Are analysts doing method validation in liquid chromatography?

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Abstract

Method validation is being applied in the reported analytical methods for decades. Even before this protocol was defined, authors already somehow validated their methods without full awareness. They wished to assure the quality of their work. Validation is an applied approach to verifying that a method is suitable and rugged enough to function as a quality control tool in different locations and times. The performance parameters and statistical protocols followed throughout a validation study vary with the source of guidelines. Before single laboratory validation, an analytical method should be fully developed and optimized. The purpose of the validation is to confirm performance parameters that are determined during method development, and it should provide information on how the method will perform under routine use. An unstable method may require re-validation. Further method development and optimization will be needed if validation results do not meet the accepted performance standards. When possible, the validation protocol should also be conducted as a collaborative study by multiple laboratories, on different instruments, reagents, and standards. At this point, it would be interesting to know how people are validating their methods. Are they evaluating all defined validation parameters? Are they indicating the followed guidelines? Is re-validation really currently used? Is validation performed by a single laboratory, or is it a collaborative work by several laboratories? Is it an evolving discipline? In this survey, we will try to answer these questions in the field of liquid chromatography.

Keywords: Method Validation; Liquid Chromatography; Survey; Guidelines; Evaluated validation parameters; Analysts

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1. Introduction

The aim of any analytical measurement is to obtain accurate, reliable, and consistent data to find the nature of a sample. These properties can be judged by the results obtained through method validation, which since long is an integral part of any good analytical practice. An analytical methodology should include besides the required data to solve the problem, at least the achievable sensitivity, accuracy, precision and range of application. Unless a method is used on a regular basis to provide confidence in its continued validity, it is essential to document that the method is still valid prior to analysis. Analytical methods should be validated, verified, or re-validated.

Analytical methods and techniques are constantly undergoing changes and improvements, since often they must stay at the cutting edge of the technology. It is also important to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte. In these instances, specific validation criteria may need to be developed for each analyte. Moreover, the appropriateness of the technique may also be influenced by the ultimate aim of the study. When sample analysis for a given study is conducted at more than one site, it is necessary to validate the analytical method(s) at each site and provide appropriate validation information for different sites in order to establish inter-laboratory reliability. Also, while validation of each method is of interest by itself, there may be situations where comparison of methods will be necessary (e.g. when more than one method have been employed in a long-term study).

Method validation (also called method performance by some authors) implies not only the definition and evaluation of the classical validation or performance parameters (or characteristics): accuracy/recovery, precision (repeatability, intermediate precision and reproducibility), linearity and application range, limit of detection (LOD)/limit of quantitation (LOQ), selectivity/specificity, robustness, ruggedness, uncertainty,

trueness, stability and system suitability studies, but also a detailed and extensive protocol on how to operate and transfer analytical methods and the involved procedures. All these validation parameters have been extensively commented and defined in the literature. Another performance parameter of an analytical method not usually included is the applicability (or scope).

Method validation is required to assure high quality and achieve acceptance of products by the international agencies. It is a mandatory requirement for accreditation as per ISO 17025 guidelines [1], and for registration of any pharmaceutical product or pesticide formulation. The main objective is to demonstrate that the procedure is suitable for its intended purpose [2–4]. In previous work, Rambla-Alegre *et al.* discussed the need of validating new developed procedures [5]. The conclusion of the authors was unequivocal: "*No doubt, the answer is clearly yes. Even more, the analytical method validation should be a mandatory step to evaluate the ability of developed methods to provide accurate results for their routine application. Indeed, without results of adequate quality or reliability, the critical decisions that will be made during routine application of the method will be untrustworthy*". In that work, a complete and detailed definition and description of method validation parameters was provided.

This work shows a literature survey on how analysts are doing validation studies during method development in liquid chromatography (LC). This is not an easy task, due to the variety of existing fields (biological fluids, pharmaceuticals, impurities, microbial, food, and botanicals, among others), and applied analytical techniques. An additional problem is the number and variety of international renowned organizations offering guidelines on method validation.

2. The validation process

The validation process starts before an instrument is placed on-line, and continues long after method development and transfer. The validity of a specific procedure should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples analyzed routinely. The preparation and execution should follow a validation protocol, preferably written in a step-by-step instruction format. Ideally, the validation proto-col should be written following a thorough understanding of themethod's capabilities and intended use. The validation protocolwill list the acceptance criteria that the method can meet. Any fail-ure to meet the criteria will require that a formal investigation beconducted.

