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From fundamentals in calibration to modern methodologies: A tutorial for small molecules quantification in liquid chromatography–mass spectrometry bioanalysis

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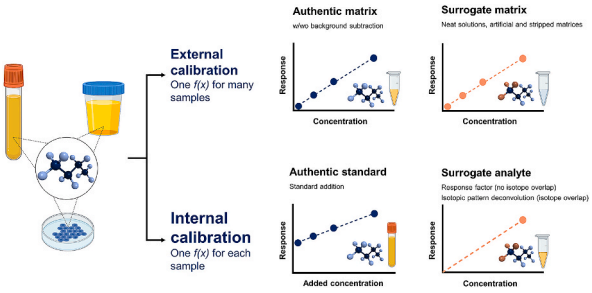
HIGHLIGHTS

- A detailed tutorial of the fundamentals of LC–MS quantification is provided.
- A collection of over 150 references on calibration is given.
- Relative, semi and absolute quantification are described.
- Methodological differences among external and internal calibrations are highlighted.
- Applications with regard to method validation guidelines are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Over the last two decades, liquid chromatography coupled to mass-spectrometry (LC–MS) has become the gold standard to perform qualitative and quantitative analyses of small molecules. When quantitative analysis is developed, an analyst usually refers to international guidelines for analytical method validation. In this context, the design of calibration curves plays a key role in providing accurate results. During recent years and along with instrumental advances, strategies to build calibration curves have dramatically evolved, introducing innovative approaches to improve quantitative precision and throughput. For example, when a labeled standard is available to be spiked directly into the study sample, the concentration of the unlabeled analog can be easily determined using the isotopic pattern deconvolution or the internal calibration approach, eliminating the need for multipoint calibration curves. This tutorial aims to synthetize the advances in LC–MS quantitative analysis for small molecules in complex matrices, going from fundamental aspects in calibration to modern methodologies and applications. Different work schemes for calibration depending on the sample characteristics (analyte and matrix nature) are distinguished and discussed. Finally, this tutorial outlines the importance of having international guidelines for analytical method validation that agree with the advances in calibration strategies and analytical instrumentation.

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Abbreviation definition

EC	External calibration
EMA	European Medicines Agency
FDA	Food and Drug Administration
IC	Internal calibration
ICH	International Council for Harmonization
IPD	Isotopic pattern deconvolution
IS	Internal standard
IsC	In-sample calibration
LC	Liquid chromatography
LLOQ	Lower limit of quantification
MIRM	Multiple isotopologue reaction monitoring
MS	Mass-spectrometry
MU	Measurement uncertainty
QC	Quality control
RF	Response factor
RR	Response ratio
SAM	Standard addition method
SIL	Stable isotope-labeled
ULOQ	Upper limit of quantification

1. Introduction

Over the last two decades, liquid chromatography coupled to mass-spectrometry (LC–MS) has emerged as an essential instrumental setup for a wide range of applications in clinical and nonclinical laboratories [1,2]. The latter has become possible due to the advent of atmospheric pressure ionization interfaces, allowing the production of gas-phase ions that can be further analyzed [3]. Compared to traditional spectroscopic detectors, such as ultraviolet absorbance, LC–MS(/MS) offers additional selectivity by determining the mass/charge ratio of ion(s) over the entire chromatogram, with the possibility of dissociating ion(s) into fragments to enhance selectivity [4]. Due to its lower limits of quantification and high selectivity, MS detection has become the gold standard for small molecule analysis, either of endogenous or exogenous origin [5–8]. Also, the hyphenation of LC to MS improves sample analysis in biological matrices, allowing analyte and matrix separation prior to detection [9–11]. Despite the fact that LC–MS systems are complex and thus require a large number of parameters to be optimized to obtain the desired performance, the possibility of performing qualitative and quantitative screening with high sensitivity and selectivity offers the best option for sophisticated analysis [12,13]. Qualitative analysis is typically performed for the detection and identification of organic molecules in study samples [14–16]. Once the qualification step has been performed, the quantitative analysis may occur to estimate the concentration of the analyte(s) of interest. Due to the possibility of providing concentration values in complex matrices, quantitative LC–MS methods are now widely applied as essential tools for biological hypothesis confirmation (bottom-up strategy) [17–20], clinical interpretation [21–23] and regulatory purposes (drugs, food, doping, pesticides, bioanalysis, etc.) [24–29].

Many articles reporting new LC–MS methods for quantification are submitted each year [30,31]. However, despite the number of articles accepted and published, the calibration and validation methodology performed, as an intrinsic part of the quantification process, often remain loosely described. Currently, as recommended in international guidelines for bioanalytical method validation, the multipoint calibration curve is the most widely used strategy to estimate the calibration function in LC–MS [32–35]. During this process, a set of calibration standards are analyzed to establish an empirical or semiempirical concentration–response function over a desired dynamic range [36,37]. Usually, a model is fitted using the least-squares regression technique and the specimen concentrations are back-calculated from the model. Depending on the availability of representative standards/matrices, the analyte nature, and potential disturbances during LC–MS analysis (matrix effect and interferences), different quantification approaches can be performed [38,39]. For example, with endogenous substances, the difficulty in obtaining a true blank matrix (i.e., a matrix without the analyte of interest) hampers a reliable quantification in terms of accuracy and precision, while a consensus in official guidelines is missing. Because the calibration methodology step plays a critical role in providing accurate and comparable results between measurement systems, some authorities have recently initiated a formal discussion on new calibration specifications to reduce the risk of misinterpretation of quantification outcomes [40–44].

A literature review showed a gap between calibration methodologies [38,45] and results quality [46,47], particularly in terms of uncertainty and validation according to recent regulatory guidelines [48]. Because no consensus has been reached in quantification methodologies for endogenous compound quantification, the calibration parameters, such as matrix or analyte nature, number and concentration of calibrators are usually selected arbitrarily for each bioanalytical scientist's preference, without considering their potential impact on the accuracy of regression [49]. The objective of the present tutorial is, therefore, to describe the principles of analytical calibration and their application considering the latest developments in LC–MS quantification methodologies, highlighting some advantages that these approaches can offer with regard to current guideline recommendations. First, a detailed summary of metrological fundamentals to correlate study sample response to concentration in LC–MS will be provided. Then, the current trends for analytical quantification will be discussed and classified into two primary categories depending on how and where the calibration function is obtained. The strengths and weaknesses of each approach will be evaluated with respect to current technology, as well as their endorsement in method validation. This tutorial aims to fill the gap in knowledge between the actual regulatory guidelines and the strategies used by the analytical community. A pragmatic approach is considered throughout the manuscript and important methodological clarifications are provided to be useful for absolute quantification in both scientific research as well as routine activities.

2. Metrological fundamentals: translate instrument analyte response into concentrations

2.1. Analytical determination approaches: relative, semiquantitative and absolute quantification

According to the nature of the calibration material used (i.e. the analyte), which can be authentic or surrogate, and the biological matrix which can be authentic, surrogate, artificial, etc., different quantification strategies (e.g., relative, semiquantitative and absolute quantification), can be considered to estimate the analyte concentration–response functions [50]. Thus, quantification is relative when a sample is analyzed before and after an alteration or compared to a control situation (e.g., sample degradation, patient with medical treatment(s)), and a simple ratio based on signal intensity fold change can be used to provide a relative estimation of the analyte(s) concentration [51]. In semiquantitative analysis, the calibration function is built using standards of similar compounds related to the analyte(s) of interest or other matrices comparable to the one of the study samples (e.g., neat solutions, artificial matrices, stripped matrices). The use of such surrogate standards and/or matrices implies a behavior similar to the authentic analyte or the study sample matrix throughout the process, including sample preparation and LC–MS analysis. In this case, the response of the instrument is translated into concentrations using another reference [52]. There is an increasing trend toward using machine learning to predict the electrospray ionization response when authentic standards and/or matrices are not available [53]. The ionization efficiency is measured in

different matrices along with a diverse array of chemical substances, as described in more detail in Section 3.1.4. Once the model is built, the concentration of similar analytes can be predicted by knowing the matrix and the eluent composition at a given retention time. Finally, absolute quantification generally relies on the use of the authentic standard and matrix for building the calibration function [45]. A schematic overview of the differences between relative, semi, and absolute quantification is summarized in Fig. 1 and more extensively explained in the next sections.

2.2. Instrument qualification

Whether the method is qualitative or quantitative, it must be developed and subsequently validated on a qualified instrument with performance that ensures the quality of the analytical results. Qualitative analysis is generally performed to identify substances based on information derived from their physicochemical properties, such as mass spectra fragmentation, and provides evidence of their presence or absence at a defined detection limit [54]. Qualitative analysis will not be discussed in this tutorial because several excellent reviews have already been proposed in this field [55–57]. In contrast, quantitative analysis is designed to determine the amount or concentration of a given analyte in a study sample, which is generally achieved by fitting an empirical analyte concentration–response function, namely the analytical calibration. In addition, an internal standard (IS) is usually added to both calibration standards and study samples to provide the best estimate of the concentration–response relationship and decrease analytical variability [58,59]. As a prerequisite, four primary components must be addressed in order to ensure proper data quality: analytical instrument qualification, system suitability tests, quality control samples (QCs) and analytical method validation [60]. The analytical instrument qualification consists of documented evidence that a measurement system is properly maintained and calibrated for its intended purpose. Calibration can also be used to characterize the measurement error and express it as deviation, bias, correction factor or calibration factor [37]. In addition to analytical instrument qualification, the Food and Drug Administration (FDA) guidelines require the preparation of a set of authentic chemical standards in neat solution used as system suitability tests to estimate the instrument performance under generic or specific analytical conditions for long-term system evaluation [41]. QCs are pooled and/or spiked study samples with known concentrations used to standardize the instrument's response and validate acquisition sequences. For quantification purposes, QCs are mandatory to assess the precision and trueness of a method assay [41]. Finally, in bioanalysis and new emerging fields such as metabolomics, pooled and diluted QCs are widely used to correct for potential intensity drift in measured signal peaks along the analytical sequence, as well as data filtering [57].

