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Method Development for Size-Exclusion Chromatography of Monoclonal Antibodies and Higher Order Aggregates

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APPLICATION BENEFITS

- Robust analysis of mAb monomer and aggregates
- High throughput SEC separation
- Consistent purity profile
- Reproducible quantitation of higher order aggregates
- Easy SEC method development

WATERS SOLUTIONS

- ACQUITY UPLC® H-Class Bio system
- ACQUITY UPLC BEH200 SEC 1.7 µm column
- Auto•Blend Plus™ technology
- Empower™ 2 software

KEY WORDS

Size-exclusion chromatography, UPLC, monoclonal antibody, method development, aggregates

INTRODUCTION

Since the early introduction of biologic based therapeutics, the presence of protein aggregates can compromise safety and efficacy.[1] Given these factors, protein aggregates are typically monitored throughout the production of a biotherapeutic. While a variety of analytical techniques have been used to analyze soluble aggregates, the dominant technique continues to be size-exclusion chromatography (SEC).[2]

While SEC has been performed with silica-diol coated columns and HPLC instrumentation, the introduction of UPLC® or low dispersion systems in combination with sub-2 µm particles has allowed for improvements in these isocratic separations, including improved resolution, higher throughput and sensitivity.[3] However, as in any SEC method, a variety of parameters can be adjusted to improve resolution and method robustness. In the following application, we will investigate the impact of some of these parameters, including mobile-phase composition, flow rate and column length on a SEC separation. Evaluation of the separation will be based on a variety of criteria such as column calibration, resolution, and aggregate quantitation.

EXPERIMENTAL

Sample Description

The protein standard (BioRad) containing bovine thyroglobulin (5 mg/mL), bovine γ –globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 mg/mL) and Vitamin B12 (0.5 mg/mL) in de-ionized water was analyzed. A murine monoclonal antibody, purified by Protein A affinity chromatography, was analyzed. The sample concentration was 10 mg/mL in 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3. Samples were not controlled for inter-experiment conditions.

Method Conditions

LC Conditions:

System:	ACQUITY UPLC H-Class Bio System with TUV and Titanium flow cell
Wavelength:	214 and 280 nm
Column:	ACQUITY UPLC BEH200 SEC 1.7 µm, 4.6 x 150 mm, part number 186005225
Column Temp.:	30 °C
Sample Temp.:	4 °C
Injection Volume:	2 µL (unless otherwise specified)
Flow Rate:	0.4 mL/min (unless otherwise specified)
Mobile Phase:	Prepared using Auto•Blend Plus technology
Final Composition:	25 mM sodium phosphate, pH 6.8, 200 mM sodium chloride, (unless otherwise specified)

