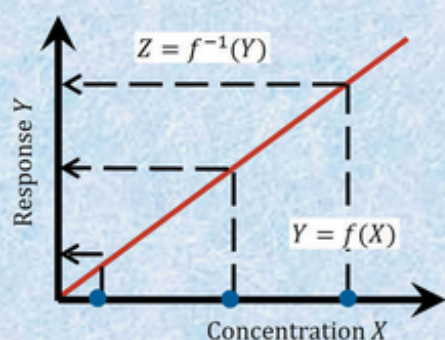


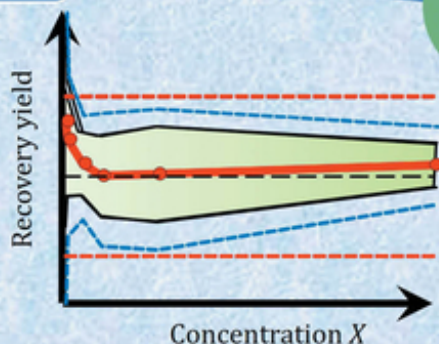
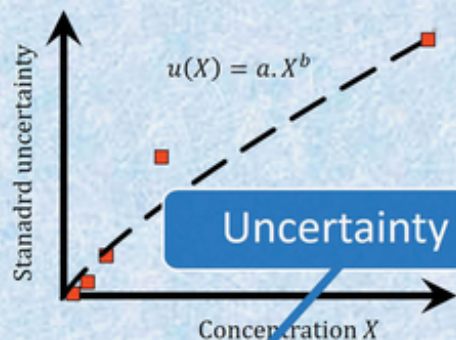
Max Feinberg and Serge Rudaz

Quantification, Validation and Uncertainty in Analytical Sciences

An Analyst's Companion



Quantification



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Preface

Why an Analyst's Companion? Millions of analyses are carried out every day in laboratories for all sectors of industry and science. Many people are willing to pay for these analyses because they are considered effective in making a scientifically sound decision. Though few publications address the economics of analytical sciences, nonetheless, a report by the European Commission concluded in 2002 that "for every euro devoted to measurement activity, nearly three euros are generated" [1]. But is it easy and simple to use an analytical result, and does it always allow you to make the right decision? Some questions illustrate the risks involved in relying on a result:

- How do you know that the laboratory used the method that gave the exact result?
- Like any measurement, analysis is subject to error. How can you estimate them?
- How can a spurious measurement be used effectively?

This is the right time to explain why and how the concept of measurement uncertainty (MU) can be used to better manage these risks. This also means that a new challenge for analysts is to develop an appropriate method for estimating MU more explicitly applicable to analytical sciences. In this perspective, a tool based on the statistical dispersion intervals called method accuracy profile (MAP) is proposed as the backbone of the book. The theoretical aspects of the MAP procedure and MU estimation are presented in several examples and template worksheets to help analysts quickly grasp this tool.

At the turn of the 1970s, three analytical chemists, Bruce Kowalski, Luc Massart and Svante Wold, conceptualized a discipline they called Chemometrics [2]. Unfortunately, they all have passed away since, but their work is still vivid. Many chemometrics books have been published, proving the added value of statistics to analytical sciences. Some are globally addressing chemometrics [3–5] other are more focused on statistics [6, 7], and others on method validation [8, 9].

This book contributes to the application of chemometrics, but the obvious aim is not to repeat what is available in many valuable publications. Only a few books precisely address measurement uncertainty in analytical sciences [10–12]. They present limited facets and do not propose a more comprehensive approach. The aim of this book is to describe a global procedure for MU estimation, easily applicable in analytical laboratories. In a recent publication, we have exposed in a condensed manner

our view of the link between validation and measurement uncertainty [13]. This book develops more extensively and practically our viewpoint.

However, it is not satisfactory to simply propose a *modus operandi* (even if it is claimed to be universal) for estimating MU when this parameter is still new in analytical sciences and not always well identified by end-users. Therefore, several chapters are dedicated to its practical use in decision-making, demonstrating its advantages. These remarks indicate that this book is primarily intended for professional analysts, although researchers and students may find it of interest.

