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# Straightforward quantification of endogenous steroids with liquid chromatography–tandem mass spectrometry: Comparing calibration approaches

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## ABSTRACT

Different calibration strategies are used in liquid chromatography hyphenated to mass spectrometry (LC-MS) bioanalysis. Currently, the surrogate matrix and surrogate analyte represent the most widely used approaches to compensate for the lack of analyte-free matrices in endogenous compounds quantification. In this context, there is a growing interest in rationalizing and simplifying quantitative analysis using a one-point concentration level of stable isotope-labeled (SIL) standards as surrogate calibrants. Accordingly, an internal calibration (IC) can be applied when the instrument response is translated into analyte concentration via the analyte-to-SIL ratio performed directly in the study sample. Since SILs are generally used as internal standards to normalize variability between authentic study sample matrix and surrogate matrix used for the calibration, IC can be calculated even if the calibration protocol was achieved for an external calibration (EC). In this study, a complete dataset of a published and fully validated method to quantify an extended steroid profile in serum was recomputed by adapting the role of SIL internal standards as surrogate calibrants. Using the validation samples, the quantitative performances for IC were comparable with the original method, showing acceptable trueness (79%–115%) and precision (0.8%–11.8%) for the 21 detected steroids. The IC methodology was then applied to human serum samples ( $n = 51$ ) from healthy women and women diagnosed with mild hyperandrogenism, showing high agreement ( $R^2 > 0.98$ ) with the concentrations obtained using the conventional quantification based on EC. For IC, Passing-Bablok regression showed proportional biases between -15.0% and 11.3% for all quantified steroids, with an average difference of -5.8% compared to EC. These results highlight the reliability and the advantages of implementing IC in clinical laboratories routine to simplify quantification in LC-MS bioanalysis, especially when a large panel of analytes is monitored.

## 1. Introduction

Endogenous molecules are significant upstream markers and sources of information for downstream biological processes [1]. Metabolites may play important roles as biomarkers in the early diagnosis and prognosis of diseases, in addition to representing great resources for the in-depth study of underlying molecular mechanisms of diseases [2–4]. In

this context, liquid chromatography hyphenated to mass spectrometry (LC-MS) is one of the most prominent techniques for clinical bioanalysis [5,6]. Despite its sensitivity and selectivity, obtaining accurate and precise metabolite concentrations relies heavily on the analytical calibration methodology [7–10].

The multi-standard external calibration (EC) is the most common approach in LC-MS bioanalytical methods, as recommended in

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international guidelines for bioanalytical methods validation [11,12]. However, in the case of endogenous compounds, a consensus for the blank matrix to build the calibration curve is missing, even if some guidelines have recently initiated a formal discussion on this topic [13]. In the Food and Drug Administration, European Medicines Agency and the last proposal of the International Council for Harmonisation guidelines, several procedures have been proposed to overcome the absence of analyte-free matrices, such as background subtraction or the use of surrogate matrices and/or analytes [14,13]. Surrogate matrices such as neat, artificial or stripped, allow the analyst to build the EC without the presence of endogenous analytes [15–17]. Conversely, EC can be performed using a pooled authentic matrix spiked with a surrogate analyte and the analyte concentration in the study sample is calculated using another reference than the authentic analyte (i.e., the surrogate analyte) [18]. In the aforementioned methodologies, samples are referred to as an external referential and a set of internal standards is commonly added prior to the sample preparation to compensate for matrix effects, as well as extraction recovery differences between the calibrants and study samples [19]. Stable isotope-labeled (SIL) standards are typically used as internal standards owing to their similar physicochemical properties with the authentic analytes [20]. For the simultaneous quantification of multiple analytes careful preparation of several calibration standards of known concentration, that may differ between analytes, is required. This can be time-consuming and probably represents the major source of interlaboratory variation, especially when covering very different endogenous concentration ranges [21].

More recently, the interest in using SIL as surrogate calibrants has been growing in the analytical chemistry community [22–24]. When a single amount of surrogate calibrant is used to obtain the study sample concentration, this methodology is referred to as an internal calibration (IC) approach. In this case, the sample standardization and the analyte concentration–response (i.e., the calibration) is performed directly in the study sample [25,26]. The IC methodology is gaining traction due to the increased number of high-quality SIL in terms of isotopic enrichment and chemical purities [27,28]. Several research groups have recently demonstrated that IC can show comparable performance to EC, with the advantage that it features a simplified analytical workflow [29–32]. The adoption of internal calibration approaches relying on the use of SIL as surrogate calibrants is of particular interest for the simultaneous quantification of a wide array of endogenous analytes, including steroids.

