

# YMC Improvement in resolution and throughput for size exclusion chromatography analysis of monoclonal antibodies by using a 2 µm Diol column

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## Introduction

Antibody drugs are expected to enhance the effect of therapies without adverse side effects. During the manufacturing processes of immunoglobulin G (IgG), which is a main ingredient of antibody drugs, its aggregates and fragments can be generated as impurities. Size exclusion chromatography (SEC) analysis is known as a common method for determination of IgG characteristics as well as quality control in the production processes of IgG. YMC has developed a new UHPLC SEC column with 2 µm particle size and with a Diol bonded phase. High resolution analysis of aggregates and fragments derived has been performed with this new SEC column. Furthermore, smooth method transfer from conventional 5 µm SEC column to this new SEC column can also be achieved while reducing analysis time by using a shorter column. In this poster, we will introduce these advantages of the new 2 µm Diol UHPLC SEC column through several examples of IgG aggregates and fragments analysis.

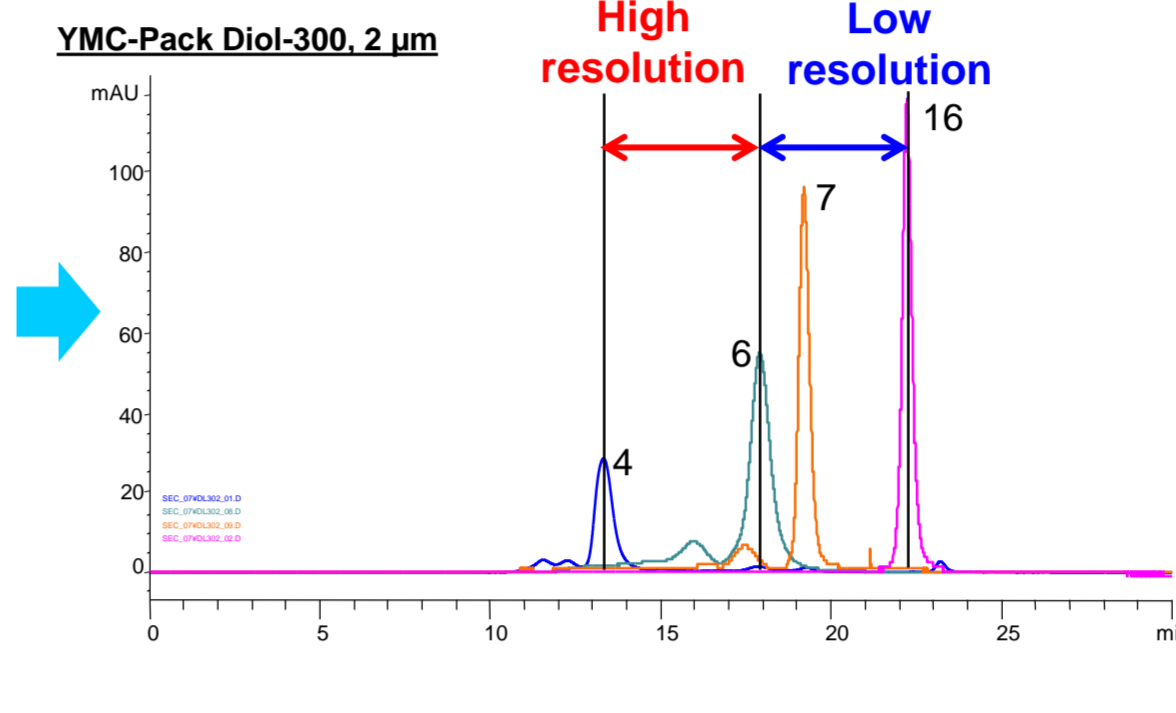
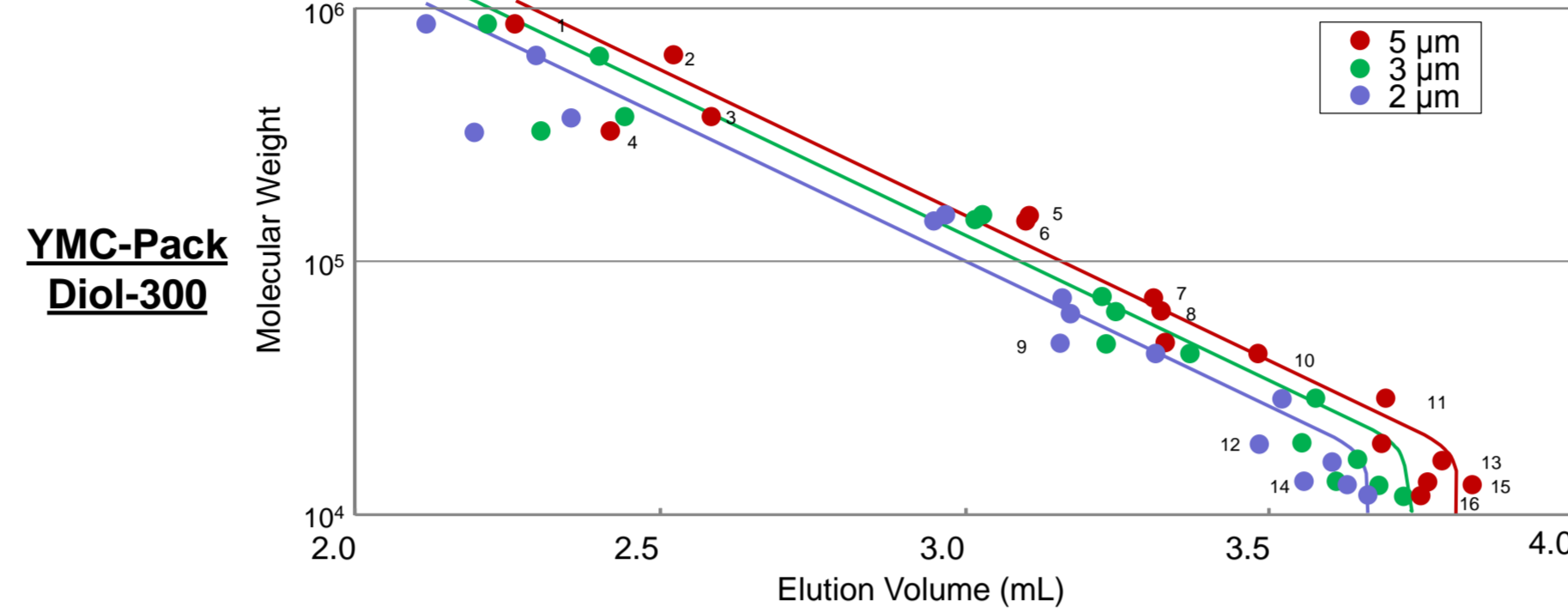
## Basic characteristics

