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Prevalidation in pharmaceutical analysis Part I. Fundamentals and critical discussion

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Abstract

A complete prevalidation, as a basic prevalidation strategy for quality control and standardization of analytical procedure was inaugurated. Fast and simple, the prevalidation methodology based on mathematical/statistical evaluation of a reduced number of experiments ($N \leq 24$) was elaborated and guidelines as well as algorithms were given in detail. This strategy has been produced for the pharmaceutical applications and dedicated to the preliminary evaluation of analytical methods where linear calibration model, which is very often occurred in practice, could be the most appropriate to fit experimental data. The requirements presented in this paper should therefore help the analyst to design and perform the minimum number of prevalidation experiments needed to obtain all the required information to evaluate and demonstrate the reliability of its analytical procedure. In complete prevalidation process, characterization of analytical groups, checking of two limiting groups, testing of data homogeneity, establishment of analytical functions, recognition of outliers, evaluation of limiting values and extraction of prevalidation parameters were included. Moreover, system of diagnosis for particular prevalidation step was suggested. As an illustrative example for demonstration of feasibility of prevalidation methodology, among great number of analytical procedures, Vis-spectrophotometric procedure for determination of tannins with Folin–Ciocalteu's phenol reagent was selected. Favourable metrological characteristics of this analytical procedure, as prevalidation figures of merit, recognized the metrological procedure as a valuable concept in preliminary evaluation of quality of analytical procedures.

Keywords: Prevalidation strategy; Standardization of analytical procedures; Quality control of laboratory methods; Performance testing; Experimental design; Method validation; Polyphenols; Folin–Ciocalteu's reagent

1. Introduction

Investigations in the field of pharmaceutical analysis and quality control of medicines require analytical procedures/methods with good performance characteristics. Reliable analytical measurements of pharmaceutical samples are an essential ingredient of sound decision involving many facets of society including safeguarding public health, improving the quality of medicines, etc. The ever-increasing volume of analytical literature on the medicines quality control sphere requires unambiguously evaluation of

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the advantages and disadvantages of an analytical procedure.

Naturally, validation of analytical procedure is unavoidable part in development of pharmacopoeial and other analytical procedures for quality control of medicines. However, validation procedure is time-consuming, expensive, and hard-working process and greatly depends on the purpose of method, the chosen technique, and the procedure in question.

For this reason, a *prevalidation* is proposed by the group of experts in SFSTP guide [1] as a first step in validation of chromatographic bioanalytical methods. The aim of the guide is to make an optimum use of the information obtained from the experiments performed during the prevalidation step in order to introduce an integrated, efficient design for the validation of chromatographic methods. The prevalidation in SFSTP guide allows the estimation of the most appropriate calibration model, the limit(s) of quantitation, and subsequently the calibration range as well as the optimum number of experiments to be performed in validation phase.

In the present manuscript, a complete prevalidation proposal based on peculiar approaches is exhibited. As an informative screening method, prevalidation should be useful for preliminary evaluation of an analytical process, with regard to reasonable need for validation and for systematically obtaining other valuable data, which cannot reveal by prescribed validation procedures, e.g. published by a number of bodies [2–7]. The key literature to this subject is valuable paper from Ermer [8], the former discussions on complete analytical procedure [9–11], and papers on standardization of quantitative analytical procedure [12–16].

An experimental design methodology e.g. fractional factorial design, star designs, Plackett–Burman design [17–20] or supersaturated design [21] could be applied to determine the set of conditions that are required to obtain a product or process with desirable, often optimal, characteristics [22]. The prevalidation phase could be skipped if consisted estimates of the performance criteria, after performed experimental design, are available to the analysts [23].

A robustness checking consists in estimating the main total effects, in detecting outliers, checking the curvature and in determining the main side effects [24]. The aim of the test is to verify the robustness of a method by identifying the factors that could be responsible for the nonrobustness of the method, i.e. the factors that cause a large change in response for a small change in their levels [21]. Although, robustness tests were performed, originally, at the end of method validation just before interlaboratory studies were executed [25–27], there is a tendency, nowadays, to apply the test much in the lifetime of a method, namely, at the end of method development or early in the validation procedure [4,28].

Suggested prevalidation procedure is not intended to replace or diminish the value of ruggedness/robustness testing. It is convenient to perform prevalidation strategy after the evaluation of reliability of analytical procedure by using experimental design approach. After performed tests, analytical procedure is proved applicable and subsequently enters in the ‘space of complete analytical procedure’.

Investigations within laboratory and simulated limited interlaboratory studies can be comprised in prevalidation process. This corresponds to the first and second stage in the development of an analytical procedure according to Conacher [29]. Namely, it was recognized that there were approximately three stages in the development of an analytical procedure: (1) estimation of acceptable performance parameters within a laboratory; (2) demonstration of successful performance in limited interlaboratory studies; and (3) demonstration of successful performance in recognized collaborative study. As a progress from the first to the third stage, the degrees of confidence that can be ascribed to the validity of particular procedure increase. The third stage represents what is generally accepted to be the highest degree of method validation, the first one can be called prevalidation, and in second stage, prevalidation and/or validation can be made. It is conceived that internal method prevalidation consists the prevalidation steps carried out within one laboratory, for instance, to prevalidate a new analytical method that has been developed or to verify that an analytical method adopted from some other source is applied sufficiently well.

Prevalidation is essential to test data validity, e.g. when validate (official) procedure might not exist, when insufficient time would be available for a full validation process, and in crisis situations. In these situations, a laboratory is required to generate analytical data on an unplanned, but urgent basis and decision

based on this data is likely to have considerable economic and/or public health consequences.

Prevalidation is rapid transfer process of value from the space of preferred (and typical) analytical characteristics to the space of real (and typical) analytical characteristics. Through this process, it was considered that any analytical method used in prevalidation process should be based on sound scientific principles and be capable of meeting certain acceptable performance parameters. Namely, submissions to compendia, e.g. [7] for new or revised analytical procedures should contain sufficient information to evaluate the relative merit of proposed procedure. However, if prevalidation procedure does not give favourable results, validation procedure will result in both increased costs and wasted time. After all, only favourable analytical prevalidation is *condition sine qua non* for analytical validation. In contrary to validation defined as a process of demonstrating that analytical procedure is suitable for its intended purpose [3,4], prevalidation is defined as *the formal evidence that an analytical system does what it is supposed to do and is continuing to do so*. This last definition has some important implications by analogy with Tranter [30], as it presupposes that there is a description of what the analytical system should do.

The aim of this work is to give the system and methods of prevalidation for establishment of figures of merit and to suggest criteria for general use in obtaining data that support method validation. Categories of prevalidation methods are given in Table 1. Moreover, the purpose of this paper is to give guidance in setting-up and interpretation of prevalidation. The proposal for the standardized system of measurement, standardized acceptance, and evaluation are made improving the known approaches [12,15]. Prevalidation is founded on good practice of descriptive and prognostic statistics.

The principles of method prevalidation presented in this paper are not applied to all types of analytical procedures occurred in practice. This prevalidation strategy is restricted and completely applicable to analytical procedures in which linear or quadratic calibration function is expected in proposed concentration range. The number of these procedures in pharmacopoeia is significant such as titrimetry, gravimetry, UV-Vis-spectrophotometry, atomic emission and absorption spectrophotometry, HPLC, etc.

Table 1
Classification of prevalidation methods

Categories	Class
Basic prevalidation measurements/strategy	1
Exploratory prevalidation	1.1
Full prevalidation	1.2
Special prevalidation methodology	2
Investigation of candidate procedures	2.1
Choice of the most suitable procedure	2.2
Acceptability of a procedure (in receiving laboratory)	2.3
Adoption of standardized analytical procedure	2.4
Sophisticate calibration of analytical procedure	2.5
Allocation problems in selectivity	2.6
Extraction of metrological characteristics	2.7
Identifying sources of trouble	3
Monitoring of analytical process	3.1
Measurement under normal, operational and environmental variables of the analytical procedure	3.2
The origin of systematic and gross errors	3.3
Checking of instruments and devices	3.4
Use and care of balances	3.5
Inter- and intra-laboratory studies	3.6

Analytical procedures for which this strategy gives as result some other nonlinear analyte–signal relationship, e.g. immunoassays and microbiological assays, are beyond the scope of this proposal. Moreover, even though the prevalidation concept is originally developed for pharmaceutical applications, it can reasonably be applied to many analytical procedures in different fields with similar specifications (toxicology, environmental, food chemistry, etc.). As a model for demonstration and presentation of prevalidation approach, Vis-spectrophotometric procedure for determination of tannins [31] with Folin–Ciocalteu's phenol reagent was randomly selected among great number of investigated and checked analytical procedures by authors. The intention of authors was to show feasibility of prevalidation strategy and to give a new impulse to the philosophy and further development toward validation for the needs of evaluation of analytical procedures and quality control in general.

1.1. Basic concept

Method prevalidation is carried out to diagnose the quality of an analytical procedure, i.e. a general process used to decide whether a method in question is capable of producing accurate and reliable data. The

aim of prevalidation is to obtain knowledge about analytical procedure and validation characteristics. This process serves to test validation parameters with the purpose of proving suitability of analytical procedure. The basis for useful prevalidation procedure is to obtain the most appropriate calibration function (often linear calibration function) covering at least a working range of one power of ten (see Section 1.7.1). The efficiency of prevalidation procedure is given by characteristic data such as constants of calibration and analytical evaluation function, standard deviation of procedure, limit of quantitation, metrological characteristics of the analytical procedure, and other.

Method prevalidation is the penultimate step in method development. In the development of reliable analytical procedures, this prevalidative work can be carried out purposefully with a few experiments and constitute the base for a next stage, i.e. validation of analytical procedure. This simple but very informative concept comprises the fixed general scheme of measurements to which a set of mathematical/statistical tests has to be applied. Investigation of dependent and independent variables, as components of analytical system, particularly relationship between them gives insight into the data quality and method's metrological characteristics. If these metrological characteristics satisfied prevalidation requirement, a validation of the analytical procedure has to be done as the following step.

