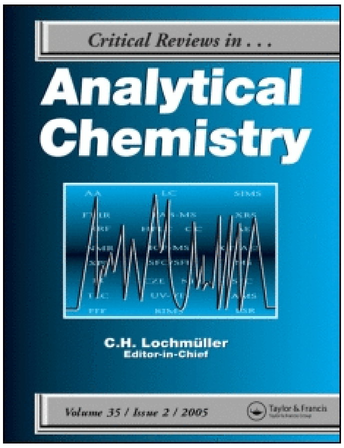

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The Role of and the Place of Method Validation in the Quality Assurance and Quality Control (QA/QC) System

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The Role of and the Place of Method Validation in the Quality Assurance and Quality Control (QA/QC) System

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One of the main developmental trends in chemical analytics is the determination of analytes at ever decreasing detection levels in samples with increasingly complex matrices. This is an extremely difficult and complicated task therefore it poses a great challenge to analytical chemists, and it requires that appropriate attention be paid to the Quality Assurance and Quality Control (QA/QC) of the obtained measurements. In this article QA/QC system is described. Following topics are discussed: (1) measurement uncertainty, (2) traceability of analytical results, (3) reference materials, (4) interlaboratory comparisons and (5) method validation. The problems and challenges during method validation process are presented.

Keywords quality control, quality assurance, method validation, validation parameters, uncertainty, traceability, reference material, interlaboratory comparison

INTRODUCTION

The basic feature of each measurement is its reliability. The numerical value representing a given physicochemical property can only be called a result of a measurement when it is reliable. In recent years, an increased interest in a wide spectrum of issues related to quality of analytical measurements has been observed (1). This is surely due to significance given to assuring the traceability of the obtained results, particularly when the "European Research Space" is concerned. In order to secure the quality of measurements, the use of proper tools is necessary. The branch of science dealing with the measurement results and measuring processes is metrology. The application of metrology to analytical measurements still encounters problems and difficulties.

From among the tools used by the "chemical metrology", the following ones are of particular interest:

- validation of analytical procedures,
- (certified) reference materials,
- interlaboratory comparisons.

Each of the above tools must be used with a full understanding of its features and in a proper way.

The priority purpose of each analytical procedure is to obtain reliable and, as much as it is possible, complete information about the researched object based on the measurement results from the analysis of the collected sample. In order to realize the above purpose the two basic conditions listed below have to be fulfiled:

- the composition of a collected sample should mirror the composition of the analyzed object (**requirement of the sample representativeness**),
- a measurement result should reflect the true content of analyte in the analyzed sample (**requirement of mesurement reliability**).

The results are obtained in the course of measuring procedures during which proper "analytical tools" (i.e. analytical procedures and measuring equipment) are used. Those tools have to be properly adopted to work, so as to obtain reliable results.

The assurance of the adequate quality of analytical measurements consists of the verification of measuring equipment reliability and establishing the range of applicability for given analytical procedures.

QUALITY ASSURANCE/QUALITY CONTROL

The measurement result is only reliable if its traceability and uncertainty have been determined (2). In order to fulfill those requirements the measurement results should be obtained by applying the analytical procedure that has been previously validated. In turn, the validation of an analytical procedure requires

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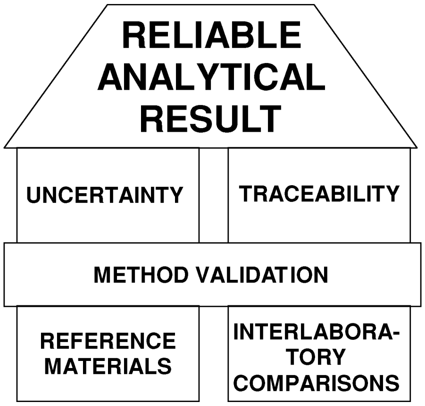

FIG. 1. Position of the elements of quality control/quality assurance system for obtaining a reliable analytical result.

the use of reference materials (preferably, of the certified type) and the participation in various interlaboratory studies.

In Figure 1 a schematic presentation of specific elements of a quality control/quality assurance system used for obtaining reliable analytical results is shown.

The following elements are included in the *QA*/*QC* system of analytical measurements:

Assurance of measurement traceability; it is a very important aspect in times of the developing and omnipresent globalization which also encrouches on science. It is not possible to conduct all the measurements in one laboratory and by one person, however only such scenario would have had guaranteed the truly comparable results. The assurance of traceability is a necessary condition which, when fulfilled, can result in comparable results obtained in different places and at various times.

Uncertainty estimation. Reliable research results should be obtained with validated procedures that include the use of reference materials and the participation in interlaboratory studies; they need to be traceable and have the estimated uncertainty. Otherwise, the measurements are not analytical results and can be treated as "random numbers."

Validation of an analytical procedure. The procedure should be characterized as precisely as possible (i.e. validated) so the highly reliable measurement results can be obtained with it.

Application of reference materials. A determination of validation parameters for an analytical procedure is only possible when they can be established from the analysis of reference materials or by comparing the obtained results with those from the reference method. Therefore the application (and production) of the appropriate certified reference materials becomes most critical.

Organization of and participation in interlaboratory studies. Such research allows to determine certified values of the produced reference materials as well as some validation parameters (reproducibility, robustness and ruggedness). In the light on the above, it is noteworthy that specific components of the *QA*/*QC* system of analytical results are interconnected.

Those elements are interrelated.

In order to secure traceability of measurements, it is necessary to use certified reference materials as well as previously validated analytical procedures.

Moreover, the following conditions should be fulfilled during the validation procedure:

- certified reference materials should be used with regard to precision estimation,
- participation in interlaboratory studies should be secured in reference to estimation of reproducibility and ruggedness,
- uncertainty should be estimated as it allows to control the whole analytical procedure.

During interlaboratory comparisons the reference materials as well as analytical procedures are being applied. On the other hand, the purpose of such studies is to determine certified values for the produced reference materials. In the production of reference materials, specifically during homogeneity and durability testing, the analytical procedures are used. One of the characteristics of a reference material is the associated uncertainty. As mentioned earlier, the uncertainty estimation is necessary in the production of reference materials.

Despite the fact that uncertainty is not a validation parameter, it is obvious that its estimation significantly improves the reliability of the obtained results. In the process of constructing the "uncertainty budget" it is necessary to determine the influence of all the possible parameters in a given analytical procedure on the expanded uncertainty estimate. This forces a precise and very detailed "investigation" of the whole analytical procedure which allows the control of it.

Interrelations among the particular *QA*/*QC* system components are schematically presented in Figure 2.

The obtaining of reliable measurement results is only possible when all the components of the *QA*/*QC* system are applied.

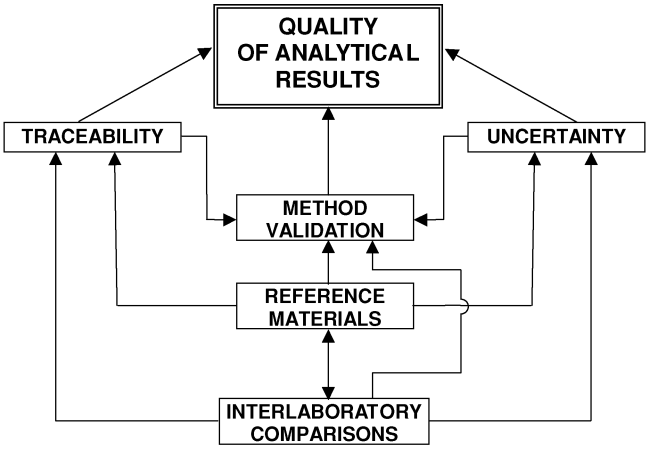

FIG. 2. Components of the QA/QC system of an analytical process.

