Quantitation and Validation in Electrophoresis: Definitions and Fundamentals

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Abstract

Definitions based on the ICH guidelines have been compiled in order to properly describe validation and performance characteristics of capillary electrophoretic methods for quantitation. The fundamentals of the underlying statistics have been outlined. Further, recent reference works about quantitation and validation in CE have been reviewed.

Keywords: Review, electrophoresis, capillary electrophoresis, quantitation, validation, definitions, fundamentals

1 DEFINITIONS

In order to ensure harmonization and comparability within the paper symposiums, this general review presents a collection of terms and their definitions. These are based on the International Conference on Harmonization (ICH) Guideline for Industry, mainly in the guideline ICH-Q2A: Text on Validation of Analytical Procedures. Details on the ICH can be found at *http://www.fda.gov/cder/guidance/anproc.htm* [1]. Moreover, the respective recently updated guidelines are available here.

VALIDATION OF ANALYTICAL PROCEDURES
The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included.

TYPES OF ANALYTICAL PROCEDURES TO BE VALIDATED
The discussion of the validation of analytical procedures is directed to three most common types of quantitative procedures in the context of electrophoresis. Type 1 are assays, which represent quantitative measurements of the major component(s). Typical assays include the measuring of the yield of a biotechnical production, the test for drug content uniformity or stability, or test for major nutritients in food analysis. Type 2 are tests for compounds of minor concentration, e.g. investigations of changes in protein expression in a certain cell or tissue (proteomics), or the test for impurities in a pharmaceutical preparation. Finally type 3 is the limit test for minor compounds, again for the control of impurities in food und drugs or in environmental analysis. In the type 3 case the concentration need not be known precisely, it must just be guaranteed that its amount is below a certain limit, e.g. due to toxicity.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

- Accuracy
- Precision
- Repeatability
- Intermediate Precision
- Specificity
- Detection Limit
- Quantitation Limit
- Linearity
- Range
- Sensitivity
- Robustness/Ruggedness

The classification of these parameters and their relation to each other is depicted in Figure 1. All parameters have to be considered for the quantitation of minor compounds (type 2). All parameters but the limits of detection and quantitation are required for assays (type 1). For the limit tests, just specificity and the detection limit must be determined.

SPECIFICITY AND ACCURACY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.
The **accuracy** of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.
These two characteristics are strongly related. Lack of specificity can lead to coevaluation of two or more analytes. This causes a systematic error and in consequence a **bias**, or a biased result.

Specificity means to measure accurately in the presence of all potential sample components. Depending on the analytical problem, these components can be matrix compounds, excipients, synthesis intermediates, process impurities or degradation products. If there is no reference material for degradation products, the analyte should be exposed to stress conditions such as light, heat (50 °C), acid and base (0.1 M HCl and NaOH) and oxidant (3% H_2O_2) [2, 3].
The ideal of specificity is rarely fulfilled; usually high selectivity can be achieved at best. A scenario where another compound would lead to the same results cannot be excluded most of the times. Lack of specificity of an individual analytical procedure may be compensated by (an) other supporting analytical procedure(s).
Specificity is an important validation parameter that should be established as early as possible. Purity of all relevant signals (e.g. peaks or spots) should be checked if possible, for example by using mass detection. In some cases the signal is still influenced by the sample matrix despite of peak purity, for instance due to sample pre-treatment operations. In order to check influences caused by the matrix, analytes in different concentrations (at least at the upper and lower end of the range) are spiked into the expected matrix. This should be blank, that means contain everything of the real sample (such as excipients, body fluids...), but the analyte. The obtained

signals are compared to signals from samples without matrix (recovery). If these signals do not significantly differ, this also gives strong evidence for accuracy.
If the matrix just causes an additional signal which is the same for all analyte concentrations, the calibration results are not negatively affected. However, a change in sensitivity caused by the matrix is occasionally observed, e.g. in UV/Vis spectroscopy and derived techniques (e.g. HPLC/UV), if the formation of complexes is hindered or favoured by the matrix [4].
Working with blank matrix, the calibration standards are spiked into the matrix to avoid false estimations. It should be checked if the same sensitivity is obtained using different batches of blank matrix.

PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.
Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements (see Eqs. 4-6). The numbers of measurements n should always be given with the number of e.g. the RSD. It is rewarding to use high numbers; the quality of statements about precision is noticeably improved from multiple measurements. Putting it another way, the confidence intervals of variability are very much improved increasing the data number. Statements about variability with $n<5$ are often meaningless (see Eq. 12, Table 1). Even a number of $n = 20$ just provides a confidence interval of about ± 30% around the measured value, that means measured 1.0% RSD can well mean a true value of 1.3% RSD. We often use $n = 60$ or more, in order to precisely measure precision.
Precision may be considered at four levels: system precision, repeatability, intermediate precision and reproducibility. In order to avoid misunderstandings and misinterpretations, it must be clearly defined what precision level is meant and under what conditions it was obtained.

a. System precision
The system precision represents the variability of the measurement process. It is obtained by repeatedly analysing the same sample.

b. Repeatability
Repeatability expresses the precision under the same operating conditions (including measurement and sample preparation) over a short interval of time. Repeatability is also termed intra-assay precision.

c. Intermediate precision
Intermediate precision expresses within laboratories variations: different days, different analysts, different equipment, etc.

d. Reproducibility
Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology).

The two last-mentioned precision levels include, additionally to the random variability of the measurement, the influence of the reference standard and especially the changes by external factors (e.g. temperature, humidity, quality of reagents, operators´ qualification etc.). The intermediate precision is characterized by variation factors within the same laboratory. However, within longer terms this precision level is approaching the reproducibility, because the a.m. factors change as well. Therefore, it is often sensible to use reproducibility as the general term, including intermediate precision.

DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Usually the detection limit (or limit of detection, LOD) is defined using the signal-to-noise ratio (Eq. 1), e.g. in European Pharmacopoeia Ph. Eur. (methods 2.2.28 und .29; [5]):

$$S / N = \frac{2H}{h_n} \quad (1)$$

Here H is the height of the measured signal, related to the average base signal (baseline). The value h_n describes the maximum spread of the baseline signal within 20 signal(peak)-widths at half height (e.g. ± 10, if there are no additional peaks neighbouring the reference peak) at both edges of the peak (see Fig. 2). The detection limit can now be defined as the concentration which guarantees a several-fold, e.g. a 2-, 3- or 5-fold, of the signal-to-noise ratio.
Unfortunately, the term detection limit is not unambiguously used in the scientific literature. Limits of detection of the same method can vary by more than the factor of 3, just from different definitions and calculations [6]. Therefore it is very important to discuss the LOD and the determination of the S/N ratio carefully.

QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.
In order to determine the limit of quantitation (LOQ), it is necessary to agree on acceptable limits for precision and accuracy. Commonly precision is given as a maximal acceptable relative standard deviation of the analytical result, $CV\%_{max}$ ($\hat{\beta}_1$ is the slope of the regression function used, see Eqs. 14-18; $\hat{\sigma}$ stands for the standard deviation of the measured values). Then the LOQ can be calculated using Eq. 2:

$$\mathrm{LOQ} = \frac{100\%}{CV\%_{max}} \cdot \frac{\hat{\sigma}}{\hat{\beta}_1} \quad (2)$$

This is also the ICH proposal. They suggest $CV\%_{max}$ as 10%.

LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.
Unfortunately, a linearity test as such does not exist. Nevertheless, significant lack-of-fit can be detected by various sensible methods. However, the sample correlation coefficient r (or $\hat{\rho}$) does not belong to the pool of these methods to assess linearity. The sample correlation coefficient may be misleading and is, despite its widespread use, to be discouraged for two reasons: First, r depends on the slope. That is, for lines with the same scatter of the points about the line, r increases with the slope. Second, the numerical value of the correlation coefficient cannot be interpreted in terms of degree of deviation from linearity. Put differently, a correlation coefficient of 0.99 may be due to random error of a strictly linear relationship or due to systematic deviations from the regression line [12].
Systematic deviations from the assumed model yield systematic patterns in the residuals and can, therefore, be detected by checking independence of the residuals (see below). Residuals plots, particularly the e_i versus $\hat{y}_i$ plot is also well suited to detect nonlinearities, since the plot will show curved patterns instead of randomness. Formal tests are also available, e.g. the ANOVA lack-of-fit test [12].

SENSITIVITY

The sensitivity of an analytical method is the slope of the calibration function which is used for the evaluation.

