CUSTOMER APPLICATION NOTE 73423

Increasing throughput of native SEC-MS in biopharmaceutical development using a tandem UHPLC setup

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Keywords: Vanquish Duo UHPLC system for Tandem LC or LC-MS, Q Exactive Plus LC-MS/MS with Biopharma option, Orbitrap, Chromeleon Chromatography Data System software, native SEC-MS, biopharmaceutical development, monoclonal antibodies, ID, aggregation and glycoform testing in one analysis, high-throughput lead selection study, productivity

Application benefits

- Reduced analysis time and lower sample consumption through acquisition of a single analysis providing informative biomolecule identification by accurate mass, aggregation, and product variants (e.g., glycoforms)
- 5-fold throughput gains were demonstrated for candidate selection studies using native tandem LC-MS over conventional methods
- Native SEC-MS offers simpler and faster information acquisition with no sample preparation
- Native SEC-MS combines gentle analysis conditions, preserving biomolecules in their near-native structure, while resulting in high quality spectral data for intact mass analysis of large biomolecules
- A higher number of sample injections can be performed in a shorter amount of time resulting in efficient sample analysis and overall lower costs
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software with the Intact Protein Deconvolution feature provides seamless data acquisition to data processing, allowing the platform to be implemented in a QC laboratory for future compliance-ready ID tests

Goal

During early biopharmaceutical development, such as early lead selection, many samples need to be investigated to find the best candidate molecule. These are typically available in low quantities (typically less than 100 µg sample in less than 100 µL sample volume).

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Consequently, it is important to maximize analytical information from the analyses performed during early lead selection. With the recent addition of native mass spectrometry (MS) to the biopharmaceutical analytical toolbox, a broad range of liquid chromatography (LC) techniques can now be hyphenated to the MS, providing new levels of versatility for intact LC-MS analysis. In this application note, the utility of size exclusion chromatography (SEC)-MS under native conditions is demonstrated and provides the following benefits:

- Reduction of analysis time per sample using a Thermo Scientific™ Vanquish™ Duo UHPLC system for Tandem LC or LC-MS
- Obtaining information of different product quality attributes (aggregation and intact MS) in a single analysis, where previously this information content was independently obtained from more than one analysis
- Platform robustness and superior data quality

Introduction

In recent years, native MS has gained significant popularity as an analytical technique for intact mass analysis of biopharmaceuticals. One of the key strengths of native MS is the chromatographic versatility, where a multitude of different chromatographic techniques (SEC, ion exchange, affinity, and more) can be hyphenated to the MS, and information from the MS can be linked directly to a chromatographic feature (a charge variant, a fragment, etc.).[1] Another key characteristic of native MS is that the average protein ion charge state is significantly reduced, and the total ion current is distributed over significantly fewer and lower charge states, compared to denaturing MS methods.[2,3] This results in detection of higher *m/z* values and higher spatial resolution between those *m/z* peaks, providing dramatically increased overall peak capacity compared to denaturing MS.[2] Furthermore, current generations of Orbitrap™-based mass spectrometers, such as the Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer with BioPharma option, provide a very high achieved MS peak resolution (Figure 1). Native MS on the Orbitrap platform