The term “reliable data” is closely related to data quality. It is the quality of a measurement and its control and assurance that allow to determine and to prove the measurement’s reliability. Different aspects of quality have specific meanings in the analytics.

Quality— a fulfillment of certain requirements; those are described by a relevant standard of the Quality Assurance System and also by any additional “internal” standards.

Analytical quality— conformance of the characteristics obtained by means of chemical analysis to the assumed target values. Information quality has four components, i.e. the quality of measurements, process quality, quality of the applied tools, and the quality of labor and work management.

Quality control, QC— an elaborated procedure used to obtain the measurements of desired quality. A quality control system consists of the following components:

- adequate professional knowledge of the employed personnel,
- proper calibration of laboratory equipment,
- good laboratory practice,
- standardized procedures.

In practice, the term “quality control” is often translated as quality assurance, here defined as methods and procedures used in order to fulfill the quality requirements (e.g. control charts, determinations on blanks, spiked samples, repeated determinations and blind samples).

Quality control and quality assurance system for measurements consists of many elements, which can be sorted into the two main groups as follows:

1. elements that allow to trace and estimate the precision of the results, such as:

- periodic analysis of control samples,
- application of control charts,
- auditing system.

2. elements that allow to evaluate the accuracy of the obtained results, such as:

- including samples of the certified reference materials in analysis,
- comparing the produced data with those obtained from the same samples by employing a reference method,
- analyzing samples spiked with a standard,
- conducting interlaboratory calibrations,
- applying control charts,
- using an appropriate auditing system.

At present, there are three systems of quality assurance designed for analytical laboratories, namely,

- Good Laboratory Practice, *GLP*,
- accreditation of a laboratory according to EN 45001 or ISO Guide 17025 (the accreditation according to ISO Guide 25 was possible until January 2003),
- certification according to the ISO 9000 series.

TRACEABILITY

Traceability is a measurement’s feature that allows a comparison of the results obtained in different locations, at various times and by means of different procedures. For the results to differ only by the content of an analyte in samples and not by varying conditions during the analysis, it is necessary that each analytical result is traceable to the established standard (3). Traceability is a parameter that allows scaling of the results obtained under different conditions by using the same point of reference (4–6). For practical reasons, it is prerequisite to have a proper traceability system in order to secure traceability (7, 8). Such system consists of documented comparisons of relevant standards to the so-called primary standard, e.g. in case of weighing, a balance calibrated with the certified standard weights should be used. Those weights have to be standardized according to national standards which, in turn, have been based on the international standard for a kilogram of mass. Such a series of comparisons forms an unbroken chain that can illustrate the idea of traceability. Its schematic presentation has been shown in Figure 3, while the rationale and meaning behind it in Figure 4.

In accordance with the recommendations of EURACHEM/CITAC Guide (9), establishing traceability for a given analytical procedure requires the following:

- determining the measured value,
- choosing the proper measuring procedure and establishing a relevant model equation,
- proving (via validation) the correctness of chosen measuring conditions and model equation,
- defining and determining the values of all parameters that may influence the final result,
- defining the strategy for achieving traceability by choosing appropriate standards and calibration procedure,
- estimating uncertainty for the applied measuring procedure.

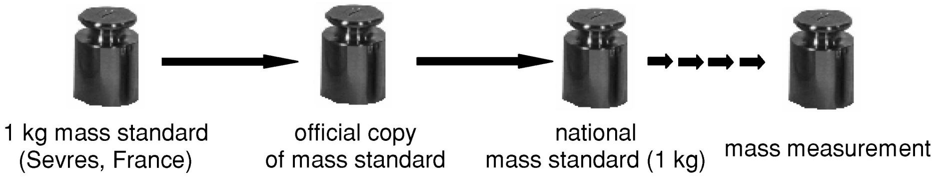

FIG. 3. The idea of traceability.

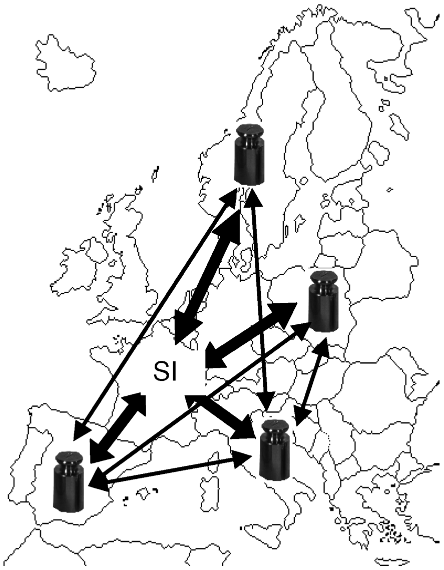

FIG. 4. Rationale and meaning of traceability

On the other hand, according to ISO, there are three basic stages for establishing traceability, as follows:

1. establishing the link between a measured value and relevant standard
2. through an unbroken chain of comparisons, and
3. estimating all the uncertainty components associated with specific links in the traceability chain.

The term "unbroken chain" needs more explanation; it means the lack of analytical information loss in connection to the multi-stage analysis. Each step of the analytical procedure requires a comparison to the reference method, reference material or directly to the SI unit.

MEASUREMENT UNCERTAINTY

Uncertainty is the basic characteristic of each measurement. Uncertainty is always present, at each step of the procedure (10, 11).

The main sources of uncertainty encountered during sample analysis by means of appropriate analytical procedure are listed in Table 1.

Uncertainty estimation results in the increased measurement's reliability, it allows to compare data from interlaboratory studies, and it helps to make decision about statistical significance of the difference between the measurement and a relevant reference value.

Measurement uncertainty is a component of all uncertainties associated with the single steps of an analytical procedure. Therefore, it is necessary to determine the uncertainty sources and values for specific stages of that procedure (12).

A flow-chart of actions to be undertaken during uncertainty estimation of the analytical result, according to the Guide to the Expression of Uncertainty in Measurement (13), has been shown in Figure 5.

TABLE 1
Possible sources of uncertainty in analysis

Uncertainty sources	
Human factors	Factors related to equipment
- erroneously or no precisely defined measured value, - non-representative sample, - incorrect application of determination procedure, - person-specific systematic reading error on analogue readouts, - lack of knowledge about all external factors influencing the analytical result, - uncertainty associated with calibration of the instrument used,	- resolution of the used measuring instrument, - uncertainty due to the standards and/or reference materials used, - uncertainty of parameters that have been determined as separate, measurements and later used for the final result calculations, e.g. physicochemical constants, - approximations and assumptions in connection to the usage of a given instrument during analysis, - fluctuations during the repeated measurements under seemingly identical external conditions,

The final analytical result consists therefore of:

- a determination of the measured value, including its unit,
- the result and its expanded uncertainty ($y \pm U$, including units for y and U),
- the value of coefficient k for which the expanded uncertainty was calculated.

Uncertainty estimate is one of the necessary components of an analytical result.

REFERENCE MATERIALS

Reference materials play a significant role in all elements of the measurement quality assurance system. They are necessary for validating the analytical procedures (i.e. accuracy determination), and often are the object of interlaboratory comparisons; they help in establishing measurement uncertainty (although such method is not always recommended) and secure traceability.

For over 20 years the European Commission has been in support of scientific programmes that focus on improving the quality of physical, biological and chemical measurements by using proper reference materials (14).

