How a Chromatographic Method is More Than a Separation

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Developing a chromatographic method is about more than separating compounds. A well-designed method complements the ultimate goals of the method-development team and helps answer research and business questions. To get to such a method, the development process must take these goals into account from the outset and follow a predefined plan.

Introduction

If chromatography is the science of separating mixtures into their components, then method development is the craft of turning separations to a useful purpose. Rarely do scientists separate compounds for the fun of it. Chromatography serves project or research goals, and developed methods must align with these aims.

Thus, separation of components is not the only requirement for an analytical method. Depending on the project, other needs might include long-term method stability, easy method transfer, high yield and purity for preparative scale-ups, detection of all impurities and degradation products, and more. The organised chromatographer identifies these needs from the beginning and designs a method-development plan that achieves both separation and other method goals.

A three-tier process (*Figure 1*) underlies this plan [1,2]:

1. Defining the project purpose
2. Matching parameters to purpose
3. Validating performance

This process ensures that complete methods are designed from the outset.

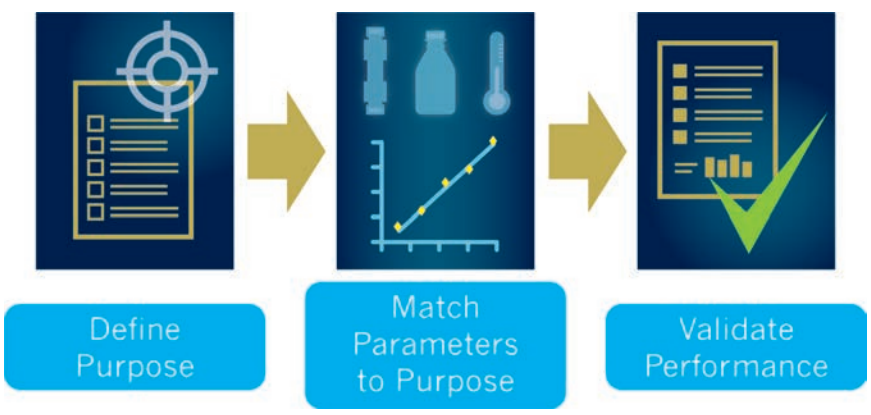

Figure 1: A method-development plan should include (i) definition of the project purpose, (ii) use of the project purpose to select and optimise parameters, and (iii) validation of performance.

Defining the Project Purpose

As discussed, the ultimate purpose of an analytical method is not to separate compounds. Separation is an important proximate purpose, but it serves a higher goal.

Too often, early-career chromatographers fail to account for the multilayered purposes in a project. They start work with only a shallow understanding, jumping into the lab after a short conversation with a colleague:

> Question: "I need to separate three impurities in this mixture."
>
> Answer: "I can do that for you."

Unfortunately, this approach leads to continuous cycles of revision. The three impurities will be separated, only for the chromatographer to learn that there are other expected impurities, which will then be separated, only to learn that an orthogonal high performance liquid chromatography/mass spectrometry (HPLC/MS) screening approach would have been the most appropriate option from the beginning.

To tease out all the needs a method must fulfill from the start, begin instead with a thorough questionnaire:

1. Is the problem fully understood?
2. Have the correct questions been asked?
3. Are all the facts at hand?

These questions reinforce that the project should be understood thoroughly. Not only at the surface - which is what often emerges in the first presentation of a problem - but with all the details of why the separation needs to be done, what is being separated, who is going to use the information, and how they are going to use it.

4. Have the customers asked the correct question?
5. Do they have all the facts?

A useful chromatographic method helps people make decisions. The list of people impacted can be long: developed methods may be used by entire teams, and even more people may rely on the method results for their work. It is therefore paramount to understand the entire user chain. For example, there is little purpose in developing a 90-minute ultra-high performance liquid chromatography (UHPLC) gradient if the final user will be a contract house with only HPLC equipment. Each person in the chain may understand pieces of the problem, but often, no one person understands the entire picture. To get a complete view of the project, speak to multiple stakeholders.

6. What approach should be taken?

Based on what has been learned so far, design an approach from first principles. Consider the expected compound structures, and use in silico tools to predict solubility, logP, logD, pKa, and degradation pathways. A logically planned approach will save time and guide the chromatographer in the right direction from the beginning.

7. How are the results reported?
8. What results are being reported?
9. What will happen to the results once reported?

Knowing why a method is being developed helps chromatographers to determine how the results should be reported. While many organisations use standard report templates, these should still be scrutinised for each project. What is the most important information for the project goals? Is that being reported? Is the provided information enough for downstream teams to make their decisions, or will they have to further interrogate the data?

