Size Exclusion Chromatography of Biomolecules – Detector Combinations to Obtain Maximum Information with Minimum Effort

Andrew Coffey, Agilent Technologies UK Ltd.

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Introduction

Background

Size exclusion chromatography is often considered to be a simple technique since it is performed under isocratic elution conditions and, under ideal circumstances, analytes do not interact with the stationary phase. In reality these simple requirements mean some of the most fundamental and important considerations may be overlooked. The elution order is based on size in solution, often mirroring molecular weight, and in fact calibration curves are usually plotted with molecular weight as ordinate (Figure 1).

However there is not necessarily a direct relationship between molecular weight and size in solution; some molecules are more compact and smaller in size, other molecules may be elongated and hence appear to be larger than expected. Furthermore, the conditions in which the analysis is performed can influence the analyte in a number of ways: the molecule can potentially change shape by adopting a different confirmation, become denatured and unfold, or perhaps begin to aggregate; all of which will influence the retention time.

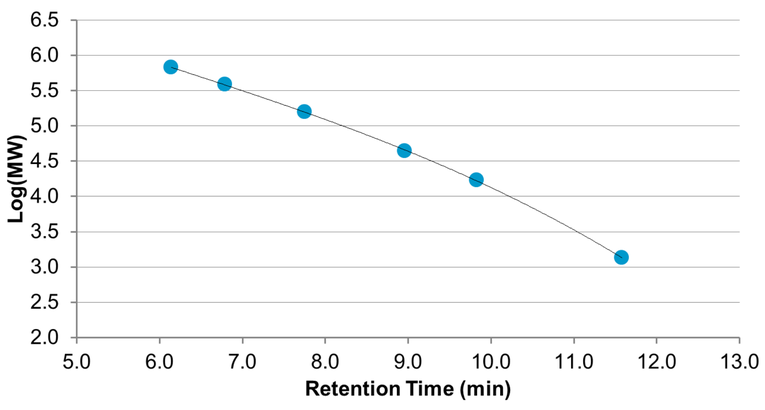

Figure 1: Calibration Curve For Standard Proteins Using Bio SEC-3 300Å 7.8x300mm Column

In order to develop robust methods for size exclusion chromatography it is essential to identify non-ideal behavior and, where possible, to eliminate causes of run-to-run variation. This may require testing a wide range of mobile phase conditions, perhaps to overcome undesirable secondary interactions. Combined with making the correct column choice for optimum resolution, this can prove a significant undertaking.

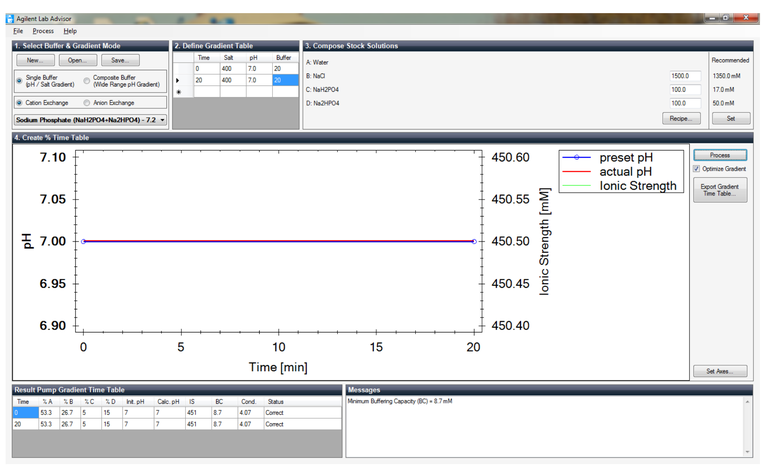

Figure 2: Screenshot of Agilent Buffer Advisor Software used to Define Quaternary Pump Parameters

Experimental

Columns: Agilent Bio SEC-3 300Å, 7.8 x 300 mm
Agilent Bio SEC-3 300Å, 7.8 x 150 mm

Mobile phase:
- Eluent A: Water
- Eluent B: 1.5M NaCl
- Eluent C: 100mM Sodium dihydrogen orthophosphate
- Eluent D: 100mM Disodium hydrogen orthophosphate

Composition adjusted using Agilent Buffer Advisor software to provide 20mM Phosphate Buffer, pH 7.0 with 0.2M, 0.3M, 0.4M, 0.5M, 0.6M NaCl as required.

Flow rate: 1.0 mL/min
Temperature: 30 °C
Injection volume: 20, 40 and 80 µL
Sample: Protein standards
- Bio-Rad Gel Filtration Standard
- Bovine Serum Albumin, 66 kDa, 2mg/mL
- α-Chymotrypsinogen A, 25.7 kDa, 4mg/mL

Detection: LS 15° & 90° / DAD (220nm, 254nm, 280nm) / RI
Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC with Agilent 1260 Infinity Multi-Detector GPC/SEC .

Results and Discussion

Non-ideal Behaviour

The most common form of non-ideal behavior is due to hydrophobic interactions between the protein and the stationary phase. The result is typically a peak that elutes much later than expected and may be broad and/or tailing.

