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Calibration and Validation of Analytical Methods

A Sampling of Current Approaches

Edited by Mark T. Stauffer

CALIBRATION AND VALIDATION OF ANALYTICAL METHODS - A SAMPLING OF CURRENT APPROACHES

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Meet the editor

Mark T. Stauffer was born in 1957. He graduated with a Bachelor of Science degree in Chemistry from the University of Pittsburgh in 1979, worked in the industry for 12 years, and then returned to Pittsburgh, receiving a PhD degree in Chemistry in 1998. He joined the Faculty of Chemistry at the University of Pittsburgh at Greensburg in 2001, receiving tenure in 2007. Since 2001, he has been collaborating with projects in archeology, foods, test kit evaluation, mine drainage, and data analysis. He and coauthors presented over 100 papers and posters at technical conferences and published 13 papers in peer-reviewed journals. He presented a short course on analytical data treatment at the annual Pittcon Analytical Chemistry Conference. He conveys his enthusiasm for research and teaching via mentoring undergraduate research and through his courses in analytical chemistry.

Contents

Preface

Ask any analytical chemist what constitutes the most important aspect of any quantitative analytical determination, and the most frequent response one might receive is "calibration" and perhaps even "calibration and validation." One might ask: "Why would this be so?" If one mulls over this matter for a moment, the answer might become apparent. Consider any analytical instrument, e.g., a UV-visible spectrophotometer, and the absorbance response produced as radiation from the spectrophotometer's source lamp passes through the solution of absorbing analyte and is absorbed to a particular degree. At face value, that absorbance has no meaning at all *unless* that signal is referenced to a *known* amount of the absorbing analyte present in the measured solution. Now, the absorbance does have meaning, as it can be expressed as a number of absorbance units per unit amount of the sought-after analyte and may potentially be used for determination of analyte concentration in an unknown solution or other types of sample. This referencing process is known as *calibration*.

The calibration process establishes a relationship between analyte signal and analyte concentration that is useful for quantitation of a sought-after analyte in a sample, using a given analytical method. A typical approach is to measure the signals produced by a series of calibration *standards* of known analyte concentrations that cover a particular range of concentration and then regress the blank-corrected signals (response variable) on the standard concentrations (predictor variable) to obtain the equation (i.e., calibration curve) that best fits the experimental calibration data and yields predicted values of the response variable. This type of approach to calibration is termed *univariate* (one response variable, one or more predictor variables) calibration and can also hold for the opposite arrangement—a response variable of concentration regressed on a predictor variable of signal. Also, included under the umbrella of univariate calibration methods is multiple linear regression (MLR), comprised of one response variable (usually concentration) regressed into two or more predictor variables (usually signal). Another broad class of calibration model, known as *multivariate* (two or more response and predictor variables each) calibration, involves the use of data arrays for the response and predictor variables, with the response matrix regressed on the predictor matrix, using the principles of linear algebra and statistics. Most multivariate calibrations are designed so that the response matrix is the array of concentration vectors for the calibration standards containing the sought-after analytes and the predictor matrix is the array of signal vectors for, e.g., spectra or chromatograms of the calibration standards at, e.g., particular wavelengths/frequencies or response times.

The *validation* process is equally important, as it verifies the signal-versus-concentration relationship acquired from the calibration samples via analysis of another, separate set of samples with known concentrations of analyte. A set of predicted analyte concentrations for the validation samples are then obtained using the calibration relationship for the method employed. The known and predicted concentrations of the validation samples are compared, and the *residuals* (errors or differences between known and predicted concentration) are analyzed statistically, e.g., by standard deviation and bias, to evaluate the accuracy and precision (i.e., the reliability), respectively, of the calibration system and, ultimately, the analytical method used for analyte quantitation. Other validation parameters assess the goodness of fit of the experimental calibra-

tion data to the values predicted by the calibration curve and also yield the limits of analyte concentration detectable by the analytical method utilized as well as the sensitivity of the method.

The focus of this book is on the roles of calibration and validation in the utilization of these techniques and their associated methods for analyte quantitation in samples. A number of approaches to calibration and validation of analytical methods will be presented in a series of selected research papers and reviews dealing with such topics as the use of the internal standard method for calibration and quantitation of proteins in biological matrices by LC-MS/MS, using a variety of data preprocessing methods and a DoE (Design of Experiments) chemometric approach to the development of calibration models for vibrational spectroscopic methods, employing DoE in conjunction with such chemometric methods as partial least squares (PLS), principal component analysis (PCA), and parallel factor analysis (PFA) for application to pharmaceutical analysis, and application of a variety of univariate and multivariate regression methods to the development of calibration models for laser-induced breakdown spectroscopy (LIBS).

This book seeks to introduce the reader to current methodologies in analytical calibration and validation. This collection of contributed research articles and reviews addresses current developments in the calibration of analytical methods and techniques and their subsequent validation. Section 1, "Introduction," contains an Introductory Chapter, a broad overview of analytical calibration and validation, and a brief synopsis of the following chapters. Section 2, "Calibration Approaches," presents five chapters covering calibration schemes for some modern analytical methods and techniques. The last chapter in this section provides a segue into Section 3, "Validation Approaches," which contains two chapters on validation procedures and parameters. This book is a valuable source of scientific information for anyone interested in analytical calibration and validation.

I am most grateful to Mr. Teo Kos, the initial Publishing Process Manager for this project, for all his efforts and support at the start of this book project and Mrs. Marina Dusevic who succeeded him for her supervision and organization of the publication of all materials; her assistance to me and the authors in the completion of our work in an easy, timely manner; and her helpful advice and guidance throughout the bulk of this project. I thank the authors for their excellent contributions to this compendium of research articles and reviews on calibration and validation schemes for quantitative analysis. I express many thanks to the technical editor who prepared these manuscripts for publication by InTech Open Access Publisher. I thank my wife, Resa, who is also an analytical chemist, for her advice, support, and encouragement; the University of Pittsburgh at Greensburg for their support; my secretary, Mrs. Valerie Kubenko, for her encouragement and assistance with this project as well as with my administrative and academic duties during my work on this book; and finally—and perhaps most importantly—my colleague, fellow administrator, and our campus statistician, Dr. Dean Nelson, for his support, encouragement, and helpful advice on statistics. Lastly, I am honored to complete this book project on the occasion of my 61st birthday—a nice "present" for the one who is embracing his later years with enthusiasm and has no intention of ceasing his work on analytical data treatment.

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Introduction

Chapter 1

Introductory Chapter: The Many Faces of Calibration and Validation in Analytical Methodology in the Present Day

Mark T. Stauffer

Additional information is available at the end of the chapter

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1. The necessary processes of calibration and validation in chemical analysis

One of the most critical aspects of any analytical method, if not THE most critical aspect, is the *calibration* of the response of the particular equipment with respect to concentration of the sought-after analyte. This aspect of the quantitative determination of an interesting analyte is necessary to understanding the interesting, and sometimes complicated, relationship between the signal produced by the desired analyte present in an interesting sample and subsequently measured by the analyst, and the amount (e.g., mass, moles, concentration) of that analyte in the sample. Without such a relationship, let alone the methods and techniques to acquire it, analytical chemistry as we know it would be impossible, if not horrendously difficult.

In this introductory chapter, the author (Editor) wishes to provide some background information for the reader, toward appreciation and understanding of the relevance and necessity of the process of calibration in quantitative chemical analysis, and the equally relevant and necessary role of the process of validation, or verification, of the calibration process. The subsequent chapters of this book will deal with the (much) finer details of analytical calibration and validation in current applications to various analytical methodologies, and thus allow the reader to see the many "faces" of calibration and validation in the realm of chemical analysis.

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1.1. What are *calibration* and *validation*, as applied to chemical analysis?

1.1.1. Calibration in chemical analysis

Calibration, in its broadest sense, may be defined as the process of bringing a task, method, procedure, or some operation in general, into conformity with a set of objectives and goals that are solidly established and highly reliable; i.e., based on information that is *precise* and *accurate* [1]. In analytical chemistry, calibration is defined as the process of assessment and refinement of the *accuracy* and *precision* of a method, and particularly the associated measuring equipment (i.e., an instrument), employed for the quantitative determination of a sought-after analyte [2]. The assessment of the analytical method and related instrumentation is based on analysis one or more *reference* samples that contain known, established quantities of the analyte(s) to be determined in the analysis. Usually, the number of reference samples, also known as *standards*, may be as few as two (i.e., a standard containing a known amount of analyte, and a *blank* or standard containing no known amount of analyte), or as many as, say, 10 (e.g., one blank and nine non-zero standards containing varying amounts of analyte). The scope of this process expands significantly when one considers the sample matrix in which the analyte(s) reside(s) and the processes needed to extract the desired analyte(s) from that matrix (if such processes are required), the instrumentation that produces and measures the analyte signal(s), and analysis of the calibration data [2].

1.1.2. Validation in chemical analysis

The term validation denotes, in general, verification of something; i.e., demonstrating by some means that an object, concept, etc. is accurate or valid [1]. In analytical chemistry, validation has the same meaning—in this case, though, the "something" to be verified is the analytical method used for analyte quantitation [3]. The calibration scheme employed in the analytical determination is particularly subject to verification, and must be, as the reliability of the analytical results produced by the determination is dependent on the reliability of the calibration expression that relates analyte signal to its concentration. Typical parameters used to validate a quantitative determination of an analyte include accuracy, precision, limits of detection and quantitation, limits of linearity of the calibration curve, dynamic range of calibration, robustness, sensitivity, and selectivity [3].

2. Rationale for calibration and validation in analytical chemistry

The rationale for performing a calibration of an analytical method may be stated as follows: *to obtain a valid relationship between the signal produced by the analyte and the quantity of analyte in two or more samples of known analyte concentration (standards), that can be described mathematically and used by the analyst to obtain quantitative information on the analyte in samples of unknown analyte concentration.* The calibration process for an analytical method involves measuring the signal produced by an analyte of interest in two or more standards (at least one blank and one non-zero standard) containing known quantities of the analyte. The measured signals from

all of the standards are total signals due to the contributions of the analyte plus other components in the standard matrix (e.g., an aqueous solution). The measured signals are then corrected for the blank signal, which is due to the other components in the standards. This blank correction of all standard signals yields a zero signal for the blank and non-zero signals for the non-zero standards, yielding in turn the signal due to the analyte only. The blank-corrected analyte signal, and the corresponding analyte concentration, are subjected to the appropriate mathematical and statistical treatment, usually linear least squares or other type of regression, to yield a mathematical equation for the best-fit line that describes the signal-concentration relationship [2, 3]. This mathematical expression may be used by the analyst to calculate the concentration of the sought-after analyte in samples of unknown analyte concentration.

The rationale for carrying out a validation study of the analytical calibration may be expressed as follows: *to verify the reliability of the calibration scheme, via assessment of the accuracy and precision of the calibration and the analytical results yielded by it.* For an analytical method to produce results that are both accurate and precise, the calibration setup employed must also be accurate and precise. The validation, or verification, process involves assessment of the calibration data, the outcome of the regression of those data, and the analytical results obtained. The assessment is accomplished by calculation of various statistics that address such parameters as the accuracy and precision of the analyte results, sensitivity of the method to the analyte, selectivity of the method for the analyte over other potentially interfering chemical species, and the lowest concentration of analyte that can be detected by the method as well as the lowest analyte concentration that can be detected with reasonable accuracy and precision [2, 3].

3. Calibration methodologies

One can say that the process of calibration has many 'faces". There is a myriad of possible approaches to the design and analysis of calibration schemes; all one has to do is peruse the published, peer-reviewed literature of analytical chemistry to get an idea of the breadth and depth of calibration methods that have been developed and subsequently implemented for a variety of quantitative analytical determinations over many years. Two aspects of the calibration process in chemical analysis, particularly, are critical to the development and implementation of calibration schemes for analytical methods. These aspects are:

- The design of the method, which would include such considerations as the number of analytes to be determined, the number of blanks and non-zero standards, the matrix of the blanks and standards, the concentration range of each analyte, and application of the method to univariate (one variable) or multivariate (more than one variable) data.
- The mathematical/statistical treatment of the calibration data (i.e., analyte signals and concentrations) that will yield a logical, workable relationship between signal and concentration.

Let us now discuss briefly the aforementioned items as they apply to calibration and validation of analytical methods.

3.1. Calibration methods: some established designs

A calibration scheme may consist of as few as two standards (a blank sample containing no known analyte plus a standard sample containing a known, non-zero quantity of analyte) to a series of standards (at least one blank sample and many standards containing known, and varying, amounts of the analyte) in which the analyte concentrations are arranged in order of increasing concentration. The resulting calibration method may be designed so that known quantities of the analyte are added to the sample matrix, or include a non-analyte chemical species that serves as an *internal* reference against which the analyte response may be ratioed to produce a relative response, or even prepare calibration standard solutions in, e.g., an aqueous medium, apart from the samples. The design of the method may be as simple as comparison of a standard sample containing a known concentration of the analyte to the unknown sample, or as complex as a series of calibration standards for one or more sought-after analytes. Let us now look briefly at some well known, widely used calibration methods employed for quantitation of interesting analytes.

3.1.1. External standard method

The external standard method is perhaps the best known and most widely used calibration method among analytical scientists. The external standard method employs a series of standards consisting of at least one blank that contains no known concentration of the sought-after analyte, and several non-zero standards containing known concentrations of the analyte and prepared in order of increasing analyte concentration. The calibration standards are prepared separate from (external to) the sample matrix, usually in a solvent, e.g., water, and containing the reagents used in sample preparation. The measured signals of the blank and non-zero standards are adjusted for the blank signal to yield a signal that reflects the signal due only to the analyte [3, 4]. The resulting blank-adjusted signals for the calibration standards are then regressed on the corresponding analyte concentrations to yield a calibration equation that is useful for determination of the concentration of the desired analyte in the unknown samples. If the analyte signal (I)-concentration (C) relationship is, e.g., first-order (i.e., "straight line") linear with a slope m, the resulting calibration function will be of the form given by Eq. (1) [3]:

$$I = mC + I_0 \tag{1}$$

The blank-corrected signals of the standards may also be plotted versus the corresponding analyte concentrations, as illustrated in **Figure 1**. The calibration equation for this plot is shown on the plot itself.

3.1.2. Standard addition method

This approach is employed mostly with samples that possess a component which yields a signal that interferes with the signal due to the analyte [3, 5, 6]. The method of standard addition involves direct addition (i.e., spiking) of known amounts of the analyte, usually as aliquots of a stock or working standard solution of the analyte, into equal-volume portions or aliquots of the sample itself. One of the sample aliquots is unspiked (i.e., no analyte added above what

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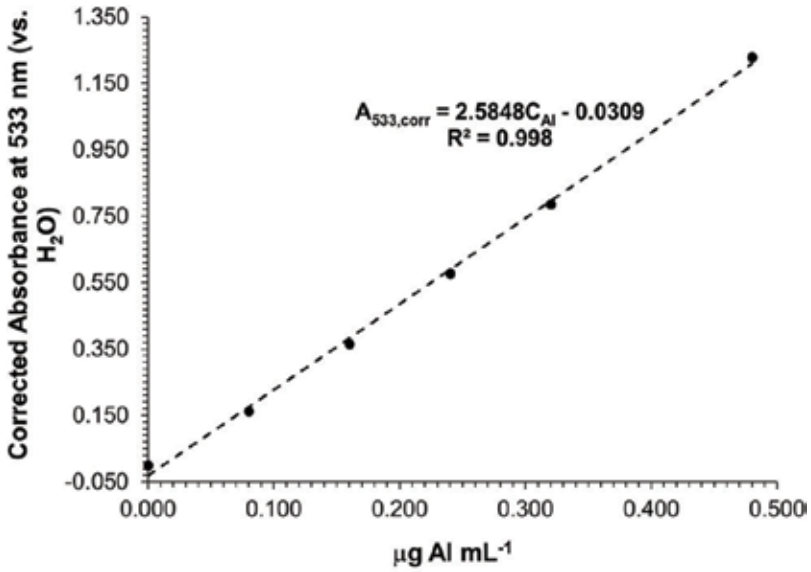

Figure 1. An example of a first-order linear calibration curve for determination of aluminum by the spectrophotometric Eriochrome Cyanine R method (courtesy of original research of the author, November 2015).

may already be in the sample), while the other aliquots are spiked with increasing amounts of the analyte, analogous to the scheme used for an external standard calibration. The effect of this addition of known amounts of the analyte to the aliquots of sample is to increase the signal due to the analyte in order to surmount the signal from the interfering species. The measured analyte signals of the unspiked and spiked sample aliquots are then regressed against the corresponding concentrations of spiked analyte to yield a calibration function that is utilized for determination of analyte concentration in the original sample by calculation of analyte concentration at zero signal. The standard addition method is used primarily to determine analytes in samples that contain chemical components which interfere with the signal produced by the analyte. An example of a standard addition plot is depicted in **Figure 2**.

3.1.3. Internal standard method

The internal standard method makes use of addition of a chemical species, different from the analyte, in a constant amount to calibration standards, blanks, and samples involved in the quantitative determination of the analyte [2, 3]. A ratio of the analyte signal ($I_{A,S}$) to the internal standard signal ($I_{IS,S}$) is calculated for the blank and each standard. Likewise, a ratio of the analyte concentration ($C_{A,S}$) to the internal standard concentration ($C_{IS,S}$) is calculated for each standard (including the blank) in the calibration set. The signal ratios ($I_{A,S}/I_{IS,S}$) are then plotted against the concentration ratios ($C_{A,S}/C_{IS,S}$) to produce a calibration curve and its calibration equation of the form given by Eq. (2):

$$\frac{I_{A,S}}{I_{IS,S}} = m\frac{C_{A,S}}{C_{IS,S}} \tag{2}$$

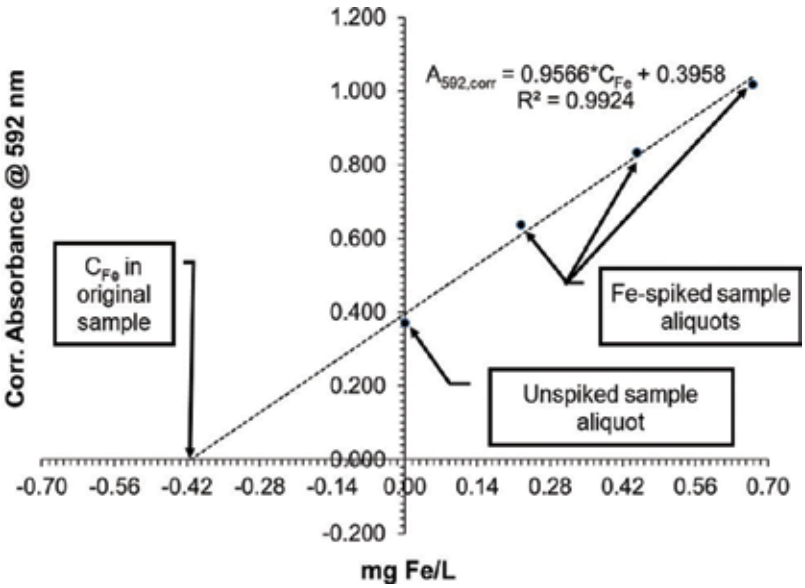

Figure 2. An example of a standard addition calibration curve for determination of iron in mine drainage by the colorimetric Ferene-S method (courtesy of original research of the author and former students, November 2010).

In Eq. (2), m is the slope of the calibration function. The internal standard method is used for irreproducible amounts of sample, varying signals from determination to determination, or losses of sample occurring during sample preparation.

3.2. Regression and analytical calibration

Regression is a statistical process in which the relationship between a dependent, or response, variable (e.g., "y") and an independent, or predictor, variable (e.g., "x") is determined and explained [7]. Through a collection of mathematical calculations, the equation that relates "y" to "x", and in essence explains it, is derived for its intended use. In a chemical analysis, the calibration process involves regression of the dependent variable (usually signal, but can be concentration regarding inverse calibration methods) on one or more independent variables; usually, concentration is taken as the independent variable, but signal is treated as the independent variable in inverse calibration methods [8–10]. The type of method employed for regression of calibration data in an analytical determination depends on how many analytes are to be determined as well as how many responses are to be measured; i.e., is the calibration model to be used *univariate* (one variable) or *multivariate* (more than one variable) in structure? This point is a good segue into the topic of univariate versus multivariate calibration, and some regression methods that are appropriate for either or both calibration models.

3.2.1. Univariate and multivariate calibration: regression methods

For sets of data/results, the term *univariate* refers to a one-variable set of data, e.g., a row or column of titration volumes. The term *multivariate* describes a set of data/results that contains

two or more variables, e.g., a group of sodium concentrations and the corresponding signal intensities measured by flame emission spectrometry—a two-variable system comprised of an independent variable and a dependent variable. In analytical calibration, however, the descriptor *univariate calibration* refers to one dependent variable (e.g., usually signal, but can be concentration) regressed on one or more independent variables (e.g., usually concentration, but can be signal), and the term *multivariate calibration* denotes two or more dependent variables (e.g., usually concentrations of two or more analytes) regressed on two or more independent variables (e.g., usually the corresponding signals) [9–13]. For each of these two broad categories of analytical calibration, there are regression methods that have been demonstrated to provide the sought-after relationship between an analyte signal and the corresponding analyte concentration [9–13]. **Table 1** provides a list of some of these well known, widely used regression methods for quantitative analytical calibration, and the type of regression. In the subsequent chapters of this book, some of the regression methods indicated in **Table 1** will be encountered, and their applications to calibration of various analytical methods illustrated.

Regression method	**Univariate/multivariate mode**
Ordinary least-squares (OLS)	Univariate (simple OLS, multiple OLS)
• Simple OLS (i.e., linear least-squares)	Multivariate (multivariate OLS)
• Multiple OLS (i.e., multiple linear regression (MLR))	
• Multivariate OLS (includes K-matrix and P-matrix methods)	
Stepwise	Univariate/multivariate
Weighted	Univariate/multivariate
Principal component (PCR)	Univariate/multivariate (usually, one dependent variable regressed on multiple independent variables)
Partial least-squares (PLS)	Univariate (PLS-1)
• PLS-1 (one dependent variable regressed on multiple independent variables)	Multivariate (PLS-2)
• PLS-2 (multiple dependent variables regressed on multiple independent variables)	
Canonical correlation analysis (CCA)	Multivariate
Ridge	Univariate
Lasso	Univariate
Regression trees	Univariate/multivariate
Artificial neural networks (ANN)	Univariate/multivariate

Table 1. Selected univariate and multivariate regression models applied to calibration data in quantitative analytical determinations [9–13].

4. Validation parameters for assessment of the reliability of calibration methods

Without the means to assess the reliability (i.e., accuracy and precision) of the calibration scheme used for quantitative determination of an interesting analyte, the calibration curve employed for quantitation of the analyte *and* the quantitative results for analyte concentration in the sample—in fact, the entire analytical method—become questionable and thereby unreliable. Thus, the need for *validation*, or assessment of the performance of the calibration for a quantitative analysis, becomes imperative for a successful analytical determination. Validation can also have more than one "face" as well.

4.1. Some examples of validation parameters

Various statistical parameters and methods have been developed over the years to accomplish the task of performing assessments of the reliability of calibration schemes used in quantitative analyses. These parameters examine such aspects of calibration schemes as the linearity of the resulting calibration curve, the goodness of fit of the regression model to the experimental calibration data, the precision of the calibration slope, and the standard errors of calibration (SEC) and prediction (SEP) [9–13], among other quantities. Such statistical parameters as the regression equation (i.e., slope (m) and y-intercept (b)), square of the Pearson correlation coefficient (R^2), and standard error of the regression (s_r) are among the assessors of calibration performance for a univariate case (i.e., an analyte signal dependent on a corresponding analyte concentration, or vice versa) [2–4, 8], and are parameters that are usually an undergraduate chemist's first exposure to calibration and validation in a quantitative analysis course. As those involved in data analysis know (all too well), there is much more to consider regarding calibration and validation methodologies. For both univariate and multivariate calibrations, parameters such as *total* and *explained residual variance* (TRV and ERV, respectively), *mean square error* (MSE), *root mean square error* (RMSE, an indicator of calibration *accuracy*), *standard error* (SE, or standard deviation of prediction errors, an indicator of calibration *precision*), *bias*, and the *coefficient of determination* (R^2, a.k.a. the square of the Pearson correlation coefficient) are widely and commonly used for assessment of calibration reliability. The formulas and descriptions for the aforementioned evaluation parameters may be found in any number of texts on chemometrics and statistics [9–13].

4.2. Validation methods: cross-validation and bootstrapping

Calibrations models are usually designed using two sets of response (dependent) variable and predictor (independent) variable data: a *training* set which, as the name suggests, "trains" or develops the model, and the *test* or *prediction*, or *validation*, set that "tests" the validity of the developed model. Assessment of the reliability of the developed calibration (training) model is made by application of that model to the validation (test) set via comparison of the predicted results to the known validation quantities; thus, it is the test set that acts as the *assessor* of the calibration model [11, 12]. Using a specific training and test set only once for development and testing of a calibration scheme may not always produce reliable results using the selected test data. Also, an insufficient amount of calibration data to yield a sufficiently large number of

predicted results can be problematical in evaluating the calibration scheme. Toward this end, two methods in particular, *cross-validation* and *bootstrapping*, have been developed to increase the number of predictions for a given calibration model.

4.2.1. Cross-validation

In cases for which there might be a paucity of data available to perform a thorough evaluation of the calibration model, a method known as *cross-validation*, that "resamples" both training and test data to produce a larger number of predictions, may be employed. Cross-validation works by splitting the total set of available calibration data into roughly equal-data segments, with one of the segments selected as the test set and the remaining segments serving as the training set. The calibration model is developed using the training set, and then tested on the test set. This process is continued until every segment has served as a test set. In this manner, the number of predictions for the model may be increased [10–13].

4.2.2. Bootstrapping

This method uses training sets with a set number of objects randomly selected from the available data set. A calibration model is developed from the training set and subsequently applied to the objects in the available data set that are not part of the training set. This process yields corresponding prediction values and their associated errors. The process is then repeated many (sometimes up to 1000) times. Two advantages of the bootstrap method are an uncomplicated approach and having the same number of objects in the training set; some disadvantages are labor-intensive calculations, the possibility of unequal consideration of all objects in the available data, and results that are sometimes overly optimistic [11].

4.3. Validation parameters for the analytical method itself

Let us not forget about other parameters that are useful for validation of the analytical method itself. These are the so-called "figures of merit" [2–4]—the accuracy (i.e., bias) and precision (i.e., standard deviation) of the analytical results, limits of detection (LOD), quantitation (LOQ), and linearity (LOL), the dynamic range (the range of concentration linear with signal from the LOQ to the LOL; i.e., LOL/LOQ), sensitivity, and selectivity [2–4]. All of these parameters for method validation are ultimately connected, and traceable, to the calibration scheme employed for quantitation of sought-after analytes using an analytical method or technique.

5. Some "faces" of calibration and validation to be found in this book

In this book on calibration and validation of analytical methods are a collection of research and review chapters on various applications and other aspects of calibration and validation in chemical analysis. In these highly interesting chapters, one can see the many and varied "faces" of calibration and validation revealing themselves to the reader, waiting to be studied and utilized by interested researchers. A quick glimpse of these "faces" should provide the reader with a preview of what is in store as one explores the content of this book:

- The impact of factorial design and machine learning strategies on pharmaceutical analysis
- Multivariate calibration methods applied to development of vibrational spectroscopic methods
- Approaches to method validation for pharmaceutical assessments, using high-performance thin-layer chromatography (HPTLC)
- A review of criteria for assessment of analytical method reliability
- Using internal standards for quantitation of proteins in biological matrices by LC-MS/MS
- Calibration methods for laser-induced breakdown spectroscopy (LIBS)
- Analytical method validation, presented in the context of laboratory competence and generation of reliable analytical results

I anticipate that the reader will find this assemblage of chapters dealing with analytical method calibration and validation useful as well as interesting, and possibly inspiring some ideas for future studies.

6. Summary

This introductory chapter to this book on calibration and validation of analytical methods was written to provide the reader with a general overview of a sort on the topics of calibration and validation as applied to problems in chemical analysis. This included a general explanation of calibration and validation, the importance of these topics in quantitative analysis, and a rationale for their use in analytical chemistry. Also presented were overviews on calibration and regression methods, and validation parameters and methods for calibration schemes and analytical results. Finally, a glimpse of the subsequent chapters in this text was given. This introductory chapter is meant to be general in scope; the reader will get much more detail in the following research and review chapters. Thus, I invite the reader to explore the following chapters to see the various "faces" of analytical method calibration and validation.

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Section 2

Calibration Approaches

Chapter 2

Analytical Calibrations: Schemes, Manuals, and Metrological Deliberations

Marwa S. El-Azazy

Additional information is available at the end of the chapter

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Abstract

Chemical measurement processes (CMPs) must be performed in a setup of controlled statistical conditions. Thus, validation of such a measurement process and assessment of its ability to accurately measure the analyte is important. Analytical calibration is the most crucial step in any analytical procedure targeting the estimation of analyte concentration. As a key component of any validation procedure, calibration must be properly conducted. To achieve that, firm knowledge with the realms of the calibration process must exist. Several jurisdictions help to build up this acquaintance, including the terminology and definitions, the international guidelines and how they differ, schemes and manuals to be used to build a calibration model, metrological considerations, and assessment procedures. Careful thinking prior to any of the previous calibration aspects is necessary and helps to improve the product of the calibration process. Throughout this chapter, aspects of the calibration assembly will be thoroughly discussed. Different types of calibration will be revealed with a focus on analytical calibration for a CMP. Steps for a successful calibration will be described. The reader will be able to use information given throughout this chapter as a guide for an effective calibration process.

Keywords: analytical calibration, terminology, regulatory agencies, multi-, one- and two-standard calibrations, calibration methodologies

1. Introduction

Millions of analytical investigations are instigated every day. Despite the massive progress and advancements implemented to the developed techniques and instrumentations, calibration stays as the most critical stage in every analytical practice leading to the estimation of the target analyte.

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An analytical measurement process is a setup with a demarcated configuration that has been carried to be statistically controlled under the designated experimental conditions. To substantiate the efficacy of analytical processes and subsequently the applicability in routine analysis, the ability of such a method to "quantify" must be assessed. Thus, and to fetch such a status of statistical management, key elements including *validation*, and hence its metrological frontier, *calibration*, must be clearly comprehended [1].

In the latest definition released by the Joint Committee for Guides in Metrology (JCGM) in their 3rd edition of the "International Vocabulary of Metrology, VIM", calibration is: "operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication" [2, 3]. Validation, in the same edition, was defined as "verification, where the specified requirements are adequate for an intended use."

Though *validation* as an idiom is already well-known, the protocols of its application are not clear for many of the analytical chemists. No need to say that validation of an already developed analytical process must be performed following a clearly written protocol and through a series of laboratory experiments. Moreover, different regulatory bodies (e.g., IUPAC, ICHQ2R1) do have different nomenclature for such a term (as well as its components) and hence dissimilar manuals, an issue that in turn leads to different performance and approval criterions [4, 5].

As a component of the validation process, calibration is also a subject of controversy in terms of vocabularies, the perception of the calibration procedure starting with method development to fitting of results obtained, implementation of the appropriate linearity testing, and hence the assessment of goodness-of-fit and deviation from linearity.

It is very important to recognize that though the existence of intrinsic discrepancies between chemical (CMPs) and physical (PMPs) measurement processes in terms of uncertainty associated with the results and the availability of reference materials; both are still treated with the same metrological approach. Yet, an imperative difference between both processes must be carefully considered which is *calibration* [6–8].

The purpose of this treatise is to shed light on the "appropriate" definition of calibration as a process that encompasses metrological/statistical as well as procedural evaluation of the analytical measurement. The different types of calibration will be revealed. Analytical calibration, across the different guidelines and with respect to definitions and terminologies, schemes, metrologies, and methodologies will be discussed.

Though in some sections of this piece complicated terminologies would be used, a reader of this chapter, even if not from the scientific community, would be able to understand information given with the help of definitions revealed in almost every section.

2. Calibration in analytical sciences: fundamentals

Several definitions exist in literature for calibration. In addition to the previously mentioned definition given by the VIM [2, 3], IUPAC definition of calibration can be viewed as a "general" description where it is given as "an operation that relates an output quantity to an input quantity" [9]. Unfortunately, these definitions instead of giving a clear-cut understanding of the term and the corresponding process have created a kind of confusion where it is common to find the wrong term being given to the wrong process or similar names given to different types of processes, etc.

However, it is noteworthy to mention that the additional "notes" given by the JCGM [3] on the definition of calibration would clarify this misunderstanding where: "A calibration may be expressed by a statement, calibration function, calibration diagram, calibration curve, or calibration table. In some cases, it may consist of an additive or multiplicative correction of the indication with associated measurement uncertainty" and "Calibration should not be confused with adjustment of a measuring system, often mistakenly called 'self-calibration', nor with verification of calibration." Furthermore, and according to JCGM, "Often, the first step alone in the above definition is perceived as being calibration."

Yet, and as per these definitions, it is important to distinguish between the different types of calibration and whether it is designed for a qualitative or a quantitative purpose. As a relation between an input quantity and another output quantity, quantitative calibration can be performed *directly* (where the measurement and the reference values are being compared

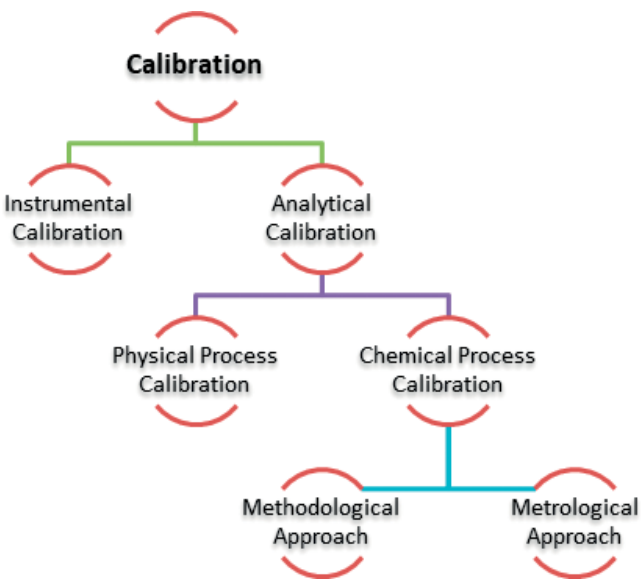

Figure 1. Schematic representation of the different approaches for calibration.

employing the same units) or *indirectly* (where the measured response is being decoded into the corresponding quantity to be determined, i.e., *analytical calibration*). Both *direct* and *indirect* calibrations can target the equipment as well as the process itself [10]. More details on these subdivisions will be given under the relevant section.

Calibration then can be tackled using different standpoints depending on its implication. In other words, is the calibration targeting the system of measurement and its quality so it is *metrological calibration* or it is an *analytical calibration* that merely describes the relation between the analyte and the corresponding response? Distinction of direct from indirect calibration and then process and instrument can be performed using the metrological maneuver. Another approach to see the calibration process would be in terms of methodologies and schemes followed to achieve such a status. **Figure 1** shows a schematic representation of the calibration process with the different approaches commonly found in literature. The following subsections will be dealing mainly with analytical calibration of a chemical measurement processes in terms of *steps and guidelines, schemes, manuals and methodologies,* and *metrological considerations*.

3. Analytical calibration

3.1. Steps and guidelines

As previously mentioned, the term *analytical calibration* is used when the calibration process cannot be performed *directly.* In general, the objective of doing calibration is to establish an experiential liaison between the instrument response signal "*y*-variable" and the reaction factors "*x*-variable." The purpose of establishing such a liaison is to be able to assess the influence of these variables on the response and hence quantify the analyte.

Surveying the literature shows that different validation strategies proposed by the different regulatory institutions usually involve quite different guidelines for analytical calibration. In addition to the differences in terminologies used to define analytical calibration and hence associated terms, other major differences can be found as follows.