10. Are these conditions going to be used long-term?
11. Is that the end of the work or is it a long-term project?

Methods should stand the test of time. Even when method life cycles are short, troublesome methods spawn unexpected problems, so it is always best to ensure robustness with thorough testing.

12. Is it fit for purpose?
13. Is this GMP, GLP, development, or nice-to-know?

Regardless of the reasons behind method development, the highest standards must always

be adhered to during the development process. This reduces downstream problems in method transfer, either in-house or to outside contractors.

Matching Parameters to Purpose

Answering these questions would be an empty exercise if the answers didn't inform method development. To turn the identified purpose into concrete method parameters, define method specifications and choose screening conditions.

Method specifications state clearly what the project needs to achieve before any work begins. The questions shared here will help to identify a broad category for the project: preparative scale-up, quantitative method development, or investigative study.

Each category has different requirements. For example, preparative scale-ups are concerned with separation purely of the compound of interest. The goal is to get high recovery and purity in a reasonable time, and other factors - like resolving individual peaks - can be sacrificed. But what constitutes 'reasonable' time or recovery? These should be defined in the specifications, depending on the information already gathered.

For quantitative method development, benchmarks must be set for quality of quantitation. If percent weight-weight is desired, pure standards must be available. There may be governmental or third-party guidelines on quantitative accuracy, or those may need to be decided, again based on the project aims.

Finally, for investigative studies (e.g., forced degradation studies), detailed information about the samples will help to design the method-development plan. It is best to work from a single combined sample, rather than multiple samples. With fewer samples, it is simpler to track compounds across multiple chromatographic conditions. Consider what impurities are probably being formed and how to determine whether the method detects all impurities. Never assume that a single UV/Vis detector, whether diode-array or single-wavelength, will capture all compounds. Systems with multiple detectors (e.g., light scattering, corona-charged aerosol, fluorescence, and MS) are more thorough.

After the specifications are set, the experimental work begins. For efficient screening and optimisation, use the information gathered in pre-development to select parameter ranges for screening.

Figure 2 shows all the parameters that affect analytical-method performance. Informed decisions should be made about all of these; selecting parameters to investigate based on guesses or past habits will only prolong method-development time.

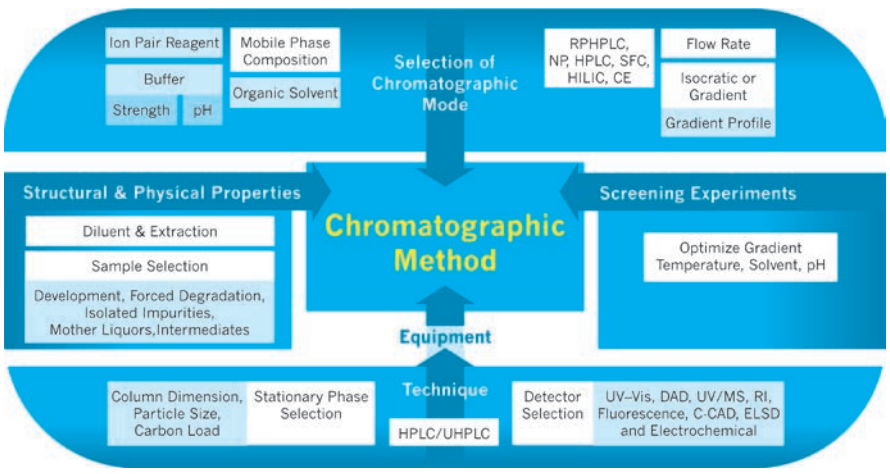

Figure 2: Many parameters affect the success of the final chromatographic method.

For example, the solvents and pHs to be screened should be selected based on analyte physicochemical properties. Often, experimental data is not readily available, so software tools can be used to help. Commercial software tools provide useful predictions such as solubility, logP, logD, and pKa.

Figure 3 shows one such tool, ACD/Method Selection Suite. The predicted logD of a compound is graphed against pH (left). Based on this, the percentage of analyte in the dominant ionic form is also graphed (right). The dominant species changes with pH, but the important point is to choose an eluent pH on the plateaus of this curve. Otherwise minor shifts in pH will vastly affect the method.

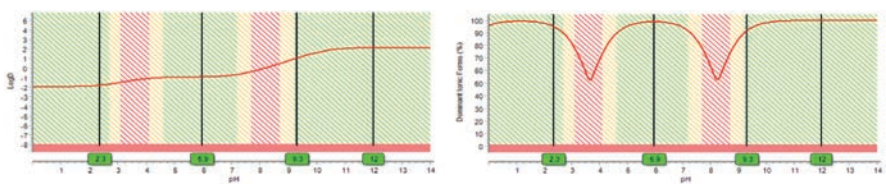

Figure 3: Predictive software can be used to visualise logD (left) and dominant ionic form (right) changes with pH.

