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Validation of Analytical Methods Based on Chromatographic Techniques: An Overview

Juan Peris-Vicente, Josep Esteve-Romero, and Samuel Carda-Broch

14.1
What Validation Is?

The purpose of any analytical method is to provide consistent, reliable, and accurate data. For this reason, the performances and the limitations of the method, as well as the external influences which may modify these features, must be determined prior to its use. Validation plays a major role in achieving this goal [1,2]. The word "validation" comes from the Latin term *validus*, meaning worth/strong, thus suggesting that something is true, useful, and reliable [3]. The most accurate definition of validation is that provided by ISO 9000:2000 as the confirmation, by means of a thorough examination and obtaining realistic and unequivocal evidences, that the procedure is effectively applicable for its indented purpose [4].

Validation is the act of proving that any approach, strategy, experimental procedure, process, laboratory staff, instrumentation, reagents, and room conditions selected for the method will function in a proper way under a fixed set of conditions. Besides, it can be used to individually evaluate the appropriateness of these factors [5]. The validation evaluates the range and conditions of applicability, and checks if every future measurement in routine analysis will provide a concentration of the analyte close enough to the true value [6]. In addition, it can also quantify the degree of coincidence of a measured concentration and the true value, by the calculation of the bias and the uncertainty associated with the result [7]. Therefore, the validation verifies if the method is suitable to be used as a quality control tool and for research support [8]. It is an essential step in method development, which must be implemented by laboratories to prove they can produce analytical data with high reliability [9].

The validation consists in the determination of well-defined quality parameters: statistical (selectivity, specificity, calibration curve, linearity, calibration range, accuracy, precision, recovery, uncertainty, limit of detection, limit of quantification (LOQ), decision limit, detection capability, robustness, stability, system suitability, and comparison with other methods) [1,2,10] and operating/economical (cross contamination, simplicity, analysis time, price per analysis, safety for laboratory staff, and environmental impact) [11,12]. The results from

method validation evince the quality and consistency of the analytical results obtained in future determinations in real samples, whereas the operational/economic parameters appraise if the method can be used for routine analysis. All these parameters are explained in more detail in Sections 14.5 and 14.6, respectively. The validation protocol is a set of directives detailing, for each parameter, the accurate meaning, the acceptance criteria, the experimental design, and the mathematical formula for its evaluation.

The fitness for purpose is the extent in which the performances of the method match the characteristics that have been agreed between the analyst and the end user of the results. If a method aims to reach a wider application, it must also meet the requirement of the government institutions and official analysis guide [13]. The procedure and the analytical requirements are not always the same, and must be individually established on the basis of the scope of the method, the analyte, the matrix, possible interfering, the kind of the sample, the expected interval concentration, and the geographic zone. The validation parameters that have to be determined and the acceptance criteria should be completely specified before starting the development of the method. A validation study must be conducted, as far as possible, considering all the effects that can be involved during the normal use of the method [14]. The final results of validation must be documented to be always available for consulting by laboratory staff, clients, and accreditation agencies, and ready to be transferred to other laboratories [15].

The validation is considered to be very closely tied to the method development. In fact, it is sometimes difficult to establish when the method optimization has ended and the validation begins. Some of the validation parameters are evaluated during the validation method, in order to optimize the experimental conditions [1]. For instance, the extraction solvent is selected to maximize the recovery, the composition of the mobile phase is chosen to maximize the specificity, and so on. Therefore, a well-developed method should be easy to validate [11]. Once a methodology is validated, it remains "validated" while applied in the same laboratory and using the same experimental conditions [16].

Many industry committees and regulatory agencies and individual researchers have published reviews and technical reports about validation strategies, quality assurance, and regulatory purposes [17]. Most of them are related to the pharmaceutical and chemical industry, which was first interested in validation [18]. Aware of its importance, a large number of international renowned organizations have offered along the years guidance about method validation: Association of Official Analytical Chemists (AOAC), American Society for Testing and Material (ASTM), Codex Committee on Methods of Analysis and Sampling (CCMAS), Cooperation on International Traceability in Analytical Chemistry (CITAC), Environmental Protection Agency (EPA), European Analytical Chemistry Group (EURACHEM), European Committee for Normalization (CEN), European Commission [10], European Cooperation for Accreditation (EA), European Medicines Agency (EMA) [19], Food and Agricultural Organization (FAO), Food and Drug Administration (FDA), International Conference for Harmonization (ICH), International Laboratory Accreditation Cooperation

(ILAC), International Organization for Standardization (ISO), International Union of Pure and Applied Chemistry (IUPAC), US Pharmacopoeia, World Health Organization (WHO), and so on [3,17]. Consequently, many validation guidelines, with different scopes, have been issued, describing the validation parameters to be studied, the way to determine each one, and their acceptance criteria. The different published documents agree about what type of studies should be done, but they show a great diversity in how the validation should be conducted [20]. Besides, the requirements are continually changing, and new guides are developed. The existence of an excessive number of protocols can confuse the analysts for their selection, and could even make the interpretation of the validation report difficult. Thus, a methodical understanding about all the aspects involving validation is essential to its correct implementation.

14.2
Need of Validation

Every day, a high number of HPLC analyses, related to the monitoring of organic compounds, are performed in thousands of laboratories around the world. These measurements are very useful in many situations: quality control of food and other consumer goods during manufacturing, processing, trading, and consumption, detection of deficient products or incorrect labeling, clinical assistance, checking the quality of drinking or waste water, forensic analysis in criminal investigations, and support for research, among others. In fact, every aspect of our life depends to some extent on analytical measurements.

Many important decisions are taken on the basis of the results: batch release or refusal, purchase of a specific product, and trademark, prescription of a medical treatment, to permit the discharge of a water stream, the outcome of a trial, interpretation of the results, and so on. In all these cases, an incorrect value can lead to a wrong decision, with awful consequences for health, reputation, and economics. Besides, the cost of making these analyses is considerable, and on occasions, the decisions arising from the results may involve a significant disbursement. Thus, it is important to determine the correct value and be sure of its reliability. For these reasons, the requirement for laboratories to use a validated method is now universally accepted [1,5,21,22].

Governments have been aware of the importance of quality control and have created legal agencies to verify that the analytical laboratories are correctly performing their work. The regulation workgroups develop guidelines indicating the official analytical methods and the criteria for validation [2]. These reference methods must be interlaboratory validated and must prove a higher reliability than other methods, because legal decisions will be taken on the basis of their results [23]. The accreditation agencies must look after the laboratories to check the correct implementation and running of the methods. Therefore, the laboratories must check the methods to verify the compliance with the national and international regulations, and then pass the accreditation [8].

The methods should be validated because of the own professional duty of the analyst. It is recognized that a laboratory must take the adequate actions to ensure that it is capable of providing coherent, interpretable, and accurate results with a known uncertainty. Thus, the analytical methods must be reliable enough to guarantee that any decision based on it will be taken with high confidence. A poorly reliable assay will probably provide false data, and then it must be removed from the laboratory's portfolio. The validation allows to prove that the analytical method is fit-for-purpose. Besides, the end users of the results must trust the values provided by the laboratory, in order to be sure that the judgment based on the values obtained can be taken with enough confidence. In case of controversy, the laboratory staff must be able to demonstrate that they have correctly performed their work [1].

Validation exercise is expensive and time-consuming, and normally disturbs the normal working of the laboratory. However, the use of a validated method eliminates testing repetitions and improves the prestige of the laboratory, attracting more clients, thus resulting in long-term profitable and time saving [24].

Summarizing, the validation of a method must be performed for the following reasons [8,20,24]:

1) Assuring high quality of the results.
2) Reaching acceptance of the products by international agencies.
3) Achieving the range of "official/reference method" approved by regulatory agencies.
4) Mandatory requirement for accreditation of the laboratory by ISO 17025 guidelines.
5) Compulsory condition for registration of any pharmaceutical product or pesticide formulation.
6) Improve the financial bottom line of the laboratory.

According to ISO/IEC 17025:2005, a laboratory must validate all the used methods [8]. The methods will be separately validated for each matrix and working range, even dealing with the same analyte. A full validation is required when implementing a new method: in-house developed, taken from a bibliographic source, transferred from other laboratories, and reference one.

To check the steadiness of the performances, many methods require a kind of day-revalidation at the beginning of the day. This normally consists in a calibration–accuracy–recovery–precision test, which can also be considered as a partial validation. The periodicity of the determination of each parameter can vary several times within a day (each determined number of analyzed samples), depending on the stability of the results and the scope of the analysis [22,25].

The cross validation is needed when two or more methods, or the same method in several laboratories, are used to obtain data within the same study. It consists in the comparison between the results provided by the analytical methodologies. The most reliable method is taken as "reference" and the other methods serve as "comparator." Furthermore, the validation is performed in other ways [1,25].

14.3
Validation History

The concept "evaluation of the performances and limitations of an analytical method" comes from the pharmaceutical industry, in order to have a tool to assess the characteristics of drug products, reaching the strong quality requirements demanded by regulatory agencies for registration and trading of the medicaments [18]. The use of mathematics to assess the quality of the new developed methods was proposed during the 1940s by the American Chemical Society and Merck & Co. However, this topic was neglected by chemists until the 1970s, where several papers were published indicating the need of establishing a reliable set of parameters to determine the characteristics of analytical methods. This paved the way for the recognition of the usefulness of method validation and its implementation in analytical laboratories [3].

Government regulatory agencies proposed compulsory guidelines for accreditation of quality control laboratories [8,10]. Several researchers also made their contribution to this topic [26]. The first report about the elaboration and processing of reliable drug products was the "Current Good Manufacture Practices," developed by the FDA in 1971. The term "validation" was not included, although accuracy and precision were stated [27]. The requirement of validation was implied in the CGMP issued in 1978, where the word "validation" was first used as a proof of suitability, and accuracy and precision were definitively stated as compulsory for regulatory submissions [28]. However, at this stage, the instructions about how to conduct validation were vague and rather incomplete. This was corrected the following years, by the preparation of many guidelines focusing on a specific scope and with a more methodical description of the procedure. A nonexhaustive list of the main validation reviews and guidelines issued, which illustrated the history of validation, is presented next.

14.3.1
The 1990s

During this decade, there was an increasing interest of implementing methodologies to ensure the reliability of the results obtained in the laboratory. Therefore, there was a worldwide outburst of publications about quality assurance guides and validation procedures, by renowned organizations and individual researchers.

Several workshops were organized to provide the opportunity to share their opinion. In 1990, the "Conference on analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies" was held in Washington DC and sponsored by the major pharmaceutical organizations: the American Association of Pharmaceutical Scientists, AOAC, FDA, International Pharmaceutical Federation (FIP), and the Health Protection Branch (HPB). It was the first major workshop dedicated to investigation of and agreement on validation procedures for analytical methods devoted to the quantification of drugs in biological samples. The conference focused on the parameters to be

studied, procedures and requirements to ensure the acceptability of bioanalytical methods. The main outcomes were the definition of the main validation parameters and the establishment of the acceptance criteria for a run. The conference report laid down broad principles to be referred to in the development of future guidelines, but it did not reach an official status. Later, in 1999, the Food and Drug Administration developed and published the official "Draft Guidance for Industry: Bioanalytical Methods Validation," based on this conference [25]. The same year, the symposium on harmonization of quality assurance systems for analytical laboratories, sponsored by IUPAC, ISO, and AOAC, was held in Budapest (Hungary). The main discussion theme was the harmonization of the different validation protocols. The discussions led to the writing of the technical report "Harmonized guidelines for single laboratory validation of methods of analysis," issued by IUPAC in 2002 [13].

The most important document published about validation was probably the "ICH Harmonized Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology, Q2(R1)," which was released in 1994, ammended in 1996, and took its current form in 2005. This ICH guideline aimed to develop consensus criteria for registration of pharmaceutical products in the US, Japan, and the European Community [29]. In 1998, the EURACHEM group published another interesting document, "The Fitness for Purpose of Analytical Methods," a laboratory guide to aid researchers to validate their analytical methodologies. This guidance became the most popular one. The second edition was issued in 2014, and incorporates the main changes in international standards and practice [1]. Other renowned organizations, such as the World Health Organization (WHO), AOAC, CDER, USEPA, US Pharmacopoeia (USP XXII 1225), British Pharmacopoeia [27], EURACHEM group, FAO, and ISO, also developed and launched several validation guidelines devoted to ensure the quality assurance in the laboratory. These guides were devoted mainly to the description of the validation parameters. Some guides focused only on specific themes. Many individual researchers also published interesting documents about the validation procedure. The more relevant events and released documents related to validation during this period are described in the Table 14.1.