2.3. Metrological approach to analytical calibration

From a metrological perspective, calibration is defined as "*an 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*" [37]. This process is clearly a two-step procedure that can be simply presented as follows:

$$\text{Calibration step}: \quad Y = f(X) + e \qquad \text{Eq:1}$$

$$\text{Inverse calibration step}: \quad X = f^{-1}(Y) \qquad \text{Eq:2}$$

where X is the "*measurement standard*" value and Y is the "*indication*". The f function establishes the relationship between X and Y. In this context X is usually the analyte concentration of standards and Y is an instrumental response that may differ in nature depending on the analytical technique. As discussed below, Y can be a simple electrical signal, a peak area or height, etc. Because uncertainties must also be considered, the random variable e is added to the first step model. The use of the inverse function f^{-1} yields its name to the second step of the procedure (i.e., inverse calibration or back-calculation). Nonlinear instrument responses can be observed due to interfering ion species in the solution such as contaminants, matrix components or instrument limitations such as limited detector linear range or ion storage capacity [61]. However, in this tutorial and for simplicity, only linear calibration curves are considered. We assume that the concentration of target analytes is sufficiently low to avoid competition effects due to a finite amount of excess charges on a droplet during electrospray ionization [62]. Considering the case of a linear calibration curve, the following equations could be considered:

$$\text{Calibration step}: \quad Y_i = \beta_0 + \beta_1 X_i + e_i \qquad \text{Eq:3}$$

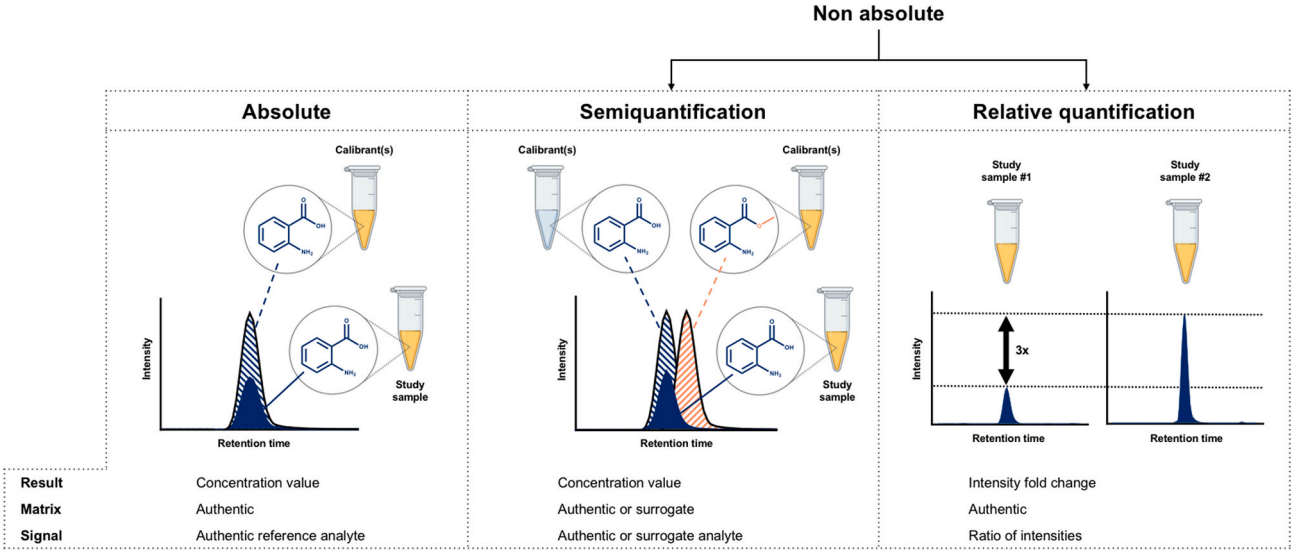

Fig. 1. Methodological and outcomes differences between absolute, semi- and relative quantification. Blue and orange lines indicate the authentic analyte and SIL responses, respectively. The yellow and the blue Eppendorf represent the authentic and surrogate matrices, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Study sample observed response : $Y_s = b_0 + b_1 X_s$ Eq:4

Inverse calibration step : $X_s = \frac{Y_s - b_0}{b_1}$ Eq:5

There is an important difference between X_i and X_s. The first is known as a calibrant that takes fixed values assigned by the analyst, while the second is unknown and computed from study samples (subscript '*s*'). The coefficients b_0 and b_1 in Eq:4 are estimations of β_0 and β_1, and may vary according to the number of calibrants and their distribution across the calibration range, the number of replicates, and the estimation technique as discussed in the last part of the present tutorial. Several approaches are available in the literature to calculate these coefficients, but the most common is ordinary least-squares regression [63], which is widely implemented in quantification-oriented software and easily applicable even if other algorithms exist, such as robust regression methods. Several authors have proposed directly fitting the 'reverse' function that links *X* to *Y*, such as Eq:6 for the linear case:

Observed concentration : $X_i = \beta_0^{'} + \beta_1^{'} Y_i + e_i^{'}$ Eq:6

Computed concentration : $X_c = b_0^{'} + b_1^{'} Y_c$ Eq:7

A basic assumption when using ordinary least-squares regression is that *X* is an independent variable (or explicative) set up by the analyst and considered error-free, while *Y* is dependent (or explained) random variable. Thus, e_i represent the errors on *Y* while the errors on *X* are assumed to be zero or, at least, negligible. In many LC–MS quantitative analyses this assumption may seem satisfactory because calibration standards are usually prepared with an uncertainty of ca. 0.1% or better which is usually lower than the instrumentation variability [64,65]. This assumption is however erroneous when study samples are measured, and thus, the computed concentration X_c is not error-free. The consequences of these results will be discussed in Section 4. Also, *X* and *Y* do not play the same role in regression models. While classical ordinary least-squares aims to minimize squared residuals in the *Y* dimension to estimate regression coefficients, reverse calibration uses the inverse relation. Thus, their impact on the accuracy of slope and intercept estimation depends on the approach chosen for the computation of the model error. When using ordinary least-squares, the precision of X_c was improved when computed with the reverse model compared to the inverse function [66]. There is also a direct link between the initial (*b*) and reverse (*b*') slope coefficients using the coefficient of determination r^2:

$$\begin{cases} b_0^{'} = \dfrac{(1 - r^2)\overline{Y} - b_0}{b_1} \\ b_1^{'} = \dfrac{r^2}{b_1} \end{cases} \quad \text{Eq:8}$$

This equation shows that both approaches give nearly similar results when r^2 is near to 1 [67].

2.4. Analytical calibration as a source of uncertainty

There is a general procedure to estimate measurement uncertainty (MU), as described in the Guide to the Expression of Measurement Uncertainty [68]. This procedure is relatively simple and consists of four steps: specify the measurand; identify the uncertainty sources; simplify and quantify the uncertainty components; and calculate the combined uncertainty. Calibration is one of the many sources of uncertainty in analytical sciences. Whatever the calibration procedure, at least two major sources are present: the calibration model selection and the algorithm used to estimate model coefficients. In some cases, calibrant uncertainty can also be accounted for. However, it is difficult to define an experimental design to separately estimate the uncertainty linked to calibration. Therefore, the role of calibration in MU can only be indirectly estimated.

