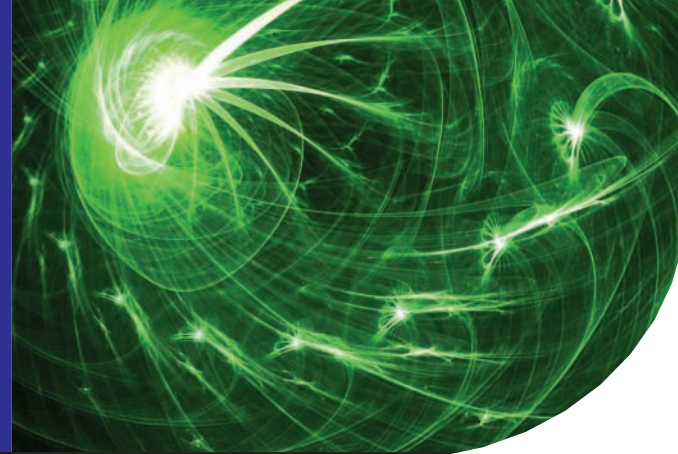


# Chromatography Focus



## Making an HPLC Calibration work (Part 1)

*Calibration of an HPLC method is necessary to give quantitative results. However there is more than type of calibration available, and each has its merits and limitations. The aim is to select the most appropriate one, and then to avoid the myriad of small pitfalls, because no-one wants almost the right answer!*

*“After the run, it is best to come back to the starting conditions over 5 minutes to give the column a snowflakes chance of keeping up with the solvent change, and to use at least 10 column volumes for re-equilibration at the starting conditions.”*

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### FIVE TYPES OF CALIBRATION

#### 1. External Standard Calibration

This involves analysing a series of standards covering the concentration range of interest. For example:

Level 1 - 40mg/l  
Level 2 - 70mg/l  
Level 3 - 100mg/l  
Level 4 - 130mg/l  
Level 5 - 160mg/l

The peak for each component is integrated and identified and the peak area is plotted against concentration to give a calibration curve (see below).

Each component should be present in the standard mix, and hence a series of calibration curves result from a single set of injections, one curve for each component.

When an unknown sample is run, the peaks are integrated and identified, and the peak areas are related to a concentration from the calibration graph.

If the calibration curve is a straight line, and it goes through zero, it is valid to use a single point calibration. For example, only the highest standard is run, and the line passes through this point and zero. If the points do not give a straight line, or the curve does not pass through zero, a multi-point calibration should be used, and normally five concentration levels are required.

Samples are run after the calibration standards, and hence it is recommended that known standards (QC Standards) are run periodically as unknowns to check the integrity of the calibration.

An external standard calibration works provided that there is no variation in injection volume, and that the HPLC conditions do not change during the run.

It is frequently asked if it is acceptable to use a single standard and inject a series of different volumes to construct the calibration. This is generally not acceptable. It is unusual for the accuracy and precision of the autosampler to be good enough, and since a constant volume of sample will be injected, the system should be calibrated in the same way.

#### 2. Internal Standard Calibration

An internal standard (IS) calibration is used to eliminate the error caused by variation in injection volume, and to compensate for losses during sample extraction. Injection volume variation is normally very small, but becomes more significant as the injection volume is reduced.

An internal standard is an additional component not naturally present in the sample. A fixed volume is added to all standards and all samples, and instead of plotting peak area against concentration for the calibration curve, we plot:

$$\frac{\text{Peak area of the sample}}{\text{Peak area of the internal Standard}} \text{ vs } \frac{\text{Concentration of the sample}}{\text{Concentration of the Internal Standard}}$$

The principle is that if a smaller or larger injection volume is used, the ratio of the peak areas will be the same, and hence the calibration is valid, even if injection volume errors occur.

