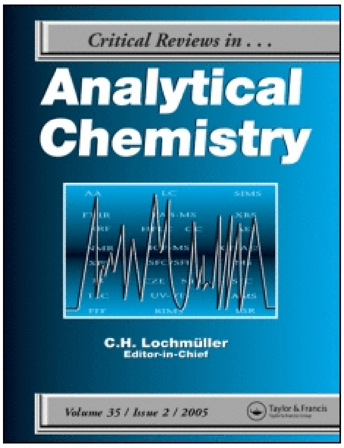

Critical Reviews in Analytical Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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To cite this Article Hewavitharana, A. K.(2009) 'Internal Standard—Friend or Foe?', Critical Reviews in Analytical Chemistry, 39: 4, 272 — 275
To link to this Article: DOI: 10.1080/10408340903001201
URL: http://dx.doi.org/10.1080/10408340903001201

Critical Reviews in Analytical Chemistry, 39:272–275, 2009
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ISSN: 1040-8347 print / 1547-6510 online
DOI: 10.1080/10408340903001201

Internal Standard—Friend or Foe?

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Internal standardization is a calibration technique that is commonly used to correct for procedural errors associated with quantitative analysis, thereby improving the accuracy of data. The commonly used protocol for internal standardization, presented through all analytical chemistry text books, relies upon a single point calibration for the compound that is used as the internal standard. Therefore, if the analyst is unaware of the linear range of the internal standard and uses a concentration that is outside the linear range, a severe loss of accuracy will occur. The severity is demonstrated through an example and precautions to prevent this problem are suggested. An alternative protocol for internal standardization that does not rely upon a single point calibration is also presented.

Keywords Internal standard, single-point calibration, ion suppression, electro-spray MS

The term "internal standard" has always been associated with the terms "accuracy" and "precision" in analysis. In theory, the internal standard method improves both the accuracy and the precision of analytical measurements. However, the internal standard protocol that is taught through typical analytical chemistry text books (1, 2), which in turn is commonly used in analytical laboratories, is prone to error—error that could reduce the accuracy rather than improve it.

Let us look at the theory behind the internal standard methodology:

We add a known amount of internal standard to the sample at the earliest possible step of the analytical procedure. Then we measure the concentration of the internal standard as well as that of the analyte at the final measurement step. The internal standard was chosen to mimic the analyte so that any change to the analyte is reflected in change to the internal standard. With mass spectrometric detection, a stable isotope labelled (SIL) analogue of the analyte molecule is often used as the internal standard and with other detection methods a structural analogue of the analyte such as an isomer has been the common choice. An increase or decrease in the concentration of internal standard (relative to the expected concentration, calculated based on the known initial amount added at the beginning of the analytical procedure) at the final measurement step implies a similar increase or decrease to the concentration of the analyte. Therefore, one would simply multiply the measured concentration of the analyte by the ratio of expected concentration of internal standard/observed concentration of internal standard at the final measurement step. Thus, the concentration of analyte has been corrected for any mishaps such as spillage or volumetric errors that occurred during the course of extraction and instrumental analysis.

Let us look at a couple of possible protocols that could be followed to achieve the internal standardisation described above:

- One could run two sets of standards: one that contains various concentrations of internal standard and the other containing various concentrations of the analyte. This would produce two separate calibration curves: one for the analyte and the other for the internal standard, covering the range of concentrations expected in samples. The final concentrations of the analyte and the internal standard in the sample could then be calculated separately, using their respective calibration curves. These final (observed) concentrations (along with the expected or the added concentration of the internal standard) will then be used in the internal standardization calculation described above.
- The second protocol, which is the protocol taught through text books, therefore commonly used in analytical and clinical laboratories, is: to run a set of standards containing various concentrations of analyte but a single concentration of the internal standard. The range of concentrations of the analyte used would cover the range expected in samples while the single concentration of the internal standard is chosen by the analyst and this is the same internal standard concentration

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> that is added to all samples. There are no warnings or guidelines available for the analyst to choose this single concentration and the loss of accuracy due to the use of wrong concentration (as described below) may not be detected in method validation step and beyond. The error added to each sample analyzed will become severe as the difference between the added and the observed concentrations of the internal standard increases.