In order to reach this goal, the book is organized around practical responses covering three major questions daily put to analysts when they develop a new method or routinely apply it to unknown samples:

- How to quantify the analyte?
- How to validate the method?
- How to estimate the measurement uncertainty?

How does this book give answers these questions? We use as a roadmap a tool based on the application of statistical dispersion intervals called MAP. The latter was initially conceived for method validation, but it can easily be used for MU estimation. While method validation is often reduced to computing a set of disconnected parameters to be estimated, the MAP approach is more global. It consists in defining the interval where the method is able to produce a given proportion of acceptable results. This perspective is in harmony with the uncertainty approach proposed by metrologists some decades ago that consists in computing the so-called coverage interval of the result.

The chapters of the book can be read independently. This may explain some redundancies in the quoted publications. But they are structured according to a reading thread illustrated in Figure 1. The thick grey arrow is the backbone. Six main chapters are characterized as rounded angle boxes. Three of them are devoted to measurement uncertainty, as it is a key issue of the book.

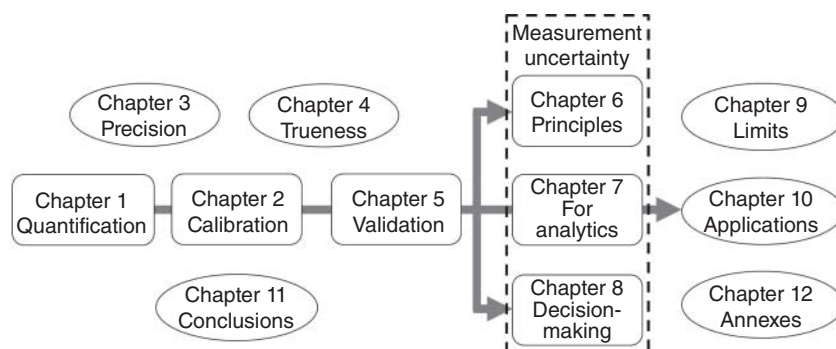


Figure 1 How to read this book.

Additional chapters appear as ellipses. They bring two kinds of information. On the one hand, theoretical background, such as precision and trueness parameter estimation and how to compute them, may be useful to better understand statistical developments involved in the method accuracy profile. On the other hand, specific examples of MU applications. One is devoted to the limits of quantification and the challenging question of controlling samples with low analyte concentration, another to method comparison.

Several data sets provide the link between the different chapters. They are used throughout for practical data handling and real software application. The aim of this data-oriented presentation is to help the analyst apply the proposed techniques in the laboratory, in keeping with the title “Companion.” This also practicality means that numerical applications for all topics covered are presented and illustrated alongside the theoretical considerations. These are based on detailed Microsoft Excel® worksheets or free equivalent, such as OpenOffice® Calc, included with the book. This software is user-friendly and does not require much explanation, and probably everyone in the laboratory knows how to use it. Although criticized by professional statisticians (for good reasons), this software is extremely helpful for quick and simple statistical computation in a laboratory, and several pitfalls can easily be avoided:

- Worksheet cell content is easily modified without any warning. Thus, once created and validated, the best initiative is to protect the worksheet or whole workbook.
- The formula inside cell is not visible unless the option to show formulas is on. To help the understanding of the template worksheets developed for this book, all formulas are made visible in the cell next to the resulting. The built-in function `FORMULATEXT` is used for this aim. It is only available in the most recent Excel releases.
- Confusion may exist between a worksheet and a text editor. Fancy presentation must be avoided, and it is better to embed a worksheet within a text editor rather than trying to do everything with a single software.

The basic use of worksheet software does not allow complex statistical calculation though it contains many built-in functions, which are used in the following examples. It is possible to use the development environment called Visual Basic for Applications coming with Excel to build more complex programs, but it requires some practice. For the most sophisticated applications, we preferred to provide Python program examples. This software is increasingly popular, and the accuracy of statistical functions is widely recognized. For instance, complex techniques, such as non-linear or weighted regression techniques, are easily implemented. Python is simpler than professional statistical software. It is developed under a free license, and there is an exceptionally large community of users who can help. The drawback is that it is a patchwork, and many additional modules must be imported to apply some methods. The simplest way to install Python is to download a free package called Anaconda [14] and select the Spyder development environment. Presented examples were programmed in this environment.

