Research Article

Pharmaceutical BIOPROCESSING

of

A high throughput ultra performance size exclusion chromatography assay for the analysis of aggregates and fragments of monoclonal antibodies

Background: Size exclusion chromatography (SEC) has been employed as an essential assay for aggregate characterization of in-process intermediates, release testing and stability studies of biologics (Q6B-ICH). Ultra-performance SEC (UP-SEC) that enables improved separation of different size species within a shorter running time than HP-SEC is highly desired. **Results:** We developed a 5-min UP-SEC assay based on BEH200 column for analysis of monoclonal antibodies on UPLC systems following screening of 13 different SEC columns. This UP-SEC assay has been evaluated with multiple antibody stability and in-process samples. The performance parameters including the resolution have been studied. **Conclusion:** This new UP-SEC method with 80% shorter running time has demonstrated better or equivalent separation efficiency than the HP-SEC method. This UP-SEC has been successfully implemented in bioprocess development and analytical testing.

Xiaodun Mou*[,‡,1], Xiaoyu Yang[‡,1], Hong Li[1], Alexandre Ambrogelly[1,2] & David J Pollard[1]

[1]Biologics BioProcess Development, Merck Research Laboratories, Merck & Co., Inc., 2000 Galloping Hill Road, Kenilworth, NJ 07033, USA

[2](Current address) Gilead Sciences, 4049 Avenida de la Plata, Oceanside, CA 92056, USA

*Author for correspondence:
Tel.: +1 908 740 5176
xiaodun.susan.mou@merck.com

‡Authors contributed equally

Size exclusion chromatography (SEC) is a molecular size base separation method. SEC is most commonly used in the pharmaceutical industry for the detection and quantitation of impurities in biologics, in particular, of **aggregates**, which is very important for each single therapeutic biologics in development and on the market [1–5]. While a number of analytical methods are used to detect and quantify high molecular aggregates, SEC remains the most commonly used method in the biotechnology industry. The simplicity and robustness of the method make it suitable for routine analysis of process intermediates as well as final products in regulated environment for release and stability tests. Although the conventional SEC is able to meet needs for most assay requirements, the low throughput of the method renders it unsuitable for applications in PAT where analytical results have to drive real-time decisions during process. A high throughput SEC assay is required for monitoring real-time purity during production process [6, 7] and for accommodating analytical needs in bioprocess automation [8, 9]. There are a few attempts to develop a high throughput SEC assay with sufficient separation efficiency, good reproducibility, and robustness [6, 10].

The need for methods with a shorter run time without compromising resolution of aggregates and low molecular weight fragments has been addressed by taking different strategies. Changing temperature, mobile phase (MP), flow rates, and use of small matrix particles, and HPLC with minimal extra-column volumes have been adapted to improve the column separation efficiency [11]. The packing of small resin particles is considered one of the most effective ways to increase column separation efficiency. The use of smaller particles yields greater column plate numbers, resulting in better **resolution** and higher sensitivity with a faster analysis time. In addition, column packing with smaller particles can be operated at higher flow rates with minimal impact on the performance, in contrast to columns with large particles (>3 µm) [12, 13]. Until recent years, the practical particle size limit was around 3 µm since the smaller particle size resulted in high backpressures above the limit of conventional HPLC systems, which created a challenge in packing a homogenous column [14, 15]. Therefore thus

FUTURE SCIENCE part of fsg

Key Terms

Aggregate: Misfolded or denatured proteins aggregate to form complexes of large molecular size during production and storage.

Column resolution: The separation efficiency of two nearby peaks resolved by the column

Monoclonal antibodies: Mono-specific antibodies are made by identical production (or immune) cells that are all clones of a unique parent cell. They recognize the identical epitope in the antigen.

far, most well established SEC assays were developed on traditional HPLC systems since early 1950s [16, 17]. The UPLC systems with a higher pressure tolerance (>10,000 psi) have been available since early 2000s and enable the benefits of sub-2 µm particles [12]. The UPLC system equipped with small tubing (<0.25 mm) and low extra-column volumes is able to further improve the separation efficiency. Numerous studies have been carried out to investigate the impacts of system pressure, MP composition, pH, and column temperature on separation efficiency [18–24]. Optimization strategies have also been investigated for analysis of specific biological products using SEC on UPLC systems in comparison to HPLC systems [1, 25–28].

As UPLC systems become available in industrial laboratories, a high throughput and sensitive SEC method becomes a possibility. A major challenge to develop the method is to find a proper commercial SEC column appropriate for ultra-performance SEC (UP-SEC) analysis of biologics drugs. Although SEC columns packed with relatively small resin particles can be purchased from several vendors including Waters, Agilent, Sepax and YMC, a comprehensive evaluation and comparison of these columns has yet to be performed. As an exception, the Waters BEH200 column with 1.7 µm particle size has been used as one of most frequently used SEC UPLC columns for comparison of its performance with high-performance SEC (HP-SEC) [13, 28–30]. The study described in this paper has screened and compared 13 UP-SEC columns with different particle size, pore size, and dimensions from four manufacturers to evaluate their separation efficiency and throughput on analysis of **monoclonal antibodies** and their in-process and stability samples. In addition, various MP buffers were tested to develop an UP-SEC method optimized for high throughput analysis of monoclonal antibodies.

Experimental procedures

Chemicals & samples

HPLC grade water, sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, 85% phosphoric acid, iso-propanol and methanol were purchased from Fisher. Sodium hydroxide solution (50%, w/w) was purchased from Mallinckrodt. Bovine serum albumin (BSA) was purchased from Sigma.

A Gel filtration standard mixture (GF-Std) that contained thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.35 kDa), was pur-

Table 1. Samples used in the development and evaluation of the UP-SEC method.

Sample	Description	Concentration (mg/ml)
GF Std Mix	Std mix	25
mAb1	Purified drug substance	51.7
mAb2	Purified drug substance	50.0
mAb3	Purified drug substance	27.0
mAb4	Purified drug substance	108.3
mAb5	Purified drug substance	39.5
mAb1-S2	mAb 1 thermal stability sample-2 month	25
mAb1-S3	mAb 1 thermal stability sample-3 month	5
mAb2-P1	mAb2 in-process sample 1	4.5
mAb2-P2	mAb2 in-process sample 2	0.5
mAb3-P	mAb3 in-process sample	5.1
mAb5-P1	mAb5 in-process sample 1	5.1
mAb5-P2	mAb5 in-process sample 2	4.3
mAb5-P3	mAb5 in-process sample 3	4.1
mAb5-P4	mAb5 in-process sample 4	6.2
mAb5-P5	mAb5 in-process sample 5	42

chased from BIO-RAD (#151–1901), and was diluted to 1 mg/ml with water or PBS before injection. The purified materials of 5 typical mAb drug substances (mAb1-mAb5) were collected at different purification steps or obtained from stability studies. These mAbs include IgG1, IgG2 and IgG4. The antibodies with various amounts of aggregates or cleavage fragments were used to assess the UP-SEC performance.

The sample information is listed in Table 1 in details. All the samples with a concentration higher than 1 mg/ml have been diluted into 1 mg/ml with water or PBS before injection.

Columns

A total number of 13 analytical UP-SEC columns that were packed with resin particles of less than 3 µm were purchased from Agilent, Sepax, Waters and YMC, and were evaluated in this study. The type and dimension of each column and their separation efficiency are described in section of Column screening. To keep the consistent elution profiles for mAbs samples, the newly purchased columns were pre-conditioned with multiple (5) injections of 1 mg/ml BSA as the pre-treatment.

UPLC

The UPLC was a Waters Acquity UPLC system with quaternary H Class pump, PDA detector, FLR detector, temperature controlled sample manager, column manager, and an external column heater/cooler (for columns longer than 150 mm) or an Agilent 1290 UPLC system with equivalent components. These two systems were used in this study to assess the different separation conditions, and performance sample analysis. Data from Waters UPLC system and Agilent 1290 UPLC system was analyzed using Empower and ChemStation, respectively.

Molecular mass analysis

The molecular size analysis of aggregate and fragments of the antibodies was performed by using SEC-MALLS with MiniDawn TriStar and OptiLab rEX RI (detector from Wyatt technology). Samples were separated on a HPLC column (YMC Diol 200, 8.0 × 300 mm) on an Agilent 1200 system at ambient temperature. The MP was 50 mM phosphate buffer, pH 7.4 with 200 mM NaCl, and the flow rate was 0.5 ml/min. The running time was 30 min and signals were recorded at 280 and 214 nm. Light scattering and refractive index signals were also collected for 25 min by ASTRA V software. High-molecular-weight (HMW) aggregates, the monomer, and the low-molecular-weight (LMW) components of the antibody were analyzed by ChemStation and ASTRA V. The molecular mass of detected components was calculated by ASTRA V.

