Internal Standard Calibration: an Alternative Approach

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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 acting always together

ISTD tricks

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

"Classic" ISTD

- *Coordinates:* **Response Ratio vs. Concentration ratio**
- *Calibration curve:* **polynomial, typically straight line through origin**
- *Prediction:* **from Response Ratio predict 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 $Ca/Cs = (ka/ks)*(Ra/Rs)^{\alpha}$;

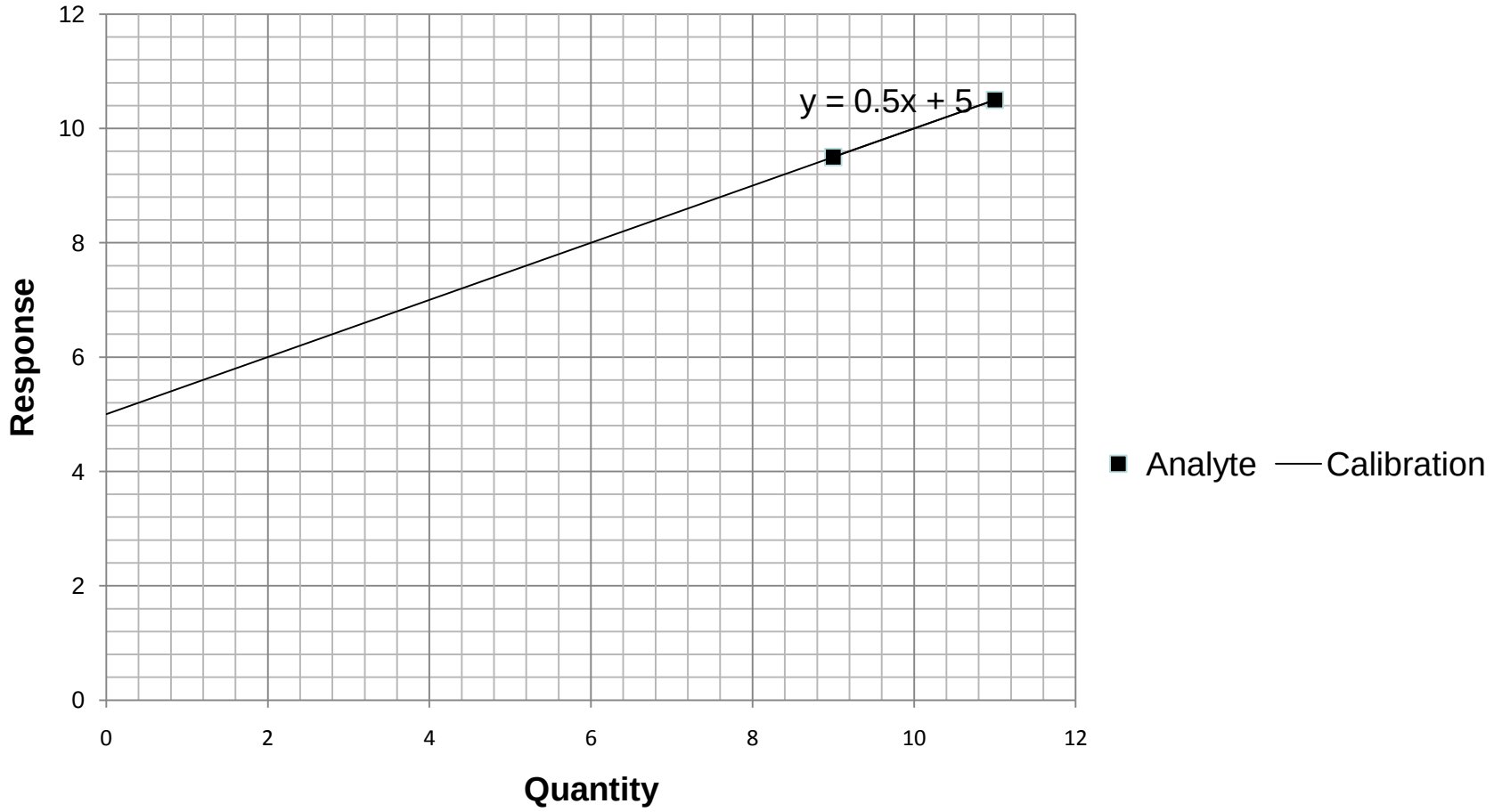
  The case of $\alpha \neq 1$ can be linearized by setting $R'=R^{1/\alpha}$
- *When it works poor:* **in most of other cases**