14.3.2
Beyond the Year 2000

The 2000s were also prolific in the publishing of validation-related documents. A large number of new guidelines, quality assurance documents, reviews, and research papers about the state of the art and specific applications were published by organizations and individual researchers. The trend was to overcome the single laboratory validation and maximize the collaboration among laboratories, thus the quality transference and multilaboratory approaches were increasingly discussed. The novelties in instrumentations were also considered. The revalidation procedure and the validation of transferred methods were also investigated.

Table 14.1 Several contributions of individuals and organizations about validation and quality assurance during the period 1990–1999.

Year	Event	Author	Ref.
1992	Development of the first issue of the "Specifications for pharmaceutical preparation" guide. This document has been upgraded each year to 2014	WHO	[30]
1993	Development of the "peer-verified methods validation program," with an exhaustive description of each validation parameter	AOAC	[31]
1994	Publication of some papers proposing the application of the life cycle approach to the validation and revalidation of methods	Hokanson	[32,33]
1994	Release of a guide about the validation of chromatographic methods	CDER	[34]
1995	Release of a validation guide for the "Resource Conservation and Recovery Act," devoted to the analysis of contaminants in environment	EPA	[35]
1995	Publication of a paper about the quantification of a theophylline in tablets using thin-layer chromatography, taking the requirements for EU multistate registration for the validation of a specific method	Renger *et al.*	[36]
1995	Inclusion of a section that describes the requirements for the validation of compendial methods. The document is upgraded in each new edition, incorporating the last advances.	US Pharmacopoeia (USP XXII 1225)	[27,37]
1996	Inclusion of the term "validation" in the British Pharmacopoeia	British Pharmacopoeia Commission	[27]
1996	Proposal of a practical guide about the validation of analytical methods, describing a set of minimal requirements for a method Most of the currently accepted validation parameters	Green	[38]
1996	Release of a protocol for validation, focusing on calibration, recovery, method comparison and robustness	Wegscheider	[39]
1997	Publication a paper describing validation procedures implemented in Japanese control quality laboratories	Seno, Ohtake, and Kohno	[40]
1997	Recommendation of the definition and application of a universal guide for the validation of analytical methods	Winslow and Meyer	[41]
1998	Publication of a guideline for the validation of analytical method for food control	FAO	[42]
1999	Release of the systems of quality assurance ISO 17028:1999, indicating the correct management of an analytical laboratory, and focusing on the sampling and on the need of method validation	ISO	[43]

Note: The standard rule ISO 17028:1999 is mandatory for the accreditation of laboratories in the EU, whereas other validation guides also offer recommendations.

The introduction of systems of quality assurance for accreditation was widely studied. In 2002, the EU Commission Decision 2002/657/EC, proposed by the European Council, was issued. It includes the requirements of the analytical methods applied to evaluate the compliance of batches of live animals and derived products, introduced or produced in the EU for food purposes. It is probably the most important validation guideline in the European Union. Its implementation is compulsory by the official quality control laboratories [10]. The requirements of validation for legal accreditation of analytical laboratories were emphasized in ISO/IEC 17025:2005, which was issued in 2005. It also includes a chapter about the testing of chemical assays. The fulfilment of this standard rule is effectively mandatory for accreditation [8]. Other organizations published documents to assist quality control laboratories to implement the standard rule ISO/IEC 17025:2005, such as FAO (focusing on food analysis and introducing the Good Laboratory Practices [44]) and AOAC [45], in 2005 and 2007, respectively.

Several workshops about several aspects of validation were organized during the considered period. In 2000, a second workshop about bioanalytical method validation, sponsored by the AAPS and FDA was held. This forum was an opportunity to share the experiences about validation over the past 10 years, and focuses on the incorporation of the new technologies, as mass spectrometry, automation, and electronics, and the study of the matrix effect [25]. The discussions of this workshop formed the basis of the guideline: "Guidance for Industry: Bioanalytical Method Validation," issued by the FDA in 2001 [46]. The same year, the "Pharmaceutical Research and Manufacturers of America Analytical Research and Development" workshop, organized by the Analytical Research and Development Steering Committee (ARDSC) of the Pharmaceutical Research and Manufacturers of America (PhRMA), was held in Wilmington, DE, USA. Approaches about the transfer of analytical procedures associated with pharmaceutical products were discussed in this workshop. The conclusions served to develop a guideline text for appropriate technology transfer, "Acceptable Analytical Practice," which was published in 2002 [47].

A list including other contributions, which provide recommendations about several aspects of validation, can be seen in Table 14.2.

The high number of documents published about validation, and the effort performed to continuously update the guidelines, is an indication of the high importance acquired by validation through these past years.

14.4
Validation Strategies

The analytical problem and the scope of the analysis must be clearly defined to properly perform the validation. The laboratory must agree with the customer on the main analytical requirements that the method must reach to solve the analytical problem (e.g., reach a determined/permitted limit, accuracy under 20% in a specified working range, etc.). Furthermore, the laboratory must state

Table 14.2 Several contributions of individuals and organizations about validation and quality assurance beyond the year 2000.

Year	Event	Author	Ref.
2000	Release of a guidance for analytical procedures and method validation for the pharmaceutical industry, which was upgraded in 2014	FDA	[48,49]
2002	Publication of the "Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals." The document is intended to present guidance for the evaluation of the initial use a method in a laboratory	AOAC	[50]
2003	Publication of a study about the implementation and validation of analytical methods, concluding that the development must be focused on an easy validation and revalidation	Breaux *et al.*	[11]
2006	Release of a guide about the transfer, comparison, maintenance, and methods for biopharmaceutical products	Krause	[51]
2009	Addition of a new chapter about transfer and validation of analytical methods.	US Pharmacopoeia	[52]
2011	Development of a guideline for the validation of bioanalytical methods, addressed to European biomedical researchers	European Medicine Agency	[19]
2012	Publication of a draft guidance for the analysis of drug foodstuffs for safety purposes	FDA	[53]

the validation parameters and the acceptance criteria, as well as other characteristics, such as economics, operation (easy to handle, able to be automated, controlled by software, etc.), work safety, and environmental. In order to provide more reliability to the method, the appropriate validation guideline must be selected [2,54]. The main guidelines have been included in Sections 14.1 and 14.3.

The adequate method must be selected considering the available instrumentation, and preferably following this priority order [17]:

- An official method. The accreditation agency has already evaluated the suitability of the method.
- An analytical method whose performances have been determined through an interlaboratory trial, according to the requirements of an official guide.
- A method from the bibliography or a previously in-house developed method.
- Develop a new method.

In any case, the selected method must be validated. The analyst must confirm that the method is ready to accomplish all the fixed analytical requirements in

the laboratory. Besides, the performance and the limitations of the method would be known, through the determination of the validation parameters. A successfully validated method is expected to produce long-term values with enough quality to meet the scope of the method. If the method characteristics do not match the minimal analytical requirements, then it must be modified, and the validation process must be repeated. This iterative process of development and evaluation should follow until the validation parameters meet the fixed requirements; then the method is fit for purpose and can proceed. To facilitate the validation procedure, a thorough validation protocol should be written, preferably in a step-by-step format, at the beginning of the process, by considering the following points [15,55]:

- Purpose of the analysis (e.g., the evaluated parameters and the acceptance criteria can be different for official food or contamination control and an in-house process control).
- What answer is required (quantitative or qualitative, sensitivity, allowed uncertainty, only under or over a maximum residue limit, etc.)?
- The aggregation state of the sample and the analytes.
- Maximum time and cost of the analysis.
- Limitation of toxic waste.

The required performances of the method must be based on the expected use. Therefore, it is not necessary to evaluate all the validation parameters, and the acceptance criteria can be modified accordingly. For instance, if the method will be used to determine if a food commodity contains a drug over a permitted limit, it is not necessary to determine the upper limit of quantification or to reach too high a sensitivity. Obviously, if the scope of the analysis is modified, a revalidation of the method might be necessary.

The analytical requirements (fixed by the validation guide or the customer) of the method are fulfilled to design the validation sequence, to establish the most important validation parameters, and the definition of the acceptance criteria. The usefulness of a specific method must be proven in laboratory assays using samples similar to those that will be analyzed. A reference certified material containing a known amount of analyte would be preferably used. If it is not available or affordable, spiked samples would be taken [10,46]. The preparation and execution of the validation must be carried out following the detailed validation protocol. The possible steps for a validation procedure may be as follows [11,54,55]:

1) Define the analytical problem.
2) Assemble a cross-functional team and assign to individual responsibilities.
3) Define the scope, the objective, and the application of the method. The two main objectives in quality control are determining the concentration of the analyte over a specified range or, if a sample contains the analyte, over a permitted limit.

4) Select the degree of validation (full, partial, or cross).
5) Select the adequate validation guideline, and establish the validation parameters and their corresponding acceptance criteria. The use of a specific guideline provides more reliability to the validation procedure and can even be mandatory, especially if the results would be used to take a legal or critical decision. The analyst can also freely select the parameters and design the validation process following his own criterion, but this questions the obtained results. An alternative practice is the combination of the two approaches. In any case, the final decision must be taken in agreement with the customer and the local regulations. The same can be applied to the acceptance values for each studied parameter.
6) Design the validation experiments. The design must be optimized to achieve the validation within a minimum number of analyses.
7) Check the relevant characteristics of the equipment and instrumentation.
8) Classify reagents, standards, and solvents, by purity, accurate amount, and stability.
9) Carry out the validation experiments.
10) Calculate the validation parameters. The results will define the performances, the limitations, and the working range of the method.
11) Compare the results with the acceptance criteria. If satisfactory values for all the parameters have been obtained, the method is considered "validated." If unsatisfactory results have been obtained for a parameter, the reasons of the failure must be discovered. Therefore, the method should be accordingly modified and the validation repeated. Another option is to decrease the analytical requirements and then consider it as validated.
12) Compare the results with those obtained by other laboratories. Discuss and resolve the possible inconsistencies.
13) Write Standard Operating Procedures (SOP) with detailed instructions to handle the sample (collection, transport, and storage) and to execute the work in routine analysis (reagents, instrumentation, experimental protocol, LC analysis, calculations, report instructions, security directives, procedures for quality control, and verification of results).
14) Define the criteria for future revalidation.
15) Describe the kind, frequency, and acceptance criteria for the evaluation of the system suitability and the quality control samples in routine analysis. These should be established to show when the method and the system are beyond the statistical control.
16) Document the validation experiments and results in a report. The selected guideline and the acceptance criteria must be indicated, to make easy its interpretation. This report should be registered and must remain available for current and future workers, clients, accreditation agencies, and partner laboratories.

14.5
Revalidation

Some of the parameters of the method would be adjusted if the performances of the method fall outside the analytical requirements, or to improve the quality of the results. The question is whether these changes require the revalidation of the method. Generally, the method should be validated if the results are excessively affected by the changes. In order to clarify this question upfront, operating ranges should be defined for each parameter, either based on experience with similar methods or else investigated during method development. These operating ranges must be verified by robustness studies (see Section 14.7.12), and must be considered a characteristic of the method. The disposal of the operating range for the instrumental parameters, instead of a unique optimized parameter, facilitates the decision making about the revalidation and reduces the experimental work for long term. Therefore, the revalidation is compulsory if a parameter is modified outside the operating range. For instance, if the optimum pH of the mobile phase is 5 and the operating range is 4 and 6, the method does not need to be revalidated if the working pH is moved to 5.5, but ought to be revalidated if the working pH is changed to 7.0 [17].

The revalidation is necessary if the scope of the method has been changed or extended (e.g., applied to other matrices, incorporation of other analytes, consideration of other possible interferences, enlarging of the working range, etc.) or the experimental parameters have been modified (other reagents, other reference standards, variation of the pH, introduction of a derivatization step, different composition of the mobile phase, detection conditions, etc.). A revalidation is also required if the instrumentation has been changed (another chromatograph, change of column or detector, etc.), or the chromatograph is moved to another location with different environmental conditions, and these modifications have not been taken into account in the first validation. The method should also be revalidated if it is to be applied by other analysts with different skills and/or transferred to another laboratory. A revalidation is recommended if the method is used after a long time without applying it or if inconsistencies have appeared during the normal use in the results [1,2]. If a variation requiring a revalidation is applied, it must follow a well-documented control system.