HP-SEC assay

For comparison with UP-SEC, the analysis of all the samples was performed using a HP-SEC assay by an Agilent HPLC system (1100 series) with UV detection at 214 nm. The auto sampler was temperature-controlled at 4°C. The chromatographic separation was performed at a flow rate of 0.5 ml/min using an YMC-Pack Diol200 column (5 µm, 200 Å, 300 × 8.0 mm) at room temperature. The MP was 50 mM sodium phosphate, 200 mM NaCl at pH 7.0. The total run time was 30 min.

Finalized UP-SEC assay

UP-SEC was optimized under various conditions for column and buffer screening as indicated in the result section. The optimized UP-SEC assay uses a Waters BEH200 column (P/N: 186005225) on a Waters Acquity UPLC system at the ambient temperature (25°C). The sampler was temperature-controlled at 4°C. The separation was performed at a flow rate of 0.5 ml/min using 100 mM sodium phosphate, 100 mM NaCl at pH 7.0 as MP. The run time was 5 min with A214 as the suggested detection wavelength, and A280 was also collected.

Calculation of selectivity, resolution & plate number

Selectivity

The time between sample injection and an analyte peak reaching a detector at the end of the column is termed the retention time (t). The time taken for the unretented species to pass through the column is called t_0. The selectivity factor, α, which describes the separation of two species (1 and 2) on the column, is calculated as the quotient of the net retention/migration times of both species t2 and t1:

$$a = (t_2 - t_0)/(t_1 - t_0)$$

For Waters BEH 200 SEC column, t0 is 1.9 min at a flow rate of 0.5 ml/min, describing a time needed to elute an unretented species in the void volume.

Resolution

Although the selectivity factor, α, describes the relative separation of two peaks, it does not take into account peak widths. Another measure of how well species have been separated is determined by the resolution. The resolution of any two separated species, 1 and 2, is defined by their retention time (t1 and t2) or elution volume and peak width (W1 and W2) at half peak height as follows.

$$Rs = \frac{1.18(t2 - t1)}{(W1 + W2)}$$

Resolution between indicated peaks was calculated manually or by using ChemStation or Empower software.

Tailing factor

The tailing factor (also called symmetry factor) of a peak was calculated by the following equation:

$$As = W0.05/2f$$

where W0.05 is the peak width at 5% of the peak maximum height from the baseline, and f is the distance of the peak maximum position horizontally to the leading edge of the peak at 5% peak height.

Plate number

The column efficiency is described by the number of theoretical plates (N). The number of theoretical plates can be measured by analyzing retention time (t) and peak width (w) at half peak height of a chromatographic peak as follows:

$$N = 5.55t^2/W^2$$

where W is the peak width at half-height. The plate number was calculated by the ChemStation or Empower software.

Results & discussion

Column screening

The separation of protein molecules on size exclusion chromatography is greatly impacted by the dimension

Table 2. Column screening with GF-Std mixture.

Brand/ type	Dimension (mm)	Cat./Code	Max FR (ml/min)	Run time (min)	*$Rs^{1\&2}$	*$Rs^{2\&3}$	$\alpha^{2\&3}$
150 mm columns							
Agilent SEC3	3 μm, 15 nm, 4.6 × 150 mm	5190-2509	0.5	7	0.92	1.47	3.50
Agilent SEC3	3 μm, 30 nm, 4.6 × 150 mm	5190-2514	0.5	5	1.99	1.95	1.48
Sepax Zenix SEC-300	3 μm, 30 nm, 4.6 × 150 mm	213300-4615	0.5	5	2.26	1.94	1.55
Sepax Zenix-C SEC-300	3 μm, 30 nm, 4.6 × 150 mm	233300-4615	0.5	5	2.01	2.08	1.69
Sepax Zenix SEC-300	3 μm, 30 nm, 2.1 × 150 mm	213300-2115	0.1	7	1.12	0.66	1.74
Waters BEH 200 SEC	1.7 μm, 20 nm, 4.6 × 150 mm	186005225	0.5	5	3.34	3.02	1.96
YMC Diol 200	2 μm, 20 nm, 4.6 × 150 mm	YT-458	0.5	5	1.36	1.18	1.74
YMC Diol 200	3 μm, 20 nm, 4.6 × 150 mm	YT-464	0.5	5	1.93	1.41	1.62
YMC Diol 300	3 μm, 30 nm, 4.6 × 150 mm	DL30S03-1546WT	0.5	5	2.11	0.83	1.26
300 mm columns							
Agilent SEC3	3 μm, 30 nm, 4.6 × 300 mm	5190-2513	0.5	12	1.03	2.69	1.67
Waters BEH 200 SEC	1.7 μm, 20 nm, 4.6 × 300 mm	186005226	0.4	15	3.81	3.46	1.80
YMC Diol 200	3 μm, 20 nm, 4.6 × 300 mm	YT-466	0.5	10	2.11	1.57	1.78
YMC Diol 300	3 μm, 30 nm, 4.6 × 300 mm	YT-467	0.6	10	2.63	1.26	1.57

*$Rs^{1\&2}$ refers to the resolution between peaks 1 and 2. *$Rs^{2\&3}$ and $\alpha^{2\&3}$ refer to the resolution and selectivity between peaks 2 and 3.

of the SEC column, and type and size of the resin. In this study, the column candidates selected have a particle size of 3 µm or less and pore size of 300 Å or less for either columns dimensions of 4.6 × 150 mm or 4.6 × 300 mm. A mixture of gel filtration standards (GF-Std), mAb1 control, and its stability sample (40°C 2 months) were used for the assessment of column performance.

Column screening with GF-Std mixture & BSA

A total of 13 different columns were tested with Gel Filtration standard (GF-Std) mixture for separation performance (Table 2). A flow rate (0.4–0.6 ml/min) was applied on each column to achieve a total run time of 5–10 min. At these flow rates, the back pressures are well within the pressure limit of the column and UPLC systems. The separation profiles of the GF-Std mixture on representative columns (4.6 × 150 mm) from each manufacturer are shown in Figure 1A, which includes Waters BEH200 column (186005225), Agilent SEC3 column (5190–2514), Sepax Zenix SEC-300 column (213300–4615) and YMC Diol200 column (YT-464).

Although the major standard peaks were all separated from each other according to their molecular mass on dif-