Example of “Classic” ISTD failure

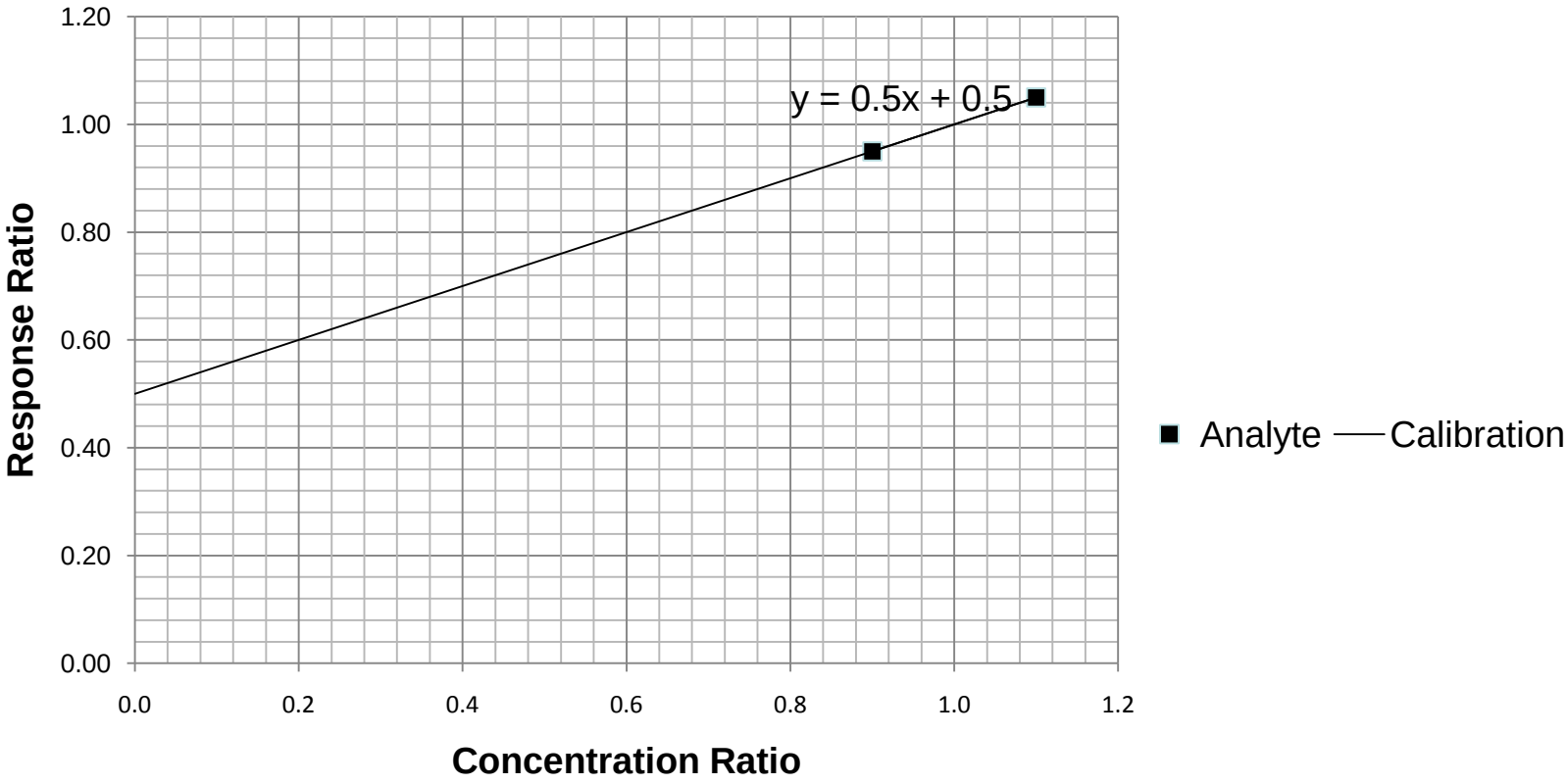
Sample	C_s	C_a	Loss, %	Q_s	Q_a	R_s	R_a	R_a/R_s	C_a/C_s	Error, %
Calibration point 1	1	0.9	0	10	9	10	9.5	0.95	0.9	0
Calibration point 2	1	1.1	0	10	11	10	10.5	1.05	1.1	0
Test analysis (calculated)	1	1	9	9.1	9.1	9.1	9.55	1.049	1.099	9.9
Volume	10									

