An Executive Summary

Sample Preparation: The Forgotten Dimension in HPLC

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Although often forgotten, sample preparation is the most important factor in achieving reproducibility from even the most advanced chromatographic methods.

Overview

Sample preparation techniques are often thought of as basic chemistry, but they may be the most important aspect of running analytical methods. In this paper, learn about some ways that sample preparation can affect the selectivity, sensitivity and reproducibility of an analysis.

Classic Sample Prep Techniques

While sample preparation is a routine task, it can be a critical factor that affects a method's accuracy and reproducibility. Reviewing the basics of sample prep, it is vital that:

1. Analysts collect samples according to standard operating procedures.
2. Solvents and glassware are chosen according to the method's needs.
3. Samples, reagents, and chemicals are handled safely.
4. Sample preparation techniques are optimized using the ideas of equilibrium and kinetics.
5. Samples are kept as clean as possible.

Sample preparation requires precise handling and manipulation of samples from the time they enter the lab until the vial is placed on the tray. One should always think about which steps of the sample handling process could enable hidden errors to creep into the method.

Several techniques are used in sample preparation, ranging from well-established procedures like weighing, dilution, and filtration, to relatively new technologies like QuEChERS. The results from two recent surveys about the popularity of sample preparation techniques are shown in **Figure 1**. Older methods are by far the most common.

The process of turning a raw sample into a form that is ready to be injected into an instrument can require numerous steps, each with its own potential for introducing variance to the method. Gravitmetric steps often have the fewest errors associated with them, assuming the balances are properly maintained and calibrated, and proper operating procedures are used. Dilutions, on the other hand, are often a neglected source of variance.

Glassware choice. It is common to do simple dilutions in volumetric glassware. It is imperative to use good techniques and avoid bad practices that can negatively quantitative analysis. For instance, one may be introducing precision and accuracy errors into the method by using smaller glassware, which is chosen for smaller samples. Larger

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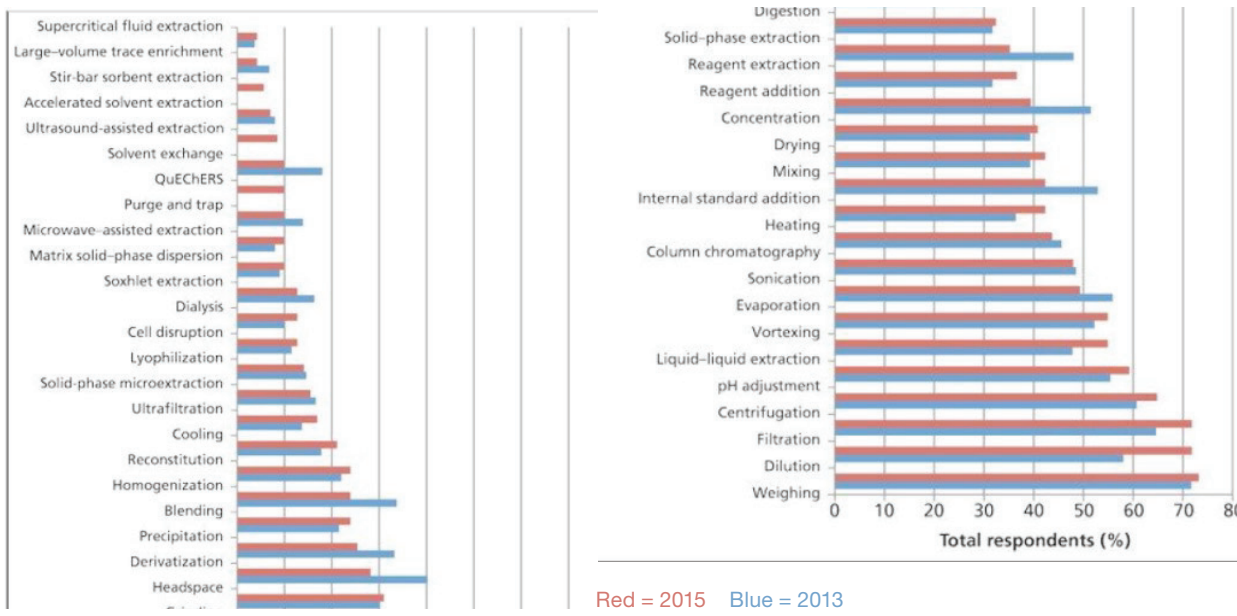
Figure 1: Popularity of sample preparation techniques.