### Specifications of novel UHPLC Size Exclusion Diol columns

Product name	Pore size	Particle size (µm)	Base material	Functional group	Pressure limit
YMC-Pack Diol-200	20 nm / 200 Å	2	Porous silica	Dihydroxy propyl	45 MPa / 450 bar / 6,530 psi
YMC-Pack Diol-300	30 nm / 300 Å				

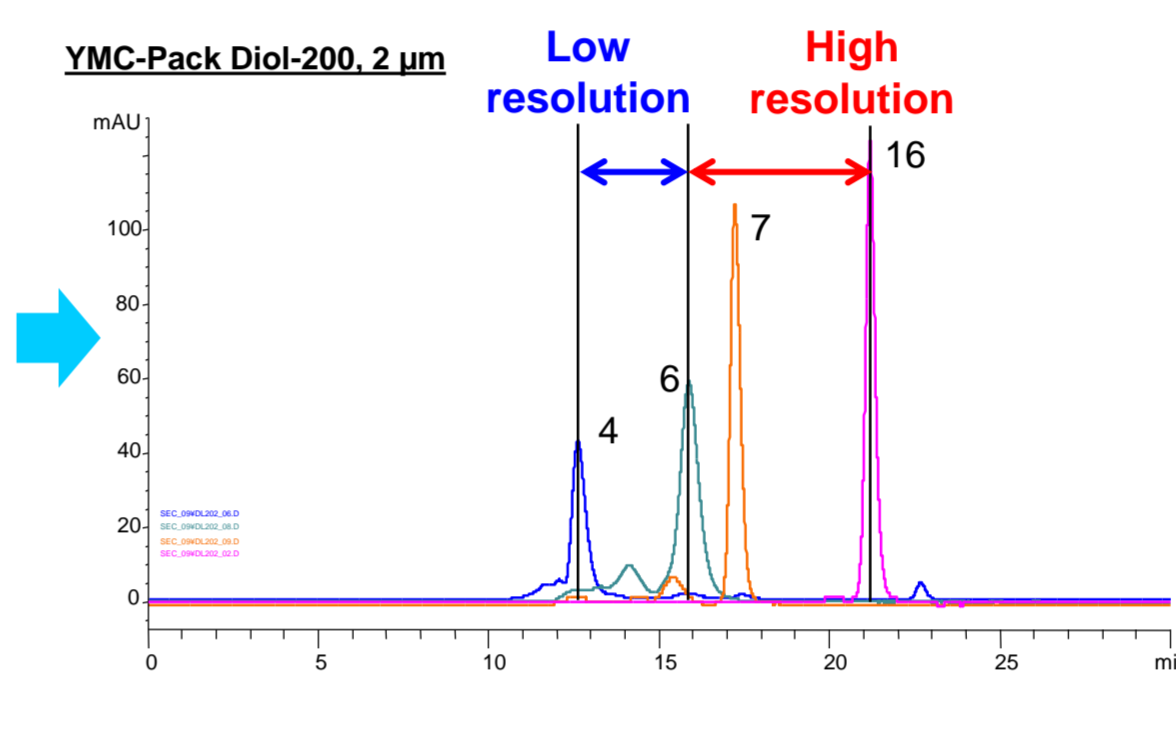
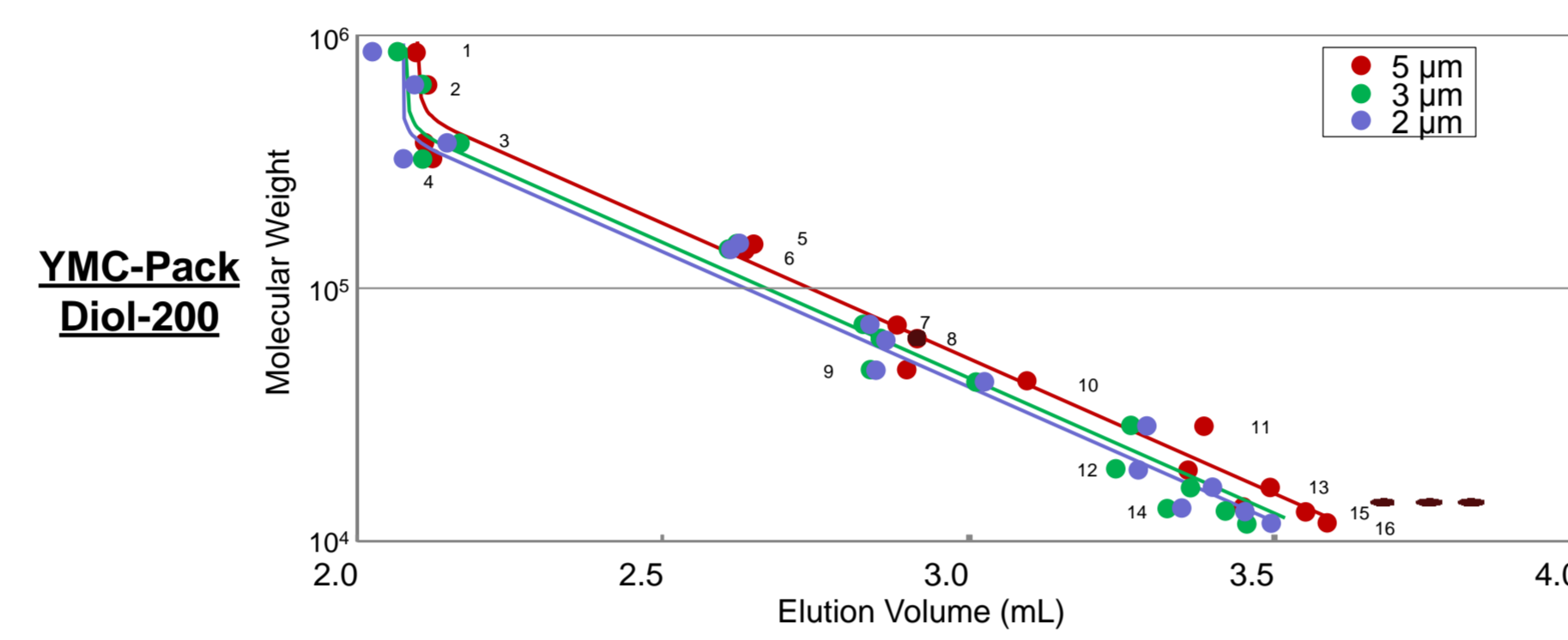
- Silica gel based size exclusion chromatography (SEC) columns bonded with dihydroxypropyl group.
- High pressure tolerance that is sufficient to withstand operating pressures required for high throughput SEC analyses
- Very low nonspecific adsorption of biomacromolecules including monoclonal antibodies

### Calibration curves and separation characteristics



#	Sample	MW	#	Sample	MW
1	IgM	900,000	9	α1-Antitrypsin	50,000
2	Thyroglobulin	670,000	10	Ovalbumin	45,000
3	IgA	390,000	11	Carbonic anhydrase	30,000
4	Fibrinogen	340,000	12	Trypsin inhibitor	20,100
5	γ-Globulin	158,000	13	Myoglobin	17,000
6	IgG	150,000	14	α-Lactalbumin	14,100
7	Transferrin	75,000	15	Ribonuclease A	13,700
8	HSA	66,000	16	Cytochrome c	12,400

Column : YMC-Pack Diol-200 or YMC-Pack Diol-300, 300 × 4.6 mm ID  
 Eluent : 0.1 M KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) containing 0.2 M NaCl  
 Flow rate : 0.165 mL/min  
 Temperature : 25°C  
 Detection : UV at 280 nm