Some strategies can select the best calibration procedure and minimize the importance of calibration as an uncertainty source. Unfortunately, the *X* concentration and *Y* response are typically highly correlated and classical statistical criteria, such as the r^2 or Akaike information criterion, are inefficient for selecting the most appropriate calibration model. The Guide to the Expression of Measurement Uncertainty recommends regrouping sources of uncertainty, and an empirical approach consists of making several estimations of the combined MU using different calibration models and regression techniques. For example, using QC results or the construction of an accuracy profile are possible holistic approaches to reach this goal [69], as explained in Section 4. Another assumption is that random errors of *Y*-values from replicate measurements of a given standard material must follow a normal (Gaussian) distribution. Ordinary least-squares also assumes that *Y*-value distributions are identical regardless of *X*. This property, called homoscedasticity, means that the variances of the *Y*-values are homogeneous. However, most LC–MS methods manage a large concentration range and *Y*-value errors often increase with analyte concentration. Weighted least-squares is an effective approach to address situations where the larger residuals observed at higher concentrations have a marked impact in the estimation of regression coefficients. Because low concentrations have smaller deviations, accuracy at the lower part of the range may therefore be compromised. In this context, different weighting schemes can be implemented to correct this heteroscedasticity issue and ensure a reliable estimation of the regression parameters. For that purpose, empirical weighting factors based either on the concentration of the calibrants or the measured response provide a simple variance approximation. The latter include weights such as $\frac{1}{\sqrt{X}}$, $\frac{1}{X}$, $\frac{1}{X^2}$, $\frac{1}{\sqrt{Y}}$, *Y*, and $\frac{1}{Y^2}$. The most adapted weighting factor could be chosen based on the relative error between the predicted and observed values of the calibration and/or QC samples [70,71]. Weighted regression coefficients can then be used for further calculations. Despite an additional computational complexity, weighted least-squares is commonly used to consider this effect, but a justification must be given to the regulatory authorities such as the FDA or the European Medicines Agency (EMA) [40,41]. Assessing the residuals distribution constitutes an efficient approach to ensure that the regression model fits the data. The most standard way to display the residuals is to plot them against the predicted values and check if they are randomly distributed in a range centered on the horizontal axis. A visual inspection is usually performed to search for potential patterns or outliers that could be a sign of a lack of fit between the chosen model and the data. In addition, dedicated statistical tests can be implemented to check the homoscedasticity and the linearity of the response with respect to concentration levels. Finally, computing regression parameters allows the value of slope and intercept of the regression function to be evaluated. However, these coefficients are least squares estimates of the true population parameters which are theoretical values that cannot be known. Thus, regression slopes and intercept coefficients are subject to inaccuracy in their estimation. For the intercept a confidence interval around the computed value can be considered, usually at the 5% level. When this interval includes zero, one cannot exclude that the value of the intercept is zero. Thus, the coefficient is considered as nonsignificant. When the confidence interval does not contain the value of zero, the intercept is considered significant.

To obtain reliable quantification in terms of trueness and precision, the analytical calibration function must take the response relationships for all relevant analytes and interferences into account. From a practical perspective, two primary prerequisites must be fulfilled: (i) the composition of calibration standards must be as close as possible to the study sample in terms of solvent and nature; and (ii) the calibration standards and the study sample must have identical behavior in the measurement system [38]. Therefore, the choice of representative standard and matrix used to build the calibration function is a critical point for the different quantification methodologies, which relies on availability and different combinations of analyte and matrix.

2.5. Sample standardization

In LC–MS analysis, the measured response Y for a given analyte can strongly vary according to the mass analyzer type, ionization modes, ion source parameters, system contamination, ionization enhancement or suppression due to sample matrix effect, along with other operational variables related to the analytical workflow. Thus, the analyte relative response must be standardized to compare performance over time and an internal standard (IS) is commonly added to the study and calibration samples at fixed concentrations. The absolute response of the analyte on the Y-axis is then normalized as a response ratio (RR) that reduces the variability:

$$Y_s = RR = \frac{Y_A}{Y_{IS}} \quad \text{Eq:9}$$

where the subscripts "A" and "IS" are related to the analyte and the internal standard measured signal (Y), respectively. This equation describes the instrument response only, without considering the respective concentrations, as with response factor calculation (see Equation (10)).

The use of IS allows for the normalization of the instrumental response and correction of the overall analytical variation in the measurement process resulting from the presence of random errors from the analytical workflow (e.g. sample preparation) and/or systematic errors originating from the presence of interfering compounds contained in the matrix [58,59]. This latter, which is known as the matrix effect, occurs from coeluting interferences such salts or undetected metabolites dissolved in the study sample or introduced during sample preparation or chromatographic analysis [72]. Both FDA and EMA advise evaluating the matrix effect when a matrix different from the study sample is used (i.e., the surrogate matrix) but only EMA provides instructions on how to do it [48]. The FDA suggests investigating this matrix by verifying the calibration parallelism between authentic and surrogate matrices [41]. Conversely, EMA recommends comparing the extraction recovery between the spiked authentic matrix and the 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 [40]. Overall, as long as the analyte and the IS are affected in the same way during the analytical process, MS signals can be standardized [73–75]. As recently emphasized by Khamis et al., IS evaluation remains a key step for sample standardization in LC–MS because the chemical nature of a compound has a significant effect on the degree of ionization and extraction recovery [47]. There are actually two primary classes of IS, isotope and structural analogs. The first class is related to the stable isotopic form of the analyte of interest, usually by replacing hydrogen (^{1}H), carbon (^{12}C) or nitrogen (^{14}N) with deuterium (^{2}H), ^{13}C, or ^{15}N [76]. Stable isotope-labeled (SIL) standards are typically used as ISs in LC–MS because they have very similar physicochemical properties compared to authentic analytes [77]. Deuterated ISs are widely used due to their lower production cost, but their lipophilicity increases with the number of ^{2}H replaced, leading to differences in their chromatographic retention with the corresponding authentic analyte. This phenomenon, known as "deuterium effect", can also impact the ISs ionization response compared to the unlabeled compounds. For example, d5-carvedilol showed a 0.9% difference in relative retention time compared to carvedilol, resulting in an inadequate matrix effect correction in plasma samples [78]. In contrast, standards containing enriched carbon or nitrogen atoms exhibit identical physicochemical properties compared to authentic analytes, thus becoming the IS of choice when available [79]. The second IS class includes compounds that share structural similarities with the analyte of interest, but they represent an inferior alternative to SIL [80]. Even if suppliers are manufacturing an increasing number of high-quality SILs, their commercial availability is limited to the most important compounds of any chemical family. When a high number of analytes must be quantified, the possibility to use one IS for multiple analytes should be carefully evaluated based on chromatographic retention and MS response [81,82]. For quantification purposes, the use of 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 study samples. Additionally, because binding equilibrium occurs in biological fluids, the IS should be introduced as early as possible in the analytical process to reach an appropriate balance between the analyte of interest and the IS spike [83].

3. Methodologies for analytical calibration/quantification

As briefly mentioned in the previous section, the nature of the analyte, the availability of the study sample material and the reference material define which type of analytical calibration is addressed (Fig. 2). The latter can be obtained as an external reference or directly inferred from the study sample. The first case refers to external calibration (EC) with the primary advantage of obtaining one function, $f(X)$, that can be used for many study samples. Conversely, internal calibration (IC) is performed when performed directly on the study samples, and one function $f(X)$ is obtained for each study sample to be quantified [38]. As detailed in Table 1, for EC, different methodologies can be implemented according to the availability of both analyte and matrix which can be used in different configurations: (1) authentic analyte in authentic matrix (Section 3.1.1); (2) authentic analyte in surrogate matrix (Section 3.1.2); (3) surrogate analyte in authentic matrix (Section 3.1.3) or (4) surrogate analyte in surrogate matrix (Section 3.1.4). Because there is no choice of matrix in IC, only the question of the analyte availability (authentic versus surrogate) remains [47,84]. Throughout this tutorial, the terms surrogate analyte and surrogate calibrant are used interchangeably because they both refer to a nominal concentration fixed by the analyst. The listed methodologies will be discussed in more detail in the following sections, including advantages and disadvantages in the context of LC-MS calibration/quantification, bearing in mind the important methodological differences between EC and IC.

3.1. External calibration (EC)

Because the EC approach allows the determination of several study samples with only one calibration function $f(X)$, it corresponds to the most rational and used methodological approach. From a methodological perspective, the first case called matrix-matched external calibration represents the highest metrological MS-based quantification approach and is extensively discussed in the major international guidelines to validate bioanalytical methods [40–42,58,59]. For exogenous compounds, the availability of blank matrices generally allows for external calibration with authentic analyte(s) in a representative matrix. However, with endogenous compounds, other approaches should be explored to overcome the absence of an analyte-free matrix, as mentioned in the FDA, EMA and the last proposal of International Council for Harmonization (ICH) guidelines [40,41]. In this context, several procedures have been proposed, such as background subtraction or the use of surrogate matrices and/or analytes [45]. In this section, we describe the multiple strategies to achieve EC, focusing on aspects that can improve the quality of the results. A discussion about the calibrations levels that the operator should use to build an EC is endorsed in Section 4.

