Application Note

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A Quick and Efficient Sample Cleanup for Biomolecule Analysis

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Abstract

Many biomolecules, such as monoclonal antibodies, are stored in buffer containing excipients, preservatives, and detergents to aid stability. These additional components can often interfere with the subsequent analysis of the biomolecule.

There are many approaches that can be taken to remove some of these undesirable components, but centrifugation using gel filtration cartridges is one of the quickest and easiest to perform. The technique can also be used to simply transfer from one buffer solution to another, with minimal loss of sample, or change in sample concentration.

In this application note, we demonstrate how to use centrifugation with gel filtration cartridges for sample cleanup. This approach is a rapid, convenient, straight-forward technique that can be used to easily avoid the problems associated with analysis of formulated monoclonal antibodies.

Introduction

When analyzing an intact, formulated monoclonal antibody by any technique involving mass spectrometry, it is highly desirable to remove unwanted components prior to analysis. Not only can they contaminate the detector, but they can also contaminate the LC column, which can lead to prolonged issues with column performance (including carry over or compromised lifetime).

Numerous approaches can be used, including comprehensive online approaches involving two dimensional liquid chromatography (2D-LC), but it is often desirable to have a simple and rapid buffer exchange or desalt of the sample prior to analysis.

A typical example is a monoclonal antibody formulated in histidine as a buffer and containing polysorbate 80 as stabilizer, among other components. We chose to investigate rituximab for this purpose. We looked at how these components interfered with intact protein analysis by liquid chromatography/mass spectrometry (LC/MS), a critical quality attribute. Using LC/MS we observed many of the glycoforms that were present as well as the excipients. But the use of AdvanceBio Spin columns allowed the undesirable components to be eliminated from the analysis. Deglycosylation and subsequent analysis of the released glycans using Gly-X InstantPC labeling enabled a more comprehensive analysis of the glycoforms present. Unfortunately, the histidine buffer contained a primary amine that can also react with the InstantPC reagent. We saw how AdvanceBio Spin columns can be used to perform a simple buffer exchange to an alternative buffer composition that did not interfere with the labeling reaction.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Formulated rituximab for analysis was purchased from Evidentic GmbH, Germany.

Sample preparation

AdvanceBio Spin columns, <100 µL (part number 1980-1103)

AdvanceBio Spin 96-sample plates (part number 1980-1104)

AdvanceBio Gly-X InstantPC (part number GX96-IPC)

Additional equipment needed:

Centrifuge with swing-out rotor (or fixed rotor for mini cartridges).

Variable volume pipette

Data processing

LC/MS data was processed by Agilent MassHunter BioConfirm software B.10.00.

Method and conditions

Table 1. Agilent HPLC reversed phase (RP) conditions for intact mAb analysis.

Parameter	Value		
Instrument	Agilent 1290 Infinity II Bio LC system		
Column	Agilent PLRP-S 1000 Å, 2.1 × 50 mm, 5 µm (part number PL1912-1502)		
Column Temp	70 °C		
Mobile Phase	A) 0.1% formic acid B) Acetonitrile + 0.1% formic acid		
Gradient Program	Time (minutes)	%B	Flow rate (mL/min)
	0	20	0.4
	1	20	0.4
	4	70	0.4
	5	70	0.4
	6	20	0.4
	9	20	0.4
Injection Volume	2 µL		
UV Detection	280 nm		

Table 2. Agilent 6545XT AdvanceBio LC/Q-TOF parameters for intact mAb analysis.

Parameter	Value
Source	Dual AJS ESI
Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer	60 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	11 L/min
Vcap	5500 V
Nozzle Voltage	2000 V
Fragmentor	380 V
Skimmer	140 V
Mass Range m/z	400 to 8,000
Scan Rate	1 spectrum/sec
Acquisition Mode	High mass range

Table 3. Hydrophilic interaction liquid chromatography (HILIC) conditions for InstantPC labeled N-glycans.

Parameter	Value		
Instrument	Agilent 1290 Infinity II Bio LC system		
Column	Agilent AdvanceBio Amide HILIC, 2.1 × 150 mm, 1.8 µm (part number 859750-913)		
Column Temp	60 °C		
Mobile Phase	A) 50 mM ammonium formate, pH 4.4 B) Acetonitrile		
Gradient Program	Time (minutes)	%B	Flow rate (mL/min)
	0	77	0.6
	45	59	0.6
	46	40	0.6
	47	40	0.6
	49	77	0.6
	60	77	0.6
Injection Volume	1 µL		
MS Detection	Parameters in Table 4		

Table 4. Agilent 6545XT AdvanceBio LC/Q-TOF parameters for InstantPC labeled N-glycans.

