TECHNICAL NOTE

A pre-concentration and online solid phase extraction setup for the LC-MS analysis of therapeutic protein mixtures

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Key words

Pre-concentration, trap and elute, desalt, MSPac, MAbPac, monoclonal antibody, mAb, Vanquish, reversed phase, mass spectrometry, Q Exactive, biopharmaceutical, biomolecules, intact protein

Goal

Demonstrate a fully automated UHPLC setup applying simple and efficient online solid phase extraction method. Showcase assay performance by using a column cartridge for sample trapping and a Thermo Scientific™ MAbPac™ RP column for separation with water/acetonitrile-based gradients. Leverage the new technologies such as the Thermo Scientific™ Vanquish™ UHPLC platform in combination with the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole Orbitrap mass spectrometer.

Introduction

Solid-phase extraction (SPE) is a commonly used laboratory technique to isolate analytes of interest from complex matrices. Because this technique is typically performed manually, it may not satisfy productivity and automation requirements for all laboratories. When repeatability between different analysts shows too much variation or the sample is subject to contamination during the manual process, an automated sample cleanup is preferable.

In the online-SPE approach for the enrichment of low abundant compounds in liquid chromatography, the sample is pre-concentrated on a trap column prior to chromatographic separation. This technique can be applied for the sample cleanup of protein mixtures often containing high amounts of nonvolatile salts, which are present in various biopharma formulation buffers. The presence of such buffers may interfere with the operation of electrospray ion sources by suppressing ionization. In this technical note, the Vanquish UHPLC setup for fully automated pre-concentration and sample cleanup is described as well as the detection of intact proteins using the Q Exactive HF mass spectrometer.

Experimental

Consumables

- Analytical column: Thermo Scientific MAbPac RP column, 1 × 100 mm, 4 µm (P/N 302695)
- Trap column: Thermo Scientific™ MSPac™ DS-10 Desalting Cartridge, 2.1 × 10 mm, 2/pack (P/N 089170)
- Thermo Scientific™ Acclaim™ Cartridge Holder (P/N 069580)
- Fisher Scientific™ LC-MS grade water (P/N W/011217)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A/0638/17)
- Fisher Scientific™ Optima™ LC-MS grade trifluoroacetic acid (P/N 10125637)
- Thermo Scientific™ Pierce™ Formic acid LC-MS grade (P/N 28905)

Sample pretreatment and sample preparation

The sample used was the commercially available monoclonal antibody rituximab (Hoffmann La Roche™, Basel, Switzerland) supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.7 mg/mL polysorbate 80, 7.35 mg/mL sodium citrate dehydrate, 9 mg/mL sodium chloride, and sterile water adjusted to pH 6.5 using sodium hydroxide or hydrochloric acid.

Solutions of 1 mg/mL alpha-lactalbumin, cytochrome c, myoglobin, ribonuclease A (Sigma-Aldrich®, St. Louis, MO, USA) in 0.1% formic acid (FA) in water and rituximab in formulation buffer were diluted with 0.1% FA in water in the ratio of 1:1:1:1:2 (w/w) to a final protein concentration of 60 µg/mL. This protein mixture represents a sample including different sizes of proteins (12–148 kDa) with various post-translational modifications like glycosylation and acetylation.

LC conditions

Instrumentation

- Vanquish Flex UHPLC system consisting of:
  - System Base Vanquish Flex (P/N VF-S01-A)
  - Binary Pump F (P/N VF-P10-A-01)
  - Quaternary Pump F (P/N VF-P20-A)
  - Split Sampler FT (P/N VF-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Variable Wavelength Detector F (P/N VF-D40-A)
  - Ultra-Low Dispersion UV Monitor Flow Cell, 45 nL (P/N 6074.0285)
  - Vanquish MS Connection Kit (P/N 6720.0405)
- Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (P/N 0726041)

Figure 1 shows the Vanquish Flex UHPLC system chosen for this setup, consisting of a quaternary low-pressure gradient pump (LPG) used as the loading pump and a binary high-pressure gradient pump (HPG) used as the analytical pump. All required capillaries and additional parts for this setup are defined in Table 1. Separation conditions are shown in Table 2.