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Glossary of Symbols

Symbol	Term
β	Coverage probability of the tolerance interval
$u(Z)$	Standard uncertainty Z
r	Coefficient of correlation
Z	Inverse-predicted concentration in the working sample
Z^*	Extrapolated sample concentration (standard addition method)
Y	Measured instrumental response
$\hat{Y}$	Predicted instrumental response
X	Concentration of the (authentic) analyte in the working sample
$\bar{X}, \bar{Z}$	Average
$\bar{\bar{X}}, \bar{\bar{Z}}$	Grand average
X_c	Concentration of the (surrogate or not) analyte in the calibrant
UR%	Relative expanded standard uncertainty
$U(Z)$	Expanded uncertainty Z
AIC	Akaike Information Coefficient
A	Variance ratio $A = s_B^2/s_r^2$
δ	Bias
E	Random error variable
f	Any calibration or uncertainty function
f^{-1}	Inverse of any function
β - γ -CTI	β - γ -Content Tolerance interval
β -ETI	β -Expectation Tolerance interval
CF	Correction factor
$p\%$	Proportional correction factor
AA	Authentic analyte (used as subscript)
IS	Internal Standard (used as subscript)

Symbol	Term
$1 - \alpha$	Level of confidence (also noted γ)
$[A-, A+]$	Acceptance interval
$u_c(Z)$	Combined standard uncertainty Z
$u^2(Z)$	Standard variance of Z
RF	Response Factor
SP	Sum of crossed products of deviations to the mean
SS	Sum of squared deviations to the mean
s_r^2	Repeatability variance
s_W^2	Within-series variance
s_R^2	Reproducibility variance
s_L^2	Between-laboratories variance
s_{IP}^2	Intermediate precision variance
s_B^2	Between-series variance
r^2	Coefficient of determination
k_{TI}	Tolerance factor of a tolerance interval
k_{GUM}	Coverage factor
k_{GUM}	Standardized coverage factor (GUM)
$a_0, a_1, a_2, \dots$	Coefficients of the calibration model
$[Z \pm U(Z)]$	Coverage interval
G_n	Input quantity of the measurement model
$[X_L, X_U]$	Measuring interval or working interval

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1

Quantification

1.1 Define the Measurand (Analyte)

The initial question for the analyst is to define what is expected to be measured. According to the International Vocabulary of Metrology [1], the “quantity intended to be measured”¹ is called the measurand, or more specifically, the analyte, when considering measurement methods applied to chemical and biochemical substances. But this simple definition may be misleading while an analyte may have variable forms during the analytical process. It is not always certain that the substance finally measured is initially intended to be measured. For example, during sample preparation, the initial organic form of the analyte may change to inorganic, and what was intended to be measured is finally modified. For instance, in living organisms, heavy metal is present combined with proteins, such as mercury to metallothionein. Still, when analyzed after mineralization, it can be transformed into sulfate, perchlorate, or nitrate.

A well-known catastrophic example is the Minamata disease; when looking for mercury in food samples, the oldest methods were based on the complete sample mineralization to obtain mercury nitrate. Soon after, it was realized that the toxic forms of mercury were organic derivatives. Hence, so-called total mercury had no great toxicological interest compared to the different organic forms. Speciation techniques in mineral analysis or chiral chromatographic methods are good examples of innovative approaches devoted to better maintaining the analyte in its expected form. Therefore, quantification in analytical sciences is often less straightforward than claimed. From the metrological point of view, the difficult traceability of chemical substances to international standards is one of these obstacles.

This is detailed in Section 6.3 as an introduction to the estimation of measurement uncertainty (MU) among many other sources of uncertainty. The encapsulated conception of modern and highly computerized instruments may also prevent the analyst from assessing what is measured. Digits displayed on the instrument screen represent what is “intended to be measured.” The paradoxical consequence is that discussing the true nature of the analyte is often avoided, while more attention

1 Definitions or quotations extracted from standards or official documents are between double quotes.

should be paid to this question. The goal of this chapter is to propose things to consider on this topic. Many examples are based on mass spectrometry (MS) hyphenated methods because several are now considered highly compliant from a metrological point of view.