RANGE

The range of an analytical procedure is the interval between the highest and lowest concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

RUGGEDNESS

A method is rugged, if it is not or only slightly affected when used in different laboratories. The ruggedness is determined in interlaboratory trials. If the interlaboratory standard deviation (see

reproducibility) is only slightly elevated compared to the repeatability, the method is rugged. Usually robust methods are rugged, and vice versa.

2 SYMBOLS AND FUNDAMENTAL EQUATIONS

2.1 Measures of central tendency and variability of data, prediction and confidence intervals [7]

A random sample, e.g. a series of measurements, can be described by measures of central tendency and measures of variability. The most popular measure of central tendency is the (arithmetic) mean $\overline{x}$ (3), the sum of all values of the sample divided by their number.

$$\overline{x} = \frac{\sum_{i=1}^{n} x_i}{n} \qquad (3)$$

In the following sums $\sum_{i=1}^{n}$ with index i=1 to n are written in the simple form $\sum$.

Another important measure is the median $\tilde{x}$. In order to obtain this parameter, all values of a random sample are ordered by size. The median is the value in the middle of this ordered list, or the mean of the two values in the middle, if the number of data is even.

As measures of variation, variance $\hat{\sigma}^2$ (4) and standard deviation $\hat{\sigma}$ (5) are most popular. The „roofing" of a variable indicates, that it is just an estimation from a random sample and not a true value. This allows for the clear distinction between the standard deviation of an entire population σ and the standard deviation of a random sample $\hat{\sigma}$. The standard deviation is descriptively given in the same unit as the measured parameter. However, the variance is the preferred measure, if total variations shall be estimated from error components, or if the contribution of error sources is considered, because variances, but not standard deviations, behave simply additive.

The percental relative standard deviation RSD% (6) is very well suited to compare the variation between different methods and techniques. This popular parameter is also often called coefficient of variation (CV). Other important measures of spread include the mean deviation d (7) and the range R (8), which is the difference between the highest and lowest value.

$$\hat{\sigma}^2 = \frac{\sum (x_i - \overline{x})^2}{n-1} \qquad (4)$$

$$\hat{\sigma} = \sqrt{\hat{\sigma}^2} \qquad (5)$$

$$RSD\% = \frac{\hat{\sigma}}{\bar{x}} \cdot 100\% \qquad (6)$$

$$d = \frac{\sum |x_i - \bar{x}|}{n} \qquad (7)$$

$$R = x_{\max} - x_{\min} \qquad (8)$$

Using these measures, statements about other, also future, members of the population are available. The value of e.g. a future measurement can be predicted with a certain error probability, using prediction intervals (9). These intervals also generally provide, in which value ranges a certain amount of the population can be expected.

Two-sided prediction interval:

$$prd(x) = \bar{x} \pm t_{n-1,\alpha/2} \cdot \hat{\sigma} \cdot \sqrt{\frac{1}{n} + \frac{1}{m}} \qquad (9)$$

One-sided prediction intervals:

$$prd(x) = \left[-\infty, \bar{x} + t_{n-1,\alpha} \cdot \hat{\sigma} \cdot \sqrt{\frac{1}{n} + \frac{1}{m}} \right] \qquad (10)$$

$$prd(x) = \left[\bar{x} - t_{n-1,\alpha} \cdot \hat{\sigma} \cdot \sqrt{\frac{1}{n} + \frac{1}{m}}, \infty \right] \qquad (11)$$

Here m is the number of measurements that will be used to determine the predicted value. This number m can (be) equal 1, if just one value is predicted. If a mean from e.g. 3 measurements is predicted, m is equal (?) 3. The t-value scales the standard deviation to the desired error probability α. This t-value is also strongly dependent from the number of data: the higher this number, the better the derivable conclusions, the smaller the prediction interval.
Two-sided problems are more common. In this case deviations to both sides of the mean can be expected (9).
If deviations to one side can be certainly excluded, then one-sided prediction intervals should be used. One-sided prediction intervals cover the whole range on one side, to $+\infty$ or $-\infty$ (Eqs. 10, 11).