Method validation is not a single event. It begins when an analyst has the initiative of implementing a new method in a laboratory, and obviously ends when the method is discontinued. The validation process is complex and time-consuming. Therefore, it must be broken down in several well-defined steps as described in Fig. 1.

A validation plan is first developed. This should include owners, responsibilities and deliverables. The first step is to define the scope of the method. This includes the compounds and concentration ranges, sample matrix, specific equipment to be used, and location where the method should be performed. Once the target analysis is known, the performance parameters, performance tests and acceptance criteria should be defined. Test protocols are then developed with all experimental details, and the tests are executed according to the protocols. Tests results are compared with acceptance criteria. Finally, routine method procedures are developed to verify constant system performance at the time of analysis. Tests may include system suitability testing and the analysis of quality control samples. All experimental conditions and validation results must be documented in a validation report.

During the course of a product development program, a defined analytical method may undergo many modifications. These evolving changes require different levels of validation to demonstrate continuity of the validity of method performance. Three different levels/types of method validation are defined: full validation, partial validation, and cross-validation. Full validation is necessary when developing and implementing an analytical method for the first time for a new product. Partial validations are performed associated to modifications of validated analytical methods that do not necessarily require full revalidations. Cross-validation is a comparison of two analytical methods and is required when two or more analytical methods generate data within the same study.

Another topic in the field of validation is remediation of validated analytical methods. This is typically triggered by the need to improve existing methods used for controlling commercial products. The improvement may be required due to an unacceptable rate of method failures related to toxicity, environmental impact, lengthy run times, obsolete instruments or consumables, changing regulatory specifications, stability testing, or business interests, among others. Frequently, old methods have to be replaced by methods using newer technologies, creating a significant challenge for the industry in providing demonstration of method equivalency and a corresponding level of validation for the methods.

The biggest advantage of analytical method validation is that it builds a degree of confidence, not only to the developer but also to the user. Although the validation exercise may appear tedious, costly and time consuming, it eventually turns out to pay for itself, eliminating annoying repetitions and leading to better time management on the long term.

Finally, one of the key requirements for method validation (which is also one of the greatest challenges) is that only well-characterized reference standards (or materials)

with well-documented purities should be used throughout the validation study. The degree of purity necessary depends on the intended use. The challenge stems from the fact that, in some cases, the tools used to characterize reference standards are being developed and validated at the same time as the reference standard itself. As part of method development, reference materials should be assessed for identity, purity, stability, and storage conditions. For all standards, the suitability for use should be ensured.

Reference standards can often be obtained from US Pharmacopeia (USP) and may also be available through the European Pharmacopoeia, Japanese Pharmacopoeia, World Health Organization (WHO), or National Institute of Standards and Technology (NIST). Reference standards for a number of biological products are also available from the Center for Biologics Evaluation and Research **(**CBER). Reference materials from other sources should be characterized by procedures including routine and beyond routine release testing as described in ICH Q6A.

If an appropriate certified reference material (CRM) is available, a single-laboratory test allows a laboratory to assess laboratory bias and method bias in combination, by analyzing the CRM a number of times. The estimate of the combined bias is the difference between the mean result and the certified value. Unfortunately, appropriate CRMs are not always available, so other materials have to be used. Materials left over from proficiency tests sometimes serve this purpose and, although the assigned values of the materials may have questionable uncertainties, their use certainly provides a check on overall bias. Specifically, proficiency test assigned values are generally chosen to provide a minimally biased estimate, so a test for significant bias against such material is a sensible practice. A further alternative is to use spiking and recovery information to provide estimates of these biases, although there may be unmeasurable sources of uncertainty associated with these techniques.

