Mass Spectrometry & Spectroscopy

Accelerating Biopharmaceutical Development with Size Exclusion Chromatography Mass Spectrometry

Dan Bach Kristensen*, Trine Meiborg Sloth, Martin Ørgaard, and Pernille Foged Jensen. Symphogen, Ballerup, Denmark
Corresponding Author - Dan Bach Kristensen dbk@symphogen.com

Intact mass spectrometry (MS) is a simple, fast and specific tool, that can provide structural insight into the proteoforms or structural variants of large protein biopharmaceuticals, including the most common class of biopharmaceuticals, recombinant monoclonal antibodies (mAbs). For instance, the glycoforms of recombinant monoclonal antibodies can be effectively analysed by intact MS. With the recent introduction and fast industrial adaptation of native MS, a multitude of chromatographic techniques can now be hyphenated to the MS for intact mass analysis, thus adding great versatility to the intact MS toolbox. Examples of chromatographic techniques used for native MS include ion-exchange chromatography, affinity chromatography (e.g., Protein A) and size exclusion chromatography (SEC). This article reports on the implementation and routine application of native SEC-MS in a biopharmaceutical development laboratory. Focus is on the application of native SEC-MS during early development (primarily lead selection studies), where analytical throughput and low sample consumption are important. Finally, emphasis is placed on the orthogonal information simultaneously provided by native SEC-MS, which is currently used as 1) an identity test, 2) a test for soluble aggregates and fragments and 3) a test for glycoform distribution.

Introduction

In recent years, native MS has gained significant popularity as a tool for intact mass analysis of biopharmaceuticals [1–7]. One of the key strengths of native MS is the chromatographic versatility, i.e., a multitude of chromatographic techniques (SEC, ion exchange chromatography, affinity chromatography and more) can be hyphenated to MS, which means that MS results can be coupled directly to a chromatographic peak (charge variants, fragments, etc.) [8–12]. For instance, native affinity MS was used to demonstrate how different mAb glycoforms bind with different affinities to the FcγRIIIa receptor, and thus elicit different antibody-dependent cell-mediated cytotoxicity (ADCC) responses [13]. Another

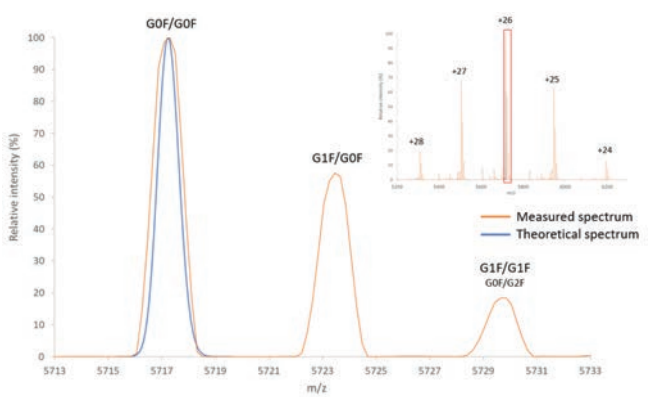

Figure 1. Raw spectra showing the charge states (upper right corner) and zoomed view of the +26 charge state of a Symphogen antibody reference sample.

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).

key characteristic of native MS is the average protein ion charge state is significantly reduced and total ion current is distributed over significantly fewer charge states, compared to denaturing MS methods [14, 15]. This results in a simpler mass spectrum with a higher mass separation and dramatically increased overall peak capacity compared to denaturing MS [14]. Modern Orbitrap mass spectrometers which have been purpose built for the analysis of intact biopharmaceuticals in native mode, such as the Q Exactive Plus/HF and Exploris 480 with Biopharma option (Thermo Scientific), have a very high effective (i.e., measured) resolution as shown in *Figure 1*. Native MS on the Orbitrap platform combines high LC versatility with high MS performance, and has become a valuable tool for biopharmaceutical characterisation.