Confidence intervals for the true standard deviation σ are also available (Eq. 12). These intervals are especially sensitive to the number of data (Table 1, compare PRECISION).

$$\frac{1}{\sqrt{F_{n-1,\infty,1-\alpha/2}}} \cdot \hat{\sigma} \le \sigma \le \hat{\sigma} \cdot \sqrt{F_{\infty,n-1,1-\alpha/2}} \qquad (12)$$

2.2 Linear regression

Often there is a known relationship between mass or concentration of standard samples and the measured value of an analytical method. Frequently this relationship is linear, e.g. because UV/Vis-absorbance measurements or techniques based on UV/Vis detectors (like in CE) are common in quantitative analytical chemistry. If BEER´s law is valid, linearity is guaranteed (Eq. 13):

$$A = \alpha \cdot c \cdot d \qquad (13)$$

A typical calibration experiment is performed by making up a series of standards, e.g. solutions, containing known amounts of analyte x_i, mostly given as concentrations. Each standard separately passes the analytical procedure. One signal y_i is generated corresponding to each x_i. The data pairs (x_i/y_i) can be plotted in a two-dimensional graph with x- and y-axes. A model function, that fits the data (e.g. a straight line), can be estimated. Now the mass or concentration of an analyte with unknown amount x_0 can be estimated by measuring the corresponding signal y_0, using the inverse function of the model function.
Consider a linear relationship (Eq. 14). The data will never exactly be matched by a line, since the deterministic linear relationship is always superimposed by measurement error ε_i. This error must be considered when a model for a linear relationship is created (Eq. 15):

$$y = \beta_0 + \beta_1 x \qquad (14)$$

$$y_i = \beta_0 + \beta_1 x_i + \varepsilon_i \qquad (15)$$

Eq. 14 is a model for the true relationship. In case of an analytical calibration, the true relationship always remains unknown. Only random samples are measured. Their number is very small compared to the number of possible measurements, which is almost infinite. Moreover, the error ε_i included in the i-th single measurement y_i remains unknown. However, we can estimate the coefficients β_0 and β_1 by a limited number n of samples. This leads to the regression line (Eq. 16):

$$y_i = \hat{\beta}_0 + \hat{\beta}_1 x_i \qquad (16)$$

Note that $\hat{\beta}_0$ and $\hat{\beta}_1$ are only estimators of β_0 and β_1. There are many different methods to obtain these estimators. They usually lead to different results. The simplest method is called **o**rdinary **l**east **s**quares (OLS). The regression line is calculated by minimizing the sums of the squares of the distances between the data pairs (x_i/y_i) and the line in y-direction. The slope $\hat{\beta}_1$ is then estimated employing Eq. 17. Because the line is always passing through the centroid $(\bar{x}, \bar{y})$, the intercept $\hat{\beta}_0$ can be estimated using Eq. 18.

$$\hat{\beta}_1 = \frac{\sum\left[(x_i - \bar{x})(y_i - \bar{y})\right]}{\sum(x_i - \bar{x})^2} \tag{17}$$

$$\hat{\beta}_0 = \bar{y} - \hat{\beta}_1 \bar{x} \tag{18}$$

The values $\bar{x}$ and $\bar{y}$ are the means of all standard amounts x_i and all signals y_i, respectively. The advantage of this approach is its simplicity. However, a number of assumptions must be valid to sensibly estimate $\hat{\beta}_0$ and $\hat{\beta}_1$ using OLS (Table 2 [2]).
It has been shown that the assumptions „x is error-free“ and „error in y is homoscedastic“ are usually invalid [2]. Nevertheless numbers for $\hat{\beta}_0$ and $\hat{\beta}_1$ are always obtained using OLS, but these may be inefficient and biased estimates of β_0 and β_1. These deviations should always be kept in mind, even if in certain cases they can be kept tolerably small by using a suitable experimental design.
If significant deviations are expected or found after checking the assumptions, the use of more advanced models is mandatory. Even if the deviations are not significant when the wrong model is used, time is always lost considering these errors and their importance. Thus the regular use of generalizing advanced statistical software is advised.
After estimates for $\hat{\beta}_0$ and $\hat{\beta}_1$ have been computed, it is elementary to estimate the mass or concentration of an analyte x_0 from its corresponding signal y_0 using the inverse function of Eq. 16, Eq. 19:

$$x_0 = \frac{y_0 - \hat{\beta}_0}{\hat{\beta}_1} \tag{19}$$

However, just the value for x_0 does not give the measurement error of this estimator. For example, if x_0 was calculated as 10 mg/L, the sample concentration could range from 9.999 to 10.001 mg/L as well as it could range from 2 to 18 mg/L. Information about random variations of x_0 and thus information about its reliability is essential. Random variations in calibration experiments are described similar to standard deviations of a series of replicate measurements (Eq. 8):

$$\hat{\sigma} = \sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n-2}} \tag{20}$$

