# How Often Should I Calibrate My HPLC?

**Part 1 – General Considerations**

This is one of the most common questions that I hear, especially when training people with less experience in an analytical laboratory setting. Unfortunately, there is not a single source of information or recommendations that can provide an answer for every user. Usually, each method provides instructions on how and when to calibrate, so let's look at some of the reasons for these decisions.

*Why do we calibrate?*

First, we need to discuss the reason for needing to calibrate. Methods and instruments do not generate quantitative answers automatically. *Every* analytical instrument requires some form of calibration.

Why?

Each method involves measuring some physical or chemical property of the sample. It might be the absorbance at a specific wavelength, the current flowing between two electrodes, or the amount of light emitted in a fluorescence experiment. There are many possibilities, but in each case the measurement produces a "response" from the instrument, in voltage, or counts, or some other property that can be converted to a number. But this number is meaningless by itself, unless we can somehow relate that number back to the amount of the sample that produced it.

This is the point where "standards" enter the discussion. Using materials of known purity, we prepare one or more standards, each containing a known amount of the substance that we are trying to measure. We can now relate the response from the system with the amount of substance in the standard. Mathematically, this means:

$$\text{Response} = RF * \text{Amount}.$$

Once we know the Response Factor (RF), we can calculate the Amount in an unknown if we know the response produced by that sample. The response factor is sometimes referred to as a calibration factor, or you could think of it as a conversion factor from instrument response units to sample amount units.

Every instrument needs a response factor. Some units have a universal RF, so that any sample can be analyzed once you have established the calibration. Balances and pH meters are good examples of this situation. Balances are usually calibrated at the factory, and we only need to "check" the calibration with a known weight. A pH meter requires a set of standards with different pH values, but once calibrated, the pH of any solution can be measured. Chromatography instruments, however, do not have universal response factors. (Yes, there are some detectors that claim to be universal, but if you read the fine print, there are always exceptions and restrictions, so I maintain that they all need to be calibrated at some point.) An individual RF is needed for a specific compound within a certain amount range, in a known solvent, using specific instrument settings. These settings make up the analytical

"method." Another compound will probably require a completely different combination of these parameters (a different method), and the RF values will be completely different.

*How do we calibrate?*

There are several ways to develop the calibration factors for a particular method with a particular compound. Options include external standards, internal standards, and process standards. For each of these options, we can calibrate using only a single level, or we can prepare standards containing different amounts, and construct a plot of response against the amount of sample (i.e., a calibration curve). The reasons for choosing a particular strategy are beyond the scope of this discussion. We will discuss them in a separate post.

*How often should I calibrate my HPLC?*

The general philosophy of Quality Control (QC) for chromatographic analysis states that a sample analysis is valid only if the system has been calibrated or the calibration has been checked recently. If the system fails calibration (or a calibration check), then any future sample analyses will not be valid. Furthermore, all results obtained since the last successful calibration are now possibly incorrect, since we do not know exactly when the instrument failed.

How often you calibrate will depend on how often you want to allow such a failure. Put another way, your calibration frequency is all about RISK! How much risk are you willing to accept that the instrument drifts out of calibration and you do not recognize the problem? Let's look at how different laboratories manage this risk.

High Value Analysis Requires Low Risk

I often use the pharmaceutical industry as an example of this situation. A production lot of a product produced under regulated conditions (e.g., Good Manufacturing Practices) may have acceptance limits of 98 – 102 % of the label claim. For example, a 200 mg tablet of ibuprofen must contain between 196 and 204 mg. These lots are also quite large, and have a large value to the producer. A mistake in production or analysis can be very costly, so all processes are tightly controlled. The analytical lab prepares calibration standards for each analysis batch, makes multiple injections of the standards, and multiple injections of the sample. The analysis sequence will have calibration standards, blanks, and system suitability (to verify performance) followed immediately by samples. Additional standards are injected after the samples. These "bracketing" standards are sometimes included in the calibration calculation or they may be used as calibration verification. The general idea of these experiments is to establish calibration at the beginning of the analysis set, immediately analyze samples, and then verify calibration at the conclusion of the set. This approach satisfies the general QC philosophy that we mentioned above but minimizes the risk of a calibration-related error.

Wide Specifications Allow More Risk

In some industries, the acceptable ranges for a compound may be relatively large (+/- 10 % or more). For other products, the method may be a simple "limit test" where the substance must be above some level or below a certain limit. Since product quality and performance are not significantly affected by these changes, the analytical requirements can be adjusted to allow less frequent calibration. Typical calibration schedules can range from once a day to once a month, depending on the relative risk levels.