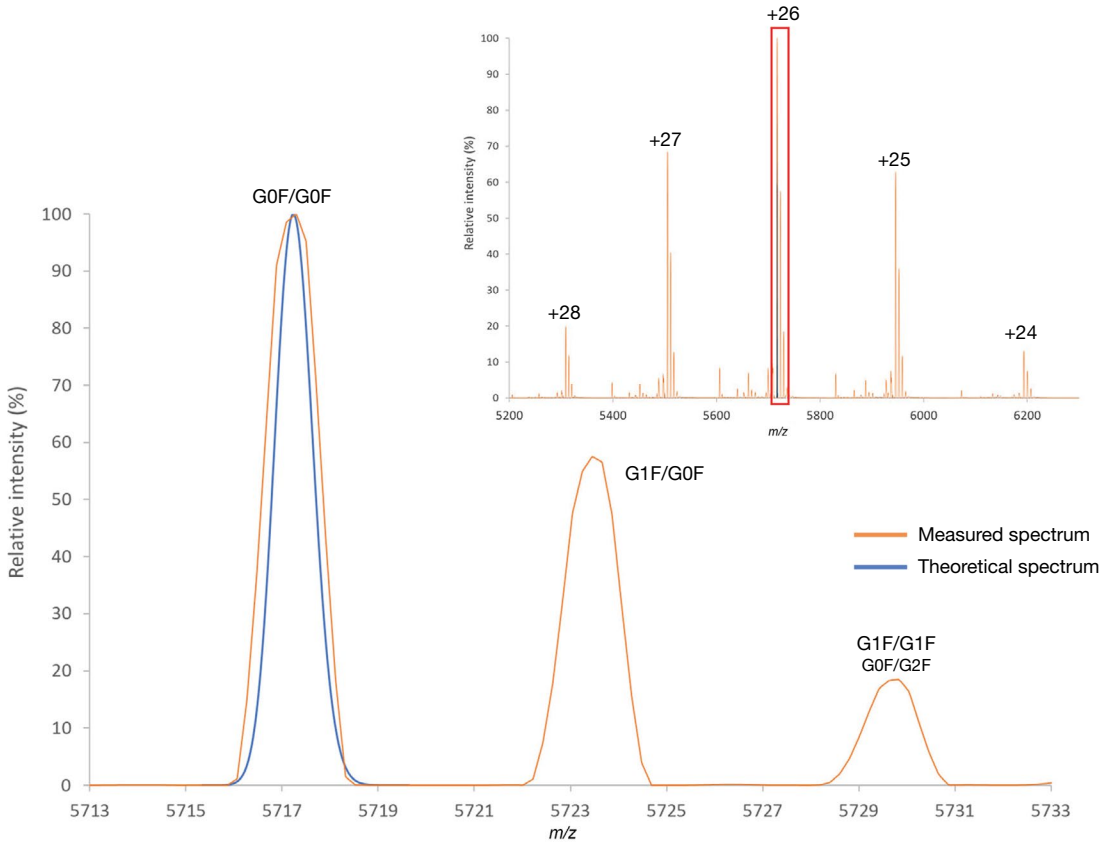


Figure 1. Raw spectra showing the charge states (upper right corner) and zoomed view of the +26 charge state of a Symphogen antibody reference sample. The reference antibody was measured by native SEC-MS on a Q Exactive Plus MS equipped with BioPharma Option. The peak shown in blue represents a simulation of the main IgG glycoform G0F/G0F (in +26 charge state) obtained using the elemental composition C6588 H10236 N1730 O2092 S46. The measured spectral peak of the G0F/G0F form wraps beautifully around the theoretical isotope distribution, illustrating the high effective resolution of the Orbitrap MS (with experimental resolution setting 35,000 at *m/z* 200).

thus combines high LC versatility with exceptional MS performance and has become an indispensable tool in biopharmaceutical development.

By combining different native chromatographic techniques with intact MS analysis, powerful insights into the proteoforms of a biopharmaceutical product can be obtained in a single experiment. For instance, charge separation via cation exchange chromatography can be coupled directly to native MS analysis and thereby provide insight into the structural nature of the charge variants (e.g. deamidation, succinimide formation, fragmentation). Native SEC-MS is particularly desirable from a chromatographic perspective, since it is based on a simple isocratic separation using a single solvent. Furthermore, native SEC-MS can provide information about soluble aggregates (from UV trace) and provide intact mass information of the monomer in a single analysis. At Symphogen, native SEC-MS has become the *de facto* standard for aggregation and intact mass analysis during early biopharmaceutical development, including lead selection studies. Native SEC-MS has replaced two independent analyses (SEC and intact MS by reversed-phase (RP) LC-MS) and has thus provided significant savings in time and sample consumption during early development. Furthermore, additional time savings and efficient utilization of MS instrument time have been obtained by implementing a Vanquish Duo UHPLC system for Tandem LC or LC-MS. In Tandem LC or LC-MS setup, one can perform analyses on two pumps and two columns all in one system at the same time. This allows the analytical gradient to be delivered on the first column with the first pump while the second pump reconditions the second column, preparing it for the next injection. Here, the implementation of native SEC-MS during lead selection studies at Symphogen is presented, with emphasis on platform robustness, data quality, as well as overall time and sample savings. The simple workflow schematic of the experimental setup is shown in Figure 2.