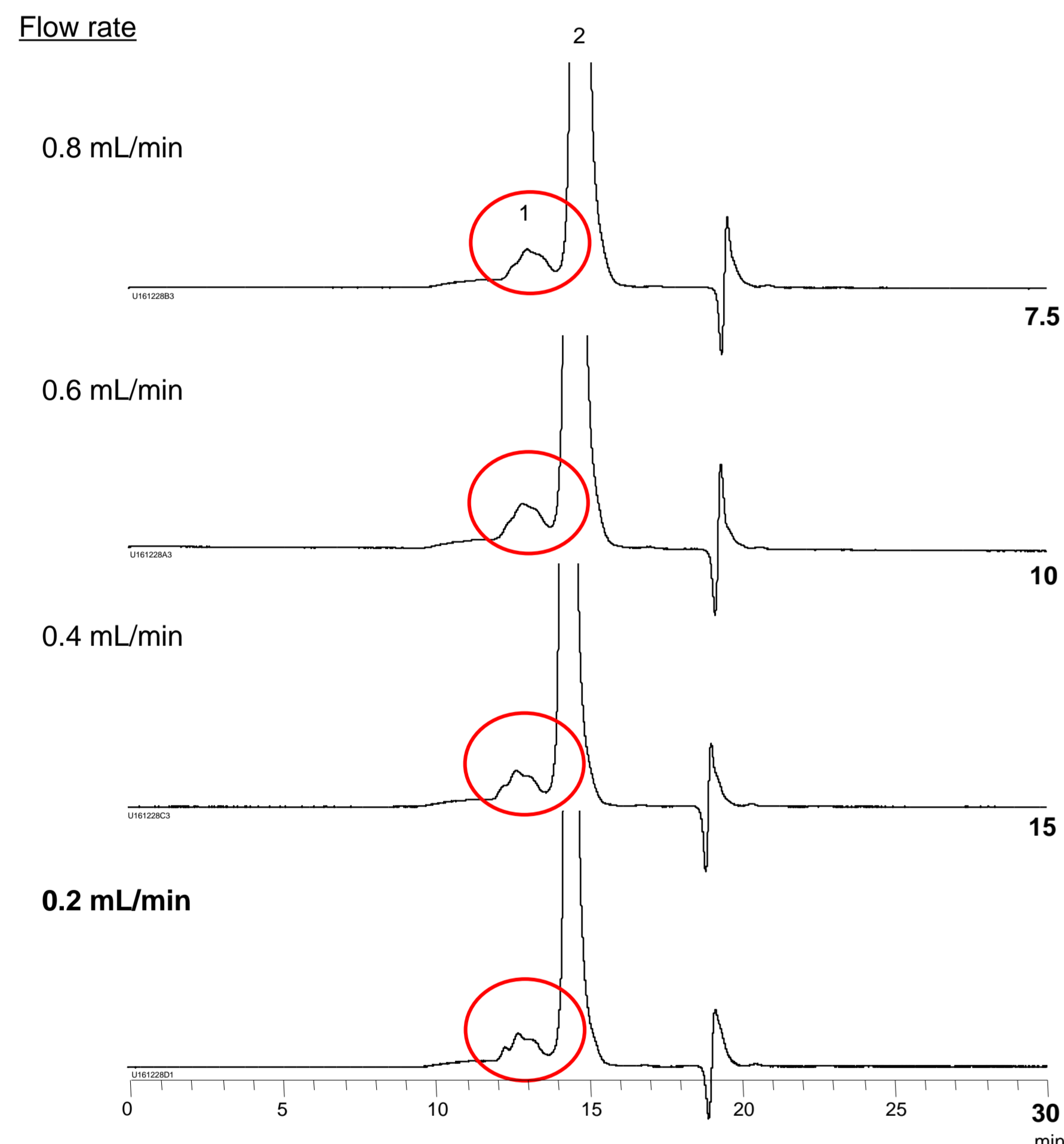


- If pore size is the same, calibration curves are identical across particle sizes (2, 3 and 5 µm). This feature allows seamless method transfer between particles (HPLC ↔ UHPLC).
- 300 Å pore material gave higher resolution on high molecular weight side, while 200 Å pore material had advantage of separation on low molecular weight side. 300 Å pore material is suitable for antibody aggregates analysis, and 200 Å pore material is better for antibody fragments analysis.