Data Management

Software:	Empower 2

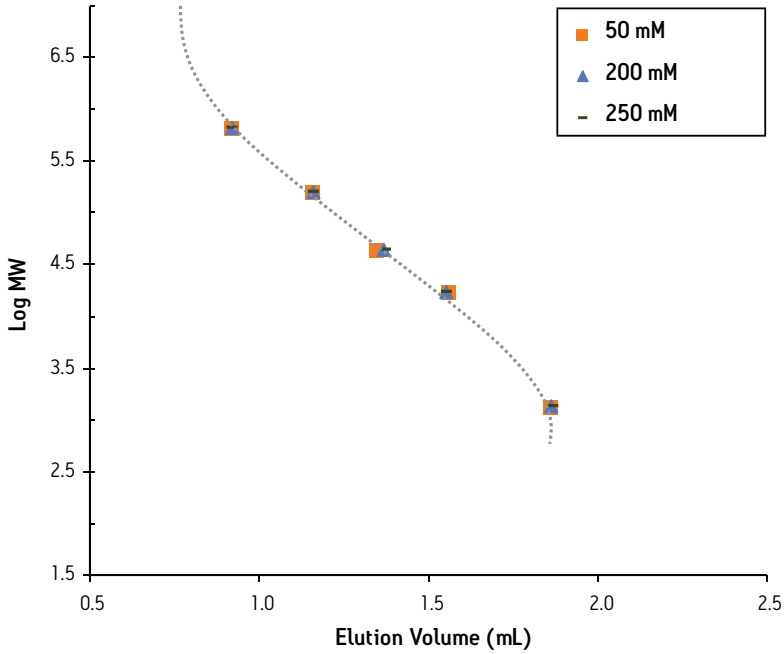
RESULTS AND DISCUSSION

A number of factors need to be evaluated in SEC method development. Ideally SEC separations are based on the size of the proteins in a solution. For this reason, size-exclusion chromatography of biomolecules is performed under aqueous, native conditions. However, the presence of mixed mode interactions can obscure size measurements.[4] More specifically, the charged sites on the packing material can interact with the proteins, resulting in an 'ion- exchange' effect. To determine the influence of these effects the mobile-phase conditions of the separation need to be evaluated. However, the conditions of the chromatographic separation can alter the protein structure and state. The concentration and identity of the salt and pH can affect the 3-D structure and the protein-protein interactions. For these reasons, evaluation of a SEC method must incorporate information of the biological activity of the biomolecule.

In the following discussion, we will outline considerations and parameters for developing a SEC method. While the SEC method development steps are illustrated on UP-SEC, the same principles apply to any HP-SEC separation. Methods will be evaluated based on peak shape, resolution, calibration accuracy, and quantitation. Optimization of the mobile-phase ionic strength and pH can easily be accomplished with a quaternary eluent management system in combination with software that can take advantage of this four eluent blending system.[5] This approach was used throughout the studies described.

Mobile-phase Ionic Strength

The ionic strength of the mobile-phase should be adjusted to minimize any secondary interactions between the packing material and proteins. To determine the effect of mobile-phase concentration on the calibration curve, a set of protein standards was analyzed at 50-250 mM sodium chloride. Sodium chloride was selected since it is the most common salt used in SEC separations. The buffer concentration (sodium phosphate) and pH were kept constant at 25 mM and pH 6.8, respectively. Over the concentration tested, the retention times for each protein were within 0.07 minutes with the greatest retention time variability observed for ovalbumin (Figure 1). These results indicate the calibration curves are not sensitive to salt concentration.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 1. Effect of sodium chloride on a SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

In addition to protein standards, the SEC separation of a murine monoclonal antibody (mAb) was evaluated at 50-250 mM sodium chloride (Figure 2). As is commonly observed with gel filtration packing materials,[2] higher ionic-strength mobile phases lead to decreased peak tailing and narrower peaks for the mAb monomer. With increasing sodium chloride concentrations from 50-200 mM, the mAb peak height increases from 0.189 – 0.289. The USP tailing factor also decreases from 1.64 to 1.22. Changes are less pronounced as the ionic strength of the mobile phase is increased from 200 to 250 mM sodium chloride (USP Tailing = 1.20).