Presently, at least several companies manufacture reference materials. However the broadest range of such materials is

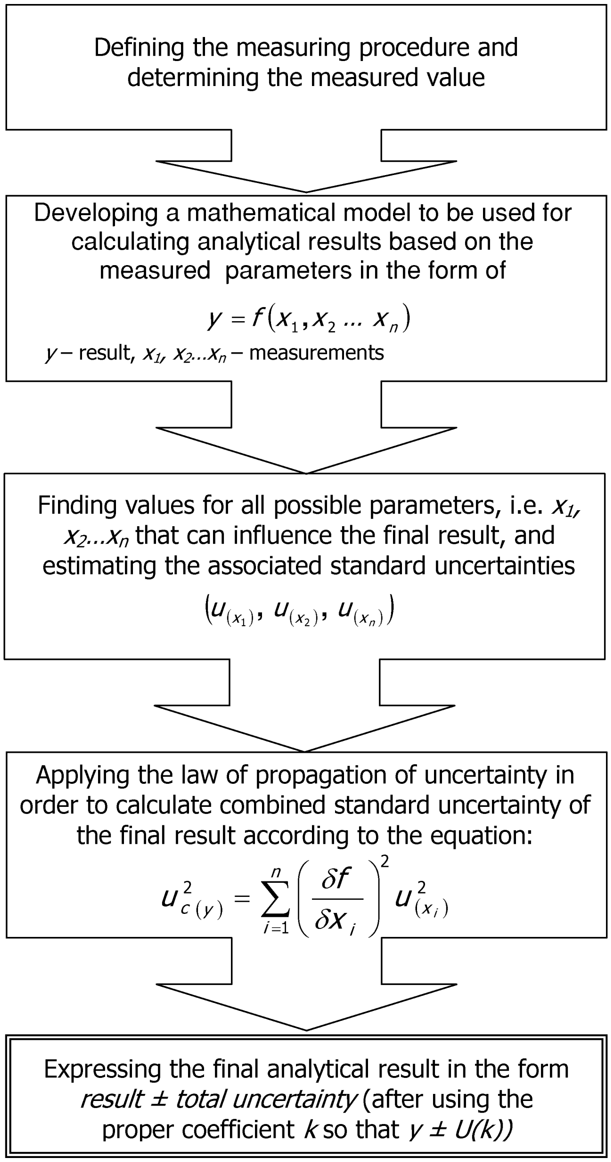

FIG. 5. Schematic presentation of the procedure for estimating the expanded uncertainty of an analytical result.

offered by NIST (USA) and IRMM within the EU framework. Most recently, the attempts are being made to introduce European Reference Materials, (ERM) (15). The general classification of reference materials is presented in Figure 6.

A general procedure for preparing reference materials is shown schematically in Figure 7.

A big challenge for both manufacturers and users is posed by gaseous reference materials, also called standard gas mixtures. In the case of preparing standard gas mixtures of toxic, reactive and badly smelling substances, the production of the exactly defined amounts of analytes via thermal decomposition of surface compounds is the original methodological approach.

This approach is based on using surface compounds that are chemically bound to the carrier's surface (e.g. silica gel, porous glass, glass rods covered with silica gel and glass fibers) and undergo thermal decomposition or chemical regrouping those results in a release of the precisely specified amount of a volatile compound. The released compounds are washed out from the reaction chamber (e.g. chromatographic injector or thermal desorber) with a stream of diluting gas which now becomes a stream of standard gas mixture. The schematic presentation of chemical modification on the carrier's surface, and creation of the exactly known volatile component that is determined during the thermal decomposition process, has been shown in Figure 8.

The present extent of the above mentioned approach's application for preparation of standard gas mixtures, which constitute a specific type of matrix-free reference materials, are presented in Table 2 (16–32).

Reference materials are necessary in each analytical laboratory, and their application falls into the following main areas:

- testing professional skills of an analyst or of a laboratory,
- routine control of accuracy (precision and correctness) of performed determinations,
- determination of validation parameters,
- laboratory accreditation,
- laboratory quality control,
- estimation of measurement uncertainty,
- traceability control and assurance,
- calibration of measuring equipment.

TABLE 2
Possible ways of producing volatile analytes via thermal decomposition of appropriate surface compounds

Carrier type	Released volatile analyte	Literature
Silica gel	CO, CO_2	(16, 17)
	CO_2	(18)
	C_2H_4	(19)
	CH_3Cl	(20, 21)
	NH_3	(22)
	CH_3NH_2	(22)
	$(C_2H_5)_2NH$	(22)
	$(C_2H_5)_3N$	(22)
	CH_2CHCH_2NCS	(23)
	C_4H_9NCS	(23)
	CH_3SH	(24)
	C_3H_7SH	(24–26)
	CH_3CHO	(27)
Rods covered with silica gel	CO, CO_2	(28)
Porous glass	C_2H_4	(29)
Glass fibers	C_2H_4	(30)
Fiberglass	CH_3Cl	(31)

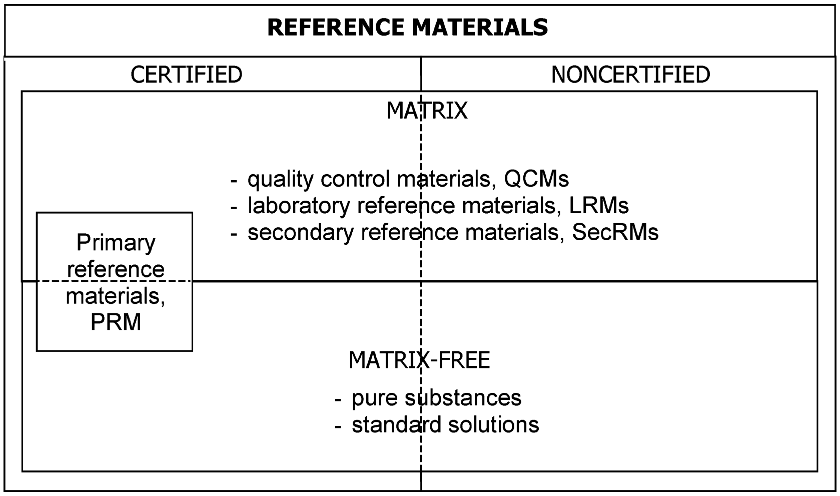

FIG. 6. Classification of reference materials.

Because of high diversity of matrices and analytes, it is not possible to prepare appropriate reference materials for all measuring procedures. Although a good knowledge of a given analytical procedure and the available reference materials allow to make the right choice. Reference materials should be used following the rules of good laboratory practice that have been established for trace analysis. Such materials should be stored under conditions that guarantee their stable composition throughout the entire usage time.

INTERLABORATORY STUDIES

Interlaboratory studies allow a comparison of measurements obtained by means of the same or similar test samples by two or a larger number of laboratories according to the earlier established conditions (33). As a result, the novel value is assigned to

- analytical procedure, i.e. measuring procedures are tested,
- laboratory, i.e. proficiency of a laboratory is checked,
- reference materials, i.e. reference materials get certified.

A generalized scheme for conducting interlaboratory studies is shown in Figure 9.

METHOD VALIDATION

One of the most important steps in the process of introducing a new analytical procedure into practice is its validation. The validation of analytical procedure consists of checking the important characteristics of this procedure. The final goal is to achieve the analysis that is reliable, precise, fully controled by the analyst, and which gives reliable results. Validation mainly allows to define a given analytical procedure.

Based on the validation parameters, it is possible to evaluate the range of applicability for a given procedure, which in turn allows to make the optimal choice of procedure.

As mentioned earlier, in order to obtain the reliable results with a known uncertainty estimate the analytical procedure which has been previously validated needs to be used.

Validation studies are most frequently conducted under the following circumstances (34, 35):

- when a new analytical procedure is being developed,
- when widening the range of applicability for a given procedure is investigated, e.g. for determining the same analyte, but in different matrix,
- when the results of the Quality Control protocol suggest that validation parameters vary in time,
- when a given analytical procedure is going to be used in another laboratory,
- when different measuring equipment will be used,
- when measurements will be conducted by another person,
- when the new analytical procedure is being compared with the known standard procedure.

A number of parameters that form a basis of the validation process depend on many factors, such as:

- type of analytical studies which will be conducted with the application of a given analytical procedure (e.g. qualitative or quantitatve analysis, single sample analysis or routine analytical work),
- requirements posed for a given analytical procedure,
- time consumption and costs associated with conducting the validation process.