3.1.1. Authentic analyte(s) in authentic matrix: matrix-matched calibration

The use of authentic matrix for multipoint EC provides an extraction recovery yield that is near to the specimen, and is commonly performed to quantify exogenous substances when a large amount of the matrix is available [85–87]. In 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

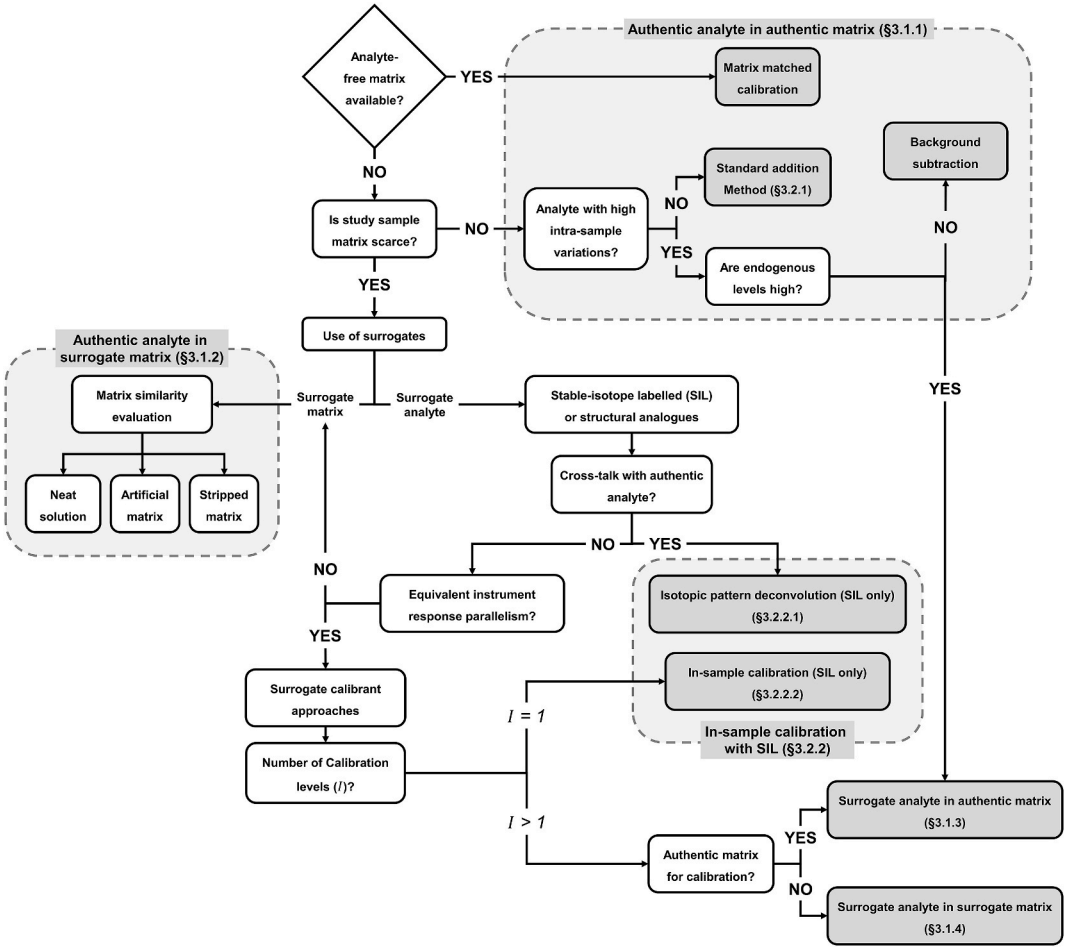

Fig. 2. Suggested workflow for the selection of an appropriate calibration strategy for LC–MS quantification of endogenous metabolites. The square box represents the starting point and grey-colored boxes indicate the calibration methodologies with their corresponding Sections in the publication.

matrix-matched EC to interpolate the concentration in the study samples. Back-calculation is performed using Equation (5), where the slope (b_1) and intercept (b_0) refer to the linear regression parameters of the added authentic standards in the pooled authentic matrix. However, the lower limit of quantification (LLOQ) is then limited by the background endogenous concentration (b_0), thus achieving poor performance with high endogenous levels because ionization competition or detector saturation may occur [88–90]. Also, endogenous metabolite concentrations may vary due to intra- and inter-sample variation, leading to highly variable results when a pooled matrix is used [91]. However, several calibration curves using different representative pooled matrices can be prepared to overcome these drawbacks and use the calibration that best covers the concentration to be analyzed [92]. Matrix-matched EC cannot always correct the matrix effect when it varies between study samples, emphasizing the importance of using an IS to correct these discrepancies, as discussed at the end of the following section (3.1.2) [93,94].

3.1.2. *Authentic analyte(s) in surrogate matrix*

Surrogate matrices are used as substitutes for authentic matrices to prepare a calibration curve with the authentic analyte(s) and can be of varying complexity, including neat solutions, as well as artificial or stripped matrices [95]. Neat solutions are generally composed of mobile-phase solvents (neat) or pure water and must prove they possess the same, or similar, extraction recovery and matrix effect as those of the original matrix before considering their use [96–99]. In contrast to neat solutions, artificial matrices are composed of salt, sugar and other simple ingredients to simulate authentic matrix properties, such as analyte solubility, extraction recovery and matrix effect. Neat and artificial solutions can be used when the study sample matrix is mainly composed of water, such as saliva, urine, tears and cerebrospinal fluid [45]. To assess the application of neat solutions and/or artificial matrices for quantification purposes, a comparison between the slopes of the calibration curves in surrogate matrices and the authentic matrix (standard addition method, SAM) should be performed [100–102]. Even if several statistical tests are available for comparing parallelism of curves, the best known is based on analysis of variance. For example, Olesti et al. evaluated the regression slopes ratio of calibration curves performed in pure water and authentic matrix to quantify 16 neurotransmitters in rat plasma and brain homogenates [96]. Although most part of the metabolites had matrix effect values inferior to 20%, the slope of five analytes in neat solution differed markedly from the SAM calibration, suggesting that the use of SIL as IS was essential to compensate differences in matrix composition. Finally, stripped matrices can be produced or available on the market, 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 estimation of calibration curves [103–105]. In contrast, the carbon-activated absorption has little effect on salts, glucose, amino acids, and other polar substances. It is important to emphasize that charcoal depletion is nonselective, and related compounds that cause matrix effect are removed, resulting in unsatisfactory matrix similarity [106]. For example, Godoy et al. evaluated the matrix effect for nucleosides by comparing calibration slopes prepared in authentic serum versus activated-charcoal-stripped serum and artificial serum. The authors observed that phosphate-buffered

Table 1
Classification of calibration methodologies based on analyte and matrix combinations used to build the analytical calibration.

Calibration function	External (out-sample)				Internal (in-sample)		
						Surrogate analyte	
Reference compound	Authentic analyte		Surrogate analyte (isotope or structural analog)		Authentic analyte	Partially labeled isotope analog	Fully labeled isotope or structural analog
Calibration matrix	Authentic	Surrogate (neat, artificial, stripped)	Authentic	Surrogate (neat, artificial, stripped)		Authentic	
Calibration methodology	Matrix-matched (with background subtraction for endogenous compounds)	Surrogate matrix	Surrogate analyte	Surrogate analyte and matrix	Standard addition method (SAM)	Isotopic pattern deconvolution (IPD)	Internal calibration (IC)
Advantages	- Matrix effect and selectivity closely to specimen	- Suitable for low concentrated compounds - Production of analyte free-matrix	- Lower LLOQ than the background subtraction	- When authentic analyte is hard to obtain	- Same matrix effect and selectivity as the specimen	- High potential for accuracy - Relying on isotopic distribution alteration	- High potential for accuracy (SIL) - Reduced numbers of calibrators
Disadvantages	- Endogenous levels defines LLOQ - Possible differences in extraction recovery and matrix effect between lots, especially with hemolyzed and hyper lipemic blood.	- Possible differences in extraction recovery and matrix effect	- Accuracy depends on surrogate specificity - Additional experiment for linearity and LLOQ	- Accuracy depends on surrogates specificity - High differences for extraction recovery and matrix to be expected	- Need for large initial specimen volume - Not suitable for high-throughput	- Accuracy depends on concentration and stability of the isotopes analogs - Additional experiment for linearity and LLOQ	- Accuracy depends on concentration and stability of the isotopes analogs - Structural analogs can not compensate differences in ionization - Additional experiment for linearity and LLOQ

saline with 2% bovine serum albumin showed a better matrix similarity with authentic serum for 8 analytes over 9. The matrix similarity of adenosine was improved from −74% to −12% by switching from stripped to artificial serum, suggesting that activated-charcoal depletion deviates considerably from the authentic matrix composition [107]. In addition to chemical depletion, the unretained fraction of sample preparation constitutes a simplified alternative to charcoal stripping. For example, Escobar-Wilches and collegues collected the SPE wash step to obtain a steroid-free urine [108]. Because these surrogate matrices do not fully represent the original matrix, the use of an IS is essential to correct for the recovery yield [109]. To correct for those matrix discrepancies, a proper evaluation should be performed as recommended by FDA and EMA guidelines [40,41]. However, only EMA specifies how to assess the matrix similarity by evaluating the instrument response slope ratio between authentic analyte in authentic matrix versus the authentic analyte in surrogate matrix. The obtained ratio should be within ±15% of the nominal value [40].