1.1.1 Quantification and Calibration

The metrology motto could be measuring is comparing. Therefore, when quantifying an analyte, the comparison principle must be previously defined. This preliminary step is usually called calibration. In modern analytical sciences, most methods use measuring instruments ranging from simple, specific electrodes to sophisticated devices; therefore, calibration procedure may enormously vary according to the nature of the instrumentation. This chapter attempts to classify the different quantification/calibration strategies applied in analytical laboratories. Because this subject is not harmonized, the employed vocabulary may vary from one domain of analysis to another and be confusing. For each term, we tried to give a definition, but it may be incomplete due to the considerable number of analytical techniques. Many suggested definitions are listed in the glossary at the end of the book.

Whatever the measuring domain, classic differences are made between direct and indirect measurement techniques. Direct method can usually refer to a measurement standard, for instance, when measuring the weight of an object on a two-pan balance with standard weights. Indirect measurements are performed using a transducer, a “device, used in measurement, which provides an output quantity with a specified relation to the input quantity.”

Reversely, with a one-pan balance, measurements are indirect. At the same time, result is obtained by means of a mathematical model linking the calibrated piezoelectrical effect on the beam to the weight. In analytical sciences, methods are usually indirect. Some exceptions are set apart, classified as direct primary operating procedures by BIPM (Section 4.2.1). For most chemical or biological analytical techniques, the measuring instrument must be calibrated with known reference items before use. Finally, quantification involves three elements, as outlined in Figure 1.1:

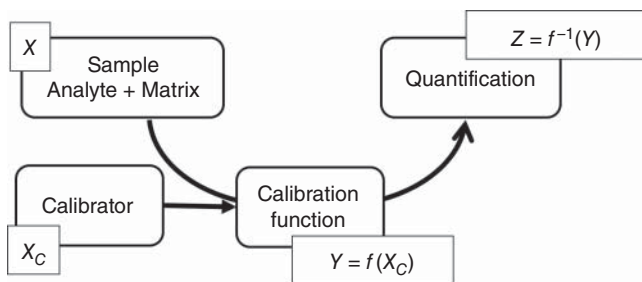


Figure 1.1 Schematic representation of the quantification principle.

- The analyte is in the working sample. Its concentration is denoted X . The searched compound (chemical or biological) is embedded within the sample matrix. It is only before any treatment that the analyte is present in the intended form. The role of sample preparation is to eliminate a large part of the matrix and concentrate on the analyte. But it may change the analyte chemical form; for instance, with the speciation of organic forms of heavy metals, sample preparation is quite different from classic mineralization.
- The calibration items are also called *calibration standards* or *calibrators*. They are prepared by the analyst to contain a known amount of a calibrant as similar as possible to the analyte. To underline this difference, it is denoted X_c . The selection of the adequate calibrant is a key-issue of quantification extensively addressed in the rest of this chapter.
- The calibration function that links the instrumental response Y to the known quantity X_c , denoted $Y = f(X_c)$.

Figure 1.1 is an attempt to recapitulate a generic quantification procedure. Most of the time, calibrators are artificially prepared and used to build the calibration function f which generally is *inverted* when analyzing an unknown sample. The three elements may be subjected to variations. Mathematical notation underlines the dissimilar roles they play for the statistical modeling of calibration and possible relationships that link the instrumental signal to the calibrant concentration. Denoting Z the predicted concentration of a sample emphasizes the role of inverting calibration function as discussed in Section 2.1. Finally, considering a given calibration dataset, distinct functions f can be fitted. A principal issue will be to select the best one because it deeply affects the global method performance. The goal of the present chapter is to describe some classical or new quantification procedures.

1.1.2 Authentic versus Surrogate

To be explicit, it is convenient to define some terms. If the chemical substance sought in the sample is called *authentic*, obviously, for many methods it is possible to prepare the calibrators with the authentic analyte. But other quantification methods exist based on a different calibration compound, which will be called surrogate standard or calibrant. It would be paradoxical to call it surrogate analyte, whereas the analyte can only be authentic. Therefore, when the analyte and the calibrant are different, it is necessary for the analyst to cautiously verify if they have equivalent analytical behavior and define an eventual adjustment method, such as a correction factor.