The parameters considered necessary for the validation of different types of analytical procedures are presented in Table 3 (34, 36).

As the number of validation parameters inceases so does the time required to conduct the validation process. Moreover, more stringent limit values (expected values) of specific parameters imply higher frequency of checking, calibrating or even re-validating of a given analytical procedure. The full validation of an nalytical procedure is not always necessary. In such

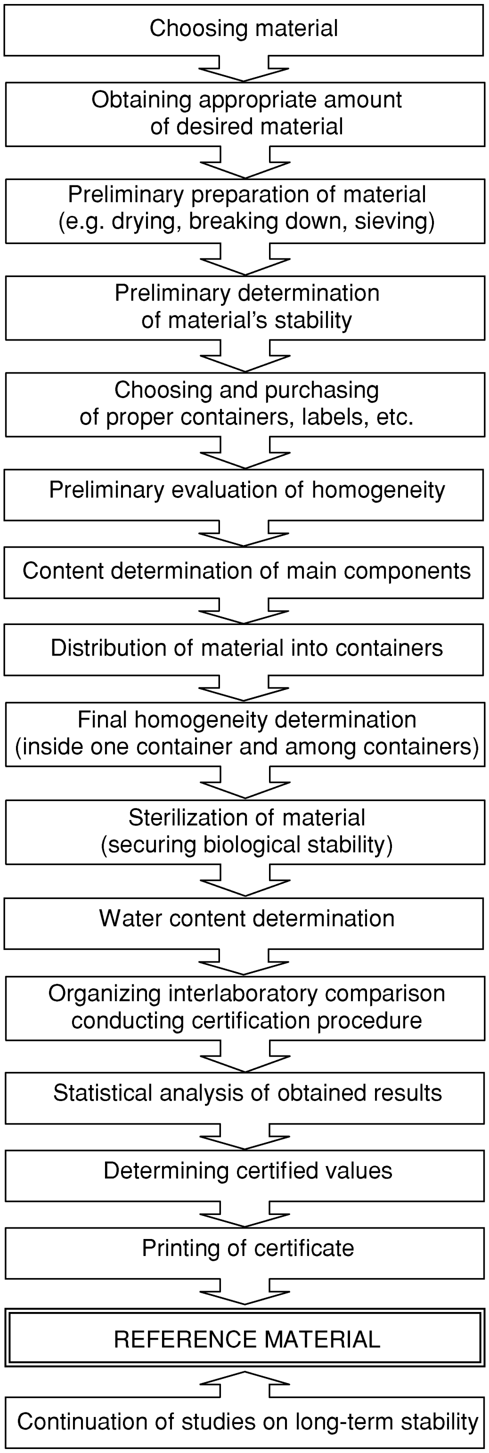

FIG. 7. A general procedure for reference materials preparation.

a case, it should be stated which parameters are included in the validation process.

Table 4 contains the parameters which, according to the recommendations of ICH (37, 38) and USP (39), should be included in the validation process.

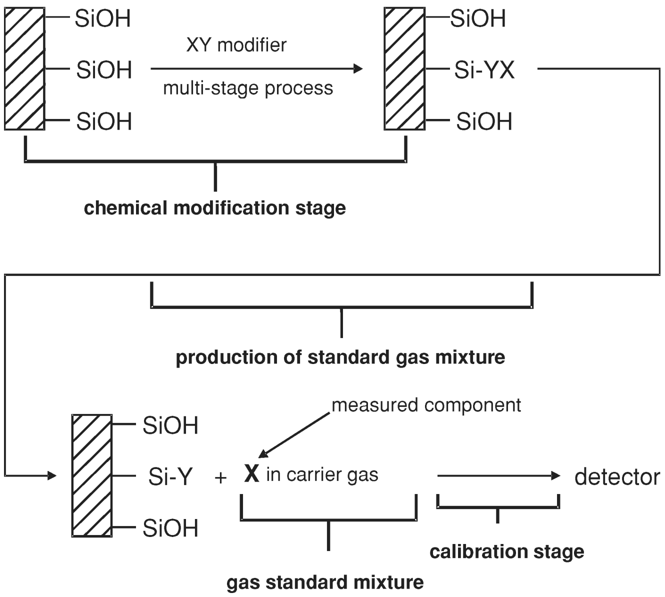

FIG. 8. Schematic presentation of standard gas mixture production by means of thermal decomposition of a compound deposited on carrier's surface.

Besides the estimation of validation parameters, the following basic characteristics of an analytical procedure should be defined before proceeding with the validation (34):

- type of the measured component (analyte),
- analyte concentration level,
- analyte concentration range,
- type of sample matrix,
- presence of interfering substances, and whether they should be determined,
- existing legal regulations and requirements which the analytical procedure should fulfill,
- type of the obtained information, i.e. quantitative or qualitative,
- required limits of detection and quantification,
- expected and required accuracy (precision and correctness) of the procedure,
- required robustness of the procedure,
- necessary measuring equipment (i.e. does a given procedure require the use of a precisely specified measuring device or any of the similar type?),
- possibility to apply the validated procedure in other laboratory.

The validation steps of an analytical procedure can be conducted in any order. However, after considering the sequence in which specific parameters are determined it seems most logical that the order should follow a flow chart presented in Figure 10 (34).

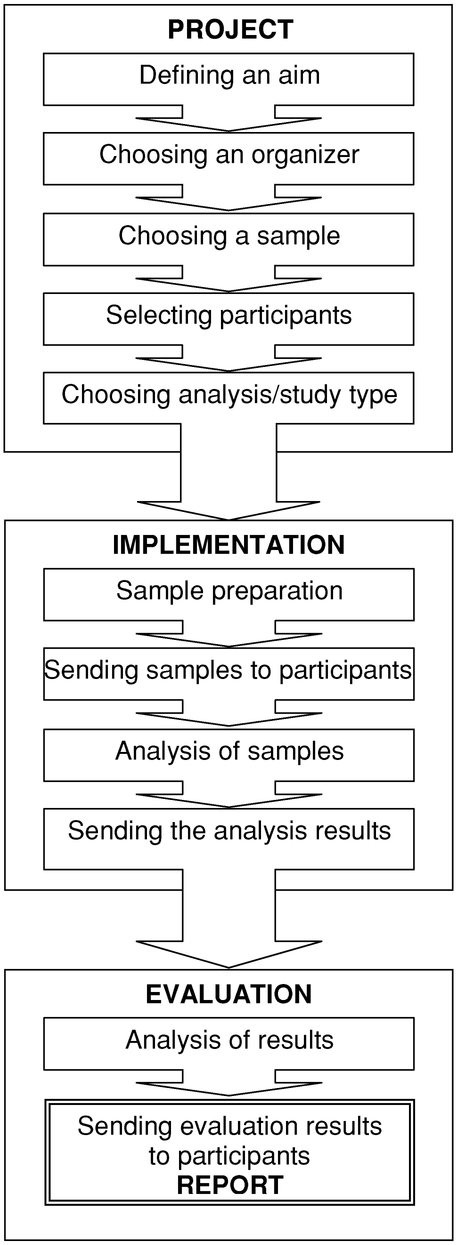

FIG. 9. A generalized outline for conducting interlaboratory studies

During the validation process various tools can be used, such as (1):

- blank samples, including reagent blanks
- standard solutions (e.g. calibration solutions and test samples),
- samples that contain a known amount of added analyte, i.e. analyte enriched samples,
- (certified) reference materials,
- repeating the procedure,
- statistical analysis of the measurement sets.