Parameter	Value
Source	Dual AJS ESI
Gas Temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	10 L/min
Vcap	2,500 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range m/z	600 to 3,000
Scan Rate	1 spectrum/sec
Acquisition Mode	Extended dynamic range (2 GHz)

Results and discussion

An effective demonstration of how a gel filtration cartridge worked is the removal of sodium chloride from solution using a conductivity detector to measure the number of sodium ions removed.

Agilent AdvanceBio Spin 96-sample plates are supplied in water containing ProClin 150 preservative, so it is recommended that this solution be removed and replaced with pure water. The outlet cap of the device was removed, and the cartridge was placed into an empty wash vessel. The inlet cap was then removed, and the cartridge was centrifuged to displace the storage solution. After emptying the waste vessel, a pipette was used to dispense water into the cartridge and then centrifuged once more. This process was repeated two or three times as necessary. Finally, the cartridge was placed in a clean collection tube and the sample applied. After centrifugation, the collected sample was ready for analysis and the spent cartridge was discarded.

Figure 1. Effective removal of NaCl from 0.8 M NaCl solution using AdvanceBio Spin 96-well plate. Sample volume as noted above each column.

Rituximab is formulated in 12.5 mM histidine buffer, pH 6.5, containing sodium citrate and polysorbate 80. In the reversed phase separation of the untreated sample, three main regions in the chromatogram were observed (Figure 2). The first peak contains impurities that are too hydrophilic to be retained by the PLRP-S HPLC column. The main peak eluting at 2.6 minutes is the intact protein. However, this peak is immediately followed by a complex pattern of peaks with m/z 44 units apart (singly charged species) or m/z 22 units apart (doubly charged species). This result is indicative of the polymeric nature of polysorbate 80.

To demonstrate the effectiveness of excipient removal, 50 µL of rituximab solution was buffer exchanged with a histidine buffer. Two AdvanceBio Spin columns (part number 1980-1103) were used according to the instructions. Initial centrifugation, to remove the storage solution, was followed by three washes with 50 mM bis-tris propane buffer. The formulated rituximab solution was then applied and collected in a clean collection tube. The buffer exchanged sample was then transferred into a high recovery glass vial insert and placed into a sample vial ready for use.