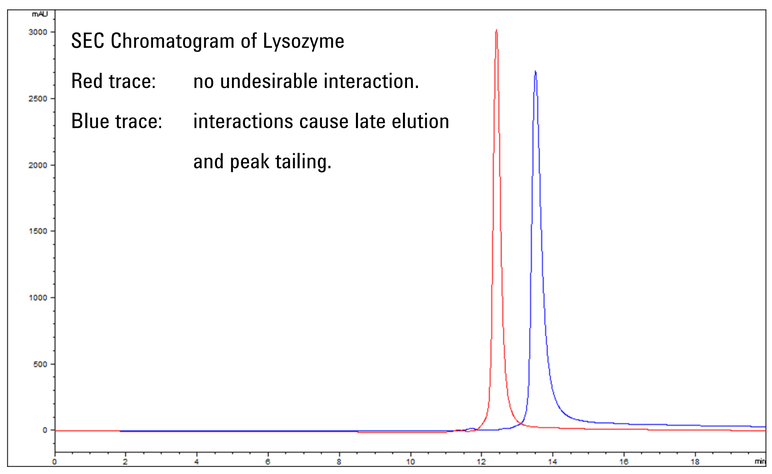

Figure 3: Non-specific Interaction Resulting in Increased Retention Time and Peak Tailing.

It is therefore essential to screen a variety of mobile phase conditions varying parameters such as pH, buffer strength and ionic strength.

In addition, as part of this investigation the effect of column length on resolution was investigated. It came as some surprise to find that α-Chymotrypsinogen A (CTG), a precursor protein of chymotrypsin containing 245 amino acids with a molecular weight of 25.7 kDa, could be partially resolved using two 30cm columns in series when using just one 15cm or one 30cm alone failed to separate the peaks:

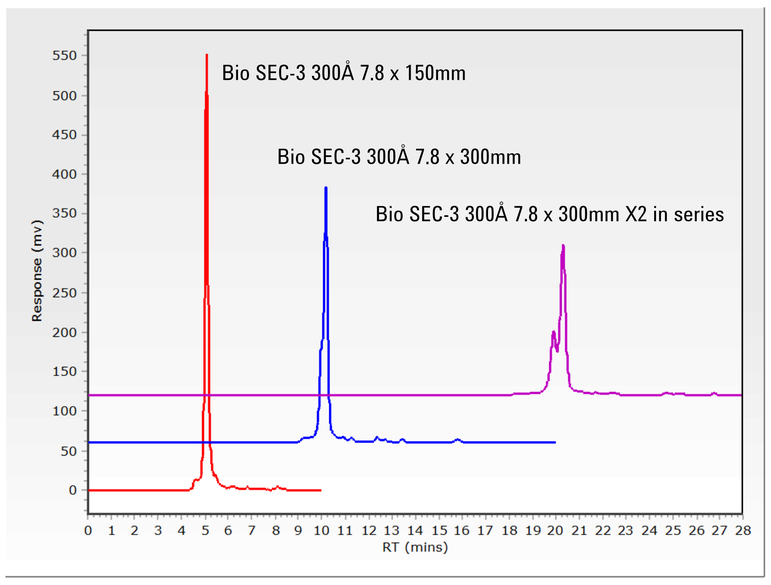

Figure 4: Resolution of α-Chymotrypsinogen A: Effect of column length

To investigate the effects of rapid screening of salt concentration the 15cm column was chosen. Initially at 0.2M NaCl concentration the peaks were not resolved however as the NaCl concentration was increased, the peaks began to separate. This work was then repeated using a 30cm column:

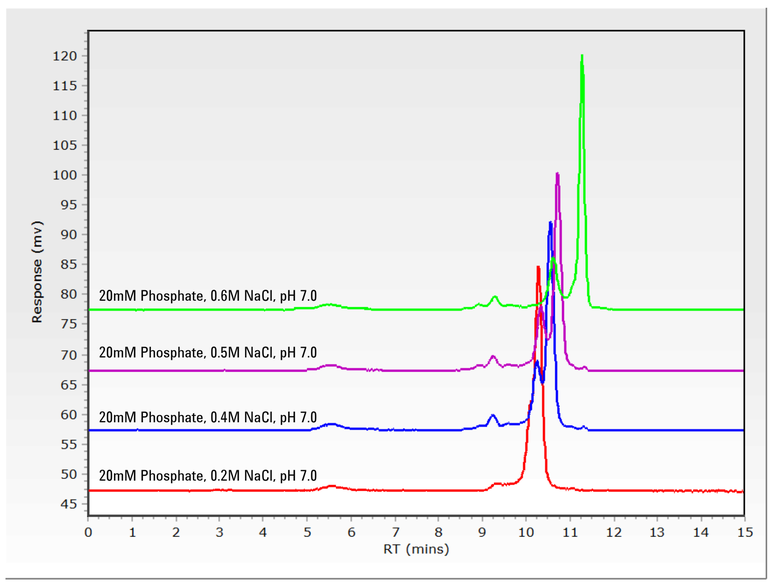

Figure 5: Effect of Increasing Salt Concentration on Separation of α-Chymotrypsinogen A (Bio SEC-3 300Å 7.8 x 300mm)

Despite the changing retention time behavior (which might be associated with hydrophobic interactions) it was noted that the peak shape improved with increasing salt concentration.