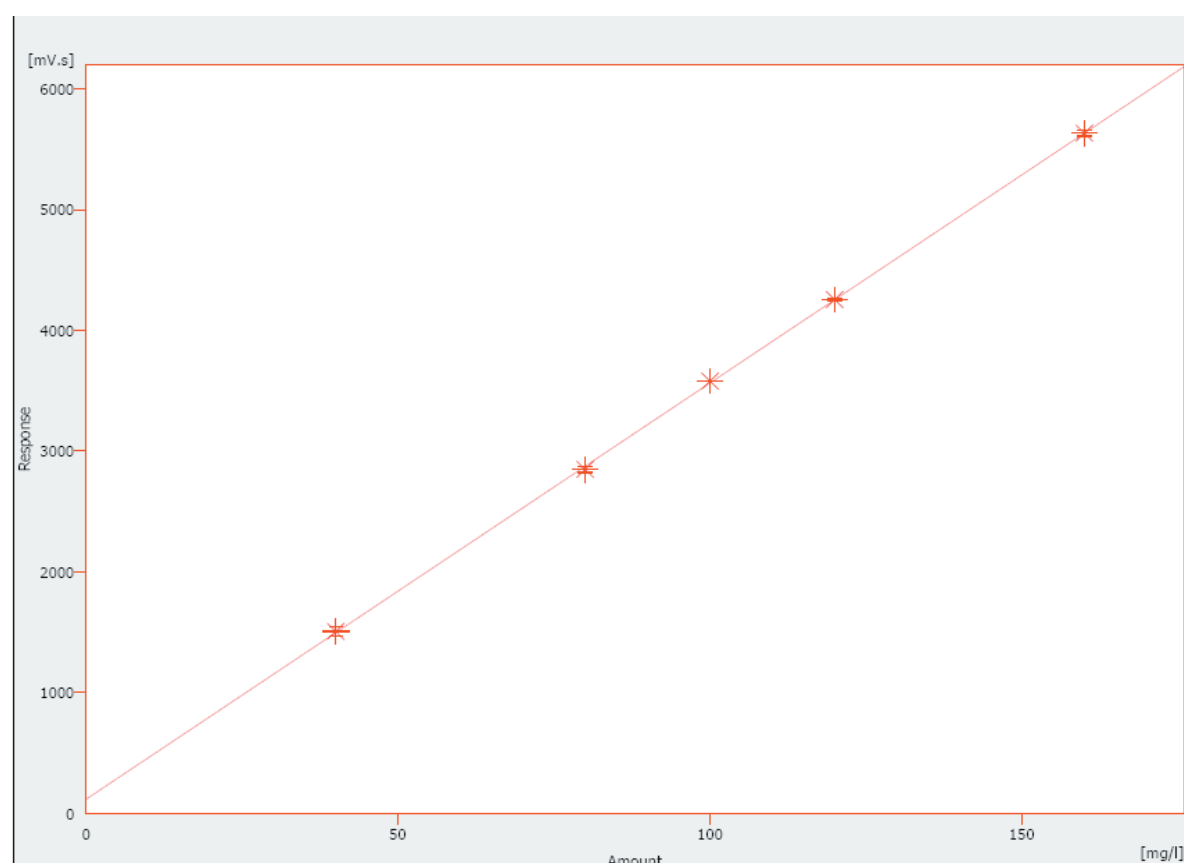
Selection of a material for use as the internal standard is very important.

- It should be a similar chemical species to the sample;
- It must be stable for long periods;
- It must absorb UV light at the same wavelength, if UV detection is to be used;
- It must be soluble in the same sample solvent and eluent systems; and
- It must not be a degradation product of the sample. For example, if analysing aspirin (acetyl salicylic acid) then salicylic acid would not be a good choice of IS.

It is also very important that a very precise volume of the internal standard can be added. Any variation will change the reported result.

When making up fresh internal standard, it is not necessary to achieve exactly the same concentration as before. However, it is necessary to re-run the calibration standards.

Using an internal standard, the same choice applies as before regarding using single point or multipoint calibration. To use a single point calibration, a straight line through zero is required. Otherwise we must use a multipoint calibration.



The concentration of internal standard that is chosen is not critical. However it is important to be able to integrate the peak easily with the integration conditions used for the samples and standards, so it is helpful if the chosen IS concentration is approximately midway between the highest and lowest calibration standard. So, for example, if the calibration range was 40-160mg/l, an internal standard concentration of about 100mg/l would be good. When running standards and samples, it is very important that the IS concentration entered into the data system is constant throughout, and many labs use a nominal value of 100. The number is not important, but the same number must be used every time.

When injecting small volumes, such as when using a microbore system, or when using a strong eluent as sample solvent, or when needed to get a straight line calibration, it is especially important to use an internal standard. The injection volume error from an autosampler will remain the same in volume terms, but this will be a much larger percentage error with a small injection volume of 1-2 $\mu$ l. Hence in these situations, it is important to select an internal standard calibration.

Another major advantage of internal standard calibration is when complex sample preparation is required, such as extraction from tissue such as blood, where sample losses can occur during extraction. The addition of the internal standard before the extraction allows losses during the extraction procedure to be excluded, assuming they affect the internal standard and the sample equally.

In some cases where the range of samples being analysed show some diversity, such as the analysis of amino acids, it is desirable to use two internal standards. This is because not all amino acids are affected in the same way by the sample preparation procedures. Using two different internal standards allows some amino acids to be calibrated with one and some with the other. Not all data systems can accommodate this.

