Size Exclusion Chromatography: Method Development

To develop a successful Size Exclusion Chromatography (SEC) method it is desired to find a column/solvent combination under which the follow conditions are met:

1. The sample is stable and has a constant relationship between hydrodynamic volume and molecular weight.
2. The column and sample do not undergo any interactions allowing for a purely size-based separation.
3. The relationship between the hydrodynamic volume of the standards and samples is as similar as possible or, at a minimum, understood.

Identifying the conditions under which these criteria are met is often challenging. This is especially true for copolymer samples were the range of possible interaction mechanisms is high. Some degree of trial and error is required. An experienced chromatographer has the advantage that they can minimize the number of cycles required to find suitable conditions and, most importantly, they have an objective set of criteria to identify success. In our opinion, a method is deemed a success when it reliably provides data which is adequate to meet the purpose of the work.

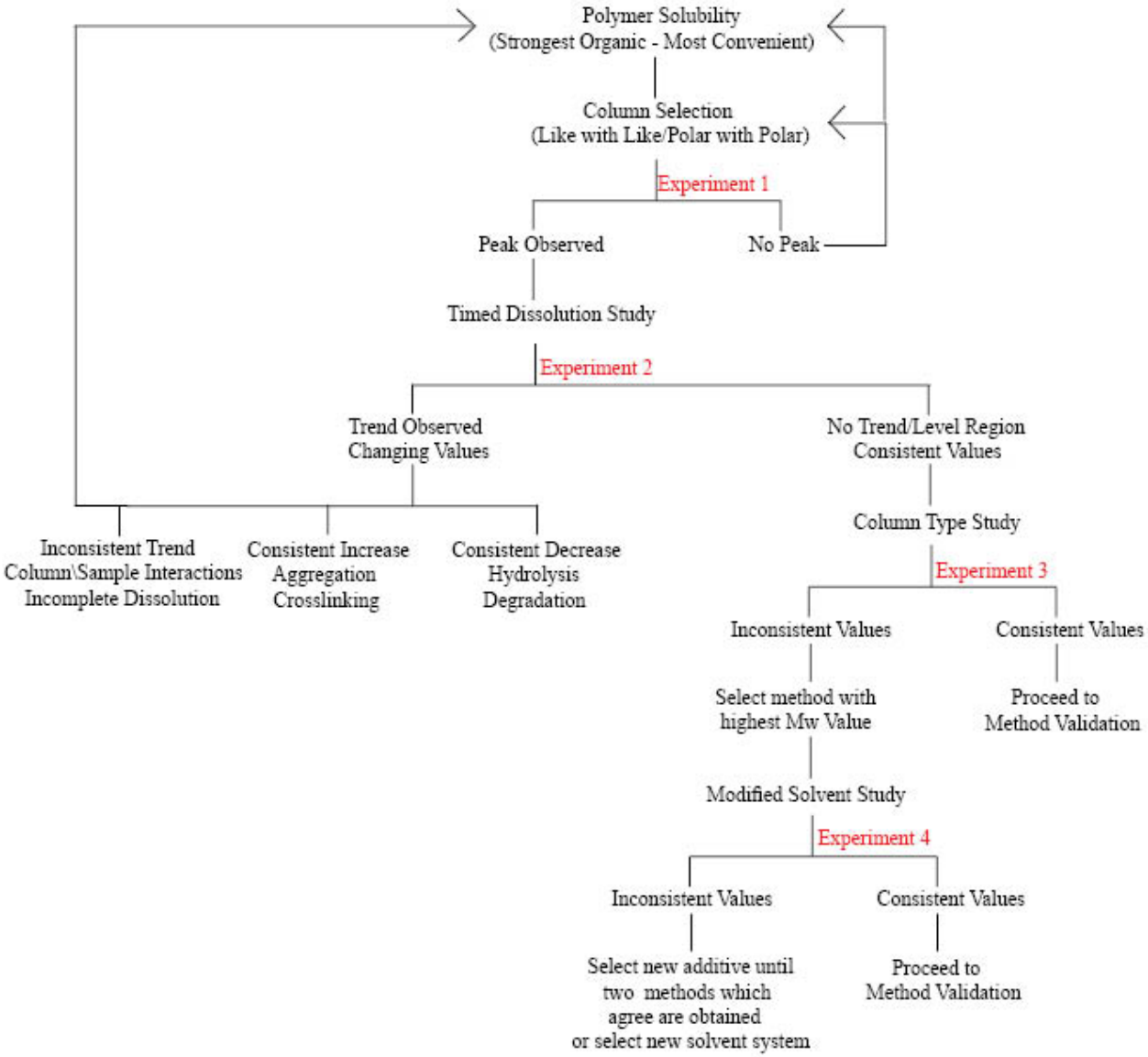
In our experience, SEC methods are typically developed to accomplish one of the two goals. The first case involves identification of changes in the molecular weight of a material for the purpose of quality control. In this instance, the degree to which the calculated molecular weight reflects the actual molecular weight is relatively unimportant. What is important is that samples with different molecular weights can be readily identified and that the method provides consistent reproducible data. In the second instance, the determination of a theoretically accurate molecular weight is desired. This approach is typically preferred when it is desired to gain a better understanding of the polymerization process and the extent of conversion.