## Analysis of a monoclonal antibody and its aggregates

### Influence of flow rate on antibody analysis

#### YMC-Pack Diol-300, 2 µm, 300 × 4.6 mm ID

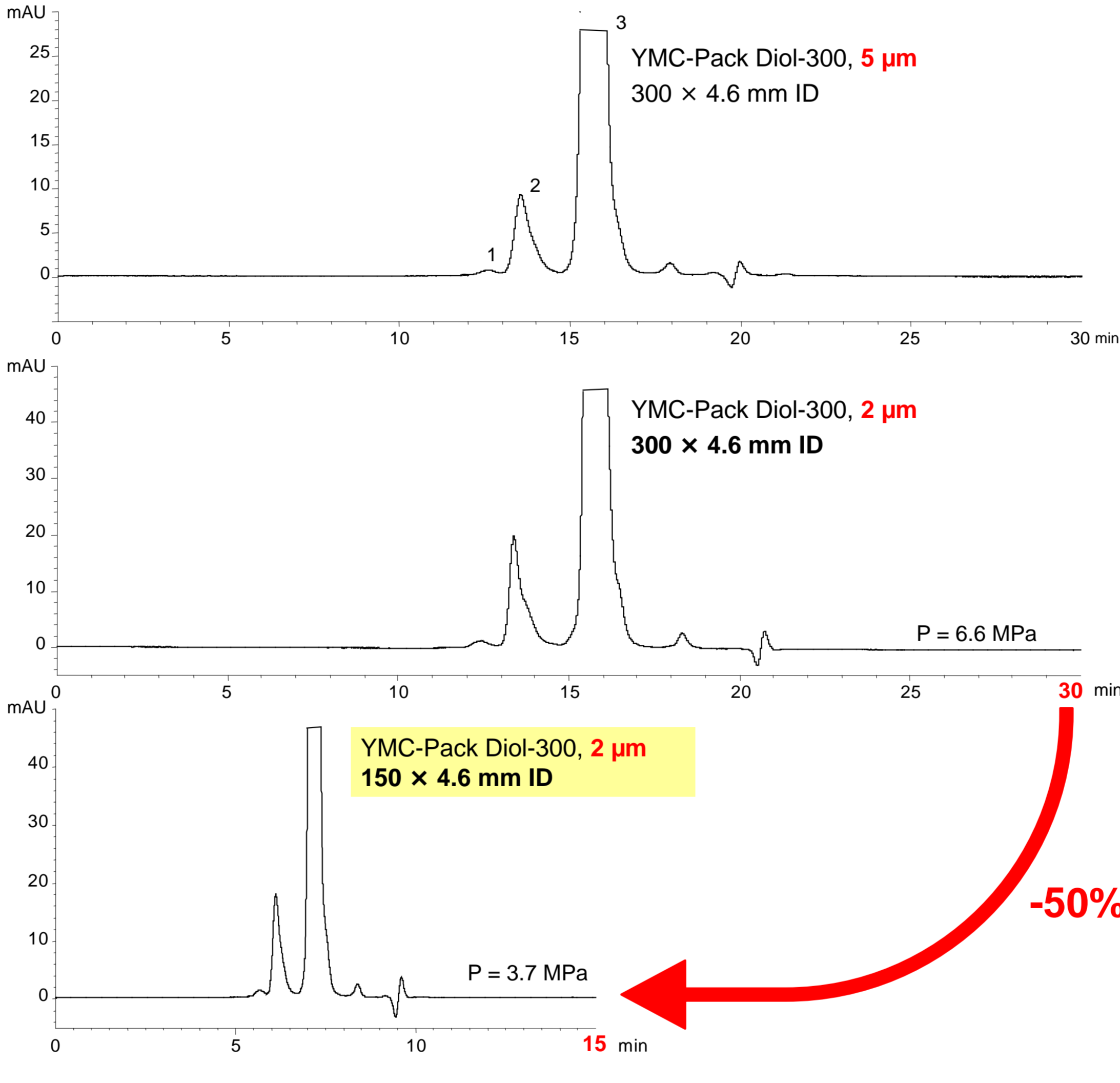


Flow rate (mL/min)	Theoretical plate number N (2)	Pressure (MPa)
0.8	7,300	28.3
0.6	8,900	20.9
0.4	9,400	14.4
0.2	15,200	7.2

Column : YMC-Pack Diol-300, 2 µm, 300 × 4.6 mm ID  
 Eluent : 0.1 M KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) containing 0.2 M NaCl  
 Detection : UV at 280 nm  
 Temperature : ambient  
 Sample : Humanised monoclonal IgG1

- Theoretical plate number and resolution of a monoclonal antibody increases as the flow rate is decreased. This suggests that lower flow rates are preferable if the aim is high resolution separation analysis. On the other hand, higher flow rates are more suitable for increased analysis throughput, especially when the resolution is sufficient.
- The backpressure at 0.8 mL/min with a column 300 mm × 4.6 mm ID is within the column pressure limit.

