Internal Standard without response ratio?

Yuri Kalambet,

Ampersand International Inc., Beachwood, OH

Yuri Kozmin

Shemyakin Institute of Bioorganic Chemistry, Moscow

Sergey Maltsev

Ampersand Ltd., Moscow;

kalambet@ampersand-intl.com
www.chromandspec.com

Abstract

An invitation to discussion is based on alternative way to account for known concentrations of one of analytes (Internal Standard) in a mixture. Alternative procedure is based on Relative Concentration calculations that can be applied to components, calibrated by External Standard Calibration procedure. Features of the new procedure:

- simple, not much more complicated than traditional Internal Standard Calibration procedure;
- has explicit calibration curve of Internal Standard;
- both External and Relative concentrations can be reported in the same peak table;
- applicable to nonlinear detectors and in wide range of concentrations of Internal Standard component;
- good error traceability;
- in the case of exact measurements results can be proven to be identical to those obtained by traditional Internal Standard Calibration procedure.

Traditional Internal Standard Calibration procedure [1-3], plotting response ratio versus concentration ratio, is formalized in multiple documents and books. The problem is in the name and formal (legal) position of the new procedure. Its aim is the same, as in traditional Internal Standard Calibration procedure: improving accuracy and reproducibility of chemical analysis by adding of component(s) with known concentration – Internal Standards. So according to this aim it has a right to be called Internal Standard procedure. In the case of exact measurements results are identical, and error traceability is good. But it does not use response ratio, mentioned in legal documents, e.g. Pharmacopoeia.

Solution cannot be found without the help of regulating authorities. Advices appreciated.

Introduction

Internal Standard (ISTD) [1-3] is a widely used chromatographic technique, aimed to compensate sample size variations, where known amount of an *internal standard* component, is added to both calibration 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.

It was demonstrated [4, 5], that this approach may cause systematic errors in the case of non-directly proportional detector response to concentration of analyte or *internal standard*; this conclusion was recently independently confirmed [7]. We implemented an alternative calculation scheme [4-6] allowing wide variations of standard and analyte concentrations and non-directly proportional calibrations of components, in which case 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:

- Calculation of Relative concentration, i.e. concentration of analyte, provided concentration of Internal Standard is known, using External Standard calibration curves.
- Construction of improved calibration curves (Relative 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 the commercial chromatographic software [6] for more than 15 years.

Calibration

- Predictive relationship between input and detector response
- Input: calibration samples – concentrations of components and injected volumes
- Output: peak area or height
- Prediction: Predict unknown input looking at response

External Standard (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, $\alpha=1$ 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 $R' = R^{1/\alpha}$