Native SEC-MS has gained good traction as a tool for biopharmaceutical characterisation [10, 11] and is particularly attractive from a chromatographic perspective, since it is based on a simple isocratic separation using a single solvent, i.e., the chromatographic setup is simple and robust. Furthermore, native SEC-MS can provide orthogonal information about 1) soluble aggregates/fragments (from a UV trace), 2) identity from intact mass information and 3) glycoform distribution from spectral ion intensities in a single analysis. The intact mass information can be effectively used for identity testing, and for identification and quantitation of biopharmaceutical proteoforms, such as the glycoforms of a mAb biopharmaceutical. To obtain a native SEC-MS spectra for recombinant reference mAb, Symphogen employed a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer with Biopharma Option, enabling confident assignment of low abundance glycoforms and glycoforms differing by as little as 25 Da. Furthermore, analysis by native MS provides information at the intact protein level, i.e., the distribution of non-glycosylated, mono-glycosylated and di-glycosylated mAb forms can be evaluated on this platform. This information is lost with alternative approaches, such as the analysis of enzymatically released glycans.

Symphogen utilise native SEC-MS as the de facto standard for aggregation and intact mass analysis during early biopharmaceutical development, including lead selection. Native SEC-MS has replaced two separate analyses [SEC and intact MS by reversed-phase (RP) LC-MS] and has thus provided significant time and sample consumption savings during early development. Furthermore, additional time savings and the efficient time utilisation of MS instruments have been obtained by implementing the Thermo Scientific™ Vanquish™ Duo UHPLC system for tandem LC workflows. 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 consumption savings.

Materials and Methods

Materials

MS grade consumables are used throughout. Water (LC-MS Chromasolv) and ammonium acetate (Optima LC/MS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Glacial acetic acid was purchased from Sigma Aldrich (St. Louis, MO, USA). A CHO-derived recombinant IgG1 mAb produced at Symphogen (QC713) was used as a system suitability reference for the native SEC-MS platform.

LC configuration and analysis

LC settings and parameters are summarised in *Table 1B*. Solvent A is prepared by adding ammonium acetate (1.93 g) and acetic acid (220 µL) directly to the purchased 1L MS grade water bottle. The water bottle is vortexed gently until ammonium acetate is completely dissolved and mixed, and the solvent used within a week.

No sample preparation is required for native SEC-MS analysis. The samples (10 µg) are loaded directly onto the SEC column, which acts as a buffer exchanger. Symphogen, when conducting lead selection studies, typically analyse between 100 and 384 lead candidates in one sequence. The system suitability reference (QC713) is analysed initially, once for every 24th sample, and after the last sample. Typically, between 5 and 17 QC713 runs are performed in one lead selection study.

Native MS data acquisition was performed using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.9.

Table 1A. MS Settings.

MS Settings	
Scan Parameter	**Setting**
Scan type	HMR – Full MS
Scan range	2500 to 8000 m/z
Fragmentation	In-source CID 130.0 eV
Resolution	35000
Polarity	Positive
Microscans	10
Lock masses	Off
AGC target	3e6
Maximum inject time	200
HESI source	Value
Sheath gas flow rate	25
Aux gas flow rate	5
Sweep gas flow rate	0
Spray voltage (kv)	4.20
Spray current (µA)	-
Capillary temp. (°C)	275
S-lens RF level	200.0
Aux gas heater temp (°C)	175

When initiating new column(s) the flow is ramped up slowly from 0.1 to 0.3 mL/min. Columns are equilibrated with at least 10 column volumes of mobile phase and until a stable UV baseline has been obtained. Sample injections are performed until stable chromatographic performance is achieved (retention time, peak shape and resolution). The column is then ready for use. If the column is not used for longer than 24 hrs it should be equilibrated with 10 column volumes of column storage solution.