Based upon our discussions, we understand that you desire to develop methods for both of these purposes. We intend to use the development of poly(acrylic acid-co-2-ethylhexyl acrylate) as a case study to demonstrate the general principles involved in SEC method development and to point out the differences in the way this process is conducted depending on the information required. We would recommend that we have a follow up discussion regarding validating this method to ensure consistent results.

Method Development

Experiment 1:

Figure I shows a flow chart which details the early stages of the method development process. The starting place for the development of any SEC method is the identification of the solubility of the polymer system. It is our approach to select what we believe is the strongest solvent (strongest organic character to increase column deactivation and most convenient based upon personal preferences) which effectively dissolves the polymer. We then perform a series of



experiments to identify a column type which will elute the polymer.

Figure I: Method development flow chart.

The column type used is a matter of trial an error. However, the general principle is “like with like.” If the sample is non-polar, then use a non-polar column. If the sample is polar, then use a polar column. This may seem counter intuitive but it is our general experience that having a column of similar polarity to the sample generally favors strong interactions between the solvent and the column. This is crucial to achieve a purely size-based separation. The concept of “like with like” works because it is generally observed that if a solvent dissolves a polymer it has similar polarity to the polymer. Thus the column and solvent will also have similar polarity. In the initial analyses, it will not be clear if the separation is a purely size-based separation. We just want to obtain a peak for the sample. These experiments provide a starting place for further exploration. In the flow chart above, this step is described as **Experiment 1**.

If a peak is not observed, then the column type should be changed and the experiment repeated. If all available column types are exhausted without obtaining a peak, then a new solvent system will need to be selected.

Experiment 2:

Assuming a peak is observed, we then proceed to perform a series of timed dissolution experiments (**Experiment 2**). The purpose of these experiments is to identify a series of dissolution times at which consistent values are obtained. We are looking for repeatability and precision and not accuracy with these experiments. The experiment is generally performed by making a single sample solution and analyzing the solution at 2, 6, 8, 24, 48, and 72 hours. It is generally observed that a dissolution time can be found above which the values will become consistent.

The primary benefit of the dissolution time experiment is that it will aid in identifying problems with sample stability. No successful method can be developed for a sample which is changing over time. If the material in question is degrading slowly or if the hydrodynamic volume is changing due to incomplete dissolution, then it is important to discover this early so that time will not be wasted trying to find a “good” column or solvent when the polymer itself is not stable.

If the molecular weight is observed to shift then it is important to analyze the observed trend in light of the known chemical structure of the polymer. Careful consideration of the trend and the sample chemistry can provide insights into the mechanism causing variability. In some cases, this problem cannot be resolved and the best option becomes analysis using a very specific dissolution time.

The preferred outcome is a repeatable molecular weight value over a range of dissolution times. If this is observed, then all additional experiments should be conducted using a dissolution time at which repeatability was observed.

Experiment 3:

The next step in this process is a series of analyses using different column chemistries in the same solvent (**Experiment 3**). All conditions should be held constant except for the column chemistry. It is the goal of this work to determine if changing the column chemistry results in changes in the calculated molecular weight. If no changes are observed, then the probability is very high that a purely size-based separation is occurring.

Interaction chromatography would be expected to change as the chemistry of the column was changed. It is highly unlikely that a polar and a non-polar surface would equally retain a sample.

