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 derivates. 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.

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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:

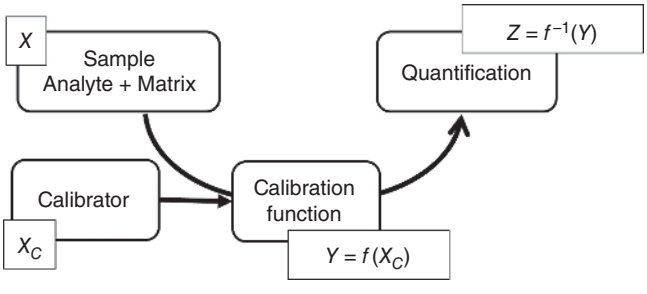


Figure 1.1 Schematic representation of the quantification principle.

- 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 inversing 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 derivates 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

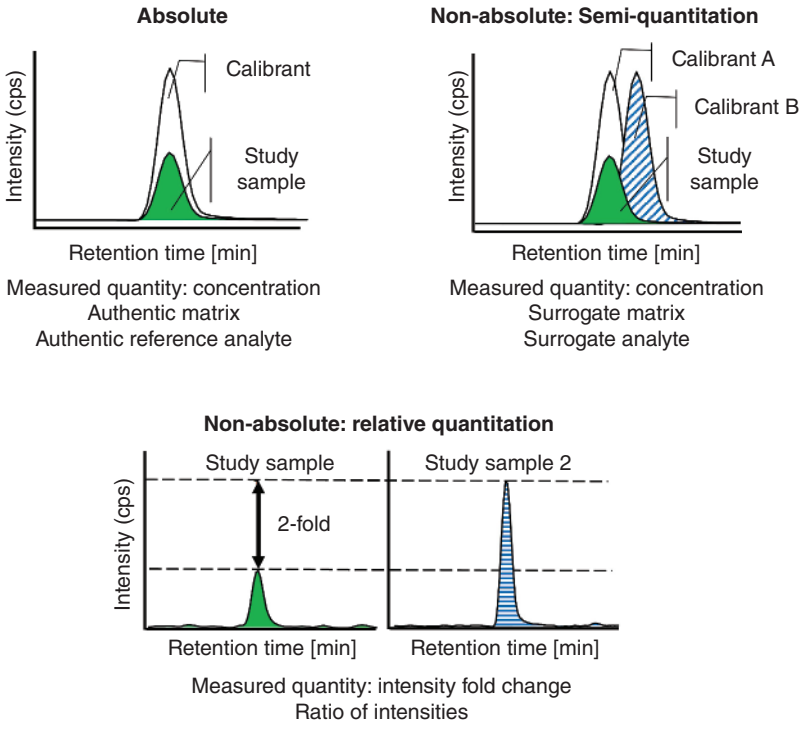


Figure 1.2 Schematic representation of absolute, semi, and relative quantification modes.

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.

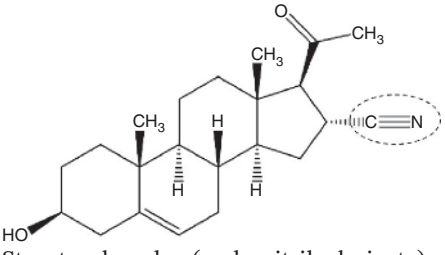
When obtaining poorly resolved absorption bands, as in near infrared spectroscopy (NIRS), the selection of one specific wavelength is difficult, and the use of a multivariate approach has been promoted. Many publications in chemometrics literature are addressing this issue. The multivariate calibration 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].

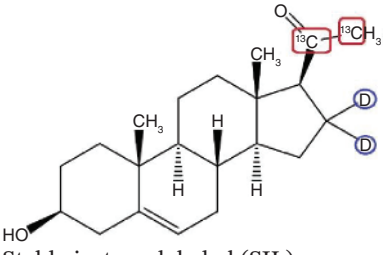
For some methods, such as MS-coupled methods, the measured response Y can strongly vary according to the detector performance, such as mass analyzer type, ionization modes, ion source parameters, system contamination, ionization enhancement or suppression due to the sample matrix effect, along with other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



Structural analog (carbonitrile derivate)



Stable isotope-labeled (SIL)

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 ^{1}H, carbon ^{12}C, or nitrogen ^{14}N with deuterium ^{2}H, ^{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 ^{2}H, 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

$$Y = \frac{Y_A}{Y_{IS}} \tag{1.1}$$

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

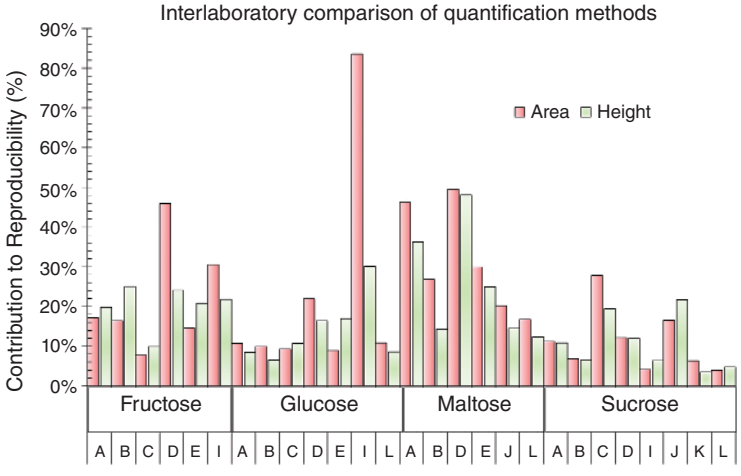


Figure 1.3 Contribution to the reproducibility of two quantification methods in liquid chromatography of saccharides.