Experimental

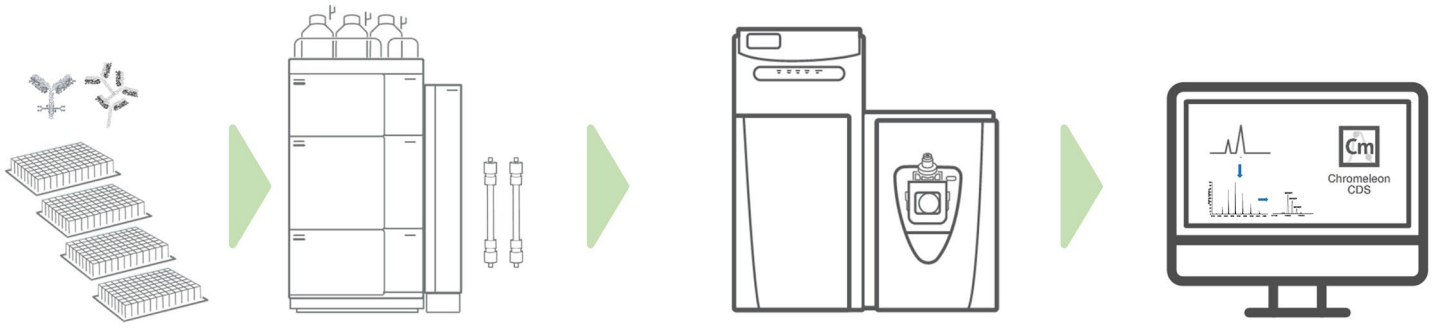
Consumables and instrumentation used in the current study are summarized in Table 1 and Table 2, respectively. No time-consuming sample preparation is required for native SEC-MS analysis. The samples are loaded directly onto the SEC column, which acts as a buffer exchanger.

Table 1. Consumables

Recommended consumables	Part number
Thermo Scientific™ Water LC-MS CHROMASOLV™	39253-1L
Thermo Scientific™ Ammonium Acetate, Optima™ LC/MS	A114-50
Sigma-Aldrich Glacial acetic acid	27225-1L-M
Commercially available SEC UHPLC column, 4.6 × 150 mm, 1.7 µm	

Table 2. Instrumentation

Instrumentation	Part number
Thermo Scientific Q Exactive Plus mass spectrometer	**0726030**
BioPharma option providing an extended mass range up to m/z 8000 for Q Exactive Plus	0726055
Thermo Scientific™ Vanquish™ Duo Horizon system consisting of:	
System Base Vanquish Horizon	VF-S01-A-02
Binary Pump H	VH-P10-A-02
Split Sampler HT	VH-A10-A-02
Vanquish Column Compartment H	VH-C10-A-02
Vanquish VWD Detector F	VF-D40-A
Vanquish Variable Wavelength - Semi-Micro Bio Flow Cell	6077.0300
Vanquish Duo for Tandem LC Workflow Kit	6036.2020
Vanquish MS Connection Kit	6720.0405



4 × 96-well plates | Vanquish Duo UHPLC System for Tandem LC or LC-MS | Q Exactive Plus with BioPharma Option | Instrument control and Data Processing in Chromeleon CDS

Figure 2. SEC-MS workflow for lead selection studies at Symphogen. In one lead selection study 4 x 96 samples are analyzed utilizing the Tandem LC setup on the Vanquish Duo system.

When conducting lead selection studies at Symphogen, a total of 384 lead candidates are typically analyzed in one sequence. A system suitability test (SST) reference is analyzed initially, then once after every 24th sample, and finally after the last sample. Typically, a total of 17 SST runs are performed in one lead selection study. Results presented here are based on the SST runs performed as part of Symphogen's lead selection studies.

LC configuration

LC settings are summarized in Table 3. Solvent A is prepared by adding ammonium acetate (1.93 g) and acetic acid (220 µL) directly to the purchased 1 L MS grade water bottle. Gently vortex the water bottle until ammonium acetate is completely dissolved and mixed. Discard solvent if unused after one week.

When installing new column(s) ramp up the flow slowly from 0.1 to 0.3 mL/min. Equilibrate the columns with at least 10 column volumes of mobile phase and until a stable UV baseline has been obtained. Perform sample injections of an SST sample until stable chromatographic performance is achieved (retention time, peak shape and resolution).