Source: D.E. Raynie, *LCGC North America,* **34**(3), 174–188 (2016).

flasks for larger samples offer better precision, at least on a percentage basis. In addition, when using non-aqueous solvents, analysts may also see some small errors in working with volumetric glassware because they were calibrated for water.

Class A glassware has a known tolerance that should be considered, and is higher for smaller volumes. For example, a 250-mL volumetric flask has a tolerance of 0.048%, while a 10-mL flask is 0.20%. Performing serial dilutions is a good way to save on the use of solvent, but introduces extra steps into the process. Each step comes with additional uncertainty, which can accumulate.

The same rule applies to pipettes, with the smallest variance resulting from the larger volumes. The type of pipette also matters. Volumetric transfer pipettes have the tightest tolerances. Graduated seriological pipettes can be an order of magnitude worse, and syringes and automatic pipettors are the worst. It is also good to keep in mind that plastic pipette tips have an added complication: there is a high surface area-to-volume ratio and a plastic surface has the possibility of adsorbing hydrophobic analytes.

Glassware condition. The condition and arrangement of glassware are also important. When flasks are shipped from the factory, they are paired with well-fitting stoppers. These items invariably become separated during cleaning and normal use. While it is not critical to keep a flask paired with its original stopper, there is enough variance that blindly picking a stopper from the drawer could result in a poor fit. If a leak occurs during the inversion and mixing step, some solvent will be lost and the concentration will be thrown off.

One way to check to see if a leak is present is to insert the lid and see if any ground glass is visible. If there is, switch to another lid until no ground glass can be seen.

Last, glassware has a finite lifespan. The volume of a heavily used flask will drift over time. If the etched labeling on the side of the glassware is no longer legible, it might be time to consider replacing it.

In a nutshell, one must remember (especially when faced with reproducibility challenges) that volumetric flasks and pipettes can be the source of small experimental errors. Such errors have the potential to add up to a potential larger error in the method that may or may not be tolerable.

Solvent mixing. Mixing aqueous and non-aqueous solvents results in a change of volume. Whenever possible, the mixing of two solvents inside a volumetric flask should be avoided or minimized. Although it adds a step, it is better to prepare the solvent mixture beforehand and then use it in the volumetric glassware.

Safety. Safety is also an important consideration in any laboratory procedure. Proper solvent handling, such as aliquoting and secondary containment, are essential. Having standard operating procedures (SOPs) in place for solvent handling has many benefits. Not only do SOPs improve safety, but they also increase the reproducibility of the analysis. This can include simple things such as aliquoting the needed solvent from the bottle rather than pipetting directly, which can lead to contamination.

Solvent choices are not absolute. There are times when a slightly sub-optimal solvent is acceptable if it greatly reduces the risk associated with a procedure.

Safety considerations become particularly important when working with pure standards of highly toxic compounds.

Phase Equilibrium and Kinetics

Many sample preparation techniques are separations in their own right, including liquid–liquid extractions (LLE), solid-phase extraction (SPE), solid-phase microextraction (SPME), head space analysis, and QuEChERS. They depend on the partitioning of the compounds between the solution and a separate phase. In some cases, the goal is to capture the analyte, and in others, it is to remove interferences. For any partitioning separation, two important parameters that must be kept in mind are equilibrium and kinetics.