The main threat to the internal standard method is the internal standard itself:

If a constant amount is not added to every sample and standard, an unnecessary error will be introduced;

If it degrades, the results will be too high;

If it is formed by sample degradation, the results will be too low; and

If it is not integrated correctly every time, errors will also occur.

For robust HPLC methods we recommend the internal standard method.

### 3. Area % Calibration

Quantitative results are obtained by adding together the areas of every peak in the chromatogram, and expressing each as a percentage of the total. The results are always given as a percentage, and its primary use is when a species has been purified, and a measure of its purity is required. In this situation, we expect one large peak and a few small ones. The purity of the large peak can be read directly from the integration results using Area %. The concentration of the injected sample is not critical – we require only that the main peak is about 90% of full scale to maximise the size of the smaller peaks for ease of integration.

Whilst a simple method, in that there are no standards to make up or run, Area% analysis requires careful preparation. For the results to be valid, all peaks must:

- Absorb UV at the same wavelength;
- Absorb with the same extinction coefficient;
- Be separated from the main peak;
- Be separated from all ghost peaks; and
- Elute from the column after the solvent front and before the end of the run.

This is quite a big ask! Why should all impurities comply with these criteria? So at best it is something of an approximation. However, it is widely used, especially amongst chemical manufacturers wishing to measure the purity of their product.

To be sure that all peaks elute from the column, and after the solvent front, it is normal to run a gradient. Ideally the gradient composition range should be as narrow as possible, starting from the highest %B (the % organic in the eluent) that will allow the first peak to elute after the solvent front, and running to the

lowest %B that will elute all peaks from the column. Unfortunately, many users simply specify 0-100% acetonitrile, and often over a very short period such as 15 minutes. Such a steep gradient is likely to result in co-elution, possibly with the main peak of interest, either from impurities or ghost peaks.

For these methods we recommend either reducing the %B range or extending the run time to maybe 30 minutes. After the run, it is best to come back to the starting conditions over 5 minutes to give the column a snowflakes chance of keeping up with the solvent change, and to use at least 10 column volumes for re-equilibration at the starting conditions. The volume of an empty column is given by  $\pi r^2 l$ , ( $r$  = column radius,  $l$  = column length) remembering to use cm as the units for  $r$  and  $l$  so that the volume is in ml. The volume of a packed column is about half the empty volume.

The problem with running a gradient is that ghost peaks elute, and the baseline is no longer flat. Ghost peaks can be seen by running the gradient without injecting a sample. They are usually due to impurities in the water, which retain on the column until the eluent becomes strong enough to elute them, when they appear as peaks. It is very important to identify the ghost peaks, because they must not be included in the Area % calculation. Bear in mind that their peak height will change depending on the length of time used for re-equilibration. For example, after leaving a system equilibrating over lunch time, much larger ghost peaks will result. It is essential that no ghost peaks co-elute with any of the peaks of interest in the chromatogram if Area% is to be used. If they do, try water from another source, use methanol instead of acetonitrile, use a different column, or change the gradient composition range or run time.

When using Area% calibration, it is necessary to run a dry blank (no injection) repeatedly until a reproducible baseline is achieved. Then run a solvent blank (inject sample solvent but no sample) to check no ghost peaks arise from the sample solvent.

It is very hard for two labs to get the same results using Area%, not least because they are likely to have different ghost peaks in their chromatograms. In the event of a dispute, look at the chromatogram, zooming in on the baseline, and check which peaks are being included and which were excluded as ghost peaks. Since a gradient baseline is rarely flat, check the integration tick marks and baselines are in the right places.

### 4. Calibration by Standard Addition

There are times when it is hard to obtain a blank which does not contain any of the sample component being analysed, such as when analysing low levels of sodium in water. There are also situations where the sample matrix may contain certain components which may affect the measured concentration, such as solutions containing formaldehyde. In these situations, using an external standard calibration may lead to errors and it may be better to consider standard addition.

A standard addition calibration consists of adding a known amount of the species of interest to the sample, and measuring the increase in detector response. Equating the increase in detector response to the amount of sample added, and assuming linearity of response, we can calculate the original concentration in the sample:

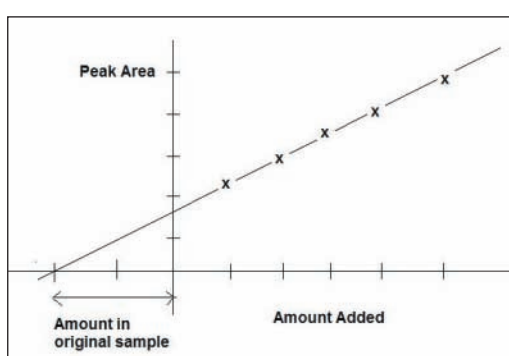
$$\text{Concentration in original sample, } O = \frac{SA \times A^{OS}}{(A^{SA} - A^{OS})}$$

Where SA = Amount of standard added

$A^{OS}$  = Area of peak without the standard addition

$A^{SA}$  = Area of peak after the standard addition

To check for linearity of response, a series of standard additions can be performed. These are then plotted as peak area vs amount added. The line will therefore cross the y axis at the area of the original sample, and the concentration in the original sample will be the intercept with the x axis, but with polarity reversed.