In this case, the calibration curve plotted is the ratio of responses (analyte/internal standard) vs. the ratio of concentrations (analyte/internal standard) or the concentration of analyte alone (1, 2). The final concentration of the analyte is calculated based on this ratio plot in which case the final concentration has already been corrected for procedural errors such as spillage and volumetric measurements.

Many analysts are unaware of the fact that there is an assumption built in to the second protocol, i.e., *that the calibration curve of the internal standard is linear within the whole range of expected concentrations of the internal standard in all samples*. This means that the concentration of the internal standard in each sample is calculated based on a single-point calibration curve. This is the fundamental flaw in this protocol which leads to serious consequences.

The choice of this single point is completely arbitrary. An analyst may choose it to be the same as the mid-point concentration of the analyte calibration or a concentration of the internal standard that produces a similar detector response to that of the mid-point concentration of the analyte. As there are no guidelines [through text books or through organizations such as International Standards Organization (ISO) (3) or Food and Drug Administration (FDA) (4)] regarding the choice of this single point, it is completely up to the analyst to decide this concentration. Further, there is no warning or recommendation (the analytical chemistry text books and the FDA guidelines included) prompting the analyst to check the linearity of the internal standard calibration around this single point before this concentration is chosen. There is a very real chance, therefore, that this single point lies within a non-linear region of the detector response curve. If this is the case, following the second protocol would reduce the accuracy rather than improve it.

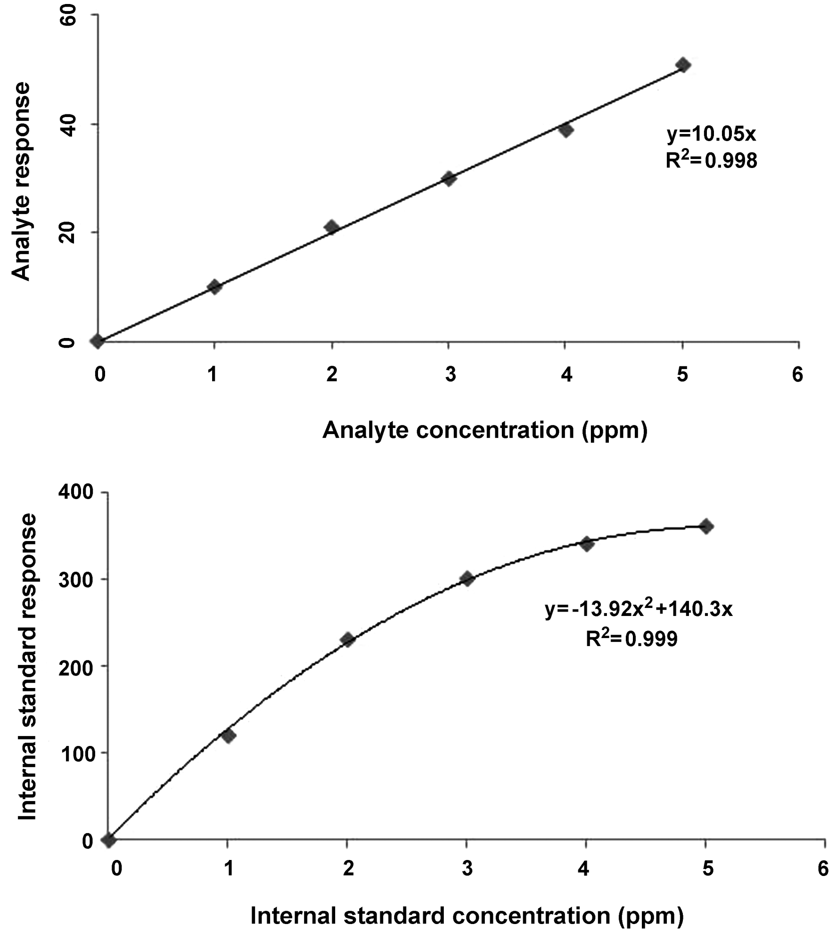

FIG. 1. Calibration plots for the analyte (top) and the internal standard (bottom) obtained for internal standardization protocol 1.