3. A little of history

Method validation has received considerable attention in the literature from industrial committees and regulatory agencies, and today is required by most regulations and quality standards that impact laboratories. It has a long and productive history in several disciplines: control of active pharmaceutical ingredients, bioanalysis, and food and environmental analysis, among others. The international renowned organizations that along the years have been offering guidelines on method validation include on a tentative exhaustive basis: the Association of Official Analytical Chemists (AOAC), American Society for Testing and Materials (ASTM), Codex Committee on Methods of Analysis and Sampling (CCMAS), US Food and Drug Administration (FDA), International Conference on Harmonization (ICH), US Pharmacopeia (USP), the European Committee for Normalization (CEN), US Environmental Protection Agency (US EPA), Cooperation on International Traceability in Analytical Chemistry (CITAC), European Cooperation for Accreditation (EA), European Analytical Chemistry Group (EURACHEM), Food and Agricultural Organization (FAO), International Laboratory Accreditation Cooperation (ILAC), World Health Organization (WHO), International Organization for Standardization (ISO), International Union of Pure and Applied Chemistry (IUPAC), among other organizations [6–18]. The selection of the guidelines will mainly depend on the type of analyzed samples.

It is clear that method validation is today a hot topic. However, reviewing the reports in the field of the analysis of pharmaceutical materials, the word validation was not mentioned in the “current Good Manufacturing Practices” (cGMP’s) of 1971, although accuracy and precision were stated as laboratory controls. The need for validation was implied only in the cGMP guideline of March 1979 [19]. This was done in two sections: Section 211.165, where the word “validation” appears, and Section

211.194, where the proof of suitability, accuracy and reliability was made compulsory for regulatory submissions.

WHO published the guideline “Validation of analytical procedures used in the examination of pharmaceutical materials” in 1992, appearing in the 32nd report of the WHO expert committee on “Specifications for pharmaceutical preparations” [20]. ICH, which has been on the forefront of developing the harmonized tripartite guidelines for analytical methods adoption in the United States, Japan and European Community, issued two guidelines under the titles “Text on validation of analytical procedures” (Q2A) in 1994, and “Validation of analytical procedure methodology” (Q2B)’ in 1995 [16]. US EPA prepared a guide for method development and validation for the Resource Conservation and Recovery Act (RCRA) in 1995 [21]. AOAC developed a “Peer-verified methods validation program” with detailed guidelines on the parameters that should be validated [22]. A review and recommendation of a strategy for the validation of analytical methods, for both in-house developed and standard methods, as well as a recommendation on the documentation that should be produced during and at the end of method validation were included.

Many journals in the field of Analytical Chemistry are publishing, since long, reports including full or partial validation of the described methods. Some examples include: Analytical and Bioanalytical Chemistry, Analytical Biochemistry, Analytical Chemistry, Analytica Chimica Acta, Analytical Letters, Biomedical Analysis, Biomedical Chromatography, Clinica Chimica Acta, Clinical Chemistry, Chromatographia, Forensic Science International, Journal of AOAC International, Journal of Chromatography A, Journal of Chromatography B, Journal of Liquid Chromatography & Related Technologies, Journal of Pesticide Science, Journal of Pharmaceutical and Food Chemistry, Journal of Separation Sciences, Therapeutic Drug Monitoring, Russian Journal of Chemistry, and the Open Access journals: Eurasian

Journal of Analytical Chemistry, International Journal of Pharmaceutical Sciences Reviews and Research, International Journal of Pharmacy and Pharmaceutical Sciences, Journal of Chemical and Pharmaceutical Research, and Scientia Pharmaceutica, among others. An example of a specialized journal in this topic is Journal of Validation Technology.

4. Literature source and protocol

The scope of this work is not approving or disapproving how people are validating their methods, but just to figure out globally the way and degree analysts are currently performing validation studies, and which parameters are considered. For this purpose, we have made a literature survey. It was deliberately chosen to work on a selection of articles obtained through the Scopus® abstract and citation database of Elsevier B.V. The initial search words were “method validation” as research topic, working on all years covered by this data base with the filtering set on document types being exclusively articles or reviews. Books, book chapters, conference papers, conference reviews, letters, editorial notes, short surveys, business articles, press and erratum were intentionally left out.