A Vanquish Duo system for tandem LC workflows is used (see *Figure 2*). With this LC system and configuration, the analysis time is 4.7 min per sample and data acquisition is active all the time. In the single column configuration, the analysis time is 8 min per sample and MS data acquisition is active 5.5 min or 69% of the time (see *Figure 2*). To eliminate time spent on loading samples and washing steps, a 'PrepareNextInjection' command is executed 3.5 min into the run in the tandem LC workflow setup (*Figure 2*), which ensures that sample injection and data acquisition is started after exactly 4.7 min.

Table 1B. LC parameters & conditions.

LC parameters & conditions	
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
Column	Waters Acquity UPLC BEH200 SEC, 4.6 x 150 mm, 1.7 µm
Injection wash solvent	20% ethanol
Sample load	10 µg (2-20 µg acceptable). Recommended max injection volume is 20 µL, although up to 100 µL has been successfully injected for dilute samples at Symphogen.
Flow	0.3 mL/min
Column temperature	Setpoint: 20.0 °C, acceptable range: 18.0 °C - 22.0 °C
Thermo statting mode:	Still air
Pre-inject wash	100 s
Post-inject wash	100 s
Max. column pressure	220 bar (3190 psi) and 300 bars (4350 psi)
Autosampler temperature	Setpoint: 5.0 °C
Detection type: Primary wavelength (reporting) Secondary wavelength (characterisation)	UV detection 280 nm 214 nm
Data collection rate	4.0 Hz
Response time	1.00 s
Narrowest peak width	0.100 min
Run time	Single column - 8 min, tandem setup – - 4.7 min

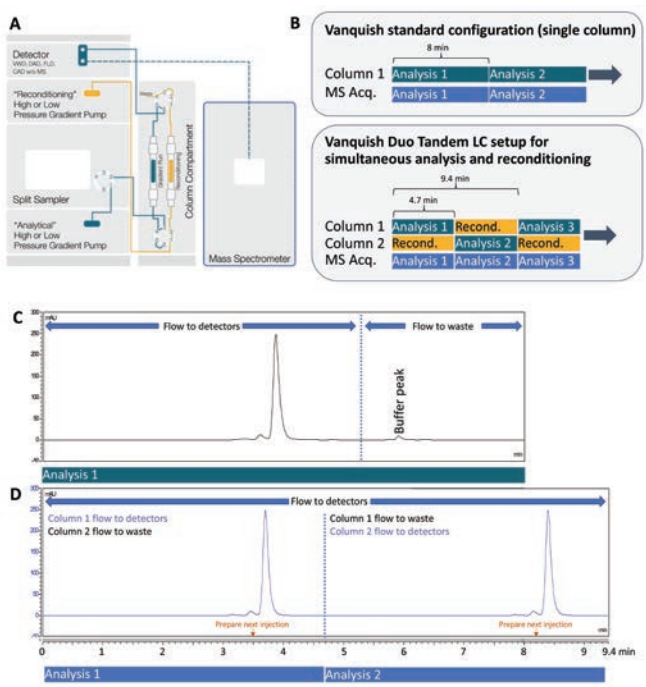

Figure 2. Vanquish Duo LC for tandem LC workflows – configuration and analysis time. A) In the tandem LC-MS workflow configuration two columns are operated at the same time. The analytical pump delivers flow to the active column, while the reconditioning pump delivers flow to the inactive column. B) 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. C) Data acquisition in single LC column configuration, MS data

acquisition is active until 5.5 min, after which the flow is switched to waste to avoid components in the buffer peak (salts, buffers, etc.) to be analysed, as these may be damaging to the MS instrument and/or have a negative impact on signal quality. D) Data acquisition in Tandem LC-MS configuration - the detector flow alternates between column 1 and column 2 every 4.7 min. In this configuration MS data acquisition is active 100% of the time and analysis time per sample is reduced from 8 to 4.7 min. In order to avoid delays between sample injections a 'PrepareNextInjection' command is executed after 3.5 min to allow the next sample to be ready for injection and the system ready for data acquisition at exactly 4.7 min.