by an uppercase letter ranging from A to L; they are saccharide-containing processed foods, such as soft drinks, baked foods, or candies. Precision for peak area appears as vertical red bars and peak height as light green bars. The role of the signal processing method is expressed as a relative contribution to the reproducibility variance. The contributions and their differences are sometimes ridiculously small, such as fructose in food C where it is below 10%. But sometimes very impressive, such as glucose in food I. If some food is not present on the diagram, the analyte was not detected. For instance, *L* is a chocolate bar that contains no fructose. Peak area is not always the best way to quantify the analyte. In the publication, an explanation is given why the discrepancies exist. It mainly depends on the resolution of peaks and their relative values.

Detecting a peak beginning and end is a contingent subject and a source of uncertainty for the surface integration, as explained in Section 4.1.2. Finally, integrator settings can be used to optimize the integration algorithm and accordingly influence the global performance of the method.

1.2 Calibration Modes

Two major calibration modes are used in laboratories, namely:

External calibration (EC)	A calibration curve is established independently from the working samples, whatever the calibrant nature and preparation. A single calibration function is used to quantify many samples. This is the most classical procedure, and several variants exist.

Internal calibration (IC)	The term is applied to diverse procedures. The calibration is achieved with a calibrant under different forms in the working samples. Conversely, one calibration function is obtained for each working sample to be quantified. Recently novel procedures have been developed for MS-based analysis and are detailed in Section 1.5.

As briefly mentioned before, the analyte nature, the availability of the working sample material and the calibration material influence the selected type of calibration. This can be summarized by this simple table leading to at least four different basic configurations.

		Matrix	
		Authentic	**Surrogate**
Analyte	Authentic	Yes	Yes
	Surrogate	Yes	Yes

Table 1.1 attempts to classify different calibration modes, external *versus* internal, commonly used in the laboratory, including the advantages (pros) and limitations (cons) for each. As illustrated, external calibration (EC) methodologies depend on the availability of both analyte and matrix. For the procedure called in-sample calibration (ISC) there is no need to select a particular calibration matrix as the working sample matrix is used. Still remains the question of the analyte's availability. The abbreviation ISC is introduced to make the difference with internal calibration.

1.3 External Calibration (EC)

1.3.1 Authentic Analyte in Authentic Matrix: MMEC

External calibration (EC) corresponds to the most often-used operating procedure because it allows the rational determination of several routine samples with one pre-determined calibration function $Y = f(X)$. The first situation, sometimes called matrix-matched external calibration (MMEC), represents a good metrological quantification approach and is extensively discussed in the major international guidelines to validate bioanalytical methods [6].

With exogenous substances, such as rare pollutant chemicals, a blank matrix is generally available and permits EC with authentic analyte in a representative matrix. On the other hand, with endogenous compounds at endogenous concentration, such as vitamins in foods, other approaches should be explored to overcome the absence of an analyte-free matrix. In this complicated context, alternative procedures have been proposed, such as background subtraction or the use of surrogate matrices and/or analytes as described below.

Table 1.1 Proposals for a classification of calibration procedures.

External calibration (EC)				
Ref.	**Authentic analyte**		**Surrogate standard[a)]**	
Matrix	**Authentic**	**Surrogate**	**Authentic**	**Surrogate**
Method	Matrix-matched (MMEC)[b)]	Surrogate matrix	Surrogate analyte	Surrogate analyte and matrix
Pros	Matrix effect and selectivity close to sample.	Suitable for low concentration compounds.	LOQ Lower than the background subtraction.	When authentic analyte difficult to obtain.
Cons	LOQ define by endogenous concentration.	Production of analyte free matrix. Possible differences in extraction recovery and matrix effect.	Accuracy depends on surrogate specificity. Additional experiment for linearity and LOQ.	Accuracy depends on surrogate specificity. High differences for recovery yield to be expected.

In-sample calibration (ISC)			
Ref.	**Authentic analyte**	**Surrogate standard (calibrant)**	
		Partially labelled isotope analogue	Fully labelled isotope or structural analogue
Matrix	Authentic	Authentic	Authentic
Method	Standard addition method (SAM)	Isotopic pattern deconvolution (IPD)	Internal calibration (IC)
Pros	Same matrix effect and selectivity as the sample.	High potential for accuracy	High potential for accuracy (SIL)
		Relying on isotopic distribution alteration.	Reduced numbers of calibrators.
Cons	Need for large initial specimen volume.	Depends on analogue concentration and stability.	Depends on analogue concentration and stability.
	Not easy implemented for high throughput.	Additional experiment for linearity and LOQ.	Structural analogues cannot compensate for differences in ionization. Additional experiment for linearity and LOQ.

a) Isotope labelled or structural analogue.
b) With or without background subtraction.