Directly proportional ESTD calibration of Standard component;
Linear calibration of Analyte

ESTD Calibration



“Classic” ISTD calibration curve



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“True” ISTD step 1: Relative Concentration

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

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

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

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

- Calculations for the above example:

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

“Universal” ISTD Calibration

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

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

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

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

- 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 “affinity” change of all calibration curves. Calibration curve will change, absolute concentration also, but not Relative Concentration

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

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

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

The only case, where Response Ratio Calibration works properly is a particular case of Simple Universal Calibration!

Full Universal Calibration

Advantages:

- Only one type of axes
- Calibration curves are suitable for 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

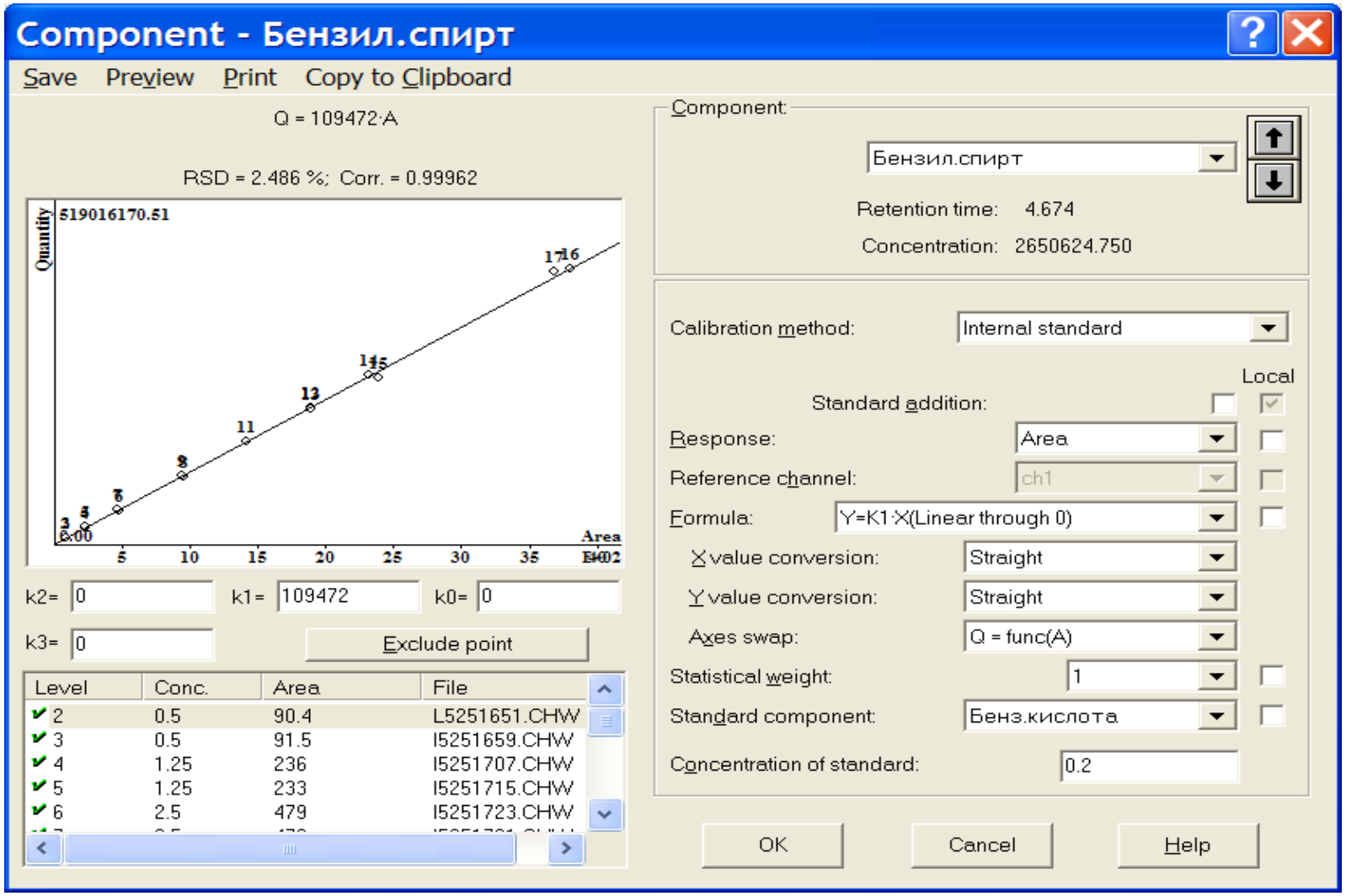
Device Drift

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

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

- Particular case: linear through origin calibration; K = k

Example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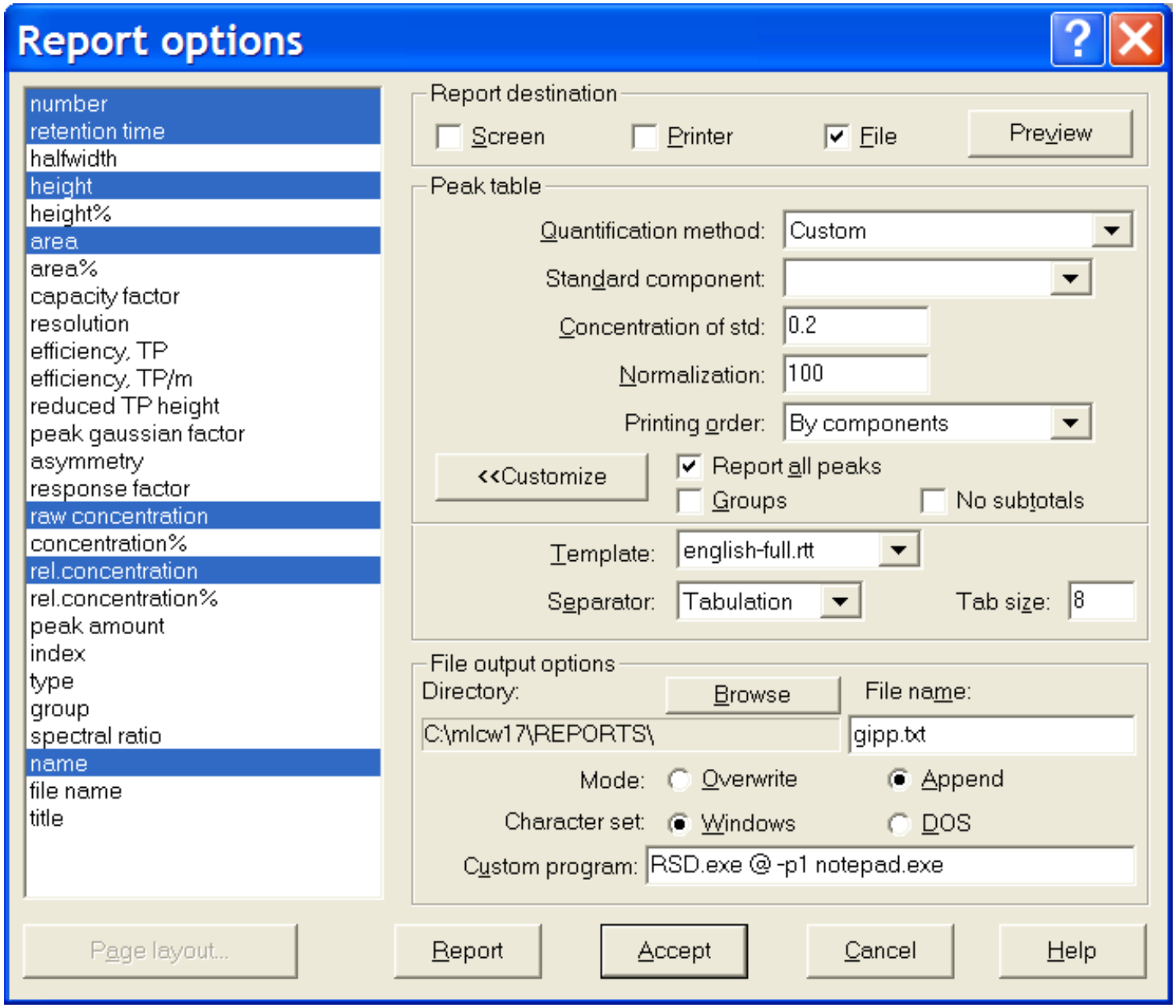
Save
Preview
Print
Copy to Clipboard
Q = 109472·A
RSD = 2.486 %; Corr. = 0.99962
519016170.51
Quantity
Area
E+02
k2= 0
k1= 109472
k0= 0
k3= 0
Exclude point
Level
Conc.
Area
File
2 0.5 90.4 L5251651.CHW
3 0.5 91.5 I5251659.CHW
4 1.25 236 I5251707.CHW
5 1.25 233 I5251715.CHW
6 2.5 479 I5251723.CHW
Component:
Бензил.спирт
Retention time: 4.674
Concentration: 2650624.750
Calibration method:
Internal standard
Local
Standard addition:
Response:
Area
Reference channel:
ch1
Formula:
Y=K1·X(Linear through 0)
X value conversion:
Straight
Y value conversion:
Straight
Axes swap:
Q = func(A)
Statistical weight:
1
Standard component:
Бенз.кислота
Concentration of standard:
0.2
OK
Cancel
Help