For several decades, the analysis of steroids has been a key tool in the diagnosis and monitoring of numerous endocrine pathologies. Steroids are a group of lipids that play a critical role in the organism at the endocrine, paracrine and intracrine regulatory levels. For example, corticosteroids (glucocorticoids and mineralocorticoids) are involved in many biological pathways such as stress and immune responses and/or regulation of inflammation and carbohydrate metabolism. Progestogens, androgens and estrogens are involved in reproductive functions including the development of secondary sexual characteristics. The simultaneous quantification of an increasing number of steroids has also become a common strategy in clinical research laboratories to cover multiple pathways of the steroidome allowing for more accurate diagnosis [33–35]. In doping control laboratories, since the introduction of the steroidal module of the Athlete Biological Passport, the quantification of a panel of steroids is also routinely used to detect potential exogenous administration of steroids [36–38]. Such laboratories are often required to analyze a large number of samples on a continuous basis, and the traditional EC is widely used as it is recommended by the most common guidelines. However, the extensive preparation of calibration curves for several metabolites with different concentration ranges brings cost implications for routine and can be challenging from a practical point of view. Indeed, each calibrator must be prepared individually as every analyte has a specific dynamic range. The preparation of calibration curves is also the major source of variability between laboratories due to the way solutions, mixtures and calibrators are prepared, along with the source of reference material purchased

[39,40]. The commutability of calibrators is particularly important for the passport approach, where each point can come from a different laboratory. Using the one-point IC instead of a complete EC decreases the number of calibrators to be prepared to a single mix, reducing the risk of inaccurate standard mixing, the time required to prepare multiple calibrators, and the instrument turnaround. Indeed, IC allows faster sample analysis without the requirement to group a large number of samples together in a batch, enabling for LC-MS random access analysis and faster data processing [27].

In this work, one-point internal calibration is shown to be a straightforward approach for multi-targeted quantification of endogenous steroids with LC-MS. The proposed workflow was applied to simultaneously quantify an extended panel of 21 steroids of interest for doping control in human serum samples from two different female populations, including phase I and phase II analytes. This dataset provides an objective benchmark to demonstrate that IC is an efficient, fit-for-purpose strategy for the direct quantification of endogenous steroids in study samples compared to time-consuming current practices, such as EC procedures.

## 2. Experimental

### 2.1. Dataset used

For the comparison between EC and IC, a complete steroid dataset from a previously published study by Salamin *et al.* was used [41]. This includes the UHPLC-MS/MS validation data acquired for the quantitative performance estimation of 14 free and 14 conjugated steroids, as well as human serum samples from two female populations showing different steroid profiles: healthy and diagnosed with mild hyperandrogenism.

The trueness, repeatability, intermediate precision, as well as lower and upper limits of quantification of the EC methodology were compared with the results obtained using IC. The reader may refer to the cited literature for a detailed description of the analytical procedure used to acquire the data for method validation and biological samples determination. This includes the chemicals and reagents used, sample preparation and UHPLC-MS/MS acquisition parameters.

The structure of the dataset, the panel of endogenous steroids quantified in the biological samples and the strategy used to compare the quantitative performance between the EC and IC methodologies are described below.

#### 2.1.1. Calibration & validation samples

The method validation data were acquired via three analytical series, with calibration curves and validation samples prepared by spiking charcoal depleted human serum at seven (conjugated) or eight (free) levels of concentration [41]. Validation samples were acquired in triplicate, whereas calibration samples were acquired in duplicate, at the beginning and at the end of each series. The reader may refer to the [supplementary material](#) of the cited article for the used concentrations of calibrators and SIL internal standards. For both EC and IC approaches, one female and one male human serum sample containing representative low and high levels of steroid profile markers were included in each analytical run as quality control [42]. Since there are no batch QCs acceptance criteria in the anti-doping field for the quantification of endogenous steroids in serum, the measured concentration values must meet the internal criteria of trueness and precision (accuracy) within  $\pm 30\%$  of the theoretical concentrations.

#### 2.1.2. Biological samples

The dataset consists of 51 serum samples and the analyte subset includes 21 steroids that were detected in biological samples within the quantification limits using EC in the original research article. The quantified analytes and the associated SIL internal standards used for the comparison between EC and IC are summarized in [Table 1](#).