Table 3. LC settings

Parameter	Value
Mobile phase	25 mM ammonium acetate pH 5.4
Column storage solution	20 mM MES, 0.1% (w/v) sodium azide, pH 6.5
Injection wash solvent	20% ethanol
Sample load	10 µg (2–20 µg). Recommended max. injection volume is 20 µL, although up to 100 µL has been successfully injected for diluted samples
Flow	0.3 mL/min
Column temperature on both columns	Setpoint: 20.0 °C, Acceptable range: 18.0–22.0 °C
Thermostatting mode	Still air
Pre-inject wash	100 s
Post-inject wash	100 s
Max. column pressure	220 bar (3190 psi) and 300 bar (4350 psi)
Autosampler temperature	Setpoint: 5.0 °C
Detection type Primary wavelength (reporting) Secondary wavelength (characterization)	UV detection 280 nm 214 nm
Data collection rate	4.0 Hz
Response time	1.00 s
Narrowest peak width	0.100 min
Length of MS data acquisition	Single column: 8 min, Tandem LC setup: 4.7 min

The column is now ready for use. If the column will not be used for more than 24 hr it should be equilibrated with 10 column volumes of column storage solution.

A Vanquish Duo UHPLC system is set up for tandem LC-MS (Figure 3). With this LC configuration, the analysis time is 4.7 min per sample and data acquisition on the MS is active all the time. In the single column configuration, the LC analysis time is 8 min per sample and MS data is acquired for 5.5 min or 69% of the time (Figure 3). To eliminate time spent on loading samples and washing steps taking up otherwise utilizable acquisition time, a "PrepareNextInjection" command is executed 3.5 min into the run for the tandem LC column setup, which ensures that the pre-injected sample introduced into the LC flow for separation and data acquisition is started after exactly 4.7 min.

MS configuration

Native MS data was acquired on a Q Exactive Plus mass spectrometer equipped with Biopharma Option using the settings shown in Table 4. For a detailed comparison on settings for the analysis under denaturing and native conditions please refer to Application Note 72348.[2] The Vanquish Duo tandem column configuration allows for 100% MS utilization by acquiring data at all times while sample flow results from alternating between columns.

Table 4. MS settings

Scan parameter	Setting
Scan type	HMR – Full MS
Scan range	*m/z* 2500 to 8000
Fragmentation	In-source CID 130.0 eV
Resolution	35,000
Polarity	Positive
Microscans	10
Lock masses	Off
AGC target	3e6
Maximum inject time	200
HESI source	
Setting	**Value**
Sheath gas flow rate	25
Aux gas flow rate	5
Sweep gas flow rate	-
Spray voltage	4.20 kV
Spray current	-
Capillary temp.	275 °C
S-lens RF level	200.0
Aux gas heater temp.	175 °C