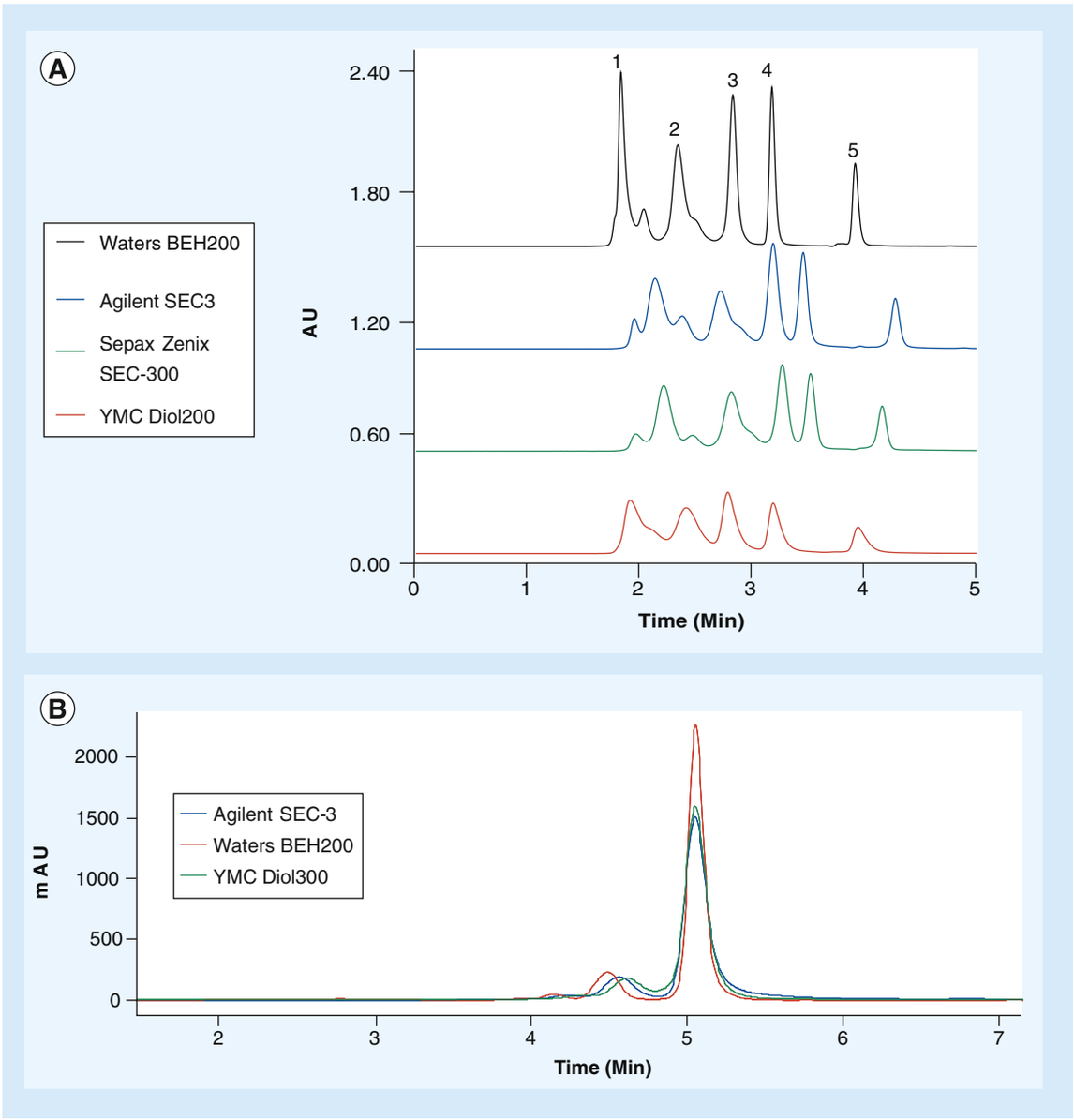

Figure 1. Separation of gel filtration standards and BSA oligomers on various UP-SEC columns using a Water Acquity UPLC system. (A) Gel filtration standards. Peak 1: Thyroglobulin, 670 kDa; Peak 2: γ-globulin, 158 kDa; Peak 3: Ovalbumin, 44 kDa; Peak 4: Myoglobin, 17 kDa; Peak 5: Vitamin B12, 1350 Da. (B) Comparison of different columns in analyzing BSA oligomers.

ferent columns, the shoulder peak between peaks 1 and 2 was best resolved on the Agilent SEC-3 and Zenix SEC-300 columns, and well resolved by Waters BEH200 on which the fronting shoulder of peak 1 was not detected (Figure 1A). These shoulder peaks were not resolved by the YMC-Diol 200 (Figure 1A). Table 2 summarizes the resolution between different peaks of the GF-Std on all tested columns. Most of the 150 mm columns were able to provide a full separation of the GF-std mix within 7 min, while the 300 mm columns were able to finish the separation within 10–15 min. The resolutions ($Rs^{1\&2}$) between peak 1 (Thyroglobulin, 670 kDa) and peak 2 (γ-globulin, 158 kDa), and $Rs^{2\&3}$ between peak 2 and peak 3 (Ovalbumin, 44 kDa), and selectivity factor (α) of peak 2 and 3 were calculated for each column. Among all the tested columns, both Waters BEH200 columns (150 and 300 mm) yielded the best resolution ($Rs^{1\&2}$ and $Rs^{2\&3}$) (Table 2). $Rs^{1\&2}$ represents resolution of MW species equivalent to monoclonal antibody oligomers (aggregates) and monomer, and $Rs^{2\&3}$ represents MW species equivalent to antibody monomer and fragment range (~50 kDa). The 150 mm BEH200 column also gave the shortest run time of 5 min. However, since the BEH200 column has the smallest particle size (1.7 µm), it yielded the highest pressure compared with other columns at the same flow rate. As a result, the 300 mm BEH200 column can only be used at a maximum flow rate of 0.4 ml/min with the longest total run time of 15 min of all the tested columns. The Zenix SEC300 and Zenix-C SEC300 columns (3 µm, 30 nm and 4.6 × 150 mm) from Sepax presented the second best separation power with high resolution and selectivity factor on both HMW and LMW species. The Agilent SEC-3 column (3 µm, 30 nm and 4.6 × 150 mm) gave comparable performance to the Zenix columns. Both Agilent SEC-3 and Sepax Zenix (3 µm, 30 nm and 4.6 × 150 mm) columns were able to resolve a leading shoulder in front of peak 1, indicating those two columns have a better resolution for large molecular mass species than Waters BEH200.

The Sepax Zenix column with a 2.1 mm diameter only allowed a maximum flow rate of 0.1 ml/min, and gave relatively poor separation on both high MW and low MW species. The YMC columns came in variant combination of particle size, pore size and column length. All the YMC columns can reasonably separate all the species, but none of them gave a better overall performance when compared with the columns from other brands.

Waters BEH200 column (186005225), Agilent SEC3 column (5190–2514), Sepax Zenix SEC-300 column (213300–4615) and YMC Diol200 column (YT-464) were further evaluated using BSA, which is a typical standard for SEC column screening. A significant difference was observed for BSA analysis among the three columns (Figure 1B). The BEH200 column displayed a sharper main peak than the Agilent SEC-3 and YMC Diol 300, and was the only column that achieved BSA dimer baseline-separation from the main peak (Figure 1B). The column screening with GF-Std and BSA both showed that Waters BEH200 column has the best performance under the test conditions, followed by Agilent SEC-3 and YMC Diol columns as second and third performers, respectively.

Column screening with stability & in-process samples of monoclonal antibodies

Three representative columns (Waters BEH200, Agilent SEC-3 and YMC Diol300) with different level of performance were selected based on the screening with GF-Std Mixtures and BSA, and were further assessed in separation of antibody aggregates and fragments on an Agilent 1290 UPLC system (Figure 2). Figure 2 shows the separation profiles of mAb1 3–month stability samples containing antibody fragments and mAb2 in-process samples with a high level of aggregates. These antibody samples represent routine standard protein, typical antibody degradation samples, and antibodies containing a large amount of aggregates, and thus are good candidates to test the column performance.

The Waters BEH200 showed a much better separation of the antibody fragment of approximately 100 kDa (F1) as determined by SEC-MALLS from the main peak in the mAb1stability sample than the Agilent SEC-3 and YMC Diol 300 (Figure 2A). The mAb2-P1 sample containing a large amount of large aggregates was also analyzed on different SEC columns. Aggregates in the sample were eluted as two relatively sharp peaks before the main peak on BEH200, and as four broad peaks with the Agilent SEC-3 and YMC Diol 300 (Figure 2B). Although the Waters BEH200 is limited in resolving the large aggregates, the aggregate peaks were well separated from the main peak, allowing accurate integration of the peaks. Compared with the YMC and Agilent columns, the Waters BEH 200 column displayed better separation of the monomer, dimer, and oligomer peak in the mAb2-P2 sample that contains a large amount of dimer (Figure 2C). Waters BEH200 column was able to detect an unknown peak between the monomer and dimer, which was not seen on YMC and Agilent columns. All the observations have confirmed the screening results from the 'Column screening with GF-Std mixture and BSA' section.

Method optimization through MP

The top performer, Waters BEH200 column (1.7 µm, 20 nm and 4.6 × 150 mm) was used to study the MP effect on separation performance, and to select the best MP composition and pH for the UP-SEC assay.

MP concentration optimization

Most common MPs for SEC are phosphate buffer. The first optimization step was to find the most suitable combination of the phosphate buffer and salt to achieve best system performance. Three MP buffers containing different phosphate and salt con-