LLE. LLE is simple, easily understood, and relatively inexpensive, although it is not always easily adapted to automation. The figure of merit for an LLE is the distribution constant (*K*), which is defined as the ratio of the concentration of the analyte in the two phases at equilibrium. When extracting from an aqueous phase to an organic phase, a high value of *K* means that the analyte will preferentially partition into the organic phase while a low value means that the concentration in the aqueous phase will be higher. If the value of the distribution constant is not known, it may be useful to do a quick experiment to get a rough approximation.

For most applications, the analyst is attempting to extract the analytes from the aqueous sample into an organic phase. For this, the value of *K* will ideally be high, but it will never be infinite. There will always be some fraction of analyte remaining, but near-quantitative extractions are possible. A low value of *K* is not necessarily a point of failure, however, as multiple extractions can be performed until sufficient analyte has been extracted, as seen in **Figure 2**. The fraction removed is shown as a function of the number of repeated extractions for several values of *K*. Sufficiently complete collection of the analyte is possible even with relatively weak extraction conditions. The trade-off is analyst time, solvent use, and the concentration of the resulting extract.

The kinetics of the partitioning must be kept in mind. The mixture should be shaken long enough for the partitioning to occur, and given enough time to settle so that the phases can reach equilibrium. Failure to do so can introduce substantial errors. Mechanical shakers can be valuable as a way of ensuring the mixtures are subjected to the same conditions each time.

It is important to remember that the distribution constant represents the concentration, not fraction removed. When the ratio of the volumes of the two phases is high (e.g., small volume of organic phase exposed to a high volume of aqueous phase), then a high value of *K* is needed or only a small fraction of the compound will be removed. Fortunately, the extraction does not have to be complete if it is reproducible.

SPE. SPE is one example where nearly all of the analyte can be captured in the bed. **Figure 3** shows an example of an SPE sample treatment. The sample is forced through the bed with either positive pressure from above or vacuum below. During the loading step, the solvent must be weak relative to the stationary phase within the column. The more strongly the analytes are bound, the tighter their band will be at the top of the column. During elution, a stronger solvent is flushed through, thus removing the solute from the column. Just as in HPLC, step gradients can be used to elute more than one band into separate fractions.

Figure 2: Multiple extractions can be performed until sufficient analyte has been extracted.