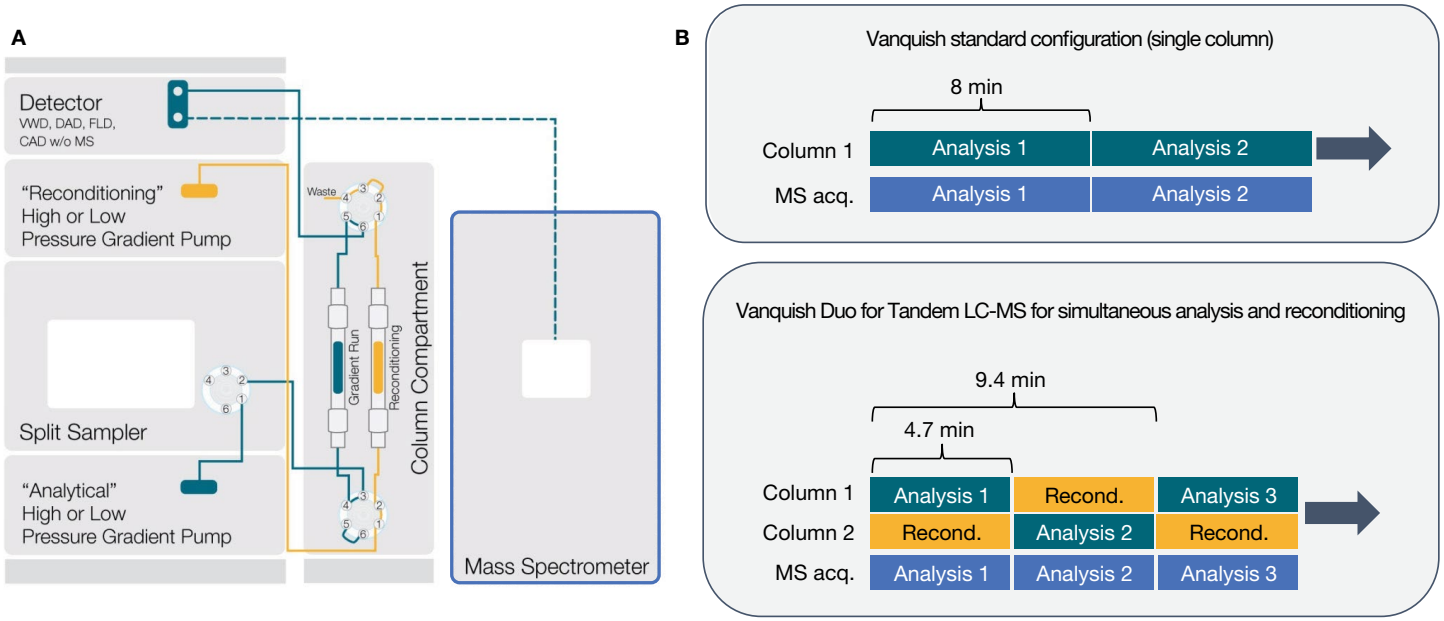


Figure 3. Vanquish Duo LC configuration and analysis time. A) In the tandem LC-MS configuration two columns are operated at the same time. The analytical pump delivers flow to the active column, while the reconditioning pump delivers flow to the inactive column. B) In a standard single column configuration analysis time is 8 min per sample. In the tandem LC configuration analysis time is 4.7 min per sample.

Chromatography Data System

Chromeleon CDS version 7.2.9 with the Intact Protein Deconvolution tool was used for MS data acquisition and processing using the settings shown in Figure 4.

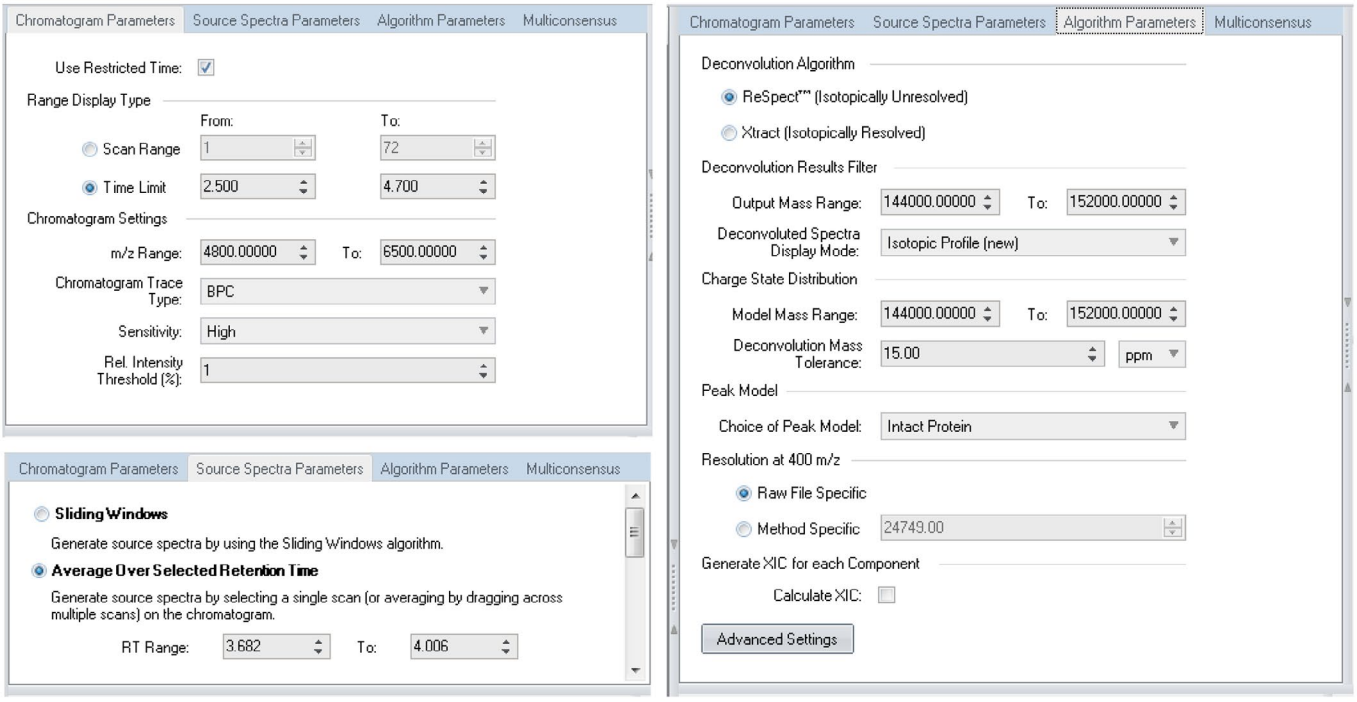


Figure 4. Processing method settings for the intact protein deconvolution of native SEC-MS data in Chromeleon CDS

Native SEC-MS provides simultaneous information on aggregation level from UV trace and identity from intact mass (Figure 5). The identity was confirmed by comparing the measured mass with the theoretical mass in a Chromeleon CDS report. The most abundant IgG species is generally the G0F/G0F glycoform for IgGs expressed in a Chinese hamster ovarian (CHO) cell-based expression system. Consequently, the theoretical mass provided for mass comparison represented the IgG G0F/G0F glycoform.