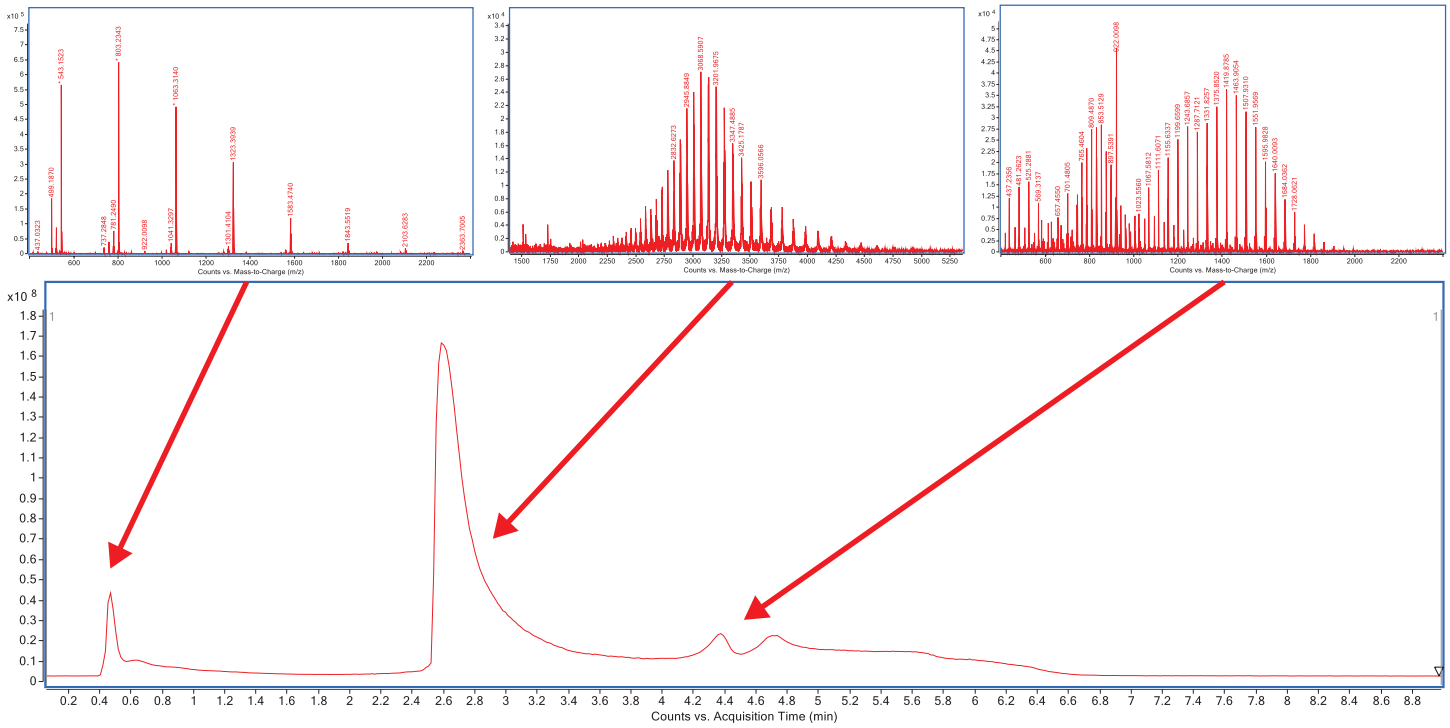

Figure 2. LC/MS analysis of intact rituximab showing regions of interest.

Subsequent reversed phase analysis (Figure 3) showed a significant reduction in the early eluting hydrophilic species, and an almost entire removal of the polysorbate 80 from the sample. The intact protein peak remained unchanged.