3.1.1. Proposing a strategy for a calibration study

Planning is the preliminary step in conducting the calibration study. The conventional scheme for performing calibration is to prepare a set of standards (plus a blank) followed by quantifying the response signal for such a set [11–13]. Common several "How" questions usually evolve as the analyst is getting ready to conduct this study:

- How many standards will be used?
- How the target of calibration will affect the composition of calibration standards?
- How the selected number of standards will be patterned and disseminated on the studied concentration range?

- How to select the concentrations that will be measured?
- How the measurement procedure would be like?
- How many times the analysis should be repeated (replications)?
- How the calibration mode will be set? (details will be discussed later)

The elements of *calibration hierarchy* according to JCGM [3] *are one or more measurement standards and measuring systems operated according to the measurement procedure*. Typically, a minimum of 5–6 calibration standards is used for this purpose. Yet, the number of standards used might vary according to the performed analytical process as well as the guidelines proposed by the supervisory body followed. The calibration standard might be *matrix-free* if the purpose is to calibrate solvents, for example, or *matrix-matched* (*MMC*) if it is expected that the presence of matrix would affect the response signal and hence the calibration outcomes. In this case, a blank sample (analyte free) should be used.

Careful distribution of the selected concentration levels over the working range is necessary for appropriate calibration. In this concern, discrimination between narrow and wide calibration ranges is essential. Attention should be paid for the case where a wide concentration range is calibrated where keeping the selected levels at very wide distances, a common approach in literature, might deteriorate the detecting system of the instrument, an issue that produces erroneous readings. The best approach is to keep the data points consistently dispersed across the selected range. Moreover, selected concentrations should be independently prepared (no serial dilutions) to avoid augmentation of error.

Selection of the concentration range to be covered should be based on the expected content of the real samples taking in consideration the matrix and the intended application of the proposed procedure [14]. According to ICH guidelines, for example, if the calibration is performed on an active ingredient or a final product, the range is usually 80–120% of the analyte concentration [4]. In case of using MMC, the blank sample (zero concentration, solvent) should be considered.

The appropriate protocol for a measurement will be the one that simulates the actual circumstances. In this itinerary, it is recommended that calibration samples are to be unevenly analyzed instead of being measured in an increasing concentration sequence. Moreover, insertion of calibration standards randomly in between the unknown samples within the measurement stream is commended.

Every experiment is associated with an error! Diminishing the random error (measurement uncertainty) and hence improving the precision is usually one of the goals when analytical calibration is performed. Replicate analysis is usually the approach. The number of recommended replicates differs according to the implemented guideline. While EMA, FDA, and AOAC indorse five replicates, ICH recommends three replicates or six replicates at a single concentration level compared by replication for 2–3 times at 6–10 concentrations evenly spaced across the linear range by Eurachem [4, 5, 14–17]. However, and due to economic considerations, triplicate analysis is the common approach.

Some guidelines impose more regulations than those previously mentioned. For example, FDA for bioanalytical method validation [18] necessitates that at least four concentrations (lower limit of quantification LLOQ, low, medium, and high) measured in six runs in duplicate/run.

3.1.2. Assembling and modeling of experimental data

Following the fulfillment of the previous checklist of "How questions," the subsequent movement will be to corroborate the liaison between the measured concentration and the equipment response. This liaison is usually established via *regression analysis* and hence *calibration graphs (commonly described as curves)*. According to JCGM [3], *calibration curve* is "expression of the relation between indication and corresponding measured quantity value", and "a calibration curve expresses a one-to-one relation that does not supply a measurement result as it bears no information about the measurement uncertainty."

3.1.2.1. Construction of calibration curve

The calibration curve is generally constructed by plotting the response values (y-axis, *dependent variable*) against the known standard concentration values (on x-axis, *independent variable, predictor)* either manually or by operating popular software like Excel®, for example. Performing regression analysis and drawing a regression line require a cautious decision on a bundle of three main components: *model, mode, and fitting technique.*

Typically, the number of predictors and so the type of response variable differ between various measurements. Accordingly, the regression pattern would be different. A common regression model is the *linear regression* where a *best-fit* straight line is drawn between x and y variables. Other types of regressions include *logistic, polynomial, stepwise (forward selection and backward removal), and ridge* regression.

In the simple linear regression, one independent variable is involved compared to more than one in case of multiple linear regression. The best-fit line is usually obtained employing the method of least squares (the most popular technique). This regression line is usually presented by the equation: $y = ax + b$, where a and b are the slope and the intercept, respectively. In this method, the line is calculated by minimizing the sum of squares of the residuals for each data point.

Regression analysis based on *principle component analysis* (*PCA*) is known as *principle component regression* (*PCR*), in which the response is regressed against a set of variables and using the PCA to find the regression coefficients. Other regression methods such as *partial least-squares (PLS)* establish a linear regression model by protruding x and y variables to a new space. This technique is mainly used when the number of data points is less than the number of variables [19, 20].

The last step after deciding upon the method and the model used is the selection of fitting technique. Adopting the case of a linear regression model being generated using the method of least squares, two approaches are commonly followed to find the best-fit line: *ordinary*

(*linear*) *least squares* (*OLS*) and *weighted least squares (WLS)* [21, 22]. As the name implies, OLS is the least-squares regression approach used when errors have a constant variance across the working range, *homoscedasticity*. That is of course in addition to the general assumptions of the OLS; errors are not correlated, conditional mean of errors is zero, and regressors are not linearly dependent (no multicollinearity). In contrary, WLS should be only used when variances are different, *heteroscedasticity*, and the working range is wide.

As an example of how to construct a calibration graph, spectrophotometric determination of tioconazole (antifungal, electron donor) using chloranilic acid (electron acceptor) via charge transfer reaction, and other calculated parameters needed to establish the regression relationship between [drug] and absorbance are shown in **Table 1**. Equations used to calculate essential regression parameters, *r (correlation coefficient)* and hence the *coefficient of determination* (R^2), *slope (a)* and *intercept* (*b*), are shown in **Figure 2**, which is the calibration graph plotted from data shown in **Table 1**.

3.1.2.2. Assessment of performance: model metrics

Evaluation of a linear relationship between concentration and response is usually performed by assessing the regression statistics, calibration graphs, and residual plots of the proposed model. Inspection of linearity is usually made *visually* by observing the calibration plot. Again, different guidelines do use different terminologies to describe the linearity and range, FDA for example uses the term calibration (standard) curve, compared to ICH guidelines which clearly defines linearity and Eurachem which uses the term working range [4, 14, 16, 18].

x_i	y_i	$x_i - \bar{x}$	$(x_i - \bar{x})^2$	$y_i - \hat{y}$	$(y_i - \hat{y})^2$	$(x_i - \bar{x})(y_i - \hat{y})$
40	0.073	−180	32400	−0.4537	0.205844	81.666
80	0.175	−140	19600	−0.3517	0.123693	49.238
120	0.276	−100	10000	−0.2507	0.06285	25.07
160	0.372	−60	3600	−0.1547	0.023932	9.282
200	0.473	−20	400	−0.0537	0.002884	1.074
240	0.582	20	400	0.0553	0.003058	1.106
280	0.677	60	3600	0.1503	0.02259	9.018
320	0.781	100	10000	0.2543	0.064668	25.43
360	0.878	140	19600	0.3513	0.123412	49.182
400	0.98	180	32400	0.4533	0.205481	81.594
Σ =						
2200	5.267	0	132000	0	0.838412	332.66
$\bar{x}$ = 220	$\hat{y}$ = 0.5267					

Table 1. Parameters needed for the calibration graph (**Figure 1**).

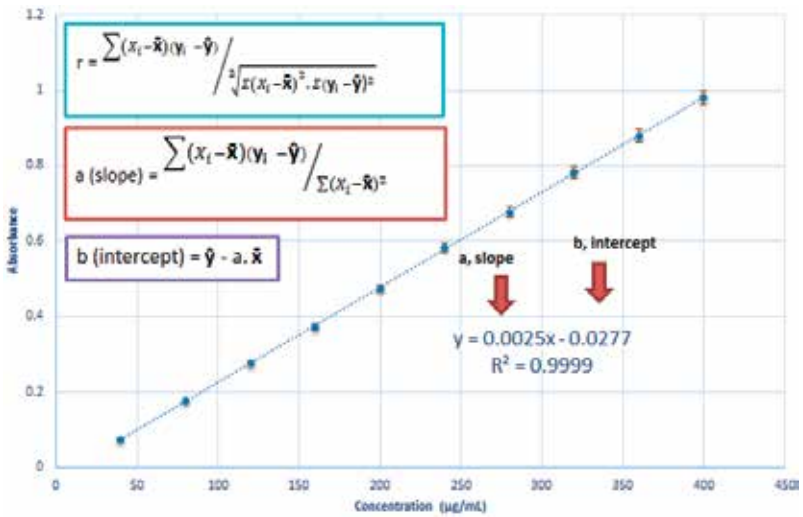

Figure 2. Calibration graph plotted from data presented in **Table 1**.

Figure 3 shows three commonly used terms to describe the range: *analytical (dynamic range)*, *working (calibration) range*, and *linear range.*

The analytical or the dynamic range is the range in which the equipment is showing a response to the tested concentration, and this response is changing as the concentration varies. This relationship might be linear or nonlinear. The calibration range, in which the liaison between response and analyte concentration has an adequate uncertainty, usually starts with the limit

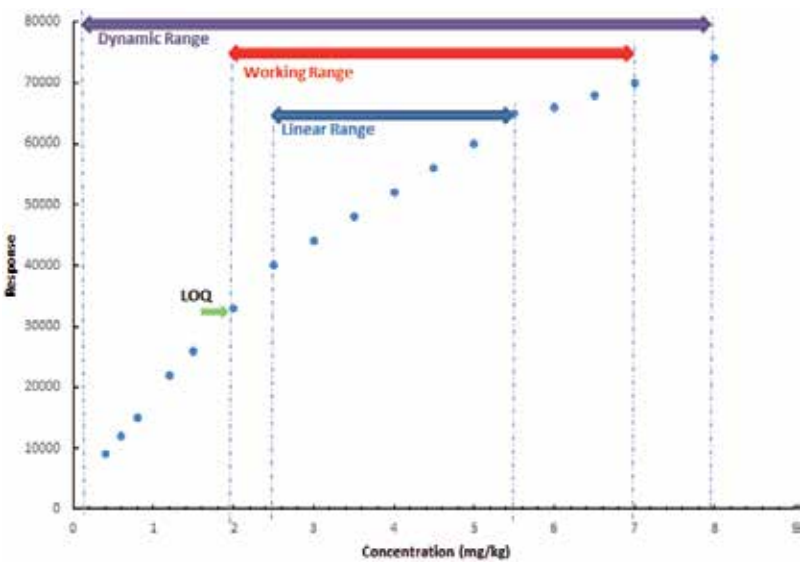

Figure 3. A demonstration of different ranges: analytical, working, and linear ranges.

of quantitation (LOQ) and ends where there is an obvious deviation from linearity. Working range is usually wider compared to the linear range. Thus, the latter can be defined as the range where there is a direct proportionality between concentration and response [14, 23, 24].

Though not being a component of the validation process, sensitivity is mentioned in variety of guidelines with the purpose of method evaluation. As a parameter, sensitivity can be easily estimated from the linear calibration graph as the function gradient. As per FDA guidelines [16], sensitivity is defined as "the lowest analyte concentration that can be measured with acceptable accuracy and precision (i.e., LLOQ)." In this itinerary, parameters such as *limit of detection* (*LoD*) and *limit of quantitation* (*LoQ*) need to be distinguished [23].

Once the status of "linearity" is established, statistical analysis is needed. Model metrics such as the correlation coefficient, slope of the regression line, and the intercept should be included (**Figure 2**). A comparison between the linearity assessment practices as per the different guidelines will be revealed in the following subsections. **Table 2** shows a comparison

Assessment approach	Recommended by	Pros	Cons	Ref.
Graphical inspection				
Residuals plot	IUPAC, NATA, INAB	Helpful together with the visual inspection in detecting linearity	Not a powerful tool in confirming linearity and needs a former experience with the different residual patterns	[14, 25–27]
Visual inspection (nongraphical)	–	Easy and useful in clear-cut situations	Subjective and cannot be used alone to indicate linearity	[16, 18]
Statistical analysis				
Analysis of variance (ANOVA)	IUPAC	$F_{calculated}$ value can be easily calculated	Not decisive	[9]
Lack-of-Fit (LOF)	IUPAC, INAB	Easy to be implemented in many software spreadsheets	Greatly dependent on the method precision, and usually several replicates are needed	[25, 27]
Mandel's fitting test	IUPAC	Easy to calculate and is mainly used when variances of two calibration functions are similar	Needs more samples compared to regular fitting tests and needs an estimation of the nonlinear model	[9]
Numerical evaluation				
Coefficients of correlation (r) and determination (R^2)	ICH, Eurachem, IUPAC, INAB, NATA	Widely used and implemented in almost all software	Sometimes deceptive and is monotonously getting higher as the number of variables increases	[4, 14, 25–27]
Residual standard deviation (RESSD)	NATA	Easy to understand and calculate	Depends on the measurement tool and different from one equipment to another	[26]

Table 2. A comparison between different linearity assessment approaches.

between the nongraphical, graphical, statistical, and numerical evaluation approaches for linearity evaluation. Contrast is shown in terms of the pros and cons of each approach as well as the recommending guideline(s).

i. *Graphical inspection:* this approach is recommended by most of validation guidelines. The preliminary step is to construct a plot between concentration and response on the x and y axes, respectively. The second step involves examining the plot visually. Majority of guidelines support using the plot of residuals as a tool to inspect linearity. Residuals can be defined as the difference between an observed value for a dependent measurement (y) and the estimated value of this measurement. As an approach, plot of residuals is a plot where calculated residuals are shown on the y-axis and the independent variable is shown on the x-axis. Linearity is confirmed when points are randomly scattered around the horizontal x-axis. Some data are not suitable candidates for plotting residuals; e.g., heteroscedastic data and outliers [14, 25–28].

ii. *Nongraphical approach*: visual inspection of data without plotting the graph or using statistical tools cannot be used as a linearity assessment tool by itself [16–18].

iii. *Statistical assessment:* statistical evaluation of data is a vital tool to confirm linearity when visual and residual plots cannot confirm a status of linearity. Generally, tests of significance are the methods used to infer whether stated claims about a sample of data extracted from a certain population are in favor or against the stipulated evidence. In other words, the significance tests are testing whether the *null hypothesis* ($H_{0)}$ is being verified or not. Examples for significance tests include the student t-test and the F-test. Significance tests reported in literature to test linearity can be summarized as follows:

- *Analysis of variance* (*ANOVA*): this test depends on calculation of combined variances (S^2) between or within a group of data replicates assembled together in a certain way. This test is only recommended by IUPAC [9]. As a significance test, $F_{calculated}$ is compared with $F_{tabulated}$. The calculated F-values is found using the following formula: $F_{calculated} = (S_{y/x}/S_y)^2$, $S_{y/x}$ is the standard error for residuals and S_y is the pure error.
- *Lack-of-fit (LOF) test:* this test is a part of IUPAC validation guidelines [25, 27]. The calculated F-value is a ratio of mean sum of squares of random error (MSS_{error}) as a measure for divergence of points from the regression line being caused by the haphazard distribution of the points following replicate measurements to the mean sum of squares due to the lack-of-fit (MSS_{LOF}) as a measure for deviation of points caused by incongruity of the calibration paradigm. A comparison between the calculated and the tabulated value is then performed. Another approach to perform LOF test is to find the probability, p-value. Having a p-value higher than 0.05 means that the lack of fit is not significant [29, 30].
- *Mandel's fitting test*: this test is used to compare between two models (one is linear and the other is nonlinear) in terms of linearity when the variances are similar. The first step is to calculate the residual standard deviation for both models [9]. Again, if $F_{calculated}$ is greater than $F_{tabulated}$, the linear model cannot be accepted.

iv. *Numerical assessment*: numerical fitting parameters are used as a measure of goodness-of-fit (GOF) in regression analysis. The following parameters are commonly used:

- *Correlation coefficient (r)* and *coefficient of determination (R^2)*: these two parameters are commonly used to express the GOF of a model. In general, R^2 is now more applicable compared to *r*, where the former measures the proportion of variance of the dependent variable being diminished by prediction of the independent variable, while the latter is just a measure for the correlation between the two variables. In general, a value of r/R^2 close to 1 is an indication for linearity [31].
- *Residual standard deviation (RESSD)*: the smaller the value of RESSD, the better the obtained fit. RESSD measures the digression of data away from a fitted regression line.

3.2. Schemes

As previously mentioned under steps and guidelines for a successful calibration, the first step is to decide on how many standards will be used for calibration? Usually, the most common approach is the use of more than one standard "multi-standard calibration." It is noteworthy to mention that the term standard can be described as "realization of the definition of a given quantity, with stated quantity value and associated measurement uncertainty, used as a reference" and in *NOTE 1A* "realization of the definition of a given quantity can be provided by a measuring system, a material measure, or a reference material" and in *NOTE 9* "The term 'measurement standard' is sometimes used to denote other metrological tools, e.g., 'software measurement standard'" [3]. Another term is usually used then to describe the measurement standard, which is *reference materials (RM).*

As per JCGM [3], RM is "material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties." The composition of RM would vary depending on the application. For example, substance RM has an individual pure component in solvent of use, compared to matrix RM, which consists of analytes prepared in a matched matrix. When RM is "accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures", it will be known as certified RM, CRM [3].

Several schemes are usually available to perform calibration depending on the number of used standards.

3.2.1. Multi-standard calibration

This is the most popular approach for calibration where a minimum of three standards is usually used. Different guidelines do have different specifications in this concern and in terms of replicates and the measurement levels (please see Section 3.1.1).

3.2.2. Two-standard calibration

This approach is usually used for investigations performed at a narrow concentration range and after the linearity of the employed function has been confirmed, probably as a continued calibration. It can be also used when the applied procedure has a background. As a condition, the analyte concentration needs to be within the range covered by the two standards.

The real [analyte] can be calculated using the formula: [anal] = [$std_{1\ or\ 2}$] + k ($y_{unknown} - y_{std\ 1or2}$), where the brackets express concentrations, k is the reciprocal of slope (sensitivity), and y is the response for the unknown and the standard, respectively [11, 12, 32]. Examples for this calibration are the pH meter and temperature sensor calibrations. A special scheme of a two-point calibration is known as bracketing calibration. In this approach, the [anal] is bracketed between the two standards assuming that a linear arithmetical interpolation can be proposed based on the knowledge of [$std_{1\ and\ 2}$]. The uncertainty associated with this approach is thus small if compared to the overall uncertainty [33, 34].

3.2.3. Single-standard calibration

As a direct calibration technique, this approach is applicable only when the calibration function linearity is established (especially in the region covering the [anal] and between the selected [std] and the origin) and if the graph intercept is zero [11, 12]. In this case, [anal] can be calculated using the calibration factor CF (which is the ratio between [std] and the average analytical response for the standard), where the unknown [anal] = CF*$y_{unknown}$. This simple calibration is generally used to test the drift from the response.

Multi-standard application then seems to be the most feasible and accurate scheme for calibration. However, this is not the case when, for example, the detector response varies with the time. In this case, the one-standard calibration is advantageous assuming that the unknown signal is within ±10–50% of the standard signal depending on whether the maximum analyte concentration limit has been surpassed or not [11, 12]. Depending on the analyte, availability of the standard, nature of the process, presence of concomitant analytes/interferences, and matrix effect, the procedure of calibration significantly varies and any of the previously reported schemes can be chosen.

3.3. Methodologies and manuals

While external and internal calibrations are the major themes, standard addition method (AC) and matrix-matched calibration (MMC) are also employed when required. Therefore, different methodologies for calibration can be proposed depending on how the RM will be applied within the course of calibration process. Through this section, emphasis will be basically on the CMPs, and the common methodologies usually followed to calibrate such a process.

3.3.1. External calibration (EC)

This approach is commonly known as "solvent/ standard calibration." As the name implies, EC is performed externally applied, i.e., the known standard solution, which is a substance RM prepared in the working solvent, is prepared and then analyzed distinctly from target samples. This approach can be applied using any of the previously mentioned schemes for calibration. The analysis protocol involves comparing the response for the unknown sample to the response for the target in the standard solution. One of the drawbacks of this methodology is the postulation that the impact of the difference between the matrices (standard and sample) can be ignored, an issue that leads to incorporation and propagation of a matrix systematic error. Nonetheless, this approach can be used when there is a minor or no contribution from the matrix effect and the instrumental drift can be ignored [11].

3.3.2. Matrix-matched calibration (MMC)

In contrast to the EC, MMC is used when the matrix has an impact on the response to the analyte. Both matrix RMs or substance RMs (together with an analyte-free matrix) can be employed for this approach. Attention should be paid that the matrix should be carefully matched. Again, the presence of analytes other than the target in the matrix could produce a matrix effect [11, 35].

3.3.3. Standard addition calibration (AC)

In this approach, known amounts of the analyte are added to aliquots of the test solution. Measurement is then performed by extrapolation of the calibration line to the zero response (no analyte). This approach can explain only certain types of the matrix effect; however; it cannot account for the effect of instrumental drift. Before the implementation of this method, the linearity of the calibration line should be confirmed over the whole concentration range. Moreover, the added concentration should be at least five times as high as the [anal] but within the linearity limits.

The actual [anal] is calculated using the equation: [anal] = CF $((y_{unknown}/y_{spiked} - y_{unknown}))$, where y_{spiked} and $y_{unknown}$ are the responses for the spiked and the unknown sample, respectively [11, 36].

3.3.4. Internal standard calibration (IC)

This approach is used to correct for both matrix effect and the drift over time. This technique is not the opposite to the previously mentioned EC; however, they can be used together. The matrix RM or as commonly known, internal standard (IS), which is structurally analogous to the analyte, is added for both unknown samples as well as the standards. The IS is selected in such a way that it can be distinguishably measured from the analyte. Moreover, there should be no interference between the IS and the analyte from one hand, and between the IS and the matrix of the unknown from the other hand. In addition to saving time and effort, the presence of the IS serves to compensate for sample loss during the preparation process [11]. The only limitation of this procedure is the availability of the ideal IS that can satisfy the previous conditions and emulate the matrix effect and the instrumental drift.

3.4. Metrological considerations

The product of the calibration scheme is usually portrayed as a mathematical model after performing the appropriate regression. Assessment of the proposed model depends on estimation of the experimental error which in turn affects linearity. Moreover, an important feature of the validation process which can be viewed as a direct calibration is the recovery studies. The concentration in the coming subsections will be on the metrological features of calibration in terms of error associated with the measurement and the recovery studies.

3.4.1. Uncertainty

As previously mentioned, the product of calibration is an experiential formula that relates the instrumental response to the analyte concentration. Thus, in other words, the actual value of

a measurement is equated with the experimental value. As a result, the uncertainty associated with the measurement needs to be determined. Principally talking about the systematic error of a measurement, and according to JCGM [3], it can be defined as "component of measurement error that in replicate measurements remains constant or varies in a predictable manner." NOTE 1 "A reference quantity value for a systematic measurement error is a true quantity value, or a measured quantity value of a measurement standard of negligible measurement uncertainty, or a conventional quantity value." As per NOTE 2 "Systematic measurement error, and its causes, can be known or unknown. A correction can be applied to compensate for a known systematic measurement error" and NOTE 3 "Systematic measurement error equals measurement error minus random measurement error."

For a linear calibration function generated from a multi-standard calibration approach using any of the methodologies of EC or IC, the linear regression line can be described by the equation: $y = ax + b$. This straight-line equation can be used to find an unknown concentration assuming that the response for this concentration is known. As the location of the regression line varies with the uncertainties associated with the regression parameters, a and b, the predicted concentration of the unknown would also be associated to uncertainty [37]. Metrologically, uncertainty of calibration is estimated using the following formula:

$$u(x_0) = \frac{S_{y/x}}{b}\sqrt{\frac{(x_0 - \bar{x})^2}{\sum_{i=1}^{n}(x_0 - \bar{x})} + \frac{1}{m} + \frac{1}{n}} \quad (1)$$

where $u(x_0)$ is the uncertainty associated with the unknown measurement, $S_{y/x}$ is the residual standard deviation, m is the number of replicates, n is the number of calibration points, $\bar{x}$ is the mean of x data points. It is noteworthy to mention that uncertainty associated with a measurement would be also sourced from the random error.

The *accuracy* and *trueness* are the terms used by majority of guidelines [1–5, 15–18]. However, there is a metrological difference between both terms. The term accuracy expresses how close an individual measurement to the real value of this measurement; however, trueness measures how close the mean of large number of values to the true value [3]. Thus, method trueness is measured as *absolute bias* or *relative bias*, which is expressed as % error and % relative error (%RE), respectively. Random error, however, affects the precision, which is calculated from the formula of standard deviation and in turn it affects the method accuracy [38]. Thus, uncertainty is affected by both bias as well as standard deviation.

3.4.2. Recovery

Generally, recovery investigations performed within the course of validation and following the calibration process can be treated as direct calibration of the proposed methods. Simply, the recovery is equal to = [found]/[actual]. It is important to declare that recovery outcome would differ per data point investigated and that the recovery value obtained at a certain value cannot be extrapolated to find the recovery at another data point.

For a linear function, the relation between recovered and actual analyte can be given as: [actual] = a [found]+ b, where a and b are the slope and the intercept, or the proportional and the additive errors, respectively [39].

4. Conclusion

Thousands of analyses and so validations are being performed every day. Calibration is a fundamental module of any analytical validation procedure. Different regulatory bodies propose different idioms and hence procedures for putting calibration in effect. Existence of a well-defined terminology for calibration and therefore a harmonized procedure would significantly improve the outcome of the analytical measurement. Appropriate selection of the calibration scheme and the subsequent methodology are the key factors for the success of analytical calibration. This chapter has outlined the process of analytical calibration in terms of appropriate designation (and considering the different releases by different documentary agencies), schemes (multi-, one-, and two-standard calibrations), and the operating manuals. Moreover, the metrological aspects of the calibration process have been revealed throughout the discussion with a focus on the recovery and uncertainties associated with analytical measurement.

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Chapter 3

Multivariate Calibration for the Development of Vibrational Spectroscopic Methods

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Additional information is available at the end of the chapter

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Abstract

Vibrational spectroscopy, namely near infrared (NIR) and Raman spectroscopy, is based on the interaction between the electromagnetic radiation and matter. The technique is sensitive to chemical and physical properties and delivers a wide range of information about the analyzed sample, but in order to extract the information, multivariate calibration of the spectral data is required. The main goal of this work will be to present in detail the available multivariate calibration strategy for development of NIR and Raman spectroscopic methods, which was successfully applied in pharmaceutics.

Keywords: multivariate calibration, vibrational spectroscopy, NIR spectroscopy, Raman spectroscopy, design of experiments

1. Introduction

The development and implementation of vibrational spectroscopic methods such as near infrared (NIR) or Raman spectroscopy has increased significantly as the use of computer technology and chemometric methods has become more available. Considering the pharmaceutical domain, these methods have been extensively applied to quantify active pharmaceutical ingredients, excipients, or physical properties either as offline method for intermediate/final product characterization [1] or as real-time-monitoring methods implemented within blending [2], granulation [3], extrusion [4], tableting [5], coating [6], or freeze-drying processes [7].

The high-throughput analysis associated with vibrational spectroscopy favored its application to gain better process understanding, sustaining the pharmaceutical product development from a Quality by Design and Process Analytical Technology point of view [8], thus enhancing the opportunity to develop well-understood, well-controlled, and continuously optimized manufacturing processes and products [5]. The nondestructive nature of vibrational spectroscopic

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methods is of great importance in the quality evaluation of production batches, as they allow the testing of a high number of samples or the entire process, depending on the type of method. Using classical methods, such as chromatography the quality of a 1–3 million tablet batch is certified on 20–30 tablets, and many functional excipients that directly influence product performance are not quantified. These limitations are exceeded by implementing process analytical instruments, such as NIR or Raman [9].

Near infrared spectra are generated by molecular vibrations that imply a change of the dipole moment (—CH, —NH, —OH, —SH) and are further complicated by overtones and combination bands that reduce the specificity of spectra. In case of Raman spectroscopy, the spectra are generated by inelastic scattering, caused by chemical groups that undergo a change in polarizability when excited with an incident light beam. These differences in molecular contribution to the generation of spectral data make the two methods complementary [10].

NIR and Raman spectra are considered a source of multivariate data, as they contain information related to physical and chemical properties of the analyzed sample. Thus, the application of chemometric methods for extracting predictive spectral variability and reducing orthogonal sources of variation is indispensable [11]. The sensitivity to both physical and chemical properties of the sample can be considered an advantage, if the analyst wants to predict several quality attributes of a drug product, such as content uniformity and crystalline structure. However, if only active content characterization is desired and polymorphism is not considered to be a critical attribute, but it is present, the calibration phase still has to include both aspects to ensure the accuracy of prediction for active content. The main disadvantage of vibrational spectroscopic methods relates to the need of an extensive calibration set that needs to include chemical, physical, instrumental, and environmental variability that is expected in future prediction sets and analysis conditions.

Vibrational spectroscopy is well suited to the means of multivariate calibration, as each observation is characterized by analytical signal/absorbance recorded at multiple wavelengths. Using multiple predictor variables instead of one wavelength overcomes some univariate calibration problems related to selectivity, precision, and diagnosis, resulting in a more robust calibration model [12].

2. Calibration set development strategies

The milestone in the development of a vibrational spectroscopic method is the chemometric model that is able to accurately predict the sample properties considered in calibration phase. Before building a model, there are several key steps that need to be considered, as they directly influence its quality and predictive performance. The first step would be the specification of responses along with variation ranges, followed by the selection of instrumental method and configuration, building a representative calibration set, recording of spectral data, data preprocessing, and developing the multivariate regression model that is further tested using external prediction sets. Each step plays an important role; however, a well-built calibration set is the best starting point to a well-performing model, as it is the source of spectral data that is used for further processing and model development.

In the calibration set development phase, the analyst has to incorporate the expected variability of future prediction sets, to ensure the representativeness of the samples. This expected variability is given first by the quality attributes that are to be predicted, for example, the concentration ranges of important formulation constituents. Frequently, this is not enough for a robust model, and other type of variability has to be included in the calibration process, such as process-induced variability or environmental variability. Production samples contain process-induced variability; however, constructing a calibration set solely on production samples is not appropriate as the factor ranges do not cover the required interval. A first option would be to prepare pilot-plant samples reproducing full-scale conditions. As the number of responses increases, the calibration set becomes larger and quickly becomes unfeasible due to the high costs of production. The second option would be to prepare laboratory samples in which the concentration ranges of desired components are varied simultaneously within appropriate ranges to avoid correlations [13].

The calibration set development strategy applied for the development of quantitative spectroscopic methods depends on the sample complexity (the number of responses and the number of interfering factors included in the calibration) and on the type of method that is developed, here considering off-line or real-time-monitoring methods. In the following section, a description of calibration opportunities will be provided starting from the simplest cases and heading toward more complex situations.

2.1. Different levels of the investigated property

The most simple calibration situations include a low number of responses, one or two, here considering a chemical and a physical property of a sample. In this case, the calibration set development strategy simply resumes to the preparation of a sample with different levels of the investigated property. Mbinze et al. developed quantitative NIR and Raman methods for the assay of antimalarial oral drops and prepared a calibration set by diluting a stock solution of quinine to obtain three concentration levels. For each level, three series with three replicates were prepared resulting in a calibration set with 27 samples [14]. Tomuta et al. used NIR to characterize meloxicam tablets by evaluating content uniformity, tablet hardness, disintegration, and friability. For content uniformity assay, the calibration set included active ingredient concentration range (five levels), days (three), and batches (three) as a source of variation, whereas in the case of physical properties assay the middle formulation was compressed on seven levels of compression force, ranging from 5 to 42 kN. Compressing the powder mixture with different forces yielded tablets with different hardness, disintegration, and friability. Different settings of a one-process factor were enough to induce variability in physical properties of the samples [15]. In a similar study, Tomuta et al. developed NIR method for physicochemical characterization of low active content indapamide tablets (2%, w/w) [1]. Virtanen et al. evaluated the crushing strength of theophylline tablets through Raman spectroscopy by considering both a process factor and a formulation factor to generate variability in tablet surface roughness. In this case, the tablets were prepared considering two particle sizes of theophylline, as raw material for the granulation phase, followed by mixing with lubricants and by compressing each granulate on five different compression forces [16].

The impact of polymorphism is a well-recognized phenomenon in the pharmaceutical industry, as the differences in crystalline structure of the same active ingredient generate different physical properties that get reflected in the quality of the final medicinal product. Croker et al. developed NIR and Raman methods to quantify FII and FIII of nootropic drug-piracetam from binary mixtures using a calibration set of 15 formulations with FII ranging from 0 to 100% [17].

Gómez et al. calibrated a Raman method for the content uniformity control of low-dosebreakscored acenocumarol tablets by under and overdosing the powdered commercial medicinal product, by adding either lactose or the active pharmaceutical ingredient to the mixture. Two commercial products with different content uniformity were considered and the two calibration sets included 7 samples in the range of 1–3% (w/w) and 12 samples in the range of 0.35–1.50% [18]. Creating calibration sets by under-overdosing samples can result in correlated concentrations between API and excipients [19]. Collinearity between concentrations leads to spurious predictions by attributing changes to the correlated formulation component instead of the real contributor [20].

Changing the production scale generates samples that incorporate different types of variability from the primary conditions through which the calibration set was prepared. As laboratory-prepared samples lack manufacturing variability, the accuracy of prediction may be affected for production prediction sets. This limitation has been exceeded by extending the calibration set with production samples [13], adjusting the sampling strategy, pre-conditioning the calibration set to future expected environmental conditions [21], or by mathematically adding process variability to laboratory samples [20].

Blanco et al. developed NIR methods to control individual steps of paracetamol tablet manufacturing, resuming to an intermediate granulation step and tableting. Prior to building a calibration model, both laboratory-prepared samples and industrial production samples were taken into account to evaluate the eventual spectral differences. In case of the granule-active content assay, the calibration set was built solely on laboratory-prepared samples, whereas in the case of tablet assay the differences between laboratory and production samples made the calibration set include both, in order to ensure representativeness. For granule particle size characterization, samples collected over a period of 2 years ensured the presence of future expected variability in prediction set [22].

Blanco et al. used NIR to characterize mirtazapine tablets in terms of content uniformity and tablet hardness. For active ingredient content, the calibration set included production tablets from 20 batches and 34 laboratory-prepared samples, whereas for tablet hardness the laboratory samples were compacted in the range of 300–740 MPa. Including production samples for both responses reduced the systematic errors and gave better predictions [13]. By adding spectra from different manufacturing scales to the calibration set, the spectral variability becomes more representative, an important aspect for prediction accuracy. As the number of manufacturing samples is lower compared to the initial calibration set, proper weighting is necessary to avoid the dominating tendency of the larger dataset. To this regard, Farrel et al. applied Tikhonov regularization as a multi-criterion-based weighting selection method to augment the performance of NIR models regarding their ability to predict production scale products [23].

Blanco et al. proposed a method to incorporate physical variability that originates from production into the calibration. The concept relies on calculating a process spectrum, which added to the laboratory sample spectra incorporates process-related physical changes. The process

spectrum represents the difference between the laboratory sample spectrum and the intermediate/final product spectra of an identical composition prepared on a different scale. The variability given by the process spectra can be further increased by multiplying the data with different coefficients [20, 24].

In situations where solid-state transformations occur within the manufacturing process, it is frequently desired to construct the calibration set with components obtained through the same method to have more representative formulations. Netchacovitch et al. used Raman spectroscopy to determine crystalline itraconazole in amorphous solid dispersions prepared by hot-melt extrusion. Calibration set included three levels of concentration and was built by using crystalline API powder, six batches of grinded extrudates with amorphous API, and placebo-grinded extrudate [25].