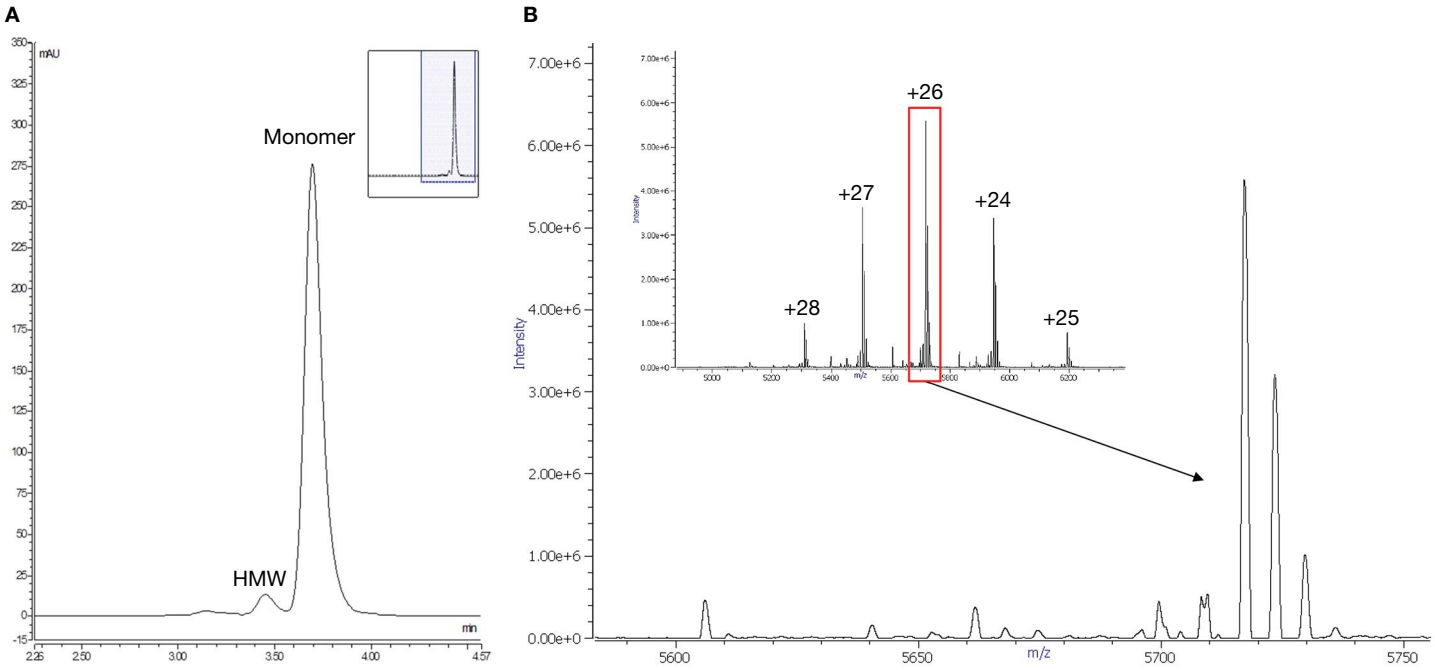


Figure 5. Native SEC-MS data for reference antibody. A) UV trace used for relative quantitation of high molecular weight (HMW) forms. B) Average mass spectrum for the chromatographic monomer peak. The non-zoomed view illustrates the different charge states of the SST antibody. A zoomed spectrum of the +26 charge state is shown for the SST antibody.

Results and discussion

Native SEC-MS robustness

Chromatographic and MS performance was evaluated for a lead selection study, which included native SEC-MS analysis of 384 lead candidates. Chromatographic performance is illustrated in Figure 6, which contains an overlay of 17 SST runs performed in a lead selection study (consisting of 423 runs in total). No normalization has been performed. Chromatographic performance was found to be robust, with RSD values well below 1% for the retention time and relative peak area of the monomer.