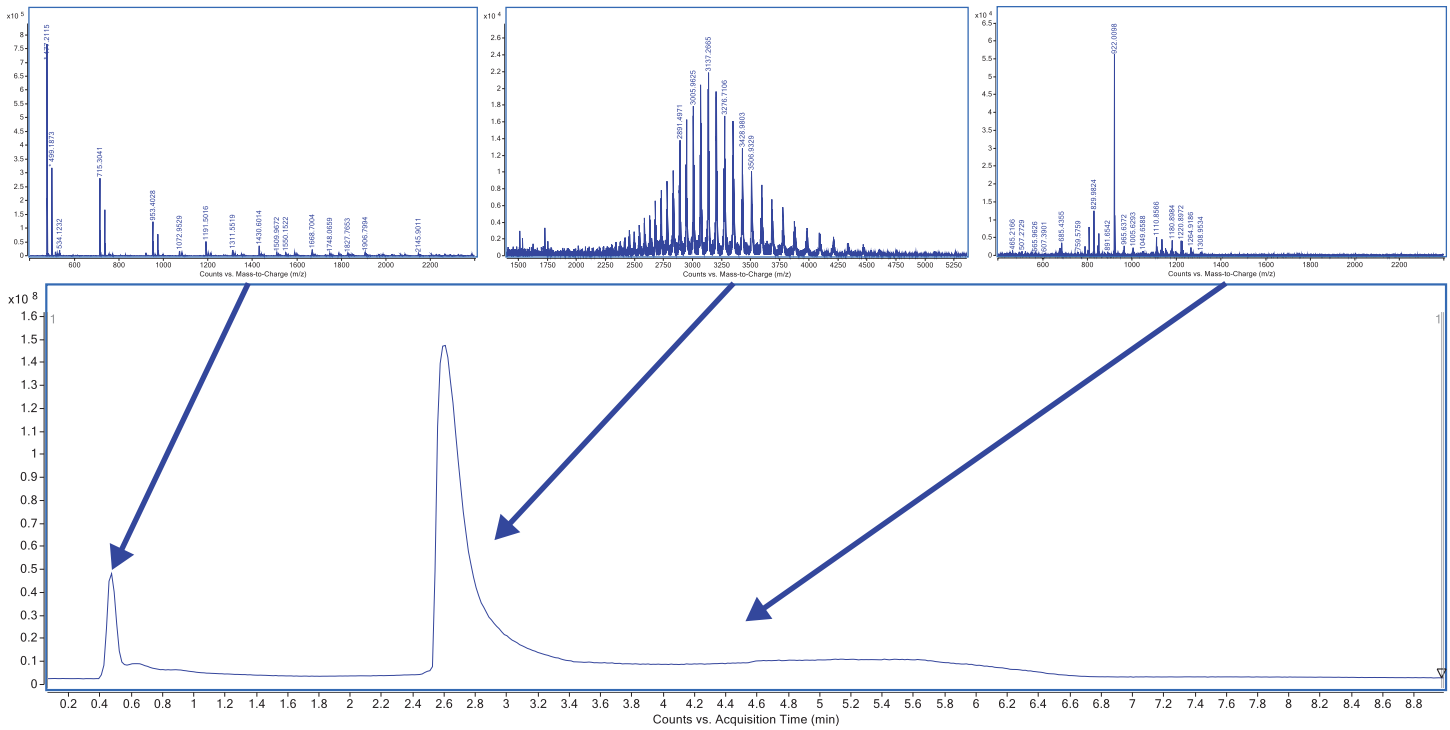

Figure 3. LC/MS analysis of rituximab following buffer exchange/desalting into histidine buffer (removal of excipients).

Using the MassHunter intact protein Maximum Entropy deconvolution of the MS signal, it was possible to observe the major isoforms of rituximab (Figure 4). Four major N-glycan combinations were observed, as expected from the possible combinations of G0F, G1F, and G2F. Several minor components were evident, but these could arise from different glycans, protein variants (such as incomplete C-terminal lysine removal) or other proteoforms. To fully understand the glycan composition, it was necessary to perform deglycosylation, and subsequently identify the individual N-glycans present.

This was achieved using the AdvanceBio Gly-X deglycosylation kit and InstantPC labeling module with subsequent analysis by LC/MS (see Table 3 and Table 4 in the method and conditions section).

