Validation of Analytical Methods:

Review and Strategy

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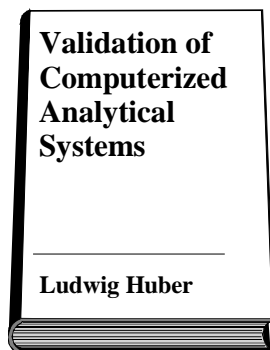
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Ludwig Huber
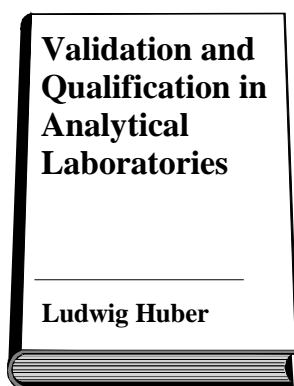
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Validation of Analytical Methods: Review and Strategy

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Abstract

The process of analytical method's validation should demonstrate that the method is fit for its purpose. The validation should follow a plan that includes the scope of the method, the method performance characteristics and acceptance limits. Parameters usually examined in the validation process are limits of detection and quantitation, accuracy, precision, selectivity/specificity, linearity, range and ruggedness. A validation report should be generated with all experimental conditions and the complete statistics. When 'standard' methods are used in the laboratory, their scope should be in line with the scope of the laboratories' method requirements and the suitability of the entire analytical system in the specific laboratory's environment should be verified for the method.

Introduction

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated

- before their introduction into routine use
- whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics
- whenever the method is changed, and the change is outside the original scope of the method.

Method validation has received considerable attention in literature and from industrial committees and regulatory agencies. The *Guidance on the Interpretation of the EN 45000 Series of Standards and ISO/IEC Guide 25* includes a chapter on the validation of methods (1) with a list of nine validation parameters. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use (2) has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics. An extension with more detailed methodology is in preparation and nearly completed (3). The United States Environmental Protection Agency (US EPA) prepared a guidance for methods development and validation for the Resource Conservation and Recovery Act (RCRA) (4). The American Association of Official Analytical Chemists (AOAC), the United States Environmental Protection Agency (USP) and other scientific organizations provide methods that are validated through multi-laboratory studies.

The United States Food and Drug Administration (US FDA) has proposed guidelines on submitting samples and analytical data for methods validation (5-7). The United States Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation (8).

There are no official guidelines referring to biological fluids. The pharmaceutical industry uses methodology published in the literature (9,10). The most comprehensive document was published as the 'Conference Report of the Washington Conference on *Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies* held in 1990 (sponsored by the American Association of Pharmaceutical Scientists, the AOAC and the US FDA, among others) (10). The report presents guiding principles for validation of studies in both human and animal subjects that may be referred to in developing future formal guidelines.

Representatives of the pharmaceutical and chemical industry have published papers on the validation of analytical methods. Hokanson (11,12) applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green (13) gave a practical guide for analytical method validation with a description of a set of minimum requirements for a method. Renger and his colleagues (14) described the validation of a specific analytical procedure for the analysis of theophylline in a tablet using high performance thin layer chromatography (HPTLC). The validation procedure in that article is based on requirements for European Union multistate registration. Wegscheider (15) has published procedures for method validation with special focus on calibration, recovery experiments, method comparison and investigation of ruggedness. The association of official analytical chemists (AOAC) (16) has developed a Peer-Verified Methods validation program with detailed guidelines on what parameters should be validated.

This article gives a review and a strategy for the validation of analytical methods for both in-house developed as well as standard methods and a recommendation on the documentation that should be produced during and at the end of method validation.

Strategy for Validation of Methods

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to the unknown samples analyzed in the routine. The preparation and execution should follow a validation protocol, preferably written in a step by step instruction format. Possible steps for a complete method validation are listed in table 1.