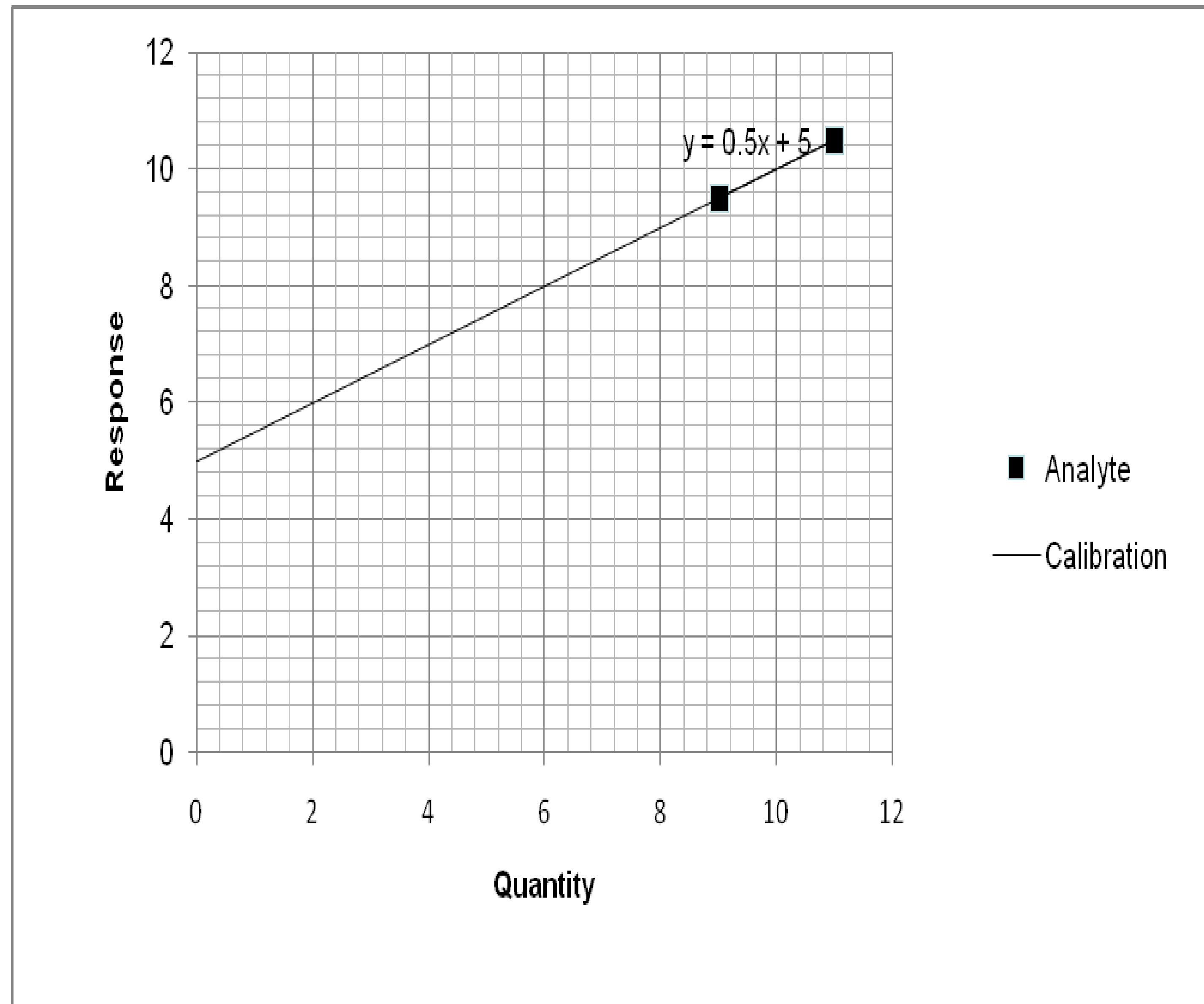
When it works poorly: in most of the other cases