The Smart Inject™ feature with sample pre-compression enables excellent column performance and significant increase in the number of injections per columns.

MS performance is illustrated in Figure 7. Reviewing the determined masses for the SST monomer across the lead selection study (17 SST runs amongst 423 total runs), the mass deviation generally fell within ±1.5 Da (±10 ppm) of the theoretical mass. Both chromatographic performance and MS performance were robust across the lead selection study.

Peak property	Average	RSD
Monomer RT	3.87 min	0.04%
Monomer relative peak area	94.1%	0.19%
HMW relative peak area	5.94%	2.94%

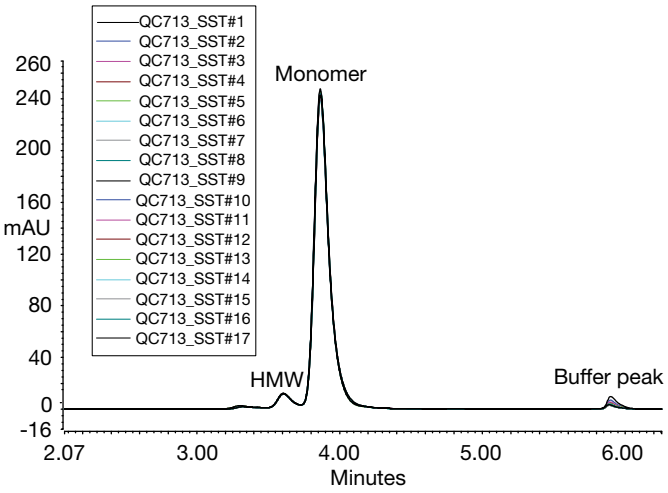


Figure 6. Overlay of 17 reference material (QC713) runs from a lead selection study. A total of 384 lead candidates were analyzed and the SST was analyzed once for every 24th sample. A total of 423 runs were performed in the lead selection study. The inserted table includes the average value and RSD for monomer retention time, as well as for relative peak areas of monomer peak and high molecular weight (HMW) peak.

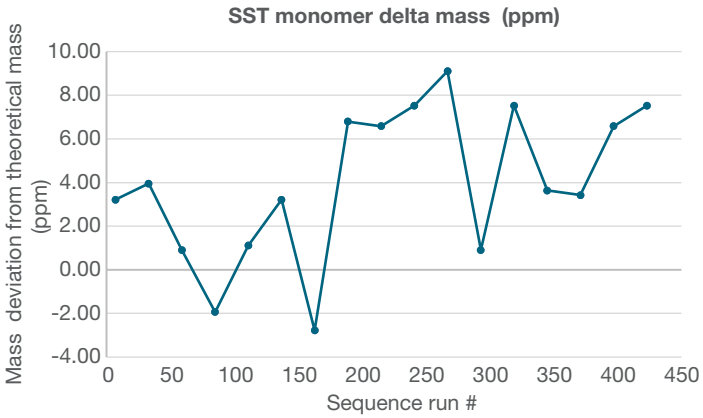


Figure 7. Results for 17 system suitability (SST) runs from lead selection study including 384 lead candidates. The lead selection study included a total of 423 native SEC-MS runs. Here the mass deviation in ppm is shown for the 17 SST runs

To date, multiple lead selection studies have been performed successfully using native SEC-MS at Symphogen, and close to 2000 unique antibodies have been successfully analyzed on this platform.

Native SEC-MS throughput and data quality

Prior to implementation of native SEC-MS at Symphogen, lead selection studies required two independent analyses; 1) SEC for aggregation and 2) intact mass analysis by RP-LC/TOF-MS. Total sample consumption was 15 µg (10 µg for SEC and 5 µg for intact MS) and total combined analysis time was 23 min (8 min for SEC and 15 min for intact MS). The SEC analysis and intact RP LC-MS analysis had to be carried out consecutively by transferring 96 microwell plates between instruments, due to limited sample quantities. With the establishment of native SEC-MS at Symphogen, a single analysis now provides the information (aggregation from UV and ID from intact mass) previously requiring two independent tests. Using a conventional single column setup native SEC-MS takes 8 min, i.e. a 65% reduction (8 min versus previous 23 min) in analysis time was achieved. At the same time sample consumption was reduced by 33% (10 µg versus previous 15 µg). Analysis time was further reduced by switching to a tandem LC configuration on a Vanquish Duo UHPLC system, resulting in an effective analysis time of 4.7 min per sample. An overview of throughput and analysis time for a single sample and an entire 384 well plate worth of samples is shown in Table 5.