CHARACTERISTICS OF VALIDATION PARAMETERS, THEIR EVALUATION, PROBLEMS AND SOLUTIONS

The discussion on determining the validation parameters should begin with the description and explanation of the nature of an analytical measurement. The main object of interest for an analyst is the analytical signal obtained as a consequence of the conducted measurement. The aim of the analyst's work is to obtain analytical information about the researched object based on the application of proper measuring procedure; the obtained signal "codes" information about the studied sample. Therefore, it is the analyst's role to decode the obtained signal, and in addition to make sure that the decoded information is reliable (40). Analytical process and the applicable analytical procedures are tools used for the decoding.

One feature of each analytical signal is its size. In case of some measurements, it is also possible to assign the location parameter. Validation parameters are determined based on the analysis of the measured values, and that is something to be kept in mind during the validation of an analytical procedure.

Specificity and Selectivity

The first validation parameter that should be determined is selectivity. It is only logical to make sure, before proceeding with the characterization of an analyte based on the measured analytical signal, that this signal has been exclusively a result of the analyte's presence in the sample.

It is quite common to confuse selectivity with specificity and vice versa, however, both terms differ in a significant way. According to IUPAC recommendation (41), selectivity should be defined as follows: "Selectivity of a method refers to the extent to which it can determine particular analytes under given conditions in mixtures or matrices, simple or complex, without interferences from other components." Moreover, IUPAC stated that "specificity is the ultimate of selectivity", and the term specificity should be used preferentially.

Selectivity therefore is the capability of a method to distinguish a given analyte from other substances. This feature is mainly a function of the described measuring technique; however, it might vary with the type of compound or matrix. A method can be called specific when it shows the highest selectivity. On the other hand, Valcárcel (42) gives a different definition of selectivity: "[it is] the capability of an analytical process to receive signals whose size almost entirely depends on the content of the measured analyte present in a sample".

The following practical definition can also be proposed (1) "specificity/selectivity is a capability of accurate and precise determination of the occurrence and content of analyte/group of analytes in the presence of other components in the real sample under given measuring conditions." Therefore selectivity is one of the main parameters that characterizes and describes an analytical procedure, particularly in the case of trace analysis (43).

From a practical point of view, an analytical measurement is considered selective when the obtained analytical signals can

TABLE 3
The parameters whose determination is necessary for difereent types of analytical procedures (34, 36)

Parameter	Qualitative analysis	Impurity test		Assay test
		Limit impurity test	Quantitative impurity test	
Precision	−[a]	−	+	+
Correctness	−	−[a]	+	+
Specificity	+	+	+	+
Limit of Detection	−[a]	+	−	−
Limit of Quantification	−[a]	−	+	−
Linearity	−[a]	−	+	+
Measuring range	−[a]	−[a]	+	+
Ruggedness	+	+	+	+

[a] It might be determined.

be discriminated, and when a value attributable to the specific analyte's feature can be assigned to each signal; this definitely depends on the type of the received signal. In case the signal is described by its intensity alone, it should be proven that the signal's size depends exclusively on the measured characteristic of a given object. For example, when weighing a sample on the analytical balance, one has to be sure that the measured value results from the real sample mass and not from unclean balance. This example shows that the topic of selectivity should also be considered in case of direct measurements.

Selectivity is dealt with differently when analytical signals have an additional attribute, i.e. location. This takes place in chromatography, where retention time is the additional characteristic of the analytical signal assigned to a given analyte. In such case, it is necessary and obligatory to determine a minimum difference of location parameters for specific analytes which allows discrimination of the signals.

TABLE 4
List of the analytical procedure parameters that should be validated according to the recommendations of ICH (37, 38) and USP (39)

Parameter	ICH	USP
Precision		
- repeatability	+	+
- intermediate precision	+	
- reproducibility	+	
Accuracy	+	+
Limit of detection	+	+
Limit of quantification	+	+
Specificity/selectivity	+	+
Linearity	+	+
Measuring range	+	+
Robustness		+
Ruggedness		+

Selectivity of the measuring process mainly depends on the composition of the analyzed sample (42). Selectivity is increasingly more difficult to achieve when:

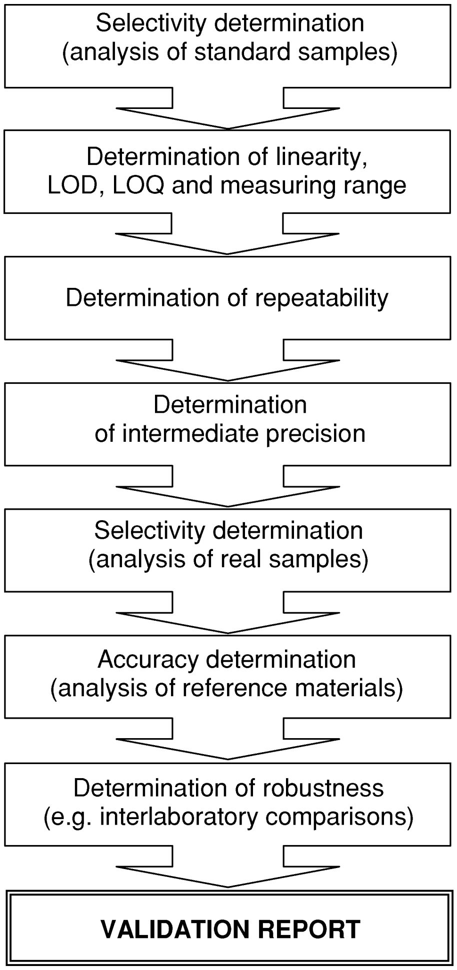

FIG. 10. The sequence in which validation parameters are determined (34).

TABLE 5
Recommended selectivity parameters (42)

Analytical technique	Selectivity parameter
Potentiometry	$E = E_0 \pm \frac{RT}{z_A F} \ln(a_A + \sum_B K_{AB} a_B^{z_a/z_A})$
Chromatography	$R_s = \frac{\Delta t}{\frac{1}{2}(W_A + W_B)}$
Non-chromatographic separation	$\alpha_{A/B} = \frac{(K_D)_A}{(K_D)_B}$

Where: E—potential, E_0—formal potential, R—gas constant, T—temperature, F—Faraday constant, K_{AB}—selectivity coefficient (from Nikolsky equation), A—analyte, B—interfering agent, z—charge of an ion, R_s—selectivity, Δt—separation (retention time difference between two peaks), W—chromatographic peak width at base, α—separation coefficient, K_D—separation constant.

- sample's composition is increasingly uncertain,
- complexity of sample matrix increases,
- matrix components do not differ much from the analyte,
- there is a bigger number of analytes,
- analyte concentration is decreasing,
- the similarity of analytes is increasing.

Increased selectivity can be achieved by:

- applying analytical methods that are selective,
- eliminating the influence of interfering components by exclusion or concealment,
- extracting an analyte from the matrix.

Depending on the type of analytical technique, selectivity can be expressed in various ways. The basic relations that allow selectivity calculations for the selected analytical techniques are presented in Table 5 (42).

Linearity

When a specific characteristic can be assigned to the analytical signal with surety then a relationship between those two parameters can be investigated. In analytical chemistry, it is a linear relationship that is the most common. In case of the majority of analytical measurements, a calibration step is used during which analyte contents are assigned to the respective values of analytical signal (44). In order to describe the relationship between the analytical signal and analyte content, the linear regression method is mainly employed. This particular method is also used for estimating some validation parameters of an analytical procedure, such as:

- linearity,
- correctness (bias estimation),
- limit of detection and limit of quantification.

Linear regression is also widely employed for calibrating the measuring instruments.

Linearity has been defined as the range of analytical procedure in which the analytical signal is proportional to the measured analyte concentration. The most common way of determining linearity is based on the use of calibration plot obtained for a given measuring instrument. To this end, samples of standard solutions at the minimum of six concentration levels are measured (usually, three replicates are used for each level). Obviously, the choice of analyte concentrations in standard solutions should reflect the expected analyte contents in the processed samples; usually, concentration range encompasses values from 50 to 150% of the expected measurements (45). Next, the linear regression method is used to estimate regression parameters.