1.2. Anatomy of prevalidation system

Once a candidate method has been obtained, it has to be shown to meet the requirements of the user, namely to measure a specific substance with a given precision, accuracy, detection limits, etc. [22]. Prevalidation can be divided into the *exploratory prevalidation* and *full prevalidation*. In the exploratory prevalidation stage, one determines, with a limited number of calibration samples, whether the analytical procedure can be considered a good candidate for its purpose. Then, the experimental plan and diagnosis on ad hoc basis is for $N = 8$ measurements. When the results are considered acceptable, a more detailed full prevalidation follows. Then, the experimental plan and diagnosis on full basis is for $N = 24$ measurements. Prevalidation acceptance criteria should be defined to indicate when the method under investigation provides results that meet objectives defined in the scope of the method. Typical acceptance prevalidation criteria are shown in Table 2.

Table 2
Acceptance criteria in prevalidation procedure

Prevalidation studies	Tasks for analytical procedure
Calibration linearity	Six levels, four replicates each[a]
Calibration function	Calculation, statistical/mathematical approach
Analytical function	Calculation, statistical/mathematical approach
Homogeneity	
ANOVA (blanks)	$F < 2.77$
Bartlett test	$\chi^2 < 20$
Precision	
Repeatability	R.S.D. < 5% (R.S.D. > 5% depending on the type of analysis and analyte concentration)
Limit of detection	$L_D = 3.3 s_{BN}/V$
Limit of quantitation	$L_Q = 10 s_{BN}/V$
Calibration range	Evaluation from linearity studies and precision
Suspect outlying values	$2.069 \leq \lvert x_i \rvert < 2.807$[b], maximum one value

[a] In spectrophotometry and other instrumental methods, it is recommended to perform two successive measurements of the same sample.
[b] Values of S^* and x^*.

Generally, an analytical procedure is established empirically and is unlikely to be optimal. To establish the optimum procedure, prevalidation methods have to be used in the first step. Subsequently, well-known validation of the analytical procedure should be performed. Prevalidation treatment specific to each analytical system may be outlined as in Fig. 1. The input of the analytical system are calibration samples, and the output, the properties of the calibration samples, which are measured to yield the analytical signals.

The response, y, must evidently be represented as the function of the amount of the sample component

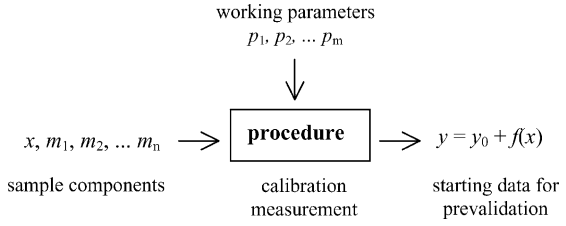

Fig. 1. Processing of data for prevalidation procedure.

to be determined, x, of the amount of the other components in the calibration sample ($m_1, m_2, \ldots, m_n$), as well as the parameters of the prevalidation treatment ($p_1, p_2, \ldots, p_m$), e.g. pH value, temperature, wavelength in a spectrum, amounts of reagents, etc. Therefore,

$$y = f(x, m_1, \ldots, m_n, p_1, \ldots, p_m).$$

The response function may in general be split into two terms:

$$y = y_0 + f(x),$$

where y_0 is random in character, originates in reference phenomena, in the fluctuations of the treatment parameters and in the perturbations of the environment [32]. The second term, $f(x)$, is the useful part of the response that is due to the component to be determined.

Further elaboration of responses represents mathematical/statistical approach, which comprises numerous of methods of mathematical statistics. These tools were used with two very different groups of tasks: (1) descriptive statistics; to compress, in descriptive way, numerous data by use of statistical characteristic figures, and (2) prognostic statistics; to derive predictions, to enable diagnosis of obtained values, which can be served in evaluation of analytical procedure/method. Complete process of extracting prevalidation characteristics of analytical procedure was presented in Fig. 2.

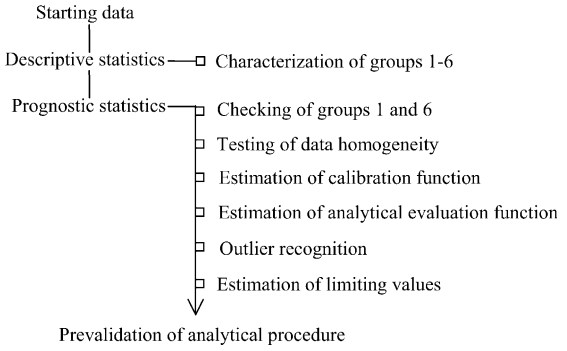

Fig. 2. Standardized evaluation anatomy of prevalidation system, $N = 24$ data.

1.3. Backing of prevalidation method

Four backings are especially important in defining the prevalidation system:

(1) The number of observations/measurements with homogenous working standards was defined in advance.
(2) The analyte matrix must be consistent within the system. Consistency presumes that samples must be so similar, physically and chemically, that the decomposition procedure is equally effective for all samples in liberating the analyte, and that the matrix measurement of all samples is effectively constant.
(3) Analytical system is defined with the most appropriate calibration function that strongly influence on further prevalidation step.
(4) It is usually assumed that most measurement data are approximated by a Gaussian or normal distribution so that well-known, common statistical techniques can be used.

1.4. Proposed and complete analytical procedure

Analytical procedures are a special group of technical and scientific measurement procedures. Modern analytical practice requires quality control of every analytical procedure based on impartial evaluation of the method's metrological characteristics. Accordingly, inauguration of a new analytical procedure inevitably calls for statistical validation.

It should be emphasized that for prevalidation process, the complete analytical procedure is very important, i.e. procedure that is defined by specifying all conditions under which the measurements must be made [9–11]. Namely, figures of merit [10] must be capable of being stated in an objective manner. Therefore, they can be given only in relation to concrete analytical procedure, not for general analytical principles/methods such as titrimetry, spectrometry, etc. Figures of merit inherent to analysis always relate to definite complete analytical procedure, which is specified in every detail by fixed working directions, and which is used for particular analytical task.

For a complete analytical procedure, everything must be previously determined: analytical task, apparatus, external and experimental conditions,

evaluation, and calibration. If any item is altered, a different analytical procedure resulted.

Until the procedure is not recorded in such a way that others can use it, it does not exist. If the description of procedure is written so that others cannot repeat the procedure faultlessly, its validity is questionable. Namely, the wording used to describe a method so that it can be produced is no less important than the science that went into its development [33].

The description of proposed analytical procedure should comprises a complete description of the analytical procedure sufficiently detailed to enable persons 'skilled in art' to replicate it [7]. The write-up should include all-important operational parameters and specific instructions such as preparation of reagents, performance of systems suitability tests, description of blanks used, precautions, and explicit formulas for calculation of test results.

1.5. Choice of calibration method

In order to evaluate from y the value for x, the functional relationship between y and x should be known. The nature of this functional relationship (calibration) is an important characteristic for prevalidation. The strength of prevalidation depends on the calibration method, which was used and selected method represents an important prevalidation characteristic. Besides, good repeatability, accuracy, and precision can only be obtained when good calibration method is used. Several methods for calibration of analytical procedure can be classified in order of decreasing effectiveness.

1.5.1. Calibration with synthetic standard samples

When it is possible to prepare standard samples, which have been synthesized from pure substances, in reliable way, then a calibration function can be established which has no bias and subsequent result will, consequently, be free from systematic error [10]. This type of calibration method is ideal for prevalidation. Since the true contents of the standard samples are known from their composition, they can be directly correlated with the measured quantities. This allows the inevitable accidental errors of the individual calibration measurements to be eliminated by taking the average from an $N = 24$ number of such measurements. Standard samples for this calibration can be prepared with reasonable expenditure only for relatively simple analytical problems, for which the type of the sample and of the components to be determined is known.

This type of calibration could be found everywhere in analytical practice where true contents of the standard samples are known and where the standard samples and analytical samples are of the same kind with respect to the analytical procedure and can be directly compared. For example, calibration of balances is recommended with this type of calibration when other methods cannot be applied.

1.5.2. Calibration with analysed standard samples

This type of calibration is especially important for analytical procedures used for complex analyses of a large series of similar samples. Calibration starts with the selection of a set of homogenous real samples, which approximately cover the range of one power of ten of species in question. The second step is the analysis of the selected samples by valid analytical procedure, whose measurement process produces the correct results, i.e. accurate measured value, also, which has been calibrated with synthetic standard samples. There are two different ways to achieving this goal: either all samples involved must be treated in such a way that, ultimately, they are of the same kind with respect to the analytical procedure used for the calibration with analysed standard samples; or the total analytical procedure must be split up into a number of different parts.

Preparation of analysed standard samples is like certification of so-called matrix reference material [34]. The certification procedure itself is the last and the most complicated step in the process of development and preparation of analysed standard samples. Certified analysed standard samples are used exclusively for pharmaceutical quality control and validation of analytical procedures.

1.5.3. Calibration by differential (standard) additions

Depending on the complexity of the effects influencing the analytical response, special calibration measures must be taken to obtain correct analytical results [35]. Matrix effects can be handled by addition of standards of the analyte to the sample. An analytical procedure can be calibrated by adding small but known amounts of the component to be determined

to the sample undergoing analysis. This calibration procedure presupposes that the added amount of the component to be determined behaves analytically in the same way as the component that was originally present in the sample. If the result is a calibration function with an easily apparent form, then it may be possible to extrapolate this function beyond the range, which was covered by the additions, and thus determine the unknown content, which was originally present in the analytical sample. This calibration is the only one, which allows quantitative determination of very small trace amounts when the basic material of the analytical sample cannot be obtained completely free from impurities.