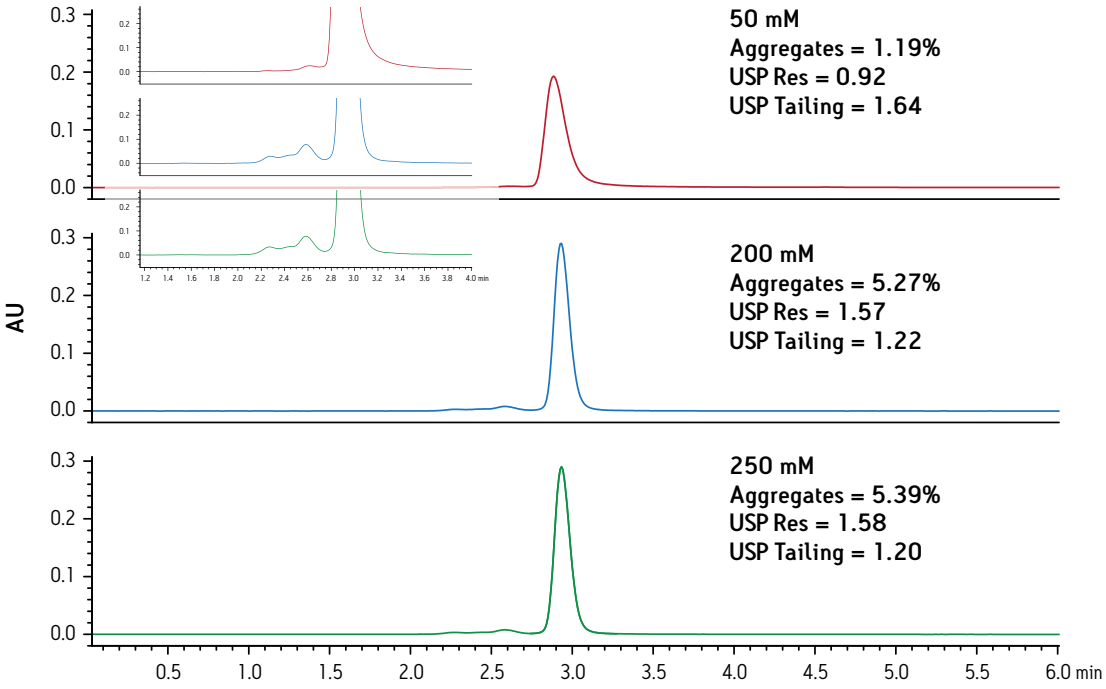


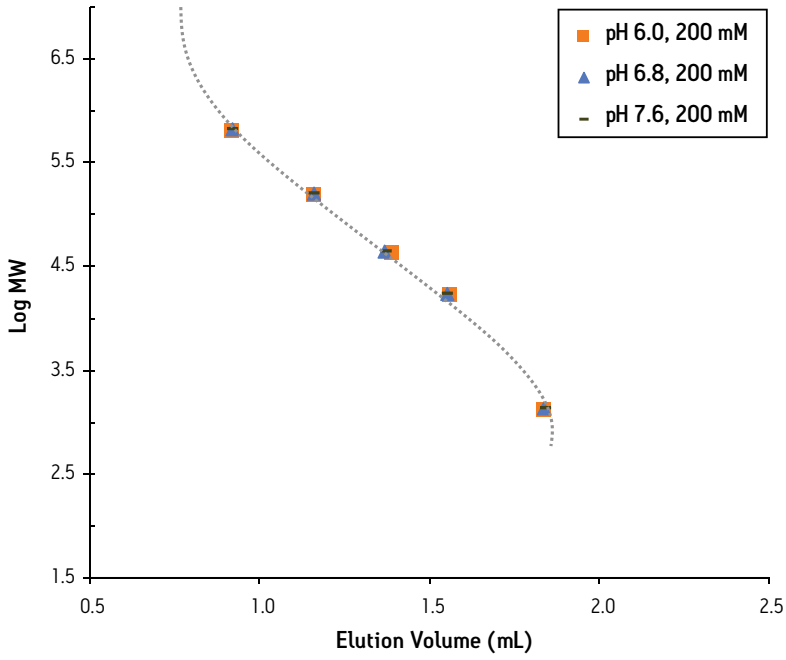
Figure 2. Effect of sodium chloride on the SEC separation of a murine mAb.

The effect of buffer ionic strength on the observed amount of aggregate was also analyzed. In the experiments previously described, increasing sodium chloride concentrations from 50 -200 mM results in greater observed recovery of aggregates (see inset). The aggregate % area increased from 1.18% to 5.27%. However, at concentrations above 200 mM sodium chloride, aggregate quantitation did not change significantly. This suggests minimal secondary interactions above this concentration.

The variability in retention time and changes in peak shape indicate secondary interactions between the protein and the column packing material, as has been observed for the materials used to prepare SEC packings. These interactions, which can lead to increased retention and irregular peak shape, are easily minimized by increasing the ionic strength of the buffer.