According to some recommendations (46), it is sufficient to calculate the regression coefficient. Then, when the coefficient values reach at least *0.999* the linearity can be assumed in the concentration range for which standard solutions had been prepared during the calibration procedure. Unfortunately, such way of proving linearity not always leads to the correct conclusions. It is possible that the obtained high value of correlation coefficient r (or determination coefficient r^2) does not indicate linearity. An extreme example illustrating such case is the Anscombe's quartet (47, 48), in which linear regression method was used for four different data sets. The obtained regression coefficients, including the slope and intercept, were the same for each set (see Figure 11). The data sets can be found on the web page (49).

The value of correlation coefficient r can be used for assuming the linearity of an analytical procedure only when standard solutions used for the calibration fulfill the following requirements (45, 50, 51):

- their concentration range encompasses the expected analyte concentrations in the measured sample(s),
- their concentration range is not higher than three times the analyte concentration,
- they evenly cover the whole concentration range.

Moreover, it is very important to plot the data and to do the visual check of the plot.

Because of the ambiguity associated with the use of correlation coefficient r as linearity measure, other methods have been proposed. Additionally, the statistical significance of the calibration plot coefficients is determined. The slope should be significantly different from *0*, while the intercept should be equal *0*. *Student's t-test* is used to test these hypotheses.

Another approach is to draw a response plot in the form:

$$\frac{y}{x} = f(x) \quad [1]$$

where,

y—analytical signal from the measuring instrument,

x—analyte concentration in the standard sample respective to the signal.

In case when the concentration range is rather wide (threefold or more), the concentration values can be plotted on a logarithmic scale. In the graph, the constant response (usually

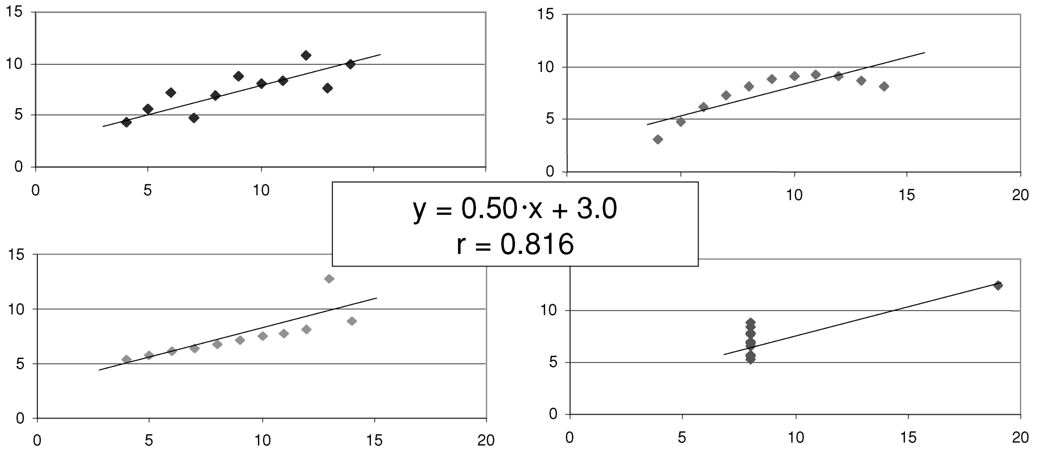

FIG. 11. The Anscombe's quartet (49).

expressed as an arithmetic mean from y/x values) is plotted as a line parallel to the X-axis together with admissible deviations (most frequently ± 5%). The values (points) that are outside the determined range denote analyte concentrations which lie outside the linear range of a measuring device.

Obviously, such method can only be applied when the intercept of the line plotted from the function $y = f(x)$ is not significantly different from *0*, and this condition is not always fulfilled. Some authors state openly that correlation coefficient r cannot be used to determine the linear association between two variables, and that it should be replaced with another statistical tool or specific tests for linearity (52). Many recommend testing for significant differences. Other methods and statistical tools can also be used, such as (53–56):

- lack-of-fit test,
- *Mandel's* fitting test,
- quality coefficient,
- *Student's t*-test.

In case when the linearity determination was based on the analysis of a series of standard solutions with a concurrent calibration plot, it would be logical to show to what degree the calibration curve reflects the values of analytical signals from standard solutions. This can be achieved by calculating relative deviations for the specific concentrations under assumptions that the analyte concentration in a standard sample is the reference value, and the measured value is the value calculated from the linear calibration function (57–59).

Linearity determination is not equivalent to stating that the function describing a relationship between the analytical signal and analyte content has just one form over the entire concentration range (the same coefficient values of the calibration curve). Linearity is a feature which describes the proportional dependence of signal on the size of the measured value, and within a given range it can be expressed by couple equations that depend on the analyte concentration level (57, 58).

The difference between correlation and regression should also be clarified. Correlation describes the degree of association between two variables, while regression the association type (52).

Limit of Detection and Limit of Quantification

Validation parameters to be determined next are the limit of detection and limit of quantification. The values of those parameters are tightly connected to measurement noise.

Signal-to-noise ratio (S/N). This parameter defines the ratio of an analytical signal to the mean noise level for a given sample; it has no unit. It can be used to determine the influence of noise level on the relative deviation of a measurement. There are various ways to calculate the S/N ratio, however, the most practical is to determine the ratio of the arithmetic mean from a series of measurements on blanks (or on samples containing very low analyte concentrations) to the standard deviation obtained for those measurements (1).

Limit of quantification, *LOQ* stands for the smallest amount or the lowest concentration of a substance that is possible to be determined by means of a given analytical procedure with the established accuracy, precision and uncertainty. *LOQ* should be estimated by using the proper standard measurement or standard sample; it cannot be extrapolated (60, 61).

Limit of detection, *LOD* is the lowest concentration that can be measured (detected) by means of a given analytical procedure with statistical significance (60, 61).

Method detection limit, *MDL* is the lowest concentration (the smallest amount) of an analyte that can be detected by using a given analytical procedure.

Instrumental detection limit, *IDL* of the used measuring device, e.g. detector is the lowest concentration (the smallest amount) of an analyte that can be detected, but not quantified, by means of a given measuring instrument.

Detection and quantification limits are both parameters that play a very significant role in the validation of analytical procedures. Although their meaning is clear, the determination of those parameters brings problems. This might result from a number of reasons, such as:

- multiplicity of definitions that describe both detection limit and quantification limit,
- practical difficulties that do not allow the unambiguous determination of the basic *LOD* parameter which is the noise level for a given instrument.

The ways of determining *LOD* depend on the following factors:

- analytical method type, i.e. manual method or method employing the appropriate measuring instrument,
- instrumental technique characteristics,
- possibility to obtain (prepare) blank samples.

Based on the above-mentioned parameters, there are couple ways of estimating *LOD* (61).

Table 6 contains the short characteristic of specific methods for *LOD* determination (61).

The choice of an appropriate method for *LOD* determination depends on the method's purpose as well as the requirements of a given analytical procedure. In the case of analytical procedure validation, it is recommended to use the method based on chemical metrology principles. Then the *LOD* value is connected to the statistical parameters such as, probability level and degrees of freedom.

In the case of individual measurements, less time-consuming methods are recommended.

It is required that the determined value of *LOD* be always presented together with the method description and relevant parameters used for its calculation. The determined *LOD* and *LOQ* values are also considered the parameters that describe the measurement quality for the used analytical procedure (62, 63).

Measuring range

Linearity and *LOQ* determinations allow to define the measuring range.

Measuring range is the range of values (analyte concentrations) within which the instrumental error is below the assumed value. In practice, it ranges from the *LOQ* value to the highest analyte concentration for which the instrument displays an increase of analytical signal. It should also be underlined that the higher limit of a measuring range can be (or rather ought to be) assumed to be the maximum concentration of analite in the standard solution for which the calibration curve had been plotted.