The measuring instrument is a transducer that converts the amount or the concentration of a chemical substance into a signal – usually electrical – according to a physical or chemical principle. How quantitative analyses are achieved varies from simple color tests for detecting anions and cations through complex and expensive instrumentation for determination of trace amounts of a compound or substance in a complex matrix. Increasingly, such instrumentation is a hybrid of techniques for separation and detection that requires extensive data processing.

The subject of analytical sciences has become so wide that complete coverage, providing clear information to an interested scientist, can only be achieved in a multi-volume encyclopedia. For instance, Elsevier published in 2022 the volume n°98 of the *Comprehensive Analytical Chemistry* handbook started in the 1980s.

The major obstacle in analytical sciences is the structural or chemical differences that exist between the analyte present in the working sample and the substance used as a calibrant. The instrument signal may depend on the authentic or surrogate structure of the analyzed substance: this dependence is marked with modern instrumentation such as mass spectrometers. On the other hand, the analyte present in a working sample is embedded with other chemicals, customarily called a matrix by the analysts. It is not always possible or easy to use the sample matrix when preparing the calibrators. These remarks lead to the definitions of four different quantification elements that can be combined to prepare or selecting calibrators and consequently obtain the calibration curve:

Authentic analyte	The same molecule or substance present in the working sample may be available for calibrator preparation, considering a high degree of purity.
Surrogate standard or calibrant	This is a reference substance that is assessed and used as a reasonable substitute for the authentic analyte. For instance, in bioanalysis, it is frequent to have metabolites or derivatives of the analyte that must be quantified without the reference molecule. Labeled molecules used in many methods involving isotopic dilution have recently been considered appropriate calibrants.
Authentic matrix	The simplest situation for using an authentic matrix is to prepare calibrants by spiking test portions of the working sample. For some applications, such as drug control, it is also possible to prepare synthetic calibrants with the same ingredients as the products to be controlled.
Surrogate matrix	This medium is considered and used as a substitute for the sample matrix. For instance, bovine serum is used in place of human serum. Then, it is assumed its behavior should be similar to the authentic matrix throughout the analytical process, including sample preparation and instrumental response.

When the surrogate matrix does not behave as the authentic or when calibration is achieved without the sample matrix, matrix effects may produce bias of trueness, as explained in Section 4.1.3. More precisely, calibration standards can be prepared with several classes of matrices. Matrix classification is widely based on analyst expertise and depending on the application domain, matrix grouping is extremely variable. For instance, broad definitions applicable to biological analysis can be as follows:

Authentic matrix (or real)	For biological analysts, serum, urine, saliva, or stool are different classes of matrices. In food chemistry, when determining the total protein, fatty and starchy foods are classified as different, or drinking water and surface water is different for water controllers.
Surrogate matrix	Matrix used as a substitute for authentic matrix.
Neat solution	Water, reagents used for extraction or elution, etc.
Artificial matrix	Pooled and homogenized samples, material prepared by weighting when the composition of the authentic matrix is fully known, etc.
Stripped matrix	Specially prepared materials are free of impurities or endogenous chemicals. They are mainly used for biomedical analysis.

It can be assumed that the combined use of surrogate standard and/or surrogate matrix may induce bias. It is necessary to cautiously verify if their analytical behavior is comparable to authentic ones. At least four combinations of the above-defined quantification elements are possible, each having pros and cons as explained later. It is possible to categorize different quantification modes depending on the selected combination:

Quantitative	Calibrators are prepared with authentic analytes and an authentic matrix. The amount or concentration of the analyte may be determined and expressed as a numerical value in appropriate units. The final expression of the result can be absolute, as a single concentration value; non-absolute, as a range or above or below a threshold.
Semi-quantitative	Surrogate standards and matrix are used. Some authors consider semi-quantitative analyses the ones performed when reference standards or the blank matrix are not readily available.
Relative	Sample is analyzed before and after an alteration or compared to a control situation. The relative analyte concentration is expressed as a signal intensity fold change. It is ratioed to another sample used as a reference and expressed as a signal/concentration.