Several laboratories are reticent to improve their methods, to avoid performing a full revalidation, especially if they are surveyed by legal accreditation agencies. A partial validation, by testing some of the several validation parameters, is allowed if the modifications are not very strong. The revalidation degree depends on the extent of the influence of the changes on the performances of the method. Therefore, the revalidation can vary from a simple accuracy–precision– recovery assay to a full revalidation. The parameters to be revalidated are determined by carrying out a system suitability test, and analyses of control quality samples are carried out. The revalidation degree is decided on the basis of the fit of the results with the predefined acceptance criteria for each parameter. A partial validation is typically allowed for a well-established analytical method, if only

a few of the following parameters have been changed: analyst, instrument within the same company, reagent purity, species within matrix, change in concentration range, chromatographic conditions, detection conditions, and sample preparation. A partial validation may also be performed if dealing with limited matrix volume or rare matrix [25].

14.6
Transferring of Validated Analytical Methods

On several occasions, a laboratory must implement a routine analytical method previously validated in another one. The method is then transferred from the laboratory that has validated the method to the one that is interested in using it. Typical instances of method transferring are from the research and development (R&D) laboratory to the quality control (QC) laboratory in the same company.

The receiving laboratory must show that it can successfully apply the method. Therefore, it must verify that the validation state is maintained, which means it is able to obtain results with the same reliability as the issuing laboratory. The competence of the receiving laboratory to use the method is demonstrated through determined assays, for instance, to repeat the critical experiments of the method validation, the same samples are analyzed both by the issuing and the receiving laboratories. The results are compared with the previously established acceptance criteria, in order to ensure the success of the transfer [56]

Nowadays, there is no official document available that can be used as a guide for the receiving laboratory to estimate the success of the method transferring. The USP has published an article describing the most common practices of method transfer [57]: comparative testing, covalidation between two laboratories or sites, complete or partial method validation or revalidation, and the omission of formal transfer, sometimes called the transfer waiver.

The transference should be controlled by a well-established procedure. The recommended steps are as follows:

1) Appoint a project owner.
2) Transfer the scientific documentation.
3) Train the analysts who receive the tasks related to the instrumentation, the method, the critical parameters, and the resolution of problems.
4) Develop a transfer plan.
5) Define and execute the tests to evaluate the success of the validation: some critical experiments of the method validation (at least two), and analysis of samples: type and number of samples (a minimum of three), replicates, and so on.
6) State the acceptance criteria (tolerated deviation from those obtained by the issuing laboratory, accepted bias, and uncertainty).
7) Describe rationale for the assays.
8) Compare the results of the tests and the acceptance criteria.

9) If the test results conform to the acceptance criteria, the transfer is successful.

10) Otherwise, the transfer is unsatisfactory, and the reasons of failure must be investigated and corrected. Afterward, the transference assay should be repeated,

11) Document the results of the transference assays.

It is important to note that the receiving laboratory has the entire responsibility to control and assess the validity of the transfer. However, the issuing laboratory should collaborate, as far as possible, during the process.

14.7
Statistical Validation Parameters

The statistical validation parameters (listed in Section 14.1) describe the performances and the limitations of the methods. They indicate the quality of the results when a single sample is analyzed. In order to determine the validation parameters, the analyst must know the meaning of each parameter and the adequate calculation process. The method is fit for purpose if the validation results are under the acceptance criteria established following the analytical requirements [1].

The validation parameters have been defined by several international organizations and have been extensively discussed in the literature. The definitions and the calculation process vary depending on the source, even if the general scope is similar. The definitions of the parameters have been taken from FDA Guidance for Industry: Bioanalytical Method Validation [46], European Commission Decision 2002/657/EC [10], IUPAC [13,58], ISO [59], and the ICH guideline [29]. The meaning provided by ICH has been taken as preferable, because this guideline has been developed for harmonization purposes.

14.7.1
Selectivity/Specificity

The terms "selectivity" and "specificity" are interchangeably used [60]. They refer to the ability to produce a signal unequivocally due to the analyte, in the presence of other compounds and under the instrumental conditions of the method. Therefore, it is the most fundamental parameter. The identification test must be able to recognize the peak of the analyte among those other peaks of the chromatogram and to discriminate between the analyte and closely related structures expected to be present in the matrix. The identification is possible only if the signal attributed to the analyte really originated from it, and not from other compounds or the instrumentation. A good selectivity is needed for both qualitative and quantitative purposes.

Interference is a compound disturbing the determination of the analyte. If two or more analytes are analyzed by the same method, each one can also be

considered as a possible interference for the others. In chromatographic analysis, we can differentiate two cases:

- A compound reacts or interacts with the analyte, enhancing or decreasing the signal, and thus causing a proportional error (matrix effect). In this case, the interference may not produce a peak in the chromatogram. The interfering substance is detectable in recovery studies. This case is not discussed here [1].
- A compound produces a chromatographic peak overlapping with the analyte. In qualitative determination, it causes a false positive (the signal of the interference is assigned to the analyte). In quantitative measurements, it causes a positive bias (the area of the interference is added to that of the analyte and then the provided concentration is higher to the true one).

The methodology to evaluate the selectivity depends on the nature of the possible interfering compounds. We can distinguish two cases:

- A compound intrinsically belonging to the sample, the matrix, and normal impurities (e.g., proteins in plasma, fish flesh compounds, etc.). The selectivity is determined by testing samples, blank and analyte-spiked, from several sources [3].
- Another additive present in the sample or a degradation product of the analyte (e.g., other drugs coadministered with the analyte in plasma, other banned drugs used for fish growing, etc.). The selectivity should be tested by analyzing samples spiked with all the possible interferences at their expected concentrations, with and without the analytes.

The method is considered "selective" if the analyte is completely resolved: the blank chromatograms do not show peaks or baseline distortions near the retention time of the analyte, and the interferences do not overlap with the analyte. If a good selectivity is not achieved, the method should be changed to avoid the interference. Possible modifications to improve the specificity are as follows:

- *Extraction*: Use an extraction method with a recuperation of 100% for the analyte and 0% for the interferences.
- *Stationary or mobile phase-composition*: Modify the stationary or mobile phase-composition in order to increase the differences between the retention times of the analyte and the consecutive peaks, and then eliminating the overlapping
- *Detector*: Modify the detection conditions or change the detector, in order to annul the signal of the interference. This changing may also vary the sensitivity.
- *Derivatization*: To strongly change the chemical properties of the analyte. This alternative must be taken as a last option, because a reaction step is a strong source of variance.
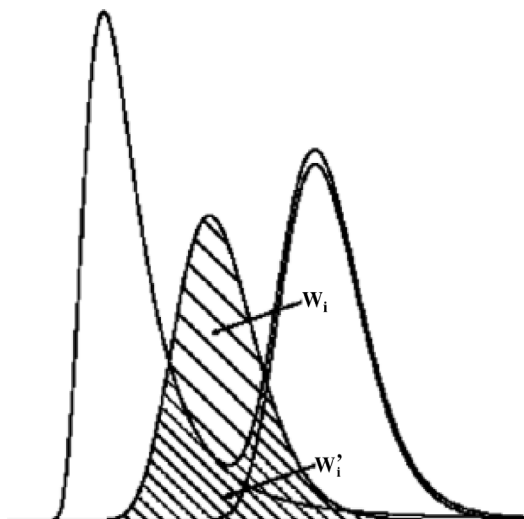
Figure 14.1 Graphic representation of the overlapping criterion.

In several cases, the interferences are at high concentration or cannot be removed by easy procedures. Thus, the laboratory must consider incorporating the interferences into the validation.

The selectivity can be quantified for each analyte by resolution measures. The resolution can be calculated focusing on a single peak (individual) or by pair peaks (elemental) [61]. All the resolution values must be calculated using the same criterion.

14.7.1.1 Individual Resolution

The individual resolution quantifies the resolution degree of a compound face to all the compounds nearby eluting. Therefore, it isolates the contribution of each compound of a mixture associating a value with each individual peak. It is calculated by the overlapping criterion (Figure 14.1):

$$r_i = 1 - \frac{w'_i}{w_i}, \tag{14.1}$$

where w_i and w'_i are the area of the peak and area overlapped with other peaks, respectively, considering that $0 < r_i < 1$.

This criterion uses both elution time and peak shape. However, it requires the drawing of the shape of the overlapped peaks, which may be an important source of error. Besides, it does not measure the separation of the peaks. An adequate resolution is reached at $r_i = 1$; inferior values (even slightly) are not accepted.

14.7.1.2 Paired Peaks Resolution

The elemental resolution is calculated between two consecutive peaks. Thus, two "elemental resolution" values are calculated for each compound: one with the
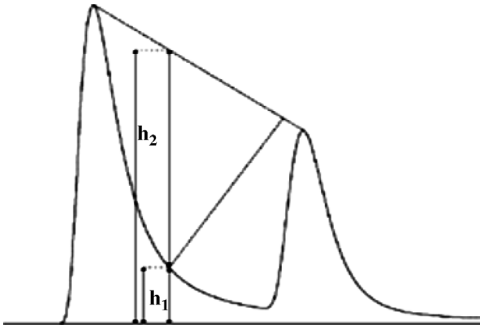
Figure 14.2 Graphical representation of the parameters of the valley peak criterion.

substance eluting immediately before and another with the compound eluting immediately after. The elemental resolution evaluates the overlapping degree and the separation. The elemental resolution can be calculated using the following criteria:

- *Modified selectivity*: It only considers the retention factors (k) and the selectivity factor (α) of the peaks:

$$r_{ij} = 1 - \frac{k_i}{k_j} = 1 - \frac{1}{\alpha}, \tag{14.2}$$

  where $k_j > k_i$ ($j = i + 1$).

  This criterion is not very useful, as it does not consider the shape of the peak. The resolution ranges from 0 (elution at the same time) to 1 (asymptote, infinite separation between the peaks) (see Figure 14.2)

- *Valley–peak criterion*: It is based on the measurement of the elevation of the baseline between two consecutive peaks. The "valley point" is the point of the baseline of the valley between the two peaks, further from the line joining the maximum of the two neighboring peaks. The resolution is calculated by the following formula:

$$r_{i,i+1} = 1 - \frac{h_1}{h_2}. \tag{14.3}$$

h_1 = distance between the x-axis and the baseline at the time of the "valley-point" (signal unity)

h_2 = distance between the x-axis and the line connecting the maximum of the two peaks.

The resolution depends on the difference of elution times and the peak shape, and is especially affected by the asymmetry. The resolution ranges from 0 (peaks fused at their maximum) to 1 (the two peaks are separated by a null baseline).
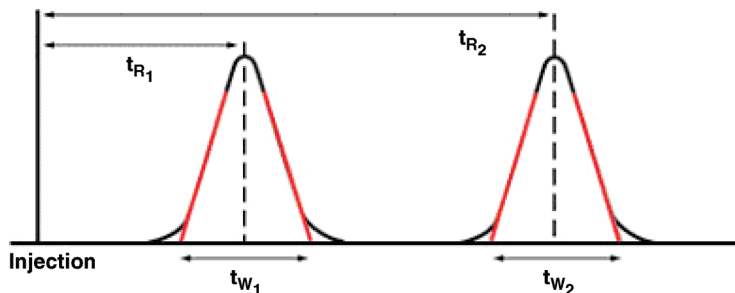
Figure 14.3 Resolution formula and graphical representation.

- *Resolution factor*: This criterion considers the retention time and the broadness of the peaks (Figure 14.3).

$$R_s = (t_{R_1} - t_{R_2})/0.5(t_{w_1} + t_{w_2}). \tag{14.4}$$

The resolution ranges from 0 (elution at the same time) to $+ \infty$ (ideally, infinite separation face to finite broadness). A $R_s > 1.5$ indicates the absence of overlapping between the peaks [24].

14.7.2
Calibration Curve and Linearity

The calibration curve is a mathematical equation relating to the area of the peak and the concentration of the analyte. In chromatographic methods, the peak area and the concentration are assumed to be related by a first-grade equation [3]:

$$A = (b \pm s_b)\,[\text{concentration}] + (a \pm s_a), \tag{14.5}$$

where $A =$ peak area; $b =$ slope, $s_b =$ standard deviation of slope, $a = y$-intercept, and $s_a =$ standard deviation of the y-intercept.

The linearity is the ability of the method to provide a signal directly proportional to the concentration of the analyte in the sample [55]. The linearity can be tested using standard solutions or spiked blank samples. This last option is preferable, because the calculated slope and y-intercept would incorporate the matrix effect. The linearity should be evaluated across the working range of the analytical method (see Section 14.5.3).