1.6. Number of measurements

Useful information that can also be obtained from the prevalidation step, based on the precision results, is the prediction of the optimal number of experiments to be realized during the validation phase [1,23]. Recommended number of validation experiments varies in literature depending on investigated method, proposed concentration range, preliminary evaluation of calibration model, etc. The number of observations can vary from 5 to 100 in particular data sets [36]. In some cases, minimum five concentrations [4], or at least three different concentrations of calibration standards measured in triplicate [37,38], as well as a minimum of five to eight calibration standards should be considered [39]. In the absence of specific guidance, IUPAC [40] recommends six or more calibration standards evenly spaced over the concentration range of interest, which should be run at least in duplicate and preferably triplicate or more in random order. In prevalidation step of bioanalytical methods and immunoassays, a minimum six calibration concentrations should be used when fitting a calibration curve to the nonlinear concentration–response relationship [1,41]. Each concentration level of calibrator should be analysed at least in duplicate [41] or triplicate [1]. The reason for replicate analysis is to provide confidence in the analytical method and sampling strategy [42]. If the values of replicate measurements are very similar, then the analyst has confidence in his results. In contrary, analyst may well decide to modify the measurement procedure. One of the most effective ways in achieving higher precision of chemical analysis is to take the average of the values from a number of repeated analyses. Simultaneously, if the number of replicates is higher, then the investigation is time-consuming and more expensive. Therefore, it is necessary to reduce the number of parallel analyses to unavoidable number of measurements.

Four types of numbers have been distinguished in connection with presented prevalidation procedure. The capital letter N represents complete number of measurements in two prevalidation processes. For full prevalidation process $N = 24$, and for exploratory prevalidation this number is 8. Number of observations covered by $N = 24$ measurements was based on six analytical groups ($J = 6$), each of the volume of four replicates of identical properties of the sample ($I = \mathrm{IV}$) [13,43]. It was shown in practice that four parallel measurements were good choice in analysis [13] and this is confirmed recently by statement that each calibration level must be treated at least as triplicates in evaluation of the limit of quantitation [1]. In instrumental analyses, in which the fluctuation of working parameters is occurred, it is recommended to perform two successive measurements of the same analyte solution (an average value of those two measurements is used for further calculation). The numbers obtained from four parallel measurements in one analytical group, used in determination of means, standard deviations, and relative standard deviations, serve for further diagnosis of the investigated analytical procedure. Since a representative estimate of the variance is required to reliably test of calibration model, all calibration standards must be prepared and analysed independently and in suggested order (1, 6, 2, 5, 3, 4).

Number of observations in ad hoc prevalidation process was based on $N = 8$ measurements, arranged in two sets ($J = 2$) of four experiments each.

Total number of measurements underlies the determination of calibration function, analytical evaluation function and standard deviation of procedure, s_M, which in turn were used in evaluation of analytical procedure.

A prevalidation procedure proposed in this paper is not intended to have predictive character about optimum number of experiments. When prevalidation results on the boundary of acceptance criteria are obtained then greater number of experiments ($N > 24$) is recommended. Furthermore, the same recommended

number of experiments per run, as a function of the appropriate between-run and within-run standard deviation estimated in prevalidation, can be found in SFSTP guide [1,23]. To include inter-series variability for the predictive character of the prevalidation, it could be interesting to perform several series (at least two) of measurement with six concentration levels with at least two replicate each. This could also be included in further development of prevalidation strategy.

1.7. Measurement samples

1.7.1. Composition of samples and analyte working range

Samples for prevalidation could contain one or more components. The purpose of prevalidation procedure is a quantitative analysis of one component or analyte, x, in sample containing or not containing other components of real samples, m, e.g. matrix, where

$$m = m_1 + m_2 + m_3 + \cdots + m_n = \sum_{i=1}^{n} m_i.$$

If analyte (which should be determined) is x and sample matrix (as a sum of other sample components) is m, then the sample amount is $x + m$, and content of analyte (which should be determined) is $x/x + m$. Therefore, the analyte working range in prevalidation process could be expressed as follows: (1) analyte working range against amount of component, x; (2) analyte working range against amount of sample, $x + m$; (3) analyte working range against content of analyte, $x/x + m$.

On the other hand, in prevalidation procedure, analytical working range should be expressed as quantity, mass, or concentration of analyte that need to be determined. In selection of analyte working range, it is necessary to take care of possibilities of analytical procedure that could be applied according to quality of measurement at lower level of analyte.

Depending on the expected result and own experience, the standard working range should be the range that ensures the constancy of basic working and technical measuring parameters, i.e. that provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The specified range is normally derived from linearity studies and depends on the intended application of the procedure [4]. For the majority of analytical techniques, the linearity of the calibration graph has to be checked and the working range restricted to the linear part of the curve, which is usually one decade [35]. When working over wide concentration range, deviation from linearity becomes more probable and must be checked and/or a new calibration function must be calculated which is valid over the entire range. This is the case for the techniques as ICP and GC-MS, which can be used over a wide concentration range [35]. It is important to realize that validated range is not necessary coincide with the useful calibration range [39,40]. For many bioanalytical methods, the concentration ranges are usually rather broad, e.g. 1–100, 1–1000 or even wider [39]. For the assay of a drug substance or a finished product, the minimum specified ranges are considered from 80 to 120% of the test substance, for content uniformity from 70 to 130%, for dissolution testing is ±20% over the specified range, etc. [4]. For validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit.

A width of the analyte working range depends on the choice of the analyte content on the lower analyte level, x_L [44–46].

The ratio 1:10 between the lower and upper analyte level could have a wide application. For example, one can select a working range from 1 to 10 μg (mg, mol, ml, % or other physical units), 2–20 or 10–100 μg, etc. Then differences between the lower and upper analyte level are 9 units, between 1 and 10; 18 units, between 2 and 20; and 90 units, between 10 and 100. As it can be seen, depending on the sample and the choice of the lower analyte level as well as on the purpose of analysis, a width of the working range can largely differ. Moreover, using above-mentioned recommendation could satisfy many real requirements. However, there are always exceptions in method standardization, which need to be particularly elaborated.

Good choice of the x_L in the sample is important because of the influence of the specific analytical signal near to blank values and proving of presumed linearity at the higher content levels of analyte, respectively. The standardized prevalidation working range of analyte covered by $N = 24$ measurements was presented in Table 3.

Table 3
Strategy of prevalidation measurements on standardized basis ($N = 24$ measurements)

Measurements as a process of obtaining results[a]	
Type of measurements	Blank measurements (B), gross measurements (y)
Number of analytical groups	$J = 6$, $j = 1, 2, \ldots, 6$
Group volume	$I = \text{IV}$, $i = \text{I}, \ldots, \text{IV}$
Total number of measurements	$N = J \times I = 24$, $n = 1, 2, \ldots, 24$
Analyte amount	x (expressed as quantity, mass or concentration)
Analyte working range	x_U to x_L (x_U and x_L are upper and lower levels of analyte, respectively): one order of magnitude; $1.0x_U = x_1 = x_U$; $0.8x_U = x_2$; $0.6x_U = x_3$; $0.4x_U = x_4$; $0.2x_U = x_5$; $0.1x_U = x_6 = x_L$
Sequence of groups measurements	1, 6, 2, 5, 3, 4
Measure (net signal)[b]	$S = y - B$
Gross signal	y
Blank signal	B

[a] For prevalidation measurements on ad hoc basis: $J = 2$, $I = \text{IV}$, and $N = 8$.

[b] The term *measure* will refer to *net signal*, i.e. the difference between the gross signal and the blank signal [47]. To facilitate the discussion we assume that in principle the quantities S, y, and B have a normal distribution, but this may be a point of dispute in actual situations.

Each examination is performed to give a block of at least two starting data: B, blank signal obtained in the measurement of blank and y, gross signal obtained in the measurement of the sample. The corrected (net) signal, S is thus obtained from gross signal value reduced by the signal value of the corresponding blank.

The measurement of standard sample with a higher concentration of analyte could result in the higher analytical result for the following measurement. This phenomenon is known as *memory effect*. To investigate this problem as a possible source of errors, standards and blanks were measured alternately as a standardized sequence of group's measurements (Table 3).

1.7.2. Types of samples

Representative samples should always be taken in such a way that a reasonably close knowledge can be obtained about the object being studied. The implementation of prevalidation methodology is based on the synthetic standard samples. Standard samples and blank samples were measured, exclusively. In analytical procedures where working parameters were varied with time, measuring of reference materials with analyte was required. A surrogate for certified reference materials could be prepared by means of the analyte addition technique to a blank. Naturally, the blank is a material identical to the laboratory/synthetic sample, but having no analyte or, more correctly, no detectable analyte amount. The blank thus includes the effects of interfering species. In addition, in control of medicines it is convenient to use special terms such as placebo, field of blank or field of matrix blank to describe the true blank.

1.7.3. Calibration step

The response function of an analytical method is, within the range, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample [1]. The response function can be linear (straight line) but some nonlinear models sometimes related to the detection method or to the particularly wide concentration range, can also be observed. In some cases, to obtain linearity between assays and sample concentrations, test data need to be subjected to a mathematical transformation prior to the regression analysis [4]. Some analytical procedures, such as immunoassays or microbiological assays, are intrinsically curved or nonlinear even after transformation. In this case, the analytical response should be described by appropriate function of the concentration (amount) of an analyte in sample (weighted, nonlinear, quadratic, etc.) [4,41,48]. Furthermore, in many instrumental analysis methods, the instrument response is proportional to the analyte concentration over substantial concentration ranges [48], but some other detection techniques do not demonstrate linearity, e.g. MS detection. Particularly common is situation where the calibration plot is linear at low analyte concentrations, but becomes curved at higher analyte levels.

For the purpose of this paper, the analytical result is an analytical signal expressed in analyte amount units, for which conversion calibration function was used. Between the analytical signal and the analytical result, calibration step is always existed. Both, analytical signal and result were random (accidentally/causally) variables. On the other hand, the analyte amount in sample is independent variable. Unfortunately, the difference between analytical result and analyte amount or concentration is often in the laboratory samples overlooked [46]. Liteanu and Rîcã were avoided this pitfall by denoting the analysis result as c and the true value as either c' [47] or $\hat{c}$ [32]. Furthermore, the difference between analyte amount, q, in the test portion and analyte concentration, c, in the laboratory sample is irrelevant to the purpose of this paper. Obviously, an analytical signal is always critical or decision value on which basis decision should be made.