1. Develop a validation protocol or operating procedure for the validation
2. Define the application, purpose and scope of the method
3. Define the performance parameters and acceptance criteria
4. Define validation experiments
5. Verify relevant performance characteristics of equipment
6. Qualify materials, e.g. standards and reagents
7. Perform pre-validation experiments
8. Adjust method parameters or/and acceptance criteria if necessary
9. Perform full internal (and external) validation experiments
10. Develop SOPs for executing the method in the routine
11. Define criteria for revalidation
12. Define type and frequency of system suitability tests and/or analytical quality control (AQC) checks for the routine
13. Document validation experiments and results in the validation report

Table 1. Steps in Method Validation

First the scope of the method and its validation criteria should be defined. These include:
- compounds,
- matrices,
- type of information: qualitative or quantitative,
- detection and quantitation limits,
- linear range,
- precision and accuracy
- type of equipment and location

The method's performance characteristics should be based on the intended use of the method. For example, if the method will be used for qualitative trace level analysis, there is no need to test and validate the method's linearity over the full dynamic range of the equipment. Initial parameters should be chosen according to the analyst's best judgment. Finally, parameters should be agreed between the lab generating the data and the client using the data.

The scope of the method should include the different types of equipment and the locations where the method will be run. For example, if the method is to be run on one specific instrument in one specific laboratory, there is no need to use instruments from other vendors or to include other laboratories in the validation experiments. In this way the experiments can be limited to what is really necessary.

Before an instrument is used to validate a method, its performance should be verified using generic standards (18,19). Satisfactory results for a method can only be obtained with well-performing equipment. Special attention should be paid to the equipment characteristics that are critical for the method. For example, if detection limit is critical for a specific method, the instrument's specification for baseline noise and, for certain detectors also the response to specified compounds, should be verified. Any

material used to determine critical validation parameters, such as reagents and reference standards, should be checked for accurate composition and purity.

If there is no or little information on the method's performance characteristics, it is recommended to prove the methods suitability for its intended use in initial experiments. These studies should include the approximate precision, working range and detection limits. If the preliminary validation data appear to be inappropriate, either the method itself, the equipment, the analysis technique or the acceptance limits should be changed. In this way method development and validation is an iterative process. For example, in liquid chromatography selectivity is achieved through selection of mobile phase composition. For quantitative measurements the resolution factor between two peaks should be 2.5 or higher. If this value is not achieved, the mobile phase composition needs further optimization.

There are no official guidelines on the sequence of validation experiments and the optimal sequence can depend on the method itself. Based on my experience, for a liquid chromatographic method the following sequence has been proven to be useful:

1. selectivity of standards (optimizing separation and detection of standard mixtures)
2. precision of retention times and peak areas
3. linearity, limit of quantitation, limit of detection, range
4. selectivity with real samples
5. trueness/accuracy, at different concentrations
6. ruggedness (interlaboratory studies)

The more time consuming experiments such as accuracy and ruggedness are put towards the end. Some of the parameters as listed under two to five can be measured in combined experiments. For example, when the precision of peak areas is measured over the full concentration range, the data can be used to validate the linearity.

During method validation the parameters, acceptance limits and frequency of ongoing system suitability tests or quality control checks should be defined. Criteria should be defined to indicate when the method and system are out of statistical control. The goal is to optimize these experiments such that with a minimum number of control analyses the method and the complete analytical system will provide long-term results that will meet the objectives defined in the scope of the method.

A validation report should be prepared that includes:

- objective and scope of the method (applicability, type)
- type of compounds and matrix
- detailed chemicals, reagents, reference standards and control sample preparations
- procedures for quality checks of standards and chemicals used
- safety considerations
- method parameters
- critical parameters indicated from robustness testing
- listing of equipment and its functional and performance requirements, e.g. cell dimensions, baseline noise, column temperature range
- detailed conditions on how the experiments were conducted, including sample preparation
- statistical procedures and representative calculations
- procedures for quality control in the routine (e.g., system suitability tests)
- representative plots, e.g. chromatograms, spectra and calibration curves
- method acceptance limit performance data
- the expected uncertainty of measurement results
- criteria for revalidation
- person who developed and initially validated the method
- summary and conclusions

Validation of Standard Methods

A laboratory applying a specific method should have documentary evidence that the method has been appropriately validated. "The responsibility remains firmly with the user to ensure that the validation documented in the method is sufficiently complete to meet his or her needs." (1) This holds for standard methods, for example, from EPA, ASTM, ISO or USP, as well as for methods developed in-house. If standard methods are used, it should be verified that the scope of the method and validation data, for example, sample matrix, linearity, range and detection limits comply with the laboratory's analyses requirements; otherwise, the validation of the standard method should be repeated using the laboratory's own criteria. The laboratory should demonstrate the validity of the method in the laboratories environment.