TABLE 6

Methods for determining detection limits: requirements, disadvantages and advantages (61)

Methods for calculating detection limits	Requirements	Disadvantages/Advantages
Visual check	Sample with known analyte content (standard solution or matrix standard)	Quick method; Estimation; Mostly used in case of classical analysis (non-instrumental); Requires vast analytical experience
Calculations based on the S/N ratio	Sample with known analyte content (standard solution or matrix standard)	Quick method; Used only for measuring equipment; It is possible to determine the S/N ratio
Calculations based on the measurements for sample blanks	Series of blanks or samples with known analyte content (standard solution or matrix standard)	Labor- and time-consuming method that does not consider the influence of calibration on LOD; Probability is used for estimating LOD
Calculations based on graphical method	Series of standard samples at 3 concentration levels; at least 6 measurements for each standard sample	Relatively quick method; It includes the influence of calibration procedure on LOD value
Calculations based on standard deviations of signals and slope of calibration curve	Series of blanks or samples with known analyte content (standard solution or matrix standard); Standard solutions for calibration curve preparation	Labor- and time-consuming method; It includes the influence of calibration procedure on LOD value; Method, "motivated" by metrology
Calculations based on limit of quantification, LOQ	Series of standard solutions; Assumed relative standard deviation for LOQ	Indirect method; LOD calculated based on the determined LOQ; LOD value (LOQ) depends on the assumed measurement precision

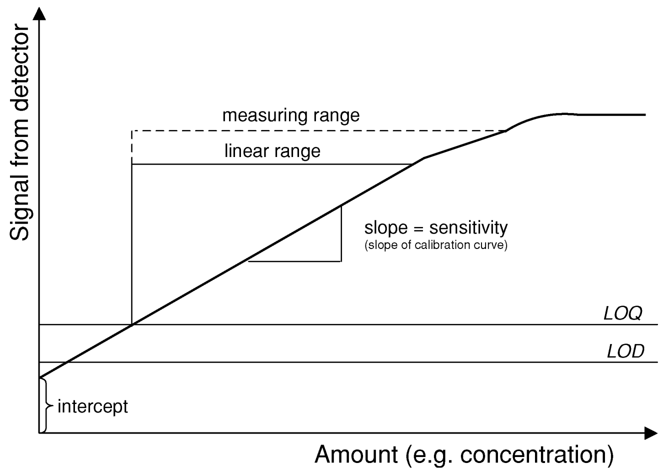

FIG. 12. Graphic representation of the definitions of linearity, measuring range, LOD and LOQ (64).

Sensitivity

Sensitivity is not included in the validation parameters set whose determination is obligatory. Its value can be simply determined based on the calibration curve parameters.

Sensitivity is a ratio of the difference in analytical signal to the change in analyte concentration that has caused it. Therefore, it describes the smallest difference in analyte content which can be determined with a given analytical procedure; it is expressed as the slope of a calibration plot, i.e. signal as a function of concentration. Some authors state that sensitivity is synonymous with *LOD*.

To summarize, a graphic representation of the definitions of linearity, measuring range, *LOD* and *LOQ* is shown in Figure 12 (64).

Precision

Precision describes the degree of agreement among the independent measurements obtained during analysis of a given sample by means of a given analytical procedure. It relates to random error, and measures the dispersion or spread around the mean value; it is usually expressed as standard deviation.

Depending on the conditions under which the results are obtained, we distinguish among repeatability, intermediate precision and reproducibility.

Repeatability means the precision of the results obtained under the same conditions (e.g. a given laboratory, analyst, measuring instrument and reagents). It is usually expressed as standard deviation of repeatability, variance, relative standard deviation or coefficient of variation.

Intermediate precision is a long-term deviation of the measuring process. In order to estimate it, the standard deviation of a series of measurements obtained in a given laboratory during the time period of couple weeks is used. Intermediate precision is a more general term than repeatability because more parameters can be changed for a given measuring procedure.

Reproducibility means the precision of the results obtained by different analysts in different laboratories by means of a given analytical procedure.

Each of the above parameters can be determined based on the standard deviation, relative standard deviation or the coefficient of variation. It is recommended that samples with various analyte contents and matrix composition should be used for reproducibility determination.

According to the *ICH* recommendations (37, 38), the value of standard deviation can be calculated as follows:

- conducting at least nine independent measurements over the entire measuring range (e.g. three independent measurements at three concentration levels)
- conducting six independent analyte determinations in standard samples at the concentration level that is relevant to the analyte concentration in the real sample
- using the results from six independent determinations for the analytes present in three different matrices and at two or three concentration levels.

On the other hand, *EURACHEM* (9) recommends 10 independent determinations that should serve as basis for the calculation of a standard deviation.

The repeatability value can be calculated for a very specific analytical procedure with the described and defined matrix composition (e.g. procedure for determining the content of analyte X in matrix Y) as well as for the one without the identified matrix composition. In the former case, the standard deviation is calculated from the measurements on samples containing the same matrix, while in the latter case, standard deviations should be estimated from the measurements on samples with different matrix compositions.

Actually, repeatability is the only validation parameter whose value can be determined without a calibration process as it can be calculated from the values of analytical signal. Repeatability calculated as the coefficient of variation, which is a relative parameter, does not require re-calculating the analytical signal size into the concentration values.

Intermediate precision is a broader term than repeatability because its value is influenced by the additional parameters such as (34, 35):

- human factors; different analysts conducting determinations as well as the lack of stability of a given analyst's performance over time;
- factors related to equipment; those stem from the fact that measurements can be conducted with the use of:
  - different instruments in a given laboratory,
  - standard solutions and reagents from various manufacturers or production batches,
  - different accessories, e.g. various *GC* columns with the same characteristics but from different manufacturers or production batches.

TABLE 7
Analytical procedure conditions to be fulfilled during the determination of repeatability, intermediate precision and reproducibility (34)

Conditions	Repeatability	Intermediate precision	Reproducibility
Equipment	C	V	V
Batch of accessories	C	V	V
Analyst	C	V	V
Matrix composition	V	V	V
Concentration	V	V	V
Batch of reagents	C	V	V
Laboratory conditions (temperature, humidity)	C	V	V
Laboratory	C	C	V

where, C – constancy; V – admissible variability.

When samples with the same analyte concentration level are used for precision determination, then the standard deviation is a sufficient parameter for performing the calculations. However, in the case of samples with different analyte content levels, the relative standard deviation or the coefficient of variation should be used. Both those parameters are used for comparing repeatability, intermediate precision and reproducibility.

The characteristics (i.e. constancy or variability) of the analytical procedure parameters that should be maintained during the determination of repeatability, intermediate precision and reproducibility have been presented in Table 7 (34).

Accuracy and Correctness

Accuracy is defined as the degree of agreement between the obtained (single!) measurement and the true (expected) value.

Correctness (**trueness**) is, on the other hand, the degree of agreement between the measurement (expressed as the mean value calculated from a series of measurements) and the expected value.

When analyzing the above definitions, it can be concluded that the term "accuracy" has been replaced with "correctness."