Pan et al. calibrated NIR method for the quantification of low-level Irbesartan Form B from pharmaceutical tablets. Form B is known to have a limited solubility and is formed from Form A via a solution-mediated process. To incorporate physical variability into the calibration set, the sample preparation procedure supposed the use of specifications similar to the manufacturing process. The robustness of the method to process induced physical variability, the effect of tablet hardness, granule size, and atmospheric humidity was evaluated. It was demonstrated that the prediction accuracy was influenced only by relative humidity, generating a positive bias in samples stored at 50%RH. Therefore, the entire calibration and validation was reconsidered by pre-conditioning the samples at 25°C and 50%RH for 20 h, prior to recording the spectra and building the model. This way, the robustness of the method was increased to future expected variations in environmental conditions [21].

2.2. Design of experiment strategy

As the number of factors increases, the calibration set becomes more complex and different strategies have to be applied to avoid correlated responses. If two formulation components C1 and C2 are correlated, a change in the concentration of C1 can be spuriously predicted as a change in C2. In DoE, factors are varied simultaneously in a systematic manner, providing orthogonality, an essential condition for estimating regression coefficients [26]. There are several design types that can be used for calibration purposes, starting from the classic full factorials down to central composite, mixture, or D-optimal designs. Considering more complex formulations, NIR spectroscopy has been applied to determine the amount of amoxicillin in the presence of seven other excipients. By applying a three-factor (API, saccharose, and other excipients) experimental design, the concentration of factors was varied orthogonally [27]. Ferreira et al. used a calibration set prepared according to a DoE with three factors: hydrochlorothyazide, cellulose, and other excipients to train a NIR method for the quantification of the active ingredient in pharmaceutical samples [28].

Li et al. calibrated Raman method to quantify active ingredient content considering the presence of different sources of variability: degradation compound, relative humidity, change of scales, and compression force. Laboratory samples were prepared based on a 3^2 full-factorial design where the active ingredient ranged between 80 and 120%, from which a subset of samples were spiked with the degradation product, added in two molar ratios. Each powder mixture was compacted at 8 and 30 kN in laboratory scale and three design points were compacted at manufacturing scale [29]. Casian et al. developed NIR and Raman methods for

the quantification of two APIs found in significantly different concentrations from immediate release tablets. The calibration set was built on a full-factorial design with two factors and five levels with a total of 25 formulations [10]. The use of full-factorial designs is feasible with two factors if five levels of variation are used [52]. Adding one more factor will generate 125 experimental runs that are impractical [26, 53].

Netchacovitch et al. calibrated a Raman method to quantify low-level polymorphic impurities in a pharmaceutical formulation through a 12-run central-composite experimental design [25]. Central composite designs are extensions of the two-level full-factorial designs that are built by adding symmetrically axial points. Dependent on the position of axial points, factors can be varied on three levels (central composite face-centered design) or five levels (central composite circumscribed) [26].

Short et al. used NIR to evaluate relative density and crushing strength of four component tablets. Compared to other studies, where only the compaction pressure was considered as a factor to induce variability in the investigated response, in this case formulation composition was varied also. The calibration set consisted of 29 formulations (mixture design) with each formulation being compressed at different pressures [30]. Lyndgaard et al. developed a Raman method to quantify paracetamol content from tablets through blisters. The calibration set included 18 formulations, selected on the basis of a ternary mixture design (paracetamol, starch, and sucrose) with each factor being varied on six levels [31]. Igne et al. evaluated the effect of API physical form, excipient particle size, different manufacturer, and changes in environmental conditions on the performance of a NIR model. The calibration samples were prepared according to a 29-run quaternary mixture design with every formulation being compressed at two of five different forces. Only changes in the particle size of lactose produced biased predictions in both ambient and chamber conditions. The authors tested variable-selection methods to increase method robustness to raw material variability [32].

Griffen et al. used Raman spectroscopy to quantify all tablet constituents, three active ingredients and two excipients. In this case, the calibration set was built on a first-order (linear) five-level, five-factor mixture design that uniformly covered the concentration ranges of the components. The concentration of individual components ranged from 1 to 85% (w/w) [33]. Mixture designs are well suited for formulation application, where the sum of all ingredients adds up to 100% and where factors cannot be manipulated independently one from another. Porfire et al. used a D-optimal design with three variables and five levels to build a calibration set with 63 formulations with the purpose of quantifying encapsulated simvastatin and two functional excipients L-α-phosphatidylcholine and cholesterol from liposomes [34]. Saraguca et al. developed an NIR method to simultaneously quantify paracetamol and three other excipients from powder blends using a calibration set constructed on a 40-run D-optimal mixture design [19].

A D-optimal design is frequently applied for a high number of factors as it gives a lower number of runs compared to factorial designs. The D-letter originates from its criterion of selecting the best subset of factor combinations from a pool of theoretically possible combinations, which relies on maximizing the $X'X$ matrix Determinant [26]. In another study, Heinz et al. trained NIR and Raman to quantify ternary mixtures of alpha, gamma, and amorphous forms of indomethacin from ternary mixtures using a 13-sample calibration set built on a cubic model experimental design [35]. Lin et al. developed an at-line blend uniformity NIR method for simultaneous

quantification of four active ingredients with structural similarity, found in different concentrations. Calibration was built on six formulations, where five factors (four APIs and one diluent) were varied on six levels while avoiding correlations. The performance of the model was improved by adding a set of spectral data from a different production scale [43].

When DoE is used, correlations are significantly reduced dependent on the type of design, number of factors, and experimental runs. However, an increased number of factors will require a high number of experimental runs to avoid collinearity, which rapidly increases the costs. Several papers have addressed the question of how many samples are needed to ensure a robust calibration [19]. The fact that models with similar performance were developed on a reduced design compared to its full-factorial counterpart suggests the presence of redundant information in full-factorial designs [36].

Saraguca proposed a method that relies on building the model on a limited number of samples and uses the remaining formulations to test the predictive performance in terms of RMSECV and RMSEP. In the following steps, the calibration set was extended by transferring one formulation at a time from the test set until the calculated cross-validation and prediction errors stabilized. The sample selection procedure focused on maximizing the concentration variability of all components [19].

Alam et al. proposed a method for calibration set development in spectral space instead of concentration space. Orthogonality in spectral response will yield a better estimation of coefficients with a minimum number of samples, while orthogonality in concentration space will not necessarily translate into spectral orthogonality, as the contribution of each component to the sample spectrum is different. The method is based on decomposing the pure component spectra of a formulation into orthogonal directions (scores), which will be varied around a model tablet score through DoE. The model tablet score represents the score of the spectra recorded on a target formulation projected onto the orthonormal basis vector of the pure components spectra. After designing the spectral space calibration set, the composition of each spectra is retrieved by mathematical means [37].

2.3. Calibration strategy for calibration *in-line* monitoring methods

The application of vibrational spectroscopy for in-line monitoring implies the use of fiber optic probes mounted at the interface of the process itself to acquire spectral data with a defined rate. The simplest way to calibrate an *in-line* method is to acquire real-time spectra through the entire process length along with collecting samples at regular intervals. The response values obtained through reference methods are correlated with the spectral data, considering the process time as a link between the two [38–40]. More extensive calibrations also evaluate the effect of sample presentation, changing process, and formulation parameters, to challenge the robustness of the methods.

For coating application, the calibration strategy relies on the linear variation of spectral response as the contribution of the coating material increases and the tablet core contribution decreases [41]. Moes et al. developed quantitative NIR method using three batches of tablets by varying the tablet core weight (240–200–160 g) and the amount of coating suspension resulting in different coating thicknesses [42]. Möltgen et al. used five full-scale experimental

runs to develop a quantitative NIR method (one run) and to evaluate the effect of changing exhaust air temperature and spray rate (two runs) and the effect of tablet density and flow motion in the coater (two runs). For quantitative calibration, samples were collected through the entire process and analyzed using reference methods [6]. For the quantification of coating thickness by means of Raman spectroscopy, Kauffman et al. calibrated the method by considering film thickness and film composition variables. Tablets were coated on five levels ranging 0.5–6% weight gain by varying their residence time in the coater. As for film composition, three different TiO_2 levels were evaluated due to the strong Raman signal of this component offering the potential for an indirect measure [41]. In the case of thin coatings, the generation of a calibration set can become a difficult task and can become limited due to the lack of reference methods. In this situation, an alternative to classical regression methods would be the Science-Based Calibration (SBC) approach, which allows the calibration without a reference method by separating spectral variability into orthogonal (covariance matrix) and predictive parts (related to the coating). Möltgen et al. applied SBC to develop quantitative NIR method for in-line evaluation of thin hydroxypropyl methylcellulose (HPMC) coatings through four experimental runs. For calibration, the pure HPMC spectrum was used as the coating response spectrum and the covariance matrix included hardware, core, water, and process-related noise. The method developed without reference samples predicted accurately coating thickness values in the range of 8–28 μm demonstrating the value of SBC [43].

In order to predict granule moisture content in a six-segmented fluid bed dryer through NIR spectroscopy, a calibration set of 20 experiments was applied. Granules were prepared with five moisture levels by varying the drying air temperature and drying time. Each moisture level had four replicates prepared on two different days [3].

Clavaud et al. developed a global regression model for moisture content estimation from freeze-dried medicine. As expected, the calibration set was extensive, including three types of active ingredient with different concentrations, different vial diameters, and excipient amounts. To include intra- and inter-product variability, 5 batches and 100 samples were used for each product [44]. Martinez et al. calibrated NIR method for *in-line* quantification of two active ingredients in a batch-blending process by investigating the influence of sample presentation. With regard to this, the high-loading API was used either in the form of a cohesive powder or in a granular form prepared by melt-extrusion. The observed spectral differences were resumed to the polymer wavelength absorption band that coincided with the water region. The offline calibration of the method was built on 13 samples which included both forms of the high-loading API [2].

Wahl et al. evaluated *in-line* the content uniformity of ternary mixtures with an NIR mounted on the feed frame of a tablet press. For calibration, the active ingredient and two excipients concentrations were varied through eight experiments selected by means of a D-optimal design and two extra runs added to ensure equidistant steps in the content of each component. Spectral data were recorded in a dynamic acquisition mode, simulating real conditions [5].

Karande et al. developed NIR method for real-time monitoring of tableting based on a 105-sample calibration set generated through a simplex lattice design with four factors (chlorpheniramine maleate, lactose, microcrystalline cellulose, and magnesium stearate). Prior to building the calibration, the effect of sampling was evaluated by recording NIR spectra in both static and dynamic conditions. The differences between measurements revealed the importance of

ensuring similar sampling conditions for calibration as for actual real-time monitoring [9]. For another application, Karande et al. evaluated the effect of different spectral-sampling strategies on the performance of an NIR model, to accurately predict blend components in quaternary mixtures. Calibration samples (24 formulations-D-optimal mixture design) were recorded in three ways: laboratory mixing and static spectral acquisition; IBC (intermediate bulk container) mixing and static spectral acquisition; IBC mixing and dynamic spectral acquisition. Dynamic sampling yielded the best calibration model with highest accuracy, demonstrating the importance of selecting similar sampling conditions to the actual testing [45].

Based on the presented examples found in literature, the most frequently applied methods to design a calibration set were as follows:

- One chemical/physical property: formulations with three to five levels of variation for the response that span the desired range of concentration/physical property.
- One chemical and one physical property: formulations with three to five levels of variation for the chemical response and for the physical property calibration are considered only for target formulation (five levels).
- Two chemical/physical properties: any type of DoE (full-factorial, central composite, mixture design, D-optimal) to avoid collinearity and spurious predictions.
- Three chemical/physical properties: simple lattice mixture designs or D-optimal designs.
- In-line methods: models built by correlating sampled product properties with in-line collected spectra. Most rigorous studies also investigated the effect of process parameters on the NIR spectra.

3. Handling chemical, physical, and environmental interferences

The dependence of the NIR spectra on the sample's chemical and physical properties caused by absorption and scatter effects can be an advantage of this type of spectroscopy, but at the same time, the scatter effects caused by sample variations or even by environmental phenomena can create a series of analytical problems. In such cases, each type of interferences has to be considered in the calibration model development. In the following section, the importance of chemical, physical, and environmental interferences will be described, providing insights on specific spectral variations produced by each category and highlighting how to handle them in order to increase model robustness [1, 2].

Generally, a quality NIR analysis should provide a model that manages a correct interconnection of the spectral variables with the samples properties of interest. At the same time, an ideal calibration model will not react to instrument variation, environmental changes, background interferences, and will be mostly focused on the information of interest. Chemometrics is the science that enables the extraction of relevant information, as well as the reduction of unrelated information as well as interfering parameters.

Spectral interferences resulting from variable physico-chemical sample properties (e.g., particle size variation and moisture content) or instrumental effects (e.g., path-length variation,

light scattering, and random noise) can be reduced, eliminated, or standardized by using spectral pretreatments, prior to the multivariate data analysis [3]. Since the correct selection of spectral pretreatment can significantly improve the reliability of the model, this topic will be discussed in the following paragraphs. The most common pre-processing techniques can be divided into two groups: pretreatments for spectral normalization and for smoothing/differentiation. The first group achieves spectral normalization through scatter-correction methods. Scatter effects are common for all spectroscopic techniques and the phenomenon appears mostly because of the physical variabilities between samples or path-length variations. Two of those pre-processing concepts are standard normal variate (SNV) and multiplicative scatter correction (MSC) which also normalize the baseline shifts of different samples [4, 5]. The second set of pre-processing methods has the capacity to reduce or remove the noise by smoothing and differentiating the spectral values. The most common spectral derivatives are based on the Savitzky-Golay (SG) [6] and the Norris-Williams algorithms [7].

In most cases, in order to obtain best results, there is the need to apply both types of pretreatment techniques one after the other. Peeters et al. tested both types of pre-processings not only to reduce light scattering effects but also to minimize peak shifts of Raman and NIR spectra. They applied SNV, MSC, and first and second derivatives obtained by calculating 15-point quadratic Savitzky-Golay filters, in order to develop a method for the off-line prediction of tablet properties [8]. Sylvester et al. developed an in-line NIR-monitoring method for a freeze-drying process using the SNV pre-processing in order to remove multiplicative interferences caused by scatter and particle size variations and the first Savitzky-Golay derivative to reduce baseline shifts and to improve the spectral resolution [9]. The successful development of a real-time method for monitoring continuous powder flow from a tableting machine feeder was described by Alam et al. Savitzky-Golay derivatization was first applied for smoothing, followed by SNV for scatter correction [10]. Environmental interferences can be caused by sample, instrument, or even laboratory variations; this type of interferences causes misalignments or shifts of the spectra and is commonly overcome by applying alignment/warping techniques to the data [3]. Those methods stretch or compress the signal in order to match it in the best way possible with a given reference spectra [11, 12].

All pre-processing methods have the purpose to reduce the undesirable variability and interferences from the data, but there is always a risk of choosing an inappropriate type or applying a severe pre-processing that would also remove valuable information. Because of this, choosing the correct technique is one of the most important steps in data pre-processing and model development.

A last useful solution to deal with problems caused by interferences is wavelength selection method. The model development can be done based on the specific spectral domain that contains the information of interest. In order to select the domain of interest or to eliminate irrelevant wavelength domains, principal component analysis (PCA) can be performed. Prior to the PCA, the collected spectra should be pre-processed and column centered, then the analysis can be performed on the data matrix. Finally, the variables should be selected according to high peak loadings obtained for all relevant principal components (PCs), and the position of the resulting features should be compared with the original spectrum to validate the selection.

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4. Data pre-processing

During the development of a multivariate calibration model, systematic variation such as baseline shifts and scatter effects, not relevant for the prediction of the response variables (Y), is present in predictor variables (X). Pre-processing methods are used in order to remove the systematic variation not related to the Y-matrix, which might impair the interpretation or predictive ability of the developed model.

The main goals of data-pre-processing are the following:

a. improvement of the robustness and accuracy of subsequent analyses;

b. improved interpretability: raw data are transformed into a format that will be better understandable by both humans and machines;

c. detection and removal of outliers and trends; and

d. reduction of the dimensionality of the data mining task and removal of irrelevant and redundant information [46].

The methods generally used for data pre-processing are divided into two categories. The first consists of classical pre-processing methods, used for normalization, smoothing, and differentiation. The second is represented by methods for variable selection and dimensionality reduction [47]. Among these methods, the most appropriate has to be chosen, such as to only remove unwanted variation, without excluding or altering chemically relevant information [48].

When used in an inappropriate way, pre-processing may introduce artifacts or cause loss of information. Thus, the purpose of the analysis is important for the selection of the most appropriate pre-processing method, because scattering is disruptive for compound identification and quantitation, but is useful to study the physical properties of the sample. As a consequence, the best pre-processing method, ensuring a correct data analysis and robust results, has to be chosen by testing and comparing the results of different methods [48].

4.1. Pre-processing methods

4.1.1. Spectral normalization

In many analytical methods, the variables measured for a given sample are increased or decreased from their true value by a multiplicative factor, which is called the scaling or gain effect. In spectroscopic methods, the scaling effect arises from path-length effects, scattering effects, source or detector variations, so the relative value of variables should be used during multivariate modeling rather than the absolute measured value. The sample normalization is one of the most important pre-processing methods, which is applied in an attempt to correct for multiplicative scaling effects, the shifts and the trends in baseline and curvilinearity, by identifying some aspect for each sample which should be essentially constant from one sample to the next, and correcting the scaling of all variables based on this characteristic [48].

Normalization methods can be subdivided into two main groups: simple normalization methods (min-max normalization, one-norm, vector normalization, standard normal variate),

requiring only the information from the spectrum to be normalized, and normalization methods requiring the presence of collective spectral data matrices or of reference spectra (multiplicative scatter correction and extended multiplicative signal correction (EMSC) [46]. Among these, the most used scattering correction algorithms include the SNV and MSC. The two pretreatments give similar results, being considered exchangeable, but the results obtained through both algorithms are compared usually, since they may be different [49]. SNV was proposed to reduce multiplicative effects of scattering, particle size, and multicollinearity changes over the NIR spectra. This approach starts with mean centering and consists of dividing mean-centered spectra by the standard deviation over the spectral intensities [50]. SNV normalizes each spectrum returning a mean of 0 and a variance of 1 spectra dataset [48]. The disadvantage is the assumption that multiplicative effects are uniform over the whole spectral range, so artifacts may be introduced by this transformation.

The de-trend method is another approach to correct for baseline shift, which removes the baseline curvature by expressing it as a quadratic function of the wavelengths. The modeled baseline is subtracted from the spectrum, so de-trend can be used after SNV to circumvent any curvilinear trend, where the baseline drift is a function of wavelength [50]. The MSC pretreatment performs a linear regression of each spectrum on a reference spectrum, which is usually the mean of all available spectra, for example, the average spectra of the calibration set, or a generic reference spectrum can also be applied [49].

4.1.2. Smoothing and differentiation

The smoothing algorithms are used in order to correct the spectral noise, while differentiation is used to enhance spectral resolution and to eliminate background absorption. The most common ways to achieve smoothing are the use of noise filters for de-noising and smoothing and Savitzky-Golay smoothing/derivative filters for smoothing/resolution enhancement. Noise filters are specific low-pass filters which can be used to reduce random noise. Their drawback is that the signal-to-noise ratio is increased at the expenses of distorting the signal. The most popular smoothing filters are the zeroth-order SG-smoothing/derivative filter, the binomial filter, and the moving average filter [46].

Derivatives are used for their capability to remove both additive and multiplicative effects in the spectra. The first derivative removes only the baseline; the second derivative removes both baseline and linear trend. The first derivative is estimated as the difference between two subsequent spectral measurement points, while the second-order derivative is estimated as the difference between two successive points of the first-order derivative spectra [51]. The most popular derivation method is SG algorithm, proposed by Golay and Savitzky in 1962 [52]. The method has the advantage that computation of the derivatives and smoothing are carried out in a single step. The algorithm used in this method is based on fitting a polynomial in a symmetric window on the raw data, in order to find the derivative at the center point. The parameters of the polynomial are calculated and the derivative of this function is found, this value being used as the derivative estimate for this center point. The same operation is subsequently applied to all points in the spectra. Two decisions are important to be made in this algorithm, i.e., the window width (width of the subset of the data) and the fitted

polynomial order. The highest derivative that can be determined depends on the degree of the polynomial used during the fitting [51].

4.1.3. Dimensionality-reduction methods

These methods rely on reducing the dimension of the predictor space spanned by a number of variables or wavelengths, in order to find the subspace mainly containing variations related to the response matrix. The orthogonal projection and the variable-selection methods are in this group. Orthogonal signal correction (OSC) and its modified version direct orthogonal signal correction ((D)OSC) are the most common among this group, developed to remove systematic variation in the descriptor matrix, that is not correlated to the response matrix. In other words, the pre-processing is performed in such a way that the removed parts are orthogonal (not linearly related) to the response matrix [53, 54]. The method has the advantage of correcting at once multiple artifacts.

An alternative OSC algorithm was developed by Trygg and Wold and is called orthogonal projection to latent structures (OPLS). The objective of OPLS is the same as of OSC, but the approach is different, i.e., the OPLS method analyzes the variation explained in each PLS component. The non-correlated systematic variation in descriptor matrix is removed, making interpretation of the resulting PLS model easier, and the non-correlated variation can be analyzed further [55].

Variable-selection techniques consist of selecting particular variables related to the response, instead of removing the interference modeled as a spectrum, the aim being to identify a subset of wavelengths that produces the smallest possible error [56]. Selecting the most correlated wavelengths may lead to better performance in PLS and PCR, but, on the other hand, selection of the most correlated wavelengths may eliminate those that correct for the influence of interfering compounds or factors [56].

4.2. Pre-processing strategy

In practical applications, combinations of pre-processing methods are usually employed in search for the best algorithm, involving more than one pre-processing step. According to Rinnan et al., several rules may serve as guidelines: scatter correction (except of normalization) should always be performed prior to differentiation; normalization can be used at both ends of the correction, but usually is easier to be assessed if it is done prior to any other strategy; MSC gives a smaller baseline correction than SNV with subsequent de-trending; it is not recommended to perform de-trending followed by SNV [51].

The ideal pre-processing strategy should only remove artifacts present in the data, without introducing any unwanted artifacts or variability in the data. When physical properties, that is, tablets' crushing strength, are evaluated through vibrational spectroscopy, typical pre-processing methods such as SNV, MSC, and the derivatives cannot be used, because they lose the baseline-shifting information, which is relevant for the physical properties. The data in this case should be modeled as such or after normalization [16]. Three approaches are described in the literature, for the selection of the most appropriate strategy: the trial-and-error approach; visual inspection and the use of data-quality parameters [57].

In the trial-and-error approach, all pre-processing methods are applied to the data and the pre-processed data are used as an input to a calibration model, which is further used to assess the quality of the pre-processing strategy by an internal measure, such as RMSEP or RMSECV [57]. For example, Karande et al. chose among various pre-processing methods through comparing the figures of merit (explained variance, R^2, RMSEC, and RMSECV) of the developed partial least-squares (PLS1) regression models, for the quantification of micronized drug and excipients in tablets by NIR spectroscopy. The raw calibration spectra were pretreated with SNV followed by first derivative and SNV followed by second derivative pre-processing. All models were developed using the entire spectral range or narrow spectral ranges. The best performance of the calibration method (highest explained variance, lowest RMSEC and RMSECV) was obtained using the whole spectral range, pretreated with SNV followed by first derivative spectral pre-processing [9] . The same approach has been used by Porfire et al. in the attempt to select the best pretreatment method in the development of calibration models for prediction of chemical composition and crushing strength of sustained-release tablets with indapamide. PLS regression was performed for non-processed spectra as well as for spectra treated by various pre-processing methods (i.e., FD, SD, SNV, MSC, FD + SNV, FD + MSC), and the most suitable pretreatment algorithm was chosen based on the results obtained for PLS model validation through cross-validation, i.e., based on its RMSECV and bias [58].

In visual inspection method, the effect of pre-processing is assessed before a model is constructed. Thus, because artifacts have been removed during pre-processing, samples should show more spectral overlap after pre-processing in visual inspection, and differences between groups of samples should be more pronounced. However, as visual inspection may be very difficult and not objective, the data are not usually inspected in "spectral mode" but in a lower dimensional space, obtained usually through principal component analysis [57]. PCA reduces the dimensionality of the problem by generating linear combinations of the original variables returning new "latent" variables. Each original variable is weighted by a loading representing the importance of the considered variable on the variance of the data. The variability of the data is expressed by new dimensions called principal components, and the projection of a pixel onto the PCs is called its score. The result of PCA is the decomposition of the pre-processed matrix in a score matrix and a loading matrix [48]. PCA is used for data overview, for example, for detecting outliers, groups, and trends among observations, for evaluating relationships among variables, and between observations and variables. In PCA, data in the matrix X are transferred into a new coordinate system defined by principal components. The direction in variable space occupied by the most varying data points will define the location of the first PC, and the second PC will be given by the largest variation orthogonal to the first component. PCs are extracted until only minor variation is left unexplained by the PC model, each component consisting of a score vector and a loading vector. Observations close to each other in a score plot have similar properties, and variables close to each other in a loading plot are correlated. Thus, the score plot is useful for the detection of strong outliers, clustering, and time trends [59] .

The detection of strong outliers through PCA is done by analyzing the score plot. The strong outliers are removed, as they may have a degrading impact on model quality. A statistic tool called Hotteling's T^2 may be used in conjunction with the score plot for the detection of strong outliers. This tool is a multivariate generalization of Student's t-test, defining the

normal area corresponding to 95 or 99% confidence. Subsequently, for a better understanding of the properties of grouped data, a splitting of data into smaller groups according to the nature of the clustering is done, and separate PCA models may be fitted. For the detection of weakly deviating observations (moderate outliers), which are not strong enough to show up as outliers in score plots, the residuals of each observation are used. The detection tool is called DmodX (a notation for distance to the model in *X*-space). A value of Dmodx is calculated for each observation, and the values are plotted in a control chart where the maximum tolerable distance (Dcrit) for the dataset is given. The plot of DmodX enables an overview of the unsystematic process variation, as moderate outliers have DmodX values higher than Dcrit [59].

Before PCA, scaling of data is usually performed, because variables have different numerical ranges so they will have different variance and they will weight differently in the data analysis. The most common approach is the unit variance (UV) scaling, consisting in dividing each variable by its standard deviation. The result is that each variable has equal variance, meaning that the "length" of each variable is identical, although the mean values still remain different [59].

Tôrres et al. used Hotelling's T^2 chart to analyze the NIR spectra of a training (calibration) set for the development of a monitoring method for the stability of captopril in tablets. Before being analyzed by PCA, NIR spectra were smoothed as described by Savitzky-Golay with a 21-point window and second-order polynomial and were processed by MSC for the correction of baseline variation due to non-homogeneity of particle's distribution [60]. The Hotelling's T^2 chart measures the distance from an observation to the center of the samples under normal operating conditions and evaluates whether a particular sample has a systematic deviation from the samples considered to be under statistical control [61]. As all samples from the training set were assumed to be normal, the training chart was not expected to identify systematic deviations in these samples in the training phase, so the number of PC retained in the model was selected to minimize the number of false alarms (false positives and false negatives) during the training phase of the control charts [60].

5. Regression methods

Regression analysis is a modeling technique used to investigate the relationship between dependent variables (responses or *y*'s variables) and independent variables (predictor, factors or *x*'s variables). According to the number of variable, three cases can be distinguished:

1. Simple linear regression—one *y* and one *x* variable.
2. Univariate linear regression—one *y* and several *x's* variables.
3. Multivariate linear regression—several *y's* and several *x's* variables [62].

The objective of a regression method can be achieved by means of a model where the observed result (dependent variables, response, *y*'s variables) is described as a function of independent variables (*x*'s variables) and the noise is left as residual.

In a regression analysis, the relationship between two data matrix X (BxK) and Y (NxM) are related to each other. A regression model can be written as in a matrix form as

$$\mathrm{Y} = \mathrm{XB} \tag{1}$$

where Y is the matrix of x's variables; X is the matrix of y's variables; B is the matrix of regression coefficient, B(KxM).

A good estimate of regression coefficient (B matrix) provides a good fit to Y and good prediction of future unknown parameters y^{T}. More, the regression coefficient vector should be of mechanistic understanding and interpretable [59, 63, 64].

A large number of regression methods were developed, all with the goal of finding the best estimation of B. In the calibration of spectroscopic methods, only multivariate regression techniques can be applied, and the most used are (1) multiple linear regression (MLR), (2) principal component regression, (3) partial least-squares regression, and (4) orthogonal partial least-squares regression (O-PLS). In the last years, some advanced regression methods as (5) Bayesian ridge regression (Bayes-RR) (6) support vector regression (SVR) or (7) decision tree regression (DTR) have started to be used.

5.1. Multiple linear regression

Multiple linear regression is an extension of simple linear regression model. In the case of MLR determination, the relationship between x's—variables and y's—variables is achieved by means of a model where the responses (y's—variables) are described as a function of analyzed factors (x's—variables) and the noise is left in the residual (ε) (Eq. (1)) [65]

$$y = f(x_1, x_2, x_3 \ldots, x_n) + \varepsilon \tag{2}$$

The function f is approximated by a polynomial equation (Eq. (3)),

$$y = b_0 + b_1 x_0 + b_0 x_2 + \ldots + b_0 x_n + \varepsilon \tag{3}$$

where bi (i = 1, 2, 3, …,n) are the regression coefficients and describe the effect of each term on the response y.

The polynomial equation (Eq. (3)) can be written in matrix way as follows:

$$y = Xb + \varepsilon \tag{4}$$

where X are the matrix of x's variables and b the vector, and the multiple linear regression is used to determinate vector b.

If there are orthogonalities between x's variables, Eq. (4) can be written as

$$b = \left(\mathrm{X}^T\mathrm{X}\right)^{-1}\mathrm{X}^T y \tag{5}$$

In this equation, matrix $X^T X$ become a diagonal matrix and b is easily calculated.

If not all the x's variables can be controlled, the number of x's variables extends the number of experimental runs or the number of experimental runs is larger than the number of x's variables, the co-linearity between x's variables arises and the orthogonality no longer exists, so the inverse of X^TX cannot be applied.

Except the cases when the calibration of spectroscopic methods is performed following the design of experiment strategy in the other multivariate calibration, the orthogonalities do not exist and the MLR cannot be applied. That is the reason why other regression methods based on latent variables as partial last squares are preferred and become popular. When the calibrations are performed based on latent variables, inside of using the original variables in the regression, a new set of orthogonal (latent) variables is calculated and leads to reduction of the original dimension of x's variables matrix and performs the least-square estimation.

5.2. Principal component regression

Principal component regression is a regression method based on principal component analysis and it is used when datasets are highly collinear. In a PCA regression, the original set of collinear variables is transformed to a new set of correlated variables. So, the principal component analysis is used to decompose the x's variables into a principal component (orthogonal basis) and a subset of components in order to predict y's variables. The basic idea of the principal component regression is to calculate the principal components and then use some of these components as predictors in a linear regression model fitted using the typical least-squares procedure [66, 67].

In the case of PCR determination, the relationship between x's variables and y's variables is achieved by means of a matrix of lower dimension (TP^T), called principal components, and a matrix of residuals (E).

$$X = 1\overline{X} + TP^T + E \tag{6}$$

where $\overline{X}$ contains X average; T is a matrix of scores that summarizes the X variables; P is a matrix of loadings showing the influence of the X variables; E is a matrix of residuals (the deviations between the original and the predicted values) [66].

The main idea of principal regression is to replace X matrix of row date to a smaller orthogonal score—loading matrix (TP^T matrix) that summarized the original X matrix, and then to relate the T-scores to y's variables.

The core of PCR is that a small number of principal components is enough to explain the variability into the data. In most of the cases, it might be found out that four to six principal components are enough to explain more that 90% of the variance into the data.

5.3. Partial least squares

The partial least-squares regression is the most popular method for the creation of models used in the development of NIR and Raman spectrometric methods and is used to develop a linear link between two matrices, the NIR/Raman spectral data and the reference values.

The PLS approach was first proposed by Herman Wold around 1975 for the modeling of complicated datasets in terms of chains of matrices, the so-called path models. PLS regression is preferable to develop calibration models because unlike MLR, it can analyze data with strongly collinear, noisy, and numerous *X*-variables, and also simultaneously model several response variables [68]. PLS was developed for situation in which the data have more independent variables than observations (the "small n, large p") or/and where collinearity is present among dataset [69].

The PLS finds a multivariate model (linear or polynomial) that describes the relationship between *Y* matrix (dependent variables) and *X* matrix (predictor variables) expressed as

$$\mathrm{Y} = \mathrm{f(X)} + \mathrm{E} \tag{7}$$

PLS may be easily understood geometrically if we imagine the matrices *X* and *Y* as *N* points in two spaces. The *X*-space with *K* axes, and the *Y*-space with *M* axes, where *K* is the number of columns in *X* matrix and *M* the number of columns in *Y* matrix. The objectives of PLS is to find a latent variable so that the best approximate *X*-space, the best approximate *Y*–space, and the greatest possible correlation between *X*-space and *Y* space.

A PLS model can be written as

$$X = 1\overline{X} + TP^T + E \tag{8}$$

$$Y = 1\overline{Y} + UC^T + F \tag{9}$$

$$T = U + H \tag{10}$$

where $\overline{X}$ contains the X average; $\overline{Y}$ contains the Y average; *T* is a matrix of scores that summarizes the *X* variables; *U* is a matrix of scores that summarizes the *Y* variables; E, F, H is a matrix of residuals (the deviations between the original and the predicted values) [12].

In a PLS algorithm, there are additional loading called weight. *P* is the matrix of weigh expressing the correlation between *X* and *U* and is used to calculate *T*. *C* is the matrix of weigh expressing the correlation between *Y* and *T* and is used to calculate *U* [12, 70].

5.4. Orthogonal partial least squares

OPLS has been developed in order to separate information in the *X* matrix that is correlated with Y matrix form Y-uncorrelated information. The idea of O-PLS algorithm was to remove systematic variation uncorrelated with the response with the goal and to reduce the number of components in order to increase interpretability of the model [55, 69, 71].

The main idea of O-PLS is to separate the systematic variation in *X* into two parts, one which is related to both *X* and *Y* (co-varying noise) and one which is orthogonal to *Y* (structured noise). Two O-PLS algorithms were developed, the first (O1-PLS) is unidirectional $\mathrm{X} \Rightarrow \mathrm{Y}$ and the second (O2-PLS) is bi-directional $\mathrm{X} \Leftrightarrow \mathrm{Y}$ and is able to separate these different types of variations in both *X* and *Y* matrices [63, 64]. The practical result of using O-PLS algorithms inside of PLS is cleaner models that are easier to display and interpret.

An O2-PLS model can be written as

$$X = 1\overline{X'} + TP^T + T_o P_o^T + E \tag{11}$$

$$Y = 1\overline{Y'} + UC^T + U_o C_O^T + F \tag{12}$$

$$T = UB_u + H_{TU} \tag{13}$$

$$U = TB_T + H_{UT} \tag{14}$$

where $\overline{X}$ is a contain de X average; $\overline{Y}$ is a contain de Y average; T is a matrix of scores that summarizes the X variables; U is a matrix of scores that summarizes the Y variables; P is a matrix of weigh that express the correlation between X and U; C is a matrix of weigh that express the correlation between Y and T; E, F, H_{TU}, H_{UT} are the matrixes of residuals.

The matrixes $\mathrm{TP^T}$ and $\mathrm{UC^T}$ hold the joint X/Y information overlap [12, 63, 64].

In the last years, O2-PLS has become the preferred regression technique for the development of calibration models in NIR and Raman spectroscopy.

5.5. Bayesian ridge regression

Another regression method recently proposed for multivariate calibration of spectroscopic methods is Bayesian ridge regression. The method presents similarities with least squares, and the estimated coefficients tend toward zero in order to avoid collinearity [44].

In a Bayes-RR regression model, higher-level prior Gaussian distributions can be introduced over α^2 and α, and the prediction can be performed by integrating over α^2, α, and the regression parameters w. Since this prior distribution is conjugate to the likelihood function, the predictive distribution is also Gaussian [72]

$$p(y|\alpha, \alpha^2) = \int p(y|w, \alpha^2) p(w|\alpha) dw \tag{15}$$

The Bayes-RR is a widely used regression technique in machine learning based on the ridge regression [73], and in the last years its performance for the development of excellent models for spectroscopic calibration has been proved [72, 74, 75].