The linearity is first evaluated by visual appreciation by plotting the average values of peak area versus the analyte amount. Furthermore, these data are treated by least-square linear regression to calculate the constants of the calibration curve and evaluate the quality of the linear relationship (the determination coefficient, r^2, and the residual sum of squares). This method aims to minimize the difference between the experimental and the calculated peak area in the tested values. A minimum of five calibration points at increasing concentrations, equally spaced, is recommended. Each calibration level should be calculated by three independent replicates. Using this statistical method, the calibration points are "naturally" weighted by the

concentration. To minimize this distortion, a $1/\text{concentration}^2$ weighting factor can be introduced if the calibration range is over two orders of magnitude. A y-intercept significantly different from 0 indicates a bias and should be further studied. A r^2 close to 1 indicates adequate linearity, whereas a r^2 close to 0 indicates the total absence of proportionality. A calibration curve is accepted at $r^2 > 0.990$ [62].

14.7.3
Calibration Range

The calibration range is the interval between the minimal (lower limit of quantification, LLOQ) and the maximal (upper limit of quantification, ULOQ) amount in samples, in which the analytical procedure provides quantitative results with a suitable level of linearity, accuracy, and precision. It is normally established by the linearity studies. The LLOQ depends on the sensitivity of the method and the ULOQ depends on the saturation of the extraction step and the detector.

Even if the logical process will be to determine the entire calibration range, the evaluation of the linearity over a range spanning 50–150% of the expected concentration or the maximum residue limit (MRL) in real samples is usually enough. Anyway, the minimal acceptable range must be taken depending on the scope of the analysis [3].

14.7.4
Limit of Detection

In chromatographic methods, the noise is the oscillation of the baseline of the chromatogram at the retention time of the analyte, when injected a blank sample. When a signal near the background noise is obtained, it must be decided if it corresponds to random responses of the blank or to the presence of the analyte. The limit of detection (LOD) is a statistical value that establishes the minimal concentration that provides a signal that can be reliably differentiated from the background noise, with a specified significance level ($\alpha = 5\%$). Therefore, signals over that produced by the LOD are assigned to the analyte, whereas inferior values are attributed to the background. Finally, at LOD, the presence of the analyte can be assessed, but not quantified with reliable accuracy and precision.

The quantification of the analyte at <LOD would provide uncertainties larger to the value itself. In this case, the result must be referred to as "concentration <LOD," instead of "concentration = 0." The LOD is logically under the calibration range. A LOD under the minimal value expected in a real sample is usually required.

The LOD must be calculated in sample matrix, as the baseline noise depends on the physical properties and chemical composition of the matrix. The ICH guideline has clearly defined several useful approaches to calculate the LOD [29]:

- *Visual evaluation*: Injection of samples containing decreasing known concentrations of the analyte. LOD is the minimal concentration providing a distinguishable peak area.

- *Signal to noise* (S/N): The minimal concentration providing a peak height three times the baseline noise.
- *Standard deviation, the 3.3s criterion*: The LOD is calculated as 3.3 times the standard deviation of the blank (s_0) divided by the slope of the calibration curve. The s_0 is the average noise ratio of the background signal (width of the baseline) at the time window in which the analyte is expected, taken with >20 replicates, or as the standard deviation of the y-intercept.

14.7.5
Limit of Quantification

The LOQ is the lowest concentration that can be quantitatively determined with accuracy and precision under the fixed acceptance criteria (normally 10–20%). The quantification of the analyte in the LOD–LOQ range is possible, but with a too high associated uncertainty. Thus, the reported confidence interval would be uninformative. Thus, the result must be simply reported as "concentration between LOD and LOQ." The LOQ would be reasonably close to the LLOQ. The LOQ must be under the minimal concentration expected in a real sample.

As for LOD, LOQ should be determined in matrix sample. ICH [29], EUROCHEM [63], and FDA [46] guidelines have proposed different approaches for the determination of the limit of quantification:

- *Visual evaluation*: Analyze a series of samples with decreasing concentrations by six replicates. The relative standard deviation (RSD, %) is plotted versus the concentration. The RSD normally increases at lower concentrations. The LOQ is the amount that corresponds to the previously defined required precision [63].
- *Signal to noise*: The minimal concentration providing a peak height 10 times the baseline noise [29].
- *Standard deviation, the 10s criterion*: Same as LOD, but 10 times the standard deviation of the blank (s_0) divided by the slope of the calibration curve [29].
- *Calibration curve*: The LOQ is taken at the LLOQ level [46].

14.7.6
Sensitivity

The sensitivity is the ability to discriminate between small variations of the concentration of the analyte. In chromatographic analysis, it is calculated as the derivative of the peak area regarding the concentration, thus the slope of the calibration curve. It is also evaluated by the LOD. The sensitivity should be calculated from data obtained in sample matrix. This parameter is usually monitored in routine calibrations [13].

14.7.7
Accuracy and Precision

The precision is defined as the closeness of agreement between the detector responses obtained by several individual measurements of a homogeneous sample, under stipulated conditions [13]. In chromatography, it is the concordance between the values of peak area obtained from independent analyses of homogeneous aliquots. The precision is provided as dispersion or variability, and quantified through the RSD of the detector response. This value is always positive (the sign of the deviation is neglected), and an RSD close to 0 means an excellent precision. The variability is due to the random errors through the method.

Accuracy and trueness, while with a different meaning [1], are interchangeably used in the literature [26], and "accuracy" is the preferred term (see Section 14.8). Accuracy/trueness is the closeness between the concentration provided by the analytical assay (calculated from the peak area through the calibration curve) and the true value [13]. The "found concentration" is taken as the average of several measurements to minimize the effect the random errors. The accuracy shows the extent to which the systematic errors affect the result, and is quantitatively stated in terms of bias. Thus, it has been highlighted as the most crucial aspect of any analytical procedure [62]. The accuracy can be reported by two ways:

- Ratio average found concentration/true concentration (maximum trueness is attained at values around 100%).
- Error, calculated as the difference of the average found concentration and the true one, divided by the true one. Maximum trueness is reached at a value of 0%.

The method shows a positive bias if the found concentration is over the true one (accuracy >100% or >0%, respectively), whereas a measured concentration under the real value (accuracy <100% or <0%, respectively) is obtained if the method has a negative bias [13].

The accuracy and precision should be evaluated during the same experimental assay and using the same solutions. These parameters should be determined at three concentrations: low (near the LLOQ or under the permitted limit), medium (near the middle of the calibration range or permitted limit), and high (near the ULOQ or over the permitted limit) [10,15]. Each level is analyzed by three to six replicates. They must be tested using traceable materials with a known concentration, ideally with a matrix close to the real samples. The tested solutions must be different from that analyzed for the calibration. The following materials are recommended, indicated in decreasing order of appropriateness:

- *Reference certified materials (CRM) close to the sample*: the concentration has been accurately determined by an accredited laboratory. However, it is an expensive material.

- *Spiked sample*: A blank sample is fortified with a known amount of the analyte. In this case, the accuracy is determined as recovery.
- *Standard solutions*: Not recommended, as the results do not include the matrix effect.
- *Real samples analyzed using a reference reliable method*: Applicable only if standards are not available.

The errors (both random and systematic) result from the combination of the errors caused by several sources, such as inherent to the procedure, specific circumstance occurring on a particular day, the analyst, the instrumentation, the laboratory, and so on. The following experimental designs have been implemented to determine the error associated with each factor:

- *Intraday measurements (within run, within batch)*: The accuracy and precision are calculated from the data obtained under the same operating conditions, by the same worker, and repeated over a short period of time (within the same day). It corresponds to the bias and variability inherent to the procedure itself, and they are the minimal values that can be obtained. Under these conditions, the precision is named repeatability. The bias and variability obtained by the validation must be compared with that described for the method and obtained by other laboratories, in order to detect inconsistencies.
- *Measurements by changing one parameter (between runs, interbatch, or within laboratory)*: Using the data obtained by changing one method factor: several days over a long period of time (interday accuracy or precision), different equipment, different purity or supply of reagents and solvents, different workers, and so on. Because of the large number of factors, only the most relevant should be studied.

Measured under these conditions, the precision is also named intermediate precision [29] or ruggedness major changes [10]. The bias and the variability express the contribution of the changed parameter.

- *Interlaboratory measurements*: the bias and variability are calculated using data obtained by several laboratories and under different operating conditions. The values are supposed to be close to the maximal variability or bias obtainable using the method. This approach is usually applied in collaborative trials for the standardization of a reference method. The precision is termed reproducibility. According to the AOAC international guidelines, the reproducibility should be calculated by a minimum of 10 independent laboratories, and each sample must be analyzed by at least 12 replicates. The samples have to be blinded and randomized [23].

The obtained values of bias and variability are compared with those established as acceptance level, in order to determine if the differences between the replicates and between the found concentration and the true value are significant.

14.7.8
Uncertainty

The uncertainty describes the fluctuations of the result of a measurement. The uncertainty has been defined as a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand (calculated magnitude) [64]. In chromatographic analysis, it characterizes the maximal distance between a measured concentration and the true value that can be obtained when analyzing a sample, with a certain significance level (α). The uncertainty quantifies the level of doubt over the concentration obtained. The result of an analytical measurement must be provided as the confidence interval: "found concentration $\pm$ uncertainty (α)," interpreted as the region around a routine analysis in which the true value resides with a probability of $(1 - \alpha)$ % [62]. The uncertainty is calculated as the standard deviation of the found concentration multiplied by the t-student factor corresponding to the significance level α with ν degrees of freedom for a two-tailed test.

The total variance on the results arise from the random errors obtained through the method from many possible sources, such as an incomplete or imperfect definition of the measure and imprecise value of physicochemical constants, storage conditions, sampling, sample preparation, purity of chemicals, matrix effects and interferences, environmental conditions, weight and volumetric equipment, incorrect reading of equipment measures, operator effects, false reference values, blank correction, limits in the discrimination or resolution of the analytical instrumentation, approximations and assumptions incorporated in the measurement method and procedure, instrumentation effects, the use of a low number of decimals in the statistical treatment of the data, and so on [7].

A low value indicates that the experimental procedure has been carefully carried out, applying good working principles. However, it is unable to detect a bias in the method. The uncertainty is estimated to judge the utility and adequacy of the result, according to the stated purpose of the analysis. A large confidence interval would have a higher probability to contain the true value, but would also be less informative. The uncertainty is closely related to the precision, but it is not the same concept. The precision refers to the variability of the detector response, whereas the uncertainty points to the variability of the concentration in the sample.

The global variance (s_0^2) must be determined by authorized laboratory staff with proven proficiency as follows:

- *Theoretical*: The total variance is calculated by combining the variance of each recognized and significant source, using a mathematical equation based on the law of propagation of errors. Although the standard deviation forms part of the validation, it is applicable to the results obtained by analysis of real samples [13,64,65]. This criterion is more rigorous, but it is rarely used, because it is too tedious, time-consuming, and some sources are difficult to evaluate.

- *Experimental*: The uncertainty is not calculated during the validation, but for each analyzed sample. Thus, real samples are analyzed by several replicates (usually three), and the standard deviation is taken from these results, and $\nu = n°$ replicates − 1. This experimental standard deviation is more representative, as it is specific for each sample. However, it provides uncertainties higher than in other methods, and increases the cost of analysis. It is the criterion more used for uncertainty measures.

14.7.9
Recovery

The recovery is the percentage of the rescue of the analyte in a sample. Using a chromatographic method, the experimental design and the calculation methodology depend on the aim: to determine the matrix effect or the effectiveness of the sample preparation. In any case, the recovery must be always determined using real samples.

14.7.9.1 Matrix Effect
The evaluation of the matrix effect through the recovery is mandatory if the linearity, accuracy, and precision have been evaluated using standard solutions. If a method developed and validated for a specific sample is used to analyze the same analyte in a closely related different matrix, the effect of the matrix change in the results should also be studied. In both cases, a sample (blank or containing the analyte) is fortified with a known amount of analyte standard and analyzed before and after spiking [16]. The recovery is calculated as follows:

$$\%\text{Rec} = \frac{(C_F - C_U)}{C_A} \times 100, \tag{14.6}$$

where C_F is the concentration detected in the spiked sample, C_U is the concentration detected in the sample before the spiking, and C_A is the true added concentration.

The recovery should be determined at the same concentrations as for accuracy (see Section 14.5.7). In order to reduce the influence of the variability, the recovery is calculated as an average value of three–six measurements.

The recovery should be ideally 100%. Recoveries under or over this value indicated a systematic error caused by the matrix effect, which must be corrected. Therefore, the method should be modified by incorporating correction strategies, such as revalidating the method using sample matrix or adding an internal standard. If the recovery is inadequate but consistent, a correction factor can be introduced.

14.7.9.2 Effectiveness of the Sample Preparation
The recovery is measured to determine the yield of the extraction procedure. Several blank samples are fortified with a known amount of analyte and analyzed. The recovery is calculated as the quotient of the peak area obtained by analyzing the

spiked sample and the peak area obtained by the direct chromatographic analysis of a standard solution containing the concentration representing a 100% recovery (thus, considering the dilution or preconcentration steps of the experimental procedure). A bias in the chromatographic step would affect the two values in a similar way, so that the difference would be caused by the sample preparation. The studied concentrations and replicates should be taken as in Section 14.5.9.2.