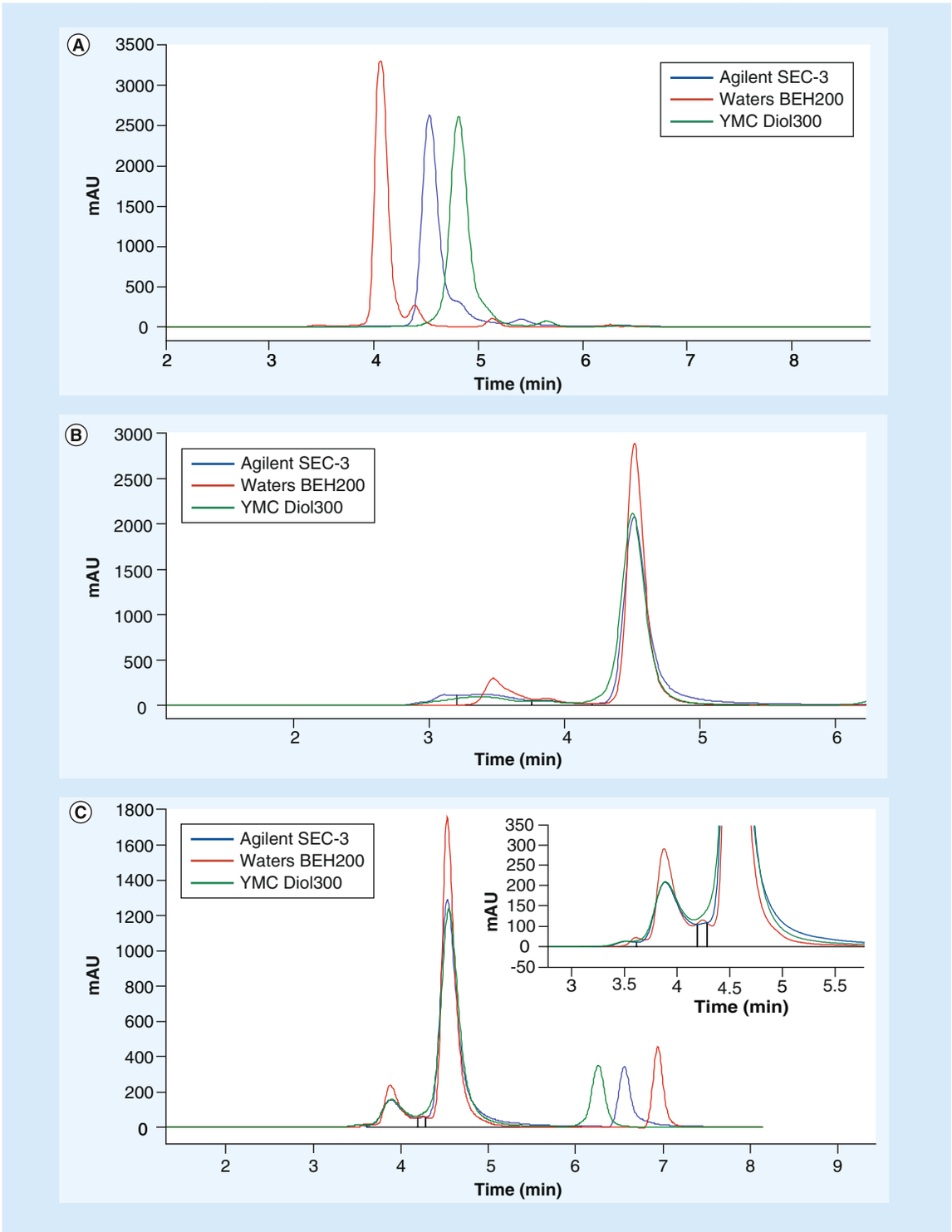

Figure 2. Separation of antibody aggregates and fragments by different UPSEC columns (4.6 x 150 mm) on an Agilent 1290UPLC. (A) mAb1-S3 stability sample, (B) mAb2-P1 in process sample and (C) mAb2-P2 oligmer sample. The flow rate was 0.3 ml/min with 100 mM sodium phosphate, 100 mM NaCl, pH 7.0 as mobile phase. The chromatograms for each column in (B) and (C) were aligned against the main peak.

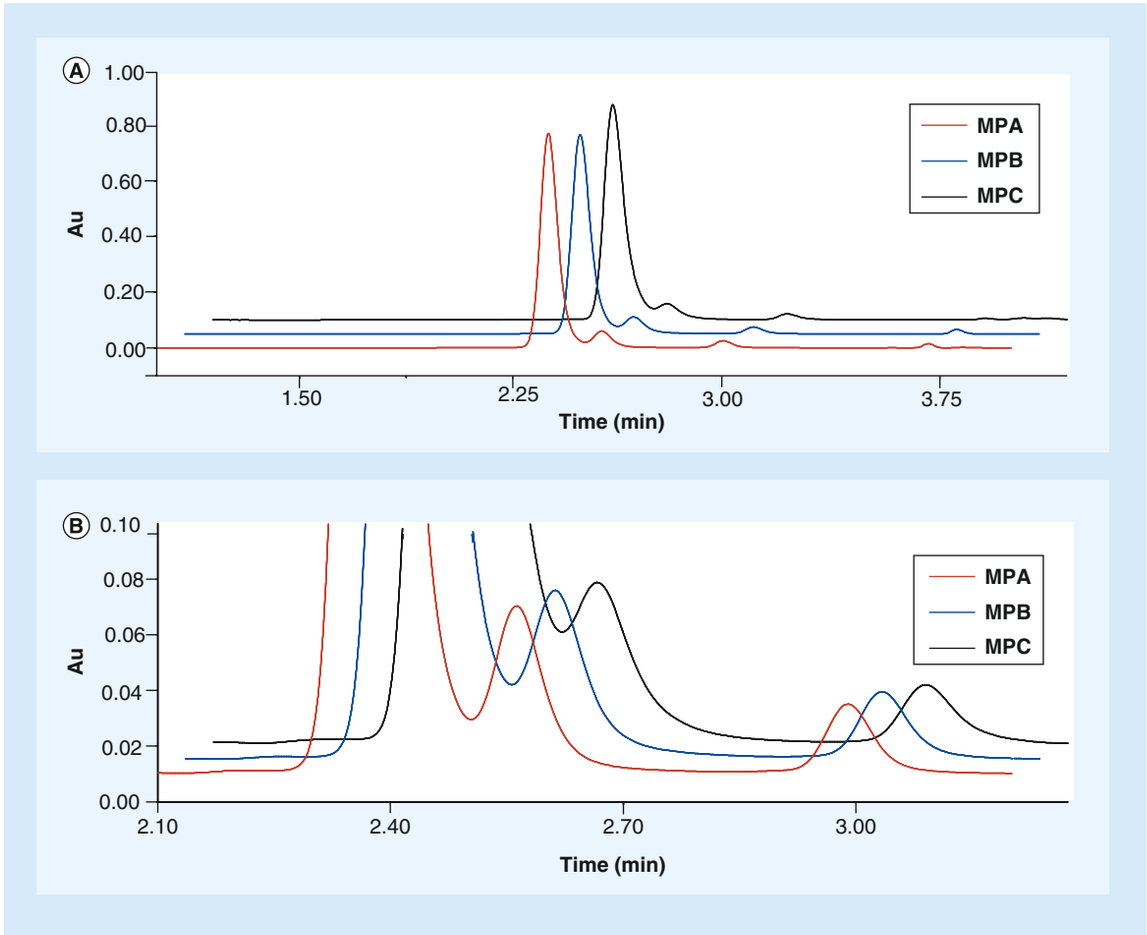

Figure 3. Analysis of mAb1-S3 stability sample on Waters BEH column using different mobile phases on Water Acquity UPLC. (A) Full sized chromatogram, (B) zoomed-in separation of fragments.

centrations were evaluated: MPA: 100 mM sodium phosphate, 100 mM NaCl, pH 7.0; MPB: 100 mM sodium phosphate, 200 mM NaCl, pH 7.0; MPC: 50 mM sodium phosphate and 200 mM NaCl, pH 7.0. The mAb1-S3 stability sample was used for MP evaluation since this sample contains a relatively high level of a 100 kDa fragment (F1) and the separation of F1 fragment from the main peak provides a good test for separation efficiency assessment. On the Waters BEH200, 100 mM phosphate buffers (MPA and MPB) yielded a better separation of the main peak and the 100 kDa fragment, compared with MPC (Figure 3). In addition, MPA yielded a slightly better separation (deeper valley) of F1 from the main peak than MPB suggesting that the salt concentration has some impact on resolution.

The total run-time, resolution between main IgG (150 kDa) and fragment 1 (F1, 100 kDa), resolution between F1 and fragment 2 (F2, 50 kDa), tailing factor and plate number of main IgG are compared in Table 3. MPA and MPB were superior MPs compared with MPC as improved resolution; peak shape and plate number was achieved in these conditions (Figure 3 and Table 3). A closer analysis also showed that MPA gave a better resolution between main IgG and F1, less tailing factor, and higher plate number for the main IgG than MPB and MPC. Therefore, MPA was selected for this UP-SEC method.

Table 3. MP screening with mAb1 stability sample.