**Table 1**  
Quantitative performance comparison between the external calibration (EC) and the internal calibration (IC) approaches. The  $\overline{RF} \pm SD$  was calculated using the dataset of the three validation days and concentration coverage was computed as described in Eq. (4). Androsterone sulfate-d4 was assigned to both androsterone sulfate and epiandrosterone sulfate (§). SIL: stable isotope-labeled, RF: response factor, IP: intermediate precision.

#	Analyte	SIL analog	$\overline{RF} \pm SD$	SIL concentration (% upper range)	EC				IC				
					Trueness (%)	Repeatability (%)	IP (%)	Linearity range (ng·mL <sup>-1</sup> )	Trueness (%)	Repeatability (%)	IP (%)	Linearity range (ng·mL <sup>-1</sup> )	Coverage (%)
1	Testosterone	-d3	0.19 ± 0.01	0.8%	92.9–107.0	1.6 - 5.4	2.4 - 9.0	0.02 - 25	79.1 - 99.1	1.3 - 2.5	1.9 - 3.5	0.06 - 25	99.84
2	Epitestosterone	-d3	1.36 ± 0.15	20.0%	94.6 - 109.8	1.3 - 5.8	3.4 - 10.1	0.02 - 10	99.1 - 115	1.3 - 4.6	1.3 - 5.6	0.075 - 10	99.45
3	Androstenedione	-d7	0.86 ± 0.04	5.0%	88.3 - 108.7	2.0 - 4.6	6.0 - 10.1	0.05 - 25	84.0 - 99.9	1.8 - 3.4	4.9 - 10.2	0.15 - 25	99.60
4	Dehydroepiandrosterone	-d5	0.97 ± 0.07	3.3%	94.0 - 106.1	2.3 - 6.8	2.4 - 12.8	0.5 - 150	92.0 - 107.4	2.4 - 5.5	3.9 - 12	5 - 150	96.99
5	Dihydrotestosterone	-d3	0.66 ± 0.03	20.0%	96.2 - 105.8	2.9 - 6.7	4.0 - 10.1	0.05 - 10	88.4 - 98.3	2.6 - 4.1	2.6 - 9.0	0.3 - 10	97.49
6	Progesterone	-d9	0.25 ± 0.01	2.0%	93.7 - 112.0	2.2 - 8.8	3.1 - 9.9	0.015 - 25	86 - 100.9	1.8 - 7.9	2.6 - 11.8	0.02 - 25	99.98
7	17α-hydroxyprogesterone	-d8	1.12 ± 0.05	40.0%	93.0 - 108.6	1.7 - 4.4	4.3 - 11.8	0.1 - 25	90.6 - 105.6	1.7 - 4.3	3.7 - 9.0	0.1 - 25	100.00
8	Cortisol	-d4	0.61 ± 0.08	25.0%	91.9 - 108.9	1.4 - 3.8	3.4 - 10.1	1 - 400	89.5 - 99.8	1.3 - 3.8	4.9 - 11.2	1 - 400	100.00
9	Corticosterone	-d4	0.44 ± 0.02	5.0%	89.7 - 107.3	2.0 - 2.7	2.5 - 4.5	0.25 - 100	82.7 - 104.3	1.7 - 2.3	4.0 - 11.3	0.25 - 100	100.00
10	Deoxycorticosterone	-d8	1.78 ± 0.05	20.0%	91.1 - 110.1	2.0 - 3.2	5.4 - 12.3	0.025 - 10	86.3 - 99.7	1.7 - 3.4	4.6 - 8.6	0.025 - 10	100.00
11	11-deoxycortisol	-d2	0.99 ± 0.18	20.0%	96.1 - 106.5	2.0 - 5.2	2.0 - 7.9	0.025 - 2.5	104.1 - 115.2	2.2 - 5.6	4.0 - 8.2	0.025 - 2.5	100.00
12	Testosterone glucuronide	-d3	0.81 ± 0.01	4.0%	96.8 - 103.3	1.7 - 7.0	2.5 - 7.0	0.05 - 25	86.2 - 95.3	1.5 - 2.3	2.5 - 7.2	0.3 - 25	99.00
13	Androsterone glucuronide	-d4	1.45 ± 0.05	20.0%	97.1 - 102.2	0.8 - 5.6	1.2 - 9.9	0.1 - 100	95.1 - 104.5	0.8 - 3.1	2.6 - 10.7	0.5 - 100	99.96
14	Etiocholanolone glucuronide	-d5	2.36 ± 0.42	20.0%	96.9 - 104.0	2.7 - 7.6	2.7 - 8.1	0.25 - 100	103.0 - 105.9	2.7 - 3.7	7.6 - 10.2	3.5 - 100	96.74
15	5βαβ-adiol-3-glucuronide	-d3	1.36 ± 0.09	100.0%	97.0 - 100.8	2.5 - 8.1	3.4 - 8.1	0.1 - 10	92.5 - 100.4	2.5 - 6.6	4.3 - 10.8	0.2 - 10	98.99
16	5βαβ-adiol-17-glucuronide	-d3	0.70 ± 0.08	100.0%	97.7 - 100.5	1.6 - 4.3	2.1 - 6.3	0.25 - 25	98.5 - 102.9	1.7 - 4.4	3.1 - 7.7	0.25 - 25	100.00
17	Testosterone sulfate	-d3	0.88 ± 0.12	8.0%	100.0 - 106.1	1.7 - 5.4	2.1 - 8.7	0.1 - 25	84.6 - 92.3	1.4 - 2.7	3.5 - 7.7	0.3 - 25	99.20
18	Androsterone sulfate	-d4 <sup>§</sup>	0.85 ± 0.04	10.0%	96.9 - 100.5	1.3 - 3.2	1.4 - 6.5	1 - 2500	94.2 - 107.1	1.7 - 3.1	2.5 - 6.8	20 - 2'500	99.24
19	Etiocholanolone sulfate	-d5	0.28 ± 0.04	10.0%	97.5 - 102.8	1.9 - 4.8	2.1 - 6.1	1 - 2500	96.8 - 109.7	1.9 - 4.8	2.1 - 7.8	5 - 2'500	99.84
20	Dehydroepiandrosterone sulfate	-d6	0.53 ± 0.01	15.0%	93.7 - 108.2	1.4 - 3.0	2.6 - 4.4	50 - 10'000	87.9 - 103.9	1.4 - 3.0	2.3 - 6.2	75 - 10'000	99.75
21	Epiandrosterone sulfate	§	0.75 ± 0.08	10.0%	100.4 - 105.8	1.3 - 2.9	1.8 - 3.8	5 - 2'500	91.2 - 111	1.2 - 2.7	1.6 - 6.0	5 - 2'500	100.00

