d. column, the gradient started at 27%B and was programmed as follows: 0.5 min - 27% B, 40.5 min - 36% B, 41 min - 80% B, 45 min - 80% B, 45.5 min - 27% B, 50 min - 27% B. For the 2.1 mm i.d. column, the gradient started at 27%B and was programmed as follows to adjust for system dwell volume (478µL): 1.7 min - 27% B, 41.7 min - 36% B, 42.2 min - 80% B, 46.2 min - 80% B, 46.7 min - 27% B, 51.2 min - 27% B. Mass analysis was carried out on a Q Exactive

HF (Thermo Fisher Scientific, San Jose, CA) operating in full scan mode. The scan range was set to 800 to 4000 m/z at 15,000 resolution, with an automatic gain control (AGC) target of 1e6 and a max ion fill time of 250 ms. HESI II (ESI source) probe depth D was used for both columns. To minimize efficiency loss due to post-column connections [17], the column outlet was connected to the HESI source inlet with 50 µm i.d. x 600 mm length tubing (MarvelXACT, IDEX, Oak Harbor, WA).

Analysis of the tryptic digest was conducted with the same LC/MS system equipped with a Halo 2.7 µm 160 Å ES-C18 column maintained at 60 °C and operated with a flow rate of either 0.4 mL/min (2.1 ×150 mm) or 0.2 mL/min (1.5 ×150 mm). Mobile phase A was composed of 0.1% DFA in water and mobile phase B was composed of 0.1% DFA in acetonitrile. For the 1.5 mm i.d. column, the gradient started at 2% B and was programmed as follows: 0.5 min - 2% B, 60.5 min - 50% B, 61 min - 80% B, 65 min - 80% B, 65.5 min - 2% B, 70 min - 2% B. For the 2.1 mm i.d. column, the gradient started at 2% B and was programmed as follows to adjust for system dwell volume: 1.7 min - 2% B, 61.7 min - 50% B, 62.2 min - 80% B, 66.2 min - 80% B, 66.7 min - 2% B, 71.2 min - 2% B. Here, the scan range for the MS was set to 300 to 2000 m/z at a resolution of 120,000, the AGC target set to 3e6 and a max ion fill time set to 50 ms. The HESI II probe depth was set to D for all columns.

Chapter 2

Comparison of mAb Separations With Columns of Varying Inner Diameter

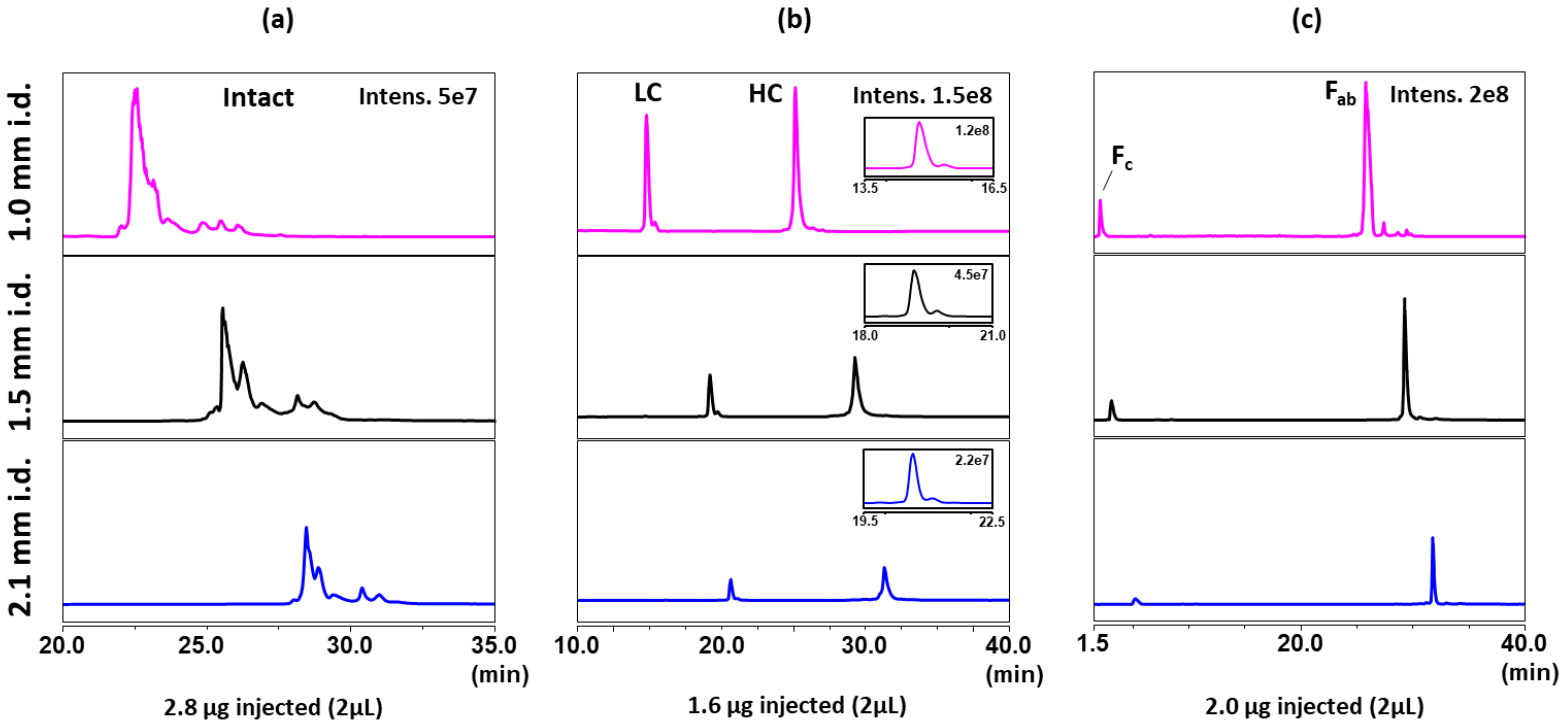
2.1 Analytical Scale Peptide and Protein Analysis by LCMS

The primary focus of this investigation was to measure differences in MS signal between 1.5 mm and 2.1 mm i.d. columns, specifically for mAb analysis with identical mass loads on column. When compared to the 2.1 mm i.d. column, the 1.5 mm column exhibited an approximately two-to-threefold increase in total ion current (TIC, 800 – 4000 m/z) integrated area counts for intact trastuzumab (G0/G0F average mass: 147,910 Da, light chain (23,446 Da, corrected for iodoacetamide groups) and heavy chain (G0F: 50,613 Da, corrected for iodoacetamide groups) trastuzumab subunits, and F_c (G0F: 25,230 Da) and F_{ab} (97,628 Da) trastuzumab subunits. The LCMS overlays exhibit evidence of this TIC increase (**Figures 2** and **3**).[2]

[2] Portions of this chapter were previously published as B.P. Libert, J.M. Godinho, S.W. Foster, J.P. Grinias, B.E. Boyes, Implementing 1.5 mm internal diameter columns into analytical workflows. J. Chromatogr. A 1676 (2022) 463207. They are reprinted here with permission from Elsevier.

Figure 2

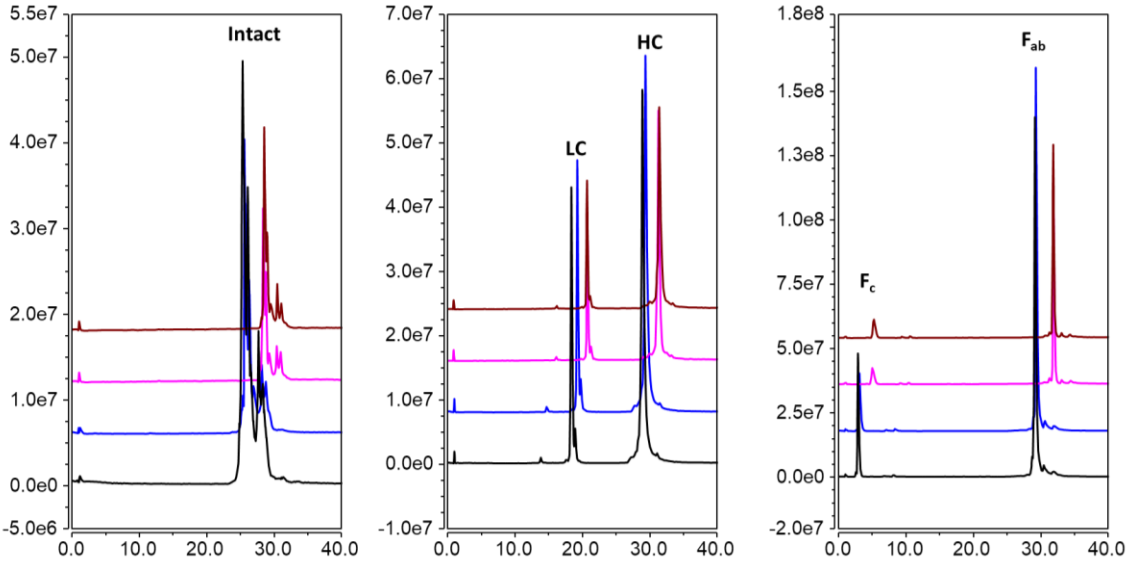
LCMS Analysis of Intact and Subunit mAb on Several Different Column Inner Diameter Formats



Note: The 1.0 × 150 mm (top, pink traces), 1.5 × 150 mm (middle, black traces), and 2.1 × 150 mm (bottom, blue traces) Halo 1000Å Diphenyl 2.7 µm columns showing MS full scan [800–4000 m/z, 3-point moving average smoothing applied] chromatograms. 2.8 µg of intact trastuzumab (a), 1.6 µg reduced and alkylated trastuzumab and inset of light-chain peak (b), and 2 µg of IdES-digested trastuzumab (c) were analyzed on each column.