Life and health sciences were intended to be the subject areas of search, focusing on method validation in LC that is really the field of expertise of the authors of this article. The search was done on February 27, 2014, and returned 12668 articles that indicated the relevance of this topic in the field. All articles included, in a greater or lesser extent, a validation of the developed procedure. The first manuscript on this topic (according to the Scopus database), published in 1975 by Björkhem et *al*. in Clinica Chimica Acta, was entitled “Validation of methods for determination of urinary estriol during pregnancy using mass fragmentography” [23]. The validation of new developed

procedures has been progressively increased in recent years, as shown in Fig. 2 for the period 2000–2013. It is obvious that analysts are more conscious of the relevance of fact of validating their LC methods. However, another reason is that the demand for validated analytical methods in LC and in other fields has grown in response to the increasing consumer market or population demands (e.g. botanical supplements, food stuff, pharmaceuticals, health, and environment, among others).

Due to the high amount of reports published under the topic in study, it was not possible to check all of them one-by-one, even for the 1543 reports published in 2013 (Fig. 2), so we just took a global vision reading some reports randomly selected along the last decade (2004-2013) [24–223]. It should be indicated that not all the reports including validation studies for the developed methods indicate this issue in the title, or even in the keywords. We have also found some rare cases that evaluate the validation parameters, but the word “method validation” is not mentioned in the text. All this rendered the survey rather laborious.

5. Results of the survey

Fig. 3 shows an overview of the frequency of the reported validation parameters. As expected, precision (98%), linearity (96%), the range of application (which is closely related to the linearity, 79.5%) and accuracy (75.5%) seem to be relevant. The frequency of LOQs and LODs is 78.5% and 61%, respectively, which depends on the type of study. Specificity and selectivity seem to have medium to low interest to the authors (39.5% and 32%, respectively). Some authors also included in their studies the matrix effect, which is related to selectivity. Recovery is sometimes used as indicative of accuracy. This is the reason why its evaluation is not too high (48.5%). Since accuracy is sometimes also termed trueness, which is stated quantitatively in terms of

bias, 8% of the surveyed articles included its evaluation. To avoid confusion, the term "accuracy" should always be used avoiding "trueness". Then, adding the 75.5% articles that studied the accuracy to the 8% calling it trueness, 83.5% of the workers were interested in measuring the accuracy. Curiously, some authors indicate that accuracy involves trueness and precision. Sample stability is also an important parameter that is not sufficiently evaluated (47%). This is typically appraised by conducting forced degradation studies. The design and execution of these studies requires thorough knowledge of the analyte being tested, as well as a good understanding of the analytical technique [5]. It is also surprising that robustness is rarely included in the validation process (18.5%). Given the importance of the variability of the results with a change in any experimental parameter in many developed methods, robustness is really a relevant validation parameter that authors should take more into consideration.

The least studied parameters were ruggedness (8.5%), system suitability (7.5%), and uncertainty (3.5%). Ruggedness evaluates the constancy of the results when external factors (not internal as robustness), such as the analyst, instruments, laboratories, reagents, and days are varied deliberately. This parameter is rarely evaluated in the reports, meaning that either it has a low impact on the quality of the results of a method or, more likely, that the method is not transferred to other locations staying where it was developed. However, it should be strongly considered when the method fails and the other validation parameters seem to be correct.

System suitability is intended to demonstrate that all constituents of the analytical system, including hardware, software, reagents, controls and samples, are functioning as required to assure the integrity of the test results. This parameter should be demonstrated by the analysis of appropriate controls at appropriate intervals. System suitability testing is an integral part of any analytical method, as specified by ICH Q2(R1) [16]. However, the guidelines are often vague and reference is made to

pharmacopeias for additional information. This can be the reason of its low consideration in a validation process.

Finally, uncertainty is a parameter associated with the result of a measurement, characterizing the dispersion of the experimental values. It is usually expressed as the standard deviation, a multiple of the standard deviation, or the width of a confidence interval. This parameter is usually referred in the calculus of accuracy or precision, thus making sense not to study it separately.

It should be highlighted that re-validation is only included in less than 1% of the studied reports. This makes sense since it should be done in specific cases, when: (i) method parameters have to be changed to maintain the original performance, and the change is outside the tolerance allowed by the followed guideline, (ii) new compounds are analyzed that are not within the method's original scope, or (iii) the sample matrix changes. In fact, only 8 reports came into view in the survey since 1991 when the search words were "re-validation" and "liquid chromatography". Also, there is not much information published about method transfer. Recently, Okamoto highlighted the relationship between transfer of analytical procedures and assay validation, focusing on analytical procedures that include LC [224].