5.6. Support vector regression (SVR)

The support vector machines (SVMs) are a set of learning methods mostly used for classification that can be used as a regression technique which is called the support vector regression. In the last years, SVM started to be used in chemometrics for NIR spectra classification and multivariate calibration. The SVR uses the same principles as the SVM and is based on finding the hyperplane maximizing the margin between classes. The hyperplane maximizing the margin is justified by statistical learning theory endowed with a probabilistic test error bound that is minimized when the margin is maximized. The regression is performed using kernel functions that transform the data into a higher dimensional feature space to make a linear

separation possible. The models obtained by SVR are more complex and difficult to interpret in comparison with those obtained by other regression techniques [44, 76, 77].

5.7. Decision tree regression

Decision tree regression is a type of decision tree algorithm that can be applied to solve regression problems. Decision trees represent one of the main techniques used for discriminant analysis, classification, and prediction in knowledge discovery. It is widely used because it closely resembles human reasoning and is easy to understand. The principle is to compute a regression in a tree structure from breaking down a dataset into smaller and smaller subsets. Recently, some applications in multivariate calibration of spectroscopic methods have been proposed [44, 77–79].

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Chapter 4

Internal Standards for Absolute Quantification of Large Molecules (Proteins) from Biological Matrices by LC-MS/MS

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Additional information is available at the end of the chapter

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Abstract

Internal standardization plays a critical role in the performance of a bioanalytical method. There has been a tremendous increase in the popularity of using liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for quantitative bioanalysis of protein molecules. Protein, being too large to be directly analyzed by LC-MS/MS, is proteolyzed and a characteristic peptide is used as a surrogate analyte for quantification. Internal standardization in small molecules' analysis is straightforward, i.e., either a stable labeled isotope (SIL) form of the analyte or a structural analogue is used. As protein quantification involves protein digestion to yield peptides, there are more options for internal standardization. Currently, internal standard selection is based on the availability of the internal standards and the sample preparation workflow. A SIL-form of the analyte protein is the ideal internal standard. However, its use is limited due to cost and commercial availability. Alternatively, a SIL form the surrogate peptide analyte or a cleavable SIL-peptide can be used as an IS. For preclinical bioanalysis of humanized IgG antibody-based drugs, a universal SIL analogue protein has been effectively used as an internal standard. This chapter focuses on internal standardization for the quantitative analysis of proteins, such as biotherapeutics and biomarkers, using LC-MS/MS.

Keywords: internal standards, protein bioanalysis, LC-MS/MS

1. Introduction

Mass spectrometry, as a quantitative tool, was largely restricted to the evaluation of small molecules until the 1990s. This was due to the lack of good-soft ionization techniques that are required for large molecule quantification. The development of soft ionization techniques such

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as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) facilitated the use of mass spectrometry for analysis of peptides and proteins [1, 2]. At the beginning of the twenty-first century, large molecule quantification was restricted to immunoassays. The advancements in mass spectrometric instrumentation and better quantification strategies have resulted in a shift of large molecule analysis from immunoassays to mass spectrometry [1, 2]. In the last decade, several methods have been reported for quantification of protein biomarkers and protein biotherapeutics. This popularity of LC-MS/MS methods can be attributed to its inherent selectivity, high dynamic range, shorter development time, and multiplexing capabilities.

Protein quantification using **targeted** mass spectrometry-based quantification from biological matrices is challenging owing to the high molecular mass and high protein background in biological matrices. Direct LC-MS/MS analysis of an intact protein analyte can be performed on proteins having molecular weights below 10 kDa. Proteins tend to ionize with multiple charges during electrospray ionization resulting in a complex spectrum with many precursor ion peaks corresponding to each charged state. Because of the complex precursor ion spectra, any single chosen precursor ion signal for quantitation will only represent a very small fraction of the total ionized analyte signal. This will result in lower signal response and reduce the sensitivity of the method. The complex ion spectrum also impacts the method specificity due to the presence of many overlapping signals arising from naturally occurring isotopes. In addition, larger protein analytes tend to be undergoing inefficient or non-existent collision-induced dissociation, in turn, impacting targeted mass spectrometric analysis. These challenges are overcome by enzymatic proteolysis of the analyte protein to yield one or more characteristic peptide fragments (i.e., signature peptides), which can be used as surrogate analyte(s) for quantification. In some methods, immunoaffinity enrichment is used for sample clean particularly for methods requiring detection limits below 100 ng/ml. These enzymatic digestions and immunoaffinity isolations are a potential source of variation and need to be controlled.

An internal standard is added to the sample during absolute quantification to compensate for variability encountered in sample processing and instrumental analysis. For mass spectrometric assays, a stable isotope labeled (SIL)-form of the analyte is an ideal internal standard it mimics the analyte throughout the method. However, SIL-proteins are difficult to produce with sufficient purity, and hence substitute internal standards such as SIL-peptides or protein structural analogues are used. In this chapter, we discuss the commonly used strategies for protein quantification using appropriate internal standards.

2. Overview of mass spectrometric-based protein quantification

Before we evaluate the different internal standardization options for protein quantification, it is essential to understand the various sources of variability during protein bioanalysis. Method variability arises during sample preparation or instrumental analysis. Protein bioanalytical quantification can encompass complex sample preparation steps such as protein analyte enrichment,

protein analyte proteolysis, and surrogate peptide analyte enrichment. Instrumental analysis comprises of chromatographic separation and mass spectrometric ionization. This section briefly summarizes various process encountered in protein quantification by LC-MS/MS.

2.1. Signature peptide selection

Selection of a signature peptide is a critical part of method development for protein bioanalysis. Peptides containing amino acid residues with potential post-translational modification (PTM) sites are usually avoided due to a potential change in peptide mass that would affect reproducible quantification. However, if the intended purpose is to quantify a post-translational modification, a tryptic peptide containing the specific PTM is selected [3–5]. Tryptic peptides containing amino acids susceptible to oxidation such as methionine and tryptophan are avoided as chemical modifications of these molecules can result in a change in its mass and thus can affect method reproducibility. Usually, tryptic peptides containing cysteine residues are avoided as they undergo iodoacetamidation. However, methods using signature peptide containing a cysteine residue have been reported after accounting for any mass change occurring prior to mass spectrometric detection [6, 7]. Missed cleavages can result inconsistent production of the signature peptide in turn impacting quantitation. Peptides containing ragged ends or dibasic ends next to each other (such as in Arg-Arg, Lys-Lys, or Arg-Lys) should be avoided as they are known to result in missed cleavages [8–10].

2.2. Sample preparation techniques for protein quantification LC-MS/MS

Biological samples, especially plasma and serum, are complex mixtures comprising of proteins, lipids, and salts in addition to the analyte molecule. Biological samples require pretreatment such as analyte enrichment or proteolysis prior to protein LC-MS/MS analysis. Sample preparation depends on the analyte physiochemical properties as well as the required level of selectivity and sensitivity. For example, proteins having a low molecular weight (<10 kDa), can be analyzed without proteolysis using protein precipitation and/or solid phase extraction. High molecular weight proteins (>10 kDa) require proteolysis to yield peptide fragments compatible for mass spectrometric analysis. These samples may require enrichment before and/or after proteolysis to achieve required detection limits. Routinely used sample preparation techniques are described below.

2.2.1. Non-selective protein enrichment techniques

For proteins smaller than 10 kDa, various non-selective protein enrichment techniques can be employed. Partial protein precipitation, using organic solvents along with surfactants, is used as a simple sample purification technique to deplete the endogenous plasma proteins. Partial protein precipitation eliminates larger proteins but leave smaller ones in solution [11–13]. Protein precipitation could have low recovery due to losses as a result co-precipitation, which would be a drawback. As it is simple but a crude clean-up technique, the resulting extracts are usually complex containing high concentration of salts and lipids. Matrix effects are commonly observed with these extracts, which can be a source of variability.

Solid phase extraction (SPE) is another purification technique that is employed solely or along with other purification techniques for sample clean up wherein the analyte is a smaller protein or peptide [12, 14–17]. Several mixed mode SPE cartridges, combining reversed phase stationary phase along with strong cation exchange or weak anion exchange, are commercially available for peptide analysis. These are usually available in 96-well microelution plate format. Microelution SPE offers many advantages including increased sensitivity due to low elution volumes, analysis of limited volume samples, significantly cleaner samples compared to extracts obtained after protein precipitation and higher reproducibility. The low elution volume avoids the need for sample extract evaporation and reconstitution, which can result in peptide instability.

2.2.2. Abundant protein depletion

Several commercial kits are available which use immunoaffinity depletion to selectively remove serum albumin, immunoglobulins, and other high abundant proteins from biological matrices [18–21]. These kits have shown to reduce protein content by up to 85% [22]. The enrichment technique is best suited for methods that have multiple protein analytes typically seen in biomarker research. The high costs of these kits and recovery issues have been the major drawback of this approach [23, 24]. Abundant protein depletion has been used in several biomarker quantification methods [23, 25, 26]. Liu et al. showed that isopropanol with 1.0% trichloroacetic acid was effective in removing 95% of the total albumin in human plasma samples while retaining 60–100% of the three analyte proteins that were evaluated. The recovery using this approach was found to be better than commercially available albumin depletion kits [27].

2.2.3. Immunoaffinity enrichment

Use of immuno (or affinity) capture for isolation of the analyte protein or its signature peptide is a highly selective enrichment technique. Combing the selectivity of an immunoaffinity capture with the selectivity of a LC-MS/MS system can allow a 1000-fold enrichment in comparison to conventional techniques [24]. Although this technique requires specialized antibodies, it provides sufficient purification to achieve quantification of low abundance proteins from plasma [24, 28–31]. Low recoveries and cross reactivities are some of the issues seen during immunocapture enrichment [32, 33]. Immunoaffinity isolations may be carried out with single or multiple antibodies depending on the availability of analyte-specific antibodies and the desired detection limits. The capture antibodies are immobilized in a 96-well plate or on to the surface of magnetic beads prior to the immunocapture. This immobilization is achieved using biotinylated antibodies and streptavidin-coated plates or magnetic beads. Alternatively, Protein A or G coated supports may be used for immobilizing the antibody using the Fc region of the antibody. The wide variety of immunocapture techniques can roughly be categorized into three categories based on the type of capture reagent.

2.2.3.1. Immunocatpture using protein-specific antibodies

The simplest approach is to use monoclonal or polyclonal antibody that is specific to the target analyte for immunocapture enrichment. Most of the protein biomarkers will have commercially

available antibodies while biotherapeutic drug molecules have specific antibodies developed for immuno-purification or screening during early pharmaceutical discovery and development. Due to the high specificity of this enrichment process, only a SIL-protein internal standard can be used an internal standard. However, in methods using a capture antibody, that has an epitope present on or near the signature peptide region of the analyte protein, an external SIL-peptide can be used as an internal standard [34].

2.2.3.2. Immunocapture using peptide-specific antibodies

Anderson et al. introduced the stable isotope standards with capture by anti-peptide antibodies (SISCAPA) strategy wherein immunocapture enrichment is directed toward a signature peptide after digestion using anti-peptide antibodies [35]. This technique allows high sensitivity and precision. However, these signature peptide-specific antibodies may not be commercially available and require inhouse development increasing method development cost and time. Some methods have employed this type of immunocapture online using specialized columns containing analyte-specific antibodies [29, 36–38]. This workflow is extensively used for multiplexed biomarker assays [24, 39]. Methods using dual enrichment, i.e., analyte protein enrichment and surrogate peptide enrichment post digestion, have also been reported. Multiple enrichment steps require the selection of an appropriate internal standard to compensate for method variability arising within each enrichment step.

2.2.3.3. Non-antibody capture of antibody-based biotherapeutic drugs

The majority of the biotherapeutic drugs are monoclonal antibodies or antibody-based molecules such as antibody drug conjugates. Antibodies have a constant tail region also known as the fragment crystallizable region (Fc region) and a variable region also referred to as antigen-binding region (Fab) region. The Fab region contains the complementarity determining region. Antibody-based drugs can be isolated using Protein A or G coated supports to bind to the Fc. This technique requires minimum time and resources for development and can achieve high throughput with adequate sensitivity. If additional selectivity is required, anti-Fc antibodies may be used depending on the analyte and the biological matrix. This technique is useful for the quantitation of humanized biotherapeutic drugs in animal models. Quantitation of humanized biotherapeutic drugs in human biological matrices requires the use of anti-idiotypic antibodies as capture agents to achieve significant detection limits. In some methods, the target antigen is used as a binding agent for the selective capture. Dubios et al. described an immunoaffinity coupled LC-MS/MS method wherein the analyte Cetuximab was isolated using its target antigen (soluble epidermal growth factor) as a capture reagent [40].

2.2.4. Enzymatic proteolysis

Most protein quantitative LC-MS/MS methods involve enzymatic digestion of the proteins to yield smaller peptides which can be easily quantified by commercially available quantitative mass spectrometers. A typical protein digestion procedure involves denaturation, reduction, and alkylation followed by proteolysis. Denaturation is carried out to unfold the protein so that it can be easily accessible to the proteolytic enzyme. Urea is the most commonly used for denaturation during protein quantification. Alternatively, denaturation has been achieved using

other chaotropic agents such as guanidine HCl, surfactants such as sodium deoxycholate, organic solvents such as methanol and heat (95°C) [13, 17, 26, 41, 42]. RapiGest SF, an acid-labile surfactant, is a Waters proprietary product that has gained high popularity for protein bioanalysis due to its compatibility with mass spectrometric detectors. This detergent is easily precipitated out by lowering the pH during the termination step of the enzymatic digestions. Reduction of the protein is carried out using dithiothreitol or TCEP (tris(2-carboxyethyl)phosphine) to break the disulfide linkages between cysteine residues. The resulting free thiol groups are then derivatized using an alkylating agent such as iodoacetamide or iodoacetic acid in order to prevent reformation of disulfide linkages.

Trypsin is the most commonly used enzyme for protein digestions primarily as tryptic peptides have a c-terminal basic residue that favors ionization. In addition, average tryptic peptides have lengths suitable for detection on commonly used quantitative mass spectrometers. However, other enzymes such Lys-C, Arg-C, pepsin, chymotrypsin have been used when a specific cleavage is required [43–46]. To improve digestion efficiency, different approaches have been illustrated including high temperature, microwave-assisted digestion, and use of organic solvents [31, 47].

The "pellet digestion" method is a simplified method in which the proteins are precipitated using an organic solvent like acetonitrile to form a pellet and the supernatant containing interfering molecules such as phospholipids are discarded. This method provides an easy, efficient way of performing a fast clean-up and has resulted in improved digestion efficiency in comparison to direct digestions [48–51].

2.3. Chromatographic separation

Liquid chromatography is used for separation of the protein or peptide mixture prior to mass spectrometric detection. For most peptide and protein analytes, reversed-phase column chemistry, typically C18 columns, allows separation of structurally and chemically similar molecules using mobile phases that are compatible with ESI. Hydrophilic interaction liquid chromatography (HILIC) has also been used to separate hydrophilic peptides. The mechanism of separation for small molecules is based on partitioning between the mobile and stationary phases. However, proteins and large peptides are not able to fully penetrate the pores and instead adsorb to the surface, and are desorbed at a critical concentration of organic solvent. Columns with larger pore stationary phases (∼300 Å) allow improved penetration of larger molecules as well as the use of higher flow rates with reduced band broadening, and therefore provide greater selectivity for some peptides and proteins.

Two-dimensional chromatographic separations, such as ion-exchange chromatography (IEC)-RPLC or RPLC-HILIC, have been utilized to fractionate and clean up samples, thus improving sensitivity of detection [16, 24, 46, 52, 53]. Additionally, many reported methods have used column trapping prior to analytical separation. The trap column retains the analytes and removes salts and other highly hydrophilic peptides [25, 28–31, 39, 54]. Some methods have used columns with antibodies for online immunoaffinity LC has been reported to achieve exceptional selectivity with minimal sample clean-up [31, 37, 38].

2.4. Mass spectrometric detection

Triple quadrupole (QQQ) and Quadrapole Ion Trap (QTrap) using the multiple reaction monitoring (MRM) mode are the most widely used mass analyzers for protein quantification. Proteins and peptides under electrospray conditions generally ionize to several charge states. For smaller peptides and proteins, $[M + 2H]^{2+}$ is usually the most abundant species, however, for larger molecules, $[M + 3H]^{3+}$, $[M + 4H]^{4+}$, and so on may also form, distributing the signal over several charge states and reducing the achievable limits of quantification. It is essential that the charge distribution across multiple samples is reproducible or will introduce method variability.

MRM mode allows measurement of multiple transitions and can be used for quantification of multiple analytes. Besides the signature peptide used for quantitative evaluation, additional characteristic peptides maybe monitored as monitoring peptides or qualitative peptides. Based on their location in the protein amino acid sequence, these monitoring peptides can provide valuable insights about the integrity of the analyte protein.

Mass spectrometric detection using electrospray ionization is highly susceptible to matrix effects. Samples with elevated concentrations of phospholipids, such as glycerophosphocholines and lysophosphatidylcholines, exhibit increase in ionization suppression when compared to normal plasma [55]. Lipid-related interference is generally not an issue with immunocapture-based methods, but it can be source of concern with methods that use non-selective isolations such as partial protein precipitation.

3. Internal standardization for protein bioanalytical methods

Internal standards need to track the analyte during all stages of sample analysis that includes sample preparation, chromatographic separation, and detection. Immunoaffinity capture, enzymatic proteolysis, and mass spectrometric ionization are the three major steps that are susceptible to variability during protein bioanalysis by LC-MS/MS. The internal standard may be added at different step(s) of the extraction process depending on the availability of the internal standards and the sample extraction workflow. **Figure 1** describes various commonly used workflows for protein quantification and the internal standard (IS) options. Protein internal standards are added prior to analyte protein enrichment. Protein analyte enrichment can be immunoaffinity isolations or a non-selective process such as partial protein precipitation, SPE, and abundant protein removal. Smaller proteins can be directly analyzed after protein analyte enrichment. Larger proteins are proteolyzed to yield signature peptide(s) that can be used as surrogate analytes. Peptide internal standards when added prior to enzymatic proteolysis can only track peptide instability and volume recovery during the proteolytic process as we well mass spectrometric ionization during analysis. Additionally, cleavable internal standard peptides may be able to track the digestion variability when added prior to proteolytic incubations. Methods requiring low detection limits utilize peptide enrichment post enzymatic proteolysis. SIL-peptides are required to track immunoaffinity-based peptide enrichment processes. Currently used internal standards for protein bioanalysis are described below.

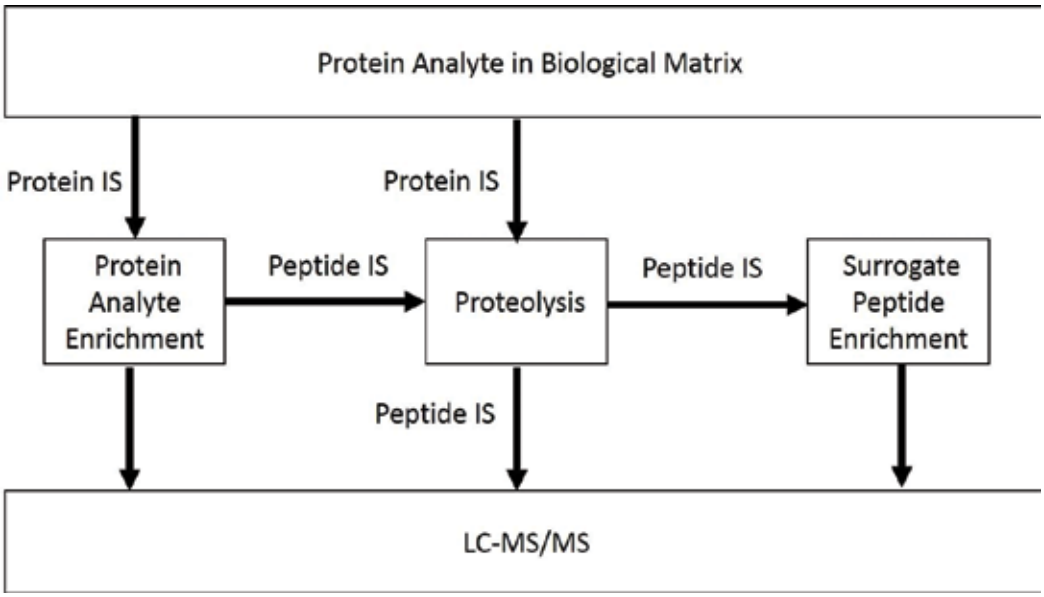

Figure 1. Internal standardization strategies for different LC-MS/MS protein quantification workflows.

3.1. Protein internal standards

3.1.1. Stable isotope labeled protein internal standard

A stable isotope labeled (SIL) form of the analyte protein is the most ideal IS for absolute quantification of proteins. As the analyte protein and SIL-protein internal standards have the same physiochemical behavior, this IS will be able to track the analyte protein throughout the entire analytical procedure. SIL-proteins are added at the start of the sample extraction and can account for immunoaffinity isolation(s), enzymatic digestion, pre-analytical treatments as well as the mass spectrometric ionization.

The EMA guidelines recommend the use of a SIL-protein IS whenever possible for LC-MS/MS methods. However, a major restriction in the use of SIL-proteins as internal standards for protein quantification is their commercial unavailability or the high cost of production. The complex structure consisting of specific intramolecular folding of amino acid chains as well as intramolecular di-sulfide linkages and presence of post translational modifications makes it difficult to synthesize these proteins in a reproducible manner. If the analyte protein is small, a SIL form of the protein can be chemically synthesized using solid-phase synthesis [16, 56]. However, for most protein analytes, the production of its isotopic labeled form requires a cellular environment. Two methods are described below for the generation of isotopically labeled proteins: metabolic labeling using whole cells and the cell-free approach using cell lysates.

3.1.1.1. Cell culture production

All cell-based labeling approaches rely on the metabolic conversion of labeled precursors into a protein. The labeled precursors used in cell-based systems may be amino acids or they may be

more fundamental precursors which serve as carbon or nitrogen sources for the synthesis of amino acids prior to their incorporation into protein.

SILAC is a popular technique for production of SIL-proteins by incorporation of SIL-amino acids into the target protein. This technique is popularly known as stable isotope labeling by amino acids in cell culture (SILAC) [57] or stable isotope labeling with amino acids (SILAA) [58]. SILAC is a straightforward procedure in which essential amino acids are left out of culture media and replaced by deuterated, carbon-13, and/or nitrogen-15 labeled variants of these amino acids. After multiple cell duplication cycles, these SIL amino acids get incorporated in the entire proteome. Heavy labeled variants of lysine and arginine that provide ample spacing between isotopic envelopes of light and heavy tryptic peptides (e.g., 10 Da using $^{13}C_6$ $^{15}N_4$-Arg and 8 Da using $^{13}C_6$ $^{15}N_2$-Lys) are the most commonly used amino acids for production of SIL-proteins using SILAC. Arginine and lysine on an average they occur at every tenth position in a protein sequence. Trypsin, the major proteolytic enzyme used in protein quantification assays, cleaves at lysine and arginine ensuring that at least one of the labeled amino is present on each tryptic peptide.

Cell culture production is highly useful for generating SIL-labeled proteins for biothereapeutics. Biotherapeutics are produced by genetically modified cell lines, yeast or bacteria. To obtain a SIL-protein, the cells producing the biotherapeutic protein are grown in a medium containing labeled precursors for the desired protein. The stable isotope labeled amino acids gets incorporated in the proteins, thus resulting in production of a SIL-protein. These SIL-proteins are then purified and can be used as internal standards.

The cell culture method is an easy process of producing labeled proteins, but requires a cell culture equipment and a sterile laboratory. A major disadvantage is that in cell culture other endogenous proteins will be simultaneously produced having the incorporated label, and hence a more elaborate purification is required. Also, incomplete labeling can occur when the pools of labeled amino acids are diluted with amino acids newly synthesized by the cell.

3.1.1.2. Cell-free production

SIL-proteins can also be made by *in vitro* protein synthesis in a cell free system [59]. Cell-free translation systems are largely supernatants obtained by centrifugation of the crude lysate of either *E. coli*, wheat germ, or rabbit reticulocytes at 30,000 g. The cell lysate supernatants, also referred to as S30 fraction, contain the cell's protein synthetic machinery consisting of ribosomes, translation factors, aminoacyl-tRNA synthetases, and tRNAs.

Cell-free systems can be operated in three different modes: batch mode, continuous flow cell-free (CFCF) mode, and CECF mode. In batch mode, the reaction is carried out in a tube to which all components of the reaction are added. It is easy to step-up and can be useful for fast and easy production of small amounts of protein. Its disadvantage is it is a closed system and hence has limited capacity.

The CFCF mode is an open system which requires the continuous supply of fresh substrates and removal of by-products by a continuous flow of a feeding solution into a reaction chamber. The CFCF mode was first developed by Spirin and co-workers [60]. The total volume of the

reaction is maintained constant by having the volume flowing out of the chamber equal to the volume flowing in. This system can be used for continuous production of labeled proteins.

The CECF mode is an open system with two chambers separated by a semi-permeable membrane. The first chamber is the feeding chamber and contains the substrates and the energy system. The second chamber is the reaction chamber and contains the enzymes and DNA. The substrates permeate through the semipermeable membrane and are converted to proteins in the reaction chamber. This can be easily set-up using a simple dialysis bag as the reaction chamber. The bag can be immersed in a feeding solution inside of a tube larger than the dialysis bag creating a simple two-chamber device.

3.1.2. Derivatized protein internal standard

Derivatization allows one to easily generate a protein internal standard that is physiochemically like the analyte protein. Winther et al. reported an LC-MS/MS method for quantification of progastrin-releasing peptide (ProGRP), a small cell lung cancer biomarker, in human serum using an acetylated form of the protein as an internal standard [30]. The IS was made in-house by specific acetylation of the lysine side chains in ProGRP (31–98) by using N-hydroxysuccinimide-based ester acetic acid N-hydroxysuccinimide (AA-NHS) as the acetylating reactant. The acetylated ProGRP (31–98) signature peptide NLLGLIEAK gets converted to NLLGLIEAKacENR, which was used as a peptide internal standard. The extraction procedure involved protein precipitation with acetonitrile followed by pellet digestion with trypsin prior to analysis.

This acetylated ProGRP internal standard mimicked the analyte ProGRP through extraction steps including tryptic digestion and hence compensated for any variations during extraction. However, acetylation of the IS-peptide, results in chromatographic differences between the signature peptide and IS-peptide. Differences in retention time can result differences in ionization due to co-eluting matrix interferences. The derivatization also caused ionization differences in the precursor ion charge states. NLLGLIEAK had +2 as the most dominant charge state while NLLGLIEAKacENR had +3 as the most dominant charge state of the precursor ion. The addition of IS to the samples improved the coefficient for both the linear and the polynomial calibration curve and the intra- and inter-day accuracy. However, the high intra-day precision values (%CV of 12–25.2%) displayed some unaccounted variability in turn questioning the performance of the internal standard in this method. An important consideration while using derivatized internal standards is to ensure that impurities of underivatized analyte protein or derivatizing reagent are not present in the purified internal standard.

3.1.3. Universal stable isotope label protein internal standard for quantification of antibody-based biotherapeutics in non-human matrix

Drug development involves bioanalytical testing in non-human species. During bioanalysis of humanized IgG antibody based biotherapeutics in animals, selected peptides from the constant region (Fc) of the antibody can be used as signature peptides. These peptides will be present in the humanized immunoglobulin (IgG)-based biotherapeutic drugs, but will not be present in antibodies found in the animal biological fluids. In methods using signature peptides obtained from the constant region, a stable labeled analogue monoclonal antibody (mAb)

can be used as an internal standard. SILu™Mab internal standard is a commercially available stable labeled IgG1 monoclonal antibody and has been metabolically labeled with $^{13}C_6$ $^{15}N_4$–Arginine and $^{13}C_6$ $^{15}N_2$-Lysine was expressed in CHO cells. SILu™Mab is used as a universal internal standard in quantitation of humanized biotherapeutics in non-human biological matrices. **Table 1** lists out the various characteristic SIL-peptides that can be generated after proteolysis of SILu™Mab with trypsin.

The universal applicability of SILu™Mab is exemplified in an immunoaffinity coupled LC-MS/MS method reported by Kaur et al. [61]. A generic method was developed for mAb-1 and its universal applicability was demonstrated with the additional six mAbs. SILu™Mab was used as the common internal standard in all seven methods. The extraction involved immunoaffinity enrichment followed by tryptic digestion. For three mAbs, the immunoaffinity capture was carried out using anti-human Fc antibody attached to magnetic beads. This immunoaffinity capture allows selective binding to a humanized mAb in nonclinical matrices. Alternatively, for remaining four mAbs, the affinity enrichment was performed using a less selective reagent, Protein A/G. After immunocapture, the analyte and IS bound to the magnetic beads were denatured, reduced, and alkylated. The isolated analyte mAb and IS were digested with trypsin prior to LC-MS/MS analysis. The method for mAb-1 in cynomolgus serum was found to have a linear response over the nominal concentration range of 0.100 to 25.0 μg/ml with high precision (%CV < 3%) and good accuracy (%DFN ± 9%). The high precision and accuracy of the method validates the effectiveness of the internal standard to compensate for any variability during extraction and analysis. The method performance was evaluated with additional six mAb as well as in rat and mouse sera. All the assays showed good precision (%CV < 20%) and accuracy (%DFN ± 20%).

The generic method, using a universal internal standard that tracks the analyte throughout extraction and instrumental analysis, circumvents the method development challenges for biotherapeutic mAbs. This internal standard use is restricted to non-human biological matrices. In human matrices, the signature peptide will lose its selectivity due to the presence of high concentrations of endogenous mAbs.

Universal SIL-peptide	Antibody isotype	Peptide location
DTLMISR*	IgG1, IgG2, IgG3, IgG4	Heavy chain
FNWYVDGVEVHNAK*	IgG1	Heavy chain
VVSVLTVLHQDWLNGK	IgG1, IgG3, IgG4	Heavy chain
NQVSLTCLVK*	IgG1, IgG2, IgG3, IgG4	Heavy chain
GFYPSDIAVEWESNGQPENNYK*	IgG1, IgG4	Heavy chain
AGVETTTPSK*	IgG1, IgG2, IgG3, IgG4	Light chain (Lamda)
YAASSYLSLTPEQWK*	IgG1, IgG2, IgG3, IgG4	Light chain (Lamda)

*Stable isotope labeled amino acid ($^{13}C_6$ $^{15}N_4$–Arginine or $^{13}C_6$ $^{15}N_2$–Lysine).

Table 1. Tryptic SIL-peptides of SILu™Mab.

3.1.4. Analogue proteins as internal standards

A structural protein analogue can also be used as an internal standard. This internal standard is the least favorable as it may not truly mimic the protein analyte during proteolysis or the signature peptide during mass spectrometric ionization. The advantage of using an analogue protein is ease of availability and low cost. As it accounts for volume loses, it can be an appropriate IS for methods that involve simple sample preparation such as partial protein precipitation. An unlabeled analogue protein is chosen based on the similarities in physiochemical properties, such as size, hydrophobicity, and isoelectric point, with the analyte protein and its surrogate peptides obtained after proteolysis. Some methods have been reported wherein the chosen internal standard was able to track the analyte efficiently throughout the method.

Halquist et al. reported an LC-MS/MS method for quantification of Alefacept, a therapeutic protein for treatment of psoriasis, in human plasma using horse heart myoglobin a protein analogue internal standard [11]. The method used partial protein precipitation to selectively precipitate background proteins while retaining the analyte protein and the internal standard protein in solution. The isolated proteins were proteolyzed using trypsin. A 20-h-incubation was chosen for proteolysis to ensure least digestion variability for both proteins. The signature peptides from analyte and IS were then separated using reversed phase chromatography and detected using tandem mass spectrometry with electrospray ionization. The chromatographic gradient conditions were adjusted to ensure the surrogate analyte peptide and the IS-peptide had the same retention time. This enabled the IS-peptide to compensate for any matrix effects during ionization.

3.2. Peptide internal standards

3.2.1. Stable isotope labeled peptide internal standard

When a SIL-protein is unavailable for use as an internal standard, a stable isotope labeled form of the signature peptide can be used. A SIL form of the signature peptide (SIL-peptide) is the most commonly used internal standard during protein quantification. SIL-IS peptides are variants of the signature peptides having one or more stable isotope labeled amino acids. Stable isotope labeled amino acids are obtained by substitution of certain atoms (N,C,H) with their heavy variants. The most frequently used stable isotopes are ^{13}C (carbon-13), ^{15}N (nitrogen-15), and ^{2}H (deuterium). A SIL-peptide can be created by using solid-phase peptide synthesis [56, 62]. Due to the widespread use of SIL-peptides for protein bioanalysis, several laboratories provide commercial services for production of customized SIL-peptides at reasonable prices.

SIL-peptides are physiochemically identical to the signature peptide but can be easily distinguished on a mass spectrometer due to the mass shift from the heavier isotopes. A minimum mass difference of at least 6 Da between SIL-peptides and the signature peptide is recommended. This will ensure an adequate resolution between the mass of SIL-peptide and signature peptide even for peptides having a dominant charge state of +2. SIL peptides can effectively compensate for extraction recovery, peptide instability and LC-MS/MS variability. However, unlike a SIL-protein it does not track immunoaffinity and proteolytic digestion

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steps. The major advantage of using a SIL-peptide is that these can be synthetized at relatively low cost.

A SIL-peptide internal standard can be added before and after proteolysis. However, if the signature peptide is suspected to undergo degradation, it is recommended to add the SIL-peptide prior to digestion. Also, if a recombinant form of the protein is not available, protein concentrations are calculated stoichiometrically, solely based on the known molar concentration of the SIL-peptide used. In such instances, the internal standard is added post digestion to obtain reproducible peptide quantification [24, 63].

3.2.2. Extended stable isotope labeled peptide internal standard

Variation in digestion efficiency can be accounted for with the use of an extended SIL-peptide which has cleavable groups flanking either side of side of a SIL-peptide [29, 64, 65]. Generally, the cleavable groups consist of three to six amino acids residues from the original protein sequence at both the N- and C- terminus [29, 65, 66]. The addition of an extended SIL-peptide IS prior to digestion provides a more cost-effective alternative to compensate for variability in digestion efficiency, peptide stability, volume recovery, and mass spectrometric ionization.

Barnindge et al. were first to report the comparison between SIL-peptide/non-cleavable peptide (NCP) and a dual cleavable peptide (DCP) or "extended SIL-peptide" as internal standards to track protein digestions [64]. They synthesized two peptides each containing a signature peptide sequence from amino acids 318 to 323 in human serum albumin (HSA). The non-cleavable peptide (NCP) was labeled with a stable isotope labeled alanine residue, i.e., NYA*EAK, whereas the other peptide had two tryptic cleavage sites and two stable isotope labeled alanine residues, i.e., DVAK-NYA*EA*K-DVFLG. Different concentrations of HSA were digested along with equimolar concentrations of NCP and DCP. Prior to digestion the samples were reduced for 30 min at 30°C using 10 mM dithiothreitol (DTT) followed by alkylation using a concentration of 30 mM iodoacetamide (IAA) with the reaction going to completion in the dark at room temperature for 30 min. Each sample was then digested with trypsin for 12 h at 30°C in a shaking water bath using an enzyme-to-substrate ratio of 1:10. The reaction is terminated after 12 h with trifluoroacetic acid. The samples were analyzed using LC-MS/MS. The results showed that a cleavable internal standard peptide could give similar results to a non-cleavable internal standard peptide. Timed digest experiments showed that the digestion rates for dual cleavable peptide and analyte protein, i.e., HSA were different with the DCP proteolysis coming to completion faster (approximately 1 min) than HSA (approximately 20 min). From these results, they concluded that although an internal standard with a cleavage site provides understanding of the digestion process, a SIL-peptide truly cannot replicate the proteolysis conditions experienced by the analyte protein.