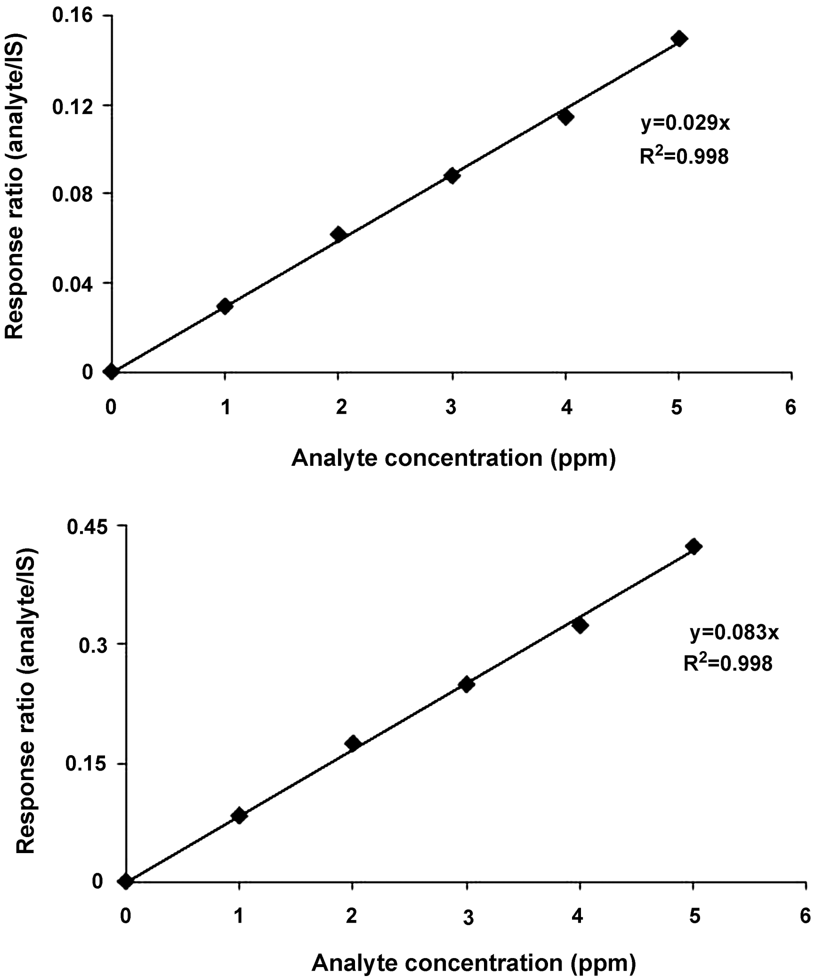

FIG. 2. Calibration plots for an internal standard concentration of 4 ppm (top) and 1 ppm (bottom) obtained for internal standardization protocol 2.

In order to appreciate the effect of this error on the accuracy let us consider a hypothetical example:

Case 1—two sets of calibration standards were prepared with varying concentrations of analyte and varying concentrations of internal standard for Protocol 1.

Case 2—One set of calibration standards was prepared with varying concentrations of analyte and a single concentration (4 ppm) of internal standard for Protocol 2.

In both cases a fixed amount of internal standard was added to all samples so that the concentration of internal standard in all samples was 4 ppm.

Figure 1 shows two separate calibration plots obtained with Protocol 1, and Figure 2 (top diagram) shows the ratio plot (Analyte response/Internal standard response vs. Analyte concentration) obtained with Protocol 1 when the internal standard concentration in all standard solutions was 4 ppm.

Now consider a sample for which the responses obtained were 25 and 350 for analyte and internal standard, respectively.

Protocol 1 gives an analyte concentration of 2.49 ppm and an internal standard concentration of 4.54 ppm. Therefore, the corrected concentration of analyte in the sample, calculated using protocol 1, is 2.19 ppm.

The ratio of responses (analyte/Internal standard) for this sample is 0.0714 so the correct concentration of analyte in the sample, calculated using Protocol 2 (Fig. 2, top diagram), is 2.46 ppm. Therefore, the error associated with the use of Protocol 2 (ratio method) is 12%.