### Improvement of resolution / throughput by using the UHPLC SEC column

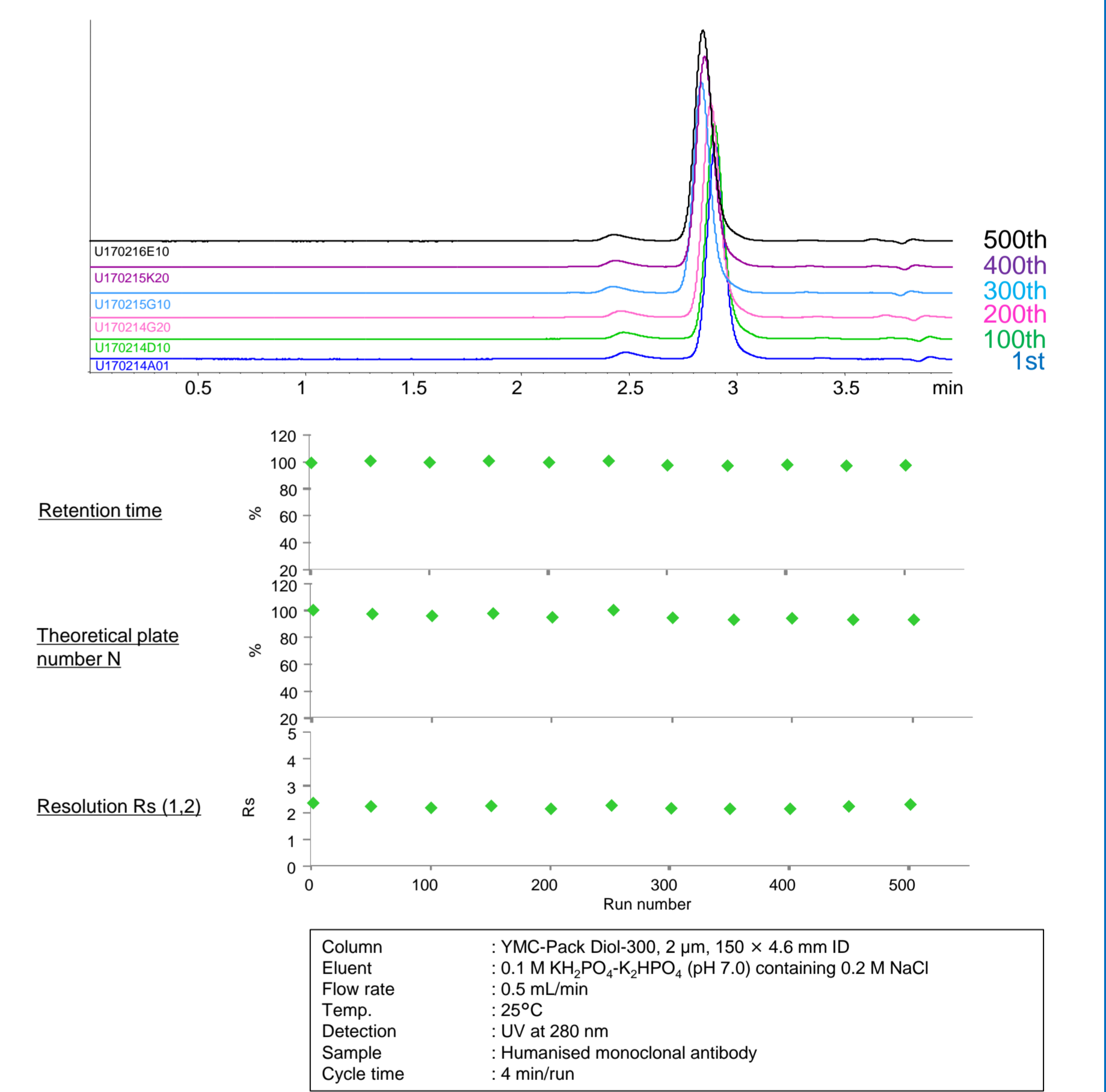


Particle size (µm)	Column size (mm)	Theoretical plate number N (3)	Resolution Rs (1,2)	Resolution Rs (2,3)
5	300 × 4.6	8,500	0.88	2.67
2	300 × 4.6	16,200	1.17	4.15
2	150 × 4.6	8,700	0.85	2.75

Column : YMC-Pack Diol-300  
 Eluent : 0.1 M KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) containing 0.2 M NaCl  
 Flow rate : 0.2 mL/min  
 Detection : UV at 280 nm  
 Temperature : ambient  
 Sample : Humanised monoclonal IgG1

- A 2 µm YMC-Pack Diol packed column greatly improved the resolution between aggregates and monomer peaks compared to a 5 µm YMC-Pack Diol of the same dimensions (300 mm × 4.6 mm ID).
- The 2 µm, 150 mm length column, gave the same resolution as that on the 5 µm, 300 mm length column for the antibody monomer and aggregates. This would suggest that analysis time can be reduced by half just by changing the column from 5 µm, 300 mm length column to 2 µm, 150 mm length column.
- The operating pressure was low enough to run on a conventional HPLC system. \*A special care needs to be taken in terms of minimizing system volume in order to avoid extra-column band spreading when using this low flow rate.