Since standardized measurements were performed with standard samples of known composition, analytical evaluation function could not be established without previous verification of linear analytical calibration function. This is because signals are dependent values and many of the procedures assume that all the errors are in the signal values and that the standard concentrations, as standard known values, are error-free [48]. Therefore, it was very important to perform suitable and complete calibration and verification of calibration function. Calibration of analytical procedure, performed with standard samples were resulted with the calibration function, $\hat{S} = f(x)$.

If there is linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by method of least squares [4]. Therefore, preliminary inspection of the relationship between signal values (namely S) and content of analyte values (namely x), in prevalidation approach, was examined by the adopted method of the least squares (R10–R12, Table 5) [48,49].

Some mathematical relations in which calculation of the parameters are based on the method of least squares are: $y = bx$; $y = a + bx$; $\log y = a + b \log x$; $y = a + bx + cx^2$, and rarely encountered is $\log y = a + \log x + c(\log x)^2$ [50]. Several conditions have to be fulfilled to justify the calculation procedure mentioned. One is that the precision of the measurement of the x values is much better then the precision of the measurement of the y values. A second condition is that values found for y, if parallel determinations are made at the same x value, have a Gaussian distribution. A third condition is the *homogeneity of variances* of the measured y values must be the same for the whole range of x values covered by the calibration curve.

The characteristic data evaluated by this standard calibration method were product-moment determination coefficient, r, slope of a line, b, intercept of a line, a, errors in the slope, s_b, and errors in intercept, s_a. The position of the grand mean of signal values $\bar{S}_N$, and the grand mean of mass of analyte $\bar{x}_N$, is known as the *centroid* of all the points. Significance of determination coefficient was checked using statistical t-test (R11, Table 5). If the calculated value of t is greater then the tabulated value, the null hypothesis is rejected and conclusion is that significant correlation does exist. Errors in the slope and intercept of the regression line were used to estimate confidence limits for the slope and intercept (R12, Table 5).

1.8. Analytical calibration function versus analytical evaluation function

A linear relationship between analytical signal and analyte content a priori assumed by the method of the least squares cannot be appropriate in any cases. Therefore, it was necessary to perform complete and deep evaluation of calibration function.

Linearity can be tested informally by examination of a plot of residuals produced by linear regression of the responses on the concentrations in an appropriate calibration set [40]. Any curved pattern suggests lack-of-fit due to a nonlinear calibration function. Furthermore, analysis of variance (ANOVA) can be used to detect lack-of-fit in a regression in order to verify whether the model chosen is correct one [22]. Another possibility to test linearity of a calibration graph is to fit a second degree polynomial to the data. The test for lack-of-fit verifies whether the straight line model adequately fits the calibration data. A straight line relationship is demonstrated if the quadratic regression coefficient is not significant. The hypothesis that the quadratic term is zero can be tested by means of the confidence interval or by means of a t-test. ISO and IUPAC include this approach, although in a different form, to evaluate the linearity of the calibration line. If nonlinearity is detected, ISO recommends either reducing the working range in order to obtain the straight

line calibration function or, if it is not possible, using the quadratic calibration function.

In practice, quadratic or cubic equations are often entirely adequate to provide a good fit to the experimental data. Polynomials with many terms are almost certainly physically meaningless and do not significantly improve the analytical results [48].

As prevalidation concept presumes that the second degree polynomial is sufficient condition for the description of analyte–signal relationship in great number of analytical procedures, Gottschalk approach [13] was used. The calculations for both calibration and analytical evaluation functions were based on sums and determinants as it was given in R13 (Table 5). Standard calculation process started with the hypothesis of the existence of a complete second degree calibration function $\hat{S} = \boldsymbol{U} + \boldsymbol{V}x + \boldsymbol{W}x^2$ (R13, Table 5). For all functions, the corresponding standard deviation of procedure (s_M), function constants (U, V, W), and their standard deviations values ($s_{\bar{U}}$, $s_{\bar{V}}$, $s_{\bar{W}}$) were calculated (R13, Table 5). Systematic t-testing of reality of constant $\boldsymbol{W}$, $\boldsymbol{V}$ and $\boldsymbol{U}$ leads to one of the following conclusions: test hypothesis $R < t$ indicates that the difference between the constant and zero is not statistically significant; in that case, the constant should be substituted with zero and the calculation procedure continued with reduced function. In case $R > t$, the constant is real. In a general scheme of logical decisions and following new calculations for reduced functions with 2 or 1 constant, the relevant constant can be obtained (Fig. 3). The persistence of constant W indicates on a curved analytical system. An analytical procedure is ideal one if ideal analytical calibration function of the type $\hat{S} = \boldsymbol{V}x$ is obtained. Any functionality other then linear one in analytical systems where linear analyte–response signal is expected could point to inappropriate analytical procedure and/or unacceptable influence of random and/or systematic errors.

According to almost the same principle, analytical evaluation function was established starting from parabolic function: $\hat{x} = U + VS + WS^2$ (R14, Table 5) using algorithms presented in detail in papers [13,43].

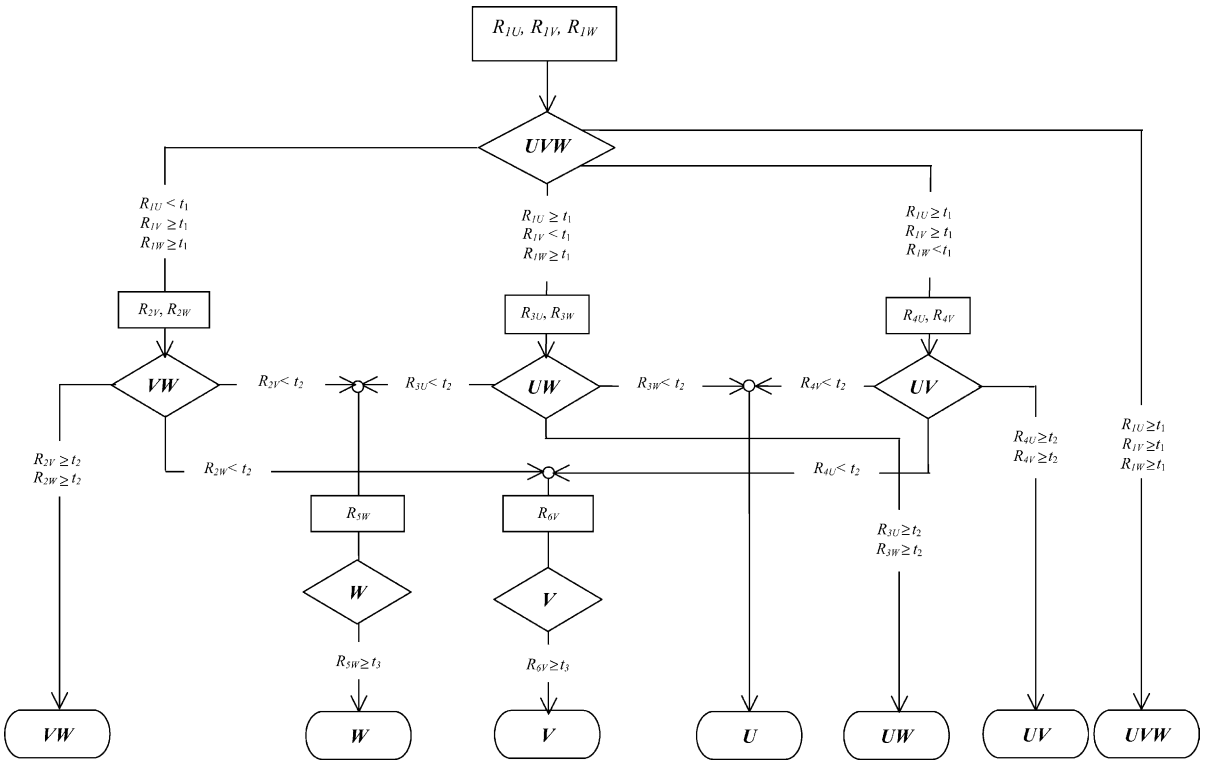

Fig. 3. Systematic evaluation of the complete calibration function.

When the ideal analytical calibration function of the type $\hat{S} = Vx$ is obtained then the standardized analytical procedure requires the ideal analytical evaluation function of the type $\hat{x} = VS$.

2. Experimental work

Among great number of analytical procedures investigated and checked with proposed prevalidation strategy by authors, for demonstration of prevalidation, the procedure for determination of pyrogallol with Folin–Ciocalteu's reagent (pyrogallol–FC system) was selected as practical and simple example. This procedure is relayed on the calibration procedure proposed by European Pharmacopoeia [31] as a part of procedure for determination of tannins in herbal drugs. Therefore, favourable analytical prevalidation characteristics are expected.

2.1. Apparatus

UV-Vis spectrophotometer Agilent 8453 (Agilent, Germany) with PC-HP 845x UV-Vis System (Agilent, Germany) and 1 cm quartz cells was used for all absorbance measurements.

2.2. Reagents

Pro analysi chemicals as well as double distilled water were used throughout the work. Analyte stock standard solution was prepared by exact weighing of 50.0 mg pyrogallol (Kemika, Croatia), dissolving in water and diluting to 100 ml with the same solvent. Secondary stock solution was made by diluting 5.0 ml of the standard solution to 100.0 ml with water. More dilute analyte working solutions were prepared by appropriate dilution (Table 4).

Folin–Ciocalteu's reagent (FC, Merck, Germany) as a chromogenic agent was used.

The solution of 29% sodium carbonate decahydrate (Kemika, Croatia) was prepared by dissolving 29.0 g in 100 ml water.