MP	Run time (min)	Rs (IgG Vs F1)	Rs (F1 Vs F2)	Tailing main IgG	Plate no. main IgG
MPA	5	1.56	3.56	1.19	6612
MPB	5	1.41	3.12	1.33	5946
MPC	5	1.32	2.92	1.31	5091

MP pH optimization

The pH effects from the MP were studied on the Waters BEH200 column. MPA containing 100 mM sodium phosphate and 100 mM of sodium chloride at different pHs was used to examine the pH impact on the column separation efficiency. The mAb1-S3 stability sample with high amounts of fragments and the mAb2-P1 intermediate with high aggregates were

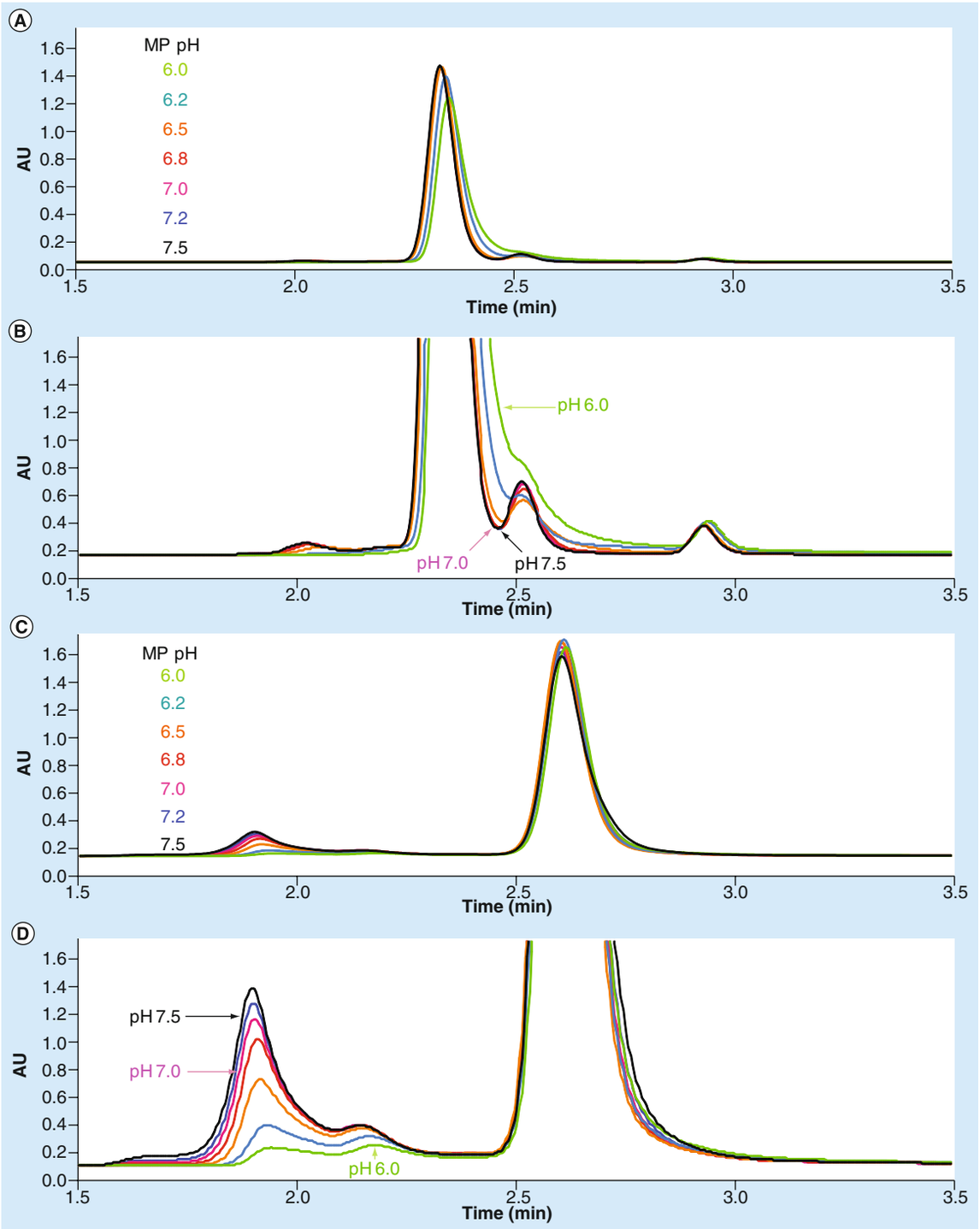

Figure 4. Comparison of mobile phase at different pHs. (A & B) Analysis of mAb1-S3 stability sample and (C & D) mAb2-P1 in-process sample by Waters BEH200 column using MPs at different pH values. (A & C) Full sized chromatograms, (B & D) zoomed-in separation of fragments and aggregates.
MP: Mobile phase.

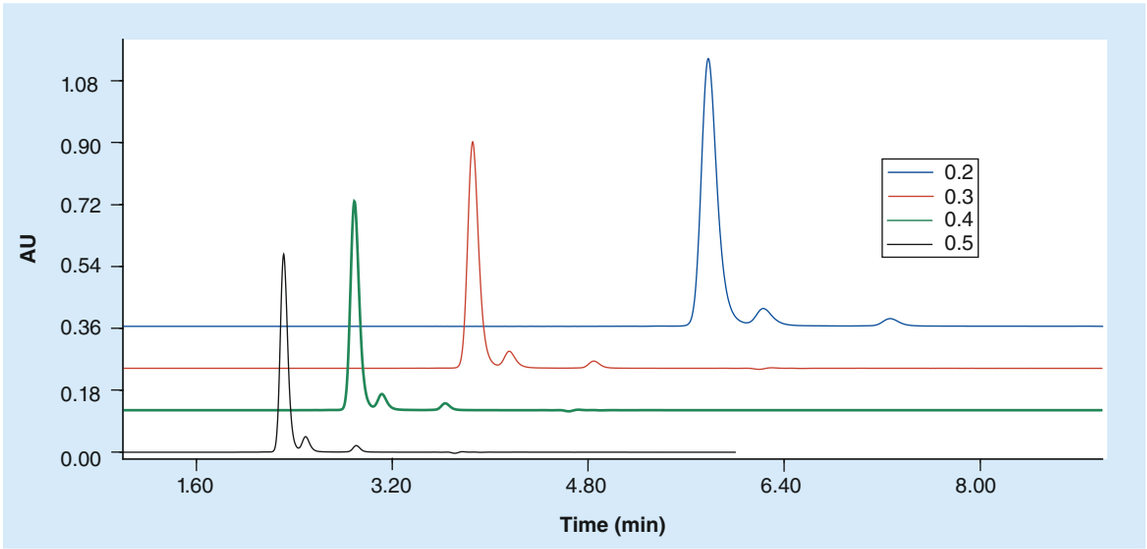

Figure 5. Comparison of the analysis of mAb1-S3 stability sample using Waters BEH200 column at flow rates from 0.2 to 0.5ml/min on a Waters Acquity UPLC system. A214 trace was normalized in the chromatograms.

Table 4. Flow rate screening with mAb1 stability sample on BEH200.

Flow rate (ml/min)	Run time (min)	Rs 1 (IgG Vs F1)	Rs 2 (F1 vs F2)	Tailing main IgG	Plate no. main IgG
0.2	15	2.03	4.36	1.37	11206
0.3	10	1.86	4.07	1.30	9572
0.4	8	1.72	3.81	1.27	8325
0.5	5	1.58	3.58	1.24	7328
HP-SEC	30	1.34	3.68	0.87	6938

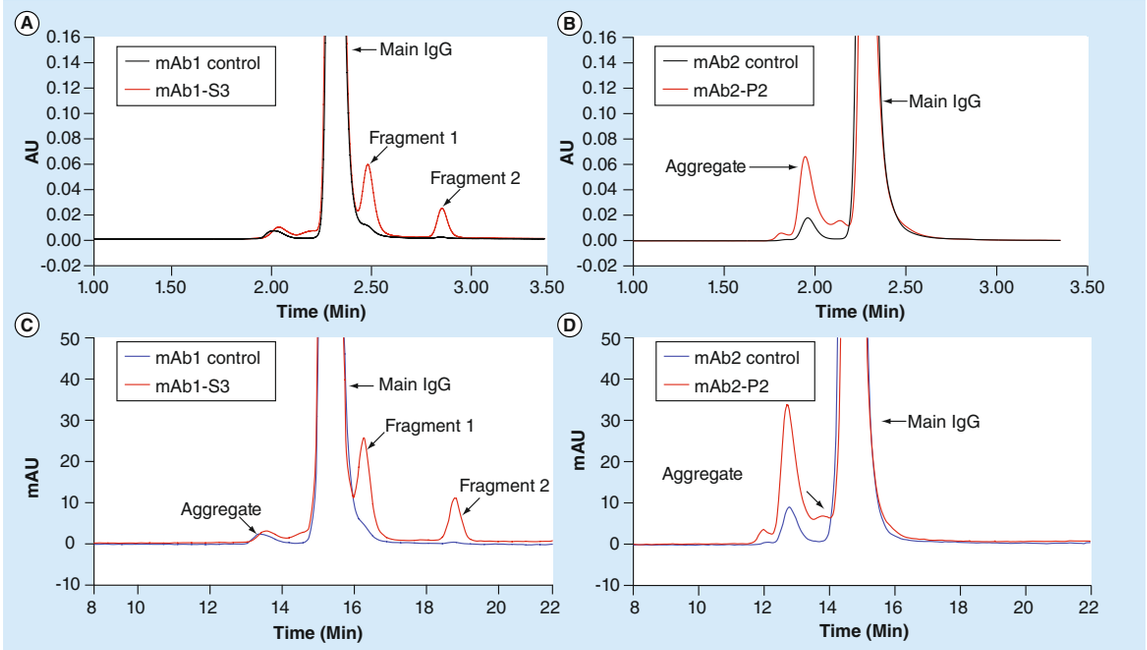

Figure 6. Comparison of HPSEC and UPSEC separation performance. (A & C) Comparison of mAb1 control and mAb1-S3 stability sample and mAb2 control and (B & C) mAb2-P2 in-process sample using (A & B) UP-SEC method on BEH200 and (C & D) HP-SEC method on YMC Diol 200.