Results and Discussion

Light Scattering Detection

To fully understand the effect of salt concentration on CTG the light scattering detector was used to determine molecular weight. Figure 6 shows a typical separation of CTG using a moderate amount of NaCl, 0.5M, in order to gain sufficient resolution to analyze both major peaks.

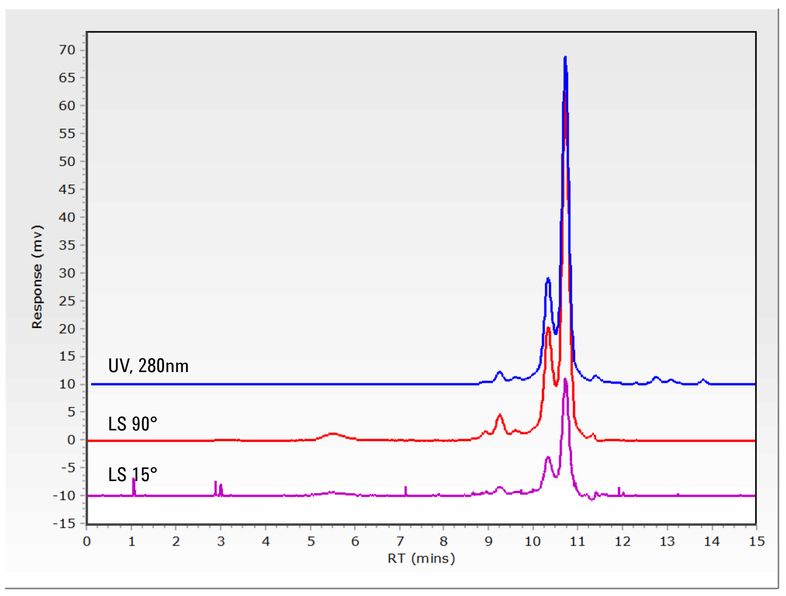

Figure 6: UV 280nm (top), LS 90° (middle) and LS15° (bottom) Traces for CTG Separation

Following calibration of the detector with BSA solution (in order to determine the inter-detector delay and system constants) the CTG was analyzed using UV 280nm signal and an extinction coefficient of 2.00. Figure 7 shows the regions analyzed and illustrates the fact that both peaks are almost identical in MW (Table 1).

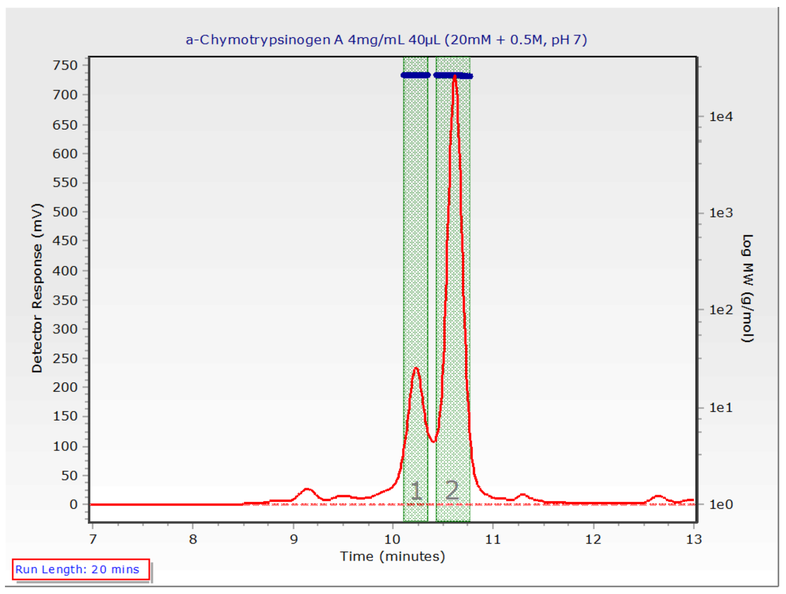

Figure 7: Light Scattering Analysis of CTG.

Peaks	Mp	Mn	Mw	Mz	Mz+1	PD
Peak 1	26102	26101	26101	26101	26101	1.000
Peak 2	25770	25771	25771	25771	25771	1.000

Table 1: Molecular Weight Analysis Results of CTG by LS

This increase in elution time does not affect the calculated molecular weight therefore it is highly likely that the CTG has undergone a change in conformation resulting in a much smaller size in solution.

Conclusions

- A systematic approach to method development is essential for robust chromatography.
- Modern tools can aid this process, greatly reducing the time spent investigating mobile phase parameters.
- Unexpected behavior can be further examined by utilizing light scattering detection.

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