Filtration of prepared sample solutions was performed by using 0.20 μm Minisart-plus membrane filter (Sartorius AG, Germany).

2.3. Execution of prevalidation (tests)

Standardized measurements were based on a set of 24 blocks of data (six sets of four experiments each) to relate measured values to blank values. Standards and blanks were measured in standard working range of one power of ten, alternately in the following group sequence: 1, 6, 2, 5, 3, 4 (Table 3).

Mathematical/statistical tests were described in groups, that is, as tests for preliminary data treatment, examinations for the variability patterns with recognition of outliers, and establishment of calibration and analytical evaluation function and minimum quantities of analyte. The model of standardized quantitative analytical procedure was presented in Table 5. The application of expert system to evaluation of spectrometric procedure for determination of tannins with FC reagent was presented in Tables 6–13. Throughout the paper, test statistic values were referred to as requirements *R*. Values *P* and *f* were designated statistical confidence level and degrees of freedom, respectively. All algorithms were quoted gradually in the paper. For own purposes, authors developed self-made computer program called ESKULAP.

3. Analysis of the results

Analytical procedure can be considered as a process in which information as a new knowledge with

Table 4
Preparation of samples

Step	Pyrogallol–FC system
1	In adequate volume of secondary stock solution of pyrogallol (2, 1.6, 1.2, 0.8, 0.4, 0.2 ml, corresponding to 50, 40, 30, 20, 10, 5 μg, respectively) 1 ml of Folin–Ciocalteu's reagent and 10 ml of water are added
2	Solution from step 1 is made up in 25 ml volumetric flask with 29% $Na_2CO_3 \cdot 10H_2O$
3	After 30 min and filtration, the absorbance at 760 nm of the final blue solution is measured
4	Blank solution is prepared and measured identically, but without analyte (through steps 1–3)

Table 5
Standardized mathematical/statistical procedure

Requirement no.	Relation	Diagnosis	References
1. Check of limiting groups 1 and 6			
R1	$\frac{\bar{y}_6}{\bar{B}_6} = AC$ $AC \geq 2$	Influence of blank dispersion on s_M	[13,43]
R2	$R = \lvert 10(AC - 1)\rvert\%$ $s_{rB1}, s_{rB6} \leq R$	Blank signal should be significantly lower then signal on lower analyte level, x_L	[13,43]
R3	$s_{ry6}, s_{rS6} \leq \pm 25\%$ $s_{ry1}, s_{rS1} \leq \pm 2.5\%$ $L_{DG} = \lvert s_M \rvert \sqrt{2} t(99, f)$ $f = N -$ number of analytical evaluation constants $\bar{L}_{DG} = \frac{L_{DG}}{\sqrt{N}}$ $s_{rL} = 100 \frac{s_M}{L_{DG}} = \frac{100}{t\sqrt{2}} = \pm 25\%$	Position of L_{DG} in relation to x_6	[12,13,43,51]
R4	$R = \left\lvert \frac{\bar{y}_6 - \bar{B}_6}{s_{y6} + s_{B6}} \right\rvert$ $R < t$ $t(P = 95, 99,$ and $99.9\%, f = I - 1{=}3)$ $R < 3.128$, poor resolution $3.128 \leq R < 5.841$, good resolution $5.841 \leq R < 12.924$, very good resolution $R \geq 12.924$, excellent resolution	Distinguishing of signals	[a]
R5	$R = 2 \left\lvert \frac{\bar{A}_1 - \bar{A}_6}{\sqrt{s^2_{\bar{A}_1} + s^2_{\bar{A}_6}}} \right\rvert$ $R < t$ $t(P = 99\%, f = 6) = 3.707; f = 2I - 2$	Preliminary linearity check of calibration function	[16,48]
2. Testing of data homogeneity Analysis of variance			
R6	Blank mean: $\bar{B}_i = \frac{1}{4} \sum_{i=\mathrm{I}}^{\mathrm{IV}} B_i$		[13,22,43,48,49]
	Grand blank mean: $\bar{B}_N = \frac{1}{6} \sum_{j=1}^{6} \bar{B}_j = \frac{1}{24} \sum_{j=1}^{6} \sum_{i=\mathrm{I}}^{\mathrm{IV}} B_{ji}$		
	Mean deviation between groups: $s^2_{Bb} = \frac{4}{5} \sum_{j=1}^{6} (\bar{B}_j - \bar{B}_N)^2$, $f = J - 1 = 15$		
	Mean deviation within groups: $s^2_{Bw} = \frac{1}{6} \sum_{j=1}^{6} s_{Bj}$, $f = N - J = 18$	Blank data homogeneity	
	$R = \frac{s^2_{Bb}}{s^2_{Bw}}$ $R < F$ $F\ (P = 95\%, f_b = 5, f_w = 18) = 2.77$ $R < 2.77$, homogeneous $R \geq 2.77$, inhomogeneous		

Table 5 (*Continued*)

Requirement no.	Relation	Diagnosis	References
Auxiliary requirements			
R7	$\bar{B}_N < 0.005\bar{y}_1$	Check of possible inhomogeneity from R6	[13,43]
R8	Total s_r: $s_{rBN} \leq \pm 50\%$ Total s: $s_{BN} = \sqrt{\frac{1}{23}18s_w^2 + 5s_b^2)}$ $f = N - 1 = 23$ or mean deviation: $\bar{s}(\bar{s}_r) = \sqrt{\sum_{j=1}^{6} s(s_r)_j^2/6}$	Mean value for $\bar{s}_B, \bar{s}_y, \bar{s}_S, \bar{s}_A, \bar{s}_{rB}, \bar{s}_{ry}, \bar{s}_{rS}, \bar{s}_{rA}$	
Barttlet test			
R9	$R = 6.116 \times \left[6\log\left(\sum_{j=1}^{6} s(s_r)_j^2/6\right) - \sum_{j=1}^{6}\log s(s_r)_j^2\right]$ $R < \chi^2$ $\chi^2(P = 95, 99, \text{and } 99.9\%, f = J - 1 = 5)$ $R < 11.07$, strongly homogeneous (s.h.) $11.07 \leq R < 15.09$, homogeneous (h.) $15.09 \leq R < 20.52$, almost homogeneous (a.h.) $R \geq 20.52$, inhomogeneous (ih.)	Check of data homogeneity for s_B, s_y, s_S, s_A, s_{rB}, s_{ry}, s_{rS}, s_{rA}	[13,32,43,52–54]
3. Establishment of analytical functions			
Analyte–signal relationship			
R10	Conventional least square method	Relationship between analyte amount and analytical signal	[48,49]
R11	$R = \frac{\lvert r\rvert\sqrt{N-2}}{\sqrt{1-r^2}} = \frac{\lvert r\rvert\sqrt{22}}{\sqrt{1-r^2}}$ $t(P = 99\%, f = 22) = 2.819$	Significance of the determination coefficient	
R12	$\pm C_b = b \pm t_{(N-2)}s_b$ $\pm C_a = a \pm t_{(N-2)}s_a$ $t(P = 99\%, f = 22) = 2.819; f = N - 2 = 22$	Confidence limits for the slope and intercept	
4. Establishment of constants of calibration function			
t-Testing for reality of constants			
R13	Sums: $S_{11} = N = 24$; $S_{12} = S_{21} = \sum_{n=1}^{24} x_n$; $S_1 = \sum_{n=1}^{24} S_n$ $S_{13} = S_{22} = S_{31} = \sum_{n=1}^{24} x_n^2$; $S_2 = \sum_{n=1}^{24} S_n x_n$; $S_{23} = S_{32} = \sum_{n=1}^{24} x_n^3$ $S_3 = \sum_{n=1}^{24} S_n x_n^2$; $S_{33} = \sum_{n=1}^{24} x_n^4$; $S_4 = \sum_{n=1}^{24} S_n^2$ Determinants: $D_1 = S_{13} \times S_{33} - S_{23} \times S_{23}$; $D_2 = S_{12} \times S_{33} - S_{13} \times S_{23}$; $D_3 = S_{12} \times S_{23} - S_{13} \times S_{13}$; $D_4 = N \times S_{33} - S_{13} \times S_{13}$; $D_5 = N \times S_{23} - S_{12} \times S_{13}$; $D_6 = N \times S_{13} - S_{12} \times S_{12}$		[12,13,43]
R13a	$\hat{S} = U + Vx + Wx^2$ $D = S_{11} \times D_1 - S_{12} \times D_2 + S_{13} \times D_3$	Hypothesis testing: statistically significant difference between constants and zero	
	$DU = S_1 \times D_1 - S_2 \times D_2 + S_3 \times D_3$ $DV = -S_1 \times D_2 + S_2 \times D_4 + S_3 \times D_5$ $DW = S_1 \times D_3 - S_2 \times D_5 + S_3 \times D_6$ $s_M = \sqrt{\frac{1}{21}\sum_{n=1}^{24} S_n^2} = \sqrt{\frac{1}{21}(\text{S4} - \text{US1} - \text{VS2} - \text{WS3})}$		

Table 5 (*Continued*)