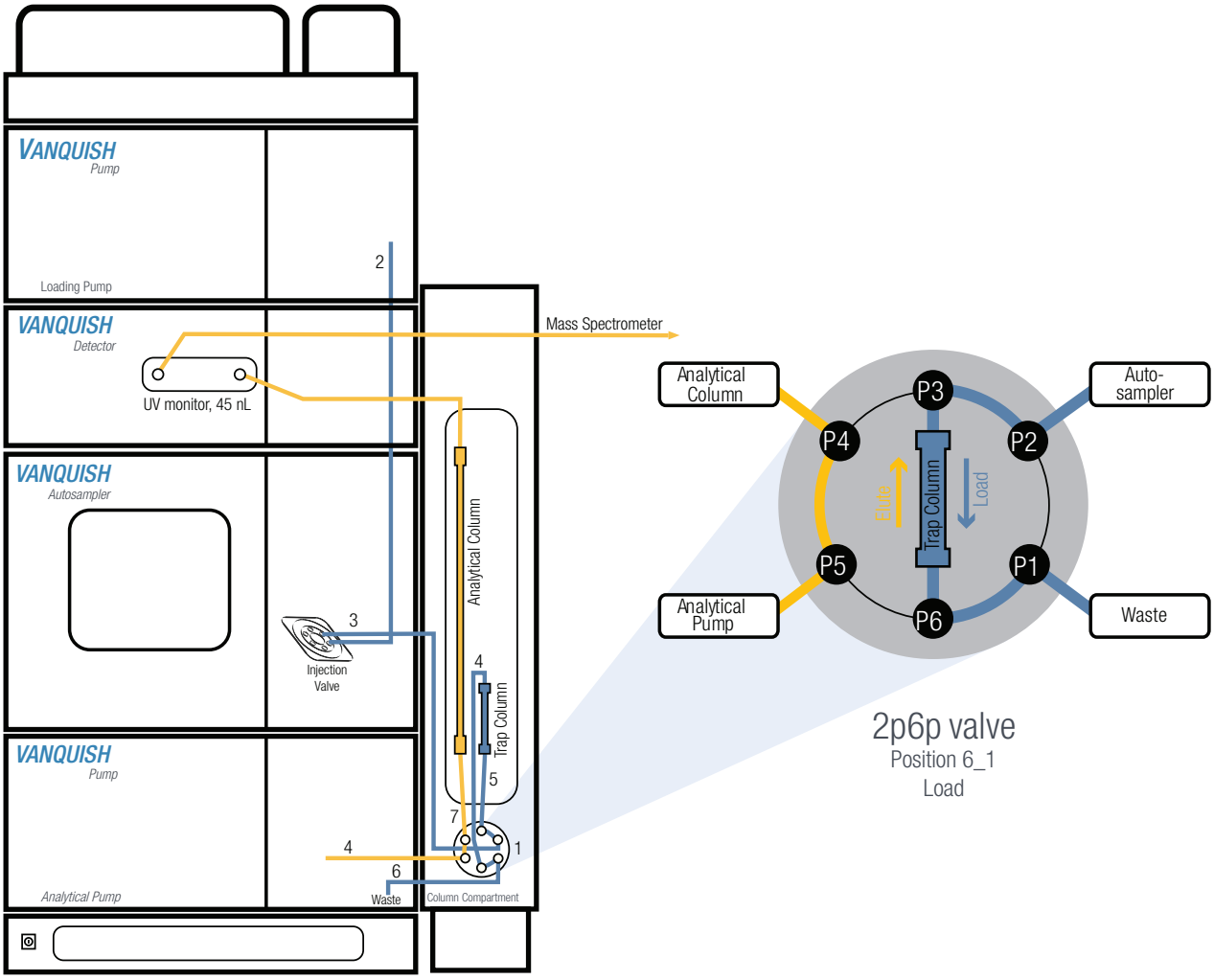

Figure 1. Vanquish Flex online SPE setup with the detailed 2-position/6-port (2p6p) valve configuration and recommended capillaries defined in Table 1.