Figure 3

LCMS Replicate Injections of Intact and Subunit mAb on 2.1 and 1.5 mm i.d. Columns



Note: The above total ion current chromatograms depict intact mAb (left panel); light chain (LC) and heavy chain (HC) mAb subunits (center panel); Fc and F(ab')2 mAb subunits (right panel). Within each panel, chromatograms depict separations obtained on the 2.1 mm i.d. column (pink and brown traces) and on the 1.5 mm i.d. column (blue and black).

The deconvoluted masses were nearly identical between the column diameters, with an average Δm of 0.7 Da for all peaks. The TIC area ratio (TIC Area Ratio = TIC $Area_{1.5mm}$/TIC $Area_{2.1mm}$) for intact trastuzumab was 3.3; for light and heavy chain subunits, the ratios were 2.4 and 2.1, respectively; and for the antigen-binding (F_{ab}) and crystallizable (F_c) region subunits, the ratios were 2.7 and 3.1, respectively. Due to the similarity between the inter-column charge state envelopes observed (**Figures 4-8**), the full scan range (800 – 4000 m/z) was used for the quantitative comparison of intact and subunit TIC chromatograms.

Figure 4

Intact Trastuzumab Mass Spectra From 1.5 and 2.1 mm i.d. Column Experiments

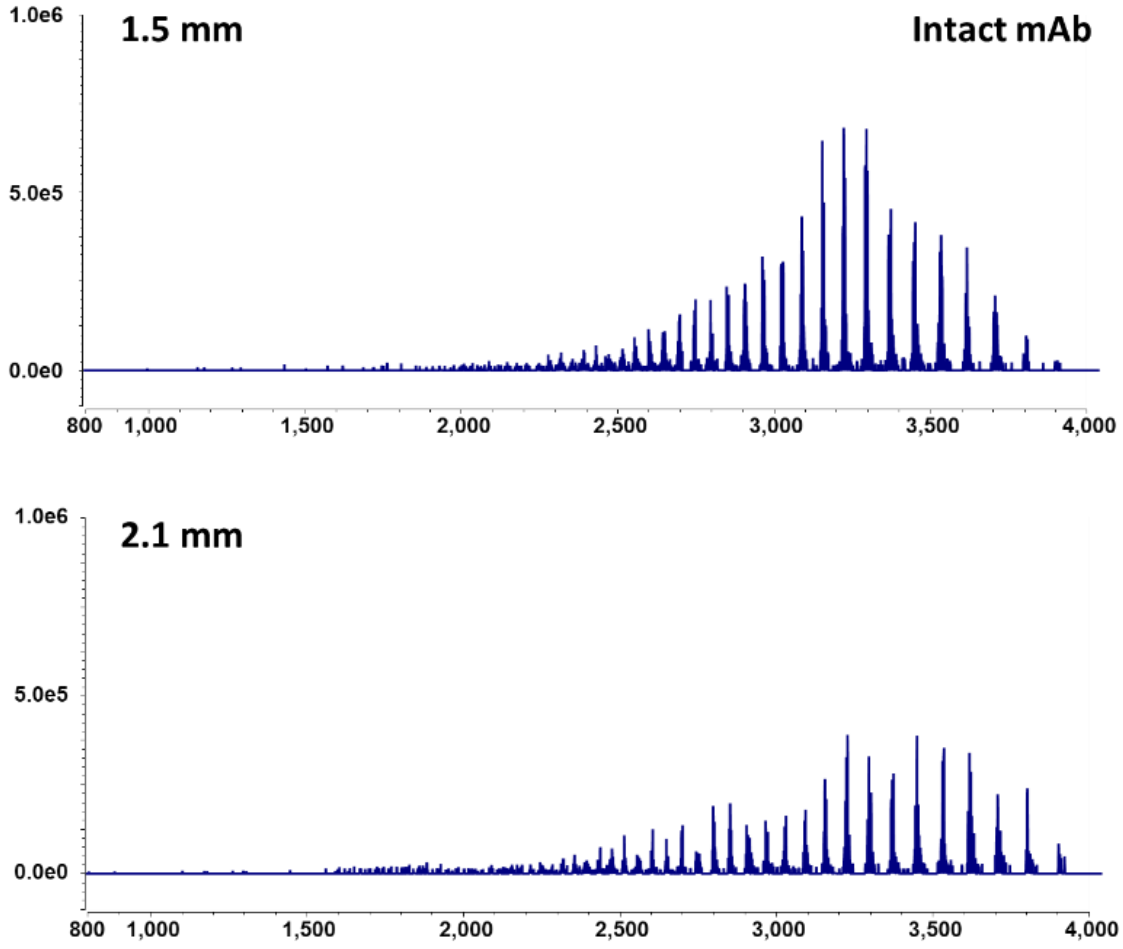


Figure 5

Trastuzumab Light Chain Mass Spectra From 1.5 and 2.1 mm i.d. Column Experiments

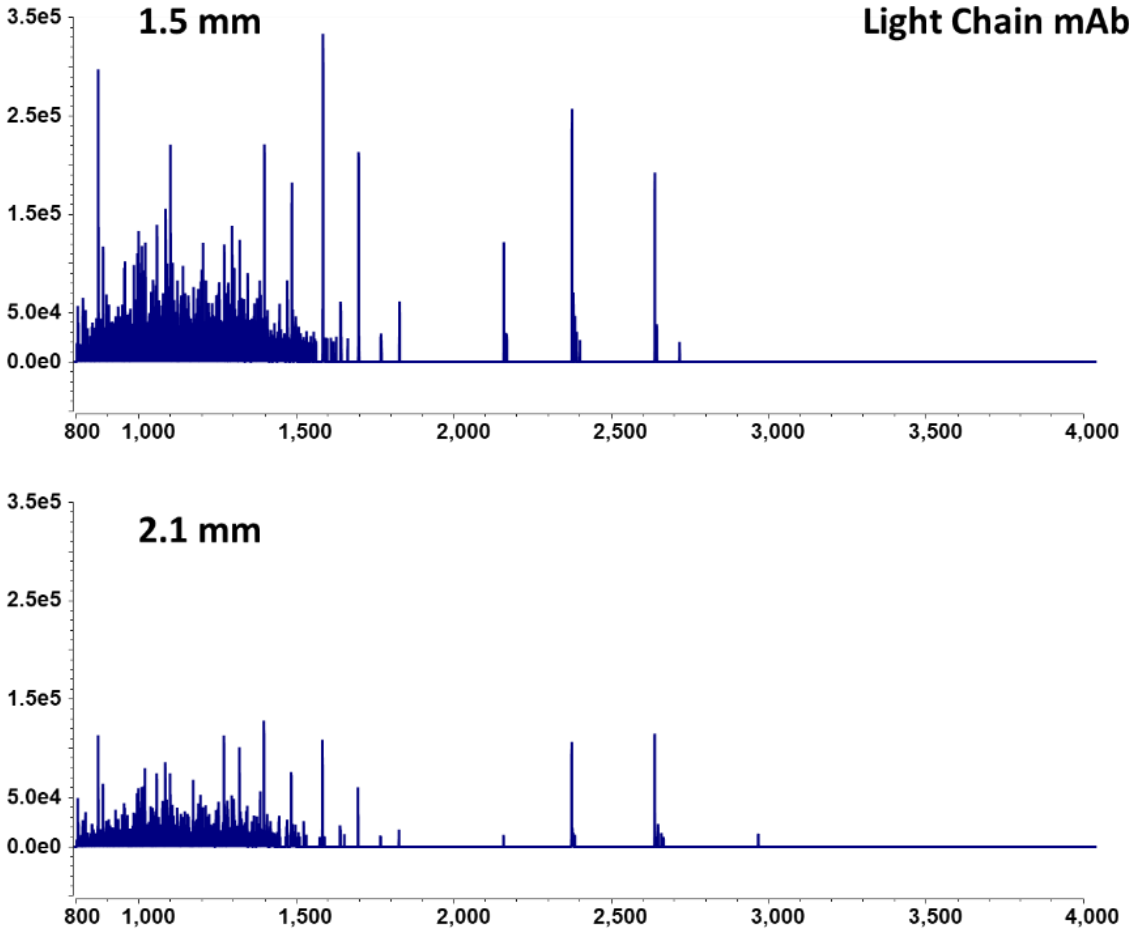


Figure 6

Trastuzumab Heavy Chain Mass Spectra From 1.5 and 2.1 mm i.d. Column Experiments

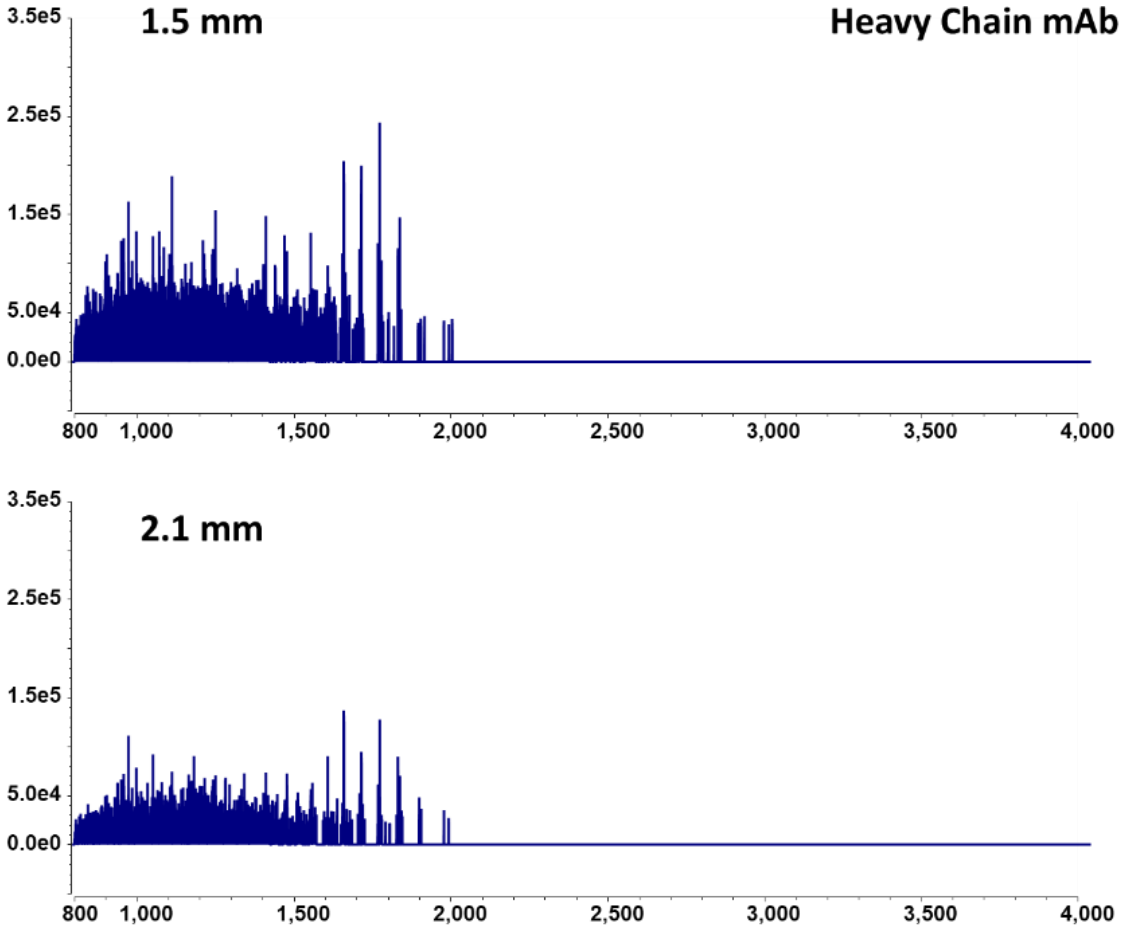


Figure 7

Trastuzumab Fc Mass Spectra From 1.5 and 2.1 mm i.d. Column Experiments

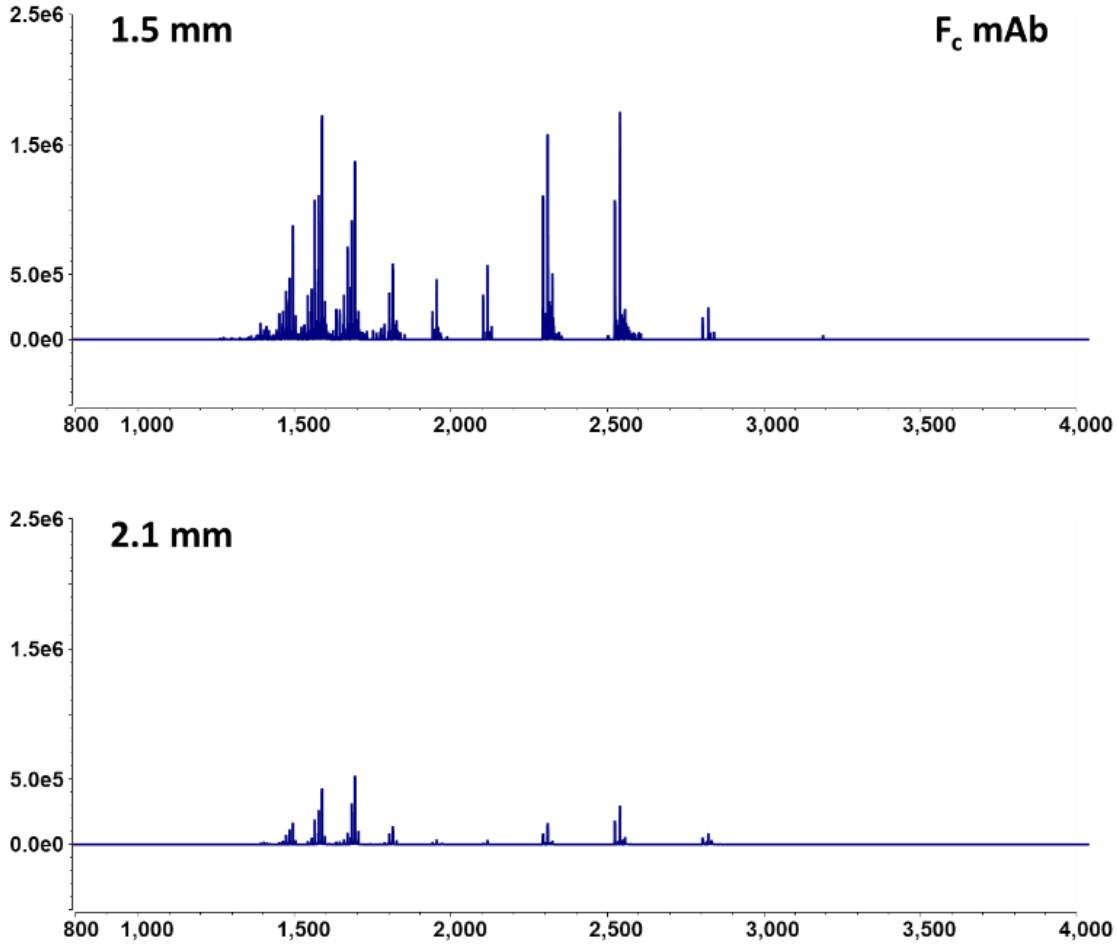
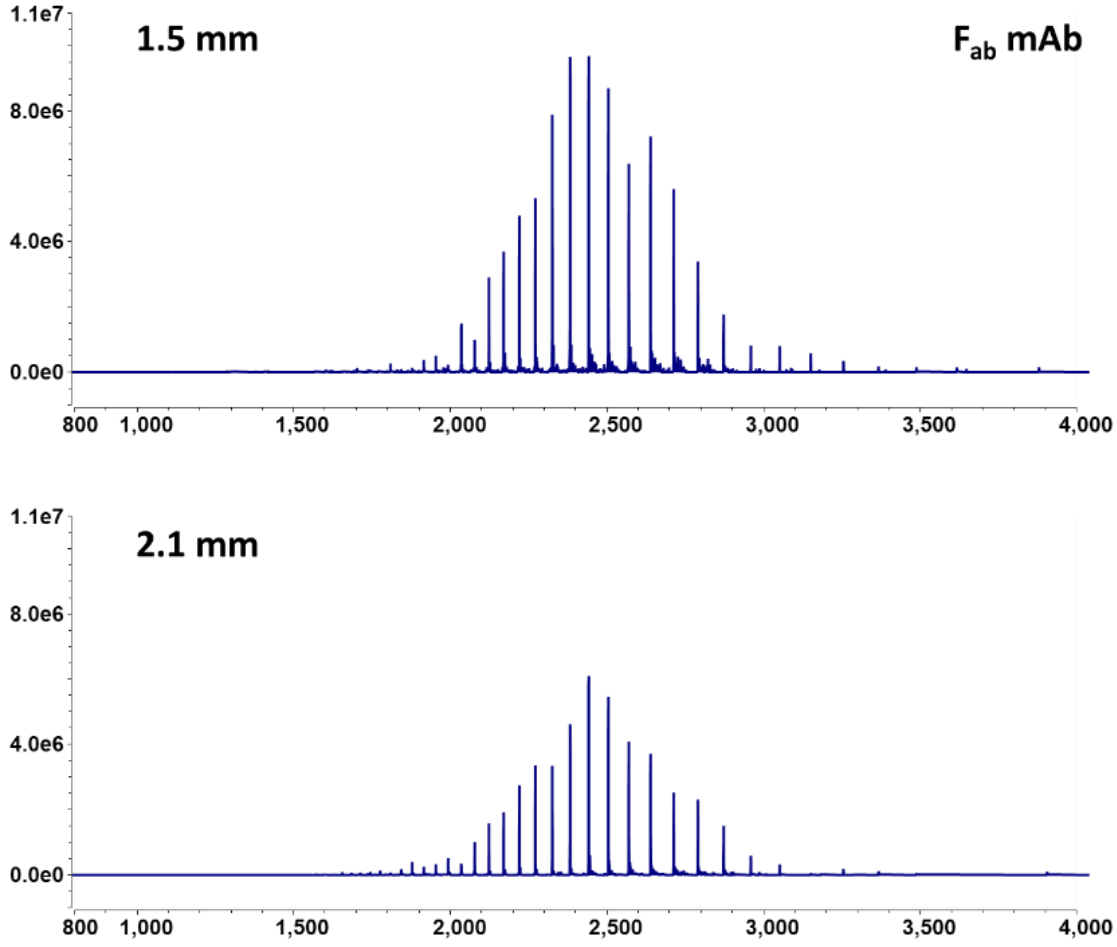