Requirement no.	Relation	Diagnosis	References
	$s_{\bar{U}} = s_M\sqrt{\frac{D_1}{D}};\ U = \frac{DU}{D};\ R_{1U} = \left\lvert\frac{U}{s_{\bar{U}}}\right\rvert$ $s_{\bar{V}} = s_M\sqrt{\frac{D_4}{D}};\ V = \frac{DV}{D};\ R_{1V} = \left\lvert\frac{V}{S_{\bar{V}}}\right\rvert$ $s_{\overline{W}} = s_M\sqrt{\frac{D_6}{D}};\ W = \frac{DW}{D};\ R_{1W} = \left\lvert\frac{W}{s_{\bar{W}}}\right\rvert$ *t*-Testing for three constants: $t_1(P = 99\%, f = 21) = 2.831$; $f = N - 3 = 21$		
R13b	$\hat{S} = Vx + Wx^2$ $D_1 = S_{13} \times S_{33} - S_{23} \times S_{23}$ $DV = S_2 \times S_{33} - S_3 \times S_{23}$ $DW = S_3 \times S_{13} - S_2 \times S_{23}$ $s_M = \sqrt{\frac{1}{22}(S_4 - VS_2 - WS_3)}$ $s_{\bar{V}} = s_M\sqrt{\frac{S_{33}}{D_1}};\ V = \frac{DV}{D_1};\ R_{2V} = \left\lvert\frac{V}{s_{\bar{V}}}\right\rvert$ $s_{\bar{W}} = s_M\sqrt{\frac{S_{13}}{D_1}};\ W = \frac{DW}{D_1};\ R_{2W} = \left\lvert\frac{W}{S_{\bar{W}}}\right\rvert$ $\hat{S} = U + Wx^2$		
R13c	$D_4 = N \times S_{33} - S_{13} \times S_{13}$ $DU = S_1 \times S_{33} - S_3 \times S_{13}$ $DW = S_3 \times N - S_1 \times S_{23}$ $s_M = \sqrt{\frac{1}{22}(S_4 - US_1 - WS_3)}$ $s_U = s_M\sqrt{\frac{S_{33}}{D_4}};\ U = \frac{DU}{D_4};\ R_{3U} = \left\lvert\frac{V}{s_{\bar{U}}}\right\rvert$ $s_{\bar{W}} = s_M\sqrt{\frac{N}{D_4}};\ W = \frac{DW}{D_4};\ R_{3W} = \left\lvert\frac{W}{s_{\bar{W}}}\right\rvert$		
R13d	$\hat{S} = U + Vx$ $D_6 = N \times S_{13} - S_{12} \times S_{12}$ $DU = S_1 \times S_{13} - S_2 \times S_{12}$ $DV = S_2 \times N - S_1 \times S_{12}$ $s_M = \sqrt{\frac{1}{22}(S_4 - US_1 - WS_2)}$ $s_U = s_M\sqrt{\frac{S_{13}}{D_6}};\ U = \frac{DU}{D_6};\ R_{4U} = \left\lvert\frac{V}{s_{\bar{U}}}\right\rvert$ $s_{\overline{V}} = s_M\sqrt{\frac{N}{D_6}};\ V = \frac{DV}{D_6};\ R_{4V} = \left\lvert\frac{V}{s_{\bar{V}}}\right\rvert$ *t*-Testing for two constants: $t_2(P = 99\%, f = 22) = 2.819$; $f = N - 2 = 22$		
R13e	$\hat{S} = Wx^2$ $S_M = \sqrt{\frac{1}{23}(S_4 - WS_3)}$ $s_{\bar{W}} = \frac{s_M}{\sqrt{S_{33}}};\ W = \frac{S_3}{S_{33}};\ R_{5W} = \left\lvert\frac{W}{s_{\bar{W}}}\right\rvert$		
R13f	$\hat{S} = Vx$ $s_M = \sqrt{\frac{1}{23}(S_4 - VS_2)}$ $s_{\bar{V}} = \frac{s_M}{\sqrt{S_{13}}};\ V = \frac{S_2}{S_{13}};\ R_{6V} = \left\lvert\frac{V}{s_{\bar{V}}}\right\rvert$	Verification of the calibration function	

Table 5 (*Continued*)

Requirement no.	Relation	Diagnosis	References
	t-Testing for one constant: $t_3(P = 99\%, f = 23) = 2.807$; $f = N - 1 = 23$		
5. Establishment of the analytical evaluation function			
t-Testing for reality of constants			
R14	Equation: $\hat{x} = U + VS + WS^2$ Desired equation: $\hat{x} = VS$ $R_V > t$ $t(P = 99\%, f = 23) = 2.807$	Verification of the analytical evaluation function	[12,13,43]
6. Recognition of outliers			
R15	Ideal calibration function: $\Delta S_n = S_n - \hat{S}_n$; $\lvert S^* \rvert = \left\lvert \frac{\Delta S_n}{s_M} \right\rvert$ Ideal analytical evaluation function: $\Delta x_n = x_n - \hat{x}_n$; $\lvert x^* \rvert = \left\lvert \frac{\Delta x_n}{s_M} \right\rvert$, $n = 1, \ldots, 24$ Maximum 1 value of 24 values with $\lvert t(95) \rvert \leq \lvert S^* \rvert < \lvert t(99) \rvert$ and $\lvert t(95) \rvert \leq \lvert x^* \rvert < \lvert t(99) \rvert$; $\lvert t(95) \rvert = 2.069$; $\lvert t(99) \rvert = 2.807$; $f = N - 1 = 23$	Recognition of outlier	[13,43,55]
7. Evaluation of limiting values			
R16	Ideal calibration function Limiting values for net signals: $S_D = \bar{S}_N + k \times s_{BN}$ Limit of detection: $L_D = 3.3 s_{BN}/V$ Limit of quantitation: $L_Q = 10 s_{BN}/V$	Position of S_D in relation to $S_6 = S_{min}$ Position of L_D and L_Q in relation to x_6	[4,22,44,48,56]
8. Extraction of prevalidation parameters			

[a] See Section 3.2.

the respect to content is transferred and retained by signals. The analytical signal, which carries information about chemical composition, can be further transformed into analytical information (*I*), if signal function $I = f(S)$ is known. The perfectness of transformation of information about the signal into analytical information is characterized by means of transinformation [57].

The analytical signal *y*, proportional to the absolute mass of the tannins present, was transformed into the corresponding absorbance value and later was used for calculation. Starting data were: mass of pyrogallol, *x*, within the given working range, absorbances obtained in measurements of the blank, *B*, and the sample, *y*, as well as the corrected absorbance, *S*.

3.1. Characterization of groups 1–6

Characterization and starting data comparison were performed by means of descriptive statistics, using two statistical criteria: the average value (arithmetic mean or mean) and the degree of dispersion (standard deviation and relative standard deviation) [48]. A standard deviation or relative standard deviation can be used as a measure of precision (or imprecision) [40,58]. A measured standard deviation can be subdivided into three categories: repeatability, intermediate precision and reproducibility [3,4]. Precision very often varies with analyte concentration. Nearly always one finds that the absolute precision of the determination (i.e. the standard deviation) increases with concentration, whereas the relative precision (relative standard deviation) decreases with concentration [38]. When the results of the linearity study are acceptable, the results for precision must be compared with the imposed acceptance requirements [39]. Available documents defining precision as validation criteria that must be assessed during the development of an analytical procedure are not precise enough. The acceptance criteria for precision depend very much on the type of analysis [59].

While for compound analysis in pharmaceutical quality control precision of better than 1% of relative

Table 6
Standard measurements for pyrogallol–FC system

Group (j)	Sample no. (i)	x[a] (μg)	B	$\bar{B}/s_B/s_{rB}$ (%)	y	$\bar{y}/s_y/s_{ry}$ (%)	S	$\bar{S}/s_S/s_{rS}$ (%)	A[b]	$\bar{A}/s_A/s_{rA}$ (%)
1	I	50.0	0.0053	0.0053/±0.0002/±3.54	0.3352	0.3400/±0.0037/±1.08	0.3300	0.3347/±0.0038/±1.13	0.0066	0.0067/±0.0001/±1.13
	II		0.0055		0.3389		0.3333		0.0067	
	III		0.0051		0.3435		0.3383		0.0068	
	IV		0.0051		0.3422		0.3371		0.0067	
6	I	5.0	0.0054	0.0059/±0.0006/±9.63	0.0374	0.0395/±0.0015/±3.92	0.0319	0.0336/±0.0014/±4.31	0.0064	0.0067/±0.0003/±4.31
	II		0.0058		0.0411		0.0352		0.0071	
	III		0.0067		0.0397		0.0330		0.0066	
	IV		0.0055		0.0398		0.0343		0.0069	
2	I	40.0	0.0054	0.0057/±0.0005/±8.91	0.2725	0.2754/±0.0020/±0.74	0.2671	0.2698/±0.0018/±0.67	0.0067	0.0067/±0.00004/±0.67
	II		0.0060		0.2770		0.2710		0.0068	
	III		0.0061		0.2768		0.2707		0.0068	
	IV		0.0051		0.2754		0.2703		0.0068	
5	I	10.0	0.0057	0.0056/±0.0002/±3.94	0.0787	0.0771/±0.0031/±4.01	0.0730	0.0714/±0.0029/±4.01	0.0073	0.0071/±0.0003/±4.01
	II		0.0055		0.0753		0.0698		0.0070	
	III		0.0059		0.0805		0.0746		0.0075	
	IV		0.0054		0.0738		0.0684		0.0068	
3	I	30.0	0.0053	0.0055/±0.0006/±10.79	0.2045	0.2060/±0.0013/±0.63	0.1992	0.2001/±0.0009/±0.46	0.0066	0.0067/±0.00003/±0.46
	II		0.0057		0.2065		0.2008		0.0067	
	III		0.0062		0.2076		0.2014		0.0067	
	IV		0.0048		0.2056		0.2008		0.0067	
4	I	20.0	0.0051	0.0056/±0.0004/±7.88	0.1395	0.1389/±0.0020/±1.44	0.1344	0.1333/±0.0022/±1.68	0.0067	0.0067/±0.0001/±1.68
	II		0.0055		0.1407		0.1352		0.0068	
	III		0.0060		0.1393		0.1332		0.0067	
	IV		0.0059		0.1360		0.1301		0.0065	
Six groups mean $\bar{s}$ ($\bar{s}_r$, %)				±0.0004 (±7.94)		±0.0024 (±2.44)		±0.0024 (±2.56)		±0.0002 (±2.56)

[a] Mass of pyrogallol.
[b] Measure of particular sensitivity, $A_n = S_n/x_n$.

