Internal Standard calculations for non-linear detectors

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Abstract

Internal Standard (ISTD) is a well-known chromatographic technique, aimed to compensate sample size variations, where known amount of a component, called internal standard is added to both standard and unknown samples. The classic Internal Standard quantification method plots the response ratio (analyte to *internal standard*) versus amount ratio (again analyte to *internal standard*). *Internal standard* component itself does not have any calibration curve. Quantification procedure uses this plot to get concentration ratio from response ratio.

We demonstrate here, that this approach may cause systematic errors in the case of non-(linear through origin) detector response to concentration of analyte or *internal standard*. We also offer an alternative calculation scheme allowing wide variations of standard and analyte concentrations and non-(linear through origin) calibrations of components. In the case of non-directly proportional calibrations it requires that External standard dependencies of both *internal standard* component and analyte are measured.

Offered scheme of Internal standard calculations splits into two independent parts:

1. Calculation of Relative concentration, i.e. concentration of analyte, provided concentration of Internal Standard is known, using External Standard calibration curves.
2. Constuction of improved calibration curves (Universal calibration) that can be simplified for the case of linear through origin dependencies.

We provide a proof, that the classic response ratio scheme for linear through origin calibration is a particular case of the presented approach.

The described calculation scheme is successfully used for Internal Standard calculations in Chrom&Spec chromatographic software for more than 15 years.

Calibration

Predictive relationship between input and detector response

- Input: calibration samples – concentrations of components
- Output: peak area or height
- Prediction: Predict unknown input looking at response

ESTD Calibration

- Response (Area or Height) versus Quantity
- Quantity is provided without error - false
- Response is measured with random normally distributed error – sometimes true

External Standard Calibration Curve

- Axes: Q – Quantity (NOT Concentration), R – Response (Area or Height)
- Independent variable: Typically Q, sometimes R
- Calibration curve: polynomial interpolation
- Prediction: either solution of polynomial equation (independent Q) or value of polynomial (independent R) – we denote either of them W(R)

Quantification:
External Standard (raw) Concentration

- Quantity of injected substance

$$Q_x = W(R_x)$$

- Concentration of initial sample

$$C_x = Q_x/V = W(R_x)/(V_{inj})$$

- V_{inj} – injection volume

ISTD Targets

Reason	*Axis*
• Sample-size variations	Q
• Effect of sample preparations	Q
• Instrument drift	R

All reasons are always acting together

ISTD tricks

- Add component with the known concentration to the analyzed sample
- Add component with the known concentration to the calibration samples

“Classic” ISTD

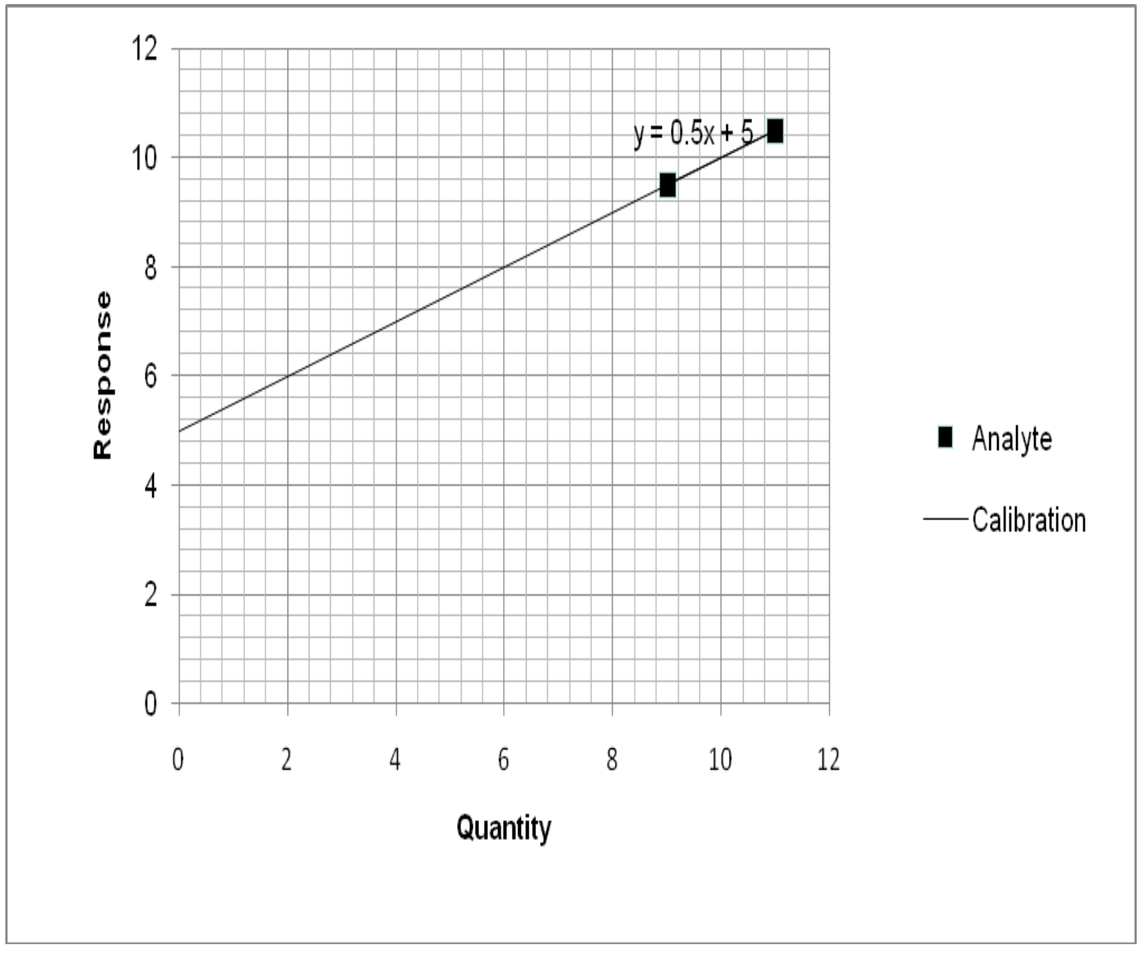
- *Coordinates:* **Response Ratio vs. Concentration ratio**
- *Calibration curve:* **polynomial, typically straight line through the origin**
- *Prediction:* **from the Response Ratio predict the Concentration Ratio**
- *Peculiarities:* **no calibration curve for Internal Standard component**
- *When it works properly:* **(ESTD) $Q=C*V=kR^{\alpha}$ with identical α for all components, α=1 being the most often case, then $C_a/C_s = (k_a/k_s)*(R_a/R_s)^{\alpha}$**. The case of $\alpha \neq 1$ can be linearized by setting $R'=R^{1/\alpha}$
- *When it works poorly:* **in most of the other cases**