The recovery must be ideally 100%. An inferior or superior value indicates loss of analytes or abnormal preconcentration, during the sample preparation. When the recovery is unsatisfactory but reproducible, a correction factor may be introduced. If the recovery is variable, an internal standard must be added, with a chemical structure related to the analyte, to compensate the variability and inefficiency of the sample preparation [46].

14.7.10
Decision Limit

This criterion was established by the EU Commission Decision 2002/657/ EC [10] to evaluate the compliance of food batches. The samples containing a concentration of an organic residue over a fixed permitted limit are discarded. Therefore, the decision limit ($CC\alpha$) is applicable when the scope of the analysis is to check if the concentration of the analyte in the sample is $\leq$ or $>$ an established permitted limit, thus the sample must be accepted or rejected. If this value has not been defined, the decision limit is calculated considering a null concentration as "permitted limit."

The decision limit is defined as the "found concentration" above which it can be concluded that the analyte is over the permitted limit with a probability less than a fixed significance level (α) to obtain a false positive. If a sample containing the analyte exactly at the permitted limit is analyzed a large number of times, the inherent variability of the method will cause that half measures will provide a "found concentration" $\leq$ the permitted limit, and half measures will provide values $>$ the permitted limit. In the first case, the laboratory will correctly accept the sample, whereas in the second case, the sample will be incorrectly rejected. Therefore, the maximal probability of a false negative is 50% (permitted limit = $CC\alpha$ at $\alpha = 50\%$). Considering that the consequences of the rejection would cause strong economic (rejecting food batches), professional (in a doping control for sportsman), and legal (drug in blood) damages, this error probability is not acceptable for a reliable quality control laboratory. Therefore, the limit value to decide the suitability of a sample is switched to a higher value by reducing the α. Therefore, a sample containing the permitted limit would provide "found concentrations" over the $CC\alpha$ only the α % of the measures and under the $CC\alpha$ at $(1 - \alpha)$ % of the measures. The maximal probability of providing a false positive is reduced to α. A legal document would mark the $CC\alpha$ as limit found concentration in a compliant sample. The decision limit depends on the permitted limit, the variability of the measure, and α. A graphical explanation of this concept can be seen in the Figure 14.4.
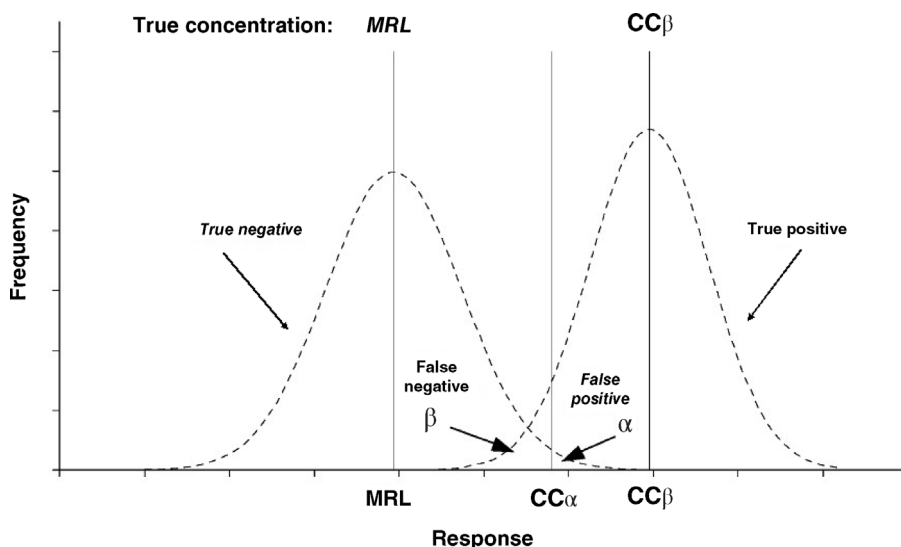
Figure 14.4 Frequency of results versus found concentration in samples containing the MRL (italic font) and CCβ (normal font). The validity of the decision taken and the associated errors has been indicated for each case.

14.7.11
Detection Capability

The detection capability was defined by the EU Commission Decision 2002/657/EC with the same scope and applicability as CCα (see previous section) [10].

The detection capability (CCβ) is the smallest content of the analyte (over the established permitted limit) at which a method is able to detect truly contaminated samples with a β probability of a false compliant result. If a sample containing the CCα is analyzed by many replicates, the random errors would provoke that half measures provide a value over the CCα (correctly rejected), and half measures provide found concentrations under the CCα (incorrectly accepted). The probability of a false negative is 50%, thus the laboratory would provide false results in 50% of the analysis. With this result, the laboratory is not really able to identify as noncompliant a sample containing CCα with enough consistency (CCα = CCβ at β = 50%). The "limit concentration in sample" from which the laboratory is really able to classify a sample as contaminated with sufficient reliability is switched to a higher value, by diminishing β. A sample containing CCβ would be measured as ≤CCα, the β % of replicates and as >CCα, the other $1 - \beta$ %. Thus, the maximal probability to make a false compliant result is reduced to β %. The laboratory must claim that it is able to detect contamination over the "detection capability," instead of over the permitted limit or the CCα. The detection capability will depend on the decision limit, the variability of the measurement, and β %. A graphical description of this concept can be seen in the Figure 14.4.

To avoid confusion, it must be stated that the CCα refers to the concentration obtained through the analysis, whereas the CCβ refers to the amount in the sample.

14.7.12
Robustness

In a laboratory, the operational parameters (factors) rarely remain exactly at the values described in the method, and they always oscillate within a realistic range. Robustness studies aim to examine the influence of the potential sources of variations in the responses of the method. The robustness is defined by the ICH as the ability of the method to remain unaffected by small but deliberate variations of the experimental conditions, likely to occur during the routine usage [29]. In chromatographic analysis, the robustness estimates the consistency of the main chromatographic parameters (tailing factor, efficiency, retention time, and peak area), when internal experimental factors fluctuate from those described in the method, and provide an indication of its stability during normal usage. Besides, the robustness evaluates, for selected factors, the range in which the modifications of the retention time and peak area are assumable, termed as operating range [66]. It must be considered that the robustness studies do not aim to find a quantitative relationship between the chromatographic parameters and the experimental conditions. The robustness is also termed "ruggedness minor changes" [10].

It is recommended to include the robustness during the appropriate step of the method validation, instead of at the end of the validation, and document all the critical results. A validation of a scarcely robust method would provide inadequate results throughout the overall validation process, and will result in loss of efficiency during routine quality control testing, with the subsequent loss of time and funds. Therefore, the robustness evaluation should be carried out before adjusting the experimental parameters. Once the operating range is known, the limit values can be included in the final method, thus providing it some flexibility. Hence, we would have a valid justification to support the modification of several experimental parameters without revalidation, if necessary [10].

Experimental conditions related to all the steps of the analytical procedures, such as the sample preparation and chromatographic analysis, can be included in the study. The first phase consists in a thorough analysis of all the method and deciding which factors are expected to have higher variability and stronger influence on the final result. The main studied experimental conditions in HPLC methods are extraction time and volume, sampled volume, pH, temperature, flow rate, injection volume, composition of the mobile phase, detector conditions, and so on [66].

Once the factors to be studied have been established, it must fix the minimal and maximal values among which the robustness has to be evaluated. The oscillation range is usually symmetrically distributed around the optimized value. The deviation is taken depending on the expected variation, according to the uncertainty associated with its measure (pH ± 1, flow rate ± 0.05 ml/min, etc.). If

the variation of the analytical results throughout the considered range is under a previously defined acceptance value, the parameter is stated as robust [10].

The robustness of each factor can be evaluated by three main ways [10,66]:

- *Sequential approach*: The parameters are evaluated one by one. The responses obtained using the minimal, optimized, and maximal value of each parameter, maintaining the others constant. This approach is quite simple and the results are easy to interpret. However, this strategy does not consider the effect on the interactions between parameters. Besides, when the number of parameters is high, the sequential approach is long, tedious, expensive, and time-consuming.
- *Considering all the combinations*: Simultaneous evaluation of all the parameters. The responses obtained by combining the minimal, average, and maximal values of all the parameters are measured. This approach evaluates the effect of each parameter and all their interactions, but the complexity of the design rapidly increase with the number of parameters. For instance, for n experimental conditions, the possible number of combination is 3^n.
- *Interpretative approach*: Simultaneous evaluation of all the parameters using a factorial design. The factorial design aims to minimize the number of experiments without excessive loss of information, by selecting a subset of combinations enough representative of the whole set. The basic idea is not to study one alteration at a time but to introduce several variations at once. A factorial design, based on that proposed by the EU Commission Decision 2002/657/EC using the Youden approach, allows the evaluation of the robustness minimizing the time and effort [10]. The combinations are selected to have a balance between the minimal and the maximal values in each experiment. An experiment, using the optimal values for each factor, is added as central point. An example of this experimental design, using seven factors, is shown in the Table 14.3.

Table 14.3 Experimental design to evaluate the robustness of an analytical method, considering seven factors.

	Experiment								
Factor	1	2	3	4	5	6	7	8	9
A	+	+	+	+	−	−	−	−	0
B	+	+	−	−	+	+	−	−	0
C	+	−	+	−	+	−	+	−	0
D	+	+	−	−	−	−	+	+	0
E	+	−	+	−	−	+	−	+	0
F	+	−	−	+	+	−	−	+	0
G	+	−	−	+	−	+	+	−	0

Symbols: (−) and (+), lowest and upper limits of the studied range; 0, optimal value.

For each chromatographic condition, the influence of the oscillation of the factors is concurrently calculated by the relative standard deviation of the measurements obtained in each experiment. This is sufficient to know the performance of the method. Therefore, the influence of the variation of the factors is estimated with an affordable and reasonable number of experiments.

14.7.13
Stability

Chemical compounds can decompose prior to chromatographic analysis, for example, during shipping, preparation of solutions, extraction, cleanup, and storage of prepared vials (in refrigerators or in an automatic sampler). The effect of degradation in the steps intrinsically belonging to the method and common for all the analysis is included in accuracy studies. However, the steps such as storage and transportation are not described in the method itself, and can strongly vary depending on the laboratory. Therefore, they should be separately evaluated though stability studies.

The stability is defined as the ability of a sample to preserve its physicochemical properties, and especially the concentration of the analyte, after several times of storage under specific conditions. Stability assays are important to estimate the maximum allowed time span between sample collection and analysis. This is especially important when dealing with bioanalytical and medical samples. This parameter is mainly studied if the analyte is suspected to degrade while remaining in the laboratory. The design and execution of these studies require a detailed knowledge of the analyte and the analysis technique.

In the four situations discussed next, the stability of the standards and the sample must be established by determining the decomposition kinetics of the analyte, measured as the reduction of its peak area through the time. The maximal transportation/storage time is considered as the time at which the peak area diminishes to a previously specified level. The following steps must be separately evaluated, applying the experimental conditions expected for the routine analysis:

- *Transportation (between sample collection and entry into the laboratory)*: This is especially difficult to study, because it is sometimes performed by external workers or the customer, and is not controlled by the laboratory. A blank sample should be spiked during the collection and analyzed when arriving at the laboratory. The shipping conditions must be accordingly modified if the concentration of the analyte significantly decreases.
- *Laboratory storage (between entrance in the laboratory and the beginning of the analysis)*: The samples are normally stored in darkness in a freezer (−20 °C), a fridge (+4 °C), or a cupboard (20–25 °C). The effect of a long-term storage and the freeze–thaw cycles may be studied by the analysis of a sample with a known concentration immediately after its preparation and at several times over a specific period. The sample is thawed before analysis,

and reintroduced in the fridge or freezer. Both long-term and short-term storage must be evaluated.

- *Laboratory storage without freeze–thaw cycle*: It is performed when the freeze–thaw cycle affects the degradation of the analyte. Therefore, the studied sample is divided into individual vials, and each vial is analyzed at separated times throughout the studied period and discarded.
- *Postpreparative (between the sample preparation and the injection in the chromatograph)*: It has to be especially studied in automated instrumentation, where each aliquot is analyzed at different time. The decomposition rate of the analyte in an aliquot depends on the injection order of the vials, and can be even different for consecutive replicates. The postpreparative stability is studied by dividing a homogeneous sample in aliquots, introducing them in the autosampler, and launching the sequence as in a real situation.