Report options
number
retention time
halfwidth
height
height%
area
area%
capacity factor
resolution
efficiency, TP
efficiency, TP/m
reduced TP height
peak gaussian factor
asymmetry
response factor
raw concentration
concentration%
rel.concentration
rel.concentration%
peak amount
index
type
group
spectral ratio
name
file name
title
Report destination
Screen
Printer
File
Preview
Peak table
Quantification method:
Custom
Standard component:
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Normalization:
100
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By components
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Report all peaks
Groups
No subtotals
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Tabulation
Tab size:
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File output options
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Overwrite
Append
Character set:
Windows
DOS
Custom program:
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Report
Accept
Cancel
Help

Conclusions

- ISTD is split in 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 account wide concentration range of Internal Standard in the case of nonlinear calibrations

Difficulties

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

About some myths concerning peak integration

- There are different integration methods (trapezoid rule, simpson's rule, etc) and results are better for more complicated rules
- Asymmetric peaks require much more points to be integrated than symmetric

Peak parameters to be "integrated"

- Area
- Height
- Retention (apex X position)
- Width at half-height
- Asymmetry (5% and 10% formulas)
- Peak baseline width

Integration formulas (Area only!)

- 121
- 1331
- 1331131133113 31 - ?
- 1331
  -1331
  --1331
  ---1331
  ----1331
  -----1331
  ……
  1468….8641

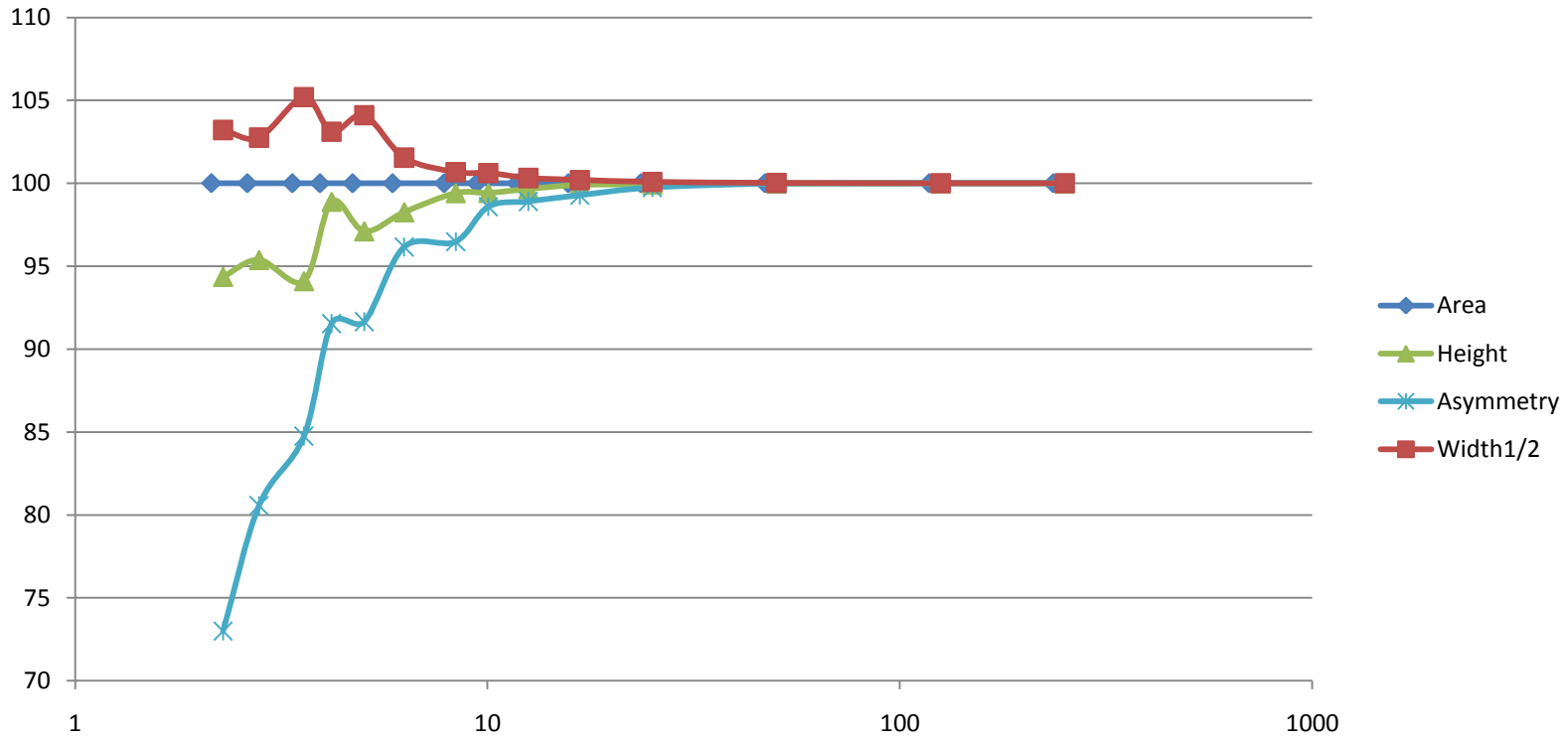
All methods are identical

- Peak boundaries are at the baseline – all values close to an end are (almost) zero! Base-to-base peaks are integrated by mere summing up of all points
- Peak baseline drop separation using Simpson's formulas is a nonsense, as "WYSYWIG" principle is violated

Why simple summation works so well?

- Integral of all derivatives over the peak region equals zero, as all derivatives are equal to sero at the end of the peak. All convex regions are exactly compensated by concave regions.

How many points are required for other parameters? EMG, asymmetry = 3.46



How many points are required for other parameters? Gaussian