Table 7
Checking of limiting groups 1 and 6

Requirement no.	Result	Diagnosis
R1	$AC = 6.73$	Significant influence of blank dispersions on S_M is not expected
R2	$R = 57.30\%$ $s_{rB1} = \pm 3.54\%$ $s_{rB6} = \pm 9.63\%$	
R3	$s_{ry6} = \pm 3.92\%$ $s_{rS6} = \pm 4.31\%$ $s_{ry1} = \pm 1.08\%$ $s_{rS1} = \pm 1.13\%$ $L_{DG} = 1.7097\,\mu g$ $\bar{L}_{DG} = 0.35$ $s_{rL} = \pm 25.19\%$	Determination limit is expected below x_6
R4	$R = 15.92$	Excellent resolution of signals
R5	$R = 0.18$	Linear calibration function is expected

standard deviation is easily achieved, for biological samples the precision is more like 15% at the concentration limits and 10% at other concentration levels [59]. For environmental and food samples, the precision is very much dependent on the sample matrix, the

Table 8
Testing of data homogeneity

Requirement no.	Result	Diagnosis
R6	$s^2_{Bb} = 1.63 \times 10^{-7}$ $s^2_{Bw} = 2.00 \times 10^{-7}$ $R = 0.81$	Homogeneous blank values
R7	$\bar{B}_N$ should be <0.0017, $\bar{B}_N = 0.0056$	Influence of blank value is not negligible
R8	$s_{rBN} = \pm 7.85\%$ $s_{BN} = 4.38 \times 10^{-4}$	
R9	$R(s_B) = 5.11$ $R(s_{rB}) = 4.80$ $R(s_y) = 4.14$ $R(s_{ry}) = 15.38$ $R(s_S) = 5.87$ $R(s_{rS}) = 17.70$ $R(s_A) = 18.26$ $R(s_{rA}) = 17.70$	s.h. s.h. s.h. a.h. s.h. a.h. a.h. a.h.

Table 9
Quality of analyte amount and analyte–signal relationship

Analyte–signal relationship		
Requirement no.	Result	Diagnosis
R10	$r = 0.99968$ $b = 0.0067$ $a = 0.0017$ $s_y = \pm 0.00017$ $s_b = \pm 0.00017$ $s_a = \pm 0.00007$ Centroid = (25.83, 0.1739)	
R11	$R = 185.25$	Significant correlation
R12	$\pm C_b = 0.0067 \pm 0.00048$ $\pm C_a = 0.0017 \pm 0.00019$	
t-Testing for reality of calibration constants		
R13	$\boldsymbol{V} = 0.0067$ $R_V = 345.14$ $s_V = \pm 0.00006$ $s_M = \pm 0.0029$ $\hat{S} = 0.0067x$	Ideal calibration function
t-Testing for reality of analytical evaluation constants		
R14	$V = 148.94$ $R_V = 345.14$ $s_V = \pm 0.4315$ $s_M = \pm\ 0.4307$ $\hat{x} = 148.94S$	Ideal analytical evaluation function

Table 10
Test for outliers

Requirement no.	Result	Diagnosis
R15	$\lvert S^*_{19}\rvert > 2.069$	One outlying value, no objection on data material
	$\lvert x^*_{19}\rvert > 2.069$	One outlying value, no objection on data material

Table 11
Estimation of limiting values

Requirement no.	Result	Diagnosis
R16	Analytical evaluation function $\hat{x} = 148.94S$ $S_D = 0.0069$	S_D is expected below S_6
	$L_D = 0.2157\,\mu g$ $L_Q = 0.6532\,\mu g$	L_Q is expected below x_6

Table 12
Data structure for pyrogallol–FC system

j	I	S	$\hat{S}$	ΔS	S^*	x	$\hat{x}$	$\bar{\hat{x}}$	$s_{\hat{x}}$	$s_{r\hat{x}}$ (%)	Δx	$\Delta x/x \times 100$ (%)	$\overline{\Delta x}$	$\overline{\Delta x}/x \times 100$ (%)	x^*
1	I	0.3297	0.3356	0.0059	1.9607	50.0	49.85	49.85	±0.56	±1.13	−0.85	−1.71	−0.31	−0.62	1.9829
	II	0.3333		0.0026	0.7986		49.65				−0.35	−0.71			0.8203
	III	0.3379		−0.0023	0.9306		50.39				+0.39	+0.78			0.9081
	IV	0.3366		−0.0010	0.4880		50.20				+0.20	+0.40			0.4655
6	I	0.0318	0.0336	0.0018	0.5686	5.0	4.75	5.01	±0.22	±4.31	−0.25	−4.92	+0.10	+2.00	0.5707
	II	0.0355		−0.0019	0.5728		5.25				+0.25	+4.91			0.5705
	III	0.0341		−0.0005	0.1985		4.91				−0.09	−1.73			0.2007
	IV	0.0342		−0.0006	0.2511		5.11				+0.11	+2.14			0.2489
2	I	0.2669	0.2685	0.0016	0.4916	40.0	39.78	40.18	±0.27	±0.67	−0.22	−0.55	+0.45	+1.11	0.5095
	II	0.2714		−0.0029	0.8434		40.36				+0.36	+0.89			0.8254
	III	0.2712		−0.0027	0.7431		40.31				+0.31	+0.78			0.7251
	IV	0.2698		−0.0013	0.6325		40.26				+0.26	+0.66			0.6145
5	I	0.0732	0.0671	−0.0061	2.0344	10.0	10.87	10.64	±0.43	±4.01	+0.87	+8.74	+0.64	+6.40	2.0297
	II	0.0697		−0.0026	0.9207		10.39				+0.39	+3.95			0.9162
	III	0.0749		−0.0078	2.5843		11.11				+1.11	+11.11			2.5795
	IV	0.0682		−0.0011	0.4262		10.18				+0.18	+1.82			0.4216
3	I	0.1989	0.2014	0.0025	0.7413	30.0	29.67	29.87	±0.14	±0.46	−0.33	−1.08	−0.13	−0.43	0.7547
	II	0.2009		0.0005	0.2191		29.90				−0.10	−0.33			0.2325
	III	0.2020		−0.0006	0.0000		30.00				0.00	0.00			0.0026
	IV	0.2000		0.0014	0.1949		29.91				−0.09	−0.30			0.2083
4	I	0.1339	0.1343	0.0004	0.0638	20.0	20.02	19.85	±0.33	±1.68	+0.02	+0.12	−0.62	−3.10	0.0548
	II	0.1351		−0.0008	0.3266		20.14				+0.14	+0.68			0.3176
	III	0.1337		0.0006	0.3547		19.84				−0.16	−0.78			0.3637
	IV	0.1304		0.0039	1.4269		19.38				−0.62	−3.09			1.4357

Bartlett test for $\hat{x}$: $R(s) = \pm 5.87$, s.h.; $R(s_r) = \pm 17.70$, a.h. Six groups mean for $\hat{x}$: $\bar{s} = \pm 0.35$; $\bar{s}_r = \pm 2.56\%$.

concentration of the analyte and on the analysis technique. It can be vary between 2% and more than 20%. The AOAC manual for the Peer Verified Methods program [60] includes a table with estimated precision data as a function of analyte concentration. According to the Washington Conference report, which is now being utilized as a basis for bioanalytical method validation, the acceptance criteria for precision is 15% and at the limit of quantitation is 20% [1,23]. For immunoassays, minimal acceptable limits for precision are 20%, i.e. 25% at the limits of quantitation [41]. According to Jenke [61], the precision criteria for general method analysis is ≤1.5% of relative standard deviation, for biological methods ≤5% and for trace analysis 5–15%. Another example of precision criteria for an assay method is that the instrument precision will be 1% and the intra-assay precision will be 2% [62]. For an impurity method, at the limit of quantitation, the instrument precision will be 5% and the intra-assay precision will be 10%.

The purpose of prevalidation strategy is not to evaluate precision, but standard deviation values obtained for all kind of absorbances in each experimental group can have predictive character in order to evaluate analytical performances of the method.

Since absorbance measurements in pyrogallol–FC system are performed with standard samples containing low level of analyte, high degree of precision was expected. Results obtained in this step were used for the further diagnosis in prognostic statistics. Standardized measurements and calculated values of pyrogallol–FC system were given in Table 6. It was shown that reasonable precision was attained for all kind of absorbances (s_{rB} from ±3.54 to ±10.79, s_{ry} from ±0.63 to ±3.92, and s_{rS} from ±0.46 to 4.31).

Table 13
Prevalidation characteristics of spectrometric determination of tannins with FC reagent

Parameter	Pyrogallol–FC system					
Working range (μg)	5.0–50.0					
Information value range (absorbance units)	0.34–0.04					
Analyte–signal relationship	$r = 0.9997$					
Calibration function	$\hat{S} = 0.0067x$					
Analytical evaluation function	$\hat{x} = 148.94S$					
Standard deviation of procedure	±0.43					
Limit of detection, L_D (μg)	0.22					
Limit of quantitation, L_Q (μg)	0.65					
Groups data						
Actual (μg)	50.00	40.00	30.00	20.00	10.00	5.00
Found (μg)	49.85	39.78	29.67	20.02	10.87	4.75
Random deviations						
$s_{\hat{x}}$ (μg)	±0.56	±0.27	±0.14	±0.33	±0.43	±0.22
$s_{r\hat{x}}$ (%)	±1.13	±0.67	±0.46	±1.68	±4.01	±4.31
Systematic deviations, $\Delta\bar{x}$						
Absolute (μg)	−0.31	+0.45	−0.13	−0.62	+0.64	+0.10
Relative (%)	−0.62	+1.11	−0.43	−3.10	+6.40	+2.00

3.2. Checking of limiting groups 1 and 6

A preliminary check of working range-limiting groups, i.e. groups 1 and 6, was the first step in the prognostic mathematical/statistical evaluation of data. The main purpose of this requirement was quality control of measurement in a group with the smallest mass of analyte, x_6. Disturbing influences that produce accidental fluctuations of the blank measurements could be of many different kinds and could seriously affect reliability of the analytical process. The requirement that ensures the blank signal to be significantly lower then the gross signal at lower analyte level, and that enables unambiguous distinction between these two signals was given by Gottschalk (R1, Table 5) [13,43]. If this requirement was not satisfied, two signals could be certainly distinguished only if standard deviations of both gross and blank signals at lower analyte level were small enough.