The use of authentic matrix for multipoint EC provides an extraction recovery yield that is close to the specimen and is commonly performed to quantify exogenous substances when a large amount of the matrix is available. In the presence of endogenous compounds, a representative pooled matrix fortified with authentic calibration standards can be prepared to estimate and remove the endogenous background signal. This approach, known as background subtraction, uses the pooled matrix-matched EC to interpolate the concentration in the working samples.

As described in Section 2.2, Z is the inverse-predicted concentration. It is obtained by inversing the equation of the calibration curve. Equation (2.24) illustrates the rationale in the case of a linear calibration curve where the slope a_1 and intercept a_0 refer to the regression parameters of the added authentic standards in the pooled authentic matrix.

However, the upper limit of quantification (ULOQ) as defined by several regulatory documents may be impaired by the blank response a_0, because detector saturation may occur. Similarly, endogenous metabolite concentrations may vary due to intra- and inter-sample variation, leading to highly variable results when a pooled matrix is used. To overcome these drawbacks, several calibration curves using different representative pooled matrices can be prepared to select the calibration model that best covers the concentration to be analyzed. MMEC cannot always correct the matrix effect when it differs between working samples, emphasizing the importance of using an IS to correct this bias.

1.3.2 Authentic Analyte in Surrogate Matrix

As stated, a surrogate matrix could be used as a substitute to prepare calibrants with the authentic analyte or a mixture of analytes. It can be of various complexity. For instance, in bioanalysis, several matrices are proposed as surrogates, namely neat solutions, synthetic or stripped matrices.

- Neat solutions: it can be the mobile-phase solvent mixture, extraction reagents or pure water.
- Synthetic matrices: they are composed of salt, sugar and simulate authentic matrix properties, such as analyte solubility, extraction recovery and matrix effect. When the working sample matrix is comparable to water, saliva, urine, tears and cerebrospinal fluid, neat and artificial solutions can be used as surrogate matrix.
- Stripped matrices: they can be in-house made or commercially available, such as depleted human or bovine serum. Charcoal stripping removes nonpolar material such as lipid-related materials, mainly hormones and cytokines, leading to an analyte-free matrix that can be used as a blank for the preparation of calibrators. It is important to emphasize that charcoal depletion is nonselective and may result in approximate matrix similarity.

Whatever the chosen solution, it must be shown it has the same, or comparable, extraction properties as the authentic matrix.

Hence, surrogate matrices may not perfectly simulate the original matrix. To correct those matrix biases, a proper evaluation should be performed as recommended

by both FDA and EMA guidelines. To assess the applicability of any surrogate matrix the classic requirement is to compare the slopes of the calibration curves calculated with the surrogate matrix and authentic matrix. Diverse statistical treatments are available, such as analysis of variance.

But only EMA specifies how to assess the matrix similarity by using the concept of acceptance. This consists in ratioing the slope between authentic analyte in authentic matrix *versus* authentic analyte in surrogate matrix. The obtained value should be within ±15% of the nominal value. Example of possible procedures is fully described in Section 2.4.3 and illustrated in the worksheet named Resource D. The standard addition method (SAM) is one other dedicated tool to achieve this goal.

1.3.3 Surrogate Calibrant in Authentic Matrix

In situations where calibration is performed using a surrogate standard, it is assumed that the physicochemical properties of both authentic analyte and the surrogate calibrant are equivalent. For instance, the extraction recovery, the chromatographic retention behavior, and the instrument response should be either identical or have acceptable differences to be fully exploited. The choice of surrogate calibrant is essential to accurately quantify the authentic analyte.

For example, ICH guidelines [7] suggest using SIL molecule as surrogate calibrant in authentic matrix, while FDA guidelines [3] do not endorse this methodology. Because the calibration reference compound does not correspond to the authentic analyte, the ratio of responses between surrogate and analyte should be investigated over the desired dynamic range. Before routinely using the surrogate calibrant, the response factor *RF* must be evaluated as an analyte-to-calibrant ratio where X_{AA} and X_{SS} are the concentrations of authentic analyte and surrogate standard, respectively, and corresponding instrument responses:

Response factor (analyte *versus* surrogate)

$$RF = \frac{Y_{AA}}{X_{AA}} \times \frac{X_{SS}}{Y_{SS}} \tag{1.2}$$

To achieve the appropriate *RF* estimation, different proportions of analyte/surrogate must be investigated. For MS methods, this step is compulsory to evaluate the ionization efficiency whereas the *RF* must be constant over the method working domain. Another way to investigate the *RF* is to check if both lines are parallel. It consists in comparing the slopes of the authentic analyte line and the surrogate, both performed in the same pooled matrix.