The differences $y_i - \hat{y}_i$ are called residuals and abbreviated e_i. $\hat{y}_i$ is called the expected mean response value. It represents the computed value obtained when a standard concentration x_i is inserted into Eq. 16; y_i is the really measured signal at that concentration. The residuals are the deviations of the standard measurements from the modelled function. Using $\hat{\sigma}$ a prediction interval for new response values can be calculated (Eq. 21), which is very similar to the one-dimensional case (see Eq. 9):

$$\mathrm{prd}\{\hat{y}(x)\} = \hat{y}(x) \pm t_{n-2}^{1-\alpha/2} \cdot \hat{\sigma} \cdot \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(x-\bar{x})^2}{\sum(x_i-\bar{x})^2}} \qquad (21)$$

When new standards x_0 are prepared and measured with the same procedure to obtain signals y_0, additional signals are expected within the prediction interval. The probability 1-α denotes the percentage of signals that will be found inside the prediction interval. Some signals will be found outside due to the random error. The value m gives the number of replicate measurements for the signal y_0 to be predicted, including m= 1 (no replicate). If m is greater than 1, the mean of all measured $y_{0,j}$ will be predicted.
However, the analyst is typically not interested in predicting new signals y_k. The principal issue, often called the „calibration problem", is the estimation of a confidence interval of the analytical result ($\mathrm{cnf}(x_0)$), which is derived from the signals y_0 of a sample with unknown concentration and the calibration line. The solution of this problem is not trivial for some theoretical reasons. All solutions depend on different assumptions, and it is still open to discussion which choice of assumptions is most appropriate. However, there is a generally accepted approach, which can be sensibly used in analytical chemistry [8-11]. A graphical explanation for this estimation of $\mathrm{cnf}(x_0)$ is given in Figure 3. The corresponding theory is discussed more detailed in [12].

$$\mathrm{cnf}\{x_0(y)\} = x_0(y) \pm t_{n-2}^{1-\alpha/2} \frac{\hat{\sigma}}{\hat{\beta}_1} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(x_0(y)-\bar{x})^2}{\sum(x_i-\bar{x})^2}} \qquad (22)$$

The value m gives the number of replicate measurements of the signal y_0 that is used to estimate $x_0(y)$. If m is greater than 1, the mean of all measured $y_{0,j}$ will be used to estimate $x_0(y)$.
The slope $\hat{\beta}_1$ plays an important role for the width of this interval. The greater $\hat{\beta}_1$, the more precisely x_0 can be estimated assuming constant signal standard deviation. The slope of a calibration function is also called its sensitivity. Because the remaining terms of Eq. 22 often are identical or much the same for similar analytical methods, the simplified term $\hat{\sigma}/\hat{\beta}_1$ is called procedural standard deviation and is often used to compare the precision of different methods.

Using an onepoint design, that means just one reference sample, an apparent sensitivity $\hat{\beta}_1^*$ is defined by $\bar{x}$ and $\bar{y}$ (Eq. 23). The analytical result x_0 is then estimated by Eq. 24 or 25 using y_0, the signal of the analyte or its mean.

$$\hat{\beta}_1^* = \frac{\bar{y}}{\bar{x}} \qquad (23)$$

$$x_0 = \frac{y_0}{\hat{\beta}_1^*} \qquad (24)$$

$$x_0 = y_0 \cdot \frac{\bar{x}}{\bar{y}} \qquad (25)$$

If x_0 significantly differs from $\bar{x}$, the main assumption permitting the use of the onepoint design is not fulfilled. However, the systematic error may be small. If a „Three-times-eight" design [2] has been used during method development and linearity was found, the systematic error E_{sys} can be estimated using Eq. 26 [7]. Here $\hat{\beta}_0$ and $\hat{\beta}_1$ are the estimates from the method development, $\hat{\beta}_1^*$ is not used.