3.1.3. Surrogate analyte(s) in authentic matrix

In situations where compound-specific standards are not available, an analytical calibration can be performed using a different reference compound following the surrogate analyte (or surrogate calibrant) approach. The latter assumes that the physicochemical properties of both authentic and surrogate analytes are comparable. Thus, the extraction recovery, the chromatographic retention behavior and the MS response should be either identical or have minimum differences to be fully exploited. The choice of surrogate compound is essential to accurately quantify the authentic analyte [110,111]. For example, ICH guidelines suggest using stable-isotope labeled (SIL) standards as surrogate calibrant in authentic matrix, while FDA guidelines do not endorse this methodology [40,41]. Because the calibration reference compound does not correspond to the authentic analyte, the ratio of MS responses between the surrogate and the 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-surrogate analyte ratio:

$$RF = \frac{Y_A}{X_A} \bullet \frac{X_{SA}}{Y_{SA}} \quad \text{Eq:10}$$

where X is the concentration, the subscripts "A" and "SA" indicate the analyte and the surrogate analyte measured signal (Y), respectively. To obtain an appropriate RF estimation, different concentration levels of analyte/surrogate solutions must be investigated. This step is necessary to evaluate the ionization efficiency and the RF should be constant over the dynamic range tested. Another way to investigate the RF is to compare the slope parallelism between the authentic analyte and the surrogate, both performed in the same pooled matrix. Aydin et al. demonstrated that the slope ratio between cortisol/cortisol-d_4 and cortisone/cortisone-$^{13}C_3$ was sufficiently close to the unit value over different days in human saliva, covering a dynamic range between 0.062 and 75.5 ng mL^{-1} for cortisol and cortisone quantification, respectively [112]. Additionally, if the RF is not constant over the tested dynamic range measurement corrections, such as LC gradient or MS/MS transitions (de)-optimization, can be evaluated to obtain a balanced response [113–115]. If the RF is not stable and SILs are used as surrogate calibrants, the analyst should investigate the potential presence of crosstalk interferences such as isotopic pattern overlap or impurities coming from SIL standards [116,117]. The RF can diverge from unit value when SILs containing enriched hydrogen atoms are used, but as long as the unit value of slope parallelism is within 15% acceptance the concerned SILs can be selected as surrogate calibrants [118]. For example, tryptophan

was successfully quantified in plasma with a relative bias between −2.0 and −8.0 using its deuterated analog, even if the response factor was 0.67 [119]. Once the RF has been established, a multipoint calibration is performed in a pooled authentic matrix and the concentration of the authentic analyte (X_s) is computed as follows:

$$X_s = \frac{\left(\frac{Y_A}{Y_{IS}}\right) - b_0}{RF \bullet b_1} \quad \text{Eq:11}$$

where the subscripts "*A*" and "*IS*" indicate the measured signal (*Y*) of the analyte and the internal standard, if any, respectively; and b_1 is the slope and b_0 is the intercept of the regression straight line performed with the surrogate analyte. As with the matrix-matched EC, the use of an IS remains strongly recommended to correct for sample preparation and matrix effect variation between study samples and calibrators, thus improving trueness and precision when dealing with real sample determination. Because the endogenous signal of the authentic analyte in a pooled matrix is stable, an interesting possibility that can be implemented with this method of quantification is to use this signal as an internal standard to normalize the instrument response of the surrogate analyte calibration, as suggested by Suhr et al. [120]. This approach, called isotope inversion by the authors, provided the same quantitative results for steroid determination using the authentic analyte in a surrogate matrix (i.e., active-charcoal stripped serum). The correlation between the two quantitative approaches showed a bias of a maximum of 4% for corticosterone, 11-deoxycortisol, 11-deoxycorticosterone and 17-OH-progesterone. In contrast, cortisol and cortisone exhibited a 20% deviation, suggesting that depleted serum might give inaccurate quantification.

Because no endogenous signal should interfere with the surrogate signal, the surrogate analyte in authentic matrix can therefore be a suitable alternative to the matrix-matched calibration when high endogenous concentrations are present and/or important intra- and inter-sample variation are observed [121].

3.1.4. Surrogate analyte(s) in surrogate matrix

Recently, the increased commercial availability of SILs has raised interest in their use as surrogates in surrogate matrices to decrease calibration preparation time. For example, Cournoyer and Dey used four labeled version of testosterone ($^2H_3$, $^2H_5$, $^2H_8$ and $^{13}C_2$–^{18}O) at different concentration levels to prepare a single tube multipoint calibrator in a surrogate matrix, 5% bovine serum albumin in water. The authors compared the performance of the proposed methodology to a multipoint EC in the same matrix for the quantification of testosterone by comparing certified serum values provided by a regulatory agency (NIST SRM 971). The obtained bias was +1.1% and −5.5% respectively, showing an improved trueness for the single tube calibrator composed of multiple and differentially labeled SILs of testosterone [122]. Where limited SIL(s) are available for the compound(s) of interest, the investigation of alternative surrogate calibrants can be carried out using structural analogs. For instance, Dahal et al. measured a set of drugs metabolites considering the difference in instrument response between the parent compound and its metabolites [123]. This semitargeted quantification approach increased interest in the use of machine learning to predict electrospray ionization, allowing the determination of the amount of target analytes without the need for authentic chemical standards [53]. Recently, Liigand and colleagues used a set of exogenous compounds as surrogate calibrants in several biological matrices such as blood, plasma, urine, cerebrospinal fluid and tissue homogenate to train the prediction model for ionization efficiencies [124]. Then, a selected group of exogenous compounds covering the LC–MS gradient distribution was spiked in the study samples. The detected analytes were quantified based on the MS response of the spiked surrogate analyte with a similar retention time and/or chemical structure. The prediction errors between nominal and computed concentrations were 2.2 and 2.0 for positive and negative ionization modes, respectively [125]. The combination of the surrogate calibrant with prediction model offers the opportunity to markedly extend the number of analytes that can be quantified in a single LC–MS run [53,114,124–126].

3.2. In-sample calibration (IsC)

In contrast to EC, the in-sample approach is distinguished by an analytical calibration obtained directly in the study sample. The standard addition method (SAM) is probably the most established IsC methodology because it is used in many fields, such as foods, environment or forensic toxicology, where different analytical matrices are considered [127,128]. In this section, two other approaches that aim to simplify the quantification stages by LC–MS are also discussed. Depending on the chemical purity and the physicochemical proprieties of SILs, two additional IsC approaches can be performed. The first methodology computes the authentic analyte concentration by altering its natural isotopic pattern with a labeled analog standard. Conversely, when no interferences between the analyte and SIL are present, the authentic analyte concentration can be determined using the internal calibration (IC) approach.

3.2.1. IsC with authentic analyte(s): standard addition method (SAM)

As a gold standard for absolute quantification, SAM allows to obtain a calibration curve in the same matrix as the study sample using the authentic analyte [45]. To determine the best calibration range, the endogenous concentration is estimated by explorative studies, such as samples spiking at a wide range of concentration levels or using neat solutions [96]. In the classic operating procedure, the study sample is divided into several 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 non spiked, and its concentration is obtained by extrapolation where *Y*-value is equal to 0. As mentioned in Section 2.4, FDA suggests performing a parallelism between SAM and surrogate matrix or analyte to justify their use, but they do not provide any information about the number of calibrants required for SAM. Considering the literature, most authors use between four and six spiked concentrations [96,129–131]. As with the EC approach, the use of IS to correct for procedural errors is recommended when a sample preparation that is different from simple "dilute and shoot" is performed [130]. 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. For example, Díez et al. showed that linear fitting provides adequate recoveries for antibiotic quantification in fruits and vegetables when concave SAM curves are obtained, while quadratic extrapolation could be used for convex or slightly concave curves [131].

Although SAM is time-consuming with the construction of one calibration curve per study sample, this approach allows interindividual differences in matrix composition to be considered and overcome matrix effects [132]. Because it provides the possibility for absolute quantification, parallelism verification is often performed with alternative matrices or analytes to verify their suitability as surrogates [133,134]. Finally, when detectors such as MS or diode-array detection allowing the recording of various physiochemical properties of the target analyte are used, several SAM calibration curves can be simultaneously acquired [135]. This multiple response monitoring, known as H-point, helps to control both proportional and constant errors, such as matrix interferences and/or detector saturation, due to the determination of converging *X*-intercept [136–138]. When MS is used as a detector, the isotope distribution or multiple fragments of the authentic analyte can be monitored using dedicated acquisition modes, as discussed in detail in Section 3.2.2.2. We suggest monitoring from two to four channels to implement the H-point methodology to ensure sufficient regression curves to determine the converging x-intercept. Thus, the SAM approach is a suitable choice for quantification when a significant amount of study

sample is available, when the surrogate analyte is difficult to obtain or when the matrix effect is highly variable from one study sample to another [139].

3.2.2. IsC with SIL(s) as surrogate calibrant(s)

The improved availability of SILs opened the possibility of employing them as ideal calibrant candidates to perform a calibration directly in the study matrix [140–142]. When SILs are used as surrogate calibrants, the analyst should first investigate the potential presence of interferences with the target analyte. When a contribution coming from the SIL is observed in the MS signal, the use of isotope pattern deconvolution (IPD) approach can be considered. In absence of significant interference, IC represents one of the most promising methodologies for modern absolute quantification in LC–MS [117]. A schematic overview of the differences between these two approaches is summarized in Fig. 3.

3.2.2.1. Isotopic pattern deconvolution (IPD). The IPD methodology is 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 on signal ratio between the isotopes of the molecule of interest and an analog with an enriched isotopic composition (i.e., SIL). The IPD is considered to be one of the most reliable and highest metrological methods and is commonly used by chemical manufacturers to calculate SIL isotopic enrichment and purity [143–146]. 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) [147]. First, the isotopic distributions for unlabeled standard and SIL as well as their combination ("convoluted" isotope distribution) can be computed (e.g. using IsoPatrn© software [148]). The labeled compound is then added to the reference material, resulting in isotopic dilution. The comparison between theoretical and experimental isotope overlap allows to determine the SIL isotopic enrichment, chemical purity and concentration [144]. Once the SIL solution has been characterized by isotope dilution mass spectrometry it can be used as a calibrant for IPD quantification.