Figure 8

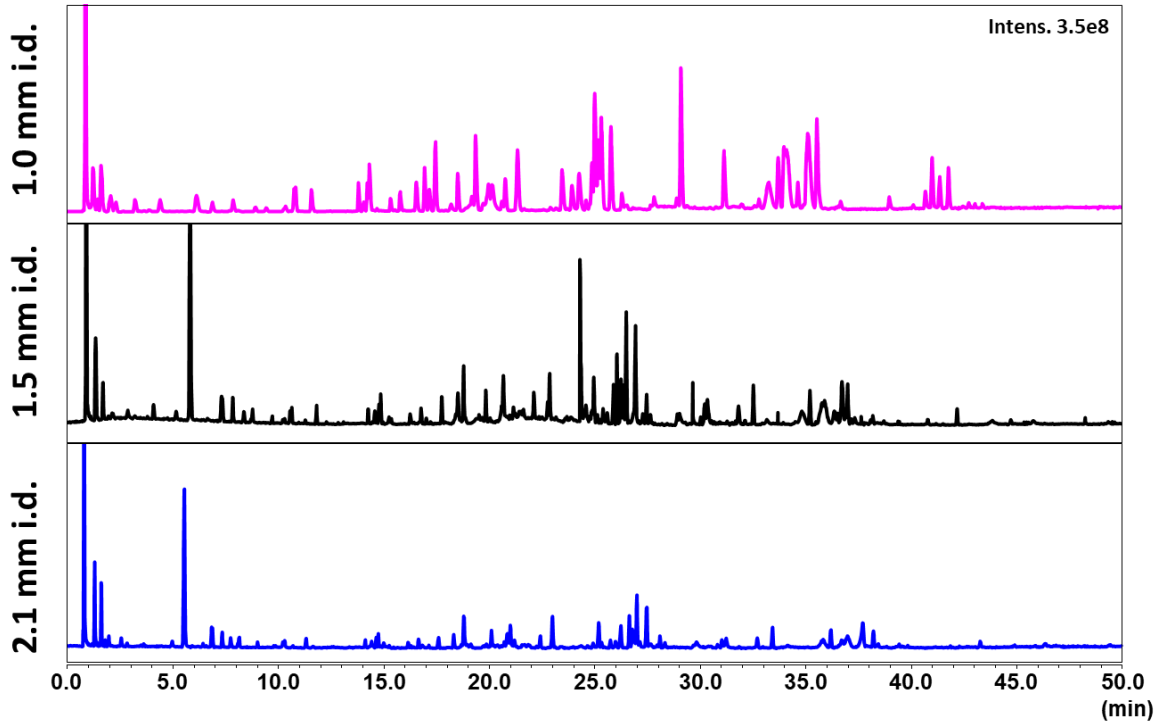
Trastuzumab F(ab')2 Mass Spectra From 1.5 and 2.1 mm i.d. Column Experiments



The deconvoluted mass results obtained from these spectra were the same on both column dimensions. Comparing the TIC chromatograms obtained for mAb tryptic peptides when using columns with different diameters was more challenging due to the complexity of the sample mixture. The MS full scan (range 300-2000 m/z) tryptic digest LCMS chromatogram (**Figure 9**) contained numerous peptides of varying sizes, many of which were not chromatographically resolved during the analysis.

Figure 9

LCMS Analysis of a mAb Tryptic Digest on Several Different Column Inner Diameters

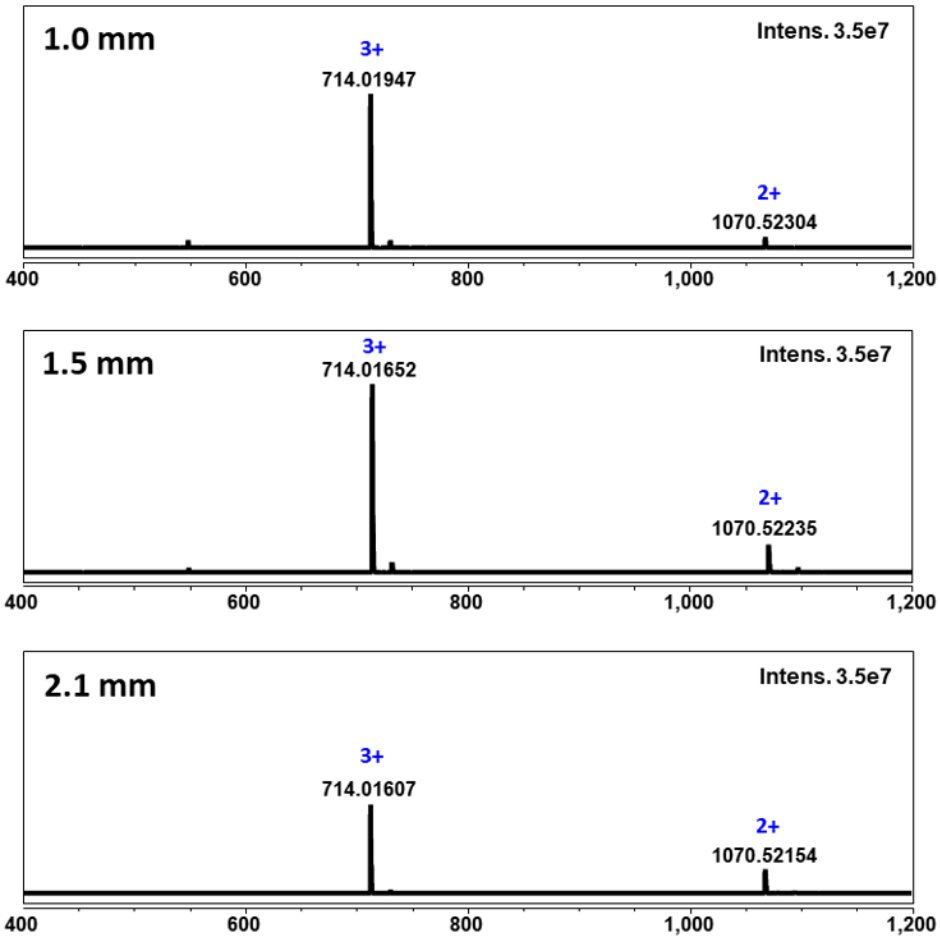


Note: Separation of 1 µg of trastuzumab digest on ES-C18 160Å 2.7 µm 1.0 × 150 mm (top, pink) 1.5 × 150 mm (middle, black) and 2.1 × 150 mm (bottom, blue) columns. Full scan total ion current chromatograms are shown (300-2000 m/z).

To simplify the comparison, inter-column peptide area ratios were calculated using extracted ion current (XIC) chromatograms. For a single given charge state and m/z value, the XIC area ratio (XIC $Area_{1.5mm}$/XIC $Area_{2.1mm}$) provided an initial comparison. However, as charge state distribution differed between each column diameter, the peak areas of the two most intense observed charge states were added to calculate a summed area ratio for several peptides from the trastuzumab tryptic digest.

Figure 10

Charge State Distribution for mAb Tryptic Peptide (R)TPEVTCVVVDVSHEDPEVK(F) on the 1.0mm, 1.5mm, and 2.1 mm i.d. Columns



Of the peptides that exhibited multiple charge states $(M+nH)^{n+}$, the 1.5 mm i.d. column often yielded tryptic peptide species with increased relative signal intensity at higher charge state occupancies for the light chain peptides (**Table 1)**.