$$E_{sys} = \frac{|x_0 - \bar{x}|}{1 + \frac{\hat{\beta}_1 \bar{x}}{\hat{\beta}_0}} \quad (26)$$

The confidence interval for x_0 is given by Eq. 27, which is a simplification of Eq. 22:

$$\mathrm{cnf}\{x_0\} = x_0 \pm t_{n-1}^{1-\alpha/2} \frac{\hat{\sigma}}{\hat{\beta}_1^*} \cdot \sqrt{\frac{1}{m} + \frac{1}{n}} \quad (23)$$

Here $\hat{\sigma}$ is the standard deviation of the signal. It is suggested to use the same number as for the endpoint design: n=6 measurements for the standards, m=3 for the analytes each. The system suitability can simply be tested by an F- and t-test comparing mean and standard deviation for the present and the previous series.

3 REFERENCE WORKS

3.1 Capillary electrophoresis

More than 800 articles have been reviewed to collect detailed information about method development, quantitation and validation in CE [13]. Here e.g. a check list for all aspects that should be mentioned in an experimental part of a paper is provided, tables of useful reagents are found, validation requirements and important aspects to accomplish them are summarized, a general strategy for method development including quantitation is described and illustrated in several diagrams.
This compilation is still ongoing in most aspects. This is confirmed in [14]. Here the repeatability in the industrial environment is estimated to be 0.7% RSD% on average, rarely above 1%. The importance of internal standards for precision in CE was again emphasized [15]. Dimethylbiguanide, diaminobenzoic acid and triaminopyrimidine have been reported to be generally suitable, in our works we often successfully used neostigmine (e.g. [16]). Using internal standards, the robustness is surprisingly little affected by the pH or the concentration of the chiral selector [17, 18]. Validation was recently discussed and reviewed in [18-20]. Meanwhile CE is included in all major pharmacopoeiae [15, 21, 22].
It should be emphasized that migration time precision, which influences peak area precision, is usually strongly related to EOF precision. Therefore the latter should be carefully investigated and reported [23]. Linearity was not seen as a real issue in CE so far; however, nonlinearities can cause underestimations of main peak areas and thus overestimations of area% for minor components. It was found that nonlinearities do not become notable below 300 mAU, usually not

critical before 1000 mAU [24]. The mentioned thresholds are instrument-dependent, some detectors even allow a higher absorbance.
Validation in the presence of complex matrices is a challenge for each analytical approach. Especially stacking is known to be matrix-sensitive. Validation of pre-concentration techniques may be facilitated using effective mobilities as indicators of matrix effects [25]. Possibly on-line pre-concentrations in the presence of matrices are easier to validate using transient ITP with suitable terminating and leading electrolytes instead of simple stacking.

3.2 Gel electrophoresis

Although quantitation and precision are important topics for gel electrophoresis as well, these have not been intensively investigated during the last years. Quantitation usually is just a side topic in publications about GE. It seems like one has settled to accept GE as a semi-quantitative techniques, where no further miracles can be expected.
There are certainly some very interesting exceptions of methodological works, either in this symposium volume, or in some recent publications which have been reviewed in the introduction of [26].

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Table 1: Confidence interval of the true standard deviation σ, dependent on the number of data n (Let $\hat{\sigma}$ = 1, α = 0.1)

n	3	4	5	6	7
Cnf_{lower}	0.58	0.62	0.65	0.69	0.71
Cnf_{upper}	4.42	2.92	2.37	1.91	1.80

Table 2: Basic assumptions to properly use ordinary least squares (OLS) linear regression models [2].