Table 5. Results of mAb1 and mAb2 samples in percent peak area by UP-SEC and HP-SEC.

Antibody	Sample	Aggregate (%)	Monomer (%)	Fragment (%)
SEC analysis on UP-SEC with Waters BEH SEC200				
mAb1	Control	0.92	98.93	0.15
	mAb1-S3	0.96	90.76	8.28
mAb2	Control	2.00	98.00	0.00
	mAb2-P2	15.85	84.15	0.00
SEC analysis on HP-SEC with YMC Diol 200				
mAb1	Control	0.91	98.91	0.18
	mAb1-S3	0.90	91.43	7.67
mAb2	Control	1.97	98.03	0.00
	mAb2-P2	15.30	84.70	0.00

separated with these different pH buffers (Figure 4). In general, a loss of the aggregates, increased peak tailing, reduced resolution of aggregates and fragments from the main peak of the mAb1 stability sample were observed at pHs lower than 6.8 (Figure 4A & B). This observation is consistent with earlier reports indicating non-specific interactions between the aggregates/fragments and resin, or disruption of formed aggregates at low pHs [31,32]. At pH 7.0, both aggregates and fragments were optimally separated. At a pH higher than 7.0, no further improvement was seen. Similar to mAb1 stability sample, mAb2-P1 in-process sample displayed a decreasing level of aggregates at lower pHs (Figure 4C & D). The aggregates detected at pH 7.0, 7.2 and 7.5 showed a similar level of relative peak area, and SEC profile at pH 7.0 was similar to that from historical results on these samples. Based on the results, the MP composed of 100 mM phosphate and 100 mM sodium chloride at pH 7.0 offered the optimized combination for resolution of aggregates and fragments from the main antibody peak.

Flow rate optimization

The impact of the flow rate on the separation was studied with mAb1 stability sample. The separation profile at different flow rates was similar as shown in the y-scale normalized chromatograms (Figure 5). Table 4 lists the total run-time, resolution between main IgG and the fragment F1, resolution between F1 and F2, tailing factor, and plate number of main IgG at various flow rates. As expected, the resolution and plate number slightly decreased when the flow rate increased, and the peak tailing reduced at the higher flow rates. Even so, the flow rate of 0.5 ml/min has achieved sufficient separation efficiency, therefore is recommended for UP-SEC of in-process samples. At 0.5 ml/min, the assay can be completed within 5 min. A lower flow rate can be used for the high aggregates content samples or for release and stability testing to achieve better separation efficiency when needed.

UP-SEC assay evaluation & application

Comparison with the HP-SEC assay

UP-SEC on Waters BEH 200 column (4.6 × 150 mm) offered the best resolution among other columns in our column test, and showed better separation efficiency than the regular high performance size exclusion chromatography (HP-SEC) even at the highest flow rate tested (0.5 ml/min) (Table 4). Therefore, a detailed analysis of the results of UP-SEC and HP-SEC were performed by using the mAb1 control with its associated 3 month stability samples, and mAb2 control with its in-process sample (Figure 6). The total run time was 5 min for the UP-SEC method using Waters BEH200 (4.6 × 150 mm) and 30 min for the HP-SEC method using YMC Diol200 (7.8 × 300 mm). The UP-SEC assay was performed using a Waters Acquity UPLC system, and the HP-SEC assay was performed using an Agilent 1100 HPLC

Table 6. Comparison on the separation efficiency between UP-SEC and HP-SEC methods.

Method	Selectivity (α)			Resolution (*Rs*)			Plate number (*N*)			
	Agg-IgG	IgG-F1	F1-F2	Agg-IgG	IgG-F1	F1-F2	Aggregate	Main IgG	F1	F2
UP-SEC	3.52	1.36	1.62	2.14	1.62	3.65	1312	7263	6297	11608
HP-SEC	1.06	1.05	1.17	1.62	1.32	3.63	1587	6829	5451	12313

Key Term

Fragmentation: Chemical or enzymatic cleavage of antibodies or proteins into small fragments during production and storage.

system. Both methods successfully separated and detected similar levels of the fragments and aggregates from the main IgG (Figure 6 and Table 5). They showed the increase of fragments in mAb1–3M stability sample and the large amount of aggregates in mAb2-F10.

The selectivity, resolution between the adjacent peaks, and plate number for both UP-SEC and HP-SEC are listed for mAb1–3M sample in Table 6. Consistent with the results in Table 4, the BEH200 column showed better selectivity between all pairs of components, and better resolution of the main IgG from the aggregate and F1. The BEH200 had similar plate numbers although its length is half of that of the YMC column in the HP-SEC assay. Increased selectivity between listed pair peaks on the BEH200 column is likely a primary factor for increased resolution, based on the fundamental resolution equation ($R_s = [N^{1/2}/4][(\alpha\text{-}1)/\alpha][k_2'/(1+k_2')]$). In addition, the small particle size in the BEH200 column creates an ultra-high pressure, which lowers the longitudinal effect and results in a narrower peak width and greater peak height compared with the traditional HPLC columns [12].

The linearity response, reproducibility, and sample recovery of the UP-SEC assay were evaluated. Results indicated that the UP-SEC assay displayed excellent response to the injection amount of protein with high reproducibility (data not shown)