MS configuration

Native MS data was acquired on a Q Exactive Plus with Biopharma option using the settings shown in *Table 1A*. Attention should be paid to in-source CID, which is critical for native MS. Further details on optimal MS settings for native and denaturing MS on the Orbitrap platform are provided by Scheffler and Damoc [14]. When using the Vanquish Duo system for tandem LC workflows the MS is acquiring data all the time, i.e., MS utilisation is 100% (see *Figure 2*).

Data processing - aggregation and identity

Evaluation of soluble aggregates from UV trace and identity testing from deconvoluted mass was performed directly in Chromeleon™ CDS version 7.2.9.

The identity is confirmed by comparing the (most intense) measured mass with the theoretical mass in the Chromeleon CDS report. The most intense mAb species is generally the G0F/G0F glycoform for mAbs expressed in a Chinese hamster ovarian (CHO)-based expression system. Consequently, the used theoretical mass is for the mAb G0F/G0F glycoform. A mass deviation < 2 Da is considered a positive identification.

Data processing - glycoform distribution

Raw data files were exported from Chromeleon CDS to a designated folder and subsequently processed in Protein Metrics Intact Mass software (https://www.proteinmetrics.com/products/intact-mass/). Protein deconvolution was performed using parameters optimised for the Orbitrap Platform and native MS mAb raw data. The spectral quality of raw native MS data produced on the Orbitrap platform is excellent, and consequently the deconvolution parameters were set to have a minimal impact on spectral data quality.

Peak assignment was based on a delta mass list containing glycoforms typically observed for mAbs in a CHO-based expression system.

Results and Discussion

Implementation of native SEC-MS and impact on sample consumption and analysis time

Native SEC-MS has replaced two analyses previously employed during early lead selection at Symphogen, namely 1) intact mass by RP LC-MS for identity testing and glycoform distribution and 2) SEC for analysis of soluble aggregates. A native SEC-MS raw data example of Symphogen's system suitability reference is shown in *Figure 3B*. A consequence of the implementation of native SEC-MS, and resulting replacement of two analyses with one, has been a reduction in sample consumption and a radical reduction in analysis time. Time savings become particular apparent when using a tandem LC workflow configuration described in Materials and Methods (*Figure 2*). A summary of sample consumption and total analysis time before and after implementation of native SEC-MS is shown in *Table 2*. A lead selection study of 384 lead candidates can now be performed in 32 hrs, compared to 158 hrs prior to implementation of native SEC-MS. In short, analysis time has been reduced from nearly 1 week to just over 1 day for a typical lead selection study.

Table 2. 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 hrs
Native SEC-MS (single column)	10 µg	8 min	56 hrs
Native SEC-MS (tandem LC workflow)	10 µg	4.7 min	32 hrs

Native SEC-MS - chromatographic performance and robustness

In addition to the reduction in sample consumption and analysis time, native SEC-MS benefits from a simple chromatographic setup (isocratic separation) and gentle analysis conditions (room temperature chromatography, aqueous solvent, near neutral pH) compared to conventional intact RP LC MS, which employs organic solvents, acids and high column temperature (typically around 80ºC). The mild analysis conditions of native SEC-MS reduce risk of analysis artefacts (e.g., cleavage of acid labile peptide bonds). The robustness of the native SEC-MS chromatography is illustrated in *Figure 3A*, which shows an overlay of 17 system suitability runs from a lead selection study of 384 lead candidates. For the monomer peak relative standard deviations (RSDs) well below 1% are observed for both retention time and relative peak area, confirming highly robust chromatography.