To this point, we should pose the following question: Is it important to state the validation guideline used? Absolutely yes. When validating a method, it is compulsory to indicate the followed guideline, since the validation criteria should be clearly established for each parameter. If this is not the case, it may be understood that the authors carried out the validation without following specific criteria, which questions the obtained results. A usual practice by some authors is just indicating the guideline used for some of the parameters included in the study (e.g. robustness or stability). In this regard, it should be said that almost half of the reports examined (49.5%) indicated

the applied guideline. Also, it was unusual to indicate the guideline in the period 2004–2006.

In all the literature surveyed, the validation was performed by a single laboratory. In a new search including the words “method validation”, “collaborative” and “liquid chromatography”, only 43 articles were found. From those, only 19 did a real collaborative interlaboratory work in method validation. With the search words “interlaboratory validation” and “liquid chromatography”, 64 articles were found. It should be noted that the purpose of a collaborative study is to determine the reproducibility of performance parameters when followed by different laboratories. Under the AOAC International guidelines, this requires a minimum of 10 independent laboratories producing valid data for 12 replicates of each sample, which should be blinded and randomized [225]. Since these studies require a high amount of participating laboratories, this could be the reason of the low number of published articles in collaborative method validation.

6. Has been method validation improved in recent years?

Scientific instruments are continuously evolving. Year after year they are becoming more sophisticated and able to perform better analyses with better results. Also, the associated software is able to do more are more calculus. In contrast, the validation parameters were established years ago, and practically have not varied. What’s new then? Neither the theory nor the practice in the validation field, but the authors and the market demand. In this context, analysts are becoming more and more conscious that reliable results can be obtained by validating their methods. This is illustrated in Fig. 2, where the reports referring validation can be seen to grow progressively.

In the reports, the validation studies may be clearly stated or hidden in the text. Unfortunately, the validation criteria are not always applied with the same rigor. Some researchers fully validate their methods, but others evaluate only some validation parameters. It should be said, however, that in all cases they are trying to improve their results. The rigor the authors adopt is often associated to the journal where the article is published. Higher impact factor journals require more complete and exhaustive method validations. However, really good work is also published in lower impact factor journals or open access journals.

In the past, some editors took the time to list what is expected in future submissions, related to some degree of method validation. However, it was never obvious or clear that a lack of such studies really has ended up in manuscript rejection. This depended on the rigorousness of the reviewers, resulting in somewhat a “luck of the draw” approach. This situation has been changing for the better, but it is not quite where it really should be today. It will change when all editors, reviewers and authors agree on the importance of doing thorough and complete analytical method validation studies.

So, considering all these points, is method validation an evolving discipline? Indeed, it evolves as authors, techniques and instrumentation do. In the past, authors did not usually follow any guideline to validate their results. They just applied the logic and evaluated some parameters, such as linearity, accuracy, precision, LOQ and LOD, but without following any specific criteria, or just following what other authors did. Some authors call these studies “optimization”, instead of validation. Thus, these studies varied from author to author. Nowadays, most authors tend to meticulously follow guidelines to validate their methods. This permits to compare results from several authors more easily. However, as in the past, it is still possible to find some authors not following any validation protocol. Therefore, theoretically, method validation has

evolved, since authors are following closely the guidelines in their reports. We say "theoretically" because validation criteria have been established for years, but not so far considered by most authors.

Before finishing, it should be noted that a clear trend in LC is miniaturization and automation, which are removing equipment validation out of the analyst responsibility putting it on the manufacturer hands. Equipment complexity is in general increasing so much that it is no more possible for the analyst using it daily to intervene on it in case of problem. Another issue in the field of validation is that the laboriousness of the validation process, at least partially, could be the reason for the low frequency of improvement or modernization of official methods of analysis. Advances in laboratory robotics and automation also are beginning to be applied to method development and validation for high-volume tests.