It must be clearly stated that it is impossible to strictly separate quantification from calibration since they are interdependent. According to the nature of the calibration standard used, which can be authentic or surrogate, and the matrix, which can be authentic, surrogate, neat, etc., different quantification strategies were

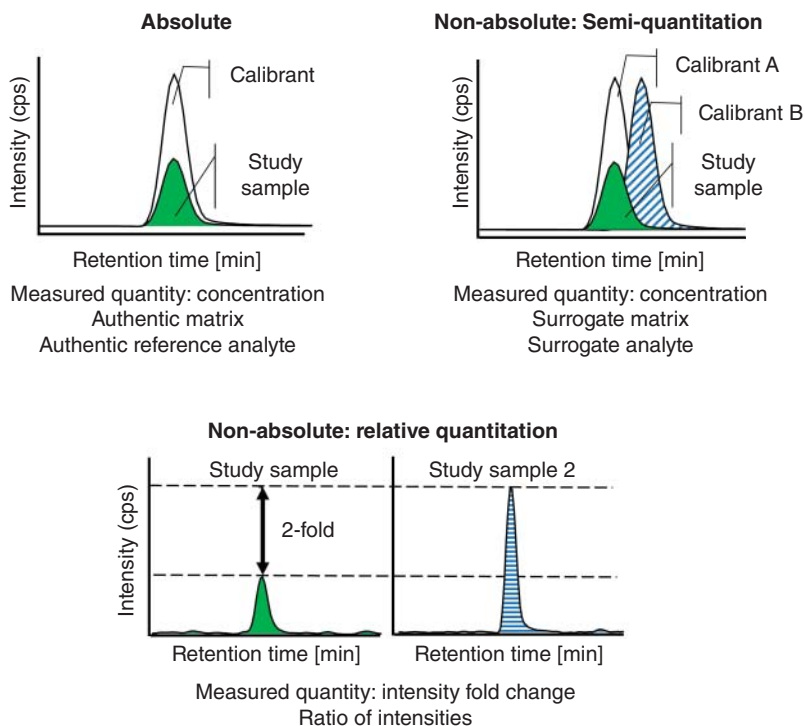


Figure 1.2 Schematic representation of absolute, semi, and relative quantification modes.

developed to obtain the effective calibration function. A schematic overview of the differences between principal quantification modes is summarized in Figure 1.2 and more extensively explained in the rest of the chapter.

1.1.3 Signal Pretreatment and Normalization

Nowadays, it is quite uncommon to use the analogic electrical signal output from the measuring instrument to build a calibration model. Digitalizing signals in modern instruments opened the way to many pretreatments, such as filtering, background correction, and smoothing. It is sometimes invisible to the analyst, although this can modify the method's performance. The outcome of many methods can be complex signals such as absorption bands or peaks in spectrophotometry or elution peaks in chromatography.

This raw information is not directly used as Y variable to build the calibration model; it is preprocessed. When dealing with absorption peaks, it is classic to select one or several wavelengths considered to be most informative. For instance, in biochemistry, protein concentration can be quickly estimated by measuring the UV absorbance at 280 nm; proteins show a strong peak here due to tryptophan and tyrosine residue absorbance. This can readily be converted into the protein concentration using Beer's law.

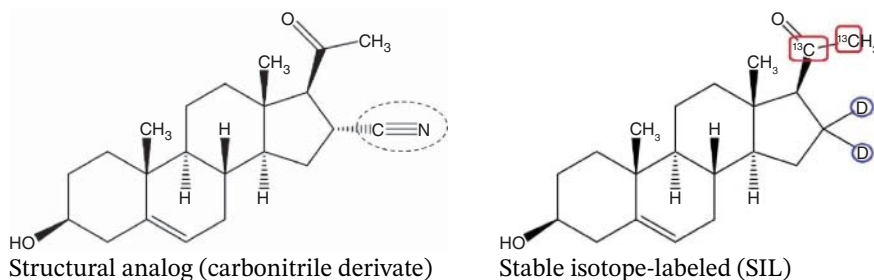
When obtaining poorly resolved absorption bands, as in near infrared spectroscopy (NIRS), the selection of one specific wavelength is difficult, and the use of a multivariate approach has been promoted. Many publications in chemometrics literature are addressing this issue. The multivariate calibration based on partial least-squares regression (PLS) has now become a routine procedure.