In another study, Faria et al. compared the performance of a SIL-peptide and extended SIL-peptide as internal standards for quantification of human osteopontin [67]. Digestion studies showed that the signature peptide production had a biphasic pattern. This pattern was attributed to the degradation of the signature peptide during digestion with trypsin due to suspected chymotrypsin-like activity. The digestion profile of the protein analyte had three phases, i.e., the "formation phase," the "transition phase," and the "degradation phase." The

formation phase was between 0 and 5 h which was dominant in the formation of signature peptide. The transition phase was between 5 and 10 h where signature peptide formation and degradation processes occur at similar rates. The degradation phase was beyond 10 h in which the degradation of the signature peptide was the most dominant phenomenon. In order to track the analyte protein digestion profile, a SIL-peptide IS and extended SIL-peptide IS were added. From **Figure 2**, we can see that the recombinant protein and the extended SIL-peptide internal standard had similar digestion profiles as they both undergo formation and degradation. SIL-peptide only undergoes degradation and hence only mimics the analyte protein digestion profile during the degradation phase. Validation studies showed that under controlled conditions and long digestion time there was no significant difference in precision when either of the internal standards was used for quantification. However, when trypsin activity was forcibly varied, the extended SIL peptide had higher precision. This difference was more pronounced when digestion was carried out at shorter time intervals.

3.3. Comparison of protein SIL-IS versus peptides IS

Li et al. evaluated the use of SIL-protein, SIL-peptide, and extended SIL-peptide as internal standard for quantification of monoclonal antibodies in preclinical biological matrix by LC-MS/MS [68]. The evaluation was carried out with four mAbs of the same IgG2 isotype as the SIL-IS: αDA-G2, (KLH)-120.6-G2 (αK-G2), 827-435-G2 (827-G2), and anti-DNP-3B1-G2 (αDB-G2). In addition, the test was extended to four more mAbs of a different isotype IgG1: anti-DNP-3A4-F-G1 (αDAG1), anti-KLH-120.6-G1 (αK-G1), 655-341-G1 (655-G1), and anti-DNP-3B1-G1 (αDB-G1). Stable isotope labeled human antidinitrophenol (DNP) IgG2 mAb was used as the IS as it is unlikely to be present endogenously in preclinical species. The whole molecule SIL-IS of clone anti-DNP-3A4-F-G2 (αDA-G2) was produced in cell culture, purified, and characterized

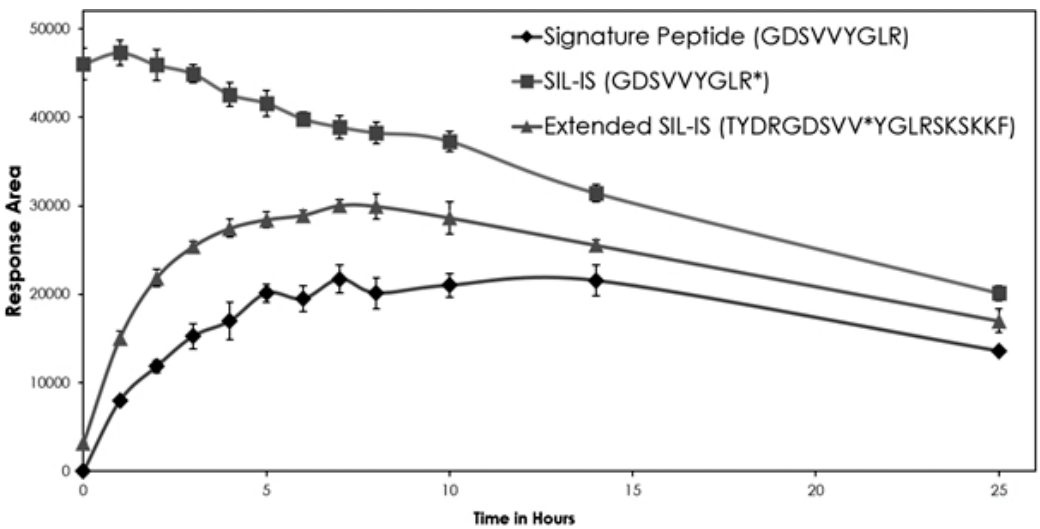

Figure 2. Comparison of digestion profiles of recombinant OPN along with internal standards, i.e., SIL-peptide and extended SIL-peptide. Reprinted from [67]. Copyright (2015) with permission from Elsevier.

prior to use. Synthetic IS peptides with stable isotopic labeled antikeyhole limpet hemocyanin leucine (L*), NQVSL*TCL*VK and REEMTKNQVSL*TCL*VKGFYPSD (six flanking amino acids), were commercially obtained and used as SIL-IS peptide and extended SIL-IS peptide, respectively. The evaluation was carried out in rat plasma and cynomolgus monkey serum.

SIL-protein IS was added to each sample prior to extraction. The analyte mAb and SIL-protein mAb were immunocaptured using an antihuman crystallizable fragment (anti-Fc) that recognizes human mAb biotherapeutics but not the endogenous immunoglobulins in the preclinical sample. After immunocapture, the analytes were eluted with 200 μL of 50% MeOH and 3% formic acid in water. Samples with IS-peptides were spiked either with SIL-peptide or extended SIL-peptide. The eluate was evaporated to dryness. SIL-peptide and extended SIL-peptide were added to samples with IS-peptides. The samples were reconstituted for reduction and alkylation. The samples were then digested with trypsin. After termination of the proteolytic reaction, the extracts were analyzed using LC-MS/MS.

For the comparison experiment, three sets of QCs each from αDA-G2, αDA-G1, or αK-G2 were analyzed in three replicates along with the three internal standards. The results can be seen in **Figure 3**. Overall, all three mAb were quantified accurately (Bias within ±20%) and precisely (%CV within 20%) using the SIL-protein IS. For the extended peptide SIL-IS, the αDA-G2 QCs were also well within ±20%. The mid and high QC values of the αDA-G1 were near the ±20% threshold but not the LQC or the QCs of the αK-G2. For the peptide SIL-IS, the QC values of αDA-G2 were marginally acceptable, with higher variability and imprecision for αDA-G1 and the worst results for αK-G2. In addition, the whole molecule IS peak response within the runs was more precise (15.5% CV) than those of the extended SIL-peptide IS (28.1% CV) or the peptide IS (27.7% CV), N = 32. From this data, we can be seen that the whole Ab IS can effectively compensate for any variability during extraction and LC-MS/MS analysis. If the immunocapture and digestion steps were optimized to have high reproducibility, then the synthetic peptide ISs may be adequate for quantification.

In another study, Bronsema et al. evaluated different internal standardization strategies for quantification of a small protein, salmon calcitonin, which could be analyzed both with and without and digestion [69]. Salmon calcitonin comprises of 32 amino acid and has a molecular weight of 3431.9 Da. Eight internal standardization approaches were compared with respect to accuracy and precision in work flows with and without digestion. Both analogue IS standard proteins (eel and human calcitonin), SIL-salmon calcitonin, SIL-salmon calcitonin signature peptide [1–11], and the cleavable SIL-salmon calcitonin peptide [1–11] were commercially obtained. ^{18}O-labeled form of the signature peptide was synthesized in-house by isotope exchange with18 O-labeled water.

The samples were extracted using three different workflows. In work flow A, the samples were extracted using only SPE prior to LC-MS/MS analysis. In workflow B, the samples were either extracted using SPE and the extracts were digested with trypsin prior to analysis. In workflow C, the samples were enriched using SPE, digested with trypsin and then derivatized prior to analysis. Derivatization was performed with a solution containing 10% of deuterated or unlabeled formaldehyde and 10% pyridine-borane complex in methanol. Waters Oasis MCX SPE cartridges were used post derivatization the clean-up prior to LC-MS/MS analysis in work

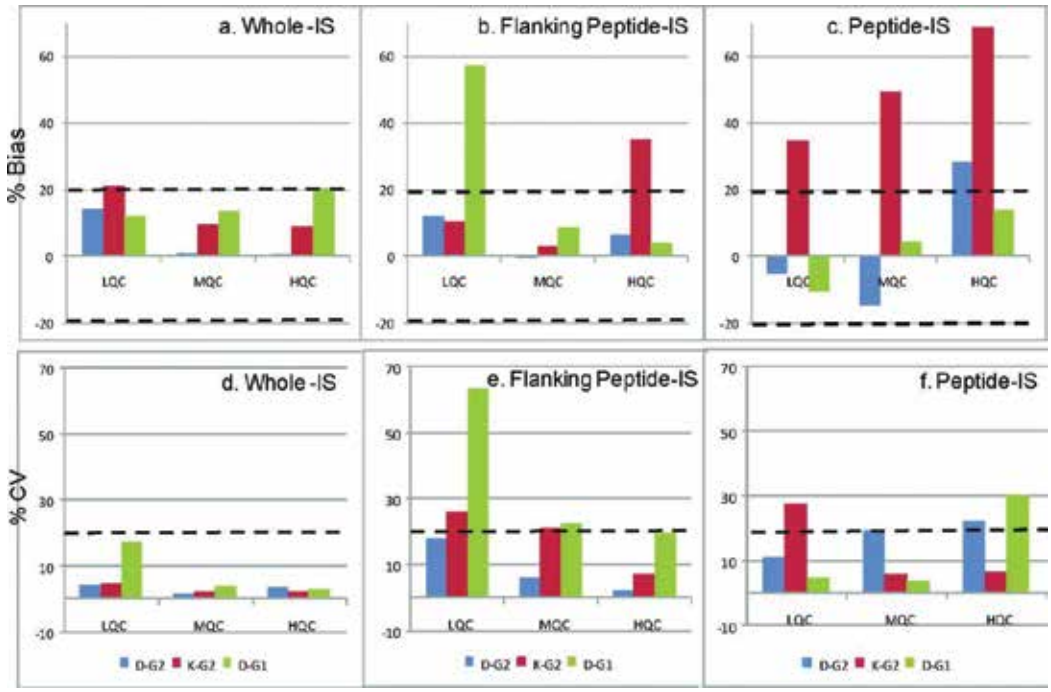

Figure 3. Accuracy and precision of QCs from 3 mAbs obtained with three different SIL-IS's. (a)–(c): Accuracy of whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively. (d)–(f): Precision of whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively. The dashed lines of 20% are the thresholds of acceptance commonly used by LBAs. αDA-G2, αK-G2, and αDA-G1 QCs are represented by blue, red, and green color bars, respectively. Reprinted with permission from [68]. Copyright (2013) American Chemical Society.

flow C. Internal standards were added at different stages depending on the workflow and internal standard characteristics. The results of precision and accuracy studies using a series of internal standardization routes as per workflows A through C, compared to the same workflows without internal standard, are illustrated in **Figure 4**.

Using workflow A (quantification of the intact analyte) without an internal standard had too much variability at 100 pg/mL, resulting in a bias outside the acceptance criterion of ±15%. When SIL-salmon calcitonin was used as internal standard, accuracy, and precision improved significantly at both high and low concentrations. This finding was consistent with the expected performance of SIL-protein IS. The first analogue protein IS, i.e., eel calcitonin (90% sequence homology) introduced high variability at the lower level. When human calcitonin (50% sequence homology) was used as an internal standard, the method performance was severely hampered. Both precision and accuracy were inferior to the results obtained without any internal standard. This illustrates that this internal standard did not correct for variability but rather introduced it into the assay.

In workflow B (quantification of the digested analyte), two internal standardization processes were used, i.e., (1) B-before referring to internal standards that are added prior to digestion

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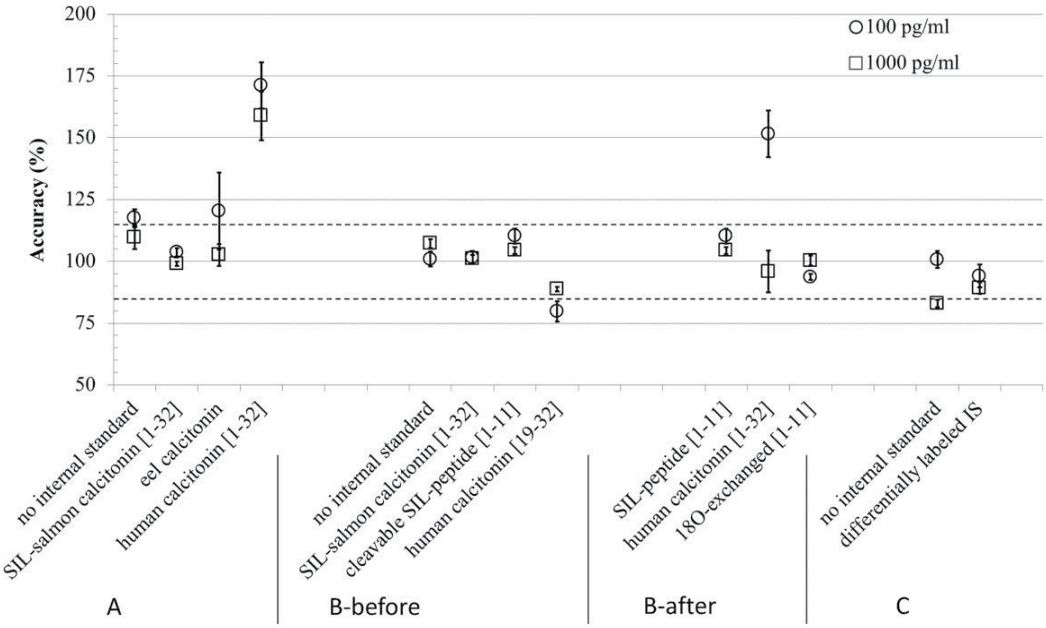

Figure 4. Precision (expressed as error bars) and accuracy for 10 different internal standardization approaches according to workflows A–C as compared to the same workflow without internal standard. The results were obtained by 6-fold analysis of the 100 and 1000 pg/mL samples. The dotted lines indicate the acceptable 15% accuracy limits as set by the FDA-guidance. In workflow B, internal standards were either added before or after the digestion step. Reprinted with permission from [69]. Copyright (2013) American Chemical Society.

and are expected to cover the digestion step, and (2) B-after referring to internal standards that are SIL or structural analogue forms of the signature peptide and will only cover the post-digestion part of the analysis (B-after). Eel calcitonin was not used in this workflow as an internal standard as it yields a signature peptide same as salmon calcitonin. Besides human calcitonin (added before and after digestion), all other approaches tested in workflow B, including the omission of an internal standard, generated acceptable results, which shows that in this workflow all steps were well under control. Again, the best results in terms of precision and accuracy were obtained when SIL-salmon calcitonin [1–32], cleavable SIL peptide internal standard and SIL peptide [1–11] were used as internal standards. Since there was no difference between the performance of a SIL-calcitonin, cleavable SIL-peptide and that of a SIL-peptide for workflow B, it was concluded that the digestion step did not negatively impact method performance. This observation possibly can be attributed to the small size of the analyte protein and absence of any tertiary or quaternary structure. The commercially obtained SIL peptide internal standard and the in-house prepared ^{18}O-labeled form performed comparably in workflow, thus indicating that the ^{18}O-labeled peptide can be used as an economical alternative to a chemically synthesized SIL peptide.

In workflow C (quantification of digested and derivatized analyte), it was seen that using a differentially labeled internal standard slightly improved assay performance compared to the results without internal standard. Precision and accuracy of this internal standardization

approach were comparable to the SIL peptide and ^{18}O-labeled peptide approaches for workflow B, which do not include a derivatization and second SPE step. Workflow C is more laborious and has multiple steps without any internal standards, and therefore will have a higher risk of experimental variability.

4. Conclusions

Precision and accuracy of bioanalysis is ultimately improved through internal standardization. The selection of an internal standard is often dictated based upon availability, time, and cost. While SIL-proteins are considered the ideal internal standard, their availability often limits their use. SIL-peptides and extended SIL-peptides are readily available, and serve as good alternatives to SIL-proteins. When using SIL-peptide, it is essential that sample preparation steps, i.e., enrichment and enzymatic digestions that are not tracked by the peptide internal standards are optimized to limit their variability. In the absence of SIL-IS standard, an analogue protein or peptide may be used as an internal standard. The use of stable isotope labeled analogue monoclonal antibody as a universal internal standard has enabled rapid development of accurate and precise methods for quantitative bioanalysis of biotherapeutics in non-human species.

Conflict of interest

The views and opinions in this chapter represent those of the authors only.

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Chapter 5

Calibration Methods of Laser-Induced Breakdown Spectroscopy

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Additional information is available at the end of the chapter

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Abstract

Laser-induced breakdown spectroscopy (LIBS) has gained great attention over the past two decades due to its many advantages, such as needless sample preparation, capability of remote measurement and fast multielement simultaneous analysis. However, because of its inherent uncertainty features of plasma, it is still a big challenge for LIBS community worldwide to realize high sensitivity and accurate quantitative analysis. Currently, many chemometric analytical methods have been applied to LIBS calibration analysis, including univariate regression, multivariate regression, principal component regression (PCR), partial least squares regression (PLSR) and so on. In addition, appropriate sample and spectral pretreatment can effectively improve the analytical performance (i.e., limit of detection (LOD), accuracy and repeatability) of LIBS. In this chapter, we briefly summarize the progress of these calibration methods and their applications on LIBS and provide our recommendations.

Keywords: laser-induced breakdown spectroscopy, sample and spectral pretreatment, calibration methods, chemometrics, calibration-free laser-induced breakdown spectroscopy

1. Introduction

Laser-induced breakdown spectroscopy (LIBS), also sometimes called laser-induced plasma spectroscopy (LIPS), has developed rapidly as an analytical technique over the past two decades. LIBS is a kind of atomic emission spectroscopy, which uses a high-energy pulsed laser as the excitation source. The laser is focused on the sample surface, thereby evaporating and atomizing the sample and generating a plasma. The light emitted by the plasma is detected by a spectrometer. One can obtain sample composition and concentration information by analyzing the plasma

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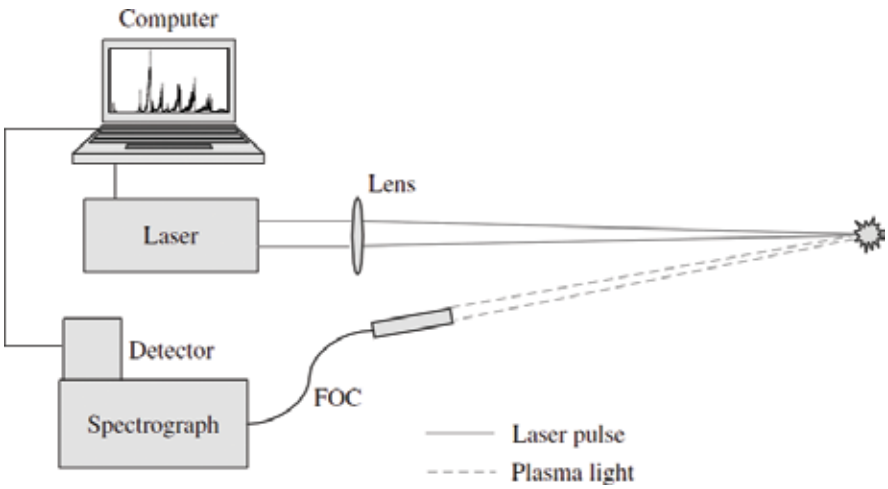

Figure 1. A schematic of a general apparatus for laser-induced breakdown spectroscopy illustrating the principal components.

emission spectra. LIBS most commonly used experimental instruments such as mainly lasers, spectrometers, detectors and computers, as shown in **Figure 1**.

The laser which is the most widely used in LIBS is the Nd:YAG solid-state lasers operated in the Q-switch mode. Typically, it is operated at the fundamental wavelength of 1064 nm, pulse energy is 30–100 mJ, pulse width is 5~15 ns and repetition rate is 1~10 Hz. In addition, the researchers tested the effects of lasers of different types and parameters on the LIBS. Trautner et al. [1] investigated polyethylene (PE) and a rubber material from tire production by employing 157 nm F_2 laser and 532 nm Nd:YAG laser ablation in nitrogen and argon gas background or in air. The effects of laser wavelength on depth resolution of thin-film solar cell are investigated by Choi et al. [2] using an ultraviolet (λ = 266 nm) and a visible (λ = 532 nm) nanosecond Nd:YAG lasers. Labutin et al. [3] summarize nearly two decades of studies on femtosecond laser-induced breakdown spectrometry (fs-LIBS). Picosecond pulse train and nanosecond pulse were compared for laser ablation and LIBS measurements by Lednev et al. [4].

Spectrometers disperse the emitted radiation of the laser-induced plasma to get a spectrum in terms of intensity as a function of the wavelength. The dominant spectrometer types used for LIBS are multichannel fiber spectrometer and echelle spectrometer coupled with an intensified CCD. The echelle spectrometer offers a wide spectral range, a high spectral resolution, and the possibility of time-resolved. The plasma parameters (plasma temperature and electron density) are constantly changing with respect to the delay time, and an echelle spectrometer with time resolution is needed when calculating these parameters. However, time-resolved broadband spectrometers are expensive and strongly dependent on external circumstances. The multichannel fiber spectrometer is robust and reliable for the use in mobile and portable LIBS instruments, providing an accredited spectral resolution, but their integration time is typically much longer than the plasma lifetime.

In recent years, with the rapid development of lasers, spectrometers and detectors and the urgent demand of in situ and online analysis, LIBS has developed rapidly. Compared with many other types of elemental analysis techniques, LIBS has obvious advantages:

1. Simple equipment: few instruments, low cost and easy integration.
2. Noncontact analysis: LIBS uses pulsed laser as the excitation source, which makes it noncontact analysis, especially in the dangerous environment or space exploration field, has broad application prospects.
3. No sample preparation: LIBS directly focused pulsed laser bombardment of the sample without processing the sample.
4. Various samples: samples can be gas, aerosols, liquids and solids.
5. Nondestructive analysis: The laser converges to the surface of the sample, and only a small amount of the sample is excited. It can be considered as nondestructive or near nondestructive.
6. Three-dimensional analysis: LIBS can collect laser at different positions on the sample surface or repeat measurements at the same location to analyze the sample surface and its different depths of the sample composition and content.
7. Total element analysis: The laser energy can simultaneously excite all the elements in the sample, so all elements in the sample can be analyzed simultaneously.
8. Remote analysis: The long-distance analysis of the LIBS can be achieved by remotely transmitting the laser energy and collecting the plasma emission spectrum through the fiber.
9. Online analysis: LIBS is a very fast technology that provides analytical results in seconds, making it particularly suitable for rapid analysis or online industrial monitoring.

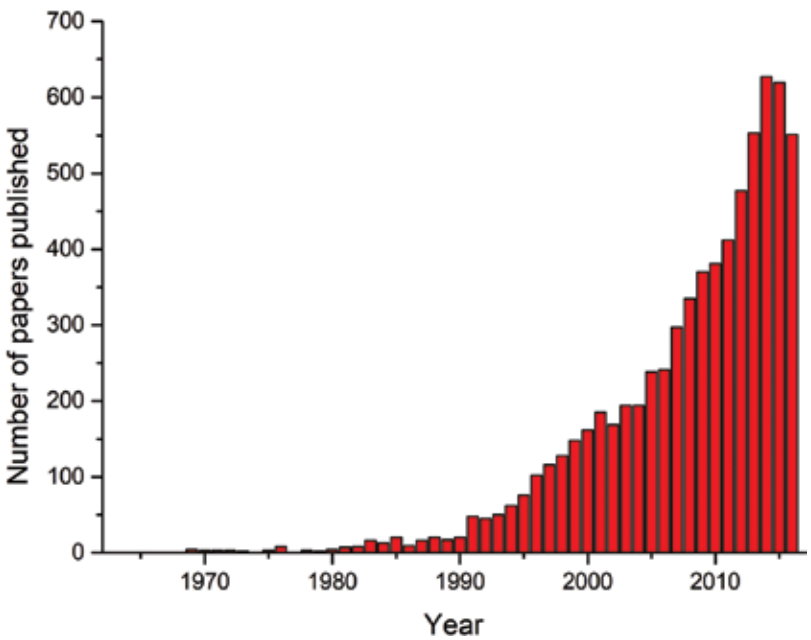

Figure 2. The number of articles published in the Web of Science search by laser-induced breakdown spectroscopy (LIBS) in 1963–2016.

Because of many advantages of LIBS, it has been applied to a number of analytical domains, for example, various alloys [5–7], slags [8, 9], soil [10], rocks [11, 12] and isotopes [13]. We searched for all scientific papers from 1963 to 2016 on Web of Science with laser-induced breakdown spectroscopy (LIBS). The statistical results are presented in **Figure 2**. It can be seen that LIBS has developed rapidly since 1990.

2. Pretreatment of samples and spectra

2.1. Sample pretreatment

One of the most widely cited advantages of LIBS is that it does not require sample preparation, but this may also be the biggest limitation for improving its consistancy. In general, LIBS performance may be enhanced using two main approaches: pretreatment of samples and spectra. Many homogeneous solid samples require no sample preparation, for example, glass, alloy and plastic. For powder samples (e.g., cement [14], soil [10] and coal [15]) which can press the cake directly, it must be consistent with the standard sample preparation process used for calibration during the pressing process. Comparing with solid samples, the direct analysis of liquid samples by using LIBS has many disadvantages: splash, less excitation and fluctuation of liquid level. The simplest way to change a liquid sample into a solid sample is to freeze it [16, 17]. Sobral et al. [18] investigated the detection sensitivity of Cu, Mg, Pb, Hg, Cd, Cr and Fe traces in water and ice samples under the same experimental conditions by using LIBS. Another effective way that can be used for liquid analysis in a solid matrix configuration consists of using an absorbent substrate, for example, plant fiber spunlace nonwoven [19], absorbent paper [20], thin wood sample [21, 22] and membrane-based filter paper [23]. Now LIBS analysis of aerosols is mainly of two categories: direct analysis and enrichment. However, the detection limit and the statistical results of the direct analysis are still relatively poor. On the other hand, the substrate-based collection does not provide as instantaneous information and does allow one to achieve lower detection limits by increasing the sample flow rates and sampling times.

2.2. Correction and removal of continuum background

The detected plasma emission spectrum at a given wavelength in a spectrum is the sum of the analyte signal and the continuum background. The analyte signal is often overwhelmed by the continuum background, which interferes the true intensity of signal and compromises spectral clarity and hence reduces the accuracy of quantitative analysis. Zou et al. [24] developed a modified algorithm of background removal based on wavelet transform for spectrum correction and applied to low-alloy steel samples. This method can effectively improve the quality of the signals and the accuracy of the regression model. Sun et al. [25] presented a method that can automatically estimate and correct varying continuum background emission. Simulations and experiments were made to successfully prove the efficiency of the method. The proposed method scarcely needs people's intervention and can automatically and flexibly estimate varying continuum backgrounds over a very wide spectrum range. Another way to deduct a continuous background is to add a polarizer to the collected light path. Penczak et al.'s [26]

research results show that the continuous background of the Al plasma emission spectra induced by 800 nm femtosecond pulse laser is strongly polarized. The use of a polarizer can effectively filter the continuous spectrum, thus improving the signal-to-noise ratio and the signal-to-back ratio of the characteristic spectrum.

2.3. Spectral normalization

In order to increase the stability of the signal, the analyte signal intensity can be normalized using a parameter representative of the actual plasma conditions. In general, there are three main standardized methods [27]: (1) normalization by using the intensity of an internal standard line; (2) normalization by using a reference signal; and (3) compensation for the plasma conditions. Castro et al. [28] used 12 different types of data normalization to reduce the interference matrix and to improve the calibration models. Their findings show that the application of normalization modes was useful to compensate for the differences among sample matrices. Models without normalization presented two- to fivefold higher errors. Karki et al. [29] studied the analytical performance of six different spectrum normalization techniques, namely internal normalization, normalization with total light, normalization with background along with their three-point smoothing methods for quantification of Cr, Mn and Ni in stainless steel. The final results show the superiority of internal normalization technique over normalization with total light and normalization with background techniques irrespective of whether it is Cr, Ni or Mn analysis. Wang et al. presented three spectrum standardized methods in order to improve the reproducibility of LIBS measurements which are named the spectrum standardization approach [30], the sampled spectrum standardization approach [31] and the multivariate spectrum standardization method, respectively. In spectral standardization, a particular example is the use of acoustic signals [32, 33] or laser-induced plasma image [34].

2.4. Automatic identification of emission lines

LIBS can excite all the elements of the spectrum of the sample, so reliable and fast identification of emission lines in laser ablation of multicomponent samples is crucial. Labutin et al. [35] applied an algorithm to automatically identify emission lines in LIBS. The algorithm is implemented by three parts: simulation of the set of spectra corresponding to different temperature and electron density, searching the best correlated pair of a model spectrum and an experimental one, and attributing the peaks with certain lines. Ukwatta et al. [36] consider the problem of element detection as a multilabel classification problem, using support vector machines (SVMs) and artificial neural networks (ANNs) for multielement classification. The proposed algorithm is evaluated by using the LIBS image obtained from the experiment. The accuracy of the machine learning method to identify the elements correctly can reach 99%. Mateo et al. [37] developed the software package SALIPS, which can quickly and semiautomatically identify the spectrum peak and give the element composition of the analytical sample. The software package simulates the spectrum by using the relative intensity of the atomic line in the NIST database. In order to facilitate visual comparison, it can present both the simulated and experimental spectra on the same plot.

3. Calibration methods of LIBS

A number of calibration methods have been applied to various research fields and physical state samples of LIBS quantitative analysis. We cannot involve all published research articles, and only a few of the most commonly used calibration methods are reviewed.

3.1. Univariate analysis

The fitting area intensity I corresponding to the transition between lower level E_l and upper level E_u of an atomic species α can be expressed as:

$$I = FN_{\alpha}^{I}A_{ul}\frac{g_u}{U_{\alpha}^{I}(T)}\exp\left(-\frac{E_u}{k_B T}\right) \tag{1}$$

where F is the experimental parameter, N_{α}^{I} is the atomic number density, A_{ul} is the transition probability, g_u is the upper-level degeneracy and $U_{\alpha}^{I}(T)$ is the partition function at the temperature T. For the same sample, if the temperature and density of each laser-induced plasma are constant, then I is proportional to the elemental concentration C. If there are a series of samples with different C, one can establish a calibration line between spectral intensity and element concentration.

$$C_i = b_0 + b_1 I_i + e_i \tag{2}$$

where b_0 and b_1 are model parameters, e_i is the random error and i is the number of samples. The parameter $\widehat{C}_i$ is the estimate value of C_i, namely

$$\widehat{C}_i = b_0 + b_1 I_i \tag{3}$$

and

$$e_i = \widehat{C}_i - C_i \tag{4}$$

In the regression analysis, the best estimate of b_0 and b_1 is obtained based on a set of I and C, which makes the $\widehat{C}$ and C to the nearest degree. For example, Bhatt et al. [38] choose Ce II 413.38, 418.65, and 439.16 nm to establish a univariate linear calibration curve, as shown in **Figure 3**.

Correlation coefficient R, also called the Pearson coefficient, is often used to denote the correlation between I and C, which is defined as:

$$\mathrm{R} = \frac{\sum_{i=1}^{n}\left(I_i - \overline{I}\right)\left(C_i - \overline{C}\right)}{\sqrt{\sum_{i=1}^{n}\left(I_i - \overline{I}\right)^2}\sqrt{\sum_{i=1}^{n}\left(C_i - \overline{C}\right)^2}} \tag{5}$$

Correlation coefficient $|R| \leq 1$, and it is closer to 1, indicating the better relevance. Most of the LIBS papers report R^2 which provides fast information about the correlation of the

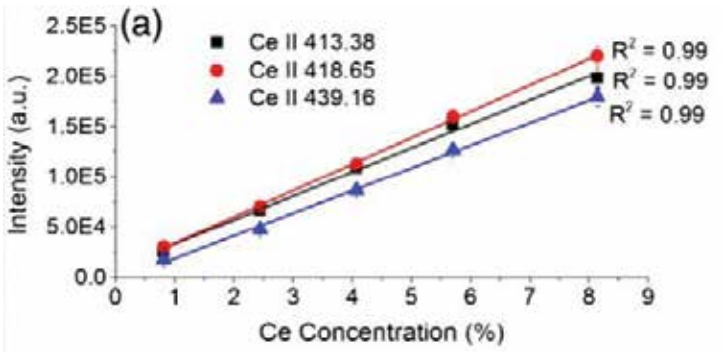

Figure 3. Simple linear regression calibration curves for Ce [38].

data and consequently a fast first knowledge about the prediction ability of the model since poor correlation necessarily implies poor predictive ability. However, it should be noticed that a model with a value of R^2 close to 1 may indeed have a poor accuracy for prediction [39].

Precision is described by the standard deviation (SD), the relative standard deviation (RSD in %) and the root-mean-square error (RMSE), which can be expressed as:

$$\mathrm{SD} = \sqrt{\frac{\sum_{i=1}^{n} \left(C_i - \overline{C}\right)^2}{N}} \tag{6}$$

$$\mathrm{RSD}(\%) = \frac{\mathrm{SD}}{\overline{C}} \times 100 \tag{7}$$

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n} \left(\widehat{C}_i - C_i\right)^2}{n}} \tag{8}$$

In order to describe the lower limits of a quantitative model, the limit of detection (LOD) can be calculated by the following equation:

$$LOD = 3\frac{\sigma C}{I} \tag{9}$$

where σ is the standard deviation of the background and C/I is the reciprocal of the slope of the calibration curve. The calculated values of LOD for different elements are presented in **Table 1**.

3.2. Multivariate analysis

3.2.1. Multiple linear regression

For LIBS, the line of an element is not one. If there are m variables and n samples, then

Elements	LOD (%)
Ce	0.098
Eu	0.052
Gd	0.077
Nd	0.047
Sm	0.25
Y	0.036

Table 1. Limit of detection (LOD) estimated for different elements.

$$C_i = b_0 + b_1 I_{i1} + \ldots + b_m I_{im} + e_i \qquad (i = 1, 2, \ldots, n), \tag{10}$$

$$Q = \sum_{i=1}^{n} e_i^2 = \sum_{i=1}^{n} \left(C_i - \widehat{C}_i \right)^2 \tag{11}$$

where I is the intensity of different spectral lines from the same element, e_i is residual, Q is the sum of squares of the residuals and $\widehat{C}_i$ is the estimate value of C_i. One can get b_0, b_1, …,b_m when Q value achieves the minimum value.

For example, Chen et al. [40] used the multiple linear regression method to quantitatively analyze chromium in potatoes. The characteristic line of Cr can be considered that the concentration of Cr (C_i) has a relationship with the intensity of Cr and/or the other corresponding elements. They normalize the quantitative analysis of Cr by considering the influence of the Ca matrix. Four independent variables (I_{Cr}, $I_{\sum Cr}$, I_{Ca} and $I_{\sum Ca}$) are used to test the performance of different linear regression methods, where I_{Cr} is the intensity of Cr I 425.43 nm, $I_{\sum Cr}$ is the sum of three Cr lines (Cr I:425.43, Cr I 427.48 and Cr I 428.97 nm), I_{Ca} is the intensity of Ca I 431.86 nm and $I_{\sum Ca}$ is the sum of five Ca lines (Ca I 422.67, Ca I 428.30, Ca I 430.25, Ca I 430.77, and Ca I 431.86 nm). Different combinations of the four independent variables were selected for unary, binary, ternary and quaternary linear regression analyses. The results of quantitative analysis of Cr element by the linear regression method with different variables are indicated in **Table 2**.

3.2.2. Principal component regression

In the LIBS quantitative analysis, the calculated concentration is affected by the lines of objective elements and other elements. In the study of empirical questions, in order to analyze the problem comprehensively and systematically, one must consider many spectral lines of many elements. Because each line reflects the information of the element concentration in varying degrees, and the lines have a certain correlation with each other, the information reflected in the calculation overlaps to some extent. In the study of multivariate problems by statistical methods, too many variables will increase the amount of calculation and increase the complexity of the problem. It is hoped that the variables involved in the process of quantitative analysis are less and the amount of information is more. Principal component analysis (PCA) is adapted to this requirement and is an ideal tool for solving such problems.