Stability studies can be performed in forced degradation conditions (exposure to high temperatures, photolysis, and moisture, exposure to the atmosphere, chemical decomposition, catalytic effect, adsorption, precipitation, etc.) to evaluate the effect of unusual storage conditions and obtain information about the chemical behavior of the sample.

14.7.14
System Suitability Testing

The system suitability testing is a set of assays allowing to check if all the components of the analytical system (instrumentation, reagents, analyst, hardware, and software) are running as required to carry out the determination for which the method has been established and validated [29]. In particular, it must be demonstrated that the instrumentation applies the conditions established by the control software, and are maintained without oscillations. It examines if the analytical method is able to preserve the essential criteria of validation through the time. Therefore, this testing should be considered as part of the validation protocol and a requirement prior to its realization, as specified by ICH Q2(R1). In fact, it can be considered as an evaluation of the analytical procedure or continuous revalidation. The system suitability test must be performed before and during a routine analytical assay, to assess that the overall system really remains useful to apply the method. The test parameters are established depending on the procedure. A typical system suitability test is to inject a standard and verify that the retention time, dead time, efficiency, tailing, and peak area are those indicated by the manufacturer [67].

This testing can be used in any measurement procedure, under which the analytical conditions can be affected by the variation of the operating conditions. It may not be limited to the final determination, but it can be planned to check the fitness of a unique step of the procedure, such as the sample preparation and chromatographic elution.

14.7.15
Comparison with Other Methods

The suitability of the method should be tested by analyzing several real samples, and to compare the results obtained with those provided by a reference method. The samples should have a concentration in the working range of both methods, and it is recommended to assay several concentrations. If possible, the two analyses must be performed using operating conditions as similar as possible (by the same worker, in the same laboratory, and in the same day), in order to minimize external differences and to assure that the dissimilar results are only due to the methods. Similar results point to an equivalence between the two methods. If the quantitative data are significantly dissimilar, the analytical procedure should be thoroughly examined to find the reason of the inconsistency. Experimental conditions or the range of applicability may be changed if the difference cannot be attributed to an operating error.

The measured concentrations in the same sample by the two methods should be compared using two statistical tools (the significance level is normally set at 5%):

- *Comparisons of paired results by t-student*: The studied factor is the analyzed method. This test is easy to carry out and can be applied for both whatever the obtained concentrations. However, the detected differences are not interpretable.
- *Plotting the results obtained by the new method versus those obtained by the reference method, and calculate the regression parameters*: This tool should be applied if the concentrations span over the working range. A sensitivity significantly different from 1 indicates a proportional error caused by the matrix effect, whereas a *y*-intercept significantly far from 0 points to a bias.

14.8
Operating/Economic Parameters

These parameters (listed in Section 14.1) are related to the practical aspects. They estimate if the method is useful for routine analysis of a large amount of samples and the suitability to be implemented in a quality control laboratory. For instance, a method with excellent performance but expensive and contaminated will not be appropriate. The method is considered as "useful for routine analysis" if it achieves the acceptance criteria marked by the laboratory. Besides, the workplace safety and the waste of toxic compounds are strongly regulated and controlled by legal institutions, and strong penalties are proposed in case of breach.

The importance of these parameters is firmly stated in quality control laboratories, and analytical researchers increasingly consider them when developing a new method [12]. The main practical/economic parameters are named and

described in this section. The maximal automation and the reduction of the number of steps in the procedure are recommended to improve the performances of all the operational parameters [12].

14.8.1
Cross Contamination

The cross contamination is defined as the modification of a sample during the analysis procedure, because of the previously analyzed sample. It can be caused by the matrix and the analyte itself. Traces of an injected aliquot could remain in the chromatographic equipment, especially in the injection needle, and slightly accumulate. Thus, it can be dragged by the mobile phase and added to the next sample. The matrix compound can increase the noise and disturb the detection of the analyte. Besides, the remaining amount of analyte is added to the analyte from the next sample, thus falsely increasing the measured concentration. This problem is caused by highly concentrated samples.

To evaluate the extent of the cross contamination, concentrated samples (near and over the ULOQ) and a blank sample are successively analyzed. If a signal is detected in the blank chromatogram at the elution time of the analyte, the cross contamination is significant, and then an intermediate cleaning step or the reduction of the ULOQ must be envisaged. Anyway, the cleaning of the injection system after each analysis is recommended to avoid cumulative effects.

14.8.2
Simplicity

The simplicity of a procedure is a subjective and qualitative parameter, which measures the probability of making a mistake. It depends on the skills of the worker and facilities of the laboratory. The complexity is increased by the difficulty of each step and the number of steps. A step can be considered more complicated if there are more variables to establish (e.g., isocratic elution is easier than gradient elution), the intervention of the operator is increased (an automated injection is easier than a manual injection), and the solvents and equipment are stable and easy to handle. A complex method would provide less reliable results.

14.8.3
Analysis Time

The analysis time per sample can be calculated in several ways:

- *Duration of the analysis of a single sample*: As the sum of the duration of the sample preparation and the chromatographic analysis.
- *Simultaneous analysis of several samples*: Applicable if a set of samples can be simultaneously processed. It is calculated as the time taken to analyze the

whole set divided by the number of analyzing samples. The analysis time per sample normally decreases if the number of analyzed samples increases.

- *Successive analysis of several samples*: If the analysis of the two sets of samples can be overlaid. For instance, a sample can be prepared while another is analyzed by the HPLC. In this case, the total analysis time is reduced. The analysis per sample is calculated as the number of samples analyzed during a specific time.

The reduction of the analysis time provokes a significant improvement in the financial bottom line of the laboratory.

14.8.4
Cost Per Analysis

The price of the analysis is highly important for a commercial quality control laboratory. It should be reduced as much as possible, without losing analytical performances, in order to compete with other laboratories. It can be calculated considering the price and the amount of consumed chemicals, amortization of instrumentation (purchase and reparation), the salary of the workers, local taxes, and the management of toxic waste.

14.8.5
Safety for Laboratory Staff

The concern about the workplace health and safety is nowadays high. This is especially important in a chemical laboratory, where hazardous compounds have to be handled. In fact, the use of the more toxic chemicals is being banned, and they are substituted by less harmful materials. The laboratories are expected to implement the mandatory safety protocols and facilities to protect the health of the laboratory staff.

The risk for the worker is a qualitative parameter. It can be estimated considering the inherent toxicity and the manipulated amount of each chemical, volatility, flammability, and the probability of skin contact or inhalation.

14.8.6
Environmental Impact

The society is nowadays very conscious about the protection of the environment. Therefore, the current tendency in analytical chemistry points to the development of more eco-friendly methods. Besides, the laboratory must implement adequate waste treatment, following the legal rules. The environmental impact of the method can be reduced by prioritizing the use of innocuous solvents and reagents, reducing the amount of toxic compounds, and diminishing the volume of the waste.

14.9
Comparison between Validation Guidelines

The most important validation guidelines are the "Food and Drug Administration: Guidance for Industry Bioanalytical Method Validation" (FDA guidance) [46], the "EU Commission Decision 2002/657/EC" [10], and the "ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1)" (ICH guideline) [29]. Even with a similar purpose, these documents have been developed for the analysis of different kinds of samples, and differ on several points, such as the parameters to be evaluated, the terminology, the methodology, and the acceptance criteria. Therefore, the suitability of the analytical process partially depends on the chosen guidance. For these reasons, the researchers must know the requirements and characteristics of the guidelines to choose and adequately implement the correct one. The differences between the guides are described below:

14.9.1
Basic Description

- The FDA guideline was developed by several departments of the FDA, such as US Department of Health and Human Services, Center for Drug Evaluation and Research (CDER), and Center for Veterinary Medicine (CVM), and was published in May 2001. The guide came from the discussions performed at the "Second Bioanalytical Method Workshop" sponsored by FDA and AAPS (see Section 14.3.2) and held in 2000. The guidance is addressed to researchers of new human and veterinary drug applications and supplements. It provides assistance to the development and validation of the bioanalytical methods required to adequately perform the pharmacokinetic studies required to establish the preclinical and clinical pharmacology and toxicology of the drugs. It must be considered that the FDA guideline offers recommendations, representing the opinion of the authors, but it does not hold a legal value.
- The European Council proposed the guideline 2002/657/EC in 2002, which provides legal directives to the laboratories to evaluate the chemical contamination of live animals and animal products for consumption, produced or exported to the EU, in order to ensure health and food safety. Maximal residue limits (MRL) have been fixed by the same organization for many contaminants in the main foodstuff, as the highest concentration that can be found in a compliant food sample [68]. The guideline lays down rules for both sampling and validation of analytical methods (studied parameters and acceptance criteria), and describes the correct approach to deal with each matrix, instrumentation, and analyte, as well as the interpretation of the data. It has been proposed to uniform the procedures and performance criteria used by laboratories approved for official residue control, in order to ensure the quality and comparability of the results. The guideline has the

status of European law and its application is mandatory for these laboratories. The validation of methods to quantify organic residues is discussed in this chapter.

- The ICH guideline was launched in 1994 by the ICH Expert Working Group ("International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland"). Several times amended, it took its last form in 2005. The purpose of the guidance is to provide assistance to the manufacturers for the application of registration of drugs produced or exported to Japan, the European Union, and the United States. It presents a discussion about the characteristics to be considered during the validation of the analytical procedures and several recommendations to accomplish the validation process. The main validation parameters are listed and defined, together with several recommendations for their determination. The document aims to connect the differences between compendia and regulators of the European Union, Japan, and the United States, and achieve a harmonization about the required validation parameters. The guideline stands only for the opinion of the organization and has not a legal value.

The three guidelines recommend performing the whole validation in sample matrix, and the use of an internal standard. The final report must include the selected guide, the methodology calculation for each parameter, the considered concentrations and number of replicates, the acceptance criteria, and the obtained data. The results should be discussed on the basis of the expected value and analytical requirements.

14.9.2
Scope

The FDA guidance is indented to be applied to the analysis of drugs and their biometabolites in both human and animal biological samples (blood, serum, plasma, urine, tissue, and skin) by gas and liquid chromatography, immunology, and microbiological procedures. The expected concentration of the drug would be low (ppb – ppm), and the accurate value should be determined with low uncertainty. The following validation parameters must be determined: selectivity, accuracy, precision, recovery, calibration curve, and stability.

The European Commission Decision 2002/657/EC guideline is appropriate to analyze undesirable organic residues by chromatography or metallic elements by electrochemistry and atomic spectrometry (not discussed in this chapter), in matrices extracted from food products. The concentration of these contaminants is usually low (ppb). The laboratory has to find if the concentration of the contaminant is under or over the maximum residue limit to accept or reject the batch, respectively. If an MRL has not been defined and the organic residue has been completely banned, the sample is noncompliant if the analyte has been detected. Anyway, the accurate concentration can also be reported to complete

the document. The guidance imposes the calculation of the specificity, trueness, ruggedness, stability, recovery, repeatability, within-laboratory reproducibility, reproducibility, CCα, CCβ, and calibration range.

The ICH guideline is proposed to reliably quantify drugs in finished products and pharmaceutical formulations for quality control and solubility studies, by any instrumentation. The target analyte is the major component and will be at a high amount. The concentration in aliquot can be adjusted, and is normally taken at ppm levels. The accurate concentration with a low uncertainty must be measured. The specificity, linearity, calibration range, accuracy, precision, detection limit, quantification limit, robustness, and system suitability should be calculated.

14.9.3
Selectivity/Specificity

The FDA requires that the analyte at the LLOQ can be detected without interferences from endogenous compounds, concomitant medication, and metabolites in all the tested biological matrices. The blank samples would be taken from at least six different sources.

The EU Commission Decision 2002/657/EC uses the term "specificity." It lays down stricter directives to identify the target analyte by chromatographic techniques. The following considerations must be studied:

- *Recognition of the analyte among the peaks of the chromatogram*: A sample must be analyzed using the suitable column, and the analyte should be eluted at a minimum of two times the dead time. A peak shall elute between ±5% of the retention time obtained by a standard solution to be assigned to the target analyte. The identification must be confirmed by comparing the characteristics of the spectrum of the sample peak with those of the standard peak, with the following tolerance margins: mass spectrum, abundance of reference ion, 10%, other diagnosis ion, 10–50%; UV-Vis absorbance by diode array detection, 10% of difference of intensity throughout the whole spectrum (>220 nm); and UV-Vis absorbance by single wavelength, detection wavelength at 10% absorptivity of the maximum wavelength.
- *Interfering compounds*: The possible interference of chemically related compounds, substances belonging to the matrix (≥20 representative sources), degradation products, and other compounds expected to occur should be considered. It must be checked if these compounds provide signals near the elution time of the target analyte.