Table 1. Additional parts needed for the online SPE setup.

#	Amount	Viper Capillary	P/N	2p6p Valve Port Assignment
1	1×	Biocompatible 2p6p column switching valve	6036.1560	
2	1×	Viper Capillary, MP35N, biocompatible, 0.1 × 650 mm	6042.2370	
3	1×	Viper Capillary, MP35N, biocompatible, 0.1 × 450 mm	6042.2350	Port 2 - Autosampler
4	2×	Viper Capillary, MP35N, biocompatible, 0.1 × 350 mm	6042.2340	Port 5 - Analytical pump; Port 6 - Trap column
5	1×	Viper Capillary, MP35N, biocompatible, 0.1 × 150 mm	6042.2320	Port 3 - Trap column
6	1×	Viper Capillary, MP35N, biocompatible, 0.5 × 350 mm	5083.2425	Port 1 - Waste
7	1×	Active Pre-heater, 0.1 × 380 mm[(a)]	6732.0110	Port 4 - Analytical column inlet

[a]Included in the System Base Vanquish Flex Ship Kit

Table 2. Separation conditions.

LC Parameters	Setting
Mobile phase A1	0.1% FA in water
Mobile phase B1	0.1% FA in water/ acetonitrile (10:90 v/v)
Mobile phase A2	0.1% TFA in water
Flow rate	See Table 3
Temperature	70 °C, forced air, unless noted otherwise in the text
Gradient	See Table 3

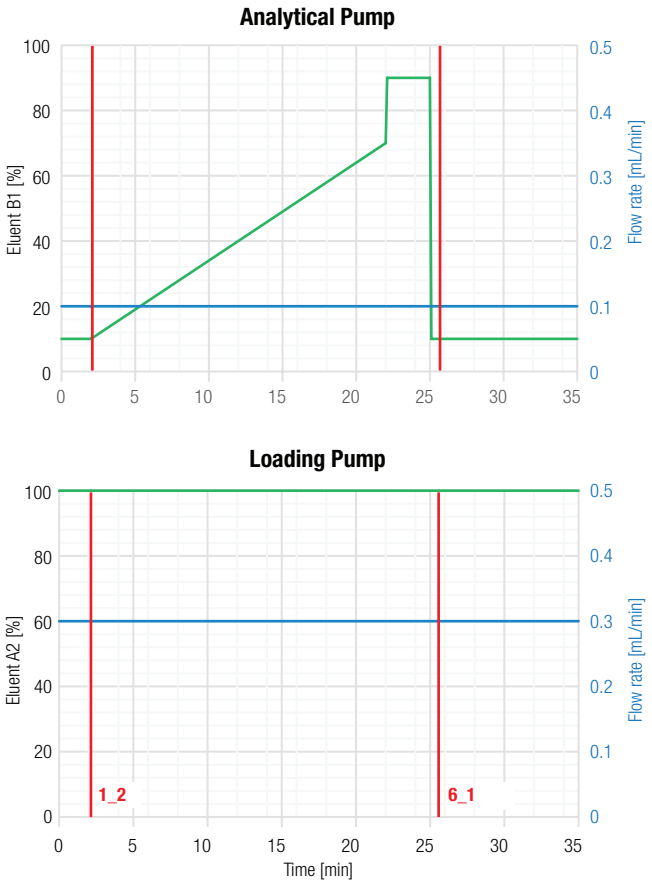

Figure 2. Valve configuration and LC gradient conditions for analytical pump / loading pump.

