**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.

**Experimental**

Columns: Agilent Bio SEC 3 300A 7.8 x 300 mm
Agilent Bio SEC 3 300A 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 1200 Infinity Bio-Inert Quaternary LC with Agilent 1200 Infinity Multi-Detector GPC/SEC.

**Introduction**

**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.

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:

**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.