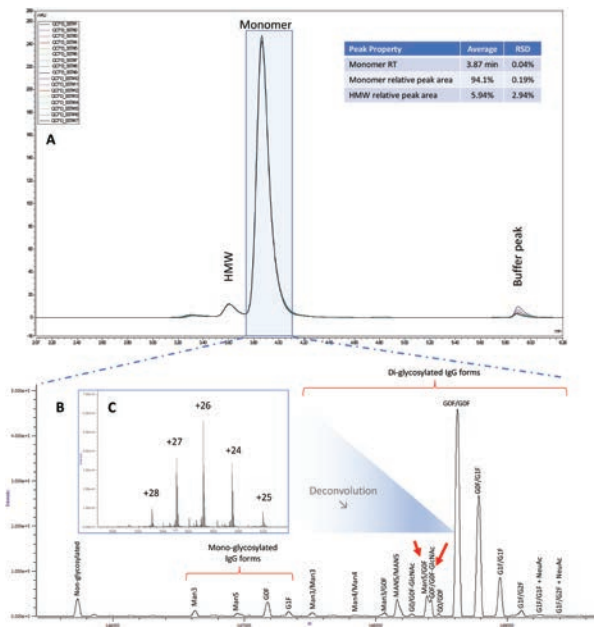

Figure 3. Data obtained from native SEC-MS experiment on Symphogen reference antibody.

A) UV trace used for quantitative evaluation of soluble aggregates and fragments. Overlaying several runs of the same analysis shows excellent chromatographic reproducibility over the time of the lead selection study. B) Deconvoluted native SEC mass spectrum of Symphogen reference mAb QC713. Native MS provides high spatial separation of spectral features and significantly increased peak capacity compared to conventional denaturing intact MS. Combined with the high effective resolution and excellent signal-to-noise ratio of the Orbitrap platform, native SEC-MS allows confident assignment of mAb glycoforms, including low abundance forms and glycoforms with small mass differences, such as the Man5/G0F and G0F/G0F-GlcNAc highlighted with red arrows (mass difference 25 Da). C) Combined MS spectrum of SEC monomer peak, from which the deconvoluted spectrum is produced through software processing.

Chromatographic robustness was furthermore evaluated over an extended period of six months based on five independent lead selection studies. The results are summarised in *Figure 4A*. The data was collected by two technicians using four independent SEC columns. The LC-MS system was not just used for native SEC-MS during this period, but also native CIEX-MS and intact RP LC-MS. A total of 2526 runs (samples, reference, blanks) were performed on the LC-MS system in the six-month period. From the data it was concluded that chromatographic intermediate precision is very good, with the average relative peak area varying no more than 1% point between lead selection studies. The precision (repeatability) is also very good within each lead selection study, as illustrated by the error bars in *Figure 4*. In conclusion, based on a large dataset collected by different technicians, using different SEC column lots, over an extended period, it can be concluded that chromatographic performance is very robust for the native SEC-MS platform used at Symphogen.

Native SEC-MS -
MS performance and robustness

A lead selection study of 384 lead candidates provided the MS ion intensity and mass deviation for the system suitability reference (QC713). QC713 was analysed once for every 24th lead selection candidate (in total 17 runs in the study) and the mass spectra were summed over the monomer peak. No falling trend was observed for ion intensity of QC713 across the lead selection study and mass deviation fell within 1.9 Da of the theoretical mass (12.6 ppm). At Symphogen, a mass deviation below 2 Da (< 13 ppm) is considered a positive identification. These results confirm that MS signal intensity and instrument calibration are maintained during a lead selection study of 384 lead candidates.

A key strength of the Orbitrap platform for native MS is the high effective resolution (*Figure 1*), which allows effective mapping of proteoforms, such as glycoforms, at the

intact MS level (*Figure 3B*). At Symphogen, native SEC-MS has become the principal analysis tool for mAb glycoform distribution on the intact level due to the high spectral data quality, short analysis time and the fact that no sample preparation is required. The robustness of native SEC-MS was evaluated across the five independent lead selection studies described above, and the results are presented in *Figure 4*. The data was acquired by two technicians over a period of six months and using four different SEC column lots. Across the five lead selection studies and all identified glycoforms the average relative intensity differed by less than 0.5% points. This clearly illustrates that native SEC-MS has a high intermediate precision for relative glycoform distribution. Precision within each lead selection study (repeatability) is high as illustrated graphically by the error bars in *Figure 4*. In summary, based on a large dataset generated by different technicians, using different SEC column lots, over an extended period, it can be unambiguously concluded the native SEC-MS platform generates robust intact MS data for evaluating relative glycoform distribution of biopharmaceutical mAbs.