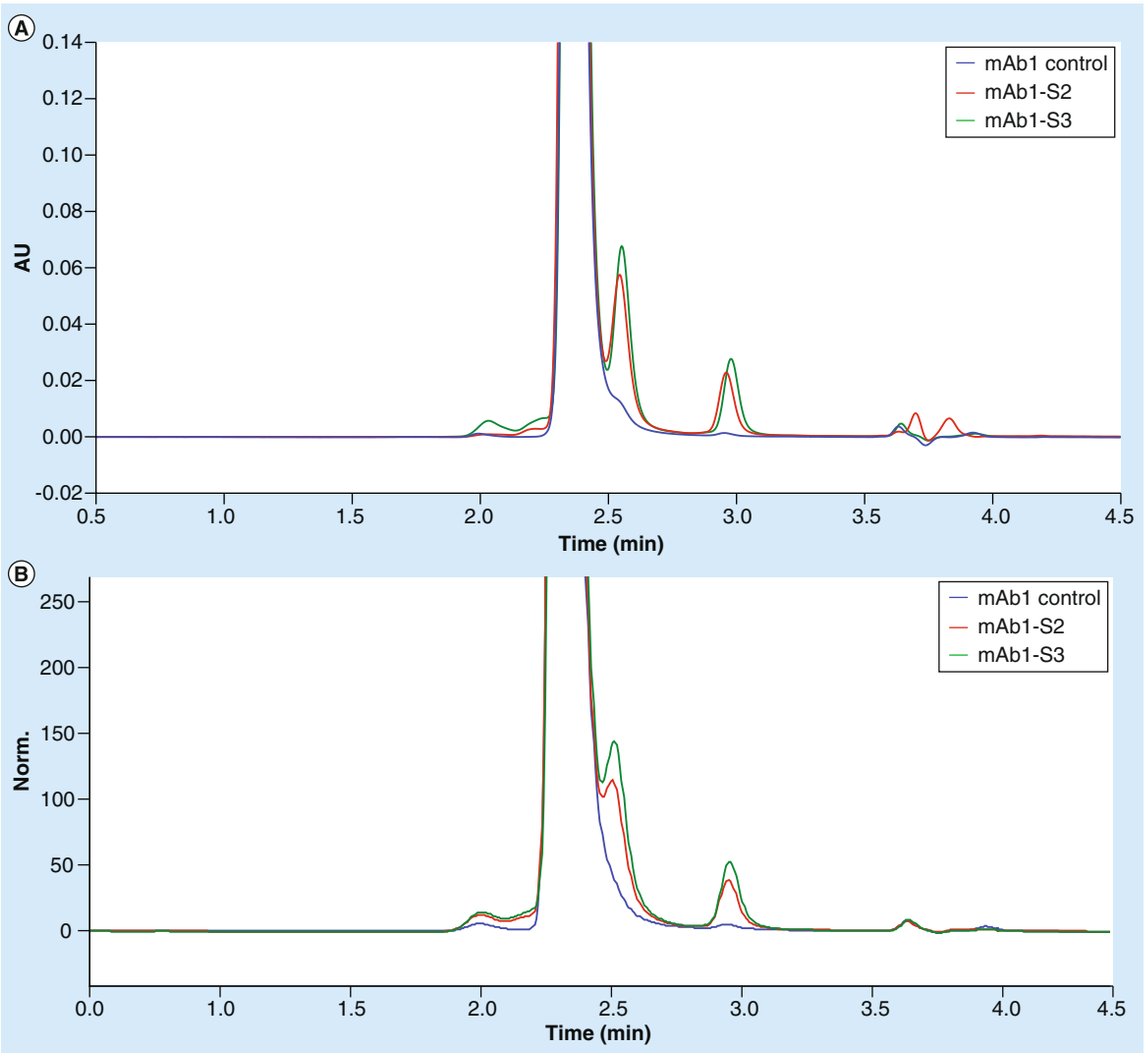

Figure 7. Comparison of Waters Acquity UPLC system and Agilent 1290 UPLC system. (A) UP-SEC results on mAb1-S2&S3 stability samples using Waters BEH200 column on Waters Acquity UPLC system and (B) Agilent 1290 UPLC system.

Table 7. Method and system comparison on percent peak area.

Method/instrument	Sample	Aggregate (%)	IgG (%)	F1 (%)	F2 (%)
HP-SEC Agilent 1100	mAb1-S2	0.76	92.81	4.85	1.58
	mAb1-S3	0.99	90.29	6.35	2.37
UP-SEC Waters Acquity	mAb1-S2	0.78	92.94	4.68	1.60
	mAb1-S3	0.97	90.72	6.10	2.21
UP-SEC Agilent 1290	mAb1-S2	0.62	92.58	5.22	1.58
	mAb1-S3	0.62	90.97	6.33	2.08

UP-SEC method using different UP-SEC systems

We also compared the UP-SEC method on different systems, Waters Acquity UPLC and Agilent 1290 UPLC using mAb1 stability samples (figure legends 2M & 3M) (Figure 7). The UP-SEC assay on both UPLC systems gave similar results on percent peak area of each component in the samples (Table 7). Results from HP-SEC assay on Agilent 1100 system are also listed in Table 7 and Table 8. Again, UP-SEC on both systems can provide similar or better separation of all the aggregate and fragments from main IgG than HP-SEC on Agilent HPLC 1100, and were able to show increased **fragmentation** in mAb1–3M sample compared with that from mAb1–2M stability sample.

Use of UP-SEC for in-process monitoring

The finalized UP-SEC assay was used to analyze a set of in-process mAb5 samples from different intermediate purification steps. As it is shown in Figure 8, clear differences in aggregation level at each step was detected throughout the purification process, where the intermediate from the 2nd process step gave the highest percentage of HMW, and the intermediate after the 4th process step gave the lowest HMW level. The results have also been confirmed with standard HP-SEC assay.

Recovery

The recovery of Waters BEH200 column was tested by comparing the total peak area with and without the column. An open-end connector was connected into the separation line to replace the analytical column when no column was used. Samples including 5 purified IgGs, 1 stability sample and 2 in-process samples were analyzed under A214 and A280. The recovery of injection on Waters BEH200 column is from 98% to 104% compared with the blank tube injections (Table 9). This suggests no loss of material on the column due to the non-specific absorption.

Discussion

Development of a high throughput and sensitive SEC method is needed as an in-process assay of samples during manufacture of biologics drugs and potentially as stability indicating and release assays. In this work, an ultra-fast (5 min) and sensitive UP-SEC method has been developed following screening 13 columns and different MP buffers using a gel-filtration standard mixture and various mAb samples. Critical variables including the selectivity (α), resolution (Rs) and plate number (N) have been assessed to compare column performance and to optimize the UP-SEC assay conditions [11].

The Waters BEH200 column (4.6 × 150 mm) packed with ultra-fine particles (1.7 µm) was chosen based on its excellent separation efficiency on both HMW aggregates and LMW fragments after comparing with 12 other UP-SEC columns. In agreement with the literature, our results indicated that columns with a smaller particle size could provide better performance

Table 8. Method and system comparison of separation efficiency.

Method/instrument†	mAb1 stability sample	Selectivity (α)			Resolution (Rs)			Plate number (N)			
		Agg-IgG	IgG-F1	F1-F2	Agg-IgG	IgG-F1	F1-F2	Aggregate	Main IgG	F1	F2
HPSEC Agilent 1100	mAb1-S2	1.04	1.07	1.17	1.62	1.34	3.68	1641	6938	6105	12715
	mAb1-S3	1.04	1.07	1.17	1.63	1.29	3.56	1603	6803	5254	12504
UPSEC Waters Acquity	mAb1-S2	3.47	1.41	1.65	2.24	1.58	3.58	1652	7328	5737	11572
	mAb1-S3	3.50	1.40	1.65	2.13	1.62	3.65	1306	7264	6286	11581
UPSEC Agilent 1290	mAb1-S2	3.36	1.40	1.65	2.14	1.34	2.99	2169	5158	5771	8297
	mAb1-S3	3.13	1.40	1.65	1.95	1.30	2.84	1546	5164	5083	8053

†HP-SEC used YMC Diol 200 column and UP-SEC used Waters BEH200 column.

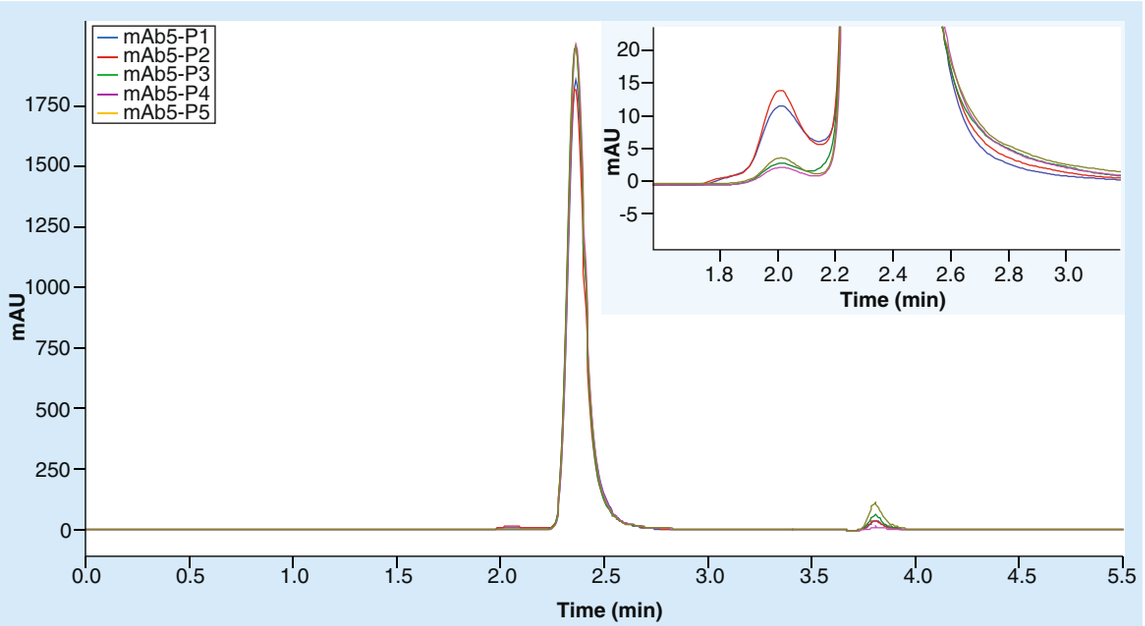

Figure 8. UP-SEC results of mAb5 In-process testing on Waters BEH200 column using Agilent 1290 UPLC system.

Table 9. Recovery under A214 and A280.

Sample	mAb1	mAb2	mAb3	mAb4	mAb5	mAb1-S3	mAb2-P1	mAb2-P2
A214	98.6	99.2	98.5	98.8	99.3	99.3	98.9	99.3
A280	101.7	102.5	101.3	101.3	101.8	102.6	103.9	100.7

under faster flow rate. The 150 mm length allowed the sufficient separation of IgG and its aggregates and fragments in typical mAb in-process and stability samples, and delivered similar or better performance compared with HP-SEC using the 300 mm column. In this study, different MP buffers were also examined and showed significant impact on the separation efficiency as they influence the state of the protein molecules and interaction between the column and the molecules.