Example of “Classic” ISTD Failure

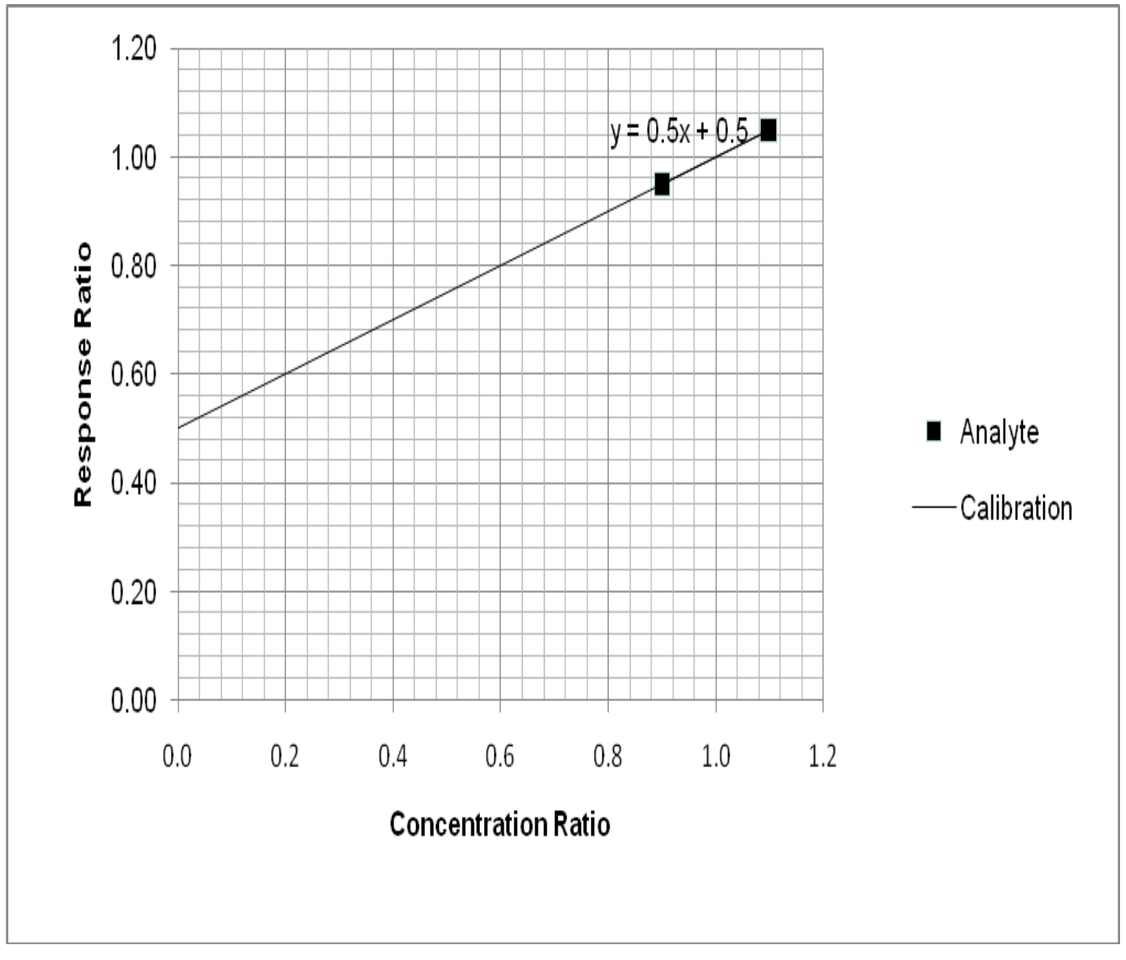
Sample	C_s	C_a	Loss, %	Q_s	Q_a	R_s	R_a	R_a/R_s	C_a/C_s	Error, %
Calibration point 1	1	0.9	0	10	9	10	9.5	0.95	0.9	0
Calibration point 2	1	1.1	0	10	11	10	10.5	1.05	1.1	0
Test analysis (calculated)	1	1	9	9.1	9.1	9.1	9.55	1.049	1.099	9.9
Volume	10									