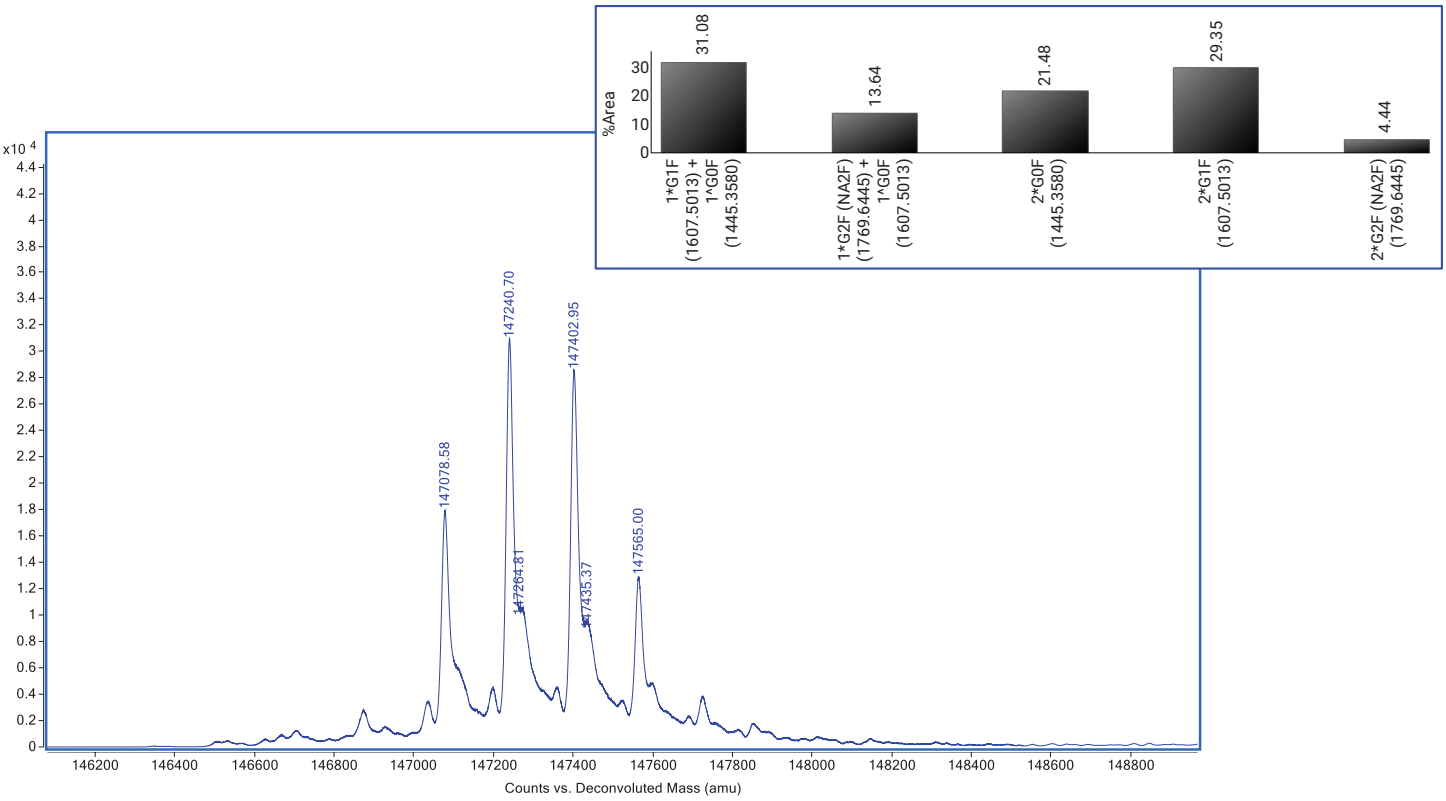

Figure 4. Deconvolution of the intact protein peak showing major glycoforms of rituximab.

Two samples of rituximab were prepared for N-deglycosylation. The first sample was at a concentration of 1 mg/mL in 12.5 mM histidine buffer. The second sample was prepared by passing the same solution through an AdvanceBio spin cartridge to perform buffer exchange into 50 mM bis-tris propane (BTP). The samples were then subjected to denaturation, deglycosylation, and labeling with InstantPC according to the instructions in the workflow, using 20 µL as the sample input (~20 µg protein). A HILIC separation using an AdvanceBio Amide HILIC column was followed by analysis using Agilent MassHunter BioConfirm software B.10.0 with a database of 145 potential targets. A selection of the most likely hits is shown in Table 5, together with their anticipated m/z values. In positive mode MS, most biantennary InstantPC-labeled N-glycans will give $[M + 2H]^{+2}$, larger sialylated will be majority $[M + 3H]^{+3}$.

Table 5. Predicted m/z values for InstantPC-labeled N-glycans from rituximab.

Glycan ID	IPC-Glycan Monoisotopic Mass	$[M + 3H]^{3+}$	$[M + 2H]^{2+}$	$[M + H]^{+}$
Man5	1495.58	499.53	748.80	1496.59
G0	1577.63	526.89	789.82	1578.64
G0F	1723.69	575.57	862.85	1724.70
G0F-GlcNAc	1520.61	507.88	761.31	1521.62
G1	1739.69	580.90	870.85	1740.69
G1F	1885.75	629.59	943.88	1886.75
G1F-GlcNAc	1682.67	561.90	842.34	1683.68
G2F5	2047.80	683.61	1024.91	2048.80
A1	2192.84	731.95	1097.42	2193.84
A1F	2338.89	780.64	1170.45	2339.90
A2	2483.93	828.98	1242.97	2484.94
A2F	2629.99	877.67	1316.00	2631.00