In 2014, Castillo et al. evaluated the IPD approach for the quantification of diclofenac and alkylphenols in wastewater [149,150]. In a first step, the natural isotopologue distributions of the analyte A_{nat} and its isotope labeled analog A_{lab} are measured. In this study, subscript indicates molecule enrichment that can be natural (*nat*) or labeled (*lab*). Then, the analyte and SIL are mixed, and the resulting isotope pattern is determined. Knowing the amount of SIL spiked, the molar fractions χ_{nat} and χ_{lab} can be deconvoluted, as shown in Equations (12) and (13).

$$\begin{bmatrix} A_{mix}^{M+0} \\ A_{mix}^{M+1} \\ A_{mix}^{M+2} \\ \cdots \\ A_{mix}^{M+n} \end{bmatrix} = \begin{bmatrix} A_{nat}^{M+0} & A_{lab}^{M+0} \\ A_{nat}^{M+1} & A_{lab}^{M+1} \\ A_{nat}^{M+2} & A_{lab}^{M+2} \\ \cdots & \cdots \\ A_{nat}^{M+n} & A_{lab}^{M+n} \end{bmatrix} \bullet \begin{bmatrix} \chi_{nat} \\ \chi_{lab} \end{bmatrix} + \begin{bmatrix} e^{M+0} \\ e^{M+1} \\ e^{M+2} \\ \cdots \\ e^{M+n} \end{bmatrix} \quad \text{Eq:12}$$

Matrix notation

$$\boldsymbol{A}_{mix} = [\boldsymbol{A}_{nat}\boldsymbol{A}_{lab}]\boldsymbol{\chi}^{-1} + \boldsymbol{e} \quad \text{Eq:13}$$

The error vector (e^{M+n}) is the parameter in the multiple linear regression that allows to compute the molar fractions. Knowing the amount of SIL (X_{SIL}) added to the study sample, a direct quantification of the analyte (X_A) can be provided without the need for a calibration curve (Equation (14)).

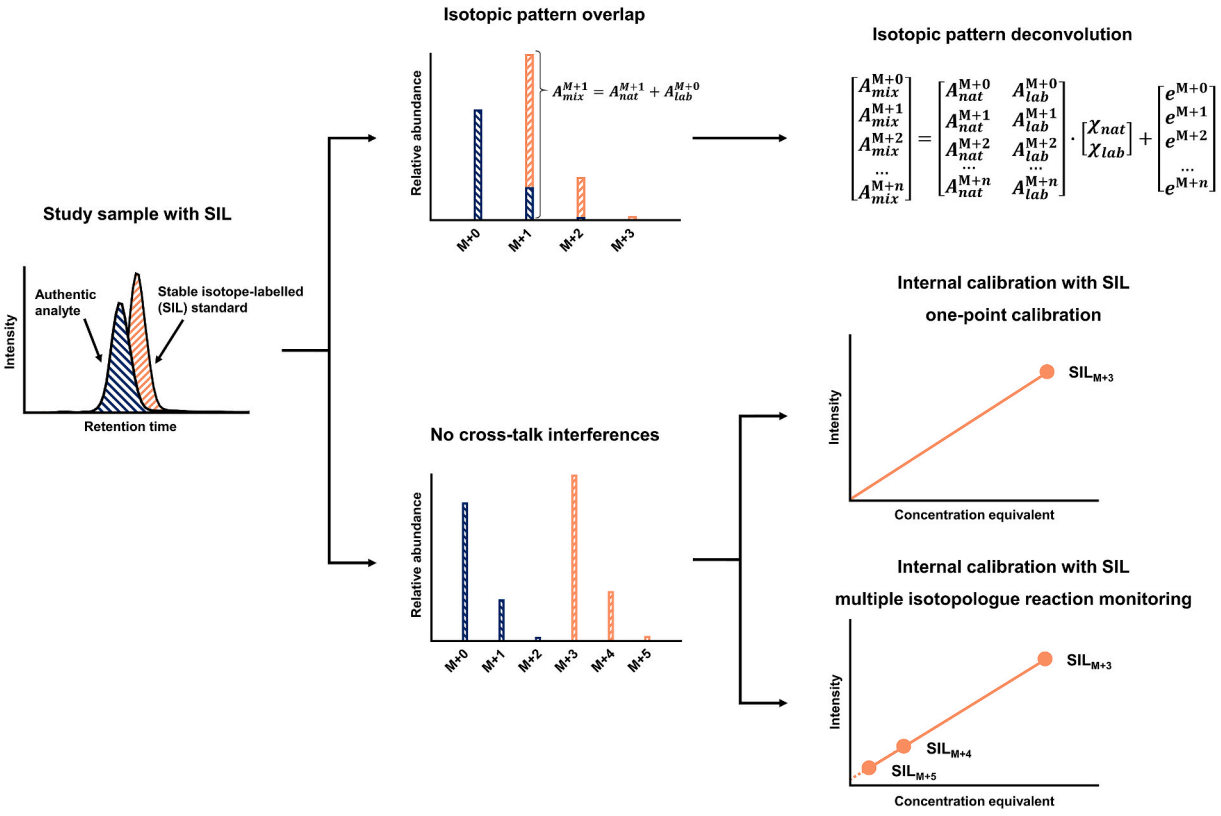

Fig. 3. In-sample calibration strategies that can be performed using SILs (§3.2.2). Blue and orange lines indicate the authentic analyte and SIL responses, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

$$\frac{\chi_{nat}}{\chi_{lab}} = \frac{X_A}{X_{SIL}} \quad \text{Eq:14}$$

However, it is necessary to have a crosstalk or isotopic overlap during MS analysis to perform the deconvolution, which 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 [151]. Thus, Castillo and colleagues used a SIL (diclofenac-d_4) with only 92.8% isotopic enrichment to increase the crosstalk effect on the natural isotopic distribution of the analyte. The IPD approach showed equal accuracy compared to a fortified calibration (SAM), achieving recoveries within 90–110% and coefficients of variation below 5% for all samples tested [149]. More recently, the same group evaluated the uncertainty of this approach in an interlaboratory study, including four different World Anti-Doping Agency accredited laboratories [152]. The IPD was performed for the quantification of testosterone in urine samples and the quantification performance compared to an EC surrogate analyte approach. The IPD showed the same accuracy and demonstrated improved reproducibility at low concentrations (2 ng mL^{-1}) with a relative standard deviation of approximately 10% and 16%, respectively. This result agrees with the high metrological quality of analytical results provided by isotope dilution mass spectrometry determination. The IPD approach was later validated with a larger pattern of steroids [153]. Then, the quantification of testosterone was improved by operating low resolution selected reaction monitoring (i.e., reduced resolution of the first quadrupole up to 8 *m/z* to transmit the entire ion cluster of the SIL and the analytes). The ions were then fragmented in the collision cell, and the resulting isotopic distribution was measured as a fragmentation pattern in the second quadrupole. This detection mode allowed more efficient ion transmission, increasing the signal of testosterone up to three times [154]. Thus, the high metrological reliability of the IPD approach can provide accurate results, with uncertainty constituted primarily from 1) experimental isotopic abundance determination (78.0%) and 2) accuracy of SIL concentration measurement (21.3%) [149]. The evaluation of these parameters involves some additional work during method development, but the IPD approach then benefits from a more accurate and faster analysis because calibration is performed with the study sample, and a traditional external calibration curve is no longer required.

3.2.2.2. Direct internal calibration with SIL (IC-SIL). The simplest quantification in LC–MS could probably be achieved when a single amount of surrogate calibrant is used to compute the study sample concentration. In this internal calibration, the authentic analyte and the surrogate calibrant, are measured together in the study sample [38], and the analyte concentration is directly obtained via the analyte/surrogate analyte area ratio [117]. Because only one concentration level is introduced in the study sample, a RF relationship should be estimated to confirm the absence of ionization competition in LC–MS between surrogate and authentic analytes, independently of the concentration. Thus, equimolar mixtures of surrogate and authentic analytes in neat, artificial and/or depleted matrices are first analyzed over the investigated dynamic range [155]. Additionally, ionization competition at non-equimolar concentrations should be investigated. Several multipoint calibrations using the authentic analyte with surrogate analyte at different concentration levels can be analyzed to study the authentic analyte response function alteration (Fig. 4) [156]. Once the RF has been empirically determined, the study sample concentration is calculated as follows:

$$X_s = \frac{Y_A}{Y_{SIL}} \bullet \frac{X_{SIL}}{RF} \quad \text{Eq:15}$$

where X is the concentration and Y the measured signal, while the subscripts "s" and "A" indicate the study sample and the analyte, respectively. This equation is a rearrangement of Equation (3), where the intercept is zero, and the slope b_1 corresponds to the RF. In Equation (15), study sample standardization is usually not considered because the computation is performed using a ratio calculated in the study sample. Indeed, the use of SIL as calibrant allows to obtain a recovery and matrix effect as close as possible to the authentic analyte [157]. Regarding the concentration level of calibrant that must be spiked, the linearity assay over the studied dynamic range to determine the RF generally helps to evaluate when crosstalk, and/or ionization competition is occurring (Fig. 4). When SIL is spiked at low concentration (i.e., 12.5% or 25% of