This simple artificial example demonstrates that the aim (compensation of the sample size variability), declared by classic ISTD calibration, is not achieved in this case. In the example ESTD calibration of the Standard component is accepted to be linear through origin, K=1 (graph not shown); calibration of Analyte linear within calibrated region, both (ESTD and classic ISTD) Analyte calibrations shown below. 9% loss of sample amount results in 9.9% error in concentration, evaluated by classic ISTD scheme. All values are within calibrated range for both calibrations. Relative concentration calculations, described below, provide precise result.

ESTD Calibration of Analyte



"Classic" ISTD Calibration of Analyte



“True” ISTD step 1:
Relative Concentration

- Accounts for systematic error due to the sample-size error and the sample loss during preparation.
- Assumption: the volume is unknown and is calculated using a known concentration of the internal standard

$$V = Q_{xistd}/C_{xistd} = W_{istd}(R_{xistd})/C_{xistd},$$

- Q_{xistd} is calculated using the calibration curve of Internal standard from R_{xistd}, C_{xistd} – declared concentration of standard in the sample
- Relative Concentration

$$C = Q_x/V = C_{xistd} \times W_x(R_x)/ W_{istd}(R_{xistd})$$

- Calculations for the above example:

$$C = Qa/V = C_s \times Q_a/Q_s = 1.0$$

“True” ISTD step 2:
Universal Calibration

- If the calibration is nonlinear, we must measure this nonlinearity, i.e. we MUST know ESTD calibration curve of ISTD component
- In the case we somehow learned this curve, we can use it to change the positions of calibration points of other components using the same trick as was used while calculating the Relative Concentration**:**

$$Q_n = C_n \times V = C_n \times W_{istd}(R_{istd})/C_{istd}$$

- If the Internal Standard predefined curve is in use, all other components get curves constructed conditionally, condition being the known calibration curve of the Internal Standard component
- Point N of the Standard calibration graph is used to calculate the “correction coefficient” for point N of all other components K=V/Va; typically, Universal calibration curves of analytes have better RSD than original External Standard Calibration curves

$$Q_n = C_n \times V = C_n \times W_{istd}(R_{istd})/C_{istd}$$

- Multiplication of Q axis of Internal Standard to any number will multiply Q coordinates of all corrected points of all components to the same number, hence causing an “affinity” change of all calibration curves. The calibration curve will change, as well as the absolute concentration, but not the Relative Concentration

$$C = C_{xistd} \times W_x(R_x)/\ W_{istd}(R_{xistd})$$

- If all calibration dependencies are linear through origin
  Q = K×R, it is possible to select a multiplication factor so that $K_{istd} = 1$ and we will get the relative response factors for all the other components (Simple Universal Calibration).