Example of “Classic” ISTD Failure

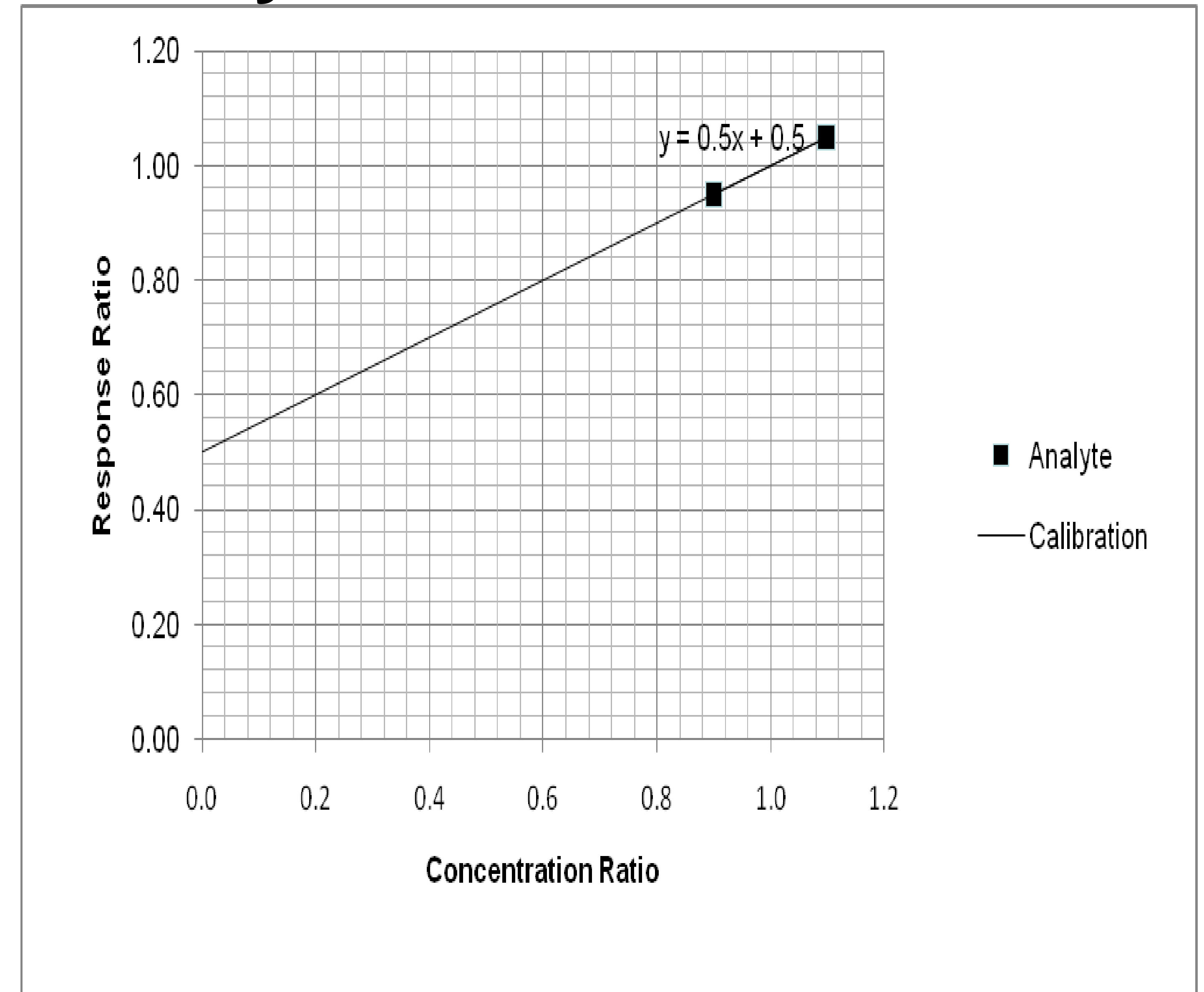
Sample	C_s	C_a	Loss, %	Q_s	Q_a	R_s	R_a	R_a/R_s	C_a/C_s	Error, %
Calibration point 1	1	0.9	0	10	9	10	9.5	0.95	0.9	0
Calibration point 2	1	1.1	0	10	11	10	10.5	1.05	1.1	0
Test analysis (calculated)	1	1	9	9.1	9.1	9.1	9.55	1.049	1.099	9.9
Volume	10									

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

Relative concentration accounts for systematic error due to the sample-size error and the sample loss during preparation in the case of known ESTD calibrations.

Nominations:

$W_{istd}(R)$ - predictive equation for the Standard component;

$W_x(R)$ - predictive equation for the Analyte;

R_{istd} - response of Standard component;

R_x - response of the analyte;

C_{istd} - declared concentration of the standard component;

V - sample volume;

The main assumption behind Relative concentration is that the sample volume V is unknown and is calculated using quantity of the standard $Q_{istd} = W_{istd}(R_{istd})$ and a declared concentration of the internal standard:

$$V = Q_{istd}/C_{istd} = W_{istd}(R_{istd})/C_{istd}. \quad (1)$$

Concentration of Analyte is obtained by dividing quantity $Q_x = W_x(R_x)$, by volume:

$$C = Q_x/V = W_x(R_x)/V = C_{xistd} \times W_x(R_x)/ W_{istd}(R_{xistd}) \qquad (2)$$

Calculations for the above example:

$C = Q_a/V = C_s \times Q_a/Q_s = 1.0 \times 9.1/9.1 = 1.0$

The result does not have any error.