The lower switching valve of the Vanquish Thermostatted Column Compartment (VTCC) was used to switch between the sample loading and sample elution configuration (Figure 3). The loading pump was utilized to load the sample for two minutes on the trap column (MSPac DS-10 Desalting Cartridge) running an isocratic flow of 0.1% trifluoroacetic acid (TFA) in water at 300 µL/min. After the switching valve was switched to the elute position (Table 4), the trapping column was part of the analytical flow path and the HPG pump was delivering a 0.1% FA in water/acetonitrile gradient (Table 3) to elute the proteins from the trap column in back-flush mode and separate them on the MAbPac RP analytical column. The column outlet was coupled to the variable wavelength detector (VWD) and to the Q Exactive HF mass spectrometer in series. At 25.1 minutes, the switching valve was again switched to position 6_1 to allow the trap column to be equilibrated with the isocratic flow of the loading pump and to be ready for the next injection. If a setup with forward-flush is required to use the trap column also as a guard column, the position of the two capillaries of Port 4 and Port 5 on the switching valve (Figure 1) have to be swapped.

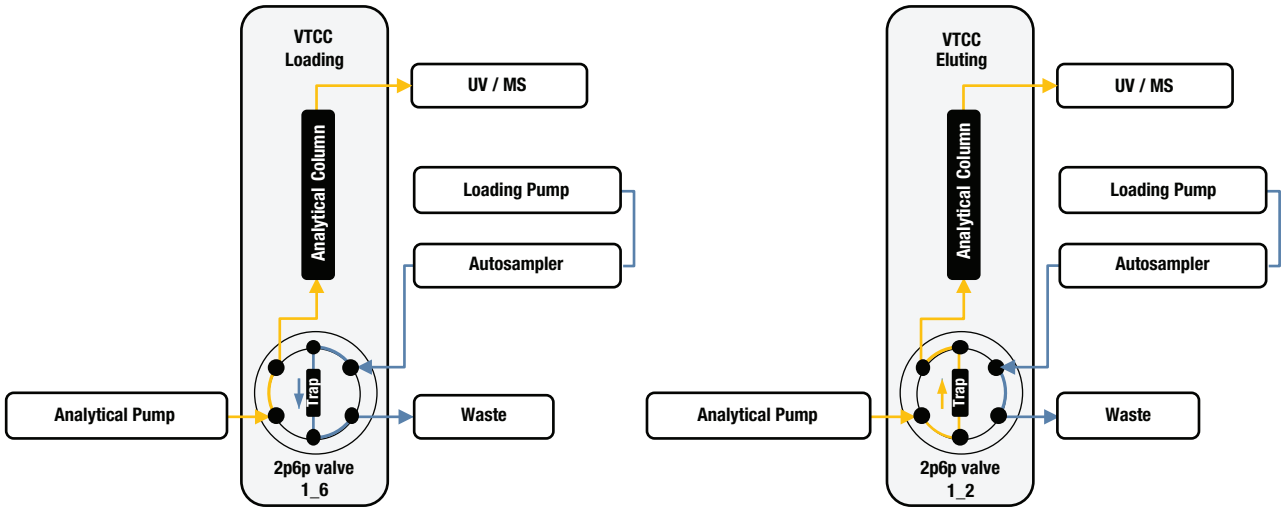

Figure 3. Valve configuration for sample trapping and sample elution/separation.

Table 3. LC gradient conditions.

Analytical Pump			
Time [min]	**A1 [%]**	**B1 [%]**	**Flow Rate [mL/min]**
0.0	90	10	0.1
2.0	90	10	0.1
22.0	30	70	0.1
22.1	10	90	0.1
25.0	10	90	0.1
25.1	90	10	0.1
35.0	90	10	0.1
Loading Pump			
Time [min]	**A2 [%]**		**Flow Rate [mL/min]**
isocratic	100		0.3

Table 4. Configuration of the lower switching valve.