Mobile-phase pH

Given the influence of pH on both secondary interactions and the structure of the protein, SEC method development should also evaluate pH and its influence, if any, on the separation and quantitation of the biomolecule. The BEH200 column was evaluated with the protein standard mix from pH 6.0 – 7.6. This analysis was performed to evaluate the effect of pH on the column calibration. The pH range was based on the buffering capacity range of the sodium phosphate buffer. The sodium chloride concentration was kept constant at 200 mM. The results show no significant shift in retention times were observed for the proteins. All of the retention times were within 0.02 minutes (Figure 3), suggesting pH has no significant affect on calibration under the conditions tested.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 3. Effect of mobile-phase pH on a SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

To test the effect of pH on a typical biotherapeutic, the mAb was analyzed under the same conditions (pH 6.0 to 7.6, 200 mM sodium chloride) (Figure 4). As the pH increases from 6.0 to 7.6, the mAb monomer peak height decreases and shifts to earlier retention time (Figure 4). However, the aggregate quantitation over the pH range from pH 6.0 – 7.6 was within 0.4% (5.7- 5.3%), indicating mobile phase pH has no effect on the measured proportion.

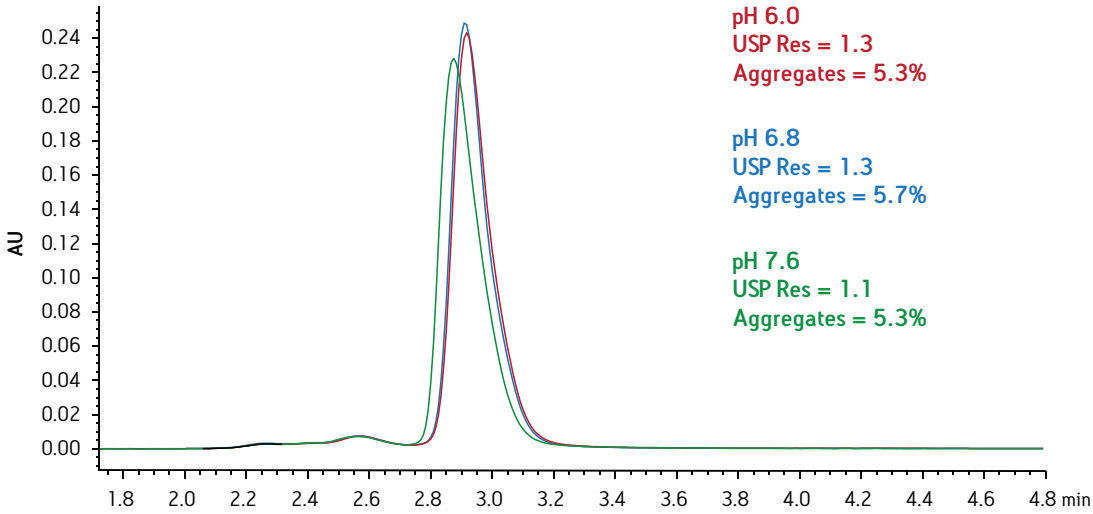


Figure 4. Effect of mobile-phase pH on a SEC separation of murine mAb. Mobile phase: 25 mM sodium phosphate, 200 mM sodium chloride pH 6.0-7.6.

The buffer pH can influence secondary interactions. In this case we observe changes for monomer elution profile but not for the dimer. This suggests a change in the hydrodynamic radius rather than a change in the secondary interactions.

Flow Rate

Resolution in size based separations can be influenced by linear velocity. Although using lower flow rates results in longer run times, the increased resolution gives greater confidence in aggregate quantitation. In addition, the use of sub-2 µm particles for this application allows the use of shorter columns. Thus, the throughput achieved with UPLC-SEC is still greater than that of traditional HP-SEC.[3]

In order to test the reliability and robustness of the method, the effect of flow rate on the SEC separation of a mAb was analyzed. Triplicate injections of the mAb were analyzed at flow rates of 0.2 and 0.4 mL/min (Figure 5). Analysis of the separations shows no significant change in aggregate quantitation with flow rate. However, decreasing the flow rate did increase the monomer/dimer resolution by 15%. While the lower flow rates allow for increased resolution, higher flow rates allow for greater throughput and faster analyses times.