Table 1

Trastuzumab Tryptic Peptides From the Light Chain mAb Fragment

Light Chain Tryptic Peptide	XIC $_{\text{Area Ratio}}$	Charge State (Z, Z+1)	XIC m/z (Z, Z+1)
(K)ADYEK(H)	0.7	1+	625.28, n/a
(R)FSGSR(S)	0.6	1+	553.27, n/a
(K)SFNRGEC(*)[-]	1.7	2+	435.18, n/a
(K)VDNALQSGNSQESVTEQDSK(D)	1.9	2+,3+	1068.48, 712.65
(K)HKVYAC(*)EVTHQGLSSPVTK(S)	1.8	3+,4+	714.69, 536.27
(K)VYAC(*)EVTHQGLSSPVTK(S)	1.6	2+,3+	938.47, 625.98
(R)ASQDVNTAVAWYQQKPGKAPK(L)	2.0	3+,4+	763.07, 572.55
(R)ASQDVNTAVAWYQQKPGK(A)	2.0	2+,3+	996.00, 664.32
(K)VQWKVDNALQSGNSQESVTEQDSK(D)	1.9	3+,4+	893.09, 670.06
(-)DIQMTQSPSSLSASVGDR(V)	1.9	2+,3+	939.93, 626.95
(R)TVAAPSVFIFPPSDEQLK(S)	2.3	2+,3+	973.51, 649.34
(K)LLIYSASFLYSGVPSR(F)	2.3	2+,3+	886.97, 591.65
(K)SGTASVVC(*)LLNNFYPR(E)	2.0	2+,3+	899.44, 599.96
(R)SGTDFTLTISSLQPEDFATYYC(*) QQHYTTPPTFGQGTK(V)	1.7	3+,4+	1396.62, 1047.72
(R)TVAAPSVFIFPPSDEQLKSGTASVVC(*) LLNNFYPR(E)	1.7	3+,4+	1242.29, 931.97

Note: *Carbamidomethyl. Z = charge number. Peptides are listed by increasing number (LC01 – LC15), from top to bottom.

Of the peptides that exhibited multiple charge states $(M+nH)^{n+}$, the 1.5 mm i.d. column often yielded tryptic peptide species with increased relative signal intensity at higher charge state occupancies for the heavy chain peptides (**Table 2).** In **Figure 10**, the XIC area ratio obtained for both of the heavy chain (HC) peptides HC06 and HC07 was 1.3 when Z=2 (*i.e.* $[M+2H]^{2+}$).

Table 2

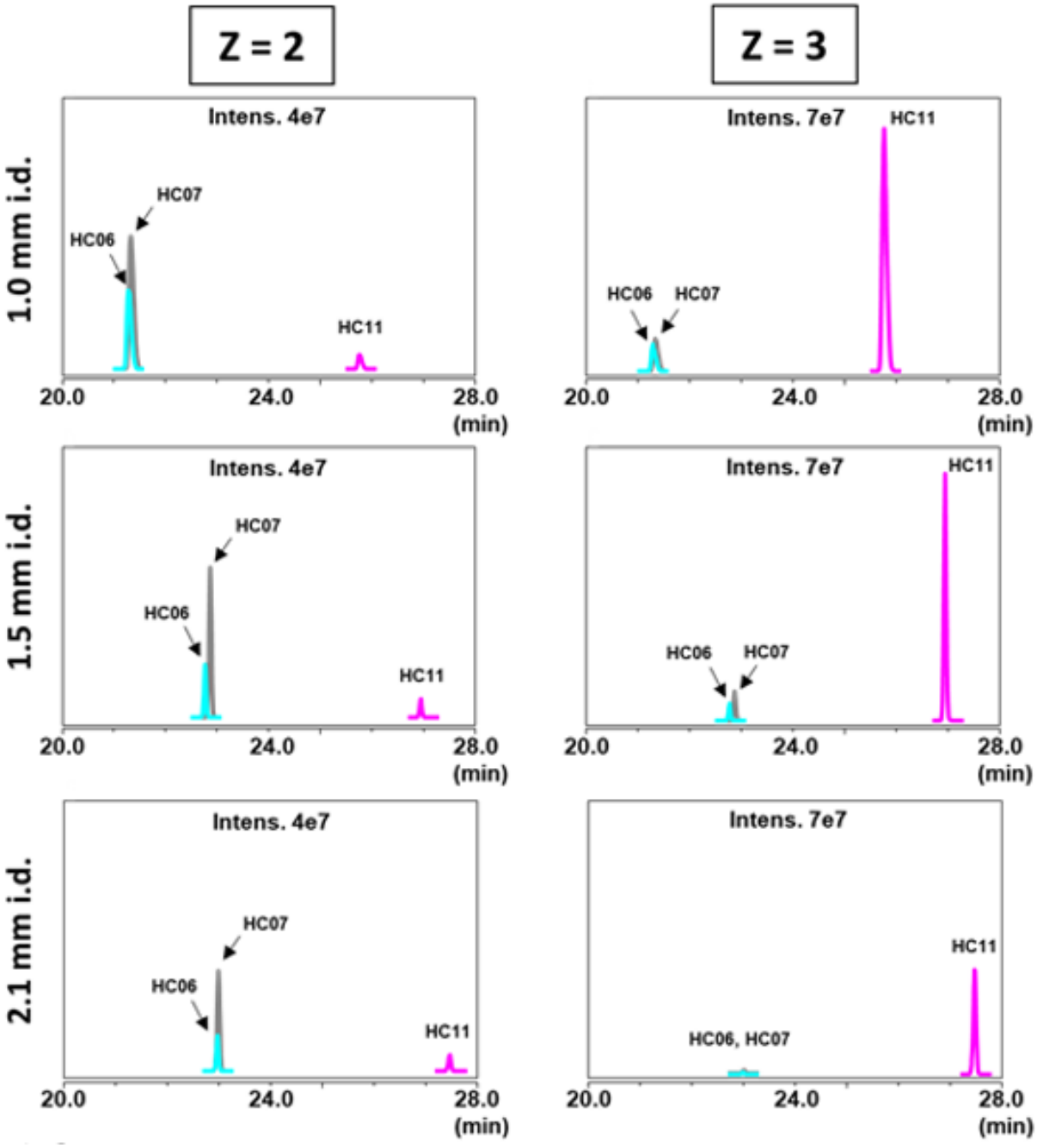
Trastuzumab Tryptic Peptides From the Heavy Chain mAb Fragment

Peptide Sequence	XIC $_{\text{Area Ratio}}$	(Z, Z+1)	XIC m/z (Z, Z+1)
(R)YADSVK(G)	0.9	1+	682.34, n/a
(R)AEDTAVYYC(*)SR(W)	1.9	2+,3+	667.78, 445.52
(K)NTAYLQMNSLR(A) oxidation	2.0	2+,3+	663.8, 442.88
(R)LSC(*)AASGFNIK(D)	1.5	2+,3+	584.30, 389.86
(R)EPQVYTLPPSR(E)	1.3	2+	643.84, n/a
[1](K)NTAYLQMNSLR(A)	1.6	2+,3+	655.84, 437.54
[2](K)STSGGTAALGC(*)LVK(D)	1.5	2+,3+	661.34, 441.22
(K)NQVSLTC(*)LVK(G)	1.6	2+	581.32, n/a
(K)GPSVFPLAPSSK(S)	1.5	2+,3+	593.83, 396.20
(R)WQQGNVFSC(*)SVMHEALHNHYTQK(S)	2.8	4+,5+	701.07, 561.05
[3](R)TPEVTC(*)VVVDVSHEDPEVK(F)	1.7	2+,3+	1070.02, 713.66
(K)TTPPVLDSDGSFFLYSK(L)	1.6	2+,3+	937.47, 625.30
(K)SC(*)DKTHTC(*)PPC (*)PAPELLGGPSVFLFPPKPK(D)	2.1	4+,5+	834.40, 667.70
(K)THTC(*)PPC(*) PAPELLGGPSVFLFPPKPK(D)	1.8	3+,4+	948.80, 711.86
(R)VVSVLTVLHQDWLNGK(E)	1.9	2+,3+	904.50, 603.30

Note: *Carbamidomethyl. Z = charge number. Peptides are listed by increasing number (HC01 – HC15), from top to bottom. [1]HC06, [2]HC07, and [3]HC11 (from Figure 10).

Figure 11

Extracted Ion Chromatograms Obtained From Z=2 and Z=3 Charge States on the 1.0 mm i.d., 1.5 mm i.d. and the 2.1 mm i.d. Columns



The XIC area ratio value increased to 8.2 and 4.9, respectively, for HC06 and HC07 when Z=3 (*i.e.* $[M+3H]^{3+}$). The summed area ratios for HC06 and HC07 were then calculated as 1.6 and 1.5, respectively. Peptide HC11 had an XIC area ratio of 0.9 at Z = 2, exhibiting similar signal on both column diameters. At the higher charge state of Z = 3, the value increased to 1.9, for an overall summed area ratio of 1.7. In general, the 1.5 mm i.d. column yielded peptides with 1.7-fold higher signal and modest increases in relative signal at higher charge state occupancies compared to the 2.1 mm i.d. column. This can likely be attributed to the lower flow rates that are used with the 1.5 mm i.d., as this parameter provides a more favorable desolvation condition for ESI, in which ion formation can proceed via an ion evaporation-like mechanism [18,19].

The value in producing peptide ions with greater intensity and at higher charge states on the 1.5 mm i.d. column may lie in the utility of performing fragmentation experiments on these higher-charge-state species. Peptide ions with $Z \geq 2$ provide structurally informative fragmentation spectra when they are collisionally-activated [20], for example, by CID or HCD. Fragmentation of peptide ions ($Z \geq 3$) by ETD also produces informative spectra [21], which can be useful in the assignment of PTM positions.