Additionally, if the *RF* is not constant over the validation domain corrections, such as LC gradient or MS/MS transitions (de)-optimization, can be investigated to obtain a balanced response. If SIL is used as surrogate calibrant, the analyst should explore the potential presence of crosstalk interferences such as isotopic pattern overlap or impurities coming from SIL standards [8]. In MS, crosstalk occurs when ions from one scan event are still present in the collision cell when a second transition is taking place. This leads to signal artifacts in the next transition's chromatogram.

The *RF* can diverge from unit value when SILs containing enriched hydrogen atoms are used, but as long as the unit value slope remains within the ±15% acceptance interval compared to the authentic analyte slope, investigated SILs can be selected as surrogate calibrants. For example, tryptophan was successfully quantified in plasma with a relative bias between −2.0 and −8.0% using its deuterated analogue, even if the response factor was 0.67 [9]. Once the *RF* has been established, a multipoint calibration is performed in a pooled authentic matrix and the concentration of the authentic analyte is computed as follows:

Corrected concentration of authentic analyte

$$Z = \frac{\left(\frac{Y}{Y_c}\right) - a_0}{RF \times a_1} \tag{1.3}$$

where:

- Y and Y_c refer to the measured signal Y of the authentic analyte and the IS, respectively.
- Coefficients a_1 and a_0 characterize the slope and intercept of the calibration line performed with the surrogate standard.

Likewise, MMEC's use of an IS remains strongly recommended to correct for sample preparation and matrix effect variation between working samples and calibrators, thus improving trueness and precision when dealing with routine sample determination. Because the endogenous concentration of the authentic analyte in a pooled matrix is stable, an exciting possibility to implement this quantification method is to use this signal as an IS to normalize the instrument response of the surrogate standard calibration.

This approach, called Isotope Inversion, provides the same quantitative results for steroid determination as using the authentic analyte in a surrogate matrix such as active-charcoal stripped serum in this application [10]. When no signal from endogenous analyte interferes with the surrogate signal, the surrogate calibrant in authentic matrix can be a suitable alternative to the matrix-matched external calibration, especially when high endogenous concentration is present and/or intra- and inter-sample variations are observed.

1.3.4 Surrogate Calibrant in Surrogate Matrix

The increased commercial availability of SILs has raised interest in their use as surrogate calibrants in surrogate matrices to reduce calibration preparation time. Numerous publications have demonstrated their benefit, especially when MS detection is considered. This semi-targeted quantitation approach could be used to determine the amount of target analytes without needing authentic chemical standards. For instance, exogenous compounds were selected as potential surrogate calibrants in several biological matrices such as blood, plasma, urine, cerebrospinal fluid, and tissue homogenate [11]. In some cases, the combination of the surrogate calibrant in surrogate matrix allows extending the number of analytes that can be quantified in a single analysis.

1.4 In-sample Calibration (ISC)

In contrast to EC, in-sample approach calibration (ISC) is characterized by an analytical calibration function obtained directly in each working sample. The SAM is probably the most established ISC procedure and popular in many fields, such as foods, environment, or forensic toxicology, where matrices are extremely variable, when the authentic analyte is available. Two other approaches also aim to simplify the quantification condition, depending on the chemical purity and the physicochemical proprieties of surrogate calibrant such as SIL. The former predicts the authentic analyte concentration by altering its natural isotopic pattern with a labeled analog standard. The latter is applicable when no significant interferences between the analyte and SIL are observed. In this case the authentic analyte concentration is directly determined.

1.4.1 Authentic Analyte: Standard Addition Method

As an operating procedure for absolute quantification, SAM consists in collecting the responses of authentic analyte additions in a series of aliquots obtained from the working sample. The simplest experimental design of SAM comprises a minimum of two runs described in Table 1.2. Notations are the same as in Figure 1.1:

- Level 0, or X_0, is the *no-addition* level and consists in recording the response Y_0 in the working sample without any addition of the authentic analyte.
- Level 1, the working sample is spiked with a known amount of the authentic analyte.

By combining the two couples of data, the corrected concentration of the working sample is given by Equation (1.4).

Corrected concentration

$$Z = Y_0 \times \frac{X_1 - X_0}{Y_1 - Y_0} \tag{1.4}$$

The short worksheet below gives an example of computation. The formula applied in cell `B5` is shown in cell `C5`. Figure 1.4 illustrates the data and shows that the corrected concentration corresponds to the extrapolation where Y-value is zero, and the line cuts the X-axis.

	A	B	C
1	**Simple SAM**		
2	Concentration *X*	Response *Y*	
3	0	32	
4	10	205	
5	Corrected result	1.8497	=B3*(A4-A3)/(B4-B3)

Table 1.2 Two-run experimental design of standard addition method.