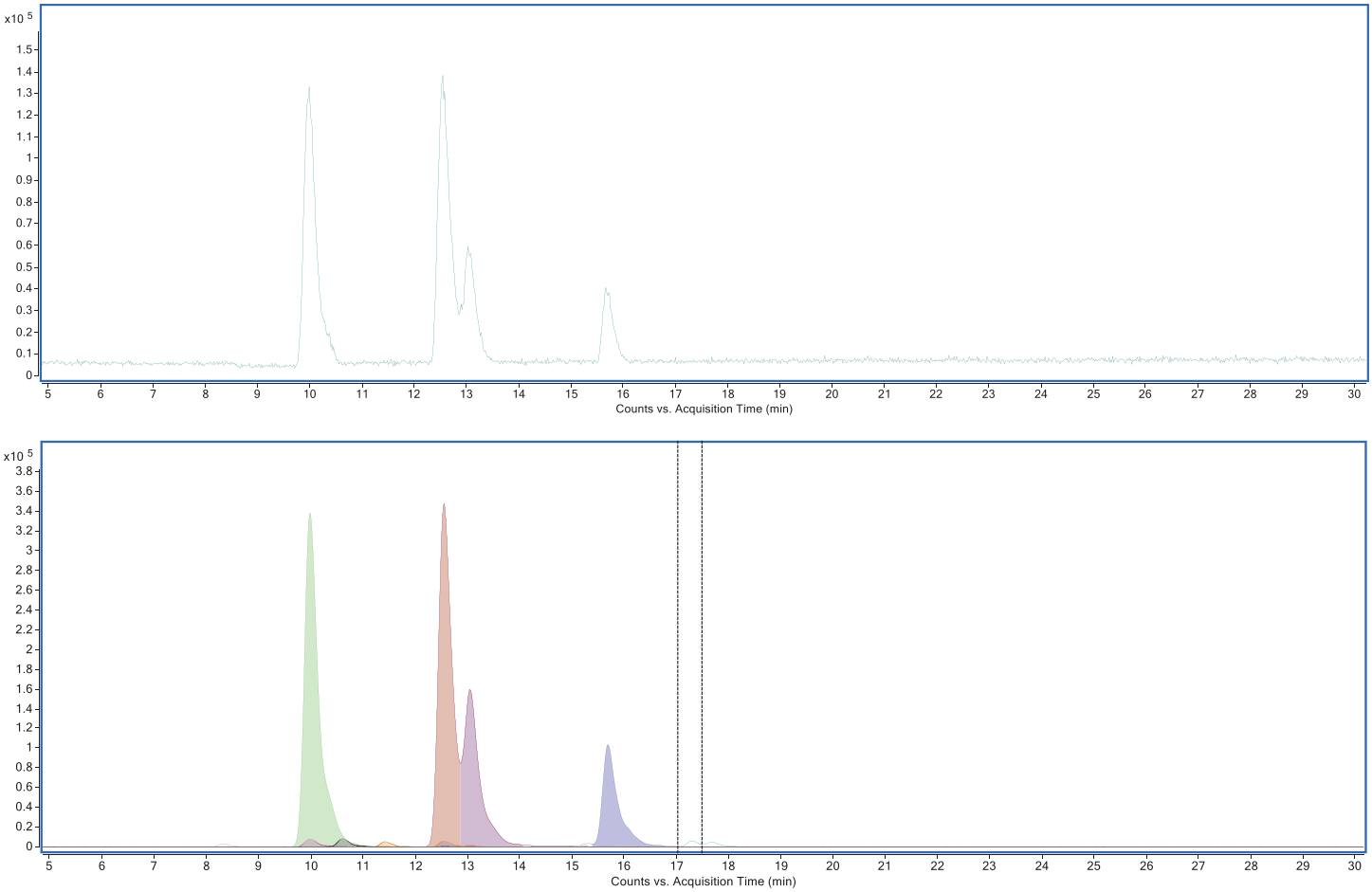

Figure 5. LC/MS Analysis of InstantPC-labeled released glycans of rituximab following buffer exchange into bis-tris propane buffer solution. The top chromatogram is a total ion chromatogram (TIC), while the bottom shows glycan peaks identified via the released glycan workflow in BioConfirm 10.

The outcome of the experiment confirmed the results of the analysis of the intact mAb. Figure 5 shows a combination of G0F, G1F, and G1F', and G2F with various other minor glycoforms present that were not detected in the intact mAb analysis. Indeed, Table 6 shows the level of individual glycans that we would expect based on the combinations detected in the intact mAb analysis.

Figure 6 shows the area% of the nine most abundant glycoforms detected and includes G0F-GlcNac and G1F-GlcNAc. These species can sometimes arise from in-source fragmentation, but this would result in the retention time matching that of the parent glycan (G0F and G1F/G1F'). In this case, the peaks were eluting earlier suggesting they were not formed as a result of fragmentation.

InstantPC is a procainamide-based reagent that reacts with primary amines of the enzymatically released N-glycans. InstantPC labeling allows for fluorescence detection and enhanced MS signal, with fluorescence being the preferred method for relative peak area % determination. Other components may also contain primary amines, such as histidine present in the mAb sample that was not buffer exchanged prior to deglycosylation. Figure 7 shows the extracted ion chromatogram corresponding to InstantPC-labeled histidine from the sample that was not subjected to buffer exchange. The sample that was buffer exchanged into BTP is shown in the lower chromatogram that is shown at 10X magnification and illustrates the effectiveness of the AdvanceBio Spin column for performing buffer exchange.

Table 6. Predicted area% of released glycans based on intact LC/MS analysis of rituximab.