Chapter 3

Compact Capillary LC Analysis of mAbs

3.1 Protein Analysis by Compact Capillary Instrumentation

Liquid chromatography (LC) continues to be a frequently employed technique for chemical analysis of natural and synthetic small molecules, polymers, biopolymers, and a variety of other analytes of interest to society. The ability to effectively separate and quantitate a broad range of analytes in complex mixtures enables chromatographers to study a broad range of applications e.g. in the pharmaceutical, environmental, clinical, forensic, anthropologic, cosmetic, and manufacturing fields of research. Over the past six decades in which LC instruments have been commercially available, the general HPLC instrument design has remained relatively constant due to its reliability, robustness, reproducibility, and instrument lifetime. The enduring format of the LC instrument speaks to the high confidence that chromatographers have in the configuration and operation of LC systems. One progression in the development of robust, reliable LC instrumentation has been the pursuit of designing smaller, more portable LC systems. Portable LC systems provide the opportunity for chromatographers to conduct experiments in ways that reach beyond the modern confines of laboratory controls and venture into the realm of acquiring field application test results under similar sample preparation and instrumental test conditions. The reduction in physical size of a portable LC device permits the chromatographer to venture directly to sample collection sites with the portable unit and conduct chromatographic analysis. This would permit access to experiments that would otherwise be off limits to many modern lab-based analytical chromatographers, assuming

the extent to which the portable unit is under control is the same as the laboratory-based unit. Compact LCs can provide the chromatographer flexibility to improve or augment the analytical-scale workflows that dominate modern LC testing environments.

Several properties have been used to define portable LC technology, including size, weight, power source and consumption, ease of operation, and waste generation [24]. Some of these factors, including reduced weight and decreased mobile phase waste generation suggest the adoption of capillary-scale LC columns for compact and portable instruments. Operating at flow rates that are 100 – 1000 times lower than typical analytical-scale LC methods minimizes the amount of mobile phase that must be carried with the instrument and the waste that is generated during analysis. Work towards achieving a completely portable LC instrument over the past decade has focused on the development of high-pressure capillary-scale pumps [25] and detectors [26]. The assembly of these components into an integrated, portable instrument resulted in the creation of the Axcend Focus LC [27]. The original column cartridge design did not include a column oven, precluding many useful protein separations when operating under typical reversed phase LCMS conditions. Therefore, an update to the portable LC unit was made to enable the analysis of proteins at elevated temperature by reversed phase LCMS. This allowed for the analysis of a therapeutic recombinant monoclonal antibody (mAb) sample by LCMS in a controlled laboratory setting. The utility of capillary LC separations can be thought of in terms of its ability to characterize small quantities of product e.g. at the discovery phase of development or during the iterative process of product definition.

Capillary LCMS analysis was performed on trastuzumab drug product using a miniaturized, portable LC system. The mass spectra and chromatographic separations

from which the mass spectra were obtained are given in **Figures 12-18**. It is important to note that some of the mass spectra that were obtained exhibited glycosylation features (**Table 3**). These glycosylation features represent a critical quality attribute of biotherapeutic drug products. Monitoring these glycosylation features is important during mAb research and development with a particular importance placed on controlling mAb glycosylation prior to the manufacturer batch release of biotherapeutic products intended for use in human health interventions. Since the presence of varying levels of mAb glycovariants that exist in a drug product formulation have consequences on the stability, potency, immunogenicity, and bioavailability of the biotherapeutic the adoption of sensitive and rapid analytical workflows for analyzing these glycovariants by LCMS is crucial to the advancement of modern biotherapeutic drug development. Furthermore, advances in the development of other mAb applications e.g. antibody drug conjugates, bispecific mAbs, polyclonal antibodies have similar critical quality attributes that would benefit from advances in LCMS technology, enabling the characterization of small quantities of research material in the early-stages of research and product development.

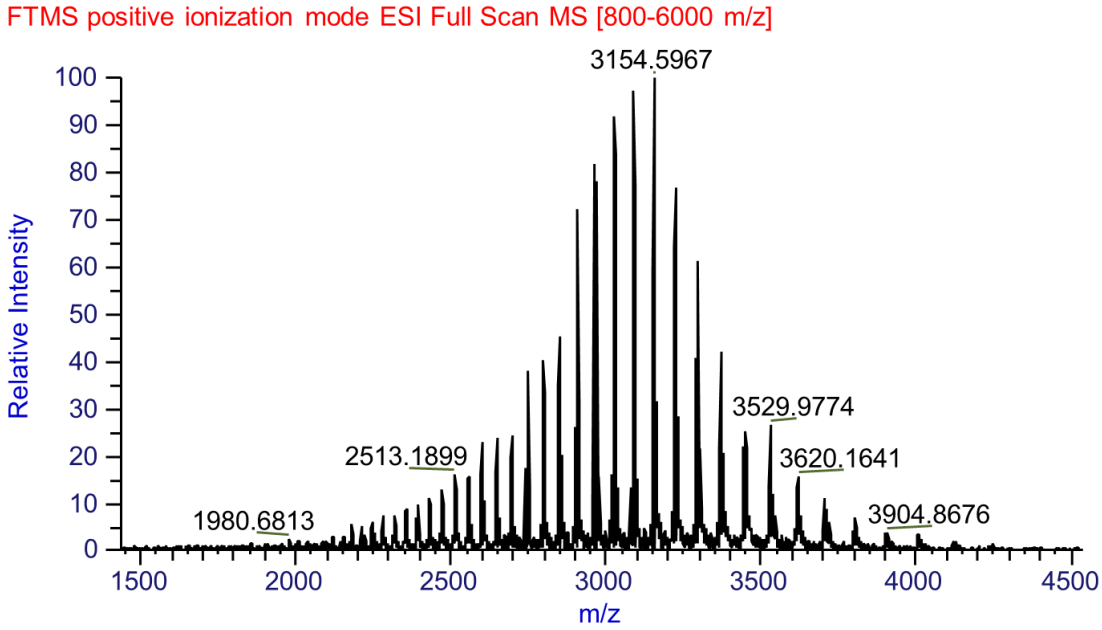
The samples were prepared as follows: IdeS control sample, (-) IdeS, was prepared at 0.5mg/mL, with 20µg of mAb in 40µL of 100mM Tris pH 7.8, 37°C for 30min; Ides sample, (+) IdeS, was prepared at 0.5mg/mL, with 20µg of mAb plus 20 units of IdeS in 40µL of 100mM Tris pH 7.8, 37°C for 30min, then the enzyme was denatured with the addition of 0.5% formic acid; 0.2mg/mL reduced mAb, 6M Guanidine HCl/10mM DTT, 100mM Tris pH 7.8, 60°C for 45min, then buffer exchanged into 5%ACN/0.1%TFA. The samples were chromatographed on a 1000Å Diphenyl

0.3x150mm 2.7µm column operated at 70°C at 7µL/min on an Axcend Focus LC (Figure 18).[3]

[3]Portions of this chapter were previously published as B.P. Libert, S.W. Foster, E.P. Gates, M. Morse, G. Ward, M. L. Lee, J.P. Grinias, Exploring Biopharmaceutical Analysis with Compact Capillary Liquid Chromatography Instrumentation. LCGC Supplements (2023) 24-27. They are reprinted here with permission from MJH Life Science.

Figure 12

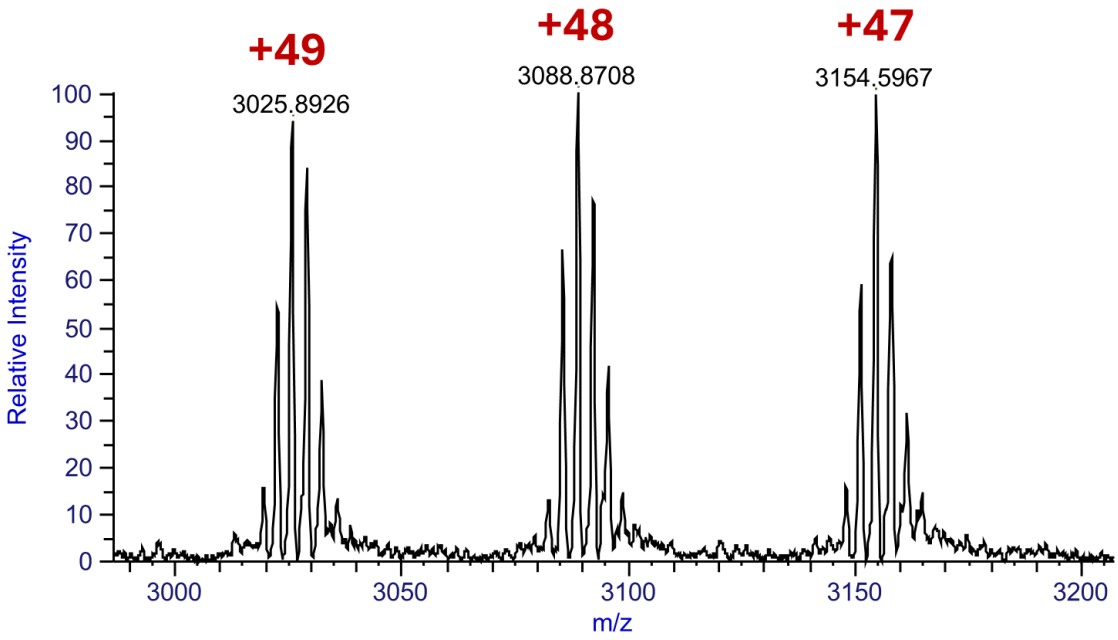
Mass Spectrum From a Capillary LCMS Analysis of Intact mAb



Note: Mass Spectra from 0.040µL injection of 0.5µg/µL (20ng on-column) intact trastuzumab separated on a 1000Å Diphenyl 0.3x150mm 2.7µm. The spectrum was obtained by averaging 25 spectra across the peak at 2.46 min. The experiment was performed on an Axcend Focus LC capillary liquid chromatography system coupled to a Thermo Q Exactive HF mass spectrometer.