Calibrate method	Input variables	R^2	Predicted value (μg/g)	Relative error (%)
Unary	I_{Cr}	0.007	S1:138.208	7.674
			S2:140.308	7.771
Binary	I_{Cr},$I_{\sum Cr}$	0.887	S1:143.649	11.913
			S2:132.129	13.147
Ternary	I_{Cr},$I_{\sum Cr}$,I_{Ca}	0.890	S1:142.413	10.950
			S2:132.687	12.780
Quaternary	I_{Cr},$I_{\sum Cr}$,I_{Ca},$I_{\sum Ca}$	0.987	S1:133.659	4.130
			S2:159.965	5.151

Table 2. Quantitative results of Cr by different linear regression methods.

Principal component analysis can transform a set of variables that may have correlation into a set of linearly uncorrelated variables by orthogonal transformation. The variable after the conversion is called the principal component. Principal component regression (PCR) is a regression analysis method for analyzing multiple regression and is based on PCA. In general, predicting the concentration by PCR can be divided into three steps: first, the PCA is performed on the data (spectral) matrix of the original independent variables, and the appropriate number of principal components is selected by finding the eigenvalue, eigenvector, variance contribution rate and cumulative contribution rate. Second, the selected principal component is analyzed by the ordinary least-squares method. Finally, the strongest possible correlations between the orthogonal PC scores and elemental composition are established. When selecting principal components by PCR, only the independent variables are taken into account, and the dependent variables are ignored. It can reduce the dimension of variables and address the problem of multiple collinearity but cannot distinguish noise when there is a lot of noise in the independent variable (signal) and lose some information of the original variables, so a better regression model will not be obtained.

Death et al. [41] applied PCR to determine the elemental composition of a series of run-of-mine (ROM) iron ore samples. LIBS spectral data were recorded in three separate spectral regions (250 nm, 400 nm and 750 nm) to measure major, minor and trace components of the iron ore sample pellets. Background stripping, normalization and spectral cleaning were applied to minimize RSD of the LIBS data. PCR analysis was used to produce calibration models of Fe, Al, Si, Mn, K and P. Independent LIBS measurement data are used to verify these calibration models. The model R^2 for Fe, Al, Si and K is 0.99, 0.98, 0.99 and 0.84, respectively. As an example, PCR calibration model of Fe is shown in **Figure 4** [41].

3.2.3. Partial least squares regression

The main purpose of PCR is to extract relevant information hidden in the spectral line and then used to predict the concentration. This approach allows one to use only those independent variables, and the noise will be eliminated, so as to improve the quality of the predictive model. However, PCR still has some defects, and some useful variables whose correlation is very small are easily missed when the principal component is selected. If we choose for

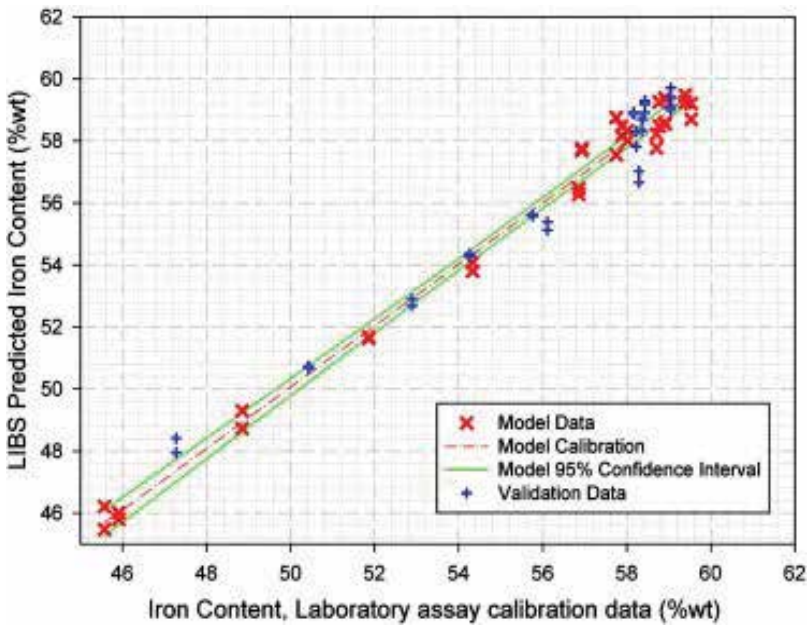

Figure 4. PCR calibration model determined for iron using the 250-nm LIBS data.

each component, it is too difficult. Partial least squares regression (PLSR) is a new multivariate statistical data analysis method. It mainly studies the regression modeling of multiple dependent variables to multiple independent variables. PLSR is more effective, especially when the variables are highly linearly correlated. In addition, PLSR solves the problem that the number of samples is less than the number of variables. Partial least squares (PLS) is the advantage of three analytical methods, which are PCA, canonical correlation analysis and

		Region I		Region II		Region III	
		8 mJ	**3 mJ**	**8 mJ**	**3 mJ**	**8 mJ**	**3 mJ**
Integrated spectra	WBC	3.47	3.27	3.27	2.74 (2)	2.72 (2)	2.63 (2)
		4	3.67	3.76	3.36	2.92	2.85
	BC	1.97	2.27	2.56	2.27	2.58 (2)	2.09 (2)
		2.22	2.54	2.03	2.67	2.78	2.57
Time-resolved spectra	WBC	3.11	2.27	3	2.02	2.73 (2)	3.96 (2)
		3.46	2.51	3.4	2.22	3.09	4.36
	BC	3.07	2.32	3.13	2.23	2.47 (2)	3.73 (2)
		3.36	2.56	3.57	3.31	2.9	4.57

Values in brackets correspond to number of factors if different from three.
WBC = without background correction. BC = with background correction

Table 3. Standard error of calibration (SEP) and prediction (SEV, in italics) estimated during the determination of silver for autoscaled data.

multiple linear regression analysis. Both PLS and PCA try to extract the maximum information reflecting the data variation, but PCA only considers one independent variable matrix, while PLS has a response matrix, so it has predictive function. PLS avoids potential problems such as nonnormal distribution of data, factor indeterminacy and unidentifiable models. PLS has two types (PLS-1 and PLS-2), and PLS-1 corresponds to the case where there is only one dependent variable. PLS-2 corresponds to the case where there are several dependent variables. Although PLSR is more complex than PCR, and the tendency of overfitting is stronger, better results can be obtained by using PLS to analyze low-precision data or high-complexity systems.

The input variable of PLS can be characteristic spectral lines [42], partial spectral region [43] or full spectrum [44]. Amador-Hernandez et al. [45] used PLS-1 to quantify gold and silver gold and silver in Au-Ag-Cu alloys. The influence of spectral region (266–340 nm, 266–269/326–340 nm and 269–313 nm), laser energy (3 mJ, 8 mJ), background correction and integration time on the quantitative analysis of PLS was studied, respectively (**Table 3**).

3.2.4. Artificial neural network

To overcome the poor precision of the calibration curve methods and the limitations of nonlinear problems, scholars have proposed the use of statistical methods for the quantitative analysis by LIBS. Artificial neural networks (ANNs) are computing systems inspired by the biological neural networks that constitute animal brains. Such systems learn (progressively improve performance) to do tasks by considering examples, generally without task-specific programming. The following outstanding advantages of artificial neural networks have attracted great attention in recent years: (1) it can fully approximate any complex nonlinear relationship; (2) all the quantitative or qualitative information is stored in the neurons in the network, so it has strong robustness and fault tolerance; (3) ANNS adopts the parallel distribution processing method, so that it can perform a large number of operations quickly; (4) ANNS can learn and adapt to unknown or uncertain system; and (5) it can handle both quantitative and qualitative information at the same time. An artificial neuron network (ANN) is a computational model based on the structure and functions of biological neural networks. Artificial neural networks usually contain input layer, hidden layer (competitive layer) and output layer, as shown in **Figure 5**.

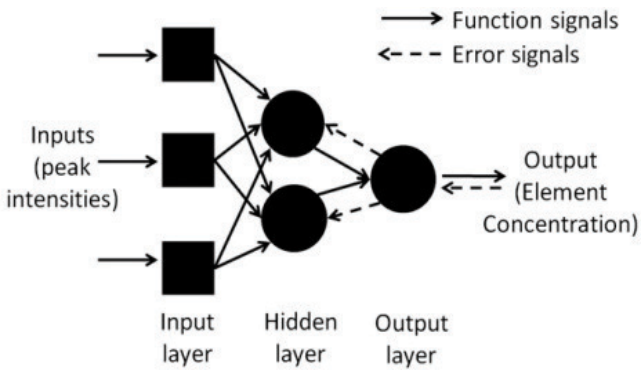

Figure 5. Schematic of the three-layer artificial neural network [46].

For example, El Haddad et al. [46] used an artificial neuron network to analyze the heavy metals in soil and predict the concentration of element. They used average relative error of calibration REC (%) and the average relative error of prediction REP (%) to evaluate the predictive quality of the ANN models. REC and REP were preferred to RMSE because they provide percentage instead of absolute values. Before using the artificial neural network, it is necessary to optimize the parameters such as the number of neurons and training times.

$$\mathrm{REC}(\%) = \frac{100}{N_c}\sum_{i=1}^{N_c}\frac{|c_i - \widehat{c}_i|}{c_i} \tag{12}$$

$$\mathrm{REP}(\%) = \frac{100}{N_p}\sum_{i=1}^{N_p}\frac{|c_i - \widehat{c}_i|}{c_i} \tag{13}$$

where N_c is the number of samples in the calibration and Np is the number of samples in the prediction sets, respectively (**Table 4**).

3.2.5. Support vector regression

The neural network structure design depends on the designer's experience and prior knowledge, while support vector machine (SVM) is based on statistical theory, which has a strict theoretical and mathematical basis. The neural network learning algorithm lacks quantitative analysis and complete theoretical support, and it also needs a lot of samples to learn. SVM is often used to pattern recognition, classification and regression analysis of small samples, nonlinear and high-dimensional data and can achieve very good results. SVM is based on the principle of structural risk minimization, which can ensure that the learning machine has a good generalization ability. SVM for regression prediction is called support vector regression (SVR). SVR also can guarantee the global optimality of the algorithm and avoid the local minimum problem that the neural network cannot solve. Therefore, when there are a small number of samples, it is better to use SVR instead of neural network. It is important to note that the selection of optimized significant penalty parameter C and the kernel parameter of RBF-δ is more sensitive for the SVR model.

For example, Gu et al. [47] used three segmental spectra of 393–397 nm, 422–423 nm and 425–427 nm as the input variables of SVR model to predict the content of Cr in soil samples. They got better predictive results that $R^2 = 0.999$ and the absolute relative error is 2.61% and the slope of the calibration curve is closer to 1, as shown in **Figure 6**.

Output	Input element	REC (%)	REP (%)
Al	Al, Ca, Ba, Fe, Ti	18.7 ± 0.8	19.3 ± 2.1
Ca	Ca, Ba, Fe, Ti	9.4 ± 0.4	15.2 ± 0.8
Fe	Fe, Ba, Ca, Ti	15.5 ± 0.6	16.8 ± 0.9

Table 4. Average relative errors of calibration (REC) and prediction (REP).

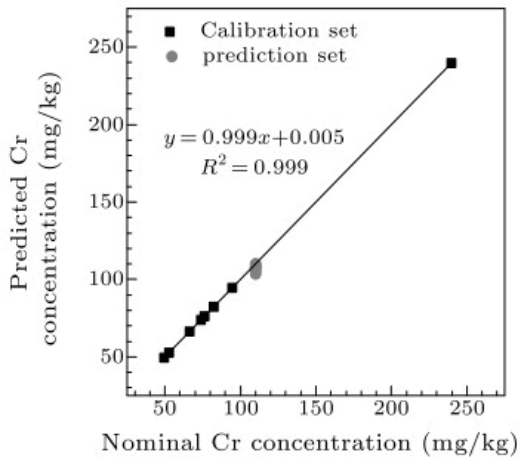

Figure 6. The calibration curve of Cr by the SVR model with segmental spectra input.

3.3. Calibration-free laser-induced breakdown spectroscopy

LIBS offers a strong potential for analysis in situ and in real time, not requiring complex sample preparation. This allows it to be applied quickly and extensively to qualitative analysis, but quantitative analysis is very difficult. Even with a given experimental configuration, the laser-induced breakdown spectrum is not only dependent on the concentration of the analyte but also dependent on the composition of the matrix and their polymerization state. Matrix effects play an important role in quantitative analysis of LIBS. In order to overcome the matrix

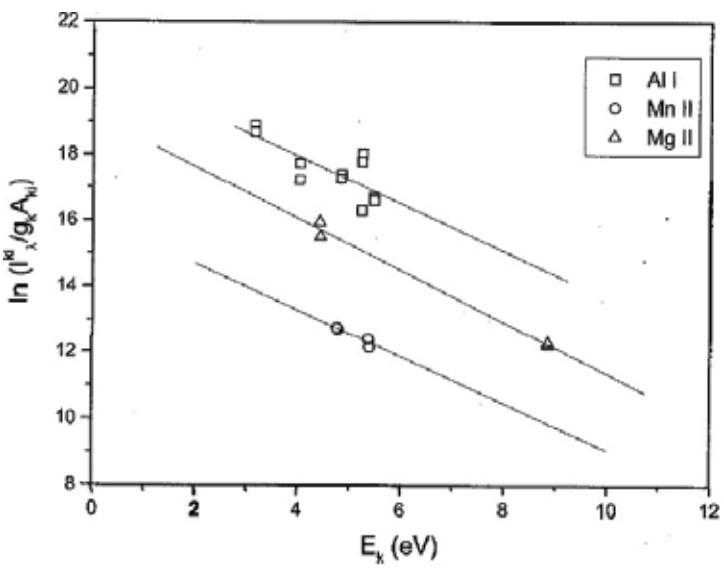

Figure 7. Boltzmann plot containing some data resulting from the analysis of an aluminum alloy. The three lines represent the results of a linear best fit of the Al(I), Mn(II) and Mg(II) data [48].

effect, Ciucci et al. [48] proposed the calibration-free laser-induced breakdown spectroscopy (CF-LIBS) approach which takes the matrix into account as a part of the analytical problem. In local thermodynamic equilibrium (LTE), excited levels are populated according to the Boltzmann distribution and ionization states are populated according to the Saha-Boltzmann equilibrium equation. Each spectral line is represented as a point in a Boltzmann plane where the slope and intercept correspond to the plasma temperature and the concentration of the corresponding element, respectively (**Figure 7**).

Ciucci et al. [48] proposed CF-LIBS for the first time and used it for quantitative analysis of the composition of metallic alloys and quantitative determination of the composition of the atmosphere. CF-LIBS has been applied to many samples, such as aluminum alloys, steel and iron alloys, precious alloys for jewelry, copper alloys, archeological copper artifacts, glasses, pigments on roman frescoes and on parchments, soils and rocks, meteorites, coral skeletons and human hair. However, the accuracy of CF-LIBS is still not high.

4. The comparison of calibration methods

LIBS is an analytical technique that can inspire all the elements in the sample. Univariate analysis uses only partial spectral information and suffers from the strong effects of plasma instability. More importantly, strong matrix effects prevented to apply simple calibration curves. There is no doubt that the multivariate analysis is superior to univariate analysis. This has been proven by numerous researchers, for instance, the analysis of rocks [49], rare earth elements [38], glass [50], cerium oxide [51], alloy steel [52], liquid steel [53], soil [54, 55], soybean oil [56], PZT (Lead Zirconate Titanate) ceramics [57], Pb in navel orange [58], Marcellus Shale [59], tailing cores [60], geologically diverse samples [49], steel melt [61], slurry [62], iron ore [63] and pellets of plant materials [64].

Many multivariate analysis methods have been applied to the quantitative analysis of LIBS, especially chemometric. Generally, the most common chemometric technique applied to concentration measurement by LIBS is PLS. It has been applied to many fields of analysis, such as soil [55, 65, 66], steel [67–69], glass [50], rock [70], iron ore [63] and coal [71, 72]. The rest of the analysis methods are PCR [50, 73, 74], LASSO [75, 76], kNN [77], ANN [78–81], SVM [82, 83] and so on. PLS has been implemented either to calculate the concentrations of a single element (PLS-1) or to simultaneously calculate the concentrations of more than one element (PLS-2). In addition to PLS, other linear (MLR [84, 85], PCR [50, 73] and LASSO [75–77]) and nonlinear regression methods (ANN [86, 87]) have been applied to LIBS quantitative analysis. In order to discern the most effective models for interpreting chemical abundances from LIBS spectra of geological samples, Boucher et al. [77] had studied nine kinds of linear and nonlinear regression methods. The advantages and disadvantages of various methods are introduced, as shown in **Table 5**. The final results show that nonlinear methods tend to overfit the data and predict less accurately, while the linear methods proved to be more generalizable with better predictive performance. The performance of different models for different oxides is different. At present, multivariate analysis, especially

Method	Advantage(s)	Disadvantage(s)	Other
PLS	Used when **X** has many collinear features and when p > > N. Provides a stable multivariate model that can account for all oxides (PLS-2).	Provides a complex model in which all coefficients are linear combinations of the original channels. Involves a complex optimization problem with no simple, closed-form representation.	Linear, uses all channels (not sparse)
LASSO	Provides an interpretable model, selects subset of predictors with the strongest effects on the response variable. Can be used for feature selection when less data are available.	Arbitrarily chooses one covariate from a group of highly collinear covariates to use in the model and discards the rest.	Linear, sparse, eliminates noisy channels
Elastic net	Performs well in the p > > N case. Provides an interpretable model that is more stable than the lasso. Useful for feature selection.	Cannot be used for feature selection in situations when less data are available because it overwhelms the data with too many model variables.	Linear, sparse, eliminates noisy channels
PCR	Decorrelates the data and reduces its dimensionality, combating the "curse of dimensionality"	Higher-order polynomial kernels tend to overfit the training set and poorly predict the testing set in this application.	May be linear or nonlinear; both use all channels
SVR	Performs well with a linear kernel. Can be either linear or nonlinear depending on the kernel.	As above, polynomial kernels tend to overfit the training set and poorly predict the testing set in this application.	May be linear or nonlinear; either uses all channels
kNN	Requires no model training other than choosing the number of neighbors, reducing run time and making it scale well to large data sets.	Tends to overfit the training data and is only as effective as the distance metric used to compare samples.	Nonlinear, uses all channels

Table 5. Comparison of various regression methods.

PLS, is our best choice. But it should be pointed out that multivariate quantitative analyses present a high risk of overfitting.

CF-LIBS can overcome the influence of matrix effect, but the poor analysis accuracy has been the fatal shortcoming of CF-LIBS. This is mainly due to the fact that the laser-induced plasma is a very complex object and its realistic description is not attainable with simple mathematical models [88]. A number of researchers have made some modifications to the CF-LIBS algorithm, such as self-absorption [89, 90]. In recent years, several research groups [91–94] began using standard samples to improve the accuracy of the nonstandard analysis. Cavalcanti et al. [92] presented and used one-point-calibration CF-LIBS to analyze a set of copper-based samples. The results show that the new method achieves similar or even higher accuracy than the calibration curve.

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Chapter 6

Linearity of Calibration Curves for Analytical Methods: A Review of Criteria for Assessment of Method Reliability

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Additional information is available at the end of the chapter

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Abstract

Calibration curve is a regression model used to predict the unknown concentrations of analytes of interest based on the response of the instrument to the known standards. Some statistical analyses are required to choose the best model fitting to the experimental data and also evaluate the linearity and homoscedasticity of the calibration curve. Using an internal standard corrects for the loss of analyte during sample preparation and analysis provided that it is selected appropriately. After the best regression model is selected, the analytical method needs to be validated using quality control (QC) samples prepared and stored in the same temperature as intended for the study samples. Most of the international guidelines require that the parameters, including linearity, specificity, selectivity, accuracy, precision, lower limit of quantification (LLOQ), matrix effect and stability, be assessed during validation. Despite the highly regulated area, some challenges still exist regarding the validation of some analytical methods including methods when no analyte-free matrix is available.

Keywords: analytical method, calibration, linearity, regression analysis, validation

1. Introduction

Calibration curve in bioanalytical method is a linear relationship between concentration (independent variable) and response (dependent variable) using a least squares method. This relationship is built to predict the unknown concentrations of the analyte in a complicated matrix. The unknown samples can be from a wide range of sources: food and agricultural, pharmaceutical formulations, forensic and the clinical pharmacology studies. This chapter is more focused on the bioanalytical methods in which an analyte is measured in blood, plasma, urine or other biological matrices. However, the main concepts are applicable to the other analytical approaches.

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The quality of a bioanalytical method is highly dependent on the linearity of the calibration curve [1]. A linear calibration curve is a positive indication of assay performance in a validated analytical range. Other characteristics of the calibration curve, including regression model, slope of the line, weighting and correlation coefficient, need to be carefully evaluated. In the following sections, each of those parameters is explained, and few practical examples have been used to further discuss the concepts.

After the calibration model is chosen, it is required to demonstrate that all future measurements will be close to the true values of the content of the analyte in the sample. This will be achieved during validation of the analytical method. There are international guidelines for the validation of the analytical methods, which need to be followed closely in order to have more consistent data throughout different laboratories and increase the chance of their acceptability by the regulatory authorities.

2. Aims

The aim of this chapter is to discuss different aspects of linearity and relevant assumption as a practical guide to develop a robust analytical method in order to predict true concentrations of the analytes in samples.

3. Calibration curve: definitions and characteristics

3.1. Regression analysis

Regression analysis is a deterministic model, which allows predicting of the values for a dependent variable (Y) when an independent variable (X) is known. The model determines the kind of relationship between X and Y. The experimental values rarely fit the mathematical model, and there are differences between the observed and the predicted values provided by the model, which are called residuals (**Figure 1**). The sum of squared residuals needs to be minimised to have the best estimate of the model parameters, and it can be done using the "method of least squares." The simplest regression model is the linear one in which the relationship between X (known without error) and Y (known with error) is a straight line, $Y = a + bX$, where a is the y-intercept and b is the slope of the line [1].

The relationship between an instrument response and the known concentrations of an analyte (standards), which is used as the calibration curve can be explained by a similar regression model. To have a robust calibration line (or curve), a series of replicates of each standard (at least three replicates of 6–8 expected range of concentration values) are recommended. The assumption for this model is that the measurement error is the same and normally distributed for each sample. If this assumption is not applicable, an extended or weighted least squares analysis will be required. The assumption regarding the measurement error must be verified to validate the results found. The distribution properties of the residuals are expected to be normal and centred on zero (Kolmogorov–Smirnov test). If the results found cannot support

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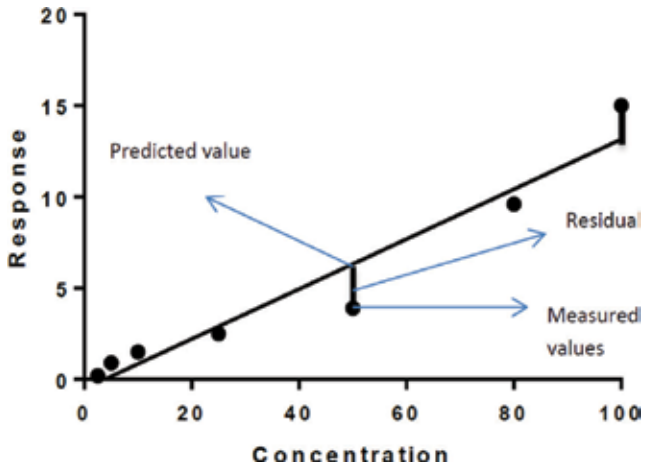

Figure 1. Linear regression model in which the differences between observed and predicted values (residuals) have been shown.

this assumption, the estimated parameters using the model cannot be used, and the model needs to be modified, e.g. using a non-linear model which requires more standard concentrations compared with having a linear relationship between concentrations and instrument response. A linear regression model between calculated standard points and the nominal ones used to evaluate the quality of the fit should have a unit slope and a zero intercept. In case of linear calibration method, the slope should be statistically different from 0, the intercept should not be statistically different from 0 and the regression coefficient should not be statistically different from 1. In case of having a significant non-zero intercept, the accuracy of the method must be demonstrated [2].

A standard 0 must be included in the calibration curve because the instrumental signal is subjected to the same kind of error for all points. The signal for the standard zero should not be subtracted from the response values for other standards before calculating the equation of the regression line because it can cause imprecision during the determination of the concentration values for unknown samples [3].

If one of the standard points deviates greatly from the calibration curve (outlier), it can be removed from the equation provided that six non-zero standards remain after removing the outlier and inclusion of that point can cause the loss of sensitivity or it clearly biases the quality control (QC) results, and the back-calculated standard concentrations deviate from its nominal value. The poor chromatography can also be considered as a justification for removing the outlier standard [4].

In order to verify the accuracy and precision of the analytical method during the period of sample analysis, quality control (QC) samples are prepared and stored frozen at the same temperature as is intended for the storage of the study samples. The calibration curve standards are prepared by spiking the reference standard solutions to the matrix (e.g. plasma or urine) either freshly or by freezing and storage with QC samples [4].

3.2. Weighting in linear regression

When the range in x-values is large, e.g. more than one order of magnitude, the variance of each data point might be quite different. However, the simple least squares method considers that all the y-values have equal variances. Larger deviations at larger concentrations tend to influence the regression line more than smaller deviations associated with smaller concentrations (heteroscedasticity) leading to the inaccuracy in the lower end of the calibration range (see the practical example 1). A simple and effective way to counteract this situation is to use weighted least squares linear regression (WLSLR) [1]. WLSLR is able to reduce the lower limit of quantification (LLOQ) and enables a broader linear calibration range with higher accuracy and precision especially for bioanalytical methods.

Two most commonly used regression models, particularly for liquid chromatography tandem mass spectrometry (LC-MS/MS) calibration curves, are linear and quadratic regression models using non-weighted or weighted least squares regression algorithm. To select the type of calibration curve and weighting, "Test and Fit" strategy is widely used due to its simplicity and lack of statistical analysis and causes inaccuracy in the regression model based on the limited set of test results. The Food and Drug Administration (FDA) guideline suggests that "the simplest model that adequately describes the concentration-response relationship should be used and selection of weighting and use of a complex regression equation should be justified" [5]. However, other experts suggested that a weighting should be used if homoscedasticity was not met for the analytical data. By neglecting the weighting for analysing data with heteroscedastic distribution, a precision loss as big as one order of magnitude in the low concentration region of the calibration curve could happen [4].

For most immunoassay methods, the response is a non-linear function of the analyte concentration, and the standard deviations (SD) of the calculated concentrations are not a constant function of the mean response; therefore, a weighted, non-linear least squares method is generally recommended for fitting dose-response data. The nonevidence-based weights (e.g. 1/Y or 1/X) are not recommended without assessment of the response-error relationship. A reference model for immunoassay data employs the four-parameter logistic (4PL) equation to fit the concentration-response relationship and a power-of-the-mean (POM) equation to fit the response-error relationship [6].

3.3. Correlation coefficient

Linearity of the calibration curve is usually expressed through the coefficient of correlation, r, or coefficient of determination, r^2. A correlation coefficient close to unity (r = 1) is considered by some authors' sufficient evidence to conclude that the calibration curve is linear. However, r is not an appropriate measure for the linearity. The FDA guidance for validation of analytical procedures [5] recommends that the r should be submitted when evaluating a linear relationship and that the linearity should be evaluated by appropriate statistical methods, e.g. analysis of variance (ANOVA). This guidance does not suggest that the numerical value of r can be used as a degree of deviation from linearity.

Other mathematical measures, including slope standard relative deviation or goodness of fit, can be used to evaluate the linearity [3]. Using residual plots is a simple way to check the

linearity. The residuals are expected to be normally distributed for a linear model, so a plot of them on a normal probability graph may be useful. Any curvature suggests a lack of fit (LOF) due to a non-linear effect. A segmented pattern indicates heteroscedasticity in data, so weighted regression model should be used to find the straight line for calibration [7].

A clear curved relationship between concentration and response may also have an r value close to one. Two statistical tests, including the lack-of-fit and Mandel's fitting tests, are suitable for the validation of the linear calibration model (practical example 2 [8]). A straight-line model with r close to 1, but with a lack of fit, can produce significantly less accurate results than its curvilinear alternative. A straight-line calibration curve should always be preferred over curvilinear or non-linear calibration models if equivalent results can be obtained and is easier to implement [8].

3.4. Slope of the curve and application in matrix effect and detection limit

Slope of the calibration curve can be used to estimate the detection limit of the assay [9]. Three times the standard deviation value of the response corresponding to the blank according to Eq. (1), obtained for seven determinations, divided by the slope of the calibration line (note that we are calculating the standard deviation of the concentration corresponding to the blank equation, and again the imprecision of the value of the slope is not taken into account) [3]:

$$\mathrm{LOD} = 3.3 \times \left(S_Y/a\right). \tag{1}$$

S_Y denotes the SD of responses, Y, for blanks or around expected LOD (limit of detection) and "a" for the slope of a linear calibration line. If the calibration curve is linear, "a" is constant, and the estimation of LOD is easy to calculate. However, when the calibration curve is not linear, e.g. in enzyme-linked immunosorbent assay (ELISA), the definition needs to be modified. In the case of ELISA, when there is a semilogarithmic calibration curve over a wide range of concentrations, the detection limit is calculated using a differential coefficient which is obtained using a computer programme [9].

It is assumed that a validated analytical method should have constant slope over the period of sample analysis. Variation in the slope might be due to the laboratory errors during sample preparations, change in the internal standard (IS) of working solution concentrations between preparations, instrument variations such as changes in mass spectrum (MS) calibrations, MS signal cross contributions between analyte and IS and matrix effect (ME) [10]. Although there is no criteria in the international guidelines to report the slope, monitoring the slope can provide valuable information regarding the quality of the sample analysis.

ME can also affect the slope of the calibration curve. Coeluting of the matrix components escaped during extraction may reduce the signal intensity and affect the accuracy and precision of the MS-based assays. The phenomenon is called ion suppression, and it has been shown that the electrospray ionisation responses of organic bases decrease with an increase in concentrations of other organic bases present in the matrix. The ME is especially dependent on the degree of sample clean-up and chromatographic separation of the analyte. When developing high-throughput assays using a short run time, a careful assessment of the ME and ion suppression is necessary [11].

3.5. Internal standard (IS)

IS is a chemical substance that is added in equal amounts to all samples, and it changes the way that calibration curve is prepared. Instead of analyte response, the ratio of the analyte to the IS signal versus the analyte concentration is plotted. The benefits of adding the IS are to correct or compensate analyte losses during sample preparation including transfer loss, adsorption loss, evaporation loss and variation in injection volume and in MS response due to ion suppression or enhancement (ME).

The IS must have similar physicochemical properties and show similar behaviour to the analyte when extracted or run through the analytical column or detection in the analytical system. An external standard also behaves similarly with the analyte, but it is run alone at different concentrations, so a standard curve can be generated. External standards do not correct for losses that may occur during preparation of the sample. Using IS is usually more effective due to lower measurement uncertainty and therefore is more common in analytical chemistry [12].

Two common types of ISs are used: structural analogues and stable isotope-labelled (SIL) ISs or isotope dilution mass spectrometry (IDMS). SIL ISs are more effective. To reduce the interferences between IS and analyte, SIL IS molecular weight is preferred to be ideally 4 or 5 Da higher than that of the analyte. Labelled SIL ISs with 13C and/or 15N are usually superior to those labelled with deuterium (2H, D or d) in terms of performance; however, the synthesis of deuterated ISs is easier and cheaper. The location of stable isotope atoms should be in a way that deuterium-hydrogen exchange is minimised during sample preparation.

A structural analogue of the analyte can be used if SIL ISs are not available or expensive. In this case, the IS should preferably have key structure and functionalities (e.g. –COOH, $–SO_2$, NH_2, halogen and heteroatoms) of the analyte with difference only being C–H moieties (length and/or position). Modifications in key chemical structure and/or functionalities cause significant differences in ionisation pattern and even extraction recovery. The IS should not be similar or converted to any in vivo biotransformed products of the analyte (e.g. hydroxylated or N-dealkylation metabolites). An appropriate structural analogue IS can be selected from the same therapeutic class as the analyte or by key chemical structure and preferably a compound that is not very commonly prescribed because those compounds may be present in pooled blank plasmas used for preparation of the calibrators and QCs. Other parameters for choosing a right structure analogue IS are physicochemical properties, such as log D (hydrophobicity), pKa and water solubility. For selection of the IS, it may be difficult to have a compound to track the analyte of interest in all the three distinctive stages of LC-MS bioanalysis, sample preparation (extraction), chromatographic separation and mass spectrometric detection. The IS should be chosen depending on which step is more critical. For example, when the extracts of samples contain coeluting matrix components that cause ion suppression, then tracking the analyte during MS detection to avoid or minimise ME becomes more important. The choice of IS is also depending on the extraction method. Tracking an analyte during a simple protein precipitation procedure would be less stringent than that for liquid-liquid extraction (LLE) or solid-phase extraction (SPE) method [13].

It is possible to develop an assay without using any IS, for example, in early drug discovery stage or when clean extracts are used. In this case, ECHO peak technique can be used where

the analyte is used as its own IS. In this method, after the injection of the sample containing the analyte of interest, a standard solution is also injected, which result in two peaks for the analyte, one from the sample and the other from the standard solution with constant concentration (an echo peak). By using their response ratio for quantitation, the ME might be compensated for because the two peaks are affected by the coeluted matrix components similarly [13].

There is no general rule for choosing the IS concentrations. However, the accuracy and precision of the method may be affected if an inappropriate IS concentration is used. As shown in practical example 3, reducing the concentration of IS can lead to the increasingly non-linear calibration curve due to chemical impurity in the reference standard or because of isotope interferences.

When choosing the IS and its concentration, the magnitude of the cross signal contribution between the analyte and IS should be considered. The IS interference signal due to its impurity or isotope interferences should be equal or less than 20% of the LLOQ response and 5% of the IS response for IS-to-analyte and analyte-to-IS contributions, respectively [14]. The minimum IS concentration required (CIS-Min) and the maximum IS concentration allowed (CIS-Max) can be calculated using Eqs. (2 and 3):

$$\text{CIS-Min} = m \times \text{ULOQ}/5. \tag{2}$$

$$\text{CIS-Max} = 20 \times \text{LLOQ}/n. \tag{3}$$

where m and n represent the % of cross signal contributions from analyte to IS and IS to analyte, respectively. As an example, if the cross signal contribution from analyte to IS is 2.5%, the minimum IS concentration calculated accordingly is 50% of the ULOQ. A high IS concentration might be useful in reducing a systemic error in the analysis of unknown samples. If the IS coelutes more closely to the analyte, it will be more effective in minimising ME.

In some cases, the analyte signal might be suppressed by the coeluting IS signal, and therefore the IS concentration must be kept low to maintain a low detection limit. However, it might be required to increase the IS concentration when the analyte suppresses the IS signal.

IS should be added as early as possible to compensate for the variabilities during sample preparation and analysis; however, if the IS structure is not very close to the analyte, it can be used to reduce the variabilities due to the ion suppression or enhancement only and not sample extraction [13].

3.6. Linearity when no analyte-free matrix exists

For making calibrators and QCs, an analyte-free matrix is required. The presence of unknown amount of the analyte in the matrix makes the quantification difficult, and different approaches have been used to overcome the problem including using stripped matrices (filtration on activated charcoal-dextran or dialysis), substitute matrices (e.g. neat solutions, artificial matrices, human serum albumin or 0.9% sodium chloride) or diluted matrices. If the actual matrix is used, various methods are followed including, background subtraction, or the standard addition method [3, 15].

One of the approaches for validation of the assay is to determine the accuracy throughout the validation step, using the biological matrix containing the endogenous compound to prepare the standard curves and all pools of six or more assays of each QC sample [3]. The amount of the analyte in the matrix (C_{basal}) can be computed using a calibration curve in the substitute matrix, and the concentration of the analyte in the QC can be calculated by subtracting the C_{basal} from the calculated one as follows, $C_{real} = C_{found} - C_{basal}$, in which C_{found} is the concentration of the analyte in the QCs calculated against a calibration curve in the substitute matrix and C_{real} is the corrected concentration [3]. When using this approach, the LLOQ of the method cannot be smaller than the endogenous concentrations of the analyte in the matrix, and therefore a lot of blank matrices need to be screened to find the suitable one.