Time [min]	Valve Configuration	Analysis Stage
0.0	6_1	Loading
2.0	1_2	Elute
25.1	6_1	Equilibration

MS conditions

Instrumentation

Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with MS source and method parameters shown in Table 5.

Data processing

The data were acquired exclusively with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR4, and Thermo Scientific™ BioPharma Finder™ software, version 2.0 was used for data analysis.

Table 5. Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer settings.

MS Source Parameters	Setting
Source	Ion Max source with HESI-II probe
Sheath gas pressure	25 psi
Auxiliary gas flow	10 arbitrary units
Vaporizer temperature	150 °C
Capillary temperature	280 °C
S-lens RF level	60
Source voltage	3.5 kV
MS Method Parameters	**Setting**
Method type	Full MS only
Full MS mass range	500–2800 / 1800–5000 *m/z*
Resolution settings	120k / 15k (FWHM at *m/z* 200)
Target value	3e6
Max injection time	200 ms
Microscans	1 / 10
SID	10 / 20 eV

Results and discussion

It has been shown that the column temperature is a critical parameter for the chromatographic separation of proteins with reversed phase chromatography[1] and thus needs to be optimized during the method development. At a column temperature of 50 °C or lower, the peak shape of mAbs is very poor, resulting from an extended elution time. However, an elevated column temperature of 70 °C results in improved peak shapes and peak widths.[2] Secondary interactions with the stationary phase provide one explanation for this phenomenon.[3] This effect also influences the trapping efficiency of proteins as shown in

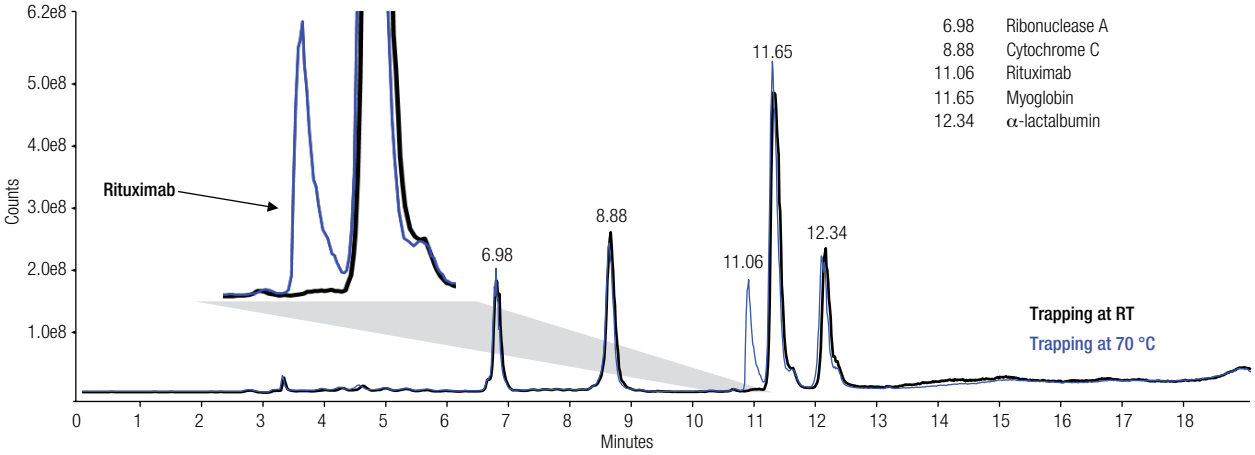

Figure 4. Analysis of a five protein mixture trapped on a MSPac DS-10 cartridge at 70 °C (blue trace) and at room temperature (black trace). Proteins were separated on a MAbPac RP column at 70 °C, showing the result for ribonuclease A, cytochrome C, rituximab, myoglobin, and α-lactalbumin A (total protein concentration: 420 ng).