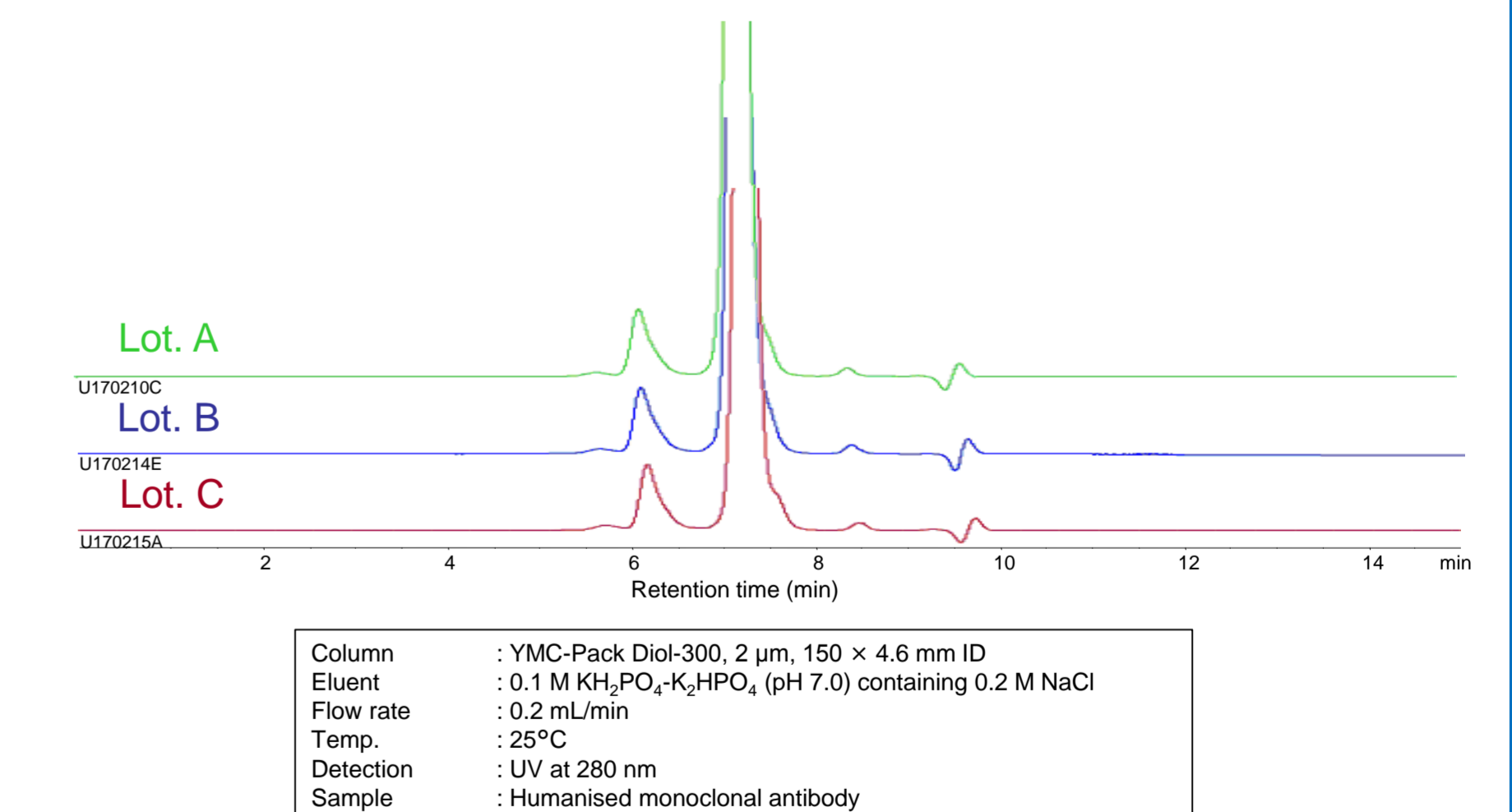
### Evaluation of column stability



Column : YMC-Pack Diol-300, 2 µm, 150 × 4.6 mm ID  
 Eluent : 0.1 M KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) containing 0.2 M NaCl  
 Flow rate : 0.5 mL/min  
 Temp. : 25°C  
 Detection : UV at 280 nm  
 Sample : Humanised monoclonal antibody  
 Cycle time : 4 min/run

- Stability of the 2 µm YMC-Pack Diol column for monoclonal antibody analysis was evaluated. Performance of the column was maintained for 500 injections, and can be expected to last for many more injections.

### Lot-to-Lot reproducibility



Column : YMC-Pack Diol-300, 2 µm, 150 × 4.6 mm ID  
 Eluent : 0.1 M KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) containing 0.2 M NaCl  
 Flow rate : 0.2 mL/min  
 Temp. : 25°C  
 Detection : UV at 280 nm  
 Sample : Humanised monoclonal antibody