Full validation of a standard method is recommended where no information on type and results of validation can be found in the standard method documentation.

Revalidation

Operating ranges should be defined for each method based on experience with similar methods, or they should be investigated during method developments. These ranges should be verified during method validation in robustness studies and should be part of the method characteristics. Availability of such operating ranges makes it easier to decide when a method should be revalidated. A revalidation is necessary whenever a

method is changed and the new parameter is outside the operating range. If, for example, the operating range of the column temperature has been specified to be between 30 and 40 °C, the method should be revalidated if, for whatever reason, the new operating parameter has been selected as 41 °C. Revalidation is also required if the sample matrix changes and if the instrument type changes, for example if a brand with significantly different instrument characteristics is used. For example, a revalidation is necessary, if a High-Performance Liquid Chromatographic method has been developed and validated on a pump with a delay volume of 5 ml and the new pump only has 0.5 ml.

Part or full revalidation may also be considered if system suitability tests or the results of quality control sample analysis are out of preset acceptance criteria and the source of the error can not be tracked back to instruments or anything else.

Parameters for Method Validation

The parameters for method validation have been defined in different working groups of national and international committees and are described in literature. Unfortunately some of the definitions are different between different organizations. An attempt for harmonization was made for pharmaceutical applications through the International Conference on Harmonization (2,3) where representatives from the industry and regulatory agencies from USA, Europe and Japan defined parameters, requirements and, to some extent, also methodology for analytical methods validation. The parameters as defined by the ICH and by other organizations and authors are summarized in Table 1 and described in brief in the following paragraphs.

- **Specificity (*)**
- **Selectivity**
- **Precision (*)**
  - repeatability (*)
  - intermediate precision (*)
  - reproducibility (**)
- **Accuracy (*)**
- **Trueness**
- **Bias**
- **Linearity (*)**
- **Range (*)**
- **Limit of detection (*)**
- **Limit of quantitation (*)**
- **Robustness (**)**
- **Ruggedness**

Table 1. Possible parameters for method validation
(*) Included in ICH publications
(**) Terminology included in ICH publication but are not part of required parameters

Selectivity/specificity

The terms *selectivity* and *specificity* are often used interchangeably. A detailed discussion of this term as defined by different organizations has been made by Vessmann (17). He particularly pointed out the difference between the specificity as defined by IUPAC/WELAC and ICH. (IUPAC: International Union of Pure and Applied Chemistry, WELAC: Western European Laboratory Accreditation Conference).

Even inconsistent with ICH, the term *specific* generally refers to a method that produces a response for a single analyte only while the term *selective* refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term *selectivity* is usually more appropriate. The USP monograph (8) defines selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix. Selectivity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength.

It is a difficult task in chromatography to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound. While in the past chromatographic parameters such as mobile phase composition or the column have been modified, more recently the application of spectroscopic detectors coupled on-line to the chromatograph have been suggested (3,5). UV/Visible diode-array detectors and mass-spectrometers acquire spectra on-line throughout the entire chromatogram. The spectra acquired during the elution of a peak are normalized and overlaid for graphical presentation. If the normalized spectra are different, the peak consists of at least two compounds.