I see these situations in petroleum laboratories, chemical additives manufacturers, and some parts of the food industry. I have even visited laboratories that only recalibrate their gas chromatographs once a year! However, I do not recommend this schedule for HPLC.

So, what schedule works for your laboratory? How would you make that decision?

In the next post we will discuss some of the specific considerations that go into deciding which schedule is best for you. If you have questions about any of these general topics, please post them here.

**Part 2 – Factors Affecting Calibration Frequency**

As we discussed in Part 1, setting your calibration frequency is about how much risk you are willing to accept. Every lab will have different thoughts about risk levels, so there is not one "correct" answer to this question.

Borrowing an example from a different topic, most laboratories will schedule routine maintenance of their instruments on an annual basis. This schedule works well for most laboratories, and there are usually few unexpected failures, as long as you are observing the usual best practices for instrument operation. However, I have been in laboratories where they perform full maintenance every six months, and some specific procedures every three months. Are their instruments failing more often, or are they doing something wrong? No. They made a decision that these instruments were a critical component that supported production, and they did not want to stop production if an unexpected instrument failure occurred. In other words, they wanted to minimize this risk of having to halt production for a critical process. They are willing to accept higher costs of maintenance to reduce the risk of production delays. This decision may not work for everyone, but it is the right one for them. The same approach applies to setting your calibration frequency.

Now we need to look at some specific factors that will have an influence on your calibration frequency decision.

**Chemistry Issues**

The fundamental chemistry behavior of your compounds may have a significant impact on your decisions. You may be able to compensate for some of these issues, but they cannot be eliminated.

<u>Stability:</u> Environmental contaminant standards do usually not require special storage, since they are, well, pretty stable (which is why they are a problem). Pharmaceutical solids and most food-related compounds are usually stable, although refrigerated storage (4 C) is typical. Volatile standards, especially if purchased as known solutions, must be stored at the coldest possible temperature to prevent evaporation.

Unstable compounds will require more frequent standard and sample preparation, and more frequent calibration. Remember that these same considerations apply to the length of time that solutions can be stored and also allowed to be in the autosampler tray. Should you re-inject vials after the septum has been punctured? It depends! Re-injection within the same run is usually not a problem. Would I inject from the same vial tomorrow? Maybe, but only if I have large tolerances and the samples and solvent are relatively less volatile. High volatility samples and solvents will limit the amount of time the

solutions are valid, and the number of times that injections can be processed from them. Having a chiller for your autosampler would extend these times, of course, but you should always perform some testing to verify that these holding times do not affect the results.

Standard Preparation: Accurate standards require careful preparation. If you have a high-value analysis and/or demanding specifications, then you need to measure out larger masses on a suitable balance, and use larger volume volumetric glassware. This process is more expensive and requires more time, so frequent calibration may not be practical. Other problems like limited solubility, slow dissolution, and complex solutions may also influence your decision. However, stability considerations must also be balanced with preparation issues.

**Laboratory Considerations**

We work in the real world and there are other non-chemistry factors that can influence our decisions. Balancing these often-competing considerations has become an important part of laboratory life.

Standard Cost: Some compounds are difficult to prepare and purify as reflected in their purchase costs. Effectively using your standards and resources becomes critical in these situations. While simply calibrating less often is an easy solution, it may not be the best choice for a high-value or demanding application. However, there are options that you can use to minimize the impact of these costs.

Stock solutions are an effective way to minimize standard waste. This concentrated solution can be stored cold, extending its lifetime, and removed only for preparation of more dilute working solutions. Of course, stability of the stock solution must be verified. The lifetime of working standards in the autosampler can be extended by replacing the cap immediately after use. Yet another option to minimize standard use is to use your autosampler to only prepare as much working standard as needed, rather than diluting into a larger volumetric flask. Download our recent poster on how to program your autosampler to make standards.

Run Time: If your run time is 45 minutes and you have six standards, calibration will require more than four hours of instrument time. Even a small number of samples will require the use of an instrument for an entire work day. Calibrating less often is certainly an option, but in this situation, the lab should really consider if a different method could provide the same quality of results in less time. Maybe you could simply increase the flow rate somewhat to decrease the analysis time. In our experience, very few analyses really need to be 45 minutes long. There are many newer column options that provide equivalent results in much less time – 15 minutes or less. Yes, this change would require some additional costs and require some downtime to validate and switch methods, but we have calculated the Return on Investment (ROI) to be highly favorable in almost every case.