## 2.2. Data treatment and statistical analysis

All analyte peak areas were automatically integrated using Target-Lynx 4.2 (Waters Corporation, Milford, MA, USA) and manually verified by the authors in [41]. No peak re-integration was performed for this retrospective study. The obtained results for the validation and biological samples using the EC were compared with the IC output.

### 2.2.1. Response factor calculation

All calculations with the IC methodology for calibration, validation and biological samples were performed with Python 3.9 using an automated in-house workflow, whose main stages are described here. The response factor (RF) was directly calculated from the calibration samples through the analyte and SIL area ratio. Because the analyzed data were produced following an EC methodology, several calibration samples were available. The RF was systematically computed with the calibration sample having the closest analyte concentration to the one of the corresponding SIL. As calibration samples were acquired at the beginning and at the end of each series, an average RF was calculated as in Eq. (1).

$$RF = \frac{1}{n} \left( \sum_{i=1}^n \frac{Y_A X_{SIL}}{X_A Y_{SIL}} \right) \quad (1)$$

where  $X$  defines the concentrations and  $Y$  the integrated areas, the subscripts “A” and “SIL” indicate the analyte and stable isotope-labeled analog, respectively;  $n$  is the number of calibration sample replicates in the series. The concentration of analytes in validation or biological samples ( $X_{A_{spl}}$ ) was calculated as in Eq. (2):

$$X_{A_{spl}} = \frac{Y_A}{Y_{SIL}} \cdot \frac{X_{SIL_{eq}}}{RF} \quad (2)$$

where  $X_{SIL_{eq}}$  is the SIL-analyte equivalent concentration;  $RF$  is the response factor, and the other terms were previously defined for Eq (1).  $X_{SIL_{eq}}$  is calculated as follows:

$$X_{SIL_{eq}} = X_{SIL} \cdot \frac{MW_A}{MW_{SIL}} \quad (3)$$

where  $MW$  is the molecular weight of the analyte A or SIL.

Before implementing the IC approach, the accepted RF variability for all steroids was assessed using the standard deviations (SD) from the three validation days (Table 1). When a new analytical run is performed, the inter-run repeatability is ensured if the currently obtained RF value obtained is comprised in the  $\bar{RF} \pm 3SD$  interval. The steroids included in our study showed RF within  $\pm 3SD$ . However, in case the RF diverged from the interval, an adaptive model based on the historical averaged RF can be implemented, as suggested by Rule and Rockwood [27].