The principles of diode-array detection in HPLC and their application and limitations to peak purity are described in the literature (20-22). Examples of pure and impure HPLC peaks are shown in Figure 1. While the chromatographic signal indicates no impurities in either peak, the spectral evaluation identifies the peak on the left as impure. The level of impurities that can be detected with this method depends on the spectral difference, on the detector's performance and on the software algorithm. Under ideal conditions, peak impurities of 0.05 to 0.1% can be detected.
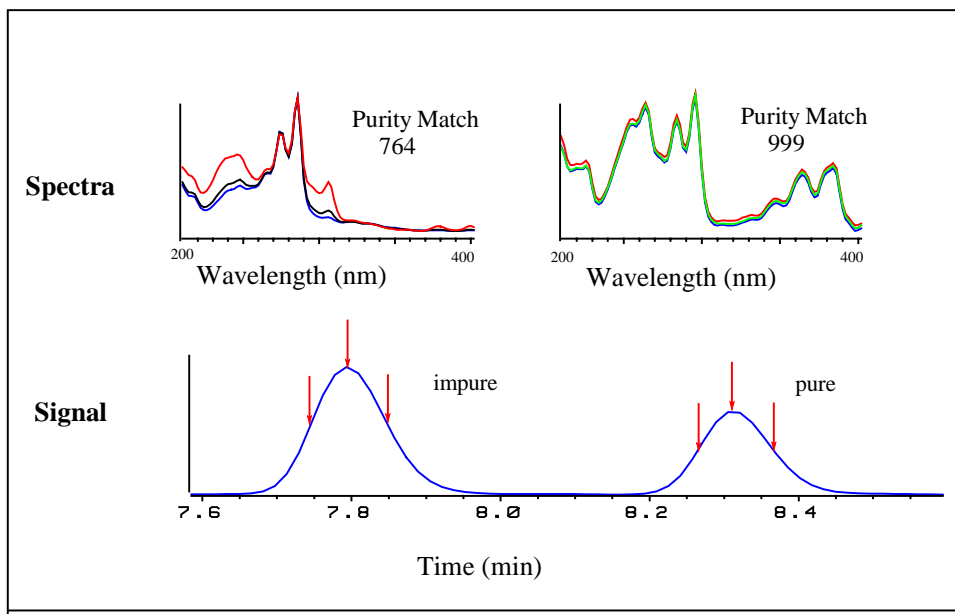
Figure 1. Examples of pure and impure HPLC peaks. The chromatographic signal does not indicate any impurity in either peak. Spectral evaluation, however, identifies the peak on the left as impure.

Precision and Reproducibility

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. The measured standard deviation can be subdivided into three categories: repeatability, intermediate precision and reproducibility (2,3). Repeatability is obtained when the analysis is carried out in one laboratory by one operator using one piece of equipment over a relatively short timespan. At least

- 5 or 6 determinations of
- three different matrices at
- two or three different concentrations

should be done and the relative standard deviation calculated. The acceptance criteria for precision depend very much on the type of analysis. While for compound analysis in pharmaceutical quality control precision of better than 1 % RSD is easily achieved, for biological samples the precision is more like 15% at the concentration limits and 10% at other concentration levels. For environmental and food samples, the precision is very much dependent on the sample matrix, the concentration of the analyte and on the analysis technique. It can vary between 2% and more than 20%.

The AOAC manual for the Peer Verified Methods program (16) includes a table with estimated precision data as a function of analyte concentration.

Analyte %	Analyte ratio	Unit	RSD (%)
100	1	100%	1.3
10	10^{-1}	10%	2.8
1	10^{-2}	1%	2.7
0.1	10^{-3}	0.1 %	3.7
0.01	10^{-4}	100 ppm	5.3
0.001	10^{-5}	10 ppm	7.3
0.0001	10^{-6}	1 ppm	11
0.00001	10^{-7}	100 ppb	15
0.000001	10^{-8}	10 ppb	21
0.0000001	10^{-9}	1 ppb	30

Table 1. Analyte concentration versus precision within or between days (Ref. 16)

Intermediate precision is a term that has been defined by ICH (2) as the long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method's intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these. The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

Reproducibility as defined by ICH (2,3) represents the precision obtained between laboratories. The objective is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts and by using operational and environmental conditions that may differ from but are still within the specified parameters of the method (interlaboratory tests). Validation of reproducibility is important if the method will used in different laboratories.

- Differences in room temperature and humidity
- Operators with different experience and thoroughness
- Equipment with different characteristics, e.g. delay volume of an HPLC system
- Variations in material and instrument conditions, e.g. in HPLC , mobile phases composition, pH, flow rate of mobile phase
- Equipment and consumables of different ages
- Columns from different suppliers or different batches
- Solvents, reagents and other material with different quality

Table 3. Typical variations affecting a method's reproducibility

Accuracy and recovery

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The true value for accuracy assessment can be obtained in several ways.