7. Conclusions

Validation tasks have been performed practically from the first published analytical reports. Whereas scientific instruments and methods have improved over the time, the rules and protocols for validation have scarcely changed. What has changed is the requirement level of the validation studies. Significant scientific and regulatory experience has been gained since the current cGMP's of 1971. This work was carried out to spotlight the question about how well analysts are doing method validation. Some analysts perform full validations, but others include only some parameters in their validation studies blindly trusting their equipment and/or employee. Some parameters are frequently evaluated, but others rarely considered. Some analysts are indeed more accurate than others when assuring the quality of their results, but in most cases we can be confident that all are doing their best to assure the quality of their methods. An

additional common challenge encountered during method development and validation in multinational companies is that methods are typically developed by the Research &Development (R&D) departments, whereas validation is typically the responsibility of a validation group. It is important that the R&D and validation groups work as one team.

Does this mean that some analysts are performing poorly? Definitively, no. Several factors should be taken into account to understand this answer. There are several guidelines to validate a method, and sometimes, authors have not enough information about which should be followed. Occasionally, the criteria of several guidelines are mixed in the studies, thinking that this will improve the quality of the results. Unfortunately, some validation parameters like robustness are usually missing, and others can be misleading (e.g. inter-day repeatability or trueness). Sometimes the researchers are not expert in the validation protocols, or it is a topic out of their field of expertise. It is also evident that important factors for performing the best are the equipment or economy of the laboratories, and the staff formation. A strong mentoring and training program in companies is an important factor for ensuring successful method development and validation.

The objectives of this work were to highlight the importance of the validation work, show to the scientific community that analysts are doing it in the measure of their possibilities, and encourage those analysts that are not yet validating their methods the advantages of doing it. People around the world are requiring highly quality results in the different products they are consuming, as well as in clinical and environmental analysis, and other fields. Therefore, the validation work should not be considered as an exercise to create more documents, neither another unnecessarily burdensome regulatory requirement, but a way to preserve/assure worldwide data quality.

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838

839 **Figure captions**

840 Figure 1. Validation steps

841 Figure 2. Number of articles published worldwide per year on the subject “method
842 validation” and “liquid chromatography” (Scopus® abstract and citation database,
843 Elsevier B.V.).

844 Figure 3. Frequency with which specific validation parameters were studied in the
845 literature survey carried out for this work.