“True” ISTD step 2: Relative Calibration

Relative Calibration starts from measuring ESTD calibration curve of the Standard component. After we measured this curve, we can use it to improve the positions of calibration points of other analytes along Quantity axis using the same trick as was used while calculating the Relative Concentration**:**

$$Q_n = C_n \times V = C_n \times W_{istd}(R_{istd})/C_{istd} \qquad (3)$$

All analytes get curves constructed conditionally, condition being the known calibration curve of the Internal Standard component. Relative calibration can be considered as a modification of the procedure that calculates ESTD curve

"True" ISTD step 3: Simple Relative Calibration

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 prediction curves. The calibration curve will change, as well as the raw concentration, but not the Relative Concentration

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

If all calibration curves are directly proportional

$Q = K \times R$,

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 Relative Calibration). That is, assuming directly proportional calibration curve of the standard component to have coefficient 1, we get relative response factors for other analytes – this behavior is almost indistinguishable from "Classic ISTD":

Comparison of Simple Relative Calibration with "Traditional" ISTD

	Simple Relative Calibration	"Traditional" 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 Relative Calibration. The only case where the Response Ratio Calibration works

properly is a particular case of the Simple Relative Calibration! However, using those weights does not improve accuracy or stability of calculations and so makes no big sense.

Device Drift

Drift model: $R = K \times F(Q)$

Drift can be compensated by Relative calibration 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

Most books on chromatography describe Internal Standard method for the case of single-point calibration, implicitly assuming direct proportionality of the calibrations. Classic Internal Standard method was established in the early days of chromatography [1-3], when detector stability was not good enough. It perfectly compensates detector sensitivity changes (detector drift), but not always variations of quantity. 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.

Our implementation of Internal standard calculations does not imply that the component used for improving calibration is the same as the component used for compensation of injection errors, so Calibration and Quantification standards may be different. More than this, improved calibration could use any of components as a standard, so it may happen that some kind of multivariate improvement of calibration dependence may give better results, than Relative calibration. The simplest implementation is calculation of injection volume using Formula 1 for every calibrated component and then averaging these volumes for using in Formula 3, so that all calibrated components become Calibration standards.

Conclusions

ISTD is split into two parts: ISTD quantification (Relative Concentration) and Relative ISTD Calibration. Parts can be applied separately.

Relative Concentration is applicable for both ESTD and Relative calibrations. It requires less often recalibration compared to raw (ESTD) concentration, as ratio of prediction functions is more stable than those functions.

Different Calibration and Quantification standards may be used.

Full Relative calibration can be used for calculation of both raw and Relative concentrations.

Simple Relative ISTD calibration can be used in the case of linear through origin calibrations instead of “Classic” ISTD calibration including imitation of user interface by calculation of the relative response factors.

Full “Relative” ISTD solution still works where “Classic” already fails, i.e. it allows to get into the account wide concentration range of Internal Standard even 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. Pharmacopoeias [8, 9] explicitly state that Internal Standard method should be implemented by Response Ratio method.

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 Pharmacopeia

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.

References

1. N.H.Ray. J.Appl.Chem., 1954, v.4, p.21
2. D.E.Willis, Chromatographia 5, #1, 42-43 (1972)
3. R.W.Yost, L.S.Ettre, R.D.Conlon. Practical Liquid Chromatography. An Introduction. Perkin-Elmer, 1980
4. Каламбет Ю.А., Козьмин Ю.П., Калибровка методом внутреннего стандарта при нелинейных калибровочных зависимостях, в кн. «Руководство по современной тонкослойной хроматографии», под редакцией Ларионова О.Г., стр. 180-184. Научный Совет Российской Академии Наук по хроматографии, Москва, 1994 // Y. Kalambet, Y. Kozmin, Internal standard calibration in the case of non-linear calibration dependencies, “Modern thin-layer chromatography manual”, ed. O.Larionov, 180-184, Scientific Council of RAS on chromatography, Moscow, (1994)
5. Каламбет Ю. А., Метод внутреннего стандарта - идея и воплощение, "Партнеры и конкуренты", № 4, 2004, 32-36 // Y.Kalambet, Internal Standard – Idea and Implementation, “Partners and Competitors”, #4, 32-36 (2004).
6. Chrom&Spec Chromatography Data System, www.chromandspec.com, www.multichrom.ru
7. A. Hewavitharana, Internal standard - friend or foe?. *Critical Reviews in Analytical Chemistry*, *39* 4: 272-275 (2009).
8. The European Pharmacopoeia 6th ed., Council of Europe European (COE) - European Directorate for the Quality of Medicineshttp:// www.edqm.eu (2007)

9. The United States Pharmacopeia–National Formulary, http://www.usp.org