If the molecular weight can be found to agree using two different column types, then the first phase of method development is complete. You can then proceed to method validation.

It is the more likely outcome that a change in molecular weight will be observed. In this instance, the method which produces the highest calculated molecular weight value is generally preferred. This is because essentially all retention mechanisms (hydrogen bonding, dipole-dipole, hydrophobic interactions) would retard sample elution and thus decrease the calculated molecular weight. If the sample elutes earlier, then it is assumed that less interaction must have occurred and the separation is closer to a purely size-based separation.

The separation which provides the highest molecular weight value is the current best method but it is not clear yet if the separation mechanism is a purely size-based separation.

Experiment 4:

To aid in identifying if the separation is purely size-based, we will now conduct a series of experiments where the solvent strength is adjusted with a mobile phase additive (**Experiment 4**). The goal of this work is to change the overall solvent composition as little as possible while using an additive to block any potential interactions between the solvent and column.

Large changes in solvent composition could have a significant effect on the hydrodynamic volume of the sample and should be avoided at this stage. Similarly, if the additive strongly changes polymer shape in solution, then the interpretation of this experiment may be unclear. Salts are particularly dangerous in this regard as they can strongly affect polymer shape. Similarly, changes in ionic character of the sample can have very large effects on polymer

hydrodynamic volume and should be avoided. Instead, it is desired that a mobile phase modifier be identified which does not change the ionic character of the polymer but which will interfere with any interactions between the sample and the column. We have listed additives which we commonly apply for this purpose in **Table I**.

Table I:

Mobile Phase Additives		
Methanol	Polar	Hydrogen Bond donor and acceptor
Acetic Acid	Polar	Hydrogen Bond donor and acceptor Hydrophobic Interactions
Triethylamine	Polar	Hydrogen bond accepting Hydrophobic Interactions
Butylamine	Polar	Hydrogen Bond donor and acceptor Hydrophobic Interactions
Tetrahydrofuran	Moderate Polarity	Hydrogen bond acceptor Hydrophobic Interactions

Generally, we will select an additive which we feel is most likely to interrupt the expected interactions between sample and column, based upon the chemistry of the polymer. To do this, we again apply the "like with like" principle. If the sample contains an acid functionality, we will add acetic acid. If the sample contains a basic functionality, we will add an amine such as TEA or butyl amine. In doing this we hope to change the polymer shape as little as possible since the polymer already contains that functionality. We also hope to effectively compete with the polymer for active sites on the column since the same group which is retaining on the column is present in the additive.

The additive selection process described above focuses primarily on polar interactions. This is generally more problematic in organic solvents such as THF. When a highly polar mobile phase (such as DMSO) is applied, then hydrophobic interactions (reverse phase retention) is the more likely problem. Using a more hydrophobic additive can be useful in such cases. Acetic acid is often a strong solvent for reducing reverse phase interactions, as is THF. Either of these solvents can be added to a polar solvent to aid in preventing these interactions.

Once an additive has been selected, the sample should be run again holding all conditions identical to the previous "Best method" except for the change in solvent composition. If the molecular weight holds constant, then we have obtained a higher level of certainty that the method in question is a purely size-based separation. If it increases in value, then it is likely that a purely size-based separation has not yet been obtained. We would then proceed to either change the solvent system entirely and repeat the process or try additional mobile phase additives to see if we can obtain a consistent value using other additives. We would be comparing these

new methods to the value obtained using the mobile phase additive (highest Mw obtained is always current best method). In the case where the molecular weight goes down, it is best to choose a new mobile phase additive and repeat the experiment. This may indicate a significant change in solvent quality.

Case Study - Poly(acrylic acid-co-2-ethylhexyl acrylate)

In the case of poly(acrylic acid-co-2-ethylhexyl acrylate), it is our understanding that the polymer in question is soluble in a range of solvents as shown in **Table I**. In addition, the columns available for method development are listed in **Table II**.