The main advantage of the standard addition procedure is that standards can be analysed in real matrices and not in clean chromatograms with only a few peaks. If required, the standard addition procedure can be used in combination with an internal standard.

### 5. Calibration using a Correlation Factor

The principle is that if it is possible to establish that a linear calibration response is achieved and that the line passes through zero, then the equation for the line is:

$$y = ax^2$$

For example, there is a fixed correlation factor relating  $x$  and  $y$  values. Running a single standard concentration we can calculate this correlation factor, and then apply this to all sample peak areas to obtain concentration values.

To use this method, it is essential to confirm linearity by running a series of dilutions which must include a zero blank. Thereafter, it is necessary only to run the single standard periodically during the sequence to confirm that the correlation factor remains constant.

When operating with a modern data system, this method offers little advantage over single point external standard calibration. However when data is exported for subsequent computation, for example in a spreadsheet, then this method is useful.

#### Should the calibration curve be a straight line?

If the detector gives a linear response to concentration, we would expect to get a straight line. However sometimes it becomes clear that the points fall on a curve rather than a straight line. We then have to decide what to do about it.

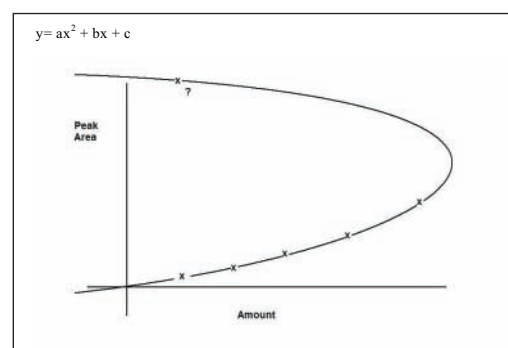
#### Option 1. Accept that the response is non-linear and use a curve.

This tends to happen when a wide concentration range is being used for the calibration. A non-linear response is also more common with some older HPLC detectors. If the data points almost fall on a straight line, it may be possible to use a line and still get reasonable correlation to the line. However if the lowest and highest data points fall on one side of the line, and the intermediate points fall on the other side, a curve will inevitably give a better fit, and hence a more accurate calibration.

If accepting a curve, there are several options available.

A quadratic equation gives rise to a parabola:

$$y = ax^2 + bx + c$$



We won't know what part of the parabola we are using. The data system simply finds values for  $a$ ,  $b$  and  $c$  in the equation above such that the curve passes through all our data points with the best fit possible. A quadratic equation is probably the safest curve to use.

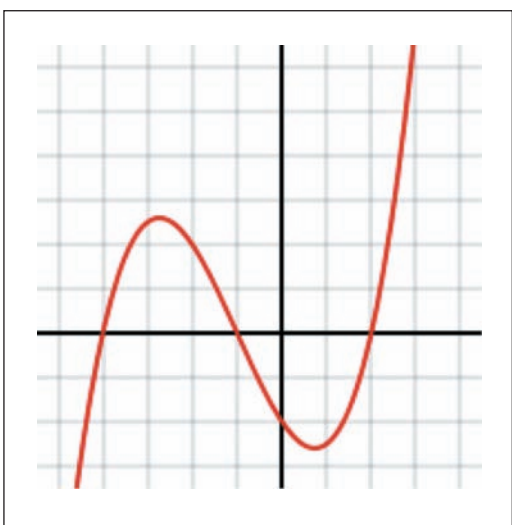
However there is a danger that if the U-shaped curve is orientated as in the diagram above, there are potentially two peak areas (on the vertical axis), which can give rise to the same concentration value.

So if a much higher concentration were found in an unknown sample, and the analyst was not looking carefully, it would get reported as a normal result when in fact it might be 100 times too concentrated.

This is a lab manager's nightmare, so if we are to accept a quadratic curve, we need to be sure that this cannot occur. If the biggest peak in our calibration almost goes off scale, it is probably safe to use a quadratic curve. But if not, there is a risk, even though it gives the best fit to the data.