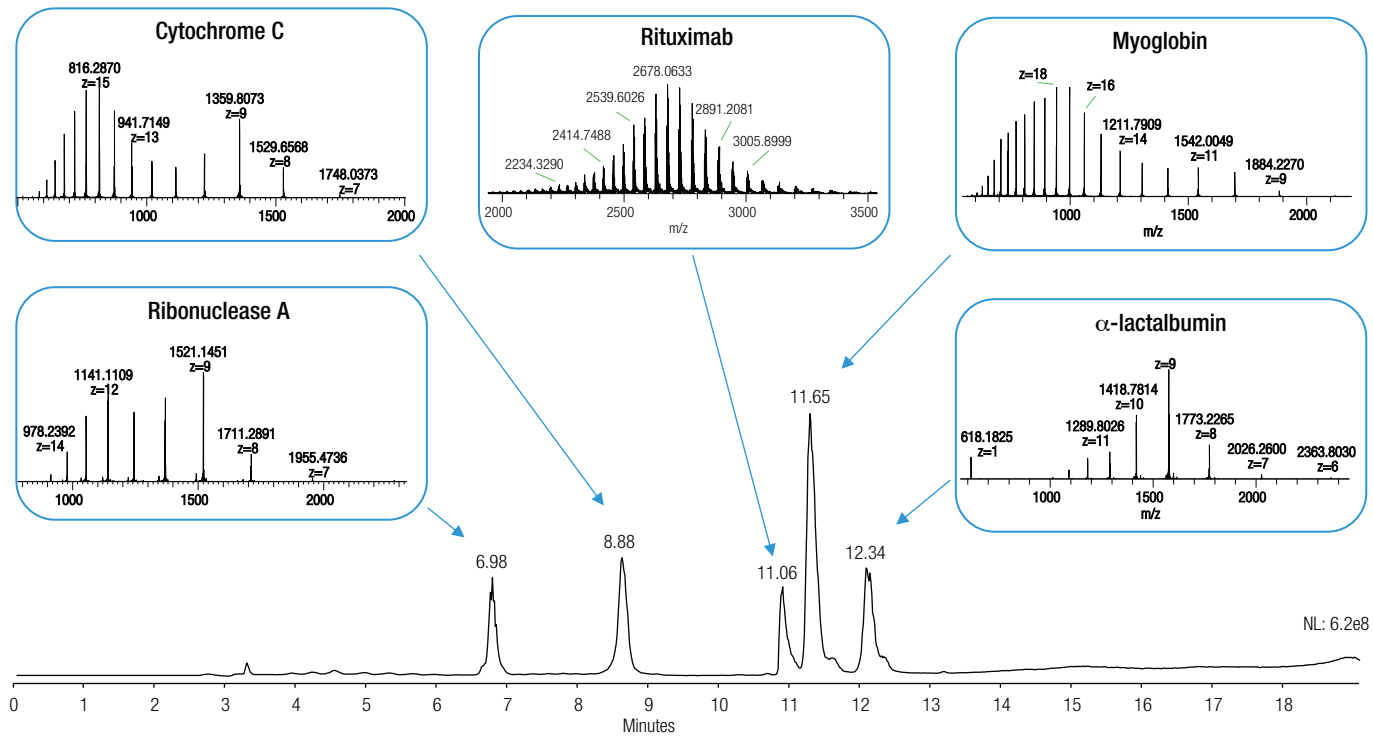

Figure 5. Analysis of a five protein mixture showing the separation and representative mass spectra for ribonuclease A, cytochrome C, rituximab, myoglobin, and α-lactalbumin A (total protein concentration, 600 ng) trapped on a MSPac DS-10 cartridge and separated on a MAbPac RP column.