Figure 1

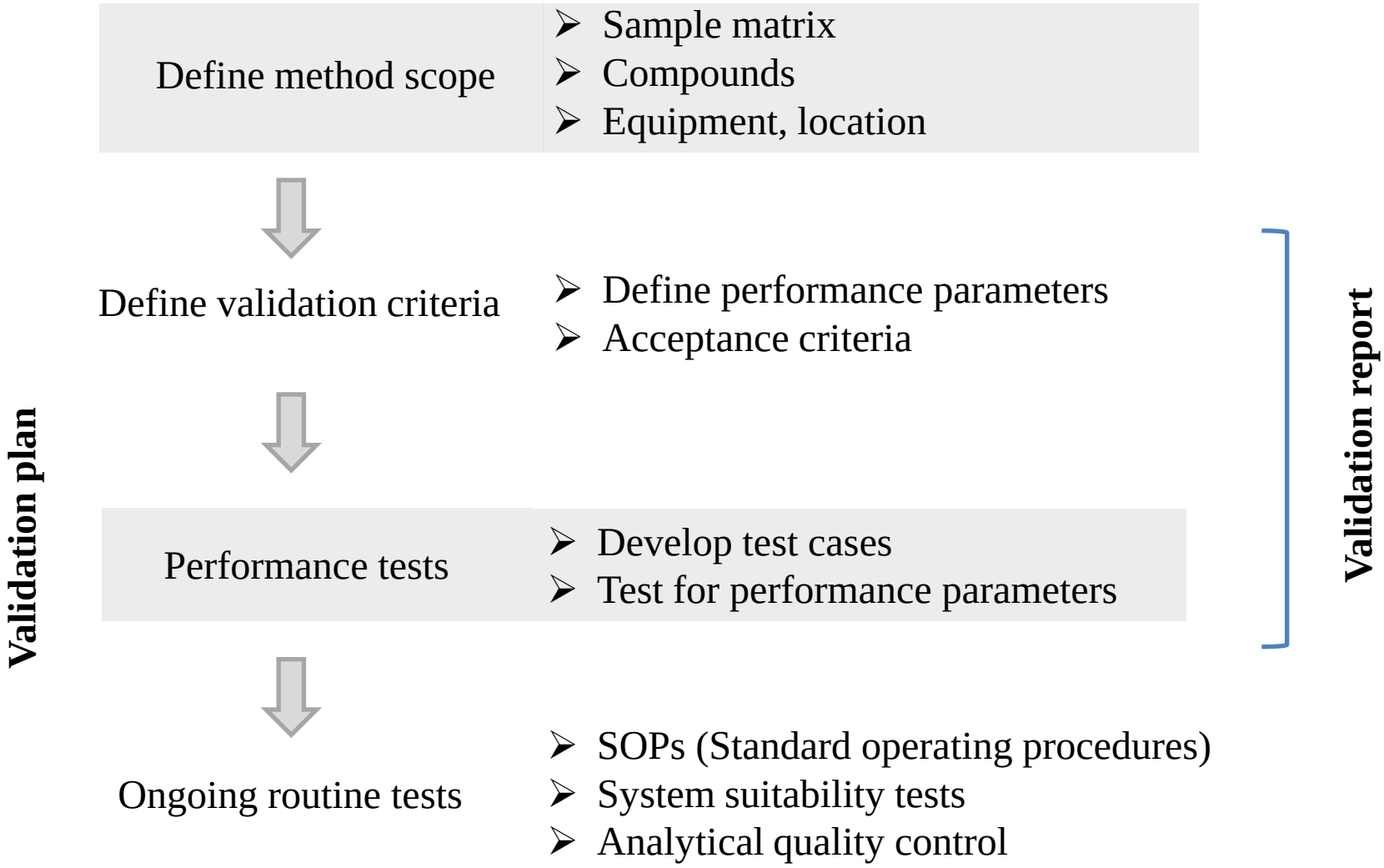

Figure 1. Ruiz-Angel et *al.*

Figure 2

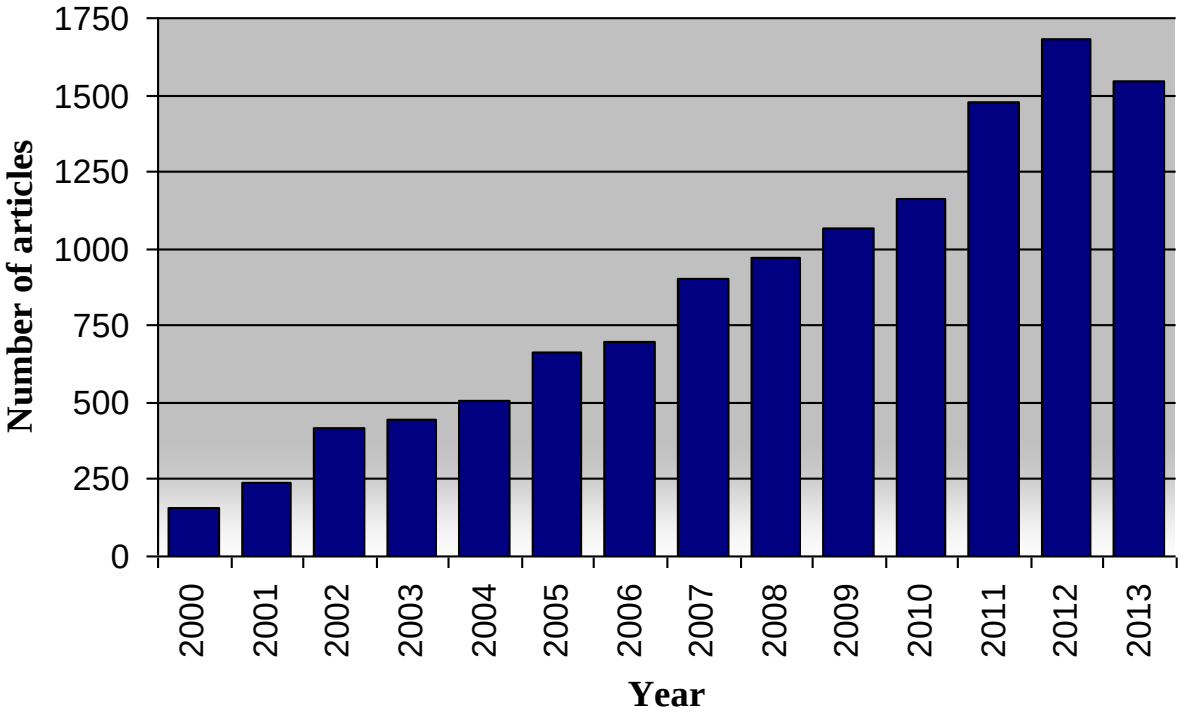