Correctness is the parameter that describes the degree of agreement between the results obtained with a given analytical

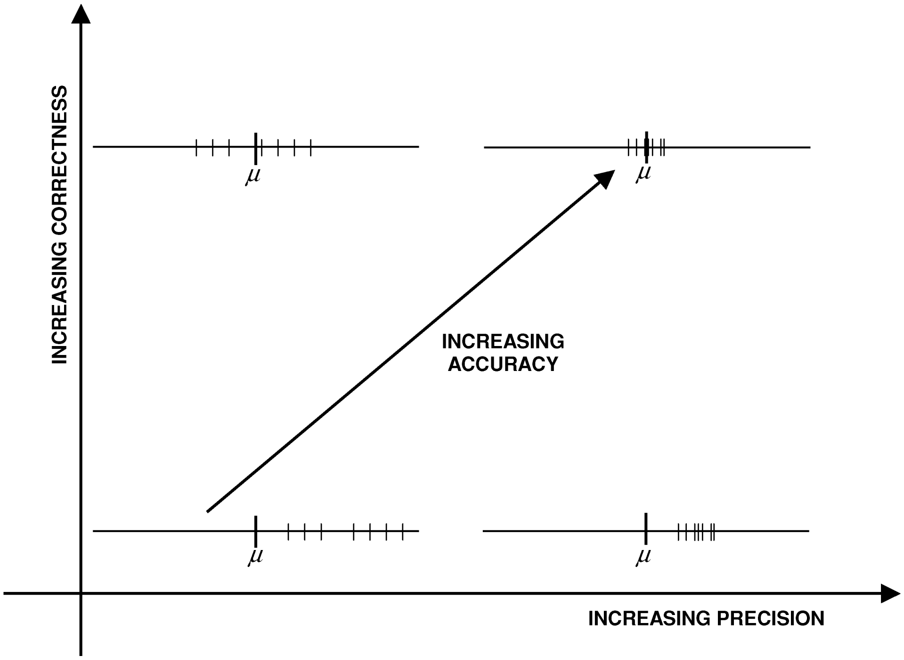

FIG. 13. Interrelations among correctness, precision and accuracy.

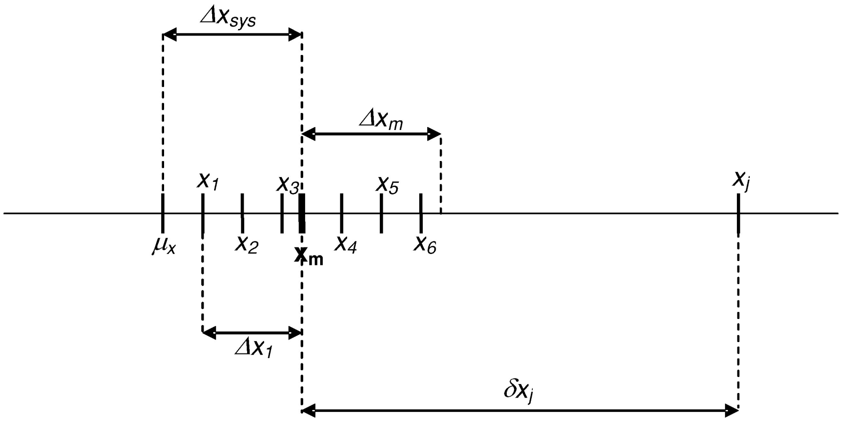

FIG. 14. Influence of specific error types on the measurement Δx_{sys}—bias of analytical methodology, Δx_i—random error of a single result, Δx_m—random error of an arithmetic mean, δx_j—gross error.

procedure and the real (expected) values. Procedure bias has the main influence on the value of correctness.

Accuracy is a combination of correctness and precision. For a given procedure, the more correct and precise the results, the more accurate every single measurement. A schematic presentation of interrelations among correctness, precision and accuracy are shown in Figure 13.

Obviously, such parameters as linearity and sensitivity also influence the accuracy of an analytical procedure.

The term accuracy is tightly associated with error terms. Depending on their type, errors have different impact on the analytical result. The influence of specific errors on the measurement is presented schematically in Figure 14.

Correctness and accuracy can be determined with the use of different approaches (65):

- analysis of an appropriate certified reference material;
- comparison of obtained result with that obtaining by applying a reference (primary, definitive) method;
- using standard addition method.

Ruggedness and Robustness

Robustness of a procedure is determined in order to estimate the influence of small fluctuations in analytical conditions on the final result. Its value has bearing on the measuring technique regime for a given analytical procedure (66). As the influence of small changes in parameters on the final analytical result increases, it becomes mandatory to maintain those parameters at the constant level. Robustness is a parameter related to the changes in internal conditions (67–69).

Ruggedness, on the other hand, is a parameter that defines the suitability of a given analytical procedure under varying conditions; it can be estimated from the reproducibility value (67–69).

Similarly to reproducibility, the robustness and ruggedness of an analytical procedure are determined via interlaboratory comparisons. However, the evaluation of an impact that the changing conditions have on the analytical procedure undergoing a validation process can be conducted within one laboratory (e.g. influence of temperature, purity and type of reagents used, pH and the conditions of chromatographic separation).

The values of both parameters for a given analytical procedure can be calculated based on the analysis of the differences among standard deviations from a series of measurements obtained while slightly changing the procedure's parameters.

SUMMARY

It has to be noted that the procedure can only undergo validation after the optimization studies have already been performed. The validation of an analytical procedure should be finished by preparing the final report that contains the following elements (1, 34):

- object and applicability of the analytical procedure (range of applicability),
- metrological basis,
- types of analytes and matrix composition,
- list of all used reagents, standards and reference materials including the specification sheets (i.e. purity, quality, manufacturer, and in the case of laboratory synthesis, its detailed description),
- description of the procedures for determining the purity of the used substances and the quality of standards,
- required rules of workplace safety,
- plan describing how a given procedure will be adopted from the laboratory conditions into routine measurements,
- procedure parameters,
- list of critical parameters, i.e. the parameters whose small variability may significantly influence the final measurement result; those parameters are defined based on the robustness of analytical procedure,

- list of all kinds of laboratory equipment including the instructions (e.g. dimensions, precision class, etc.) and schematic block diagrams in case of more complex devices,
- detailed description of conditions for conducting a given analytical procedure,
- description of the applied statistical methods including appropriate equations and calculations,
- quality control procedure in case of routine analyses,
- appropriate figures and graphs, e.g. chromatographs and calibration curves,
- conformity of the determined validation parameters with the target limit values,
- uncertainty of the measurement result,
- criteria to be fulfilled during the re-validation process,
- name and surname of the person who had performed validation,
- list of cited literature,
- summary and conclusions,
- confirmation and signature of a person responsible for checking and accepting of the validation process.

Validation of an analytical procedure is a necessary and prerequisite, although not sufficient, condition for obtaining reliable measurements (1, 34, 35). For several years the EU legislature has been introducing appropriate standards and directives in relation to the quality control/quality assurance of analytical results. Those legal acts are also becoming a necessity in Poland. However, there are obstacles on the way to implementing appropriate standards and rules into the laboratory practice; the lack of proper understanding of the purpose and reasoning behind the validation as a procedure preceding routine use of an analytical procedure is one of such obstacles. Validation is a complex process, therefore it is not easy to conduct. Main problems asocciated with incorrect validation processes can be divided into the following three groups:

- "conservatism", here meaning the attachment to old ways of checking the parameters or to old names,
- "terminology"; ambiguous definitions resulting from the so-called "free" translation, most frequently from English (70),
- "tools"; precisely defined procedures for determining of specific parameters are lacking.

The purpose of the project is an attempt to eliminate difficulties associated with the validation process which, in most cases, result from the lack of precise descriptions of how validation parameters are determined. It is well known that the determination of a validation parameter is tightly connected to the characteristics of a given analytical procedure. Therefore, the following aspects are very important:

- clear, non-ambiguous and fully understandable definitions of validation parameters,
- description of precise algorithms for determining of validation parameters,
- specification of potential problems that might occur during the validation process,
- defined procedure on how the conclusions about the quality, applicability and characteristics of an analytical procedure can be drawn based on the obtained validation parameters values,
- understandable description of estimating the uncertainty of an analytical result including a non-ambiguous and logical way to record the final determination,

During the validation process of an analytical procedure various tools are used, such as:

- appropriate standards and standard solutions,
- (certified) reference materials,
- participation in interlaboratory studies.

The quality of the obtained results depends on the metrological quality of standards and standard solutions that had been used in the validation process of the applied analytical procedure.

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