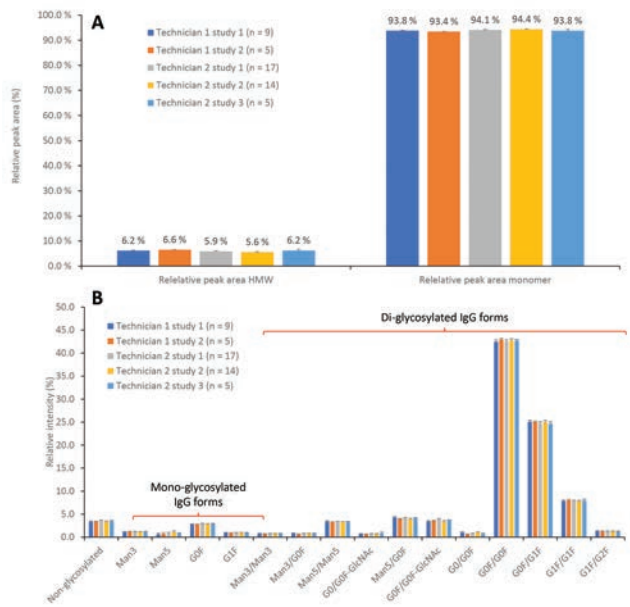

Figure 4. Native SEC-MS system robustness and reproducibility presentation across lead selection studies.

A) Average relative peak areas of system suitability reference (QC713) from five independent lead selection studies are shown. QC713 was analysed initially, then once for every 24th lead selection sample and after the last lead selection sample. The number of QC713 runs varied between 5 and 17, reflecting the number of lead candidates analysed in each study (between 96 and 384). The five studies were conducted over a period of six months by two technicians and using a total of four SEC columns. A total of 1100 native SEC–MS runs (samples, QC713 and blanks) were conducted in the five studies. (HMW: high molecular weight.) B) Average relative glycoform intensities obtained for the system suitability reference mAb (QC713) from five independent lead selection studies. Glycoforms are sorted after increasing mass from left to right.

Conclusion

At Symphogen, the implementation of native SEC-MS has replaced conventional SEC and intact MS by RP LC-MS for identity testing, aggregation/fragment analysis, and analysis of glycoform distribution. This has resulted in radically reduced sample consumption and analysis time during early lead selection studies. Although not covered here, the presented native SEC-MS platform can also be applied for subunit analysis (e.g., Fab2 subunit generated from Ides digestion). Robust chromatographic and MS performance has been clearly demonstrated for the implemented native SEC platform. Finally, excellent native MS spectral data quality is obtained on the employed Orbitrap platform. This allows for highly effective detection and relative quantitation of biopharmaceutical quality attributes, as exemplified by the analysis of mAb glycoform distribution in the current paper. Thus, native SEC–MS can be used to characterise multiple quality attributes with a single high resolution, robust and fast analysis.

References

1. B. J. Kullberg, J. D. Sobel, M. Ruhnke, P. G. Pappas, C. Viscoli, J. H. Rex, J. D. Cleary, E. Rubinstein, L. W. P. Church, J. M. Brown, H. T. Schlamm, I. T. Oborska, F. Hilton, M. R. Hodges, Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: A randomised non-inferiority trial. Lancet. 366, 1435–1442 (2005).

2. M. Aulí, A. Domènech, A. Andrés, M. Orta, M. Salvà, J. Descotes, N. Prats, Multiparametric immunotoxicity screening in mice during early drug development. Toxicol. Lett. 214, 200–208 (2012).