Figure 4. Trapping and separating the proteins at 70 °C results in a baseline-resolved chromatogram for all five proteins. Changing the trapping temperature to room temperature and keeping the analytical column at 70 °C results in a loss of the rituximab peak (#3 -RT 11.06 min). The supressed elution of the mAb from the trapping column at room temperature causes this missing rituximab peak in the chromatogram and leads to a slightly increased baseline between 11 and 16 minutes. The trap column temperature has a major influence on the loading efficiency of monoclonal antibodies, but a less pronounced effect for smaller proteins (10–20 kDa). This example outlines the importance of elevated temperatures for protein trapping as well as protein separations.

Using the described online SPE setup for protein samples allows a very fast and simple way to efficiently clean up, enrich, and separate proteins for LC-MS analysis. Figure 5 shows a representative total ion current (TIC) chromatogram for the baseline separation of the five protein mixture. The sample was loaded to the trap column and the actual separation started at 2.0 minutes. The peak at 3.2 minutes

shows the eluting TFA, retained to the trap column during the loading step, and other very polar compounds eluting from the analytical column. All proteins of the mixture could be separated and the corresponding averaged mass spectra are shown in Figure 5. The rituximab spectrum consists of 15 averaged scans and all other spectra are an average of 87 scans. The mass spectrum for rituximab was acquired with a resolution of 15,000 and 10 microscans. For all other protein spectra, a resolution setting of 120,000 and 1 microscan were used to acquire isotopically resolved spectra for the determination of the monoisotopic mass.

Using the binary HPG pump for the chromatographic separation enables very fast separations, due to the very low gradient delay volume of this pump. Despite the larger gradient delay volume the quaternary LPG pump, it is sufficient for loading under isocratic conditions and can be used in this setup also for very fast separations.

The intact mass of the five proteins in the mixture and the four most abundant glycoforms of rituximab are obtained after the deconvolution of the full MS mass spectra with the BioPharma Finder software. Theoretical and measured masses are shown in Table 6 with the individual mass deviation for each protein/isoform. The calculated mass deviation for all proteins is below 5 ppm and demonstrates the remarkable mass accuracy of the Q Exactive mass spectrometer.

Table 6. Theoretical monoisotopic / average molecular weights (MW), measured MW, and mass deviation for the proteins and associated variants.

Protein	UniProt Accession Number	Theoretical MW (*monoisotopic*/ average) [Da]	Peak # - Retention Time [min]	Measured MW (*monoisotopic*/ average) [Da]	Mass Deviation [ppm]
Ribonuclease A (bovine)	**P61823**	*13673.260*	Peak 1 – 6.98 min	*13673.236*	**1.76**
Cytochrome C (bovine)	**P62894**	*12222.200*	Peak 2 - 8.88 min	*12222.180*	**1.64**
Rituximab + 2 G0F glycans	–	**147074.60**	Peak 3 -11.06 min	**147074.63**	**-0.14**
Rituximab + G0F/G1F glycans	–	**147236.74**	Peak 3 -11.06 min	**147237.31**	**-3.81**
Rituximab + G0F/G2F or (G1F)2 glycans	–	**147398.89**	Peak 3 -11.06 min	**147398.17**	**4.89**
Rituximab + G1F/G2F glycans	–	**147561.03**	Peak 3 -11.06 min	**147560.97**	**0.41**
Myoglobin (horse)	**P68082**	*16940.960*	Peak 4 – 11.65 min	*16940.956*	**0.24**
α-lactalbumin A (bovine)	**P00711**	*14168.747*	Peak 5 – 12.34 min	*14168.728*	**1.36**

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Conclusion

The method shown here for the separation of a five protein mixture demonstrates the applicable online SPE setup using Vanquish UHPLC systems for biopharma samples of medium complexity. The setup can be used for fully automated sample cleanup and enables direct injection of untreated samples. For the analysis of low abundant compounds, the setup can also be used for analyte enrichment, with the possibility of high volume injections. In comparison to time- and labor-intensive manual offline SPE, this automated method is faster and less prone to errors. The LC-MS system with single point Chromeleon CDS control fulfills GMP/GLP requirements and is a turn-key solution for fully integrated and automated sample handling.

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