A cubic curve has an S-shape:

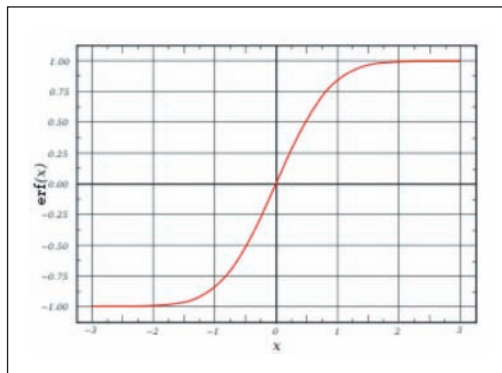
$$Y = ax^3 + bx^2 + cx + d$$



A cubic curve is asking for trouble. Even if one of the data points was a blank, you may still be able to get a curve to fit. My recommendation is not to use one, and if you do, to be very very careful.

A sigmoid curve also gives an S-shape, but one which looks more like a pKa plot:

$$y = \frac{1}{1 + e^{-x}}$$



If we get calibration data which fits a sigmoid curve, the concentration range is really far too wide.

The flat section at the top and bottom represent the points where we are above and below the limit of detection. So rather than use this, a much narrower concentration range should be used.

**Option 2. Don't accept the curve and make changes to give a straight line calibration**

Question is, what changes can we make! There are three options:

**1. An extra dilution step**

If a range of 20-200mg/l proves to be too wide to get a straight line, a 1:10 dilution step will result in a much narrower range (2-20mg/l) which may well give a straight line response. Be aware that as the dilution factor increases, small errors in sample preparation become amplified.

**2. Smaller injection volumes**

If we were injecting 20ul, try injecting 2ul instead. The result will be the same as for option 1, but it eliminates the additional dilution step. However, the injection accuracy and precision will be worse, so an internal standard calibration would then be required.

**3. Plot the log of the peak area against the log of the concentration.**

Using the log function is often the answer to all known curves! It doesn't require a dilution step or an internal standard. But it can make the calibration less sensitive to small changes in concentration.

Part 2 will appear in ILM October/November 2009

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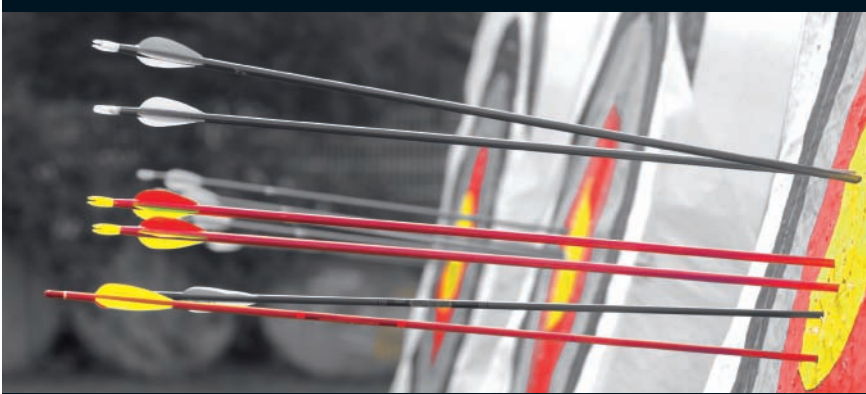
Hichrom also offer a free technical help desk, manned by experienced chromatographers, who will help you specify, identify and source the LC accessory that you need for your application. The help desk can be contacted on +44(0) 118 930 3660 or by email at: [technical@hichrom.co.uk](mailto:technical@hichrom.co.uk).




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## From Technology Overview to Focused Applications

Malvern Instruments online web seminar series introducing triple detection techniques for size exclusion chromatography/gel permeation chromatography (SEC/GPC) provides information for experts and beginners alike.

The series on SEC/GPC triple detection, which has become the preferred method for characterising natural and synthetic polymers and proteins, kicked off on 16th July with a basic overview. It continues monthly for the rest of the year with specific application examples covering both protein and polymer characterisation.

The web seminars are presented by technical and applications specialists from Viscotek, a Malvern company, and registration is free to worldwide audiences at <http://www.malvern.com/webinar>

Gel Permeation Chromatography (GPC), also known as Size Exclusion Chromatography (SEC), is a technique that employs specialised columns to separate natural and synthetic polymers, biopolymers and nanoparticles on the basis of size. As the sample is separated and elutes from the column, it can be characterised by a single concentration detector or series of detectors.

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Malvern's series of more than 120 web seminars in 2009 covers many different aspects of the company's technologies and products for materials characterisation.

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