The finalized UP-SEC assay in the study has been extensively evaluated against the HP-SEC assay by running multiple different mAb samples. The results showed similar peak area percentages by both UP-SEC and HP-SEC assays. However, UP-SEC consistently showed better resolution between F1 and main peak of antibodies. The resolution of the 100 kDa fragment has always been challenging by regular HP-SEC during the stability evaluation. Therefore, better separation efficiency and much shorter assay time offer great advantage of UP-SEC over HP-SEC.

Future perspective

The UP-SEC assay developed in this study consistently demonstrates much higher throughput capability and greater separation power for analyzing aggregates and fragments in biological protein or antibody samples. This method can dramatically facilitate the work flow of bioprocess development and help making timely in-process decisions. This paper provides multiple examples of using UP-SEC for sample monitoring at different purification stages, and is expected to lead a trend of application of UP-SEC in both biopharmaceutical industry and academics in the near future. Considering that the UPLC systems become available in most analytical laboratories, a high throughput UP-SEC method is expected to become a widely implemented assay in both early stage and late stage analytical development and testing of biological protein samples.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- We screened as many as 13 different size exclusion chromatography (SEC) columns and developed a high throughput SEC assay that can be completed within 5 min, a more than 80% reduction of time compared with HP-SEC.
- The new UP-SEC based on Waters BEH200 column delivered better or equivalent separation efficiency of mAb samples compared with HP-SEC different and showed nearly 100% sample recovery.
- The new UP-SEC method was implemented as a routine analytical method for in-process samples, and stability studies, and produced similar sample component profiles as HP-SEC.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Yu C, Mun S, Wang N. Phenomena of insulin peak fronting in size exclusion chromatography and strategies to reduce fronting. *J. Chromatogr. A* 1192(1), 121–129 (2008).

2 Luo Y, Matejic T, Ng CK *et al.* Characterization and analysis of biopharmaceutical proteins. *Separ. Sci. Technol.* 10, 283–359 (2011).

3 Cordoba-Rodriguez R. Aggregates in mAbs and recombinant therapeutic proteins: a regulatory perspective. *Biopharm. Int.* 21(11), 44–53 (2008).

4 Jing Y, Borys M *et al.* Identification of cell culture conditions to control protein aggregation of IgG fusion proteins expressed in Chinese hamster ovary cells. *Process Biochem.* 47(1), 69–75 (2012).

5 Hughes H, Morgan C, Brunyak E *et al.* A multi-tiered analytical approach for the analysis and quantitation of high-molecular-weight aggregates in a recombinant therapeutic glycoprotein. *AAPS J.* 11(2), 335–341 (2009).

6 Bareither R, Pollard D. A review of advanced small-scale parallel bioreactor technology for accelerated process development: current state and future need. *Biotechnol. Prog.* 27(1), 2–14 (2011).

7 Alvin K, Ly J *et al.* Technology improvements to accelerate process development of biologics. *Amer. Pharma. Rev.* 16(4), 8–15 (2013).

8 Jeong J, Zhang T, Condon R *et al.* Ultra-high pressure LC (UHPLC) for therapeutic protein characterization. *Amer. Pharma. Rev.* 14, 44–51 (2011).

•• **Stated UHPLC as the next generation instrument could be used in routine assays for therapeutic protein development and production control.**

9 Moore J, Patapoff T, Cromwell M. Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody to vascular endothelial growth. *Biochemistry* 38, 13960–13967 (1999).

10 Berkowitz S, Engen J, Mazzeo J, Jones G. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. *Nat. Rev. Drug Discov.* 11(7), 527–540 (2012).

11 Snyder L, Kirkland J, Glajch J. Chapter 2 and Chapter 11. *Practical HPLC Method Development (Second edition)*. John Wiley & Sons, Inc., NJ, USA. (1997).

12 Swartz M. UPLC: an introduction and review. *J. Liq. Chromatogr. Relat. Technol.* 28, 1253–1263 (2005).

13 Diederich P, Hansen S, Oelmeier S, Stolzenberger B, Hubbuch J. A sub-two minutes method for monoclonal antibody-aggregate quantification using parallel interlaced size exclusion high performance liquid chromatography. *J. Chromatogr. A* 1218(50), 9010–9018 (2011).

• **Previous work on high throughput size exclusion chromatography (SEC), but with longer run time and more complex instrument settings.**

14 Engelhardtm H, Ahr G. Optimization of efficiency in size-exclusion chromatography. *J. Chromatogr. A* 282, 385–397 (1983).

15 Hagel L, Lundström H, Andersson T, Lindblom H. Properties, in theory and practice, of novel gel filtration media for standard liquid chromatography. *J. Chromatogr. A* 476, 329–344 (1989).

16 Lathe G, Ruthven C. The separation of substances on the basis of their molecular weights, using columns of starch and water. *Biochem. J.* 60(4), xxxvi (1955).

17 Lathe G, Ruthven C, Biochem J. The separation of substances and estimation of their relative molecular sizes by the use of columns of starch in water. *Biochem. J.* 62(4), 665–674 (1956).

18 Arakawa T, Ejima D, Li T, Philo J. The critical role of mobile phase composition in size exclusion chromatography of protein pharmaceuticals. *J. Pharm. Sci.* 99(4), 1674–1692 (2010).

19 Martin M, Guiochon G. Effects of high pressure in liquid chromatography. *J. Chromatogr. A* 1090(1–2), 16–38 (2005).

20 Kopaciewicz W, Regnier F. Nonideal size-exclusion chromatography of proteins: effects of pH at low ionic strength. *Anal. Biochem.* 126(1), 8–16 (1982).

21 Golovchenko N, Kataeva I, Akimenko V. Analysis of pH-dependent protein interactions with gel filtration medium. *J. Chromatogr. A* 591(1–2), 121–128 (1992).

22 Fallas M, Neue U, Hadley M, McCalley D. Investigation of the effect of pressure on retention of small molecules using reversed-phase ultra-high-pressure liquid chromatography. *J. Chromatogr. A* 1209, 195–205 (2008).

• **Provided guidance on UPLC method development and how to overcome ultra small particle size effect and instrument limitation.**

23 Fallas M, Neue U, Hadley M, McCalley D. Further investigations of the effect of pressure on retention in ultra-high-pressure liquid chromatography. *J. Chromatogr. A* 1217, 276–284 (2010).

• **Provided guidance on UPLC method development and how to overcome ultra small particle size effect and instrument limitation.**

24 Popovici S, Schoenmakers P. Fast size-exclusion chromatography–theoretical and practical considerations. *J. Chromatogr. A* 1099(1–2), 92–102 (2005).

25 Ejima D, Yumioka R, Arakawa T, Tsumoto K. Arginine as an effective additive in gel permeation chromatography. *J. Chromatogr. A* 1094(1–2), 49–55 (2005).

26 Oliva A, Farinã J, Llabrés M. Development of two high-performance liquid chromatographic methods for the analysis and characterization of insulin and its degradation products in pharmaceutical preparations. *J. Chromatogr. B* 749(1), 25–34 (2000).

27 Tantipolphan R, Romeijn S, Engelsman J *et al.* Elution behavior of insulin on high-performance size exclusion chromatography at neutral pH. *Pharmaceut. Biomed.* 52(2), 195–202 (2010).

28 Fekete S, Veuthey J, McCalley D, Guillarme D. The effect of pressure and mobile phase velocity on the retention properties of small analytes and large biomolecules in ultra-high pressure liquid chromatography. *J. Chromatogr. A* 1270, 127–138 (2012).

• **Thorough study on the UHPLC analyte retention time from the effect of pressure and mobile phase velocity.**

29 Hong P, Koza S, Bouvier E. A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. *J. Liq. Chromatogr. Relat. Technol.* 35, 2923–2950 (2012).

• **A comprehensive review on SEC analysis and the importance of aggregation analysis in biotherapeutics proteins.**

30 Liu H, Gaza-Bulseco G, Chumsae C. Analysis of reduced monoclonal antibodies using size exclusion chromatography coupled with mass spectrometry. *J. Am. Soc. Mass Spectrom.* 20(12), 2258–2264 (2009).

31 Golovchenko N, Kataeva I, Akimenko V. Analysis of pH-dependent protein interactions with gel filtration medium. *J. Chromatogr. A* 591(1–2), 121–128 (1992).

32 Gaza-Bulseco G, Liu H. Fragmentation of a recombinant monoclonal antibody at various pH. *Pharm. Res.* 25(8), 1881–1890 (2008).