Alternatively, the endogenous concentration of the analyte in the matrix can be subtracted from the added concentrations and uses the subtracted concentrations to build the calibration curve. Using the actual biological matrix for making the calibrators and QCs reduces the recovery and matrix effects between samples and calibrators. Again, the limitation of this method is that the increase in background peak area after spiking with standards has to be at least 15–20% of the background peak area, and the LLOQ is limited by the endogenous background concentration even if much lower concentrations can be detected by the method. Another difficulty is when multiple analytes with different endogenous compounds need to be quantified [15].

Alternatively, the background concentration in the blank matrices can be lowered by dilution of the blank matrices before spiking with standards. However, by diluting the matrix, the composition of the matrices in the study samples versus calibration curve is different leading to different recoveries of the analytes. Therefore, the extraction recoveries of analytes between the matrix and diluted matrix should be determined before using this method [15].

3.6.1. Surrogate matrices

Surrogate matrices can vary widely from a simplest form, mobile-phase solvents (neat) or pure water to a synthetic polymer-based solution. Some biological matrices, e.g. cerebrospinal fluid or tears, are difficult to obtain. The surrogate matrix should simulate the authentic matrix in terms of composition, salt content, analyte solubility, recovery and ME. For example, phosphate-buffered saline (PBS) or bovine serum albumin (BSA) in PBS (20–80 g/L) has the similar protein and ionic strength as human plasma.

To use neat solutions as surrogate matrices, extraction recovery and ME are required to be com parable with the original matrix. For example, thromboxane B2 and 12(S)-hydroxyeicosatetraenoic acid were quantified in human serum using mixture of water/methanol/acetonitrile (80:10:10, v/v/v) as a surrogate matrix, and the ME and recoveries of the analytes were demonstrated to be comparable.

3.6.2. Stripped matrices

Biological matrices can be stripped from particular endogenous components to generate analyte-free surrogate matrices. Adding activated charcoal, for example, can adsorb and remove the analyte from the matrix, but the charcoal must effectively remove from the matrix before

spiking the analyte. Some analytes, e.g. homocysteine, cannot be removed by the charcoal and also the composition of the matrix may change or cause batch-to-batch variation after adding the charcoal leading to the altered analyte recovery and ME. Some light-sensitive analytes can be decomposed by heat or exposing to the light and therefore removed from the matrix.

3.6.3. Method of standard addition

In the standard addition method, every study sample is divided into aliquots of equal volumes, and the aliquots are spiked with known and varying amounts of the analyte to build the calibration curve. The sample concentration is then calculated as the negative x-intercept of the calibration line. This method is very accurate because it allows direct quantitation of endogenous analytes without manual subtraction of background peak areas. The disadvantage of the method is that it requires a large amount of sample and is very time-consuming and labour intensive. Examples of using this method when the analyte-free matrices are not available include measuring abscisic acid, a phytohormone from plant leaves and the emission of polycyclic aromatic hydrocarbons from petroleum refineries. Standard addition can also be used when some matrix components produce MS signals that interfere with the analytes of interest.

We have used this method by some modifications to measure homocysteine and pyridoxal 5-phosphate in samples of human serum and whole blood, respectively [16, 17]. The matrix was first spiked with different concentrations of the analytes, and the endogenous concentrations of the analytes were estimated using the negative x-intercept of the calibration line. Then, the endogenous concentrations were added to the spiked concentrations, and new calibration curves with real concentrations were constructed (practical example 4). QCs were prepared in both actual and surrogate matrices, and the sample volume reduced to only 20 μL to minimise the matrix effect.

3.7. Validation

All the developed analytical methods need to be validated to make sure that each measurement of the content of the analyte in the sample in routine analysis is close to the true values [7]. There are international guidelines for validation of the analytical methods including FDA [6], European Medicines Agency (EMA) [14], International Union of the Pure and Applied Chemistry (IUPAC) [18] and Association of Official Analytical Chemists (AOAC) International. The major parameters need to be validated including linearity, accuracy, precision, specificity, selectivity, sensitivity, ME and stability testing.

3.7.1. Selectivity and specificity

Selectivity is the ability of a method to determine a particular analyte in a complex matrix without interference from other ingredients of the matrix. Specificity, however, is the ultimate in selectivity, and it means that no interference is expected to occur, but these two terms are used interchangeably in the literature. If a method has specificity for an analyte, it means that either you have it or you do not. Selectivity can be graded as low, high, partial, good or bad, but the selectivity refers to 100% selectivity (or 0% interference) [19].

Selectivity can be calculated by comparing the chromatograms obtained after injection of a blank sample with and without the analyte or analytical solutions and with and without the matrix components.

3.7.2. Accuracy

Accuracy (or trueness or bias) is the most important aspect of validation and should be addressed in any analytical method. Accuracy shows the extent of agreement between the experimental value (calculated from replicate measurements) and the nominal (reference) values. Accuracy is a measurement of the systematic errors affecting the method. To estimate the accuracy of a method, the analyte is measured in comparison with a reference material or by spiking known amount of analyte in the blank matrix (QC samples) and calculating the percentage of recovery from the matrix. It can also be estimated using the comparison of the results from the method by a reference method [19].

The guideline for validation of analytical methods by the EMA [14] recommends checking the accuracy within run and between runs by analysing a minimum of five samples per four QC levels (LLOQ, low, medium and high) as a representative of the whole analytical range in at least two different days. The accuracy needs to be reported as the percentage of the nominal concentrations and the mean concentration should be within 15% of the nominal values for all QC levels, except LLOQ, which should be within 20% of the nominal values [14].

3.7.3. Precision

The term precision is defined as the closeness of repeated individual measurements of an analyte under specified conditions. This term is demonstrating the repeatability and reproducibility of the method and expressed as the coefficient of variation (CV). Precision should be measured for LLOQ, low, medium and high QC samples in the same run that accuracy is testing. The acceptance criteria are also similar to the accuracy evaluation [14, 19].

3.7.4. Uncertainty

To make sure that a method is correctly fit for the purpose of measurement, "uncertainty" of the method is required to be evaluated [7].

A detailed list of all possible sources of uncertainty needs to be prepared. A preliminary study may identify the most significant sources of uncertainty. Typically, the two sources of uncertainties are Type A or random error and Type B or systematic error. Random error is caused by unpredictable variations and gives rise to variations in repeated observations. The random error can generally be minimised by increasing the number of observations. Systematic error, however, is a type of errors, which remain constant, or its variation is predictable and therefore independent of the number of observations. The result should be corrected for all recognised significant systematic errors. The steps involved in uncertainty estimation are identification of uncertainty sources, quantification of uncertainty components and calculation of combined and expanded uncertainty. The main sources of uncertainity are sampling, environmental conditions, method validation, instruments, weighting and dilutions, reference materials,

chemicals and in high-performance liquid chromatography (HPLC) are repeatability of peak area, dilutions factors, reference materials and sampling. Sampling, calibration and repeatability were the most significant sources, which affect combined uncertainty [20].

3.7.5. LOD and LLOQ

The LOD is generally defined as the lowest amount of an analyte in a sample that can be detected by a particular analytical method. LOD is usually evaluated using the calculation of the signal/noise relationship considering the assumption that data normality, homoscedasticity and independency of residuals are met. The signal-to-noise ratio is determined by comparing the analytical signals at known low concentrations compared with those of blank sample up to a concentration that produces a signal equivalent to three times the standard deviation of the blank sample [19]. Determination of the LOD is not necessary during the validation, because the assay may have high variability in that level.

On the other hand, the lowest concentration of an analyte in a sample, which can be reliably quantified is defined as the LLOQ. The analyte signal at the LLOQ level should be at least five times the signal of blank sample and the accuracy and precision within 20% of the nominal concentrations. The LLOQ should be selected based on the expected concentrations in the study. For example, for bioequivalence studies the LLOQ should not be higher than 5% of the maximum concentration of the analyte in the samples (C_{max}) [14].

3.7.6. Matrix effect (ME)

ME measurement is necessary for validation when the analytical method uses mass spectrometry as the detector due to the ion suppression or induction caused by the matrix components. The ME evaluation required spiking the analyte (at low and high concentrations) in six lots of matrix obtained from individual donors. First, the ratio of the peak area in the presence of matrix to the peak area in the absence of the matrix is calculated to achieve the matrix factor (MF), followed by the calculation of the IS normalised MF by dividing the MF of the analyte of interests by the MF of the IS. The CV of the IS-normalised MF is calculated from the six lots of the matrix and should be ≤15% (practical example 5). In some cases that this method is not practical (e.g. online sample preparation), the variability of the response should be assessed by analysing at least six lots of matrix spiked at low and high levels. The overall CV should not be greater than 15%. The ME is also recommended to be tested in haemolysed, hyperlipidaemic matrices or plasma collected from renally or hepatically impaired patients depending on the target population of the study [14].

3.7.7. Stability

Stability testing must be planned based on the conditions applied to the samples during processing. The stability is tested using spiked concentrations of the analyte to the matrix at low and high QC levels (six replicates at two levels are generally sufficient). Short-term stability at room temperature (2–8 h depending on the latest period of time required for sample processing), long-term stability at storage temperature (e.g. at −20°C or −80°C), freeze and

thaw and stock solution stabilities are the most common tests. The stability of QC samples are analysed against a freshly prepared calibration curve, and the calculated concentrations should be within 15% of the nominal concentrations. The stability of processed samples in the autosampler temperature also determines how long samples can be stored in the autosampler without the analyte been degraded [14]. Any other variation during sample processing which can potentially affect the stability of the analyte of interest needs to be tested during validation.

4. Practical examples

4.1. Practical example 1: impact of weighting

See **Table 1**.

4.2. Practical example 2: linearity assessment

In **Table 2,** it shows that the linear regression model (LRM) must systemically be rejected at the 95% confidence level ($F_{crit,95\%} = 4.53$) for lack-of-fit test and at 99% confidence level ($F_{crit,99\%} = 10.56$) for Mandel's fitting test. Thus, despite the fact that r and quality coefficient (QC) are greater than 0.997 and lower than 5%, respectively, the linearity of the calibration lines was rejected based on the F-tests. So, the r is not a good measure of the linearity assessment. Even with a QC value less than 3%, the LRM is rejected at the 95% confidence level (**Table 2**). Alternatively, the residual plots give useful information to validate the chosen regression model.

The residual plot can be used to check if the principle assumptions, i.e. normality of the residuals and homoscedasticity, are met when evaluating the goodness of fit of the regression model. The U-shaped residual plot usually shows that a curvilinear regression model is a better fit than an LRM. In order to correct the non-linearity, a quadratic curvilinear function ($f(x) = a + bx + cx^2$) can be chosen. The "lack of fit" tests for the quadratic regression model

Concentration (ng/mL)	Area ratio	Accuracy (no weighting)	Accuracy (1/x weighting)
0	0.002	0	0
6	0.006	125	92.3
18	0.019	104	94.1
37.5	0.400	98.5	94.1
75	0.836	101	99.3
300	3.320	98.5	98.9
480	5.290	97.8	98.5
600	6.890	102	103

Table 1. Increasing the accuracy of the lower end of the calibration curve by applying the weighing.

Linear regression model				Quadratic regression model	
LOF	**Mandel's test value**	**QC (%)**	**r**	**LOF**	**P-value on second-order coefficient**
11.08	51.46	3.93	0.9982	0.63	0.0000
19.42	56.84	4.23	0.9978	1.58	0.0000
7.13	26.29	3.67	0.9985	0.94	0.0006
6.99	37.73	3.79	0.9984	0.18	0.0002
11.43	58.21	4.03	0.9981	0.31	0.0000
29.91	53.02	3.53	0.9986	4.08	0.0000
49.80	71.07	3.76	0.9984	5.69	0.0000
23.77	73.86	3.19	0.9989	1.66	0.0000
31.95	63.37	3.24	0.9988	3.55	0.0000
7.49	33.50	2.92	0.9991	0.54	0.0003
9.99	55.19	3.95	0.9983	0.15	0.0000
10.71	28.65	4.70	0.9975	1.89	0.0005
25.21	79.60	3.34	0.9987	1.62	0.0000
13.16	35.74	3.37	0.9987	1.93	0.0002

For the quadratic regression model, the F-value of the lack-of-fit test and the P-value for testing significance of the second-order coefficient for the quadratic regression model are represented. The significance value at the 95% confidence level is underlined (reproduced from Van Loco et al. [8] with permission from Springer-Verlag).

Table 2. The F-value of the lack-of-fit (LOF) test and Mandel's fitting test is compared with the quality coefficient for several linear calibration lines of Cd.

(QRM) are summarised in **Table 2**. The test for lack of fit indicates that this QRM fits the calibration data at 99% confidence level in all cases except one. To check the suitability of the order of polynomial regression model, the significance of the second-order coefficient needs to be estimated. The P-value on the second-order coefficient, shown in **Table 2**, is systemically smaller than 1%, and therefore a lower order model should not be considered. Moreover, residual plots (**Figure 2**) were constructed for the QRM, and the residuals were randomly scattered within a horizontal band around the centre line. Therefore, the QRM was selected as the reference model. It is noted that an increase of the variance is observed at higher concentrations [8].

As a summary, in this example, a linear model with $r > 0.997$ and QC < 5% but with lack of fit (LOF) yielded predicted values for a mid-scale calibration standard that significantly differ from the nominal ones. The accuracy was overestimated, while the precision on the results was comparable in both LRM and QRM [8].

4.3. Practical example 3: IS concentration and the linearity

The role of IS concentration on the linearity of the calibration curve has been demonstrated by Tan et al. [13]. They presented a case in which decreasing concentration of the IS from 100%

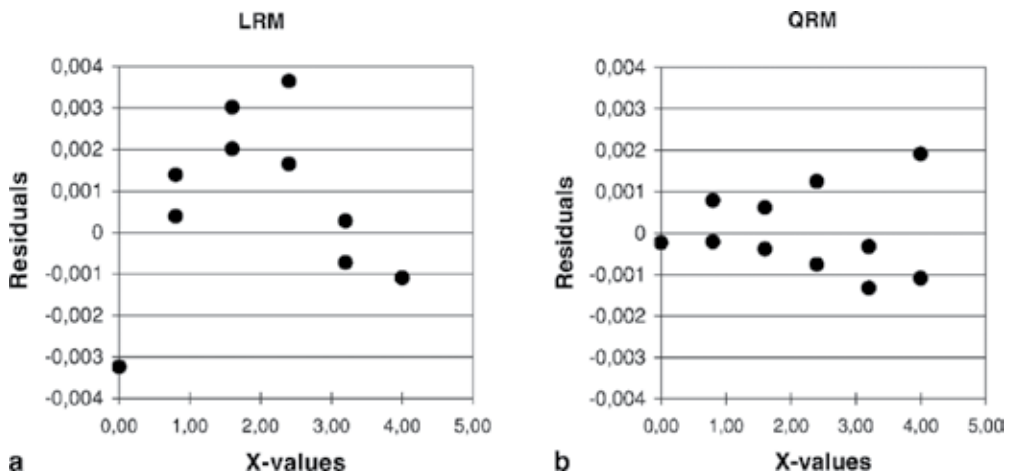

Figure 2. Plots of residuals for (a) the linear regression model (LRM) and (b) the quadratic regression model (QRM) versus predicted values (adopted from Van Loco et al. [8] with permission from Springer-Verlag).

to 5% ULOQ made the calibration curve non-linear. In that case, the cross-contribution from the analyte to the IS is equivalent to 5% of the concentration of the analyte. The cross-signal contribution from the analyte to the IS is either due to the isotope interference or chemical impurity in reference standard [13].

4.4. Practical example 4: method of standard addition for homocysteine calibration curve

Table 3 shows the calculated calibration curve data for homocysteine standard solutions spiked into a pooled human serum.

To estimate the endogenous concentrations of homocysteine in the sample of pooled human serum, the negative x-intercept of the curve is calculated:

$$x = \frac{-0.262}{0.000598} = 438\ \text{ng/mL}. \quad (4)$$

Spiked concentrations (ng/mL)	Calculated concentration (ng/mL)
0	N/A
50	30.87
600	632.76
1100	1107.05
1600	1652.48
2100	2167.02
2600	2584.02
3100	3031.75

Regression equation: y = 0.000598 x + 0.262.

Table 3. Homocysteine calibration curve: the x-axis is representing the spiked concentrations.

Then, the nominated concentration is changed to the spiked + endogenous concentrations, and a new calibration is constructed (**Table 4**).

Now, by comparing the detector response for the unknown samples with the second calibration curve, the unknown sample concentrations can be calculated.

4.5. Practical example 5: ME calculations

Table 5 is representing the analyte peak area spiked in six different lots of human plasma. The MF has been calculated by dividing the area of analyte (or IS) in each matrix to the average peak area of the analyte (or IS) in the pure solutions. The IS-normalised MF is the ratio of the MF for the

Spiked + endogenous concentration (ng/mL)	Calculated concentration (ng/mL)
0 + 438 = 438	381.51
50 + 438 = 488	469.28
600 + 438 = 1038	1071.34
1100 + 438 = 1538	1545.77
1600 + 438 = 2038	2091.35
2100 + 438 = 2538	2606.98
2600 + 438 = 3038	3023.15
3100 + 438 = 3538	3471.01
Regression equation: y = 0.000598 x – 0.000762.	

Table 4. Homocysteine calibration curve: The x-axis is representing the spiked + endogenous concentrations.

Analyte of interest			IS			IS-normalised MF
Peak area		Matrix factor	Peak area		Matrix factor	
Spiked	Pure (mean of three replicates)		Spiked	Pure (mean of three replicates)		
1,095,000	1,210,000	0.905	4,320,000	6,343,333	0.681	1.33
1,050,000		0.868	6,240,000		0.984	0.882
1,110,000		0.917	5,780,000		0.911	1.01
1,120,000		0.926	5,660,000		0.892	1.04
1,100,000		0.909	5,770,000		0.910	0.999
1,130,000		0.934	5,170,000		0.815	1.15
Mean						1.07
SD						0.154
CV%						14.4

Table 5. Calculation of ME.

analyte to the MF for the IS. The CV% in this example was 14.4%, which is within the acceptance limit for the matrix effect by the EMA guideline for validation of bioanalytical methods [14].

5. Key results

- Calibration curve is a regression model between an known concentration of an analyte and the response from an instrument enabling the estimation of the concentration of the analyte in an unknown sample.
- Weighted least squares linear regression (WLSLR) is necessary when the standard deviations across the standard range are not consistent. Weighting improves the sensitivity and accuracy of the lower end of the calibration range.
- Coefficient of correlation is not a suitable measure for the linearity of the calibration curve, and the linearity should be evaluated using an appropriate statistical analysis.
- Stable isotope-labelled compounds are the most preferable internal standards. However, carefully chosen structural analogues with similar functional groups and physicochemical properties can contribute to generation of comparable analytical methods.
- The concentration of the internal standard may affect the linearity of the calibration curve due to the cross signal contribution between the analyte and the internal standards.
- When an analyte-free matrix does not exist, the amount of endogenous analyte in the matrix can be estimated using the negative x-intercept of the regression equation and adding this value to the spiked concentrations of the analyte to calculate the actual concentrations of each standard.
- During validation of an analytical method, selectivity, specificity, accuracy, precision, uncertainty, LLOQ, matrix effect and stability are the minimum criteria to be evaluated.

Abbreviations

4PL	Four-parameter logistic
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CIS-Max	Maximum IS concentration
CIS-Min	Minimum IS concentration
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FDA	Food and Drug Administration

HPLC	High-performance liquid chromatography
IDMS	Isotope dilution mass spectrometry
IS	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
LC–MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid–liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOF	Lack of fit
LRM	Linear regression model
ME	Matrix effect
MF	Matrix factor
MS	Mass spectrometry
PBS	Phosphate buffer saline
POM	Power of the mean
QC	Quality control
QRM	Quadratic regression model
SD	Standard deviation
SD	Standard deviation
SIL	Stable isotope labelled
SPE	Solid-phase extraction
ULOQ	Upper limit of quantification
WLSLR	Weighted least squares linear regression

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Section 3

Validation Approaches

Chapter 7

Validation of Analytical Methods

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Additional information is available at the end of the chapter

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Abstract

Method validation is a key element in the establishment of reference methods and within the assessment of a laboratory's competence in generating dependable analytical records. Validation has been placed within the context of the procedure, generating chemical data. Analytical method validation, thinking about the maximum relevant processes for checking the best parameters of analytical methods, using numerous relevant overall performance indicators inclusive of selectivity, specificity, accuracy, precision, linearity, range, limit of detection (LOD), limit of quantification (LOQ), ruggedness, and robustness are severely discussed in an effort to prevent their misguided utilization and ensure scientific correctness and consistency among publications.

Keywords: method validation, accuracy, precision, linearity, LOD, LOQ

1. Introduction

Analytical method validation is an essential requirement to perform the chemical evaluation [1–3]. Method validation is a procedure of performing numerous assessments designed to verify that an analytical test system is suitable for its intended reason and is capable of providing beneficial and legitimate analytical data [4–8]. A validation examine includes testing multiple attributes of a method to determine that it may provide useful and valid facts whilst used robotically [9–11]. To accurately investigate method parameters, the validation test ought to consist of normal test conditions, which includes product excipients [11–14]. Therefore, a method validation examine is product-specific.

2. Procedure

2.1. Parameters to be checked for method validation

- Selectivity/Specificity
- Precision

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- Accuracy
- Linearity
- Range
- Stability
- Limit of Detection (LOD) and Limit of Quantitation (LOQ)

2.1.1. Selectivity/specificity

Selectivity of an analytical method is its ability to measure accurately an analyte in the presence of interferences that may be expected to be present in the sample matrix.

Selectivity is checked by examining chromatographic blanks (from a sample that is known to contain no analyte) in the expected time window of the analyte peak. And the raw data for selectivity will be recorded in the raw data in approved formats.

2.1.2. Precision

Precision of a method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings.

Precision is measured by injecting a series of standards or analyzing series of samples from multiple samplings from a homogeneous lot. From the measured standard deviation (SD) and Mean values, precision as relative standard deviation (% rsd) is calculated.

$$\%\text{rsd or CV} = \frac{\text{SD}}{\text{Mean}} \times 100 \tag{1}$$

The raw data for precision will be recorded in the approved format and the acceptance criteria for precision will be given in the respective study plan or amendment to the study plan.

OR

Precision can be also calculated by using Horwitz equation:

The acceptable percent of relative standard deviation results for precision may be based on the Horwitz equation, an exponential relationship between the among-laboratory relative standard deviation (RSD_R) and Concentration (C): [15]

$$\%\text{RSD}_\text{R} = 2^{(1-0.5\log\text{C})} \tag{2}$$

For estimation of repeatability (RSDr), is modified to:

$$\%\text{RSD}_\text{r} = \%\text{RSD}_\text{R} \times 0.67 \tag{3}$$

The Horwitz curve has been empirically derived and has been proven to be more or less independent of analyte, matrix and method of evaluation over the concentration range C = 1 (100%) to C = 10^{-9} by the evaluation of vast numbers of method precision studies. The

modified Horwitz values for repeatability CV given under may be used for guidance. If measured repeatability is outside those values, suggested explanation must be submitted for consideration. The details were presented in **Table 1**.

2.1.3. Accuracy

The accuracy of an analytical method is the degree of agreement of test results generated by the method to the true value.

Accuracy is measured by spiking the sample matrix of interest with a known concentration of analyte standard and analyzing the sample using the "method being validated." The procedure and calculation for Accuracy (as% recovery) will be varied from matrix to matrix and it will be given in respective study plan or amendment to the study plan.

2.1.4. Linearity

The linearity of an analytical method is its capability to elicit check consequences which might be at once, or with the aid of well described mathematical adjustments, proportional to the concentration of analytes in within a given range.

Linearity is determined by injecting a series of standards of stock solution/diluted stock solution using the solvent/mobile phase, at a minimum of five different concentrations in the range of 50–150% of the expected working range. The linearity graph will be plotted manually/using Microsoft Excel or software of the computer (Concentration vs. Peak Area Response) and which will be attached to respective study files.

2.1.5. Range

The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity using the set method. This range will be the concentration range in which the Linearity test is done.

Percent of analyte	**Proposed acceptable % RSD_r (Horwitz value × 0.67)**
100.00	1.340
50.00	1.490
20.00	1.710
10.00	1.900
5.00	2.100
2.00	2.410
1.00	2.680
0.25	3.300

Note: The unmodified Horwitz equation is used as a criterion of acceptability for methods collaboratively tested by CIPAC.

Table 1. Details of Horwitz values.

2.1.6. Stability

Many analytes readily decompose prior to chromatography investigations, for example during the preparation of the sample solutions, during extraction, clean-up, phase transfer, and during storage of prepared vials. Under these circumstances, method development should investigate the stability of the analyte. Accuracy test takes care of stability. It is required to mention in the method how long a sample after extraction can be stored before final analysis, based on the duration taken for accuracy test.

2.1.7. Limit of detection and limit of quantitation

The term LOD is defined as the lowest concentration at which the instrument is able to detect but not quantify and the noise to signal ratio for LOD should be 1:3. The term LOQ is defined as the lowest concentration at which the instrument is able to detect and quantify. The noise to signal ratio for LOQ should be 1:10.

Determination of Limit of Detection (LOD) and Limit of Quantitation (LOQ) from Detector Linearity experiments (applicable to only instrument sensitivity).

LOD and LOQ values are calculated manually by taking Noise to signal ratio of a lowest/ known concentration of linearity samples and it will be expressed in µg/ml or ppm. To calculate in %, values of LOD and LOQ will be multiplied by 100/lowest or known concentration of test item (mg/L) taken for analysis of that particular a.i. or impurity analysis.

Calculations of LOD and LOQ values for instrument sensitivity:

$$\mathrm{LOD\,(mg/L)} = 3 \times \frac{\mathrm{Noise}}{\mathrm{Signal}} \times \text{Lowest concentration of the linearity samples}$$

$$\mathrm{LOQ\,(mg/L)} = 10 \times \frac{\mathrm{Noise}}{\mathrm{Signal}} \times \text{Lowest concentration of the linearity samples}$$

Calculations of LOD and LOQ values for method:

$$\mathrm{LOD\,(\%)} = \frac{\mathrm{LOD\,(mg/L)}}{\text{Test item conc.used for quantification}} \times 100$$

$$\mathrm{LOQ\,(\%)} = \frac{\mathrm{LOD\,(mg/L)}}{\text{Test item conc.used for quantification}} \times 100$$

OR

2.1.8. Mathematical derivations

2.1.8.1. Determination of limit of detection (LOD) and limit of quantitation (LOQ)

Prepare a series of standard solutions (minimum five concentrations covering working concentrations used for routine analysis) and analyze each solution minimum twice and record the instruments response.

- Using the concentrations and corresponding instrument response, LOD and LOQ can be calculated as follows:

Let the linear regression equation be $Y = a + bX$.

Where, X and Y are the variables (data of two parameters). Generally, X is called the independent variable and Y, the dependent variable.

Take concentration on X-axis and instrument response on Y-axis.

"a" and "b" are the regression constants. Further, "a" is known as the intercept and "b," the slope of the line.

Let (X_1, Y_1), (X_2, Y_2), $(X_3, Y_3)\ldots(X_n, Y_n)$ be the set of values required to be fit in the linear equation.

a. Method of arriving at "a" and "b" $\overline{y}$

i. Tabulate as given below:

X_1	Y_1
X_2	Y_2
.	.
.	.
.	.
X_n	Y_n
Mean, $= \overline{X} = \Sigma X/n$	$\overline{Y} = \Sigma X/n$

ii. Calculate the following parameters:

$$\Sigma xx = \Sigma\left(X - \overline{X}\right)^2 = \Sigma X^2 - (\Sigma X)^2/n$$
$$\Sigma yy = \Sigma\left(Y - \overline{Y}\right)^2 = \Sigma Y^2 - (\Sigma Y)^2/n$$
$$\Sigma xy = \Sigma XY - (\Sigma X)(\Sigma Y)/n$$

iii. Calculate the slope "b," and intercept "a" as given below:

$$b = \frac{\sum xy}{\sum xx}$$

$$a = \overline{Y} - b\overline{X}$$

b. Method of calculation r (correlation coefficient)

$$r = \frac{\sum xy}{\sqrt{\sum xx . \sum yy}}$$

c. Method of calculation standard deviation for "a" and "b"

The standard deviation of the individual deviations of measured values in Y, above and below the linear line (fitted line) is:

$$Sy.x = \sqrt{\frac{\left(\sum yy - \left\{(\sum xy)^2 / \sum xx\right\}\right)}{n-2}}$$

From this, the standard deviation for "a" and "b" are calculated.

Standard deviation

for "a," represented = $Sy.x\sqrt{\frac{\sum X^2}{n\sum xx}}$

as S_a

Standard deviation.

For "b," represented = $Sy.x\sqrt{\frac{1}{n\sum xx}}$

as S_b

2.1.8.2. Application of a, b, and S_a to obtain limit of detection and limit of quantitation

When S_a is obtained for a linear calibration line, then it provides a clear information on the standard deviation of the "Blank" (or Control) response from the instruments.

The LOD and LOQ can be worked out, as given below:

$$\text{LOD} = \frac{|a| + 3S_a}{b}$$
$$\text{LOQ} = \frac{|a| + 10S_a}{b}$$

Note:

- The above calculations can be programmed in a computer but before every use, the computer program must be validated using the example given in section
- The above procedure can also be used for obtaining LOD and LOQ of the method from recovery test results by taking fortified concentration on X-axis and obtained concentrations on Y-axis.

3. Example

In this example, the linear regression equation is employed to find out the extent of linear response of an Detector to a reference analytical standard in the concentration range of about 0.2–3.0 ppm.

Each of these working standards is injected thrice (1 μl per injection), and the peak area counts corresponding to the active ingredient peak are given below.

From the peak areas corresponding to each concentration level, the mean, standard deviation (SD) and coefficient of variation (%CV) are also calculated. The details were presented in **Table 2**.

Fitting the data of concentration of standard solution and mean detector response (peak area counts) in a linear equation

Let the equation be $Y = a + bX$.

Where, Y = Mean peak area counts and X = Concentration of standard solution, μg/ml.

The calculations were presented in **Table 3**.

Conc. of standard solution (μg/ml)	Peak area			Mean	SD (n − 1)	%CV
	1	2	3			
0.1956	32,827	33,299	32,731	32,952	304	0.923
0.4890	87,783	88,480	87,446	87,903	527	0.600
0.9780	176,037	174,675	177,203	175,972	1265	0.719
1.467	246,212	250,786	246,849	247,949	2477	0.999
1.956	319,143	319,615	315,316	318,025	2358	0.741
2.934	415,059	410,773	418,407	414,746	3827	0.923

%CV = SD × 100/Mean: The coefficient of variation (CV) shows that the Injection variation is less than 1%.

Table 2. Calculation details of mean, SD, and %CV.

Sl. no.	Y	X
1.	32952	0.1956
2.	87903	0.4890
3.	175972	0.9780
4.	247949	1.4670
5.	318025	1.9560
6.	414746	2.9340

Table 3. Calculation details of additional parameters.

$$\sum Y = 1277547 \qquad \sum X = 8.0196 \qquad \sum XY = 2424193.441$$
$$\overline{Y} = 212924.5 \qquad \overline{X} = 1.3366 \qquad n = 6$$
$$\sum Y^2 = 3.7441177 \times 10^{11} \qquad \sum X^2 = 15.820245$$

Using the above parameters, calculate the following

$$\begin{aligned} \sum xx &= \sum X^2 - (\sum X)^2/n \\ &= 15.820245 - (8.0196)^2/6 \\ &= 5.101248 \end{aligned}$$

$$\begin{aligned} \sum yy &= \sum Y^2 - (\sum Y)^2/n \\ &= 3.7441176 \times 10^{11} - (1277547)^2/6 \\ &= 1.0239070 \times 10^{11} \end{aligned}$$

$$\begin{aligned} \sum xy &= \sum XY - (\sum X)(\sum Y)/n \\ &= 2424193.441 - (1277547)(8.0196)/6 \\ &= 716624.12 \end{aligned}$$

Calculation of a, b, and r

$$\begin{aligned} b &= \frac{\sum xy}{\sum xx} \\ &= \frac{716624.12}{5.101248} \\ &= 140480.16 \end{aligned}$$

$$\begin{aligned} b &= \frac{\sum xy}{\sum xx} \\ &= \frac{716624.12}{5.101248} \\ &= 140480.16 \end{aligned}$$

$$\begin{aligned} a &= \overline{Y} - b\overline{X} \\ &= 212924.5 - 140480.16 \times 1.3366 \\ &= 25158.718 \end{aligned}$$

$$r = \frac{\sum xy}{\sqrt{\sum xx \cdot \sum yy}}$$
$$r = \frac{716624.12}{\sqrt{1.0239070X10^{11}X5.101248}} = 0.99157$$

Note: Sometimes r^2 is also used to express the goodness of fit.

Calculation of standard deviation for a and b:

$$\begin{aligned} Sy.x &= \sqrt{\frac{\sum yy - \left\{(\sum xy)^2 / \sum xx\right\}}{n-2}} \\ &= \sqrt{\frac{(1.0239070X10^{11}) - \left\{(716624.12)^2/(5.101248)\right\}}{6-2}} \\ &= 20731.806 \end{aligned}$$

The standard deviation for a is calculated as:

$$\begin{aligned} S_a &= Sy.x\sqrt{\frac{\sum X^2}{n\sum xx}} \\ &= 20731.806\sqrt{\frac{15.820245}{6\times 5.101248}} \\ &= 14905 \end{aligned}$$

The standard deviation for b is calculated as

$$\begin{aligned} S_b &= Sy.x\sqrt{\frac{1}{n\cdot\sum xx}} \\ &= 20731.806\sqrt{\frac{1}{6\times 5.101248}} \end{aligned}$$

Note: Assay procedures vary from highly exacting analytical determinations to subjective evaluations of attributes. Therefore different test methods require different validation schemes.

Category I

Analytical methods for quantitation of major excipients and/or active ingredients, and preservatives in finished goods.

Category II

Analytical methods for determination of impurities or degradation compounds in finished goods. These methods include quantitative assays and limit tests, titrimetric and bacterial endotoxin tests.

Category III

Analytical methods for determination of performance characteristics, e.g., sterility testing, dissolution and drug release for pharmaceutical products.

Data Elements Required for Assay Validation.

Details of required validation parameters of assay presented in **Table 4**.

Analytical parameters	Assay category 1	Assay category 2 quantitative	Limit Test	Assay category III
Assay accuracy	Yes	Yes	*	*
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	*
Limit of detection	Yes	Yes	Yes	*
Limit of quantitation	Yes	Yes	No	*
Linearity	Yes	Yes	No	*
Range	Yes	Yes	*	*
Robustness	*	*	*	*

*May be required depending on the specific test.

Table 4. Validation parameters of assay [16].

4. Conclusions

Analytical validation data playing a fundamental role in pharmaceutical industry, pesticide industry for releasing the economic batch and long term stability information consequently, the records must be produced to suited regulatory authority requirements.

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Chapter 8

Method Validation Approaches for Pharmaceutical Assessments – Highlights with High Performance Thin Layer Chromatographic (HPTLC) Techniques

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Additional information is available at the end of the chapter

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Abstract

Method validation is an important activity for pharmaceutical evaluations to ensure that analytical methods are suitable for their intended use. With particular focus on active ingredient and impurities, the implementation of different categories of method validation are explained for qualitative and quantitative methods. Detailed explanations with example approaches are provided for the key aspects of method validation, namely specificity, accuracy, linearity, limits of detection/quantitation, precision, robustness, and method range. While all of the sections outlined for method validation are generally applicable for a variety of techniques commonly used in pharmaceutical analysis (i.e., UV and HPLC instrumentation), focused attention is provided for examples that have been implemented using high performance thin layer chromatographic techniques.