	Concentration *X*	Response *Y*
Level 0 (no addition)	$X_0 = 0$	Y_0
Level 1 (spiked)	X_1	Y_1

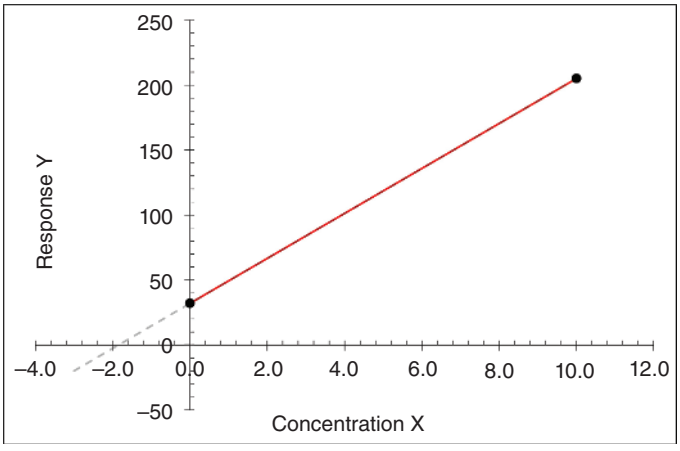


Figure 1.4 Two-run standard addition method.

This simplified experimental design can be routinely applied when each sample may have a specific matrix effect. For instance, when analyzing surface waters, it is classic to use simple SAM for each sample because the composition is recognized as highly variable. In this case, the result is obtained by combining two measurement values that are not replicated. A discussion about the role of replication in reducing MU is presented in Section 8.4.3.

Even simplified SAM is time-consuming with preparing and measuring two test portions per working sample. The benefit is to consider interindividual differences in matrix composition, to overcome matrix effects, and avoid building an EC curve. In that respect, it can be asserted as an absolute quantification method, as far as the response is exactly proportional to the concentration, in other words, linear.

As mentioned before, the FDA suggests applying SAM in a more complex experimental design to verify if using a surrogate matrix or analyte is justified. It calculates two calibration lines: one prepared by spiking several test portions of the working sample, the other by preparing calibrators with the surrogate matrix, which can be neat.

In the classic operating procedure, the working sample is divided into four and six identical aliquots, and a fortified calibration curve is obtained by spiking increasing known amounts of the authentic analyte, e.g. 50, 100, and 200% of the expected endogenous concentration. Only the first aliquot remains nonspiked, and

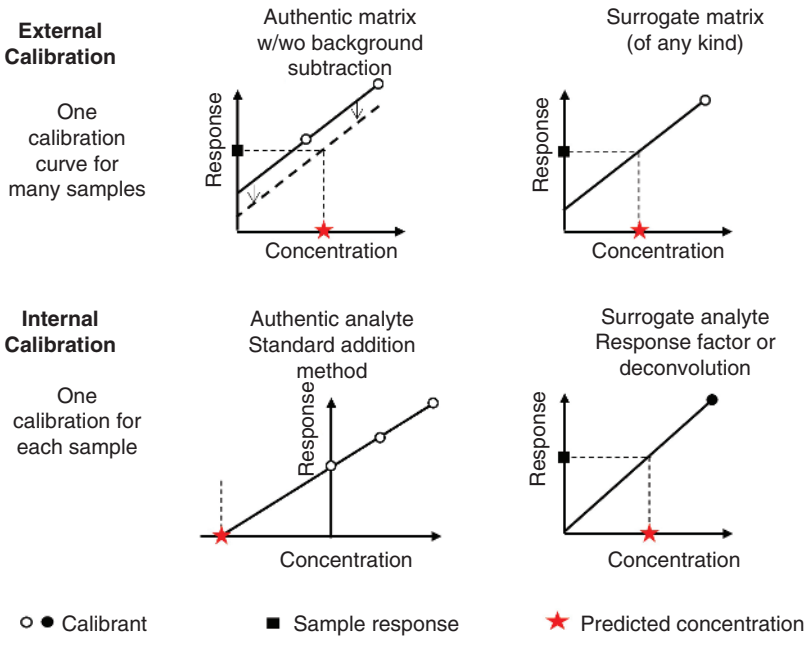


Figure 1.5 Calibration modes in analytical sciences. Source: Adapted from Visconti et al. [13].

its concentration is obtained by extrapolation where Y-value is equal to 0. This other protocol is illustrated with the example of Section 2.4.3. When the number of spikes is significant, SAM can also be applied when the calibration curve is polynomial, particularly when high endogenous signals affect the linearity of the response due to detector saturation.

When multiple signal-based detectors, such as MS or DAD, can record several physicochemical properties of the target analyte, more than one SAM calibration curve can be simultaneously acquired for the same working sample. This multiple-response monitoring leads to the possibility of dealing with the H-point standard addition method (HPSAM).

This new procedure is effective to control both proportional and additive biases (defined in Chapter 4), such as matrix interferences and/or detector saturation, when all calibration lines are converging at almost the same X-intercept. A comprehensive example is presented in Section 10.1. If the calibration lines are not correctly converging, a revised HPSAM was proposed including chemical modifiers [12]. Figure 1.5 is an attempt to propose a schematic overview of the diverse quantification/calibration strategies described in this chapter.

1.5 Some New Quantification Techniques

As stated, this chapter does not aim to give an exhaustive description of all possible quantification modes. However, it is valuable to describe some new insight on

a class of analytical techniques involving MS detection hyphenation, while many novel quantification modes were recently developed thanks to the improvement of modern MS instrumentation. More details are available in a recent review [13]. MS-coupled methods have progressively emerged as a one of the key instrumental components for numerous applications in laboratories, thanks to the development of new instruments and the reduction of costs.