According to the ICH guideline, the ability of the method to identify the target drug should be proven by analyzing chemically similar materials or closely related to the analyte to confirm that a negative response is obtained. Besides, it strongly focuses on ensuring that the drug could be analyzed in the presence of excipients, degradation products, and impurities. Thus, the drug product should be spiked with the standards of the possible interfering compounds. Besides, the

degradation products can be *in situ* produced by relevant stress conditions: photolysis, heat, humidity, acid/base hydrolysis, and oxidation. Furthermore, it must be demonstrated that the target drug elutes at an elution time different from the impurities and degradation products. If impurity standards are not available, samples containing impurities must be analyzed by another reference method, and the quantitative results and impurity profiles must be similar.

In the three cases, the specificity should be proven by showing the adequate chromatograms.

14.9.4
Calibration Curve

The FDA guidelines and EU Commission Decision 2002/657/EC require a reproducible relationship signal/concentration, but not necessarily linear. The ICH guideline clearly states the need of a linear relationship, directly or after mathematical transformations. In the three cases, the parameters of the calibration curve and the goodness of fit should be provided, but a minimal value is not required. The FDA guideline proposes the use of six–eight calibration points throughout the working range, whereas the EU Commission Decision 2002/657/EC and the ICH recommend only five points.

14.9.5
Calibration Range

In the three cases, the working range of the curve must match the expected concentration of the target analyte in the sample.

The FDA guideline indicates that it must be verified if the LLOQ fits the acceptance criteria for accuracy and precision. Besides, the ability to dilute samples initially over the ULOQ should be demonstrated.

The ICH states the need to cover the 80–120% of the expected concentration to perform a verification of the purity of a finished drug product and a solubility study, whereas 70–130% is recommended to analyze a pharmaceutical formulation.

14.9.6
Limit of Detection and Limit of Quantification

In the three cases, the LOD and the LOQ are indented to be either under the minimal expected concentration of the analyte in the sample or under the permitted limit.

The FDA guidance does not provide any recommendations to calculate the LOD, and states that the LLOQ must be taken as the LOQ. The EU Commission Decision does not provide any recommendation. Surprisingly, the ICH provides a thorough description of the calculation criteria of LOD and LOQ: visual absorptivity evaluation, signal to noise ratio, and standard deviation of the response and the slope. The analyst is free to select the approach.

14.9.7
Accuracy

The FDA guidance states that accuracy must be determined using quality control (QC) samples with a known amount of the target analyte. The purity of the analyte standard must be accurately known. Three types of reference standards are usually used, by decreasing order of preference: certified reference standards, commercially supplied reference standards from a reputable source, and in-laboratory synthesized standards by a reliable noncommercial establishment. The source, lot number, expiration date, certificates of analyses, and identification and purity tests should be furnished for each standard batch.

Three concentrations should be studied: three times the LLOQ (low QC), near the middle of the working range (medium QC), and near the ULOQ (high QC). Each level should be analyzed by five replicates.

The EU Commission Decision 2002/657/EC uses the term "trueness" and strongly recommends the use of reference certified materials, or spiked samples if CRM are not available. Three concentrations should be studied: 0.5×, 1×, and 1.5×MRL; or 1×, 1.5×, and 2×minimum working amount, stated as the LOQ, if an MRL has not been defined. Each level should be analyzed by six replicates.

The ICH guideline suggests the determination of accuracy using standard drugs mixed with the expected amount of impurities, excipients, and degradation products. If they are not available, real samples can be used and analyzed by other methods to determine the "true" concentration. The accuracy should be determined by a minimum of nine total measurements, preferably three concentrations and three replicates. The studied levels should cover the entire working levels, but no values are indicated.

Each guideline has set its own maximum tolerable deviation for accuracy, which depends on the concentration. The FDA guideline, ±15% for levels >LLOQ and 20% for the LLOQ, whereas the EU Commission Decision 2002/657/EC states: for <1 ppb, −50–20%; between 1 and 10 ppb, −30% to +10%; and >10 ppb, −20–10%. The ICH guideline does not indicate acceptance criteria.

14.9.8
Precision

The three guidelines recommend the determination of the precision at three stages: repeatability, intermediate precision, and interlaboratory reproducibility. The same experimental design (studied concentrations and replicates) proposed for the accuracy can be applied to the precision. Only the ICH guidance provides the possibility to evaluate the precision solely at the expected concentration (that marked by the manufacturer) for finished drug product, by six replicates. The term "intermediate precision" appears in the ICH guideline, whereas the EU Commission Decision 2002/657/EC uses the terms "ruggedness major changes" or "within-laboratory reproducibility" and the FDA guidelines has taken the expression "between-run or interbatch precision" for the same concept.

The acceptance criteria are established on the basis of concentration. The FDA has set RSD < 20 at the LLOQ and <15% for higher amounts. The EU Commission Decision 2002/657/EC accepts an RSD for the interlaboratory reproducibility up to the value indicated by the Horwitz equation:

$$\text{RSD} \,(\%) = 100 \times 2^{(1 - 0,5 \log C)}, \tag{14.7}$$

(minimal value = 23), where C is the concentration in g/g.

The repeatability and the within-laboratory precision would typically be under $2/3 \times$ above value. In a validation procedure, the obtained within-laboratory RSD shall be under the interlaboratory one. The ICH guideline has not recommended any limit value.

14.9.9
Recovery

The three guidelines do not require the calculation of the recovery to determine the matrix effects (Section 5.9.1). Indeed, as the entire validation is carried out in sample matrix (in CRM or fortified blank sample), the matrix effect is already included in the results. In fact, the accuracy is measured as the recovery, and the EU Decision Commission 2002/657/EC and the ICH guidelines interchangeably use both terms.

The FDA guidance separately considers "accuracy" and "recovery", and demands the determination of recovery to evaluate the effectiveness of the sample preparation (Section 14.5.9.2). In fact, biological samples contain compounds, such as proteins, which can bind the analyte. The analyst must check that these interactions have effectively been broken, prior to the injection. Besides, the low volume usually available and the complexity of the matrix complicate the processing of the sample, thus increasing the risk of obtaining inadequate recuperations. Therefore, the guideline proposes the evaluation of the suitability of the sample preparation, independent of the LC run. The concentrations-to-evaluate and acceptance criteria are the same as those proposed for accuracy.

14.9.10
Robustness

The EU Commission Decision 2002/657/EC and the ICH guideline recommend a robustness study considering minor variations of the factors that can influence the final observations, during both sample preparation and chromatographic run. The EU Commission Decision 2002/657/EC recommends the use of a factorial design to measure the robustness, even if a sequential approach is also feasible, whereas the ICH does not indicate any recommendation for the measurement. According to both guidelines, the confidence interval, a factor significantly influencing the final results, must be measured and the instrumental condition must be especially controlled and its surveillance should be included in the suitability testing. The FDA guideline does not mention the robustness.

14.9.11
Stability

The FDA guidance recommends the evaluation of the stability of the analyte in the sample matrix throughout the same timeline as the incurrent samples: collection and handling, freeze–thaw cycles (three cycles, 24 h frozen and 24 h room temperature), short-term storage (up to 24 h at room temperature), long-term storage (under the indented storage conditions), and postpreparative (anticipated residence time in the autosampler). The stability of the analyte in both standard solutions and in spiked samples should be evaluated over a time period equal to the duration of the whole analysis at room temperature. This serves to differentiate the loss of analyte because of the method or the normal degradation through time. The stability should be studied by triplicate.

The EU Commission Decision 2002/657/EC requires the determination of the stability of the analyte under the most usual storage conditions, in order to determine the maximum storage time: in darkness at −20 °C, in darkness at −4 °C, in darkness at room temperature, and under light at room temperature. The degradation study finishes when the diminishing of the analyte peak in the chromatogram is significant. The stability should be studied in two chemical environments: standard solution and matrix, using preferably incurrent or, if not available, spiked samples.

As seen, the FDA guidance recommends the study of the sample stability under more situations than EU Commission Decision 2002/657/EC. That is because bioanalytical and clinical samples from biological origin are usually unstable and the target drug can undergo undesirable side reactions. Therefore, they are stored under strict conditions and strongly controlled. However, both guidelines have not set acceptance criteria. On the other hand, the ICH guideline does not allude to stability.

14.9.12
Decision Limit

The CCα is required only by the EU Commission Decision 2002/657/EC, and is calculated as follows:

a) In the case of substances for which no MRL has been established, α should be 1%:
   - *Calibration curve procedure*: The corresponding concentration of a signal equals to the y-intercept plus 2.33 the standard deviation of the y-intercept. The calibration curve must have been determined using sample matrix.
   - *Blank analysis*: The concentration calculated from signal of three times the width of the baseline at the time window in which the analyte is expected, taken as the average value of 20 replicates.
b) For substances with an established permitted limit, $\alpha = 5\%$:

- *Calibration curve procedure (if measured in sample matrix)*: Permitted limit plus 1.64 the standard deviation obtained in precision studies at the permitted level, divided by the sensitivity.
- The permitted limit plus 1.64 the standard deviation associated with the "found concentration," obtained by analyzing ($n = 20$) a blank sample spiked at the permitted limit.

14.9.13
Detection Capability

The $CC\beta$ is only required by the EU Commission Decision 2002/657/EC. It is normally calculated taking a $\beta = 5\%$, as follows:

- Calibration curve procedure (only if the calibration curve has been obtained in sample matrix): the $CC\alpha$ plus 1.64 the standard deviation obtained during the precision studied at the $CC\alpha$, divided by the sensitivity.
- The decision limit plus 1.64 standard deviation associated with the "found concentration," obtained by analyzing ($n = 20$) a blank sample spiked at the $CC\alpha$.

14.9.14
Main Differences between Guides

The main differences of the above described guidelines are indicated in Table 14.4.

14.10
A Survey about Validation of Chromatographic Methods across the Scientific Literature

It would be interesting to know if analytical researchers working on liquid and gas chromatography are nowadays really validating the methods that they develop, and which parameters they include. This would provide an idea of the current awareness among scientists about the importance of validation, and the parameters considered as more significant. For these reasons, a survey over the literature was performed. We worked on a selection of original research documents obtained through Scopus® citation database (Elsevier B.V., Amsterdam, The Netherlands), over the 2004–2014 period.

The search was separately performed for HPLC and GC. The search focused on the area "life, health and physical sciences" and the search topics were "validation," "liquid" or "gas," and "chromatography" in the fields "article title, abstract, or keywords". Only "articles," "articles in press," and "conference paper" were considered, as these documents are those preferably chosen by the researchers to report their original results. The reviews, books, editorial, short

Table 14.4 Main differences between the three compared validation guidelines.

Item	FDA [46]	EU Comm. Dec. [10]	ICH [29]
Purpose	Assist researchers of new drug applications to perform the analysis required to determine the clinical properties of the drugs	State rules for the official residue control laboratories to monitor contaminants in animal products for human consumption in the EU	Assist manufacturers to fulfill the requirements of the regulatory agencies from USA, Japan, and EU for the registration of pharmaceutical products
Scope	Drug research	Food safety	Drug trading
Analyte	Drug	Any organic contaminant	Drug
Matrix	Biological fluids and tissues	Foodstuffs	Pharmaceutical formulations
Expected concentration	Low/moderate (ppb, ppm)	Low (ppb)	High (ppm)
Objective of the analytical method	Quantify the analyte for clinical studies	Determine if the contaminant is (or) the permitted level to decide the compliance of the sample	Quantify the active principal and determine the impurity profile in a medicament
Addressed to	Researchers of new drugs applications	Official quality control laboratories	Drug manufacturers
Legal value	None	EU law	None
Selectivity/ specificity	• Selectivity • Possible interference of endogenous and concomitant drug compounds • Test > 6 matrices	• Specificity • Retention time < 5% from the expected value • Possible interference of related or likely-to-occur compounds • Test > 20 matrices	• Specificity • Possible interference of related compounds and degradation products
Calibration curve	• Reproducible curve • Six–eight calibration levels	• Reproducible curve • Five calibration levels	• Linear • Five calibration levels
Calibration range	• Cover the expected amounts • Dilution of samples > ULOQ	Cover the permitted limit	Cover 70–130% of the expected concentration
LOD: calculation	Not indicated	Not indicated	• Visual evaluation, • $S/N > 3{:}1$ • 3s criterion
LOQ: calculation	LLOQ=LOQ	Not indicated	• Visual evaluation • $S/N > 10{:}1$ • 10s criterion