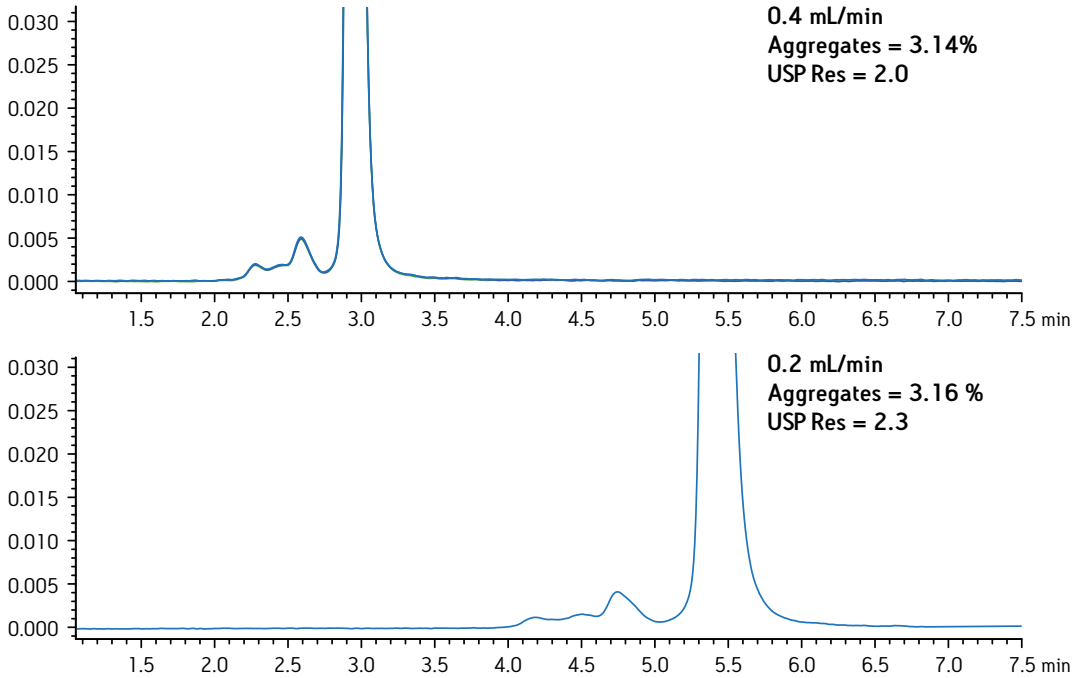
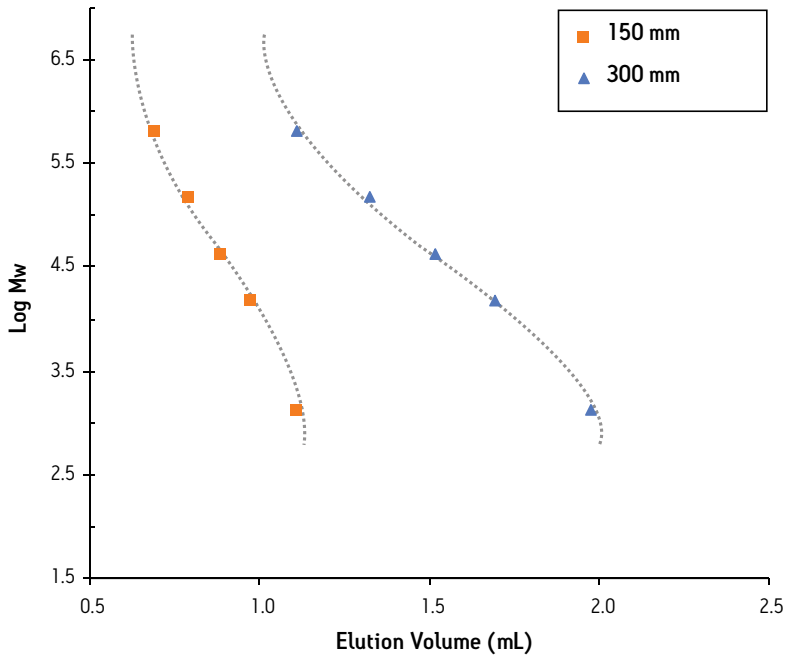


Figure 5. Effect of flow rate on a SEC separation of a murine mAb.