When repeating the same with a lower concentration of internal standard added, 1 ppm instead of 4 ppm (in all samples and standards) so that the concentration is within an approximately linear region of the internal standard calibration (Fig. 1, bottom diagram), a sample with an analyte response of 25 and internal standard response of 100 gives the corrected analyte concentrations: 3.22 ppm (Protocol 1) and 3.01 ppm (Protocol 2—using Fig. 2, bottom diagram). The error associated with ratio method in this case is 6.5%.

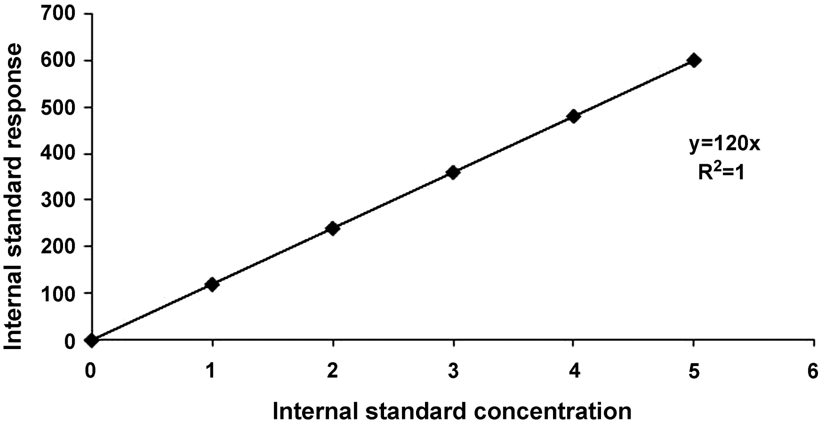

FIG. 3. Calibration plot of an internal standard with a wide linear range.

Note that the internal standard response for this sample (100) is only slightly different from the expected response of 120, suggesting a minor change to the sample during extraction and analysis. Even with this minor change and using a concentration of internal standard within a nearly linear calibration range, the error is significantly high. This shows the danger of using a single point calibration for the internal standard even when the internal standard concentration is only slightly different from the ideal and in an almost linear region of calibration.

Now let us consider what would have happened if the internal standard response is perfectly linear above and below the fixed concentration used in the samples and standards (i.e., the bottom diagram of Fig. 1 becomes Fig. 3):

In this case, the same sample (with an analyte response of 25 and internal standard response of 100) gives the corrected analyte concentrations: 2.98 ppm (Protocol 1) and 3.01 ppm (Protocol 2). The error associated with ratio method in this case is only 1.0%.

This example clearly demonstrates the importance of ensuring the linearity of internal standard calibration when using Protocol 2. Even a slight deviation from linearity can cause an error as high as 6.5%.

As most of us analysts who have been involved with complex extraction procedures and/or instrumental techniques such as mass spectrometry know, the range of concentrations of internal standard observed in a batch of samples is not always narrow. Use of a single point calibration, therefore, is not good laboratory practice (GLP). The severity of this problem is most prominent with the increasingly popular detection method, electro-spray mass spectromety where the ion suppression effects caused by the sample matrix differ widely from sample to sample. This gives rise to a wide range of detector responses for the internal standard. As the SIL internal standards are heavily relied upon to correct for these ion suppression effects, relying on a single point calibration to calculate the observed internal standard concentration has dire consequences.

The first protocol, rarely followed by analytical laboratories, is not based on an assumption such as the second so it will produce an accurate value for the concentration of the internal standard in each sample, thereby improving the accuracy of the analyte concentration obtained for each sample. If one were to use (the commonly used) Protocol 2, the only way to obtain data as accurate as Protocol 1, is to ensure linearity around the chosen single point. The linearity should be ensured for higher as well as lower concentrations around the point: with the commonly used sample extraction procedures in biological samples where protein precipitation follows a drying and reconstitution step, the concentration of the internal standard can be higher than expected; therefore, the observed concentration may be above the single point chosen.

It is time, therefore, in the advent of complex sample extraction procedures and the ion-suppression complications encountered in techniques such as liquid chromatography-electro spray mass spectrometry, to prompt analysts to check the linearity of their internal standard calibration (around the concentration added to all samples and standards) to ensure that the assumption made in the commonly used Protocol 2 is valid. This could be added as part of a method validation protocol administered through guidelines such as FDA and ISO (3, 4). This will ensure that the internal standard works for, rather than against, the analyst.

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