Figure 2. Ruiz-Angel et *al.*

Figure 3

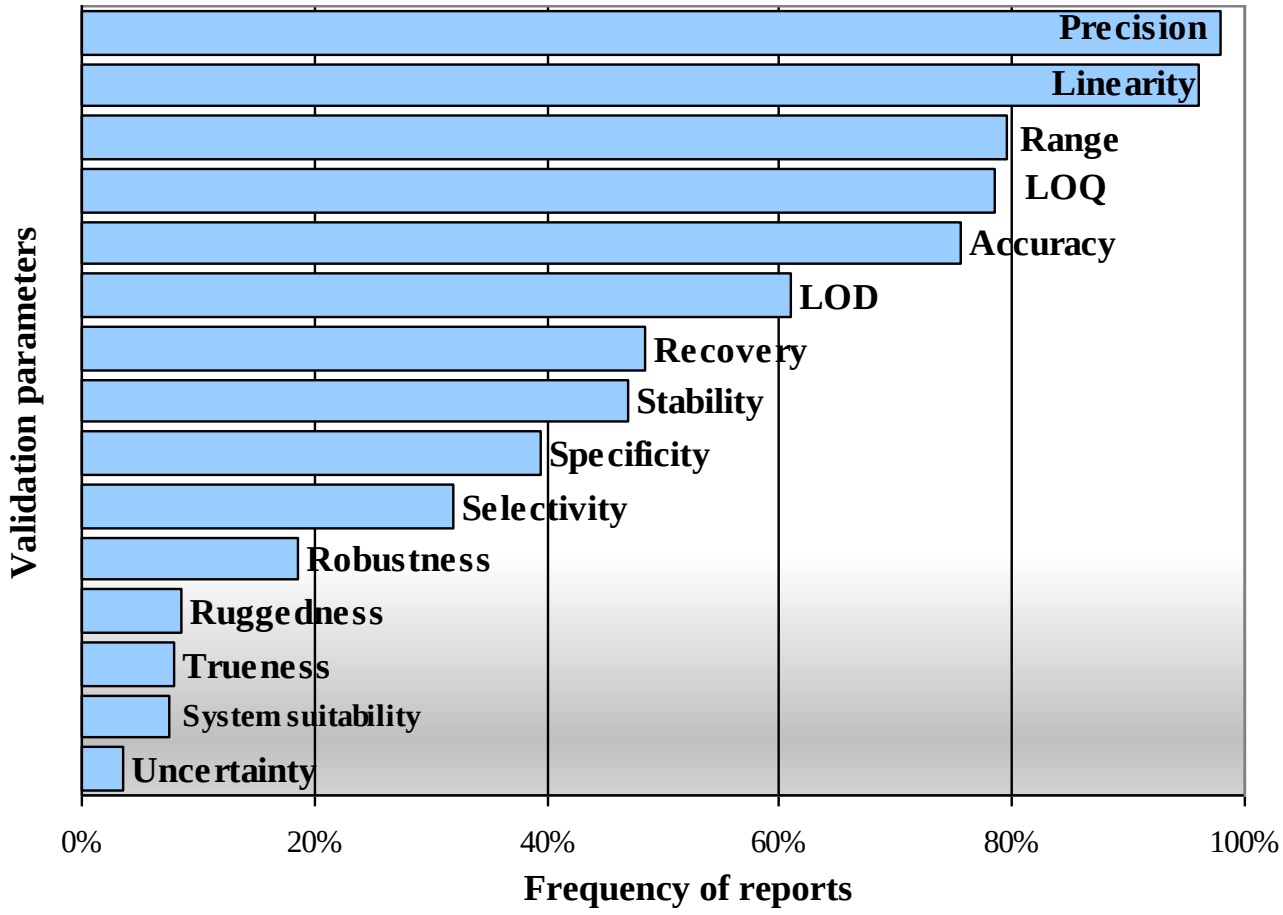

Figure 3. Ruiz-Angel et *al.*