If the output signal is time-resolved, such as liquid or gas chromatographic peaks, they are always pretreated by an integrator. Initially, it was a separate device, but now it is included in the monitoring software. It can determine several parameters characterizing the elution peak, such as retention time at the highest point, skewness, peak height, but mainly peak area. The peak area is in the favor with analysts. But several publications demonstrated that for some methods, peak height is preferable to peak area and that when standardizing a method, the integration conditions must be carefully harmonized [2].

For some methods, such as MS-coupled methods, the measured response Y can strongly vary according to the detector performance, such as mass analyzer type, ionization modes, ion source parameters, system contamination, ionization enhancement or suppression due to the sample matrix effect, along with other operational variables related to the analytical workflow.

Thus, the analyte relative response is standardized to compare performance over time. A common operation is adding an internal standard (IS) to the study and calibration samples at fixed concentrations. For instance, two official inspection bodies advise evaluating the matrix effects when a complex surrogate matrix is used [3, 4]. For the latter, the Food and Drug Administration (FDA) suggests investigating the matrix effect by performing parallelism testing between linear calibration curves computed with the authentic and surrogate matrices. This method is not always effective, while parallelism statistical testing is conservative, i.e. depending on the data configuration significant difference may be considered nonsignificant and only applicable to linear models.

Conversely, the European Medicines Agency (EMA) provides full instructions on how to do it and recommends comparing the extraction recovery between the spiked authentic matrix and surrogate matrix used for the calibration, along with the inclusion of IS as an easy and effective method to correct biases between these two matrices. When the analyte and the IS are affected similarly during the analytical process, instrument signals can be correctly standardized. A comprehensive approach is proposed further using the method accuracy profile (MAP); it is also an effective approach to detect and control matrix effects.



Two main categories of IS, namely structural analogs and stable SIL, can be identified. The molecule of pregnenolone is used to exemplify this. The first category, visible on the molecule on the left, is related to compounds that generally share structural or physicochemical properties similar to the authentic analyte.

The second category, exemplified by the molecule on the right, includes stable isotopic forms of the analyte, usually by replacing hydrogen ^1H , carbon ^{12}C , or nitrogen ^{14}N with deuterium ^2H , ^{13}C , or ^{15}N , respectively. Obviously, using labeled IS requires the coupling to a mass spectrometer. Deuterated IS are widely used due to their lower cost. Still, their lipophilicity increases with the number of substituted ^2H , leading to differences in their chromatographic retention times with the corresponding authentic analyte. This phenomenon, known as *deuterium effect*, can also impact the instrumental response or behavior (e.g. the electrospray ionization process in MS) compared to unlabeled compounds.

Even if an increasing number of high-quality SIL are commercially available, they are limited to the most commonly used chemical compounds. When many analytes must be simultaneously quantified, the possibility of using one IS for multiple analytes should be carefully evaluated. For quantification purposes, using one IS per target compound is generally recommended when available because they are assumed to compensate for specific differences in matrix effect and extraction recovery between the calibration methodology and working samples.

To complete this rapid overview, when compatible with the analytical method, the use of standards linked to the International System of Units (SI) is a convenient means of standardizing the instrumental response and correcting the overall variation in the measurement process resulting from diverse sources of uncertainty, such as sample preparation or interfering compounds, also known as the matrix effects. The absolute instrumental response is then normalized as a response ratio:

Normalized response ratio

$$Y = \frac{Y_A}{Y_{IS}} \quad (1.1)$$

In this formula, Y_A and Y_{IS} are the responses obtained with the analyte and the IS, respectively. This formula gives a relative instrumental response but does not consider the respective concentrations. To be more in harmony with Figure 1.1, Y_{IS} is equivalent to Y_c . This new notation is used because the IS is a particular example of a compound used for calibration.

The influence of signal preprocessing, such as peak integration, was experimentally demonstrated during an interlaboratory study on determining fructose, maltose, glucose, lactose, and sucrose in several foods by liquid chromatography [5]. A specific experimental design was developed to achieve this demonstration. Participants were requested to send their results calibrated as both peak heights and areas. Considering the mean values obtained with the two approaches, differences ranged from -18% up to $+5\%$. This indicates that trueness may be affected by the quantification mode. Precision, expressed as the reproducibility variance, was computed using both sets of results.

More details about this common parameter of precision are given in Section 3.2.1. In Figure 1.3, a subset of interlaboratory results is reported. Food types are indicated