Figure 13

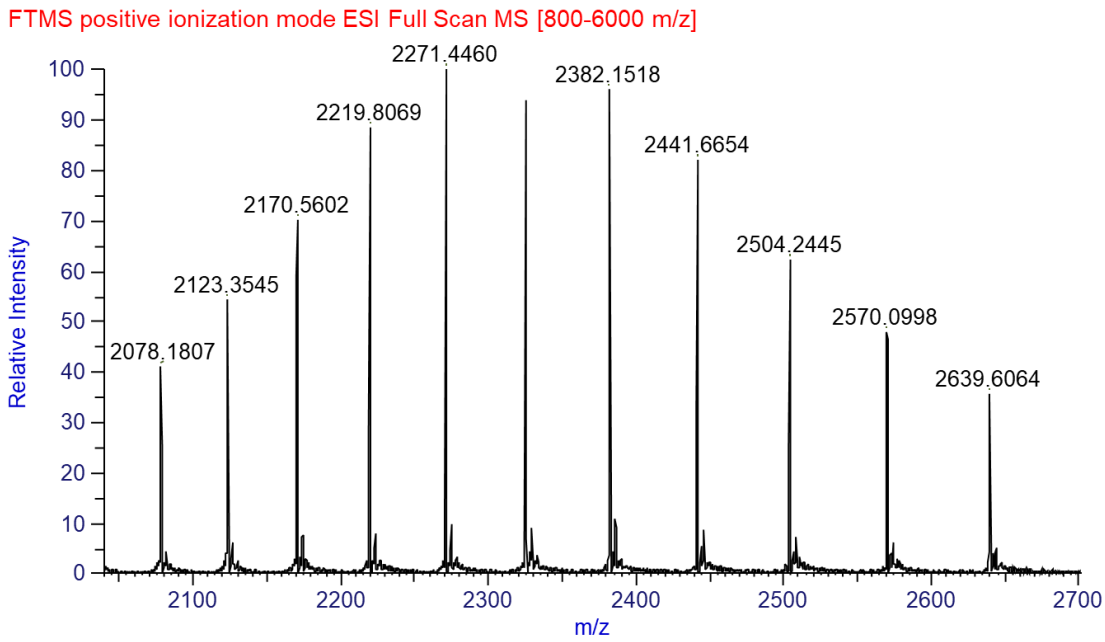
Mass Spectrum From a Capillary LCMS Analysis of Intact mAb



Note: The mass spectrum from 0.040µL injection of 0.5µg/µL (20ng on-column) intact trastuzumab separated on a 1000Å Diphenyl 0.3x150mm 2.7µm shows the glycosylation features that are present within each charge state. The spectrum was obtained by averaging 25 spectra across the peak at 2.46 min. The experiment was performed on an Axcend Focus LC capillary liquid chromatography system coupled to a Thermo Q Exactive HF mass spectrometer. Each charge state (+49, +48, +47) exhibits finger-like spikes of ion intensity within each charge state representing discrete glycosylation features that are present on the mAb (see Table 3).

Figure 14

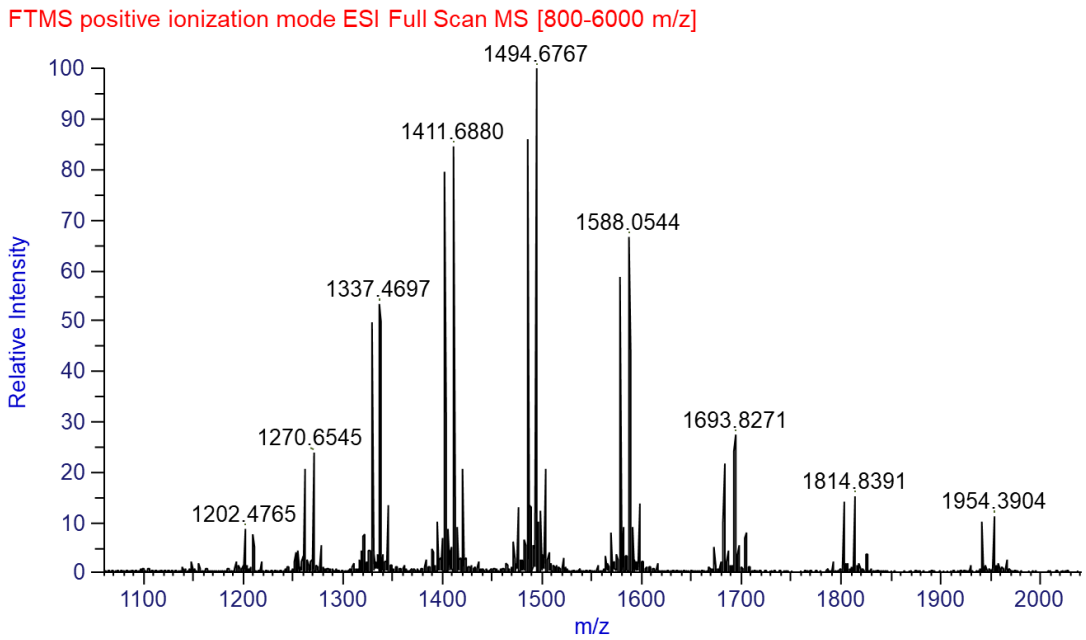
Mass Spectrum From a Capillary LCMS Analysis of mAb F(ab')2



Note: Mass Spectra from trastuzumab F(ab')2 subunit separated on a 1000Å Diphenyl 0.3x150mm 2.7µm. Average of 95 spectra displayed for peak at 4.10 min. The experiment was performed on an Axcend Focus LC capillary liquid chromatography system coupled to a Thermo Q Exactive HF mass spectrometer.

Figure 15

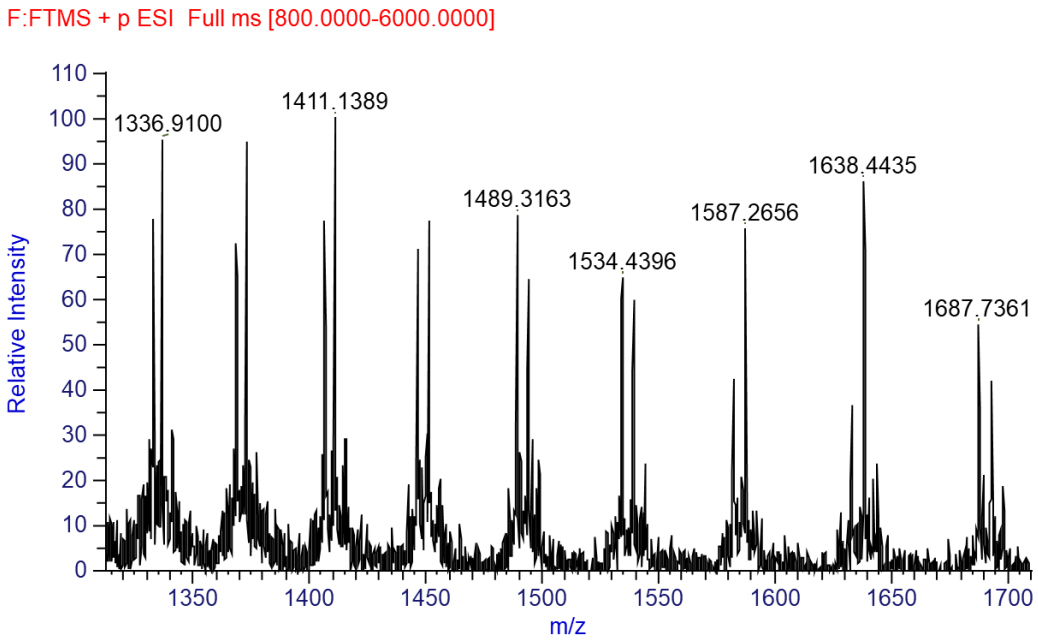
Mass Spectrum From a Capillary LCMS Analysis of mAb Fc Subunit



Note: Mass Spectra from trastuzumab Fc subunit separated on a 1000Å Diphenyl 0.3x150mm 2.7µm. Average of 40 spectra displayed for peak at 2.98 min. The experiment was performed on an Axcend Focus LC capillary liquid chromatography system coupled to a Thermo Q Exactive HF mass spectrometer.