The latter has become possible due to the advent of atmospheric pressure ionization interfaces, allowing to produce gas-phase ions that can be further analyzed. Compared to traditional spectroscopic detectors, such as UV absorbance, mass spectrometers offer additional selectivity by determining the mass/charge ratio of ion(s) or transition. An increasing number of articles reporting new MS-coupled methods for quantification are submitted each year [14].

In the field of MS-coupled methods, the greater availability of SILs opens the possibility of novel calibration procedures. They can mainly be employed as ideal surrogate calibrants to directly perform the calibration in the study matrix. Obviously, if they are used for this purpose, the analyst must first investigate the potential presence of interferences with the authentic analyte. When a contribution coming from the SIL is significant and modifies the signal, the application of isotope pattern deconvolution (IPD) was proposed as a corrective approach. In the absence of significant interference, internal calibration represents one of the most promising methodologies for modern absolute quantification.

1.5.1 Isotopic Pattern Deconvolution (IPD)

Isotope dilution mass spectrometry (IDMS) is a well-known technique applicable both to organic as inorganic analysis. It is because all isotopes of one element show almost the same chemical properties but mass differences between isotopes that IDMS allows quantifying the analyte by mass spectrometry. There are different IDMS operating procedures offering also various levels of precision. In many routine applications simple and fast operating procedures can be applied. The IPD is one of these high precision procedures based on the natural isotopic pattern alteration of a standard using a minor isotope labeled analog. In contrast to traditional analytical methods that rely on signal intensity, IPD is established by ratioing the signals between the isotopes of the molecule of interest and an analog with an enriched isotopic composition (i.e. SIL).

The IPD is sometimes claimed to be one of the most reliable and highest-quality metrological methods and is commonly used by chemical manufacturers to calculate SIL isotopic enrichment and purity. The isotopic abundance and concentration of the isotope labeled analog can be obtained by reverse isotope dilution mass spectrometry, i.e. a calibration against a high purity solution of the natural analyte prepared from a gravimetric solution of a suitable reference material.

First, the isotopic distributions for unlabeled standard and SIL as well as their combinations are computed using dedicated software: this is the convoluted isotope distribution. Free-access software is available coded with R to achieve the deconvolution. The labeled compound is then added to the reference material, resulting in

isotopic dilution. Then, the comparison between theoretical and experimental isotope overlap allows us to determine the SIL isotopic enrichment, chemical purity, and concentration.

Finally, once the SIL solution has been characterized by isotope dilution mass spectrometry it can be used as a calibrant for IPD quantification [15]. The more detailed procedural aspect is as follows, where variable A is the measured isotopic abundance, subscript *nat* comes for natural, *lab* for labeled and *mix* for mixed.

- Step 1. The natural isotopologue distributions of the analyte X and its isotope labeled analog X_{SIL} are measured. Let us remember that isotopologues only differ in their isotopic composition and have the same chemical formula. Superscripts $M0$, $M1$, etc. used in following formulas to indicate isotopologues.
- Step 2. Authentic analyte and SIL are mixed, and the resulting isotope pattern are determined. The basic concept is to say that the pattern of mixed solution is a linear combination of natural and labeled patterns weighted by the molar fractions q_{nat} and q_{lab}, respectively:

  Deconvolution model for IPD

$$A_{mix} = q_{nat}A_{nat} + q_{lab}A_{lab} + E \tag{1.5}$$

The vector of random error E is added to account for the errors in the isotopic determinations. It is called a deconvolution model because it is slightly different of the classic calibration model, such as Equation (2.6), where there is only one predictive variable, the calibrant concentration usually noted X as explained in Sector 2.2. In this case there are two predictive variables A_{nat} and A_{lab}. Once the isotopic abundances are measured, we have a set of equations:

Isotopic patterns

$$\begin{aligned}
A_{mix}^{\mathrm{M0}} &= q_{nat}A_{nat}^{\mathrm{M0}} + q_{lab}A_{lab}^{\mathrm{M0}} + E^{\mathrm{M0}} \\
A_{mix}^{\mathrm{M1}} &= q_{nat}A_{nat}^{\mathrm{M1}} + q_{lab}A_{lab}^{\mathrm{M1}} + E^{\mathrm{M1}} \\
A_{mix}^{\mathrm{M2}} &= q_{nat}A_{nat}^{\mathrm{M2}} + q_{lab}A_{lab}^{\mathrm{M2}} + E^{\mathrm{M2}} \\
&\cdots \\
A_{mix}^{\mathrm{Mn}} &= q_{nat}A_{nat}^{\mathrm{M}n} + q_{lab}A_{lab}^{\mathrm{M}n} + E^{\mathrm{M}n}
\end{aligned}$$

They can be rewritten in a more condensed matrix form (the term matrix is used with its mathematical meaning) clearly showing this a multiple regression model with two variables and no intercept:

Multiple regression model

$$\mathbf{A}_{mix} = [\mathbf{A}_{nat}\mathbf{A}_{lab}]\mathbf{q}^{-1} + \mathbf{E} \tag{1.6}$$

- Step 3. Apply least-squares multiple linear regression to get the solutions of model 1.6; i.e. the estimates of the molar fractions q. With Excel this can be achieved using the `LINEST` built-in function. This function usage is described in Section 2.3.1. In this chapter, `LINEST` is applied to estimate the three coefficients of a quadratic model. Model in equation 1.6 is also a 3-coefficient model, with one coefficient equal to 0.