Applicability of this requirement was also extended to the recognition of influence of blank values dispersion on the standard deviation of the procedure (s_M) through R2. The reason of high relative standard deviations at lower analyte level could be *memory effect*.

For the standard measurement, it is also required that s_r values for both gross and corrected measurements at x_U and x_L lie below ±2.5 and ±25%, respectively (R3, Table 5). This heuristic requirement gives preliminary information whether L_{DG} is to be expected below x_L. Moreover, s_r values higher than ±25% point to unacceptable fluctuation of the measurements at x_L. The same is true for blank measurements with $s_{rBN} > \pm 50\%$. According to the same author, the determination limit L_{DG}, should lie below the lower level of analyte, x_L (R3, Table 5) [13,43].

Additional checking of quality of signal resolution was based on the modified *t*-test for comparison of two mean values according to Eckschlager and Štěpánek [63] (R4, Table 5). This requirement gives information about the possibility in distinguishing of gross and blank signal at, x_6. In the pyrogallol–FC system, gross and blank signals were excellent distinguished (R4, Table 7).

The preliminary linearity check of the calibration function was based on an a priori assumption of linearity. For this purpose, Lutteroti and Grdinić have proposed the statistical test for the comparison of the means of two independent samples [16], as the difference from the modified *t*-test proposed by Gottschalk

[13,43]. This concept was applied to A values (particular sensitivity values, $A = S/x$) for limiting groups 1 and 6 (R5, Table 5). Since obtained value (R5, Table 7) was below tabulated value t for this requirement, linear calibration function is expected.

Since all requirements (R1–R5, Table 7) were fulfilled, it was proceed with the evaluation procedure, i.e. with examinations of data homogeneity.

3.3. Testing of data homogeneity

Simple analysis of variance, applied to the six groups of blank values, leads to an objective decision about homogeneity of the respective data material [13,22,43,48,49]. The relevant data include dispersion within groups, s^2_{Bw}, dispersion between groups, s^2_{Bb}, and total standard deviation of blank signals, s_{rBN} (R6 and R8, Table 5). A ratio s^2_{Bw}/s^2_{Bb} bigger then the chosen F value indicates inhomogeneous blank data where random error could not be excluded. Dispersion within individual groups statistically not different from dispersions between groups speaks in favour of data homogeneity. Analysis of variance applied to pyrogallol–FC system indicated high homogeneity of blank values (R6, Table 8). Random and systemic errors could be present although high homogeneity data was obtained. The reason for this are high values for both s^2_{Bw} and s^2_{Bb}, and therefore small value of s^2_{Bw}/s^2_{Bb}.

Gottschalk [13,43] also introduced some auxiliary criteria (R7, Table 5). For the system under study, influence of blank value is not negligible (R7, Table 8). The influence of inhomogeneity of blank values can be taken negligible if they are small enough in relation to information obtained at the upper analyte level, and if s_{rBN} was not exceeded ±50% (R8, Table 5). If requirements R6, and/or R7, and R8 were established than y values could be corrected with grand blank mean, $\bar{B}_N$. For pyrogallol–FC system, influence of blank values on results of analysis could be excluded. This imposes the need of correcting each y value with grand blank mean in pyrogallol–FC system.

The effects observed in the analysis of blank data could be occasionally controversial. Contrary to conclusions on negligible effects of blank dispersion on s_M (R1 and R2) and homogenous blank values obtained from the analysis of variance (R6), the value from additional checking of blanks (R7) could point to the possible influence of blank values on analytical method reliability. Moreover, the possible source of high s_{rBN} values could be negative blank values and these could be a misleading factor in diagnosis. Therefore, it seems reasonable to assume that s_{rBN} are a relatively limited value in judging of the data quality.

Barttlet test, used for analysis of more then two variances, precisely six variances in the case of the standardized procedure was worth attempting (R9, Table 5). The homogeneity of deviations provides an insight into the data structure and enables quick recognition of the source of error. Barttlet test applied to s and s_r values for B, y, S, A values (R9, Table 8), as well as on the values of the apparent mass of analyte, $\hat{x}$ (Table 12) pointing to high data homogeneity, except for A values. However, when applied to s_r values for the y, S, A and $\hat{x}$ data, the test indicated a lower level of homogeneity (R9, Table 8).

3.4. Relation between signal and concentration

For system under study, significant correlation between signal and amount of analyte was existed (R10–R12, Table 9). Moreover, both ideal calibration and analytical evaluation functions were found. The characteristic data evaluated from this standardization and evaluation procedure were the constants of the calibration and analytical evaluation function; the mean errors of the constants and the standard deviation, s_M, of the analytical procedure in the given working range (R13 and R14, Table 9).

It was possible to evaluate apparent signal values, $\hat{S}$, from the final calibration function and apparent masses of analyte, $\hat{x}$, from the final analytical evaluation function, enabling the recognition of outliers, as well as analyte limiting values. Only with defined analytical evaluation function, it is possible to conclude on accuracy as a total error of analytical procedure. Analysis of variance, the Barttlet test, reality of analytical function and agreement of actual, x, and appropriate, $\hat{x}$ values was given information on accuracy of the analytical procedure. The data structure for the pyrogallol–FC system was given in Table 12.

3.5. Outlier recognition

Outlier is a measurement that appears to differ unreasonably from the others in the set of results. Statistical techniques may be used for their identification.

Zero and negative measurements were often considered as outliers, but when working near the limit of detection, a certain number of analyses by chance alone were expected to be zero [64]. When outliers were discarded from the data set, it is important that they were revealed and statistically checked.

Outliers inevitably arise in calibration experiments, just as they occur in replicate measurements, but it is rather harder to deal with them in regression statistics [48]. Although the individual signal values in calibration experiment are assumed to be independent of one another, the residual $(S_n - \hat{S}_n)$ are dependent of one another, as they sum is always zero. Therefore, it is not normally permissible to take the residuals, as they were a conventional set of replicate measurements, and apply some generic and largely recommended tests to identify any outlier, e.g. Grubbs' or Dixon's test.

For the regression analysis, clear distinction between outliers with the respect to the model (regression outliers) and outliers in the replicates at one concentration level must be made. [39]. A possible approach to detect outliers in the replicates is to apply the single and paired Grubbs' outlier test. For the identification of regression outliers, several diagnostics have been proposed [22]. The simplest one consists in a comparison of the absolute value of the standardized residual with cut-off value, which is generally equal to 2 or 3. It is based on the fact that the probability for the residual to have a value as large as 2 or 3 times the residual standard deviation is very small.

In this paper, similar test for checking outliers proposed by Gottschalk was used [13,43]. Testing for the outlier was done by comparison of $|S^*|$ and $|x^*|$ values with the t-values of confidence intervals for $P = 95$ and 99% confidence level (R15, Table 5). Accordingly, one outlying value is tolerable within the 24-data population. Inspection of results indicates that, despite of one outlying value, there is no objection on the homogeneity of the data material in pyrogallol–FC system (Table 10).

3.6. Estimation of limiting values

According to Gottschalk, L_{DG} is a minimum amount of analyte significantly different from zero that could be determined by analytical procedure. This approach is preferred in systems where more then one constant of analytical function was present. This calculation was based on s_M value of analytical evaluation function and for the system under study gives the value of $L_{DG} = 1.71\,\mu g$ of pyrogallol. This calculated value being bellow the respective x_6 level was confirmed the correctness of preliminary test R3.

Up-to-date recommendations [4,22] were also incorporated into the standardized measurement model (R16, Table 5). These recently adopted concepts of limiting values (L) comprise the slope of the analytical calibration function (sensitivity), $\boldsymbol{V}$; the constant of ideal analytical evaluation function, V; and k stands for suggested numerical factor of 3.3 and 10 for the limit of detection, L_D, and limit of quantitation, L_Q, respectively. Limit of detection was derived from the smallest measure S_D, that is, analyte's signal at the detection limit that can be detected with reasonable certainty for a given analytical procedure. All estimated limiting values were significantly lower then the mass of analyte at lower analyte level, x_6 (Table 11).

These investigations confirmed the usefulness of pyrogallol–FC system under study. The procedure were characterized by L_Q value of $0.65\,\mu g$ of pyrogallol and by systematic deviations ranging from -0.62 to $+6.40\%$. It is likely that small deviations of blank and gross values are the principal generator of random deviations ranging from ± 0.46 to ± 4.31. The extensive prevalidation metrological characteristics, critical for selection of methodology for Vis-spectrophotometric determination of tannins, are summarized in Table 13.

4. Conclusions

It is desirable to have preliminary knowledge or a priori on the performance characteristics of the method before properly starting the validation stage. Therefore, a very simple, useful, and informative prevalidation concept for analytical procedure/method evaluation was established. Full prevalidation, as a part of prevalidation strategy, was based on systematic and sophisticated approach for calibration and establishment of constants of calibration and analytical evaluation function, as well as on elaborative system of prevalidation diagnostics for each prevalidation step. As results of the full prevalidation process, prevalidation characteristics were obtained. One characteristic of the present paper is that linear

relationship between response and amount of analyte have been discussed. Relationships that are more complex may be required in some practical situations. However, the present paper seems justified for at least two reasons: the number of analytical procedures based on linearity is frequently occurred in practice, and prevalidation procedure is seemed as a serviceable assistant to method validation.

Good metrological characteristics obtained for presentation model (pyrogallol–FC system) using prevalidation strategy showed that this metrological procedure has proven valuable for evaluating the power of model spectrometric methods and assessing the analytical protocols fitting the task of producing data of appropriate quality.

This prevalidation proposal was recommended for solving problems that arise from evaluation and application of analytical procedure. Moreover, methodology described in full prevalidation could be approached to *interlaboratory control* by procedure performed in one laboratory.

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