Table I

Solvent	Polarity	Potential Interactions	Monomer Favored
Toluene	Non-polar	pi-pi interactions Hydrophobic Interactions	2-ethylhexylacrylate
Tetrahydrofuran	Moderate	Hydrogen bond accepting Hydrophobic Interactions	2-ethylhexylacrylate
MEK	Moderate	Hydrogen Bond acceptor Hydrophobic Interactions	Neither
Ethyl Acetate	Moderate	Hydrogen Bond acceptor Hydrophobic Interactions	Neither
DMAC	Polar	Hydrogen bond accepting	Acrylic acid
Isopropanol	Polar	Hydrogen bond accepting Hydrogen bond donating	Acrylic Acid

Table II

Columns	Chemistry	Column Polarity	Potential interactions
Jordi DVB	DVB	Non-polar	pi pi interactions hydrophobic interactions
Polymer Labs	Polystyrene/divinyl benzene	Non-polar	pi pi interactions hydrophobic interactions
xStream	Polyamide	Polar	Hydrogen bond acceptor Hydrophobic interactions
Shodex PVOH	PVOH	Polar	Hydrogen bond donor and acceptor

Experiment 1:

We would recommend that the initial experiment performed for the GPC analysis of poly(acrylic acid-co-2-ethylhexyl acrylate) be performed primarily using THF. This mobile phase is preferred by many GPC analysts as it is an excellent solvent for a wide range of polymers and is appropriate for use with a wide range of standards including polystyrene and polymethylmethacrylate. In a departure from the normal, we would also recommend that acetic acid be added to the THF to suppress the potential for dissociation of the acidic groups. Thus our selection for the first solvent choice would be 95%/5% THF/Acetic acid.

The initial column selection is not obvious for this polymer system given the polar/non-polar combination of monomers (acrylic acid = polar, 2-ethylhexyl acrylate = non-polar). Based upon our experience, we believe that a PS/DVB or DVB column will not be successful in pure THF. We recommend starting with a single xStream Mixed Bed GPC column with the appropriate guard. This column matches best with the acrylic acid component of the sample and since this is also the most retentive component in the polymer, it makes sense to focus on it. Thus the conditions for the first experiment would be:

SOLVENT	THF/Acetic Acid
FLOW RATE	1 mL/min
INJECTION VOLUME	200 µL
COLUMN TEMPERATURE	35C
CONCENTRATION	2.5 mg/mL
COLUMN	Jordi xStream Mixed Bed DVB 25cm x 10mm
DETECTOR	RI
STANDARDS	Poly(methyl methacrylate)

The results of the first experiment will be judged based upon weather a peak is obtained. Proceed to Experiment 2 if a peak is observed. Otherwise, a new column type should be selected (DVB) and the experiment repeated.

Experiment 2:

Once a peak is obtained, we recommend that you proceed to performing a timed dissolution study. In this experiment, the sample will be placed into solution and then analyzed at 2, 6, 8, 24, 48 and 72 hours. This study can be performed by using time delays on an autosampler system. The purpose of this experiment is to identify a time frame at which stable values for the molecular weight can be obtained. The result which is desired is to identify the time beyond which the values of the analysis are consistent. Assuming a consistent value can be obtained,

then proceed to Experiment 3. If the values are not satisfactory, then we would recommend proceeding to changing the solvent composition. We do not expect the polymer to degrade or crosslink based upon the chemistry indicated. This would suggest that changes in hydrodynamic volume are the primary mode of failure if one is observed.

Experiment 3:

If the values from Experiment 2 are observed to be consistent, then proceed with Experiment 3. We would recommend using a DVB or PS/DVB column for comparison purposes. This is not ideal given the likely outcome, but it is our understanding that no other column type is available to you. In our facilities, we would proceed to use a Jordi Fluorinated GPC column for this experiment.

If the sample results are found to match for the two column systems, then the method development project is complete. If it is not found to be consistent, then proceed to Experiment 4.

Experiment 4:

If the two column sets do not produce consistent results, then proceed to adding a mobile phase additive. Based upon the chemistry of the sample, methanol and acetic acid both appear to be excellent options as mobile phase additives. Given our choice of initial solvent, we would recommend the addition of methanol to the existing THF/acetic acid solution. A 95/5/5% solution seems a good choice.

The purpose of this analysis is to confirm that the calculated molecular weights remain constant for the two conditions under which the polymer is analyzed. If the initial analyses were performed in THF, this may not be observed due to changes in polymer coil shape with the addition of acetic acid. We recommend comparing values in THF/acetic acid 95/5 to values obtained in THF/acetic acid/methanol 90/5/5.