One alternative is to compare results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known. Secondly, accuracy can be assessed by analyzing a sample with known concentrations, for example, a certified reference material, and comparing the measured value with the true value as supplied with the material. If such certified reference material is not available, blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible.

The concentration should cover the range of concern and should particularly include one concentration close to the quantitation limit. The expected recovery depends on the sample matrix, the sample processing procedure and on the analyte concentration. The AOAC manual for the Peer Verified Methods program (16) includes a table with estimated recovery data as a function analyte concentration.

Active Ingred. [%]	Analyte ratio	Unit	Mean recovery [%]
100	1	100%	98-102
>=10	10^{-1}	10%	98-102
>=1	10^{-2}	1%	97-103
>=0.1	10^{-3}	0.1 %	95-105
0.01	10^{-4}	100 ppm	90-107
0.001	10^{-5}	10 ppm	80-110
0.0001	10^{-6}	1 ppm	80-110
0.00001	10^{-7}	100 ppb	80-110
0.000001	10^{-8}	10 ppb	60-115
0.0000001	10^{-9}	1 ppb	40-120

Table 4. Analyte recovery at different concentrations (Ref 16)

Linearity and calibration curve

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in samples within a given range. Linearity is determined by a series of three to six injections of five or more standards whose concentrations span 80-120 percent of the expected concentration range. The response should be directly or by means of a well defined mathematical calculation proportional to the concentrations of the analytes. A linear regression equation applied to the results should have an intercept not significantly different from zero. If a significant nonzero intercept is obtained, it should be demonstrated that there is no effect on the accuracy of the method.

Frequently the linearity is evaluated graphically in addition or alternatively to mathematical evaluation. The evaluation is made by visual inspection of a plot of signal height or peak area as a function of analyte concentration. Because deviations from linearity are sometimes difficult to detect two additional graphical procedures can be used. The first one is to plot the deviations from the regression line versus the concentration or versus the logarithm of the concentration, if the concentration range covers several decades. For linear ranges the deviations should be equally distributed between positive and negative values.

An other approach is to divide signal data by their respective concentrations yielding the relative responses. A graph is plotted with the relative responses on the Y-axis and the corresponding concentrations on the X-axis on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn in the graph corresponding to, for example, 95 percent and 105 percent of the horizontal

line. The method is linear up to the point where the plotted relative response line intersects the 95 percent line. Figure 2 shows a comparison of the 2 graphical evaluations on the example of caffeine using High-Performance Liquid Chromatography.
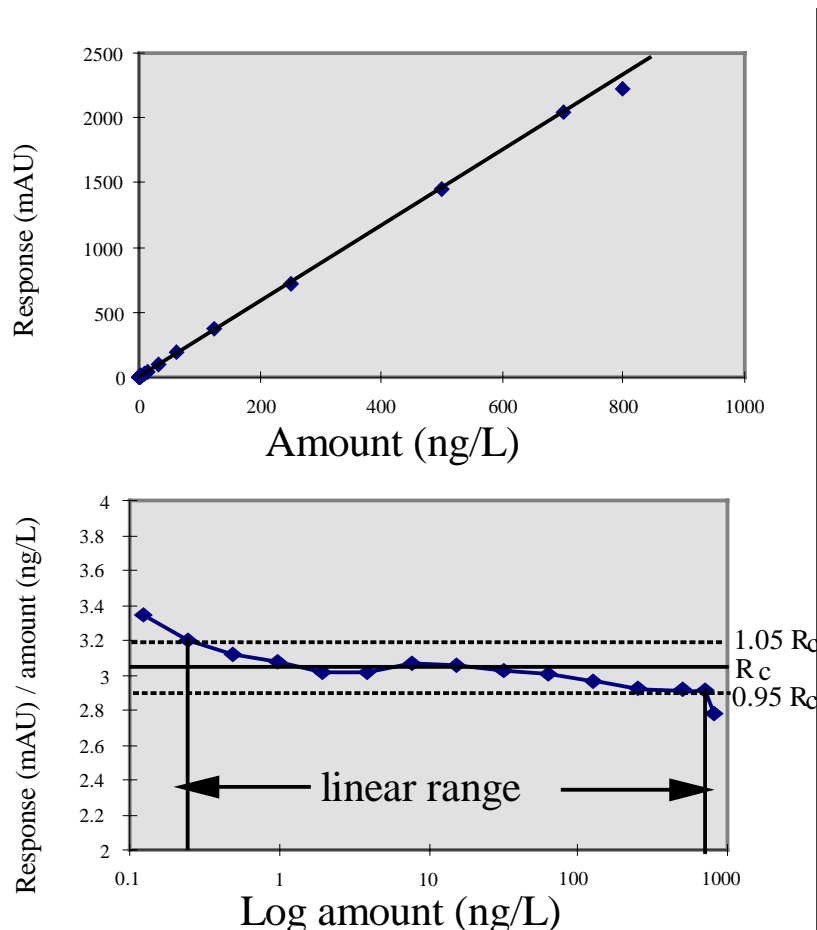