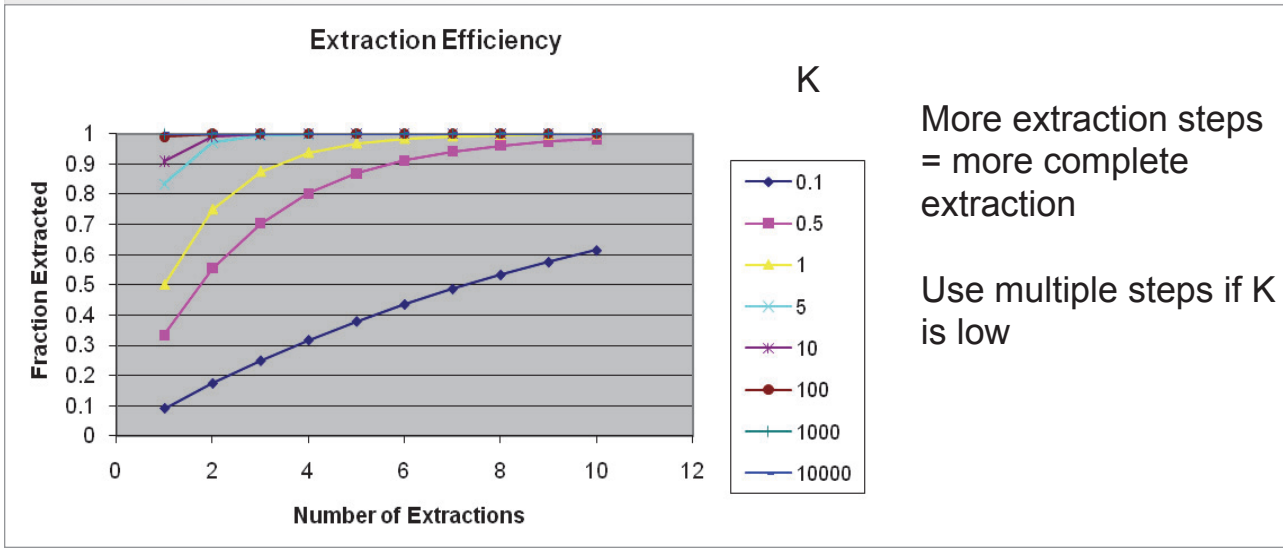
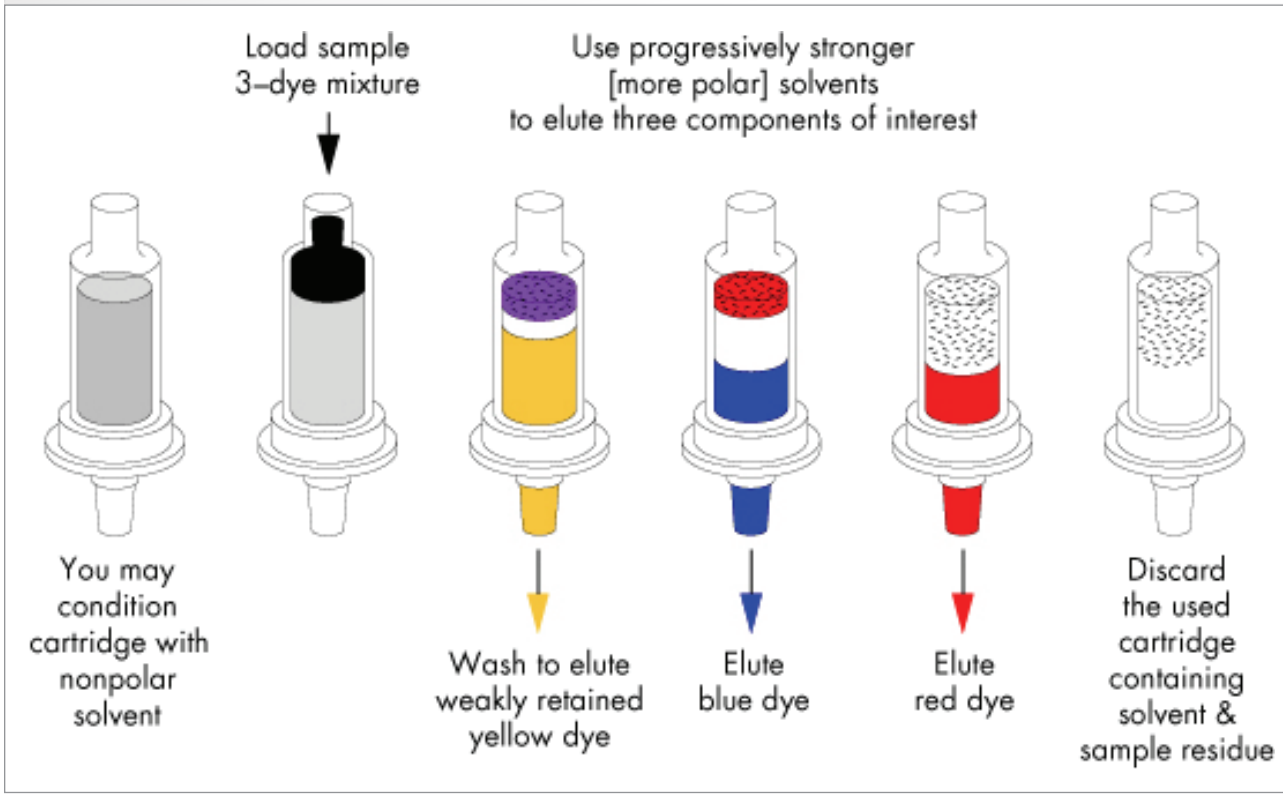


Figure 3: Example of a solid-phase extraction sample treatment.