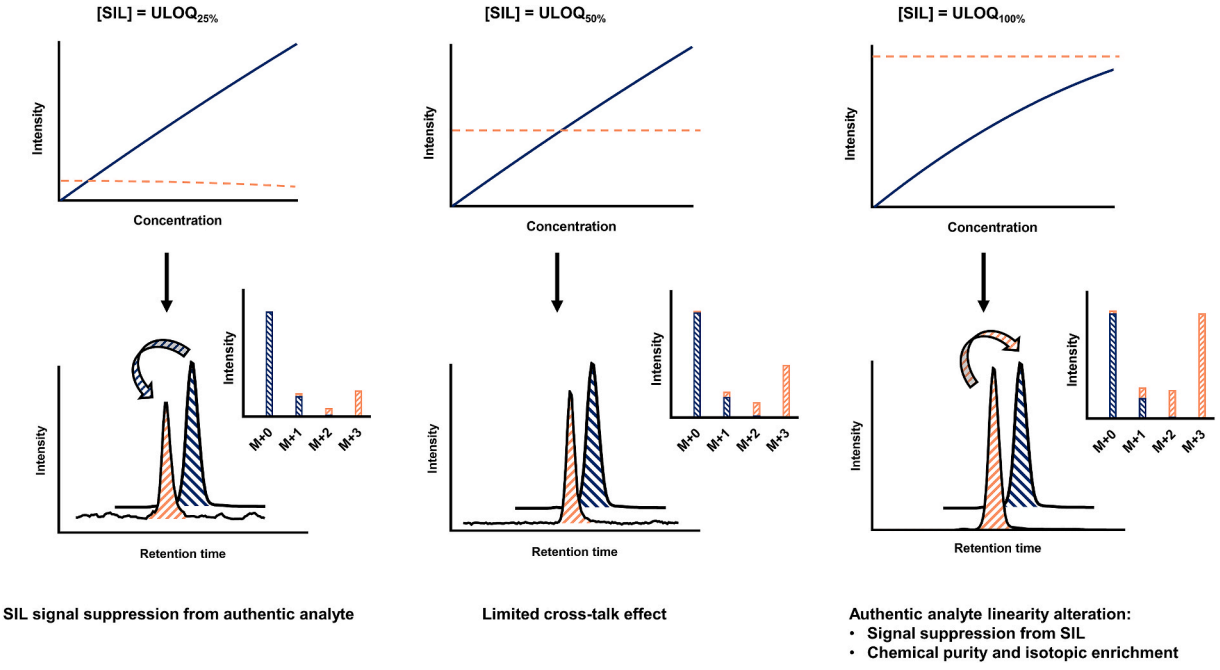

Fig. 4. Linearity assay of authentic analyte calibration curves using different concentration levels of SIL, namely 25%, 50% and 100% of ULOQ. Blue and orange lines indicate the authentic analyte and SIL responses, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the upper limit of quantification (ULOQ) level), marked competitive ion suppression occur due to the concomitant presence of the analyte at higher concentrations in ionization source. Conversely, when the SIL concentration is fixed in the highest range of the response function, the influence of the surrogate signal on a low concentrated analyte can be detrimental and generate significant bias [158]. The crosstalk on the analyte's signal from SIL chemical impurities becomes relevant when the latter is at 75% or 100% of ULOQ. In addition to SIL synthesis purity and isotopic enrichment degree, mass isotopologue distributions should be carefully evaluated to minimize signal interferences [159]. Once the crosstalk has been evaluated, the RF is estimated in the same way as described in Section 3.1.3. Khamis and colleagues evaluated the influence of setting the SIL concentration at 1.3%, 16.7% and 66.7% of the ULOQ level to correctly quantify amino acids in urine. For dansylated-tryptophan, the authors observed that instrument response over the concentration range was affected when the respective SIL, dansylated-tryptophan-$^{13}C_2$, was spiked at 1.3% and 16.7% of ULOQ, suggesting that two mass-unit differences of SIL were insufficient to be excluded from the isotopic envelope of the authentic analyte. Thus, by spiking the SIL at the highest concentrations (66.7% of ULOQ) the isotopic contribution coming from the authentic analyte was markedly reduced and the respective response function was not compromised [156]. To translate the analyte (A) response into concentration, the determination of the SIL concentration equivalent ($X_{SIL_{eq}}$) should be calculated as follows:

$$X_{SIL_{eq}} = X_{SIL} \bullet CP_{SIL} \bullet IE_{SIL} \bullet \frac{MW_A}{MW_{SIL}} \quad \text{Eq:16}$$

where MW is the molecular weight, CP is the chemical purity as percentage and IE represents the isotopic enrichment, expressed as the probability of finding a labeled atom at any single site.

Because the calibration curve with one concentration level is naturally forced through zero, the IC approach may provide suboptimal results when the contribution coming from the intercept is predominant at very low concentrations. Recently, Gu et al. introduced one-point calibration methodology using SIL as calibrant and their isotopes to extend the LLOQ [160,161]. To perform this analysis, the authors used a triple quadrupole instrument and a particular acquisition method named multiple isotopologue reaction monitoring (MIRM). By monitoring the SIL isotopic fragmentation abundances, a regression was constructed by plotting the surrogate analyte (SIL) concentration equivalent on the abscissa (X) and the instrument response of the corresponding MIRM channel on the ordinate (Y). Then, the authentic analyte concentration can be calculated using the regression parameters (i.e., slope and intercept) [162]. The MIRM approach provided additional regression points to reduce the intercept significance, but the obtained regression could not be considered a conventional $Y = f(X)$ function because the relationship is obtained with multiple signal contributions from a single concentration of the calibrant [163]. This approach was used to improve the quantification accuracy for daclatasvir in human serum compared to matrix-matched multipoint EC, reducing the observed bias from −5.1% to −1.1% at the lowest tested concentration (1 ng mL^{-1}) [162,164,165]. In a recent study, the MIRM approach was used to reduce the bias in testosterone quantification from 38% to 8% compared to the SIL one-point calibration, where the serum reference concentration was 162 pg·mL^{-1} [116].

Overall, the IC approach with SIL as calibrant, which is conceptually straightforward for absolute quantification with modern MS instrumentation, requires additional steps during method development, such as the experimental determination of the RF and, with the MIRM approach, isotopic abundance determination [166]. However, once the method has been developed, it is markedly faster in routine analysis because a calibration curve is no longer required, and similar results to EC can be obtained [167]. Currently, the IC methodology is raising interest due to the increased number of high-quality SILs commercially available, even if they remain limited to the most important compounds of any chemical family. To overcome this limitation, isotope standards can be generated by derivatizing authentic analytes with labeled $^{13}C_2$-dansylchloride and $^{13}C_2$-dansylhydrazine. Recently, Cifuentes Girard et al. used this strategy combined with a fully automated derivatization strategy to improve reproducibility and to control the reaction kinetics [168]. This approach was developed and validated to quantify 40 endogenous polar metabolites in human urine, opening the possibility performing multi-analyte quantification for accurate targeted endogenous analyte determination.

4. From error estimation to uncertainty quantification

4.1. Error estimation on back-calculated values

Since the 1990s, metrologists have developed what they call the 'Uncertainty approach' in opposition to the traditional 'Error approach' [68]. To estimate the measurement uncertainty of any reported result of analysis, an effective solution consists of applying the uncertainty approach and considering calibration as one of the many sources of uncertainty participating in the combined measurement uncertainty (MU) of an analytical result, noted $u_c(X_s)$. As described above, many studies have investigated the influence of different improvements when applied to calibration, such as weighted least-squares regression, replication of measurements or optimized distribution of calibrants within the calibration interval. If there are beneficial, they can be evaluated due to the reduction of $u_c(X_s)$. It remains difficult to make a definite statement in absence of a consensus accepted by all analysts. If there are benefits, they can be evaluated by the reduction of $u_c(X_s)$, considering that a harmonization of both the MU estimation procedure and the compliance decision still need to be achieved. Many examples applicable to a specific operating procedure or field of analysis have been published. In the specific domain of application addressed in this paper, no paper on MU estimation is available, therefore it is not possible to describe a common procedure. However, as stated in Section 2.3, the role of calibration in MU can be examined.

According to some international guidelines, calibration standards could be prepared with surrogate analytes and/or matrices if the calibration performance is guaranteed using QCs samples prepared by spiking reference standards into a matrix identical to that of the study samples [40–42]. The inverse function f^{-1} used for the back-calculation of an unknown concentration X_s is not directly applicable to QCs and real samples. Despite the inconsistency of the term 'inverse calibration' and the demonstrated performance of the reverse approach, guideline recommendations are primarily based on the two-step procedure. This can be explained via the linear regression and the fact that the computation becomes more problematic when another model is selected, such as a second-order polynomial or logistic function [169].

4.2. Measurement of uncertainty

The MU corresponds to the inverse-computed concentration X_s which is not error-free, while the analytical calibration with standards is assumed to be error-free when applying least-squares regression techniques. Despite this contradiction, an estimation of the variance s_{X_s} of the inverse-computed concentration X_s was proposed by Miller as reported in Equation (17) [170].

$$s_{X_s} = \frac{s_e}{b_1}\sqrt{\frac{1}{J} + \frac{1}{I} + \frac{(Y_s - \overline{Y})^2}{b_1^2 \sum_i (X_i - \overline{X})^2}} \quad \text{Eq:17}$$

where s_e is the residual standard deviation; I is the number of calibrants $1 \leq i \leq I$; and J is the number of replicate measurements of the unknown samples that must be predetermined. If no replicate is present, the resulting quotient is $1/J = 1$. X_i is the calibrant concentration, and Y_s is

the measured instrument signal of the test sample. As usual, $\overline{X}$ and $\overline{Y}$ are their mean values, respectively [171].