Figure 16

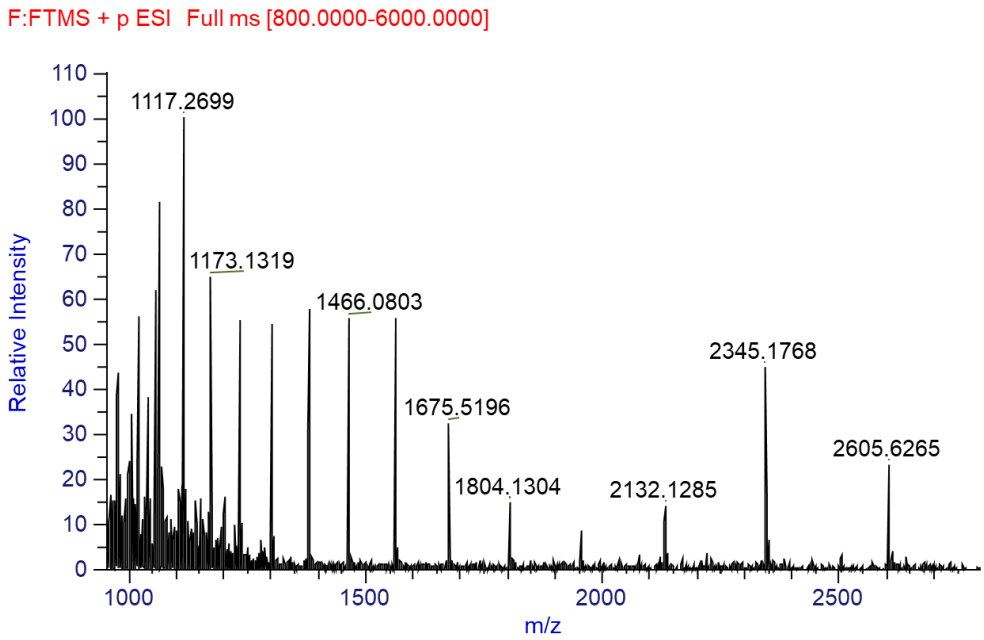
Mass Spectrum From a Capillary LCMS Analysis of mAb Heavy Chain Subunit



Note: Mass Spectra from trastuzumab heavy chain subunit separated on a 1000Å Diphenyl 0.3x150mm 2.7µm. The average of 43 spectra is displayed for the peak at 5.54 min. The experiment was performed on an Axcend Focus LC capillary liquid chromatography system coupled to a Thermo Q Exactive HF mass spectrometer.

Figure 17

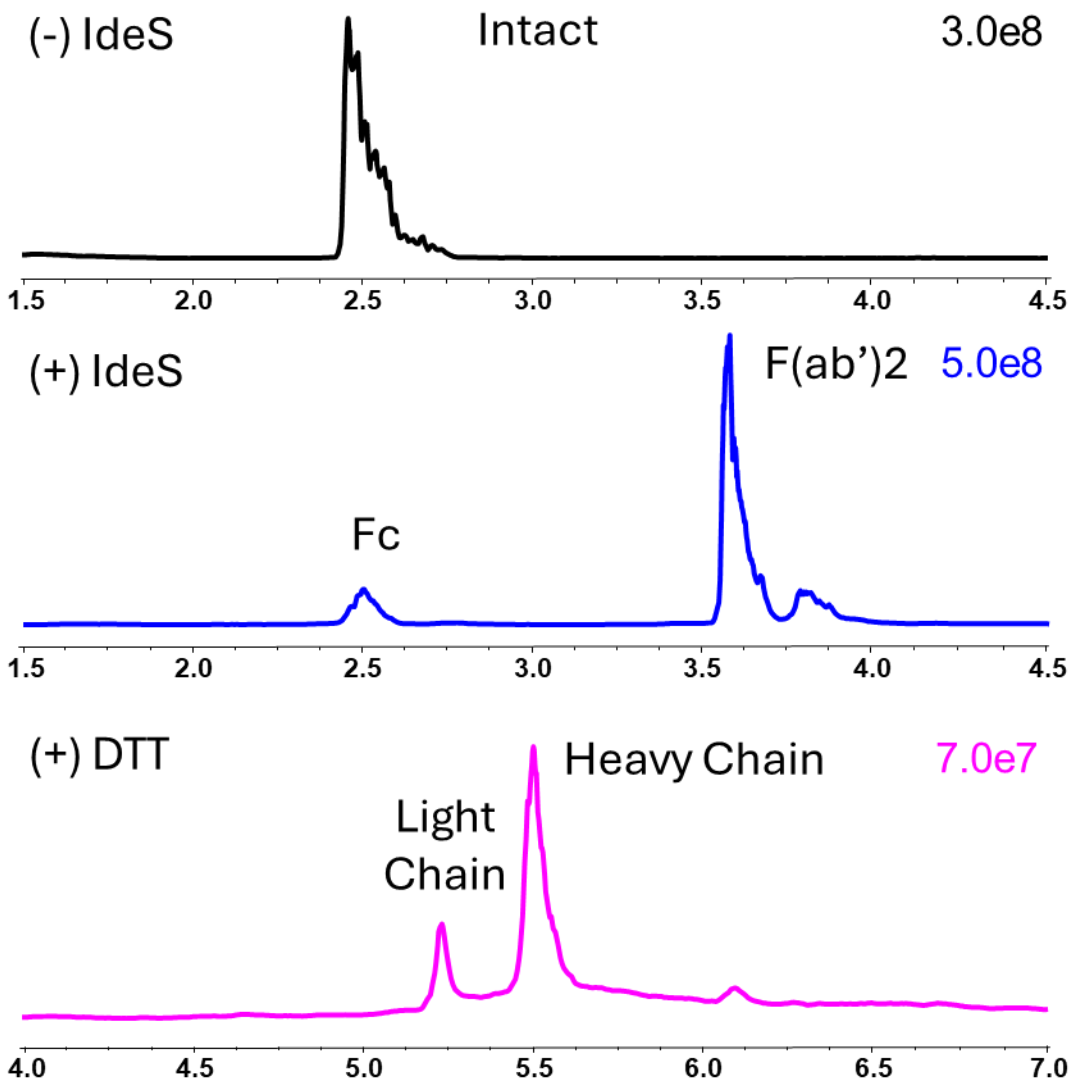
Mass Spectrum From a Capillary LCMS Analysis of mAb Light Chain Subunit



Note: Mass Spectra from trastuzumab light chain subunit separated on a 1000Å Diphenyl 0.3x150mm 2.7µm. Average of 30 spectra displayed for peak at 5.22 min. The experiment was performed on an Axcend Focus LC capillary liquid chromatography system coupled to a Thermo Q Exactive HF mass spectrometer.

Figure 18

Total Ion Current From a Capillary LCMS Analysis of Intact and Subunit mAb



Note: Total Ion Current chromatograms from intact and subunit trastuzumab separated on a 1000Å Diphenyl 0.3x150mm 2.7µm column. The experiment was performed on an Axcend Focus LC capillary liquid chromatography system coupled to a Thermo Q Exactive HF mass spectrometer. The capillary LC system was operated at 7 µL/min with gradients of 25-52.5%B over 5 min for (+) IdeS, 20-50%B over 7 min for (-) IdeS, and 10-67%B over 10 min for (+) DTT.

Table 3

Comparison of Trastuzumab Mass Spectral Deconvolution Results From Capillary and Analytical-Scale LCMS Analyses

	Capillary 0.3x150mm	Analytical 1.5/2.1x150mm
Intact mAb (G0/G0F)	147,905	147,910
IdeS digest, F_c (G0/G0F)	25,231	25,230
IdeS digest, F_{ab}	97,628	97,628
Light Chain	23,441	23,446
Heavy Chain	50,601	50,613

Note: Results of spectral deconvolution (the operation used for image or signal processing to obtain the molecular weight information from the mass spectrometry experiment) reported in units of Daltons [22-23].

Chapter 4

Conclusions

By solely reducing the HPLC column internal diameter from 2.1 mm to 1.5 mm and scaling the mobile phase flow rate to the column's optimal linear mobile phase velocity, maintaining all other system settings and instrument connections the same, a two-fold increase in UV area count for a selection of small molecule standards as well as a two-fold or greater increase in total ion current area counts was observed for therapeutic mAb analysis by LCMS. A two-fold average increase in the XIC was also observed for tryptic peptides when the contribution from the two highest peptide charge states was used to calculate a summed area ratio. By reducing the column internal diameter to 1.5 mm and operating the mobile phase flow rate at the optimal linear velocity, solvent consumption decreased by half. The ease of implementing 1.5 mm columns into analytical workflows to gain a two-fold or greater increase in UV signal or XIC/TIC for bottom-up and top-down LC/MS, can address the challenges of establishing robust methods for clinical diagnostic, drug discovery and industrial HPLC testing.

Capillary-scale chromatography columns also find utility in HPLC workflows, specifically those that evaluate small amounts of sample injected on the column. These chromatography tools have benefited researchers in the fields of drug discovery, clinical diagnostic testing, as well as many other niche areas of research including the engineering and design of the chromatography columns themselves. While scaling down the HPLC column experiment to smaller column internal diameters significantly reduces the chromatographic mobile phase consumption, one must also take into consideration the physical limitations of HPLC operation at reduced mobile phase velocities as well as the

limitations of sample loading on smaller packed beds. Reductions in the physical size of the instrumentation used for capillary column chromatography are likely to produce added benefits for further reductions of sample and mobile phase.

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