Kinetics are an important factor in SPE. The packed beds use larger particles than HPLC, meaning the rate of mass transfer is much slower. Driving the solution through the bed too quickly will result in some analyte passing completely through before it can be captured by the stationary phase. Sometimes, the difference between high and low reproducibility comes down to the strength of the vacuum system used to pull samples through the SPE cartridges.

When preparing samples for HPLC, the obvious choice might be to pick an SPE cartridge with a similar chemistry to the LC column. While this kind of cleanup has value, using a chemistry that is orthogonal to your subsequent separation is more likely to selectively remove compounds other than the intended analyte.

One often overlooked advantage of normal-phase or ion-exchange SPE is that the sample is eluted in a highly aqueous phase. When injected onto a reverse-phase HPLC column, the sample will stack on the head of the column, thus providing a significant boost in sensitivity. Vendors have excellent resources available for the selection and use of SPE cartridges.

SPME. In SPME, the extraction phase is typically a thin layer on a fiber. The phase ratio is so high that only a small fraction of the solute is captured by the extraction phase. In this regimen, achieving reproducibility depends entirely on reaching equilibrium every time and avoiding variations in sample matrix or laboratory conditions that could affect the partition coefficient. One must pay attention to kinetics and ensure that the fiber is in contact with the solution long enough. If one is unsure, this is a good place to consider internal standards.

In the past 10 years, there have been numerous advances in commercially available SPME systems. They work well with small samples and are well suited for automation and high throughput, including working with 96-well plates. Because the process involves partitioning the analytes out of the sample solution, dirty or particle-laden samples are much less of a problem.

Pipette tip SPE is a growing technique, in which the sample is pulled into a pipette tip packed with the solid phase. It lends itself well to small samples and is relatively easily adapted to automation. Unlike traditional SPE, the solution must pass through the bed in two directions; first being drawn in and then flushed out. This places somewhat tighter restrictions on the need to have a high partition coefficient. An analyte that is only weakly bound will tend to be flushed out again. However, this feature is arguably an advantage when there are large amounts of a weakly bound interferant. In addition, clogging issues are somewhat alleviated as the head of the column is effectively backwashed each time.

QuEChERS. In recent years, a new technique has become quite popular known as QuEChERS, an acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe. In a typical

application, the sample is homogenized and extracted using an organic solvent such as acetonitrile or ethyl acetate. The organic phase is then isolated and can be dried with magnesium sulfate. As a final step, derivatized particles are mixed with the sample to selectively capture unwanted sample components and then separated via centrifuge. Several surface chemistries of the particles are available, including primary secondary amines for sugars, organic acids, and lipids; graphitized carbon black for aromatic and conjugated compounds such as carotenoids and chlorophyll; and C18 for highly nonpolar compounds.

Unlike many of the other methods, the SPE is being used as a one-way means of removing what is not wanted from the sample. The QuEChERS method has quickly been adapted to such wide-ranging applications as pharmaceutical drugs in blood; preservatives in pet food; and acrylamide in fried food.

Conclusion

Sample preparation is very often the biggest source of variance for a method, making it the most important factor to control and design well. It is often neglected because it constitutes the cheaper, more mundane part of any method. One cannot always rely on the column to make up for an insufficiently selective sample preparation. Likewise, even a mass spectrometer with the highest resolving power is still susceptible to ion suppression from an insufficiently purified sample matrix. Separations of any kind work best with clean samples, and instruments require less maintenance. Well-established and standardized techniques can minimize variance, whether day-to-day, person-to-person, or lab-to-lab.

Many sample prep techniques are separations in their own right, whether LLE, SPE, SPME, QuEChERS, or another solid-phase method. Treating them as a potentially orthogonal dimension to your primary separation is a useful way to simplify an otherwise complex sample. As with all separations, being mindful of both the equilibrium and the kinetics of that separation is important to both the sensitivity and reproducibility of your method.