Even if it is not completely suitable for MU estimation, Equation (17) can provide valuable information regarding the optimization of calibration performance in EC, in parallel with bioanalytical guideline recommendations:

1. The first term of Equation (17) concerns s_e which estimates the random errors in the *Y*-direction. The possibility of minimizing these errors is provided with the use of an appropriate IS, as recommended by all regulatory agencies.
2. The second term b_1 concerns the slope of the calibration curve. The higher the slope is, the lower the errors in the inverse-computed concentration X_s. As defined by Currie, it corresponds to the sensitivity of an analytical calibration, the latter being defined as the ratio between the response variation of the analytical calibration and the analyte amount variation [54]. An analytical calibration can thus be considered sensitive when a small variation in the calibrant induces a large variation in the response. This definition differs from international guidelines such FDA and EMA, where sensitivity is described as the lowest measurement range with acceptable accuracy and precision [40,41].
3. At least two strategies for calibrant selection and distribution can be considered to improve (i.e., narrow) the confidence interval of X_s. First, the number I of calibration standards used to build on the regression line must be increased. According to the most commonly used guidelines, approximately six calibration points will be adequate in many experiments, and smaller values are not allowed. Conversely, the use of many calibration samples means accurately preparing many standards. The second strategy relies on the increase in the number of replicates J, which reduces the width of the confidence interval.
4. As the study sample measurement response Y_s approaches mean $\overline{Y}$, the third term inside the square root converges to zero, thus reducing the s_{X_s} value. In the conventional least squares approach, a calibration will therefore give the most precise results when the measured signal corresponds to a point near to the centroid of the regression line. One can thus expect that the prediction errors are not equal for all points. In the ordinary least-squares, this result simply indicates that the precision of inverse computed concentration is better when the response is located near to the center of the calibration curve rather than at the edges. Because the dosing range is often large in bioanalytical determination, the instrumental response heteroscedasticity often legitimates the application of weighted least-squares regression. Also, applying a weighting factor does alter the precision of the back-calculation values, where it remains optimal when the signal measured signal is close to the weighted centroid of the regression line. For these models the situation can be different, explaining why authorities recommend a justification of the weighting factor.
5. Term $\sum_i (X_i - \overline{X})^2$ indicates that it is more valuable to distribute calibrants at the edges of the dosing range to maximize it. One of the well-known properties of least-squares regression is that better estimations of the regression coefficients are obtained when two levels of concentration are distributed far away from each other in the calibration interval. International guidelines in analytical validation, such as FDA and EMA recommend performing a calibration with at least six calibration levels, including the ULOQ, LLOQ and 3*LLOQ in the calibration scheme. However, both FDA and EMA do not specify the other calibrants level distribution, and EMA only raised the interest of performing replicates of calibration [40,41]. In this context, Tan et al. evaluated the impact of calibration schemes to quantify triamcinolone, rosuvastatin and valsartan in human plasma. The authors used different calibration schemes of concentration levels and replicates, such as eight concentration levels in duplicate and two-concentration levels in octuplicate. The batch failure rate, defined as ≤2/3 individual QC replicates accepted and/or unacceptable CV/mean bias at one or more QC levels, was marginally better when using the two-calibration scheme [172]. Also, before using this calibration scheme the linearity of instrument response must be verified using a multipoint calibration curve, as ionization competition or detector saturation may occur. Thus, instead of using several calibration standards at various concentrations, it is better to make repetitions measurements at the lower end of the dosing range, (i.e., with minimum analyte concentration (LLOQ or 3*LLOQ) and replicate measurements at the ULOQ).

Official guidelines have integrated most of the concepts that lead to good inverse-computation when dealing with EC in the linear case in their recommendations. While only a few formal expectations are provided for other types or models of analytical calibration, when the method of standard additions (SAM) is used, the calculated concentration is subject to error in a similar way to the EC approach (Equation (17)). However, in this case, X_s is not predicted from the measured Y but is always extrapolated to the point on the *X*-axis, where $Y = 0$. Also, the same formula to estimate the standard deviation, s_{X_E}, of the extrapolated *X*-value X_E becomes:

$$s_{X_E} = \frac{s_e}{b_1}\sqrt{\frac{1}{I} + \frac{\overline{Y}^2}{b_1^2 \sum_i (X_i - \overline{X})^2}} \qquad \text{Eq.18}$$

Some analogies can be observed with Equation (17), such as increasing I or b_1 again improves the precision of the estimated concentration. It has been suggested that the single spiked sample should have an analyte level at least five times that of the test sample, but linearity must be demonstrated to obtain accurate results [127]. If the response of the system is nonlinear, the extrapolation involved in the SAM approach becomes more problematic to estimate. To our knowledge, no formal indication about the number of calibration standards, their range or proportionality toward the test sample is mentioned. The validation process is therefore only based on the quantitative method performance obtained on QC samples, similar to EC. The latter also applies to IPD and IC, as these calibration approaches are relatively new and not yet endorsed by official guidelines.

Thus, when considering the MU estimation of a quantitative method, the calibration methodology remains one of the key steps to reliably assess the performance characteristics. However, it is impossible to estimate the role for calibration in MU independently. Only a comparative approach is applicable using different calibration models or algorithms [173].

The multipoint matrix-matched EC is the approach recommended by international guidelines for the validation of bioanalytical methods [40,41,44]. However, some guidelines (i.e., ICH guideline M10 step 5 on bioanalytical method validation) have recently initiated a formal discussion on alternative calibration approaches to overcome the lack of blank matrices (Table 2) [40]. The 2018 FDA document on bioanalytical method validation guidance for industry included endogenous compounds in the additional issues section, suggesting the use of surrogate matrices for calibration and stating that "the FDA encourages the development and use of new bioanalytical technologies" [41]. This mention from international guidelines suggests a growing interest and need for new analytical calibration practices. Currently, the possibility of cross validating the results obtained with an alternative analytical method to a reference methodology allows the analyst to investigate alternative quantification methodologies without sacrificing performance. However, if official recommendations are based on the regular criteria for calibration strategy evaluation (e.g., the number of concentration levels and coefficient of determination) and not on the obtained results, the analyst will not be able to present alternative methodologies to the most important authorities [174]. The implementation of

Table 2
Regression parameters in calibration methodologies and their inclusion in international guidelines for bioanalytical method validation. N/A: not applicable.

	Quantification methodology	Matrix	Slope b_1	Intercept b_0	Number of calibrants	Endorsed by bioanalytical guidelines
External calibration	Authentic analyte in authentic matrix	Authentic - analyte-free - background subtraction	Analyte response	Signal blank	Multiple	FDA (2018), EMA ICH M10 5 (2022)
	Authentic analyte in surrogate matrix	Surrogate				FDA (2018), EMA ICH M10 5 (2022)
	Surrogate analyte in authentic matrix	Authentic	Response factor			EMA ICH M10 5 (2022)
	Surrogate analyte in surrogate matrix	Surrogate				No
In-sample calibration	Standard addition method	Study sample	N/A	Sample blank	Multiple or single	EMA ICH M10 5 (2022)
	Isotopic pattern deconvolution			N/A		No
	Internal calibration		Response factor	Zero	Single	No

renewed criteria to evaluate the calibration strategy, including a focus on the observed results and their respective uncertainties, as well as a harmonization of common practices that must be investigated will help the analyst in the validation process, leading to more robust and comparable results between laboratories [48,175]. This process is probably the most pragmatic way to use innovative quantification methodologies implemented for modern LC–MS instrumentation.

5. Conclusion and perspectives

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 real official guidelines and strategies used by the analytical community prevents consensus about exactly how validation should be performed. Recently, the introduction of in-sample calibration approaches allowed the analyst to perform the calibration in the authentic 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 of few samples or even a single sample. With these unique advantages, internal calibration strategies have great 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.

This paper does not completely address the subject of calibration and several topics remain to be explored. The following points require more attention from analysts:

1) Too many guidelines still confound calibration curve linearity and trueness linearity and the requirement to using a linear calibration curve is abusive. Statistical software is easily available or even embedded within the instrument control software. Sophisticated and approved algorithms can be applied to complex calibration models, such as non-linear, with weighted least-squares regression methods.
2) The question of MU estimation will certainly become crucial in the coming years and the role of calibration is an important issue. It is disappointing to consider that almost no publication for MU applied to new LC–MS technique is available. As stated, selecting the best calibration method is difficult when using the classical statistical tools but can be achieved by computing the combined MU for different models. This raises the question of the harmonization of a procedure for estimating MU in the analytical sciences. Too many confusing approaches exist which do not give out the same estimates. Depending on the working range, it is possible to promote different strategies. For large working ranges, the estimation of the uncertainty function derived from the accuracy profile is very promising. For small working ranges, the use of QC data is another possible issue. But the answer must come from the analysts themselves.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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