The introduction of native SEC-MS and tandem LC configuration in summary leads to significant reductions in analysis time and reduction in sample consumption. In addition, at Symphogen the transition from TOF MS to Orbitrap MS has had a major impact on spectral data quality. Current generation Orbitrap instruments, purposely built for analysis of large biomolecules, provide intact protein mass spectra with exceptional achieved resolution and mass accuracy (Figure 1, Figure 5, and Figure 7). As a consequence, more spectral features can be assigned with increased confidence, particularly for low abundant

Table 5. Analysis time and sample consumption during early lead selection at Symphogen

Technology platform	Sample consumption	Analysis time min/sample	Analysis time lead selection study 384 lead candidates
SEC + intact RP LC-MS (pre-native SEC-MS)	15 µg (10 + 5 µg)	23 min (8 + 15 min)	158 hr
Native SEC-MS (single column configuration)	10 µg	8 min	56 hr
Native SEC-MS (tandem LC-MS configuration)	10 µg	4.7 min	32 hr

spectral features, which tend to get lost in the signal background typical for ToF-based mass spectrometers. Due to the low noise level typical for Orbitrap-based MS spectra, differences in the overall quality and in particular the appearance of peaks and peak patterns in the spectra before and after deconvolution are minimal. In conclusion, the deconvolution step has only minimal effects on resulting spectra (Figure 8).

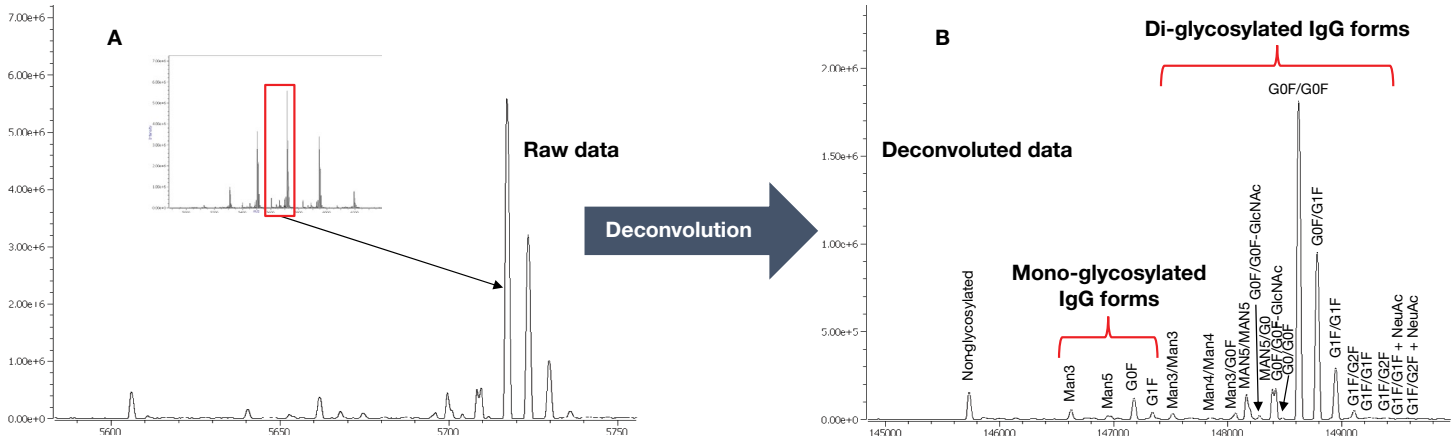


Figure 8. Native SEC-MS acquired and deconvoluted data for reference antibody. A) Spectrum of the +26 charge state of the SST antibody with high quality spectral data (e.g., low background, baseline resolution between glycoforms). B) Processed spectrum of the acquired data showing minimal effects of deconvolution on the spectral quality.

Conclusion

At Symphogen the implementation of native SEC-MS using the Vanquish Duo UHPLC system has radically reduced analysis time during early lead selection studies. Simultaneously, intact MS data of excellent spectral quality is obtained on the Q Exactive Plus MS system with BioPharma Option. This application shows how a high-throughput candidate lead selection study of 384 mAbs can be reduced from 158 hours of analysis time, down to just 32 hours of analysis time; almost 5-fold throughput gain on a single LC-MS system. Choosing Chromeleon CDS, seamless data acquisition and data processing are achieved, allowing the platform to be transferable into a QC laboratory for future compliance-ready ID tests.

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