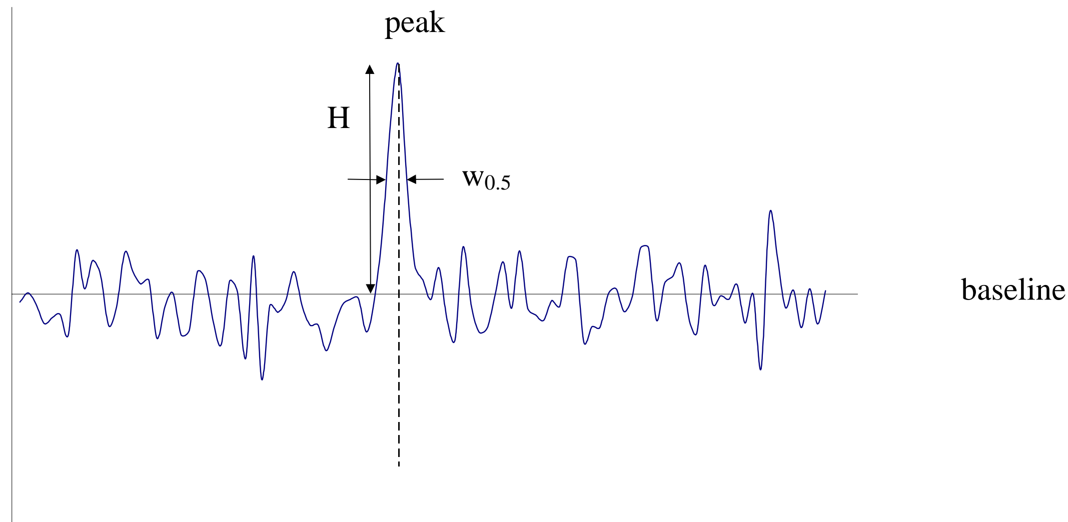
Assumptions	**violated if e.g.:**
Linearity	non-linear relationships, e.g. enzyme kinetics, TLC calibrations or other saturation effects
x is error-free	error in preparing standard solutions
Error in y is normally distributed	transformed raw data, counts data
Homoscedastic[1)] error in y	use of UV/Vis-detectors, volume dosage of standard solutions (e.g. HPLC injectors)
Error terms have zero mean (no systematic errors)	wrongly prepared standards
Error is uncorrelated	use of several dilutions from one stock solution to obtain calibration standards; memory effects; trends with time, e.g. varying UV lamp intensity, temperature-dependant drifts, instability of samples or chemicals

1) compare glossary

Fig. 1: Description of analytical quality and classification of the major performance parameters. Error impairs precision, which is strongly related to the sensitivity and the LOQ, while bias degrades accuracy. The robustness of a method can be influenced by both random error and bias.

Fig. 2: Determination of the detection limit (LOD) using Eq. 1, according to Ph. Eur., V 2.2.28, 2.2.29 [5].

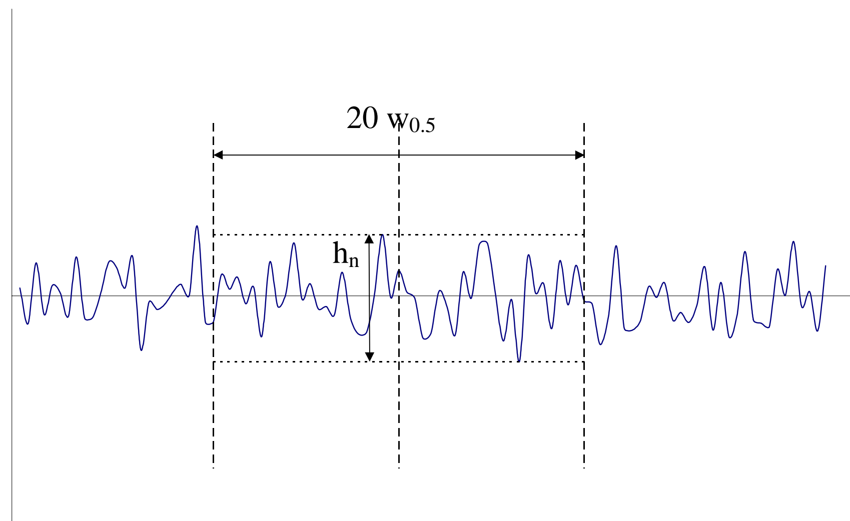
A) Chromatogram with peak signal



H Peak height from top to baseline (best straight line through noise)

$w_{0.5}$ Peak width at half height

B) Determination of noise from the chromatogram of a blank sample



20 $w_{0.5}$ Region corresponding to the 20fold of $w_{0.5}$. This region can be symmetrical to the signal of interest (as shown here), or asymmetrical, if required due to matrix signals

h_n maximum amplitude of the baseline noise in the 20-fold-$w_{0.5}$-region.

Fig. 3. Graphical explanation for the estimation of cnf(x_0) using Eq. 22 [2]; compare [12].