Production/profit: Yes, money drives many decisions that labs have to make. However, the smart labs only make compromises that do not impact both long-term success and product quality. Consider the pharmaceutical example in Part 1. The product and method requirements are demanding, and there are not many calibration changes that can be tolerated without a loss in data quality. Any efficiencies need to come from decreased run times as well as solution preparation improvements.

Other laboratories may be able to tolerate more calibration risk because of the need for a faster answer. A multi-step process may require an answer on an intermediate product within, say, two hours. Full calibration will not be possible, so the lab will use calibration checks to monitor system performance. For a stable process and a well-designed HPLC method, some labs can successfully use calibration checks for an extended time – days or weeks – if necessary. However, these calibration checks must still be performed at regular intervals, to minimize risk. Ideally, at least one check standard is analyzed with every sample, or at least daily. See the next section on Practical Choices for a discussion of this issue.

**Practical Choices**

As you can see from this discussion, there are many considerations and many possible options for setting your calibration frequency. In this section we will try to give you a little more general guidance to help with your decisions. We do not want to give you many absolute "rules," but there really are a few things that all labs should do.

Absolute (sort of) calibration rules:

- Calibrate your HPLC when you first load a method and then at regular intervals. You can decide what "regular" means, but you must do it according to some schedule.
- If you do not calibrate with every sample set, then check the calibration with a known standard (i.e., a check standard) between full calibrations.
- Every sample set should have standards injected both before and after samples. The standards can be calibrations injections or check standards, but they must be present. With this approach you calibrate/verify both before and after samples. This situation produces a very low risk of calibration errors.

Of course, now we will talk about exceptions to these "absolute" rules.

- If you only have one sample in a set, performing a full calibration would be very wasteful. Check standard(s) would be acceptable in this case. If you have relatively wide tolerances and a stable system, you might consider eliminating the "after" injection as well, but this does increase your risk.
- If the instrument is running continuously at method conditions, and there are time gaps between samples (say, every two hours), it might be acceptable to simply inject the samples without any calibrators. You will get a faster answer, but of course your risk of error will be greater. Under these conditions, a regular calibration check should be performed at regular intervals. For example, in a production facility operating 24 hours a day, the calibration could be checked at the beginning of each shift, or maybe once a day at the beginning of one designated shift.

Comments? Post them here!

In the next post, we will discuss other specific events that create a need for recalibration.

**Part 3 – Events That Mean Recalibration Is Required**

In the two previous posts we discussed general recommendations and guidance for routine method calibration. For well-designed methods and instruments, the regular use of check standards is the most efficient approach to regular method calibration. As we discussed, method recalibration is performed at regular intervals or when a check standard fails. In addition, there are other common events that require some level of recalibration immediately after the event.

The following situations represent activities that can have a direct impact on the observed chromatography and therefore should result in full recalibration.

New pre-mixed mobile phase: if you use a premixed mobile phase, the new solution may have a slightly different composition, resulting in retention time changes. Small changes in resolution and/or response are also possible.

New mobile phase solution: buffers and other additives are added to aqueous and organic solutions for a variety of chromatographic reasons. Usually the added components will adjust retention times, peak shape, or selectivity. Small changes in these solutions between batches can then be expected to affect the chromatography. Recalibration will help to adjust retention and responses as appropriate.

New column: as a column ages, you may see changes in retention, efficiency, peak shape, or selectivity. Your method should include some limits that tell you when to change the column, and the method must then be adjusted for the new column's performance.

Routine maintenance or repair: any changes to the physical instrument mandate method recalibration, since every module in the instrument contributes to the overall performance, which includes calibration. It should be obvious that modifying major components like the detector lamp, pump seals, or injector components could have an impact on the method. However, even small changes, like changing the tubing between modules, can have a significant impact on method performance if you do not use the same size (diameter and length) of tubing.

The following situations represent smaller changes in the system, and a full recalibration may or may not be necessary. However, I highly recommend that additional calibration checks be performed before beginning any analysis sets. If you are following the rules we have outlined in this series, then at a minimum you would be analyzing check standards at the beginning and end of the set anyway. The additional checks should be performed before beginning these formal analyses.

A different method is used on the instrument: most instruments are used for multiple methods. If the previous method used significantly different conditions or solutions, traces of those solutions can remain in the system for an extended time. Ion pairing methods are particularly troublesome in this regard, as the ion pairing agents often remain in the column for a long time, even with washing and cleaning. Many labs will use separate column in these situations. (Fortunately, ion pairing is not very common, having been replaced by HILIC options over the last 20 years.)