– Step 4. Knowing the SIL concentration, noted X_{SIL} or equivalently X_c, used for spiking the working sample, direct quantification of the analyte Z can be provided without the need for a calibration curve as shown in Equation (1.7).

Estimated sample concentration

$$Z = X_{SIL} \times \frac{q_{nat}}{q_{lab}} \tag{1.7}$$

To correctly achieve this procedure and be able to perform the deconvolution, it is essential to have a crosstalk or isotopic overlap. This is possible when SIL chemical purity and/or isotopic enrichment is less than 100% or when there is only a small mass-unit difference between the isotope labeled standard and its analogous compound. IPD reproducibility was estimated based on an interlaboratory study, including four different World Anti-Doping Agency (WADA) accredited laboratories, and compared to a more traditional EC calibration method using surrogate standards. More details on interlaboratory precision parameters are available in Section 3.1.

The IPD shows the same accuracy and demonstrates improved reproducibility at low concentrations (2 ng/ml) with a relative standard deviation of reproducibility ranging approximately from 10 to 16%, respectively [16]. This result shows that isotope dilution mass spectrometry determination analytical methods are of high metrological quality. To confirm the high metrological reliability of the IPD, MU was estimated the same manner it is presented in Section 6.4 for LEAD example.

Uncertainty budget shows that MU is mainly dependent on the experimental determination of isotopic abundance (78.0%) and SIL concentration measurement (21.3%). Reducing these two sources of uncertainty involves some additional work during method development, but the IPD procedure then benefits from a better performing and faster analysis because calibration is performed within the working sample, and no traditional EC curve is required.

1.5.2 Direct Internal Calibration with Labeled Calibrant (IC-SIL)

When possible, the simplest quantification procedure is probably achieved when an in-sample single amount of surrogate calibrant is used to compute the working sample concentration. With this procedure, authentic and surrogate standards are simultaneously measured. The estimated analyte concentration, Z, is directly obtained via the peak area ratio of the sample *versus* the surrogate calibrant. Because only one concentration level is introduced in the working sample, a response factor relationship must be first established to confirm the absence of ionization competition between surrogate and authentic analyte, independent of the concentration. Thus, equimolar mixtures of surrogate and authentic analyte in neat, artificial and/or depleted matrices are first analyzed over the investigated calibration range. Additionally, ionization competition at nonequimolar concentrations should be investigated. Thus, several multipoint calibrations using the authentic analyte with surrogate standard at different concentration levels can be analyzed to study the authentic analyte response function alteration. Once the *RF* has been empirically determined, the working sample concentration is calculated as follows:

Working sample concentration

$$Z = \frac{Y}{Y_{SIL}} \times \frac{X_{SIL}}{RF} \tag{1.8}$$

This equation is a reorganization of Equation (1.3), where the intercept is zero, and the slope a_1 corresponds to the *RF*. When SIL is spiked at low concentration, such as 12.5 or 25% of the ULOQ, marked competitive ion suppression occurs due to the concomitant presence of the analyte at higher concentrations in ionization source. Conversely, when the SIL concentration is fixed in the highest bound of the response function, the influence of the surrogate signal on a low concentrated analyte can be detrimental and generate a significant bias. A correction procedure was proposed by determining the SIL concentration equivalent, noted X^*_{SIL} obtained with the following formula:

SIL concentration equivalent

$$X^*_{SIL} = X_{SIL} \times P_{SIL} \times E_{SIL} \times \frac{MW_A}{MW_{SIL}}$$

where *MW* is the molecular mass of authentic analyte and SIL surrogate, *P* the chemical purity as percentage, and *E* the isotopic enrichment, expressed as the probability of finding a labeled atom at any single site [17]. New reagents and improved instrumentation give opportunities to develop novel and faster quantification procedures exhibiting high metrological quality parameters. For instance, the one-point calibration method using SIL as calibrant and their isotopes was introduced to extend the lower limit of quantification (LLOQ).

To perform this analysis, a triple quadrupole instrument was used and a particular acquisition method named multiple isotopologue reaction monitoring (MIRM) was developed. By monitoring the SIL isotopic fragmentation abundances, a regression model was constructed by plotting the surrogate standard concentration equivalent on the abscissa and the instrument response (peak areas) of the corresponding MIRM channel on the ordinate. Then, the authentic analyte concentration can be calculated using the regression parameters [18]. This is just an example of regularly active literature.