Keywords: method validation, pharmaceuticals, HPTLC, assay, active pharmaceutical ingredient, impurities

1. Introduction

Method Validation (MV) is a development process undertaken to establish, within acceptable statistical bounds, that an assessment procedure or method consistently yields a "true" result both in "within laboratory" and "among laboratories" testing. Pharmaceutical product quality assessments are focused on methods for the active pharmaceutical ingredient (API) and related impurities. Being able to perform methods of analysis to assess product quality is critical in law enforcement and regulating commerce. In addition, for new drug products, these quality determinations are surrogate performance indicators for assuring the safety and efficacy of a

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pharmaceutical product. The safety and efficacy of a pharmaceutical product are established with a "pivotal lot" production of the product and the characterization of this lot with well validated methods with acceptable performance characteristics is critical to assure that future production lots have the same quality characteristics as the "pivotal lot", thereby assuring they have equivalent safety and efficacy.

In the United States of America (USA), there are both private and public standards; the private standards are created through a USA Food and Drug Administration (FDA) approval process of industry method submissions that can be used for law enforcement, and public standards, which are promulgated in the monographs of the United States Pharmacopeia (USP) [1], that may be used in law enforcement or to support commercial agreements. The private standards, which are not publicly available, are private agreements between the approving government body and the submitting industry on the methods and standards to be used in law enforcement. The method validation protocols for the establishment of private standards are provided in the guidance of the "International Conference On Harmonisation Of Technical Requirements For Registration Of Pharmaceuticals For Human Use" (ICH) which have been incorporated into the laws and regulations in the European Union, Japan and the USA; these procedures are required for the assessments of new drug entities [2]. The method validation protocols for the establishment of monographs to support public standards are provided in USP <1225> [3] and ICH Q2 [4]. Both protocols cite the same analytical performance characteristics and test procedures except that the public standard must be able to be applied to all legally marketed products containing the specific API whereas the private standard applies only to the approved API in the specific product.

The analytical performance characteristics which must be assessed in both the ICH and USP are Accuracy, Precision (both Repeatability and Intermediate in ICH), and Specificity. Detection Limit, Quantitation Limit, Linearity and Range depending on which attributes are to be assessed. The USP presents the characteristics as noted below [3]:

"Category I — Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

Category II — Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.

Category III — Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release, etc.).

Category IV — Identification tests."

These can be categorized further into Assay procedures for Category I, Category II, impurity determinations, and Category III (dissolution and drug release are different procedures for preparing a solution of the API), all performance characteristics except Detection Limit must be validated. For the Category II limit tests only the Specificity and Detection Limits must be validated and for Category IV, Identification Tests, only the Specificity needs to be validated. The following sections will provide approaches toward the various aspects of method

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validation. Although the approaches are generally applicable to common techniques used in pharmaceutical analysis (such UV-VIS and HPLC quantifications), particular emphasis will be placed on high performance thin layer chromatography (HPTLC) techniques.

2. Specificity

Specificity is the ability of a method to distinguish an analyte from all substances that are present or likely to be present in test samples [3, 4]. When possible, these substances should include future degradation products and other ingredients (i.e., excipients). An analytical procedure is specific when placebo and impurity spots do not overlap partially with and are not buried under the analyte spot. In addition, the calculated amount of analyte does not depend on the quantity of other substances.

Various approaches are possible when evaluating method specificity [5–8]. Ideal demonstration of specificity for an HPTLC analytical procedure requires chromatographing simultaneously three types of samples: sample type 1 is the pure analyte or its reference standard, sample type 2 is the analyte mixed with a representative blank and all likely impurities, and sample type 3 is the representative blank mixed with all likely impurities. Likely impurities include degradation products, reagents, intermediates, excipients, side products, and analyte isomers. The mixtures can be created by spiking test samples (API substances or finished products) or placebos with likely impurities.

In practice, the unavailability of one or more of these types of samples can pose a significant challenge. In some cases, it is often difficult to know all likely impurities. There can be several sample deficiency scenarios. If the pure analyte or its reference standard is not available, demonstration of specificity can be quite challenging if not impossible. If a representative blank is available, but some or all likely impurities are missing, the typical test sample is subjected to stress testing environments. It should be noted however that stress testing is unlikely to produce some analyte isomers, reagents, intermediates and side products. If a representative blank is unavailable, but some likely impurities are available, spiking the typical test sample with impurities can show that increasing impurities will not change analyte signal. In addition, efforts should be made (perhaps by contacting the manufacturer), whenever possible, to create a representative blank even if it's not exactly in the same dosage form as the test sample. If neither a representative blank nor impurity standards are available, the typical test sample is subjected to stress testing to alleviate some of the deficiencies. Once again, the limitations of stress testing should be acknowledged because it may not produce all likely impurities, it may not account for impurities that are completely buried under the analyte signal, and it may not indicate whether some excipients or impurities can react with the analyte.

In general, stress testing, impurity spiking, and peak-purity analysis are the common tools used to address certain sample deficiencies. To demonstrate method specificity, validation reports typically discuss several measures of performance. One measure of specificity is resolution of the analyte spot relative to the closest non-analyte spot. For HPTLC, the resolution should be a least 1 [5].

Analyte peak purity is another measure of specificity that is typically reported. Often, analyte peak purity in the analyte reference standard is compared to analyte peak purity in the other test samples mentioned above. The analysis is performed by comparison of peak spectra at the start, apex, and end of the analyte peak. Some authors use correlation coefficients [9] as a measure of peak purity, and others rely on software algorithms that may involve Matrix Algebra. It should be noted that while peak purity can detect the presence of some impurities in the analyte peak, it does have some limitations. For example, peak-purity analysis does not account for missing impurities that could overlap with the analyte peak, and it does not account for impurities having a spectrum that is similar to that of the analyte. In addition, peak-purity analysis is not applicable for detectors that do not register the entire analyte spectrum for each time point.

A third measure of peak purity is an overlay of chromatograms. This measure is especially useful for showing the analyte peak stability during impurity spiking or stress testing. For example, the chromatograms of a finished pharmaceutical product, before and after accelerated aging, can be overlaid to support method specificity.

3. Accuracy

A succinct definition of accuracy is "nearness to truth". The ICH guidelines [4] provide the following definition:

"The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found."

In other words, accuracy of a method represents the agreement between an expected value and the value generated by the candidate method (the method value). Therefore, accuracy determination involves determining the expected value, finding the method value and calculating the agreement between the two values [3, 4].

In pharmaceutical testing, accuracy is mainly relevant to quantitative methods, such as assay, content uniformity, dissolution, and impurity quantitation. To determine the accuracy of a quantitative HPTLC method, there are typically four major options, which differ mainly on how the expected value is determined. Unfortunately, the most preferable options are not always feasible due to the non-availability of appropriate reference standards or placebo samples. For each option, we will explain how to determine the expected value and the method value. Agreement between the two values will be addressed later.

3.1. Options for determining the expected value and the method value

3.1.1. Option 1 (using a certified reference standard)

The first option involves using a representative, certified reference standard. We say representative because the certified reference standard needs to have a chemical matrix that is the same as the matrix of a typical unknown sample. So, if the method is intended for API quantitation

in an API substance, the reference standard could contain only the API. However, if the method is intended for finished product testing or for impurity quantitation in API substance, the reference standard should contain the appropriate amount of all the substances typically found in the finished product or API substance. The reference standard should be prepared by an ISO certified reference material manufacturer.

3.1.1.1. Determining the accepted value

If a representative certified reference standard is available, the accepted value is the certified amount of analyte (e.g. API) per given sample of the reference standard.

3.1.1.2. Determining the method value

To obtain the method value, at least nine reference standard solutions are prepared and tested as if they were unknown samples, using the candidate HPTLC method. The average and standard deviations of the results will represent the method value. The standard solutions should contain the following analyte concentrations:

- The first three standard solutions should contain analyte concentrations between 50 and 90% of the analyte's label claim (or quantitation limit for an impurity).
- The next three standard solutions should contain analyte concentrations between 90 and 110% of the analyte's label claim.
- The last three standard solutions should contain analyte concentrations between 110 and 150% of the label.
- Due to the unavailability of representative certified reference standards for most pharmaceutical products, option 1 is rarely used in method validation.

3.1.2. Option 2 (Using a representative blank)

The second option involves using a representative blank, which means a substance or mixture that contains all the chemical components of a typical unknown sample except the analyte. It is important to note that the chemical composition of a representative blank depends on both the analyte and the composition of a typical unknown. For assay, or content uniformity, the representative blank is a placebo. For API quantitation in the API substance, the representative blank is typically the solvent used to dissolve the standard. For impurity quantitation in an API substance, the representative blank is typically the API substance. For impurity quantitation in the finished product, the representative blank is a mixture of the placebo plus all the APIs plus all the typical impurities. During testing, the representative blank should be treated the same way as an unknown sample would be. Care must be taken so that only the absence of analyte distinguishes the representative blank from a typical unknown sample.

3.1.2.1. Determining the accepted value

If a representative blank is available, at least nine samples are prepared by spiking the blank with various amounts of analyte. The accepted value can be represented as the average and

standard deviation of all the amounts of analyte spiked to the representative blank samples. At least nine difference samples should be tested covering a minimum of three different concentrations across the expected range of analyte concentration (50–150% of label claim or impurity limit).

3.1.2.2. Determining the method value

Once the spiked blank samples are prepared, they can be analyzed in parallel using the candidate HPTLC method. The average and standard deviation of the results (expressed in the same unit as the accepted value) can represent the method value.

3.1.3. Option 3 (Using a reference method)

If options 1 and 2 are not feasible, a reference method can be used to determine the accuracy of a candidate method. The reference method must be independent of the candidate method, have been well validated with a stated accuracy, and have the same intended use as the candidate method.

3.1.3.1. Determining the accepted value

To obtain the accepted value for option 3, the reference method can be used to test 6 or more unknown samples. The average and standard deviation of the results will represent the accepted value.

3.1.3.2. Determining the method value

To obtain the method value, each of the samples used to determine the accepted value is tested using the candidate method. The average and standard deviation of the results will represent the method value.

3.1.4. Option 4 (Using standard addition to unknown)

In lieu of option 3, method accuracy can be estimated using the standard addition method [10]. In this case the test sample is an unknown finished product or an API substance, whose analyte amount has been predetermined using the candidate method.

3.1.4.1. Determining the accepted value

To obtain the accepted value for option 4, at least 6 or more stock solutions of unknown samples should be prepared and tested per the candidate method. The average and standard deviation of the results will represent the accepted value.

3.1.4.2. Determining the method value

To obtain the method value, each of the stock solutions used to determine the accepted value is tested once again using the standard addition method [10]. So, each stock solution should have its own standard addition curve with 5 or more data points. The average and standard deviations of the absolute values of the x-intercepts will represent the method value.

3.2. Agreement between expected and method values

Several calculation methods are used to determine the agreement between the expected and method values. The percent recovery method is the simplest. It involves dividing the average method value by the average expected value and multiplying the result by 100. Although this method is considered acceptable in the ICH guidelines [3, 4], and it is found in many publications, it does not take the standard deviations into account.

One method that takes variation into account is the expanded uncertainty interval method [11]. It involves combining the expected and method uncertainties to obtain the expanded uncertainty, which is then compared to the difference between the average expected value and the average method value. If the expanded uncertainty is greater or equal to the difference, the candidate method is considered accurate.

A more statistically rigorous method to calculate accuracy is the t-test for two equal means [12]. It can be performed using MS Excel or other statistical software, but it requires an understanding of hypothesis testing. Interested readers can consult any general Statistics book for more details on t-test and hypothesis testing.

The concept of accuracy profile, which is different from the concept of accuracy described herein, is described by Shewiyo et al. It aims to describe method performance using a single statistic [13].

4. Linearity

Linearity evaluations demonstrate measurements from a test method are proportional to the amount of analyte within a particular concentration range [3, 4]. Responses from samples containing different amounts of analyte are obtained from the test method. Generally, a minimum of five different concentrations should be used where multiple (i.e., ≥3) responses are obtained at each analyte level. The method response (y-axis) is plotted as a function of the analyte concentration (x-axis) for subsequent analysis with linear regression techniques, where slope, intercept, and correlation coefficient are reported. The concentration range should cover the upper and lower levels anticipated during an analysis.

In the following example, a graphical representation of a linear calibration model is demonstrated, where the raw data is provided in **Table 1** and **Figure 1** shows the corresponding linear regression curve.

[Concentration], w/v	[Response], Instrument reading (triplicate results)
2	0.06, 0.06, 0.06
4	0.12, 0.12, 0.11
6	0.17, 0.17, 0.16
8	0.22, 0.23, 0.22
10	0.28, 0.28, 0.28

Table 1. Data to demonstrate a linear calibration model.

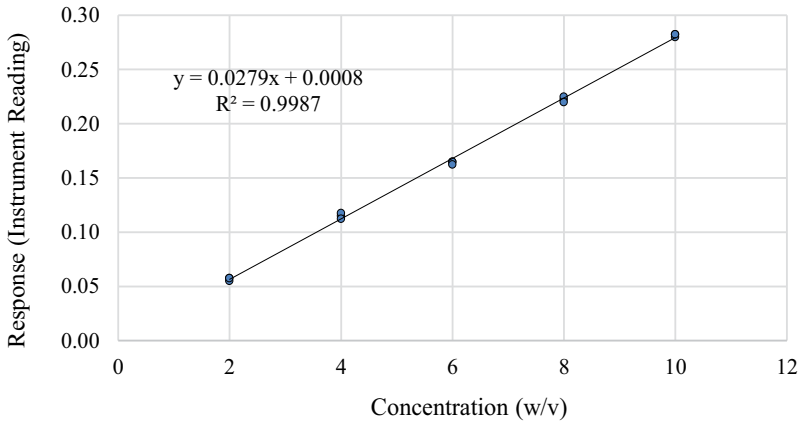

Figure 1. Graphical representation of linear calibration model data.

However, a linear model may not be the best calibration fit for the data as is the case of the data listed in **Table 2**, and plotted in **Figure 2**. When the linear model is applied to the data, the resulting correlation coefficient ($R^2 = 0.98$) is less than ideal.

Further examination of the data indicates that a polynomial fit can provide a better calibration model from the data (**Figure 3**). It should be noted that most pharmaceutical analysis methods commonly use a one-point standard during routine use of the method (after validation has been established).

Variable	**Data**							
[Concentration], w/v	1	2	3	4	5	6	7	8
[Response], Instrument reading	10	20	30	41	46	55	60	65

Table 2. Data to demonstrate a non-linear calibration model.

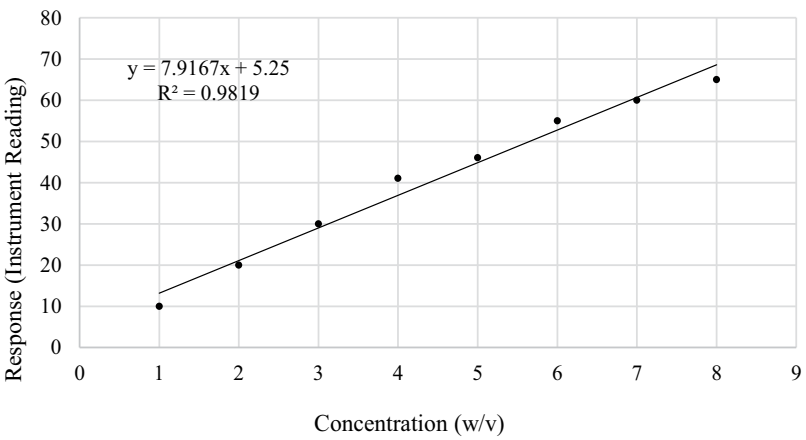

Figure 2. Graphical representation of a less than ideal linear calibration model.

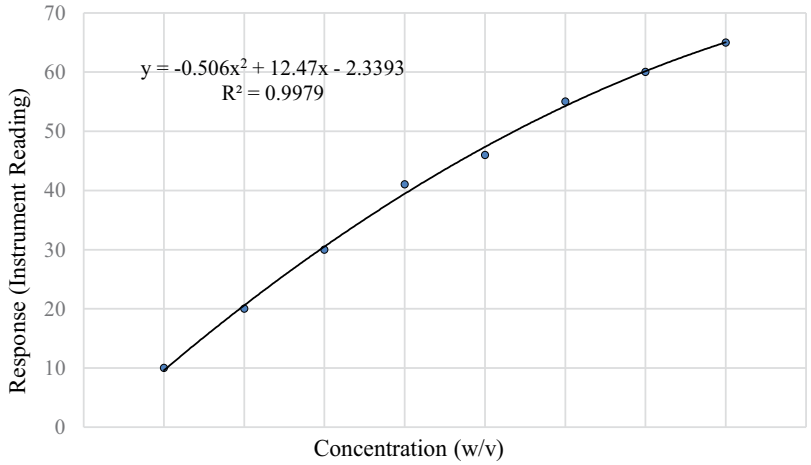

Figure 3. Graphical representation of polynomial approach to calibration model.

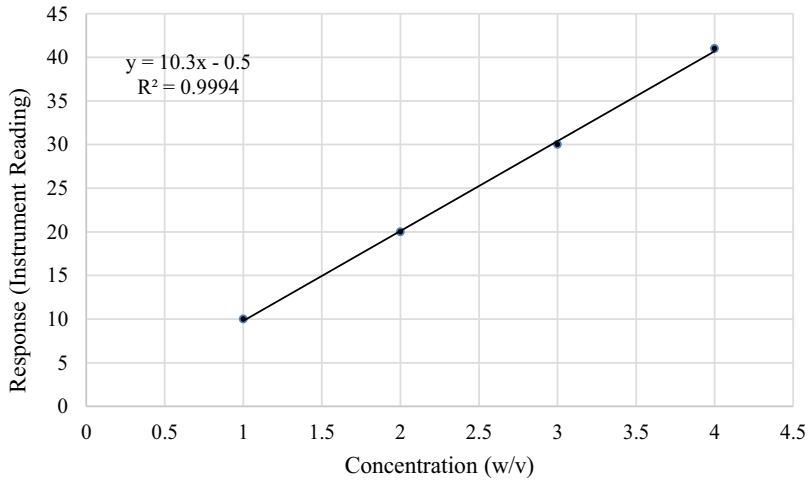

Figure 4. Graphical representation of the more linear range of the preceding polynomial calibration model.

If the analysis range for the method only requires concentrations from 1 to 4 (w/v), a linear model for just that concentration range provides a r^2 of 0.9994 (**Figure 4**) and would be easier to implement in future analysis (note-an additional standard should be added within that range during final validation).

5. Limits of detection/quantitation

Various options are possible for determining limits of detection (LOD) and limits of quantitation (LOQ) [3, 4, 14, 15]. The section below will provide some key example approaches for tests that generate instrument based responses.

The signal to noise ratio can be used to determine both the LOD and LOQ, where responses are obtained from blank and from an array of samples at lower concentrations. A ratio of signal (from analyte samples) to noise (from blank) of 3 is an accepted concentration level for the LOD. Likewise, a concentration level that provides a signal to noise of 10 can be used as the LOQ.

Another approach first involves the determination of the standard deviation of the response and the slope of calibration (linearity) curve. Although other options are possible [3, 4, 14, 15], the standard deviation of the response can be estimated from replicate injections from blank samples or from the standard deviation of y-intercepts from multiple regression lines. Multiplying the ratio of the standard deviation of the responses to the slope of the curve by 3.3 or 10 provides the LOD or LOQ, respectively.

6. Precision

For an analytical method, precision is an assessment of the consistency of results obtained with multiple measurements from the same sample [3, 4, 16]. There are three categories of precision for an analytical method, namely repeatability, intermediate precision, and reproducibility, which can be assessed through variations with different equipment, testing times (conducted on different days), analysts, and/or laboratories.

Repeatability is often evaluated with replicate measurements of a sample on the same day in the same laboratory, where the analyst and equipment are not changed. Intermediate precision can be evaluated from replicate measurements of a sample within the same laboratory, but with systematic variations with different analysts, times of analysis, and equipment (such as different instruments). Reproducibility is commonly determined from replicate measurements of the same sample but within different labs, which will inherently incorporate different analysts, equipment, and time of analysis.

ICH Q2 provides several recommendations for number of replicates and concentration levels for each of the three types of precision [4]. Recommended approaches for repeatability are at least nine measurements that span the method's range (such as three replicates for each of three analyte levels) or at least six measurements at the target analyte level. ICH Q2 does not specify a minimum number of samples for intermediate precision and reproducibility but encourages that the effects of variables (i.e., analysts, days, instruments) be systematically evaluated. The following section will provide possible approaches for evaluating repeatability and intermediate precision, followed by references for examples for reproducibility will be provided.

To perform the appropriate precision assessments, the following equations are indicated [16] and will be used for further development of subsequent examples. The average ($\overline{x}$) of n replicates is provided in Eq. (1),

$$\overline{x} = \frac{\sum_{i=1}^{n} x_i}{n} \tag{1}$$

where x_i represents the individual replicates measurements. The standard deviation (s) of a data set can be determined through Eq. (2),

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \overline{x})^2}{(n-1)}} \tag{2}$$

and the % relative standard deviation (%RSD) is provided in Eq. (3).

$$\%\text{RSD} = 100 \times \left(\frac{s}{\overline{x}}\right) \tag{3}$$

The %RSD is often used in method validation assessments because it normalizes the standard deviation to the average.

From Eqs. (1)–(3), an evaluation of repeatability can be determined. In the following example, assume that an analyst has performed six replicate analysis (within the same laboratory) from a method capable of quantifying the amount of active ingredient in a pharmaceutical product in units of % label claim (assay) and obtained the following results (102.1%, 100.5%, 98.2%, 99.1%, 101.8%, 99.8%). Using Eqs. (1)–(3), the average ($\overline{x}$), standard deviation (s), and %RSD would be 100.25%, 1.52%, and 1.52%, respectively, where s (or more commonly %RSD), is a measure of method repeatability.

Intermediate precision involves an evaluation of variations "within runs" and "between runs" [17]. Consider the data in **Table 3** containing replicate runs (n = 3, indexed with j) obtained on each of multiple days (p = 5, indexed with i), where each day used a different analyst with separate solution preparations but using the same method as above for the repeatability analysis.

With each entry in the data representing a separate x_{ij}, the repeatability or within-run standard deviation (days) can be determined using Eq. (4), where $\overline{x}_i$ is provided in Eq. (5).

$$s_r = \sqrt{\frac{\sum_{i=1}^{p}\sum_{j=1}^{n}\left(x_{ij} - \overline{x}_i\right)^2}{p(n-1)}} \tag{4}$$

n = 3(j)/p = 5(i)	Day 1	Day 2	Day 3	Day 4	Day 5
Replicate 1	102.2	98.7	99.3	101.9	102.1
Replicate 2	100.3	101.8	98.1	100.1	101.4
Replicate 3	99.9	102.3	98.7	99.1	101.2
$\overline{x}_i$	100.8	100.9	98.7	100.4	101.6
$\overline{\overline{x}}$			100.5		

Table 3. Example data for intermediate precision determination.

$$\overline{x}_i = \frac{\sum_{j=1}^{n} x_{ij}}{n} \tag{5}$$

The between-run standard deviation (days) can be calculated with Eq. (6), where $\overline{\overline{x}}$ is provided in Eq. (7).

$$s_B = \sqrt{\frac{\sum_{i=1}^{p} \left(\overline{x}_i - \overline{\overline{x}}\right)^2}{p-1} - \frac{s_r^2}{n}} \tag{6}$$

$$\overline{\overline{x}} = \frac{\sum_{i=1}^{p}\sum_{j=1}^{n} x_{ij}}{pn} \tag{7}$$

Subsequently, the intermediate precision standard deviation can be calculated with Eq. (8).

$$s_{IP} = \sqrt{s_r^2 - s_B^2} \tag{8}$$

From the data presented in **Table 3** and using Eqs. (4)–(8) [17], the standard deviations for repeatability (within-run), between-run, and intermediate precision are calculated as 1.26, 0.80, and 1.49, respectively.

Evaluations for reproducibility utilize interlaboratory trials, and are commonly employed when a procedure requires further standardization for use among a more extended array of laboratories. ISO 5725 [18] provides the necessary approach and management structure needed to properly plan, conduct, and interpret the results of an interlaboratory trial that will involve multiple laboratories conducting replicate analysis of a sample(s) at a particular analyte level(s). Approaches are provided to graphically (Mandel's statistics) and quantitatively (Cochran/Grubb) identify outliers so that the most accurate assessments of repeatability and reproducibility variance (standard deviations) are possible. The calculations involved in these types of trials are fairly extensive. Several examples are provided within ISO 5725, and Vander Heyden et al. provides a detailed example for an interlaboratory trial for an HPLC procedure [19].

Overall, desired levels for precision for pharmaceutical analysis are commonly on the order of ~2% RSD. However, different ranges can be necessary depending on the concentration level of the analyte (i.e., higher levels of %RSD can be allowed as the analyte concentration decreases) [17].

7. Robustness

Robustness is a measure of how much a method is impacted by deliberate (small) changes in method conditions [3, 4]. The following are a listing of the types of parameters that can be

evaluated to assess method robustness; solution stability (to heat and or time), extraction conditions during sample preparation (time, temperature, mechanical shaking time, sonication time), type of filters used during final standard/sample preparation, minor adjustments in mobile phase composition, and adjustments in other chromatographic conditions (flow rate, different suppliers of columns, temperature). Commonly, robustness is evaluated during the development stages of the method.

An approach for evaluating robustness could be to compare an analysis using the primary method compared to an analysis where a certain parameter is adjusted. Depending on the method and sample type, adjustments in parameters that generate less than ~2% difference relative to the primary method can provide a reasonable measure of how sensitive the method is to various types of adjustments. During the development of the method for example, a study could be conducted to evaluate the sensitivity of the method on the type of filter by comparing the results from a sample solution that was centrifuged (without filtration) to those filtered with different filter types (PTFE, PVDF, nylon) from different manufacturers. Solution stability could be evaluated by comparing results from freshly prepared solutions compared to the same solutions stored at room temperature over several days.

There are a variety of approaches that can be incorporated to evaluate method robustness. Dejaegher and Vander Heyden provide an extensive review for a variety of approaches to systematically evaluate method robustness (ruggedness) [20].

8. Range

The range of the method corresponds to the lower and upper analyte concentration where satisfactory levels of linearity, precision, and accuracy have been achieved during the method validation process. The range is indicated in the same units as that of the results obtained from the method.

For analysis of pharmaceutical products [3, 4], the following ranges (in percentage relative to the target level) are often required for the respective types of tests; assay (80–120%), content uniformity (approximately 70–130%), impurities (approximately 50–120% of the acceptance limit), dissolution (± 20% of the required range).

9. Application with HPTLC techniques

High-performance thin layer chromatography (HPTLC), an extension of TLC, is a robust, simple, rapid, and efficient tool in quantitative and qualitative analysis of compounds [21], and a variety of applications can be found in the literature [22–29]. In this section an overview of applications of HPTLC in typical pharmaceutical testing protocol is highlighted with examples. HPTLC is one of the sophisticated instrumental techniques based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization,

selective detection principle, minimum sample preparation, hyphenation, and so on enable it to be a powerful analytical tool for chromatographic information of complex mixtures of pharmaceuticals, natural products, clinical samples, and food stuffs [21]. HPTLC is one of the ideal TLC technique for the analytical purposes because of its increased accuracy, reproducibility, and ability to document the results, compared with standard TLC. Because of this, HPTLC technologies are also the most appropriate TLC technique for conformity with GMPs [30].

9.1. Identification test

In a pharmaceutical testing protocol, identification tests are intended to ensure the identity of an analyte in an API or finished pharmaceutical product sample. This is normally achieved by comparison of a chromatographic behavior of unknown sample to that of a reference standard. The identity of the test substance is confirmed if the migration distance of the test substance matches that of the reference substance. Thin layer chromatography experiments are among the key identity tests in most pharmacopeia monographs. Pharmacopeia standards are typically used by industry as a basis for meeting QC requirements and current good manufacturing practices (cGMPs). Many identification tests in the major pharmacopeia (e.g., USP, Ph. Int., and Ph. Eur. [1, 31, 32]) use planar chromatography (TLC), however HPTLC is a superior technology. **Figure 5** below represent a typical densitogram obtained in the identification of sulfamethoxazole (SMX) and trimethoprim (TPM). In this example, the migration distances are 0.35 and 0.90 for TMP and SMX respectively.

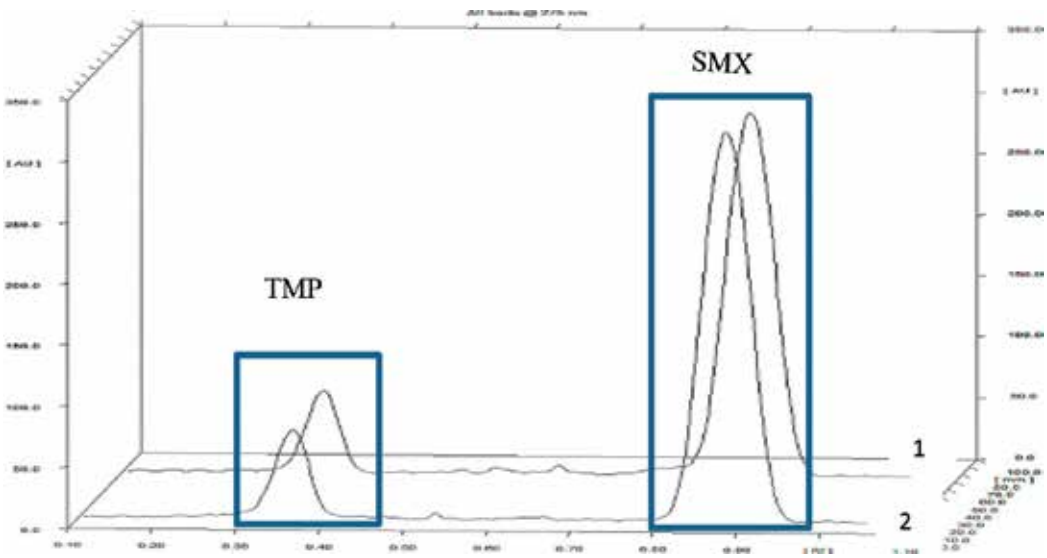

Figure 5. An example of overlaid densitogram for identification of sample 1 and a reference 2 containing sulfamethoxazole (SMX) and trimethoprim (TPM). Conditions Mobile Phase: (Methanol: Ethyl Acetate: Toluene 6: 9:15 v/v) Detection Wavelength: 275 nm and Application Volume: 5 μl and aluminum plates precoated with silica gel 60 F254 as the stationary phase.

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9.2. Assay content determination

A second most important critical quality attribute for pharmaceutical products testing is assay or determination of content. The procedure intended to measure the analyte present in a sample. In this context, the assay represents a quantitative measurement of the major component(s) in the drug substance. This is done by comparing the area under the peak of test substance to that of reference standard material. For a drug product, containing paracetamol an overlaid densitogram is presented in **Figure 6**. Similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution) [33].

9.3. Impurities and related substances

The principal requirement is that an analytical method for assessing impurities should be a stability indicating and meeting specificity criteria described in Section 2 above. Stability indicating method (SIM) is defined as a validated analytical procedure that accurately and precisely measures the active ingredients (drug substance or drug product) free from process impurities, excipients and degradation products. This can be demonstrated by forced degradation study of the drug substance and subjecting the resultant solution to the chromatographic conditions [34].

Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Quantitative tests for impurities are meant to quantify the exact amount of impurity. This is

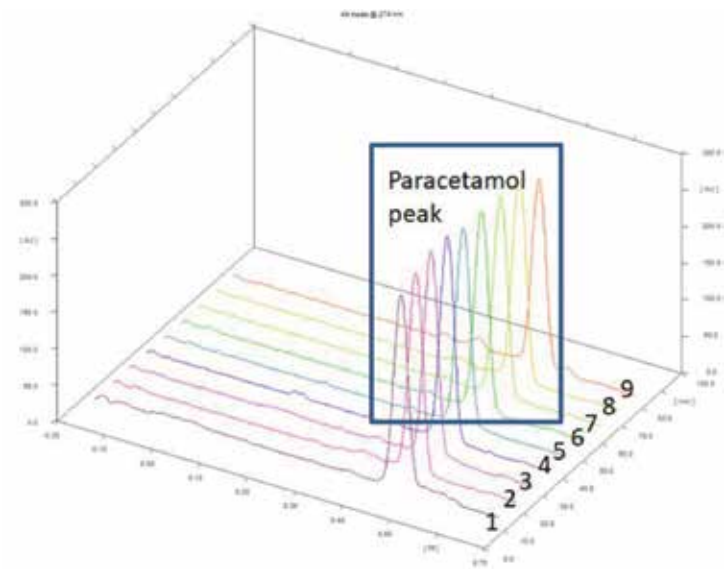

Figure 6. An example of overlaid densitograms for assay of paracetamol in sample tracks 2, 3, 5, 8 and 9, and a reference in tracks 1, 4 and 7. Conditions: Mobile Phase: Acetone; Methanol; Toluene: 6:6:16 v/v/v acidified with three drops of Glacial Acetic Acid, Detection Wavelength: 274 nm, Application Volume: 5 μl and aluminum plates precoated with silica gel 60 F254 as the stationary phase.

done by comparing the response from a single or multi-level calibration curve. Whereas the limit test is an estimative test where the impurity is controlled not to exceed certain limit. In this case an impurity standard is prepared at the control level and compared to the response from the sample (which should not exceed this level).

With the improved resolution powers of HPTLC (enhanced by reduced particle sizes), it is possible to perform both tests by using HPTLC. In the literature, there are many stability indicating method for various drug substances for example pseudoephedrine and cetirizine in pharmaceutical formulations [35], clopidogrel bisulphate [36] timolol maleate [37] simultaneous determination of ezetimibe and simvastatin [38], piroxicam [39], and estradiol [40].

9.4. Dissolution testing

Dissolution testing is a performance characterizing test and a requirement for all solid oral dosage forms and is used in all phases of development for product release and stability testing [41–43]. It is a key analytical test used for detecting physical changes in an active pharmaceutical ingredient (API) and in the formulated product. It is a multi-unit test and multi-point sampling, making it very tedious. HPTLC offers a multi-channel capability where a total of 18–25 samples can be applied on one plate in form of bands and analyzed simultaneously. One lot of a product will require 6 units (tested in duplicate), plus calibrators (in triplicates at single or multiple levels). HPTLC methods have been successfully deployed for monitoring dissolution profile of diclofenac and acetaminophen [44], and the stability of rifampicin in dissolution medium in presence of isoniazid [45].

9.5. Content uniformity

The test for Content Uniformity (CU) is the assay of the individual content of drug substance(s) in a number of individual dosage units to determine whether the individual contents are within the set limits [46]. Multiple capsules or tablets are selected at random and each are analyzed to determine the active ingredient in each capsule or tablet. The performance efficiency of this method can benefit from the HPTLC multi-channel capabilities. HPTLC has been successful applied in content uniformity of atorvastatin calcium tablets [47], diazepam tablets [48], diosgenin and levodopa [49], nicorandil tablets [50] and rosiglitazone in tablets [51]. All of these HPTLC method examples provide a faster, more cost efficient approach to quantitative testing for routine analysis.

10. Conclusions

Classic method validations for pharmaceuticals involve techniques such as UV-VIS, TLC, and HPLC. This chapter highlights different examples with High Performance Thin Layer Chromatography (HPTLC). General approaches are provided for method validation, as applicable to pharmaceutical assessments, outlined for each of the key aspects (i.e., specificity, accuracy, linearity, limits of detection/quantitation, precision, robustness, and range). Although classical application of pharmaceutical method validation uses techniques such as UV-VIS or HPLC,

important examples are provided using HPTLC techniques that provide high accuracy/precision with minimal use of reagents and other resources.

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This book seeks to introduce the reader to current methodologies in analytical calibration and validation. This collection of contributed research articles and reviews addresses current developments in the calibration of analytical methods and techniques and their subsequent validation. Section 1, "Introduction," contains the Introductory Chapter, a broad overview of analytical calibration and validation, and a brief synopsis of the following chapters. Section 2 "Calibration Approaches" presents five chapters covering calibration schemes for some modern analytical methods and techniques. The last chapter in this section provides a segue into Section 3, "Validation Approaches," which contains two chapters on validation procedures and parameters. This book is a valuable source of scientific information for anyone interested in analytical calibration and validation.

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