Figure 2. Graphical presentations of linearity plot of a caffeine sample using HPLC. Plotting the sensitivity (response/amount) gives clear indication of the linear range. Plotting the amount on a logarithmic scale has a significant advantage for wide linear ranges. Rc = Line of constant response.

Range

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (e.g. percentage, parts per million) obtained by the analytical method.

Limit of Detection and Quantitation

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. In chromatography the detection limit is the injected amount that results in a peak with a height at least twice or three times as high as the baseline noise level.

The limit of quantitation is the minimum injected amount that gives precise measurements , in chromatography typically requiring peak heights 10 to 20 times higher than baseline noise. If the required precision of the method at the limit of quantitation has been specified, the EURACHEM (1) approach can be used. A number of samples with decreasing amounts of the analyte are injected six times. The calculated RSD% of the precision is plotted against the analyte amount. The amount that orresponds to the previously defined required precision is equal to the limit of quantitation.
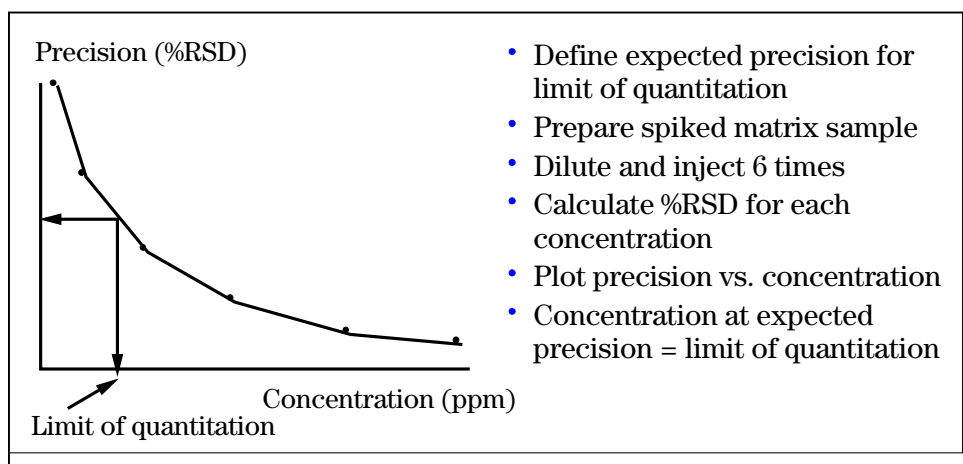

Figure 3. Limit of quantitation with the EURACHEM method.

Robustness

Robustness tests examine the effect operational parameters have on the analysis results. For the determination of a method's robustness a number of chromatographic parameters, for example, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition are varied within a realistic range and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range. Obtaining data on these effects will allow to judge whether a method needs to be revalidated when one or more of parameters are changed, for example to compensate for column performance over time. In the ICH document (3) it is recommended to consider the evaluation a methods robustness

during the development phase, but it is not required to be included as part of a registration application.

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