Overall, internal calibration with SIL as calibrant is conceptually straightforward for absolute quantification with modern MS instrumentation, but requires additional steps during method development, such as the experimental determination of the *RF* and, with the MIRM procedure, isotopic abundance determination. However, once the method is developed, it is markedly faster in routine analysis because a daily repeated calibration curve is no longer required, and comparable results to EC can be obtained. Currently, the IC is raising interest due to the increased number of high-quality SILs commercially available, even if they remain limited to the most classic compounds. To overcome this limitation, isotope standards can be generated in-house by derivatizing authentic analytes with labeled $^{13}C_2$-dansylchloride and $^{13}C_2$-dansylhydrazine.

As an illustration of the selection of quantification procedure, Figure 1.6 presents a flowchart applicable to LC-MS methods. Some parts of this flowchart are transferable to other methods of analysis and/or detection modes. Possible strategies are

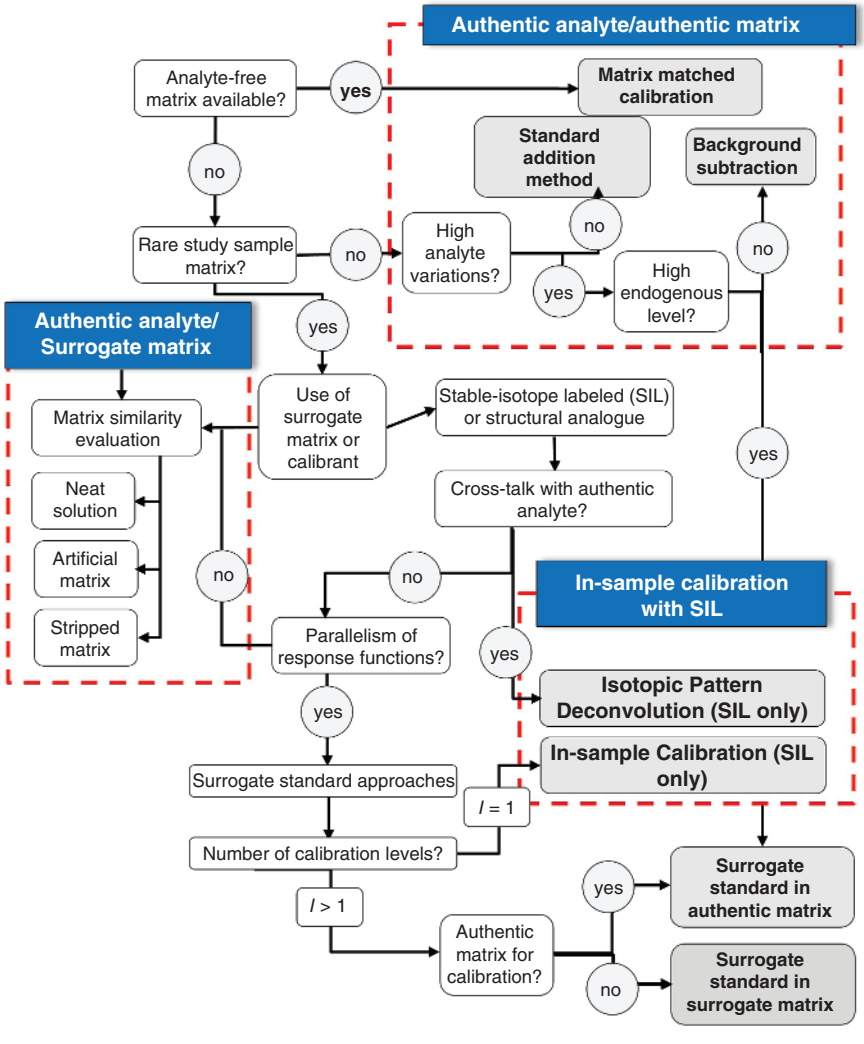


Figure 1.6 LC-MS on endogenous metabolites: proposed workflow for selecting a calibration operating procedure. Source: Adapted from Visconti et al. [13].

identified by square corner grey boxes, namely, authentic analyte/authentic matrix, authentic analyte/surrogate matrix, and ISC with SIL. For each case, different calibration procedures are appropriate, depending on complementary information about the analyte, the sample matrix availability, or the presence of endogenous analyte in the matrix.

More details are given in [13] and the rest of the chapter. In recent decades, advances in analytical calibration methodologies, instrument technology and enlarged SIL availability have contributed to improving the accuracy and throughput of quantitative analysis. However, the gap in knowledge between published official guidelines and strategies used by the analytical community prevents consensus about exactly how validation should be performed.

The introduction of innovative calibration approaches allowed the analyst to perform the calibration in the authentic working sample matrix, overcoming different bottlenecks such as the lack of blank matrices, the extraction efficiency, and matrix effect between the external calibration curve and unknown samples. Scientific interest is growing around direct internal calibration with SIL due to its analytical process simplicity and quickness to provide quantitative results from a few samples or even a single sample. With these unique advantages, internal calibration strategies have enormous potential to be widely applied for various quantitative applications and may even change the landscape of quantitative analysis, although these methodologies are still not officially endorsed by international guidelines for analytical method validation.

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