IgG Glycoform	%Area
2 * G0F	21.48%
G0F / G1F	31.08%
2 * G1F	29.35%
G1F / G2F	13.64%
2 * G2F	4.44%

Released Glycan	Predicted Area%
G0F	37.02%
G1F / G1F'	51.71%
G2F	11.26%

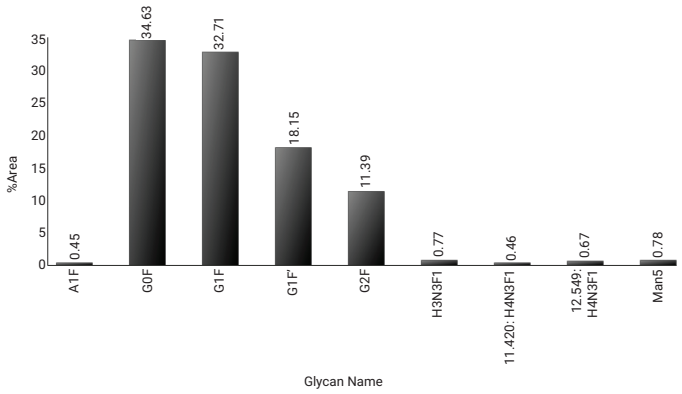

Figure 6. Area% of major InstantPC-labeled released glycoforms, using MS detection.

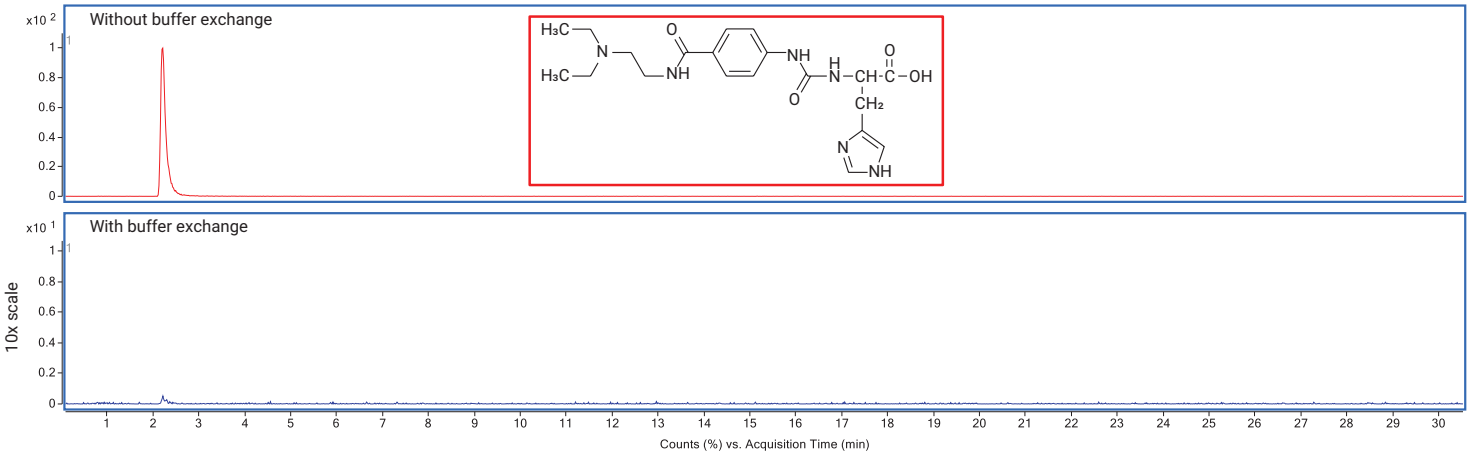

Figure 7. Showing InstantPC-labeled histidine detected in rituximab samples without buffer exchange (above) and with buffer exchange (below).

Conclusion

AdvanceBio Spin columns and 96-sample plates are a rapid and convenient tool for performing desalting and buffer exchange. This tool also allows for simply separating large biomolecules from inconvenient small molecule components that may otherwise interfere with the analysis.

Excellent levels of salt removal or buffer exchange can be seen with minimal loss of sample or change in sample concentration in minutes. This approach was compared to other approaches that take considerably longer to perform or result in a dramatic change in sample concentration.

The process can also be used to remove sample components that we do not wish to introduce into our MS detector, including sugars, nonvolatile salts, or surfactants and detergents.

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DE49916998

Published in the USA, April 1, 2024
5994-7239EN

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