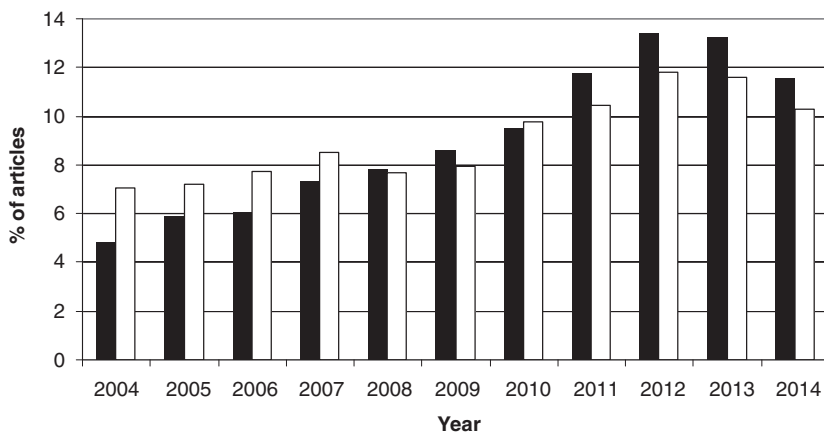|	Underexpected values	Under the permitted limit	Under the expected value
LOD/LOQ: acceptance criteria			
Accuracy: evaluated levels	Three levels (low, medium, and high throughout the working range)	• Termed: trueness • Three levels (near the permitted limit)	• Nine measurements or • Three levels × three replicates (no values indicated)
Accuracy: acceptance criteria	<20% LLOQ <15% for values over LOQ	<1 ppb: −50 to 20% 1–10 ppb: −30 to +10% >10 ppb: −20 to 10%.	None
Precision: evaluated levels	Same as accuracy	Same as accuracy	Same as accuracy or six replicates at the expected amount
Precision: terms used for each degree	• Repeatability • Between-run or interbatch precision • Interlaboratory reproducibility	• Repeatability • Ruggedness major changes or within-laboratory reproducibility • Interlaboratory reproducibility	• Repeatability • Intermediate precision • Interlaboratory reproducibility
Precision: acceptance criteria (RSD, %)	<20% LLOQ <15% for values over LOQ	• Interlaboratory: $100 \times 2^{(1 - 0.5 \log C)}$ (min. 23) • Others: two-thrids times the interlaboratory	None
Recovery	Study the effectiveness of the sample preparation (experiments and acceptance criteria same as for accuracy)	Same concept as accuracy	Same concept as accuracy
Robustness	Not mentioned	Termed: "ruggedness minor changes" • Study variation of the main factors	Study the variation of the main factors
Stability	• Collection and handling • Freeze–thaw cycles • Short-term storage • Long-term storage • Postpreparative	• Short-term storage • Long-term storage	Not mentioned
CCα	Not mentioned	Yes	Not mentioned
CCβ	Not mentioned	Yes	Not mentioned

Figure 14.5 Ratio of articles published each year/the total period on the subject "validation" and "liquid chromatography" (black bars) and "gas chromatography" (white bars) (Scopus® abstract and citation database, Elsevier B.V.), during the 2004–2014 period.

surveys, notes, erratum, and other documents were discarded because they usually discuss already published methods, and then the information obtained would be duplicated. Only documents written in English have been considered.

The search was carried out on March 16, 2015, and returned 15 406 documents for liquid chromatography and 3098 for gas chromatography. All these articles describe the development and validation of an HPLC or GC analytical method that proves the high importance of this topic. The year-to-year distribution can be seen in Figure 14.5.

The evolution of the number of articles dealing with validation is similar for both HPLC and GC. As it can be observed, the inclusion of the validation process has increased in the 2004–2011 period, and remains roughly constant from 2012–2014. These results demonstrate that the concept of validation has been progressively accepted through the last decade by analysts and researchers. Thus, they are nowadays completely conscious that validation is an essential step in the development of their analytical methods. Another reason is that the public have increasingly been demanding a stricter control of materials that are directly or indirectly related to their health, such as foodstuff, cosmetics, clinical samples, pharmaceutical formulations, and environmental samples, among others, in order to assure that the traded samples fit the regulations. For these reasons, customers, manufacturers, and control agencies demand the use of validated methods with clearly stated performances. This has a special incidence in chromatography, because it is the technique of choice for the analysis or organic compounds in samples from a wide range of different matrices.

It is also important to determine the parameters that are included in the validation process, in order to figure out what the researchers are currently considering as "validation." Therefore, the survey was extended by determining the frequency of each validation parameter. The search was repeated, by successfully

Table 14.5 Percentage of articles including each validation parameter.

Validation parameter	HPLC	GC
Accuracy	56.1	37.0
Trueness	1.7	2.2
Precision	45.0	30.7
Uncertainty	3.9	9.2
Recovery	39.0	36.5
Sensitivity	39.5	34.5
Limit of detection	48.9	46.2
Limit of quantification	35.4	28.3
Linearity	29.9	19.5
Calibration range	36.2	21.7
Selectivity	12.0	9.4
Specificity	35.8	24.5
Stability	35.5	16.4
Robustness	10.2	4.6
Ruggedness	3.5	1.7
Decision limit	4.3	3.2
Detection capability	3.8	3.3
System suitability	3.3	1.7

adding each validation parameter as "research topic" throughout all the manuscript. The validation parameters studied were "selectivity or specificity," "linearity," "calibration range," "LOQ," "LOD," "sensitivity," "accuracy or trueness," "precision," "recovery," "uncertainty," "robustness," "ruggedness," "decision limit," "detection capability," "stability," "system suitability," "revalidation," "collaborative or interlaboratory," and "guideline or guidance." Because of the high amount of published articles, it was not possible to check all of them one by one, so that we looked for a overall vision reading some documents randomly taken. The articles including "validation" in the title, abstract or keyword effectively detail the development and validation of a new method. However, it is also possible that some authors perform validation studied without including the word validation in the article, abstract, or keywords. The results are shown in the Table 14.5.

These results indicate that the relative importance of each statistical validation parameter is nearly similar for both techniques. The validation parameters with higher interest among analysts are those related to the concepts more representative of the performance of the method and match with the definition of validation itself: to ensure that a single measurement will provide a value close to the true one (accuracy, trueness, precision, and recovery) and the determination of the concentrations in which the method can be applied, especially of the minimal detectable amount (sensitivity, LOD, LOQ, linearity, and calibration range).

A good result in both cases is crucial to establish the suitability of the method in a specific application. Both terms "accuracy" and "trueness" are used, but the first one is largely preferred. The meaning of the term "recovery" in a specific work is difficult to interpret, as it would have been used as indicative of the accuracy or to measure the matrix effect. Despite its importance, the uncertainty is barely incorporated into validation studies (<10%). This parameter is closely related to the precision and is not separately calculated. Besides, this parameter is rather calculated when analyzing real samples, instead of during the validation.

The identification of the analyte is evaluated by the 47.8 and 33.5% of the HPLC and GC analysts, respectively. In both techniques, two-third parts of the papers use the term "specificity" and the other third part use "selectivity," to name this concept. As the identification of the analyte is a basic aim in chromatographic analysis, we expected a proportion near 100%. We think that many authors optimize the separation conditions to detect the analyte, but they do not include the terms "selectivity" or "specificity" in the report. The stability is evaluated by 35.5 (for HPLC) and 16.4% (for GC) of the researchers, principally under storage conditions.

Robustness (<11%) and ruggedness (<4%) have been barely studied for HPLC- and GC-based methods. That means only a few researchers consider that the within-laboratory variation of the experimental factors, starting from the optimized ones, will have a strong impact on the results. Besides, many researchers consider that the instrumentation and an already optimized method should be enough stable to invest funds and time in their determination. However, these parameters are usually considered when a successfully validated method fails in a routine application. Besides, the term "ruggedness" has two major meanings: as "ruggedness minor changes," it is equivalent to the robustness, and "ruggedness minor changes" is calculated as the "intermediate precision." Therefore, it can be concluded that the researchers may calculate the ruggedness, but show the results under the label "robustness" and "intermediate precision." The term "robustness" is preferably used to avoid confusions.

The decision limit and the detection capability have been calculated only by <5% of the chromatographic researchers. Indeed, these parameters were proposed by the EU Commission Decision 2002/657/EC not long ago (since 2002), which is applied only in Europe and EU countries, and their use is not extended among analysts. Besides, they are specifically evaluated when the scope of the analysis is to determine if samples are noncompliant, instead of quantifying the analyte. The uncertainty is barely incorporated in validation studies (<10%). This parameter is closely related to the precision, and is not separately calculated. In fact, this parameter is rather calculated when analyzing real samples, instead of during the validation. The system suitability is evaluated in few reports (<4%), in spite of its importance. In fact, the suitability of the instrument system is normally evaluated before and through a run in quality control. The system suitability testing depends on the facilities of each laboratory, and then its inclusion in a general research article would not be very informative. The main guidelines are frequently vague about the way to carry it out. These can be the reasons of its low relevance in the validation process.

The revalidation has been considered only in seven articles for HPLC and two articles for GC during the surveyed period. The revalidation is related to modification of the scope or the procedure, and transfer of methods to other laboratories. However, there themes are closely related to routine analysis laboratories, which do not issue their achievement, whereas analytical researchers prefers dealing with the development and entire validation of new methods. For these reasons, we do not expect a large number of articles mentioning the revalidation. Interlaboratory collaborative trials have been performed to validate newly developed method in only 4.4 and 3.6% of the surveyed papers for HPLC and GC, respectively. Although this study provides relevant data and more reliability to the method, it is costly and requires a high number of participating laboratories. Besides, few researchers are interested in upgrading their methods to a reference degree. The guideline selected for the validation has been indicated in the 35.1 (for HPLC) and the 16.0 (GC) % of the reports. We must consider that the use of a specific guideline, although recommended, is not mandatory. In some cases, the validation is really based on a guideline, but several modifications are applied, such as the evaluation of a fraction of the described parameters, the variation of the acceptance criteria, or the adaptation of the experimental design. Besides, the combination of several guidelines is also possible.

14.11
Conclusions

Nowadays, the analytical methods have acquired a high importance, due to the critical decisions taken on the basis of the analytical results. This is especially true for liquid or gas chromatography-based methods, because they are applied in several topics with a high impact in our society. Therefore, the need of the disposal of consistent analytical methods is unquestionable to protect the population. Validation allows to state the characteristics of the method, and then to verify if it is applicable to its intended purpose. The results of the validation would be taken to judge the quality, reliability, and consistency of the future measurements performed by the studied method. An inadequate value during validation is useful to detect and correct the limitations of the method, and can be considered as a step of the development.

For many years, quality control laboratories have been implementing the validation of the used methods as an integral part of a good analytical practice. In fact, these laboratories are strongly controlled by accreditation agencies, which require the validation to fit their regulations and quality standards. At one time, analytical researchers were less involved in validation, but during the past few years, they have been more and more aware of the importance of validation and have progressively incorporated it in their work. It is especially useful for ambitious research laboratories, which aim to upgrade their developed methods to "reference method."

Validation is not a fixed protocol, but an evolving and mutable process The protocol can be modified depending on the characteristics of the method, such

as the status (reference or in-house), the objective of the analysis, the analyte, the sample, the need of the customer, the possible applications, and the requirement of regulatory agencies. The preparation of the validation protocol must consider these factors. Several authors also recommend the performing of a daily partial validation, instead of a full validation for the implementation of the method. Both approaches are correct and can even be combined.

Analysts must have a thorough understanding of the validation parameters, in order to correctly establish and execute the experimental design and select the adequate statistical tools. A large number of reviews and validation guidelines have been published by regulatory agencies to assist analytical researchers through this process. However, these guidance documents differ on several points, such as the scope, parameters to be studied, terminology, methodology, and acceptance criteria. The analysts must select the appropriate guideline and correctly interpret it to ensure its adequate implementation. Therefore, it is essential to have clear definitions of all the terms involved in the validation procedure.

Validation topic is faced with several outstanding challenges for the future. First of all, a higher degree of harmonization between the validation guidelines would be desirable. The different regulatory agencies should standardize their criteria to consider a method as "suitable." Especially, the used terminology must be homogenized. This will facilitate the interpretation of all the guidelines, thus encouraging the researchers to undertake the validation process and making easy the judgment of the results by the customers. In fact, agencies are aware of this problem, and several symposia have been organized since the 1990s with harmonization purposes. Besides, the operational parameters should be progressively incorporated in the forthcoming editions of the validation guidelines. Thus, the analytical researchers would take into account the suitability for routine analysis, when developing a new analytical method. This would get close to the interests of analytical researchers and those of quality control laboratories, and would encourage collaboration among them.

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