	Simple Universal Calibration	Response Ratio
Axis X	$R_s * C_a / C_s$	C_a / C_s
Axis Y	R_a	R_a / R_s
Direct proportionality coefficient Y=KX	$K = \frac{\sum_i X_i Y_i}{\sum_i X_i^2} = \frac{\sum_i R_{si} R_{ai} \frac{C_{ai}}{C_{si}}}{\sum_i \left(R_{si} \frac{C_{ai}}{C_{si}} \right)^2}$	$K = \frac{\sum_i X_i Y_i}{\sum_i X_i^2} = \frac{\sum_i \frac{R_{ai}}{R_{si}} \frac{C_{ai}}{C_{si}}}{\sum_i \left(\frac{C_{ai}}{C_{si}} \right)^2}$
Quantification formula	$C_a = \frac{1}{K} C_s \frac{R_a}{R_s}$	
Weighted regression coefficient	$K = \frac{\sum_i w_i X_i Y_i}{\sum_i w_i X_i^2} = \frac{\sum_i \frac{R_{ai}}{R_{si}} \frac{C_{ai}}{C_{si}}}{\sum_i \left(\frac{C_{ai}}{C_{si}} \right)^2}; w = \frac{1}{R_{si}^2}$	

Quantification formulas for both methods coincide, and coefficients become identical in the case of weighted Simple Universal Calibration. The only case where the Response Ratio Calibration works properly is a particular case of the Simple Universal Calibration!

Full Universal Calibration

Advantages:

- Only one type of axes
- Calibration curves are suitable for the calculation of both Absolute and Relative concentrations

Disadvantages:

- ESTD Calibration curve of Internal Standard is required
- Recalibration has to be made as often as for ESTD calibration

Device Drift

- Drift model: $R = K \times F(Q)$
- Drift can be compensated completely, if exists such a k, that

$$K \times F(Q) = F(k \times Q)$$

- Particular case: linear through origin calibration; K = k
- “Classic” scheme can completely compensate device drift in the case of exact injected amount even for nonlinear calibrations!

Discussion

Classic Internal Standard method was established in the early days of chromatography [1, 2], when detector stability was not good enough. It perfectly compensates detector sensitivity changes, but using this method for variations of quantity may lead to problems. The difference between these two cases is straightforward: for improved calibrations in the case of detector sensitivity change we should move the points along the Response axis, and when injected quantity is varied, we should move the calibration points along Quantity axis. In the case of linear through origin calibrations these two ways of correction may give similar final results, but for nonlinear calibration each case deserves its own math.

Conclusions

- ISTD is split into two parts: ISTD quantification (Relative Concentration) and Universal ISTD Calibration. Parts can be applied separately.
- Relative Concentration is applicable for both ESTD and Universal ISTD calibrations.
- Full Universal ISTD calibration can be used for both ESTD and ISTD calculations.
- Simple Universal ISTD calibration can be used in the case of linear through origin calibrations instead of “Classic” ISTD calibration including imitation of user interface.
- Full “Universal” ISTD solution still works where “Classic” already fails, i.e. it allows to get into the account wide concentration range of Internal Standard in the case of nonlinear calibrations.

Difficulties

- User habits. The more people are used to the “Classic” ISTD method, the more difficult it is to change their minds. Typical argument: -“The old approach works. We typically use linear through origin calibrations. Why should we change the way we work?”.
- Formal documents. Pharmocopoeias state that Internal Standard method is implemented by Response Ratio method.

References

1. N.H.Ray. J.Appl.Chem., 1954, v.4, p.21
2. R.W.Yost, L.S.Ettre, R.D.Conlon. Practical Liquid Chromatography. An Introduction. Perkin-Elmer, 1980

EU Pharmacopoeia

- *External standard method.* The concentration of the component(s) to be analysed is determined by comparing the response(s) (peak(s)) obtained with the test solution to the response(s) (peak(s)) obtained with a reference solution.
- *Internal standard method*. Equal amounts of a component that is resolved from the substance to be examined (the internal standard) is introduced into the test solution and a reference solution. The internal standard should not react with the substance to be examined; it must be stable and must not contain impurities with a retention time similar to that of the substance to be examined. The concentration of the substance to be examined is determined by comparing the **ratio of the peak areas or peak heights** due to the substance to be examined and the internal standard in the test solution with the **ratio of the peak areas or peak heights** due to the substance to be examined and the internal standard in the reference solution.
- *Calibration procedure.* The relationship between the measured or evaluated signal (y) and the amount (concentration, mass, etc.) of substance (x) is determined and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte by means of the inverse function.

US Pharmacopoeia

- Reliable quantitative results are obtained by external calibration if automatic injectors or autosamplers are used. This method involves direct comparison of the peak responses obtained by separately chromatographing the test and reference standard solutions. If syringe injection, which is irreproducible at the high pressures involved, must be used, better quantitative results are obtained by the internal calibration procedure where a known amount of a noninterfering compound, the internal standard, is added to the test and reference standard solutions, and **the ratios of peak responses of drug and internal standard** are compared.
- Assays require quantitative comparison of one chromatogram with another. A major source of error is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe. The effects of variability can be minimized by addition of an internal standard, a noninterfering compound present at the same concentration in test and standard solutions. The **ratio of peak response of the analyte to that of the internal standard** is compared from one chromatogram to another.