The instrument is powered off: in addition to the fact that some modules will need time to "warm up," a power cycle is a significant event that can have unexpected consequences. Turning off a detector lamp should probably be included in this category as well. Recalibrate.

The pump flow is stopped: there is some debate about this issue, and I am hesitant to include it here. In regulated laboratories, this event would generally require that the entire analysis begin again. This is a very conservative approach that minimizes problems, but it is quite wasteful and may not be necessary in every situation. In labs with wider specification ranges, one could argue that, as long as the instrument has been "on" and only the pump flow was interrupted, then there would be little risk of changes in the behavior of the system. Such an assumption will not be true in every case, and there are many examples where the next one or two injections are affected, until the system becomes fully stable again. If these next injections include your calibration checks, then your entire analysis set may be invalid. At a minimum, injection of an additional blank is recommended.

Alternatively, you could maintain pump flow, but at a much slower rate. Many systems are kept on at a flow rate of 0.1 mL/min. for extended time periods between samples. Mobile phase usage is much less under these conditions. Adjusting the flow to method conditions should result in faster equilibration. However, there are some column and detector combinations where this activity will result in an extended equilibration time due to baseline changes. The user must assess the relative merits of reduced solvent use against the time required to have the instrument available again.

I hope these posts have helped you think about what you do now for calibration and whether you might want to change what you do. We would love to hear examples and questions from your laboratory on this topic. Feel free to post them here are write to us directly at info@accta.com.

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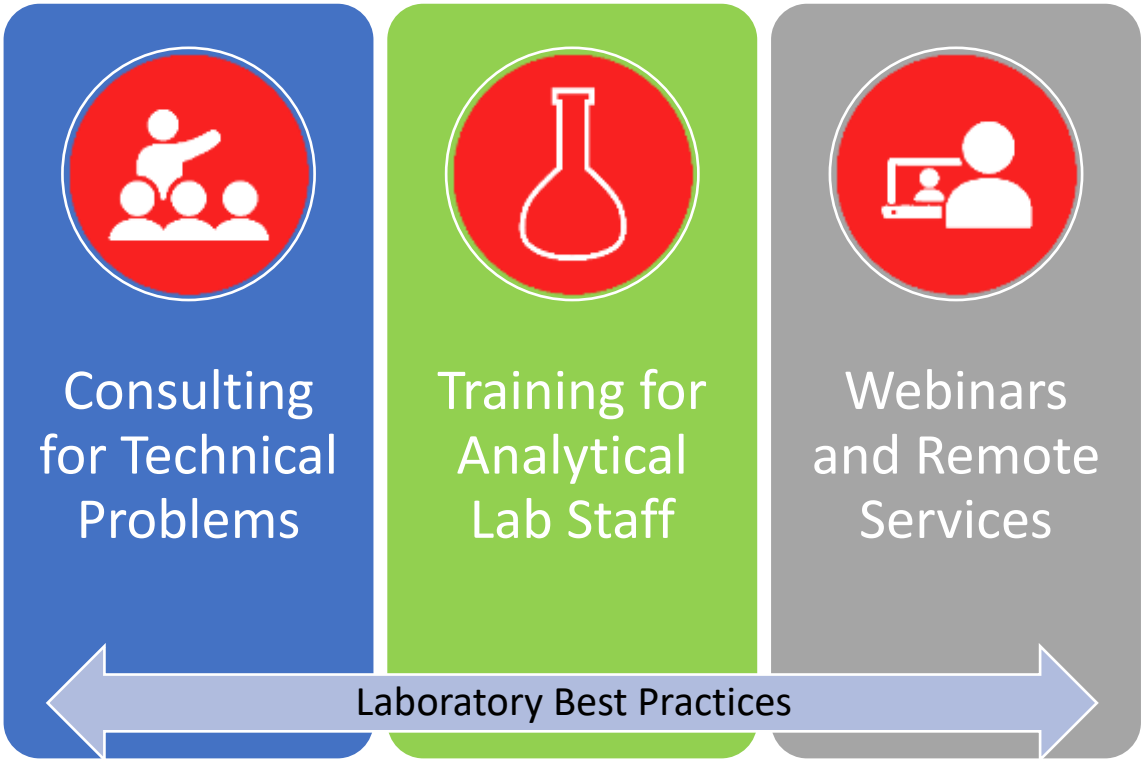


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Internet: www.accta.com

Email: info@accta.com

Phone: +1-651-731-3670