Column Length

Improvements in SEC resolution can also be gained by increasing column length. SEC separations are based on diffusion into and out of the pores of the column's packing material. The larger proteins cannot access the pores and thus elute earlier. The smaller the protein, the longer the residence time within the pores, which results in longer retention times. These principles allow for greater resolution with longer column lengths.

To demonstrate these effects, a set of protein standards were run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Comparison of the calibration curves reveals a shallower slope for the 300 mm column as compared to the 150 mm, demonstrating the higher resolving power achievable on a longer column (Figure 6).



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 6. Effect of column length on SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

The effect of column length was also tested for the SEC separation of a murine mAb run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Under the same conditions, the longer column provided improved resolution for the monomer/dimer (2.07 to 2.80) (Figure 7) with comparable aggregate quantitation. The improved resolution is also apparent in the monomer peak tail, in which a small, lower molecular weight peak is partially resolved on the 300 mm but not on the 150 mm column. However, the improved resolution is accompanied by an increase in retention time (from 3.0 to 6.0 minutes).

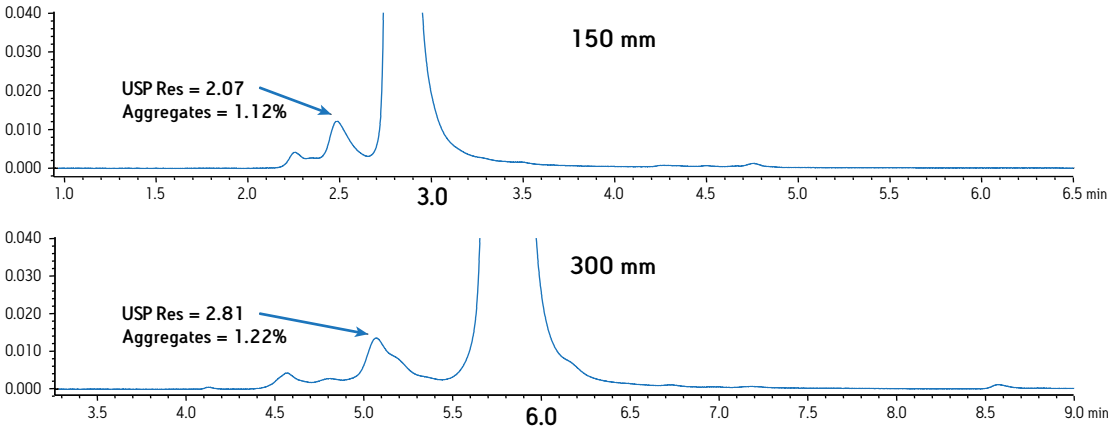


Figure 7. Effect of column length on a SEC separation of murine mAb.

These results indicate that column length can be a useful tool in method development. Depending on the method requirements, column length can be chosen to either provide improved resolution or higher throughput. For example, in a manufacturing environment a longer column allows for improved resolution. While in discovery or development, a shorter column allows for faster analysis time and high throughput.

CONCLUSIONS

Size-exclusion chromatography continues to be a standard technique for the analysis of monoclonal antibodies and their aggregates. However, as in any SEC method, a thorough evaluation needs to be performed to develop an optimum separation. While HP- SEC can be time consuming, the use of UP-SEC allows method optimization to be predicted in less time with a high level of efficiency and higher degree of confidence. In addition, the use of Auto•Blend Plus technology makes it easier and less labor intensive to systematically examine the effects of mobile phase on protein structure and on secondary interactions.

As described, optimization should evaluate a number of conditions, including mobile phase (pH and ionic strength), flow rate, and column length. In addition - although not described in detail - injection volume, mass load and temperature can also affect SEC separations. Therefore, a suggested set of experiments should evaluate:

1. ionic strength
2. pH
3. column length
4. flow rate
5. other variables (mass load, injection volume, temperature, etc.)

These experiments should incorporate information on the biological activity of the protein. If factors affecting the proteins biological activity are limited, PBS is the recommended starting mobile phase.

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