To take another example, column selection depends upon both compound properties and project considerations. Compound properties obviously affect stationary phase selection: non-polar analytes should be separated on C8, C18, or similar stationary phases; low-to-medium polarity analytes on diol, amino, cyano, phenyl, or C4; and medium-to-higher polarity analytes on silica, amino, or cyano. But the project purpose should also be considered. What will happen to the method after development? If it will be transferred to a lab with only HPLC, then it would be pointless - and even counterproductive - to develop the method on a UHPLC column. If the method is going to be used long-term, then ideally the original column type should be available for the entire duration. Even with the same column type, batch-to-batch variation in column packing can affect separation. Before finalising method conditions, it is a good idea to run the separation on multiple batches to monitor resolution and specificity changes.

Detector choice is yet another factor that is influenced by both analyte physicochemical properties and other requirements. Obviously, a detector should be chosen based on expected compound properties, e.g., whether a compound is UV-absorbing. (Eluent properties should also be considered, as they may influence compound properties. For example, absorbance spectra shift with solvent pH.) But project purpose also enters the equation: if measuring degradants is important, there should be some understanding of how the compound might degrade and how that might affect measured signals in different detectors. It is often a good idea to start a development project with multiple detectors. This ensures that all compounds are detected and thus mass balance can be achieved.

A factor-by-factor explanation of all the parameters in *Figure 2* is beyond the scope of this paper. Yet as these examples illustrate, informed consideration is necessary for efficient method development.

Optimising Parameters

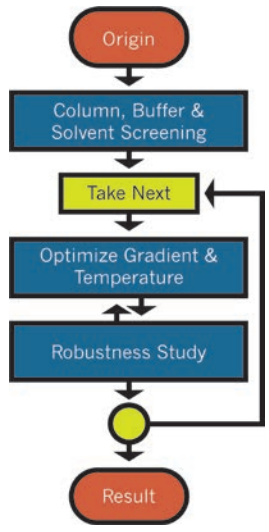

Figure 4: An example optimisation workflow.

Picking parameter ranges for screening is only the start of the experimental work. A rational approach to method development explores the selected parameter space to find optimal and robust areas.

Figure 4 shows a standard screening and optimisation plan. Rather than optimising all the variables at once, the chromatographer handles them in waves - a more efficient and manageable approach.

In the first wave, the column, buffer, and solvent are screened. This step should narrow down choices, though it needn't leave only one option. If two or more columns perform to the determined specifications, it may be worthwhile to take multiple columns forward.

Once column, buffer, and solvent are selected, optimise gradient and temperature. Simulation tools can help here. Given a few chromatograms collected at different gradients and temperatures, software can simulate chromatograms across the parameter space and find areas of optimal resolution.

This stepwise process will make method development more efficient, but the experienced chromatographer will keep some perspective on the entire project. Certain steps taken early on will make methods more likely to pass later steps of validation and risk assessment. For example, start working with limit-of-detection samples early. This will flag any issues with sensitivity before work is sunk into a method that will never meet specifications.

By the end of optimisation, the method-development team should have settled on a complete set of method parameters.

Validating Performance

Approaching pre-development and development methodically should put chromatographers in a good place for validation - with a method that is likely to pass validation and robustness testing.

Before validation, assess the forced degradation results (if applicable) and check mass balance. Write up a risk assessment: a living document that addresses the most likely points of failure. This document should be regularly reviewed and updated, especially with the results of robustness and method-transfer exercises.

Then move to validating the method, checking:

1. Sample concentration and injection volume
2. Analytical-wavelength selection
3. Relative-response-factor determination
4. Analytical solution stability
5. Specificity, resolution, linearity, reporting limit, and limit of detection
6. Method of quantification

During the validation process, draw upon earlier data where reasonable. A large quantity of data is generated during development. If development experiments are well designed, and the resulting data is properly processed, annotated, and stored, it can be used to answer validation questions. For example, development data can be used to investigate peak-shape changes or retention-time drifts over time, reducing the number of validation experiments required.

If performance can be proven, then the method-development project has been a success. But so long as a method is in use, development never really ends. Whenever the use case changes - if raw materials change, the synthesis pathway is altered, or the samples are different - reassess the method.

Conclusion

Method development is about more than separating compounds. To develop a method that will achieve the ultimate goals of a team or organisation, a chromatographer should start with a thorough understanding of the method purpose. The information gathered at this step feeds into downstream steps of planning and parameter selection. Starting the development process intelligently makes optimisation more efficient and validation more likely to be passed. When a chromatographer understands and implements all steps of this process, they are on the way to a complete method.

References

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2. S. Sule, S. Ambadekar, D. Nikam, A. Sule, S. Bhure. World J. Pharm. Res. 3 (2014) 258.