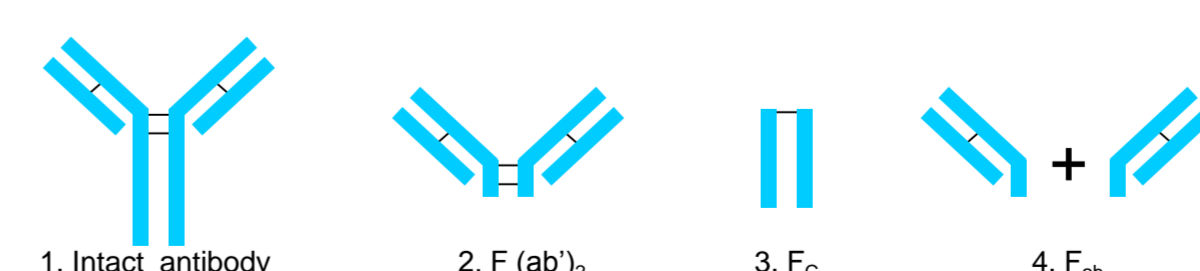
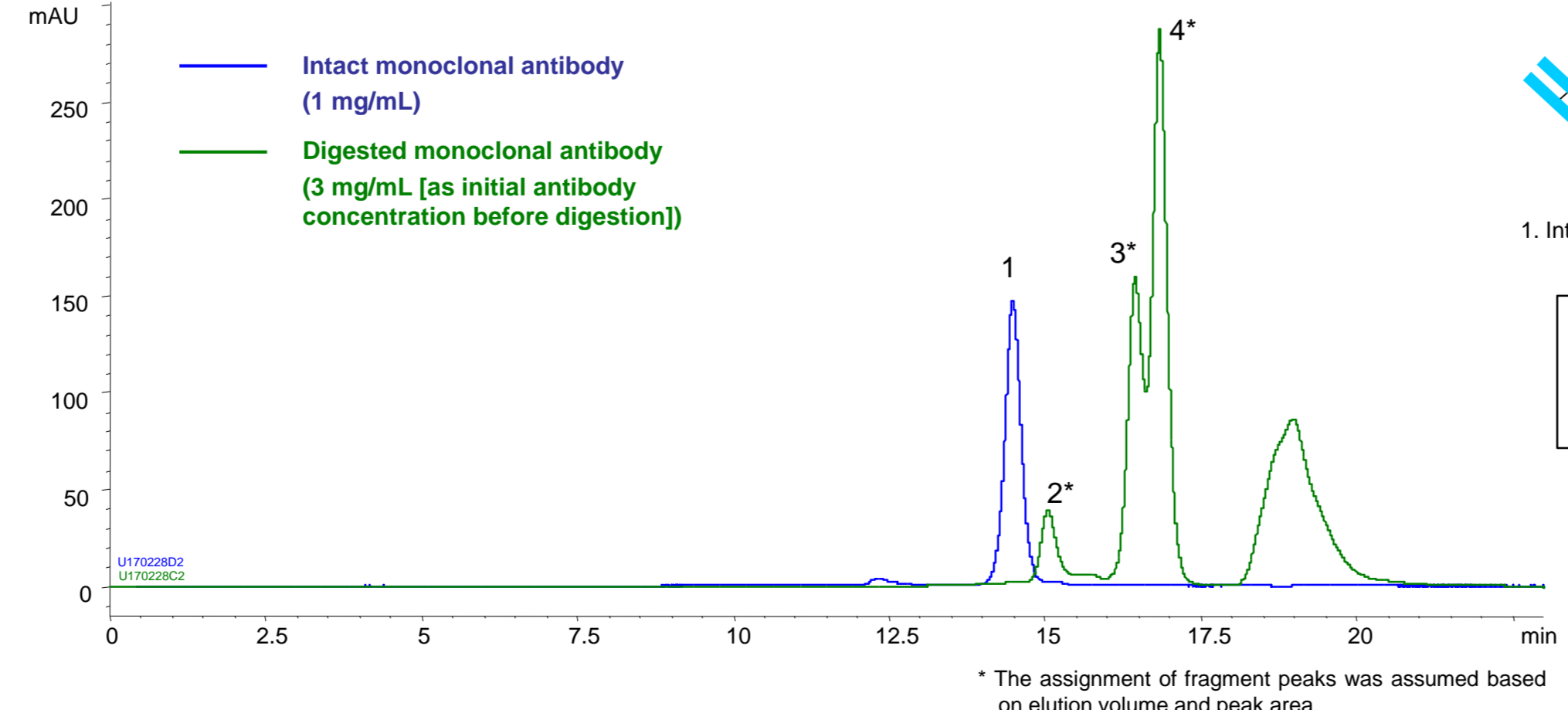
- Separation reproducibility for 2 µm YMC-Pack Diol columns from different manufacturing lots was evaluated. Separation was identical between lots which results in robust method development.

## Analysis of monoclonal antibody fragments

### Method of Papain digestion

- 50 mM KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> (pH 7.5) buffer was prepared.
- Papain and other components were added to the buffer to give the following concentrations:
  - Papain 0.1 g/L
  - NaCl 0.15 mol/L
  - L-cystein 0.01 mol/L
  - EDTA 0.01 mol/L
- A monoclonal antibody was added to the solution prepared at 2) to give 3 mg/mL.
- The solution was left for 17.5 hours at 25°C.

### Overlaid chromatogram of the intact and the digested monoclonal antibody



Column : YMC-Pack Diol-200, 2 µm, 300 × 4.6 mm ID  
 Eluent : 0.1 M KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) containing 0.2 M NaCl  
 Flow rate : 0.2 mL/min  
 Detection : UV at 280 nm  
 Temperature : ambient

- The YMC-Pack Diol-200 UHPLC column was used for the antibody fragments analysis. Intact antibody and fragments were well separated making this a good column for QC work.

## Conclusions

The new 2 µm YMC-Pack Diol UHPLC SEC columns have been shown to improve resolution and/or throughput of monoclonal antibody analyses.

This offers significant advantages for antibody analysis of aggregates and control of fragments in research as well as quality control of pharmaceutical products.