3. M. Tassi, J. De Vos, S. Chatterjee, F. Sobott, J. Bones, S. Eeltink, Advances in native high-performance liquid chromatography and intact mass spectrometry for the characterization of biopharmaceutical products. J. Sep. Sci. 41, 125–144 (2018).

4. G. Terral, A. Beck, S. Cianférani, Insights from native mass spectrometry and ion mobility-mass spectrometry for antibody and antibody-based product characterization. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1032, 79–90 (2016).

5. S. Rosati, N. J. Thompson, A. Barendregt, L. J. a Hendriks, A. B. H. Bakker, J. De Kruif, M. Throsby, E. Van Duijn, A. J. R. Heck, Qualitative and semiquantitative analysis of composite mixtures of antibodies by native mass spectrometry. Anal. Chem. 84, 7227–7232 (2012).

6. T. Botzanowski, S. Erb, O. Hernandez-Alba, A. Ehkirch, O. Colas, E. Wagner-Rousset, D. Rabuka, A. Beck, P. M. Drake, S. Cianférani, Insights from native mass spectrometry approaches for top- and middle- level characterization of site-specific antibody-drug conjugates. MAbs. 9, 801–811 (2017).

7. F. Füssl, A. Trappe, K. Cook, K. Scheffler, O. Fitzgerald, J. Bones, Comprehensive characterisation of the heterogeneity of adalimumab via charge variant analysis hyphenated on-line to native high resolution Orbitrap mass spectrometry. MAbs. 11, 116–128 (2019).

8. R. Gahoual, A.-K. Heidenreich, G. W. Somsen, P. Bulau, D. Reusch, M. Wuhrer, M. Haberger, Detailed Characterization of Monoclonal Antibody Receptor Interaction Using Affinity Liquid Chromatography Hyphenated to Native Mass Spectrometry. Anal. Chem. 89, 5404–5412 (2017).

9. T. H. Chen, Y. Yang, Z. Zhang, C. Fu, Q. Zhang, J. D. Williams, M. J. Wirth, Native Reversed-Phase Liquid Chromatography: A Technique for LCMS of Intact Antibody-Drug Conjugates. Anal. Chem. 91, 2805–2812 (2019).

10. Y. Yan, T. Xing, S. Wang, T. J. Daly, N. Li, Coupling Mixed-Mode Size Exclusion Chromatography with Native Mass Spectrometry for Sensitive Detection and Quantitation of Homodimer Impurities in Bispecific IgG. Anal. Chem. 91, 11417–11424 (2019).

11. J. Jones, L. Pack, J. H. Hunter, J. F. Valliere-Douglass, Native size-exclusion chromatography-mass spectrometry: suitability for antibody–drug conjugate drug-to-antibody ratio quantitation across a range of chemotypes and drug-loading levels. MAbs. 12 (2020), doi:10.1080/19420862.2019.1682895.

12. B. Wei, G. Han, J. Tang, W. Sandoval, Y. T. Zhang, Native Hydrophobic Interaction Chromatography Hyphenated to Mass Spectrometry for Characterization of Monoclonal Antibody Minor Variants. Anal. Chem. 91, 15360–15364 (2019).

13. S. Lippold, S. Nicolardi, E. Domínguez-Vega, A.-K. Heidenreich, G. Vidarsson, D. Reusch, M. Haberger, M. Wuhrer, D. Falck, Glycoform-resolved Fc RIIIa affinity chromatography–mass spectrometry. MAbs. 11, 1191–1196 (2019).

14. K. Scheffler, E. Damoc, Full characterization of heterogeneous antibody samples under denaturing and native conditions on the Q Exactive BioPharma mass spectrometer. Appl. Note 72348 (2017) (available at https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/AN-72348-LC-MS-Heterogeneous-Antibody-Samples-AN72348-EN.pdf).

15. A. O. Bailey, The Quest for Highly Effective Resolution: Therapeutic Protein Characterization via Separations-Coupled Native MS. Am. Chem. Soc. (2018), pp. 1–15.