### 2.2.2. Quantification approaches comparison

Passing-Bablok regressions, based on a robust nonparametric model, were used to evaluate EC and IC agreement in serum study samples using Python 3.9 in-house code. The model is based on the hypothesis that if the confidence interval at 95% (95% CI) for the intercept includes the zero value and the 95% CI for slope includes the one value, there are neither constant (intercept) nor proportional (slope) differences between both methods [43].

The results of the quantitative performance obtained with validation samples in charcoal-depleted serum were compared using the following criteria: trueness, repeatability, intermediate precision, linearity range (i.e., lower and upper limit of quantification), as well as the concentration coverage of the IC approach with respect to the EC (Eq. (4)). The precision (repeatability and intermediate precision) and trueness were verified with validation samples at seven (conjugated) or eight (free) concentration levels spiked in charcoal-depleted serum extracted in triplicate over three validation assays. The intraday precision (repeatability) was expressed as the coefficient of variation (CV%). Using one-

way ANOVA decomposition, repeatability was calculated as the within-group precision, while intermediate precision was obtained as the square root of the sum of squares of the within-group and between-group precisions [12]. Trueness was calculated as the average of relative bias over the three validation assays. The method was considered accurate and precise when CV values were below 15% and trueness in the range 70–130% [44–46]. The lower and upper limits of quantification were defined when the total error (i.e., the sum of bias and precision) was higher than 30% in the accuracy profile [47]. The concentration coverage was defined as the ratio of linearity ranges (i.e., where 95% of the results fall within the 70% – 130% limits) between IC and EC:

$$\text{Concentration coverage (\%)} = \frac{ULOQ_{IC} - LLOQ_{IC}}{ULOQ_{EC} - LLOQ_{EC}} \cdot 100 \quad (4)$$

where LLOQ and ULOQ are the lower and upper limits of quantification, respectively.

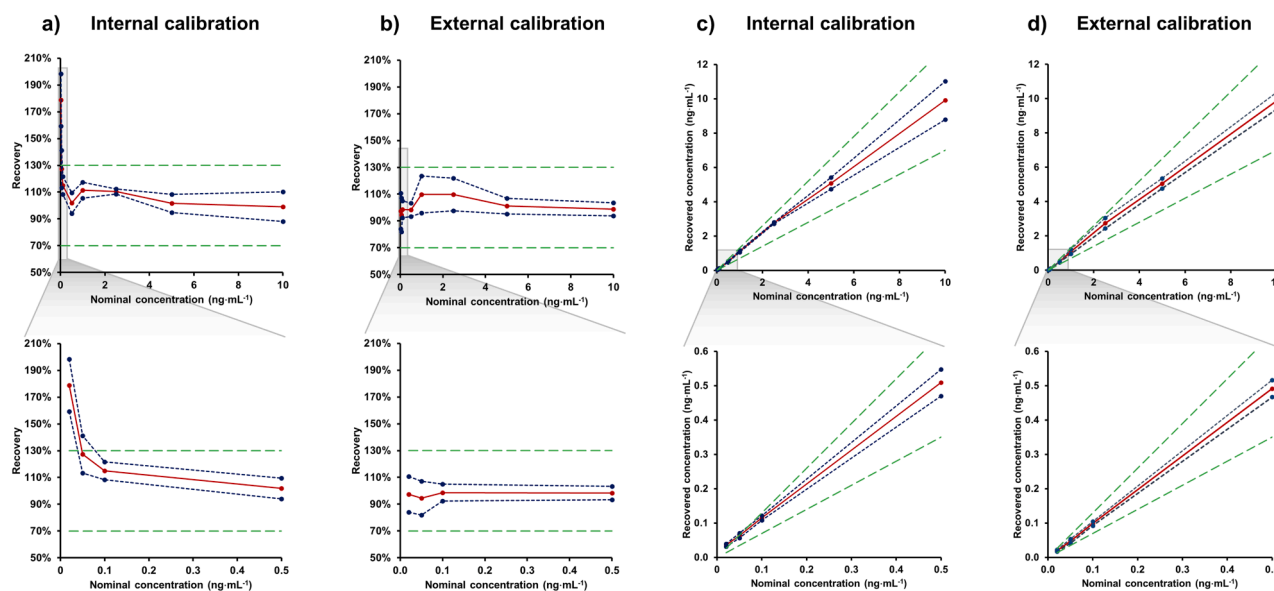
## 3. Results and discussion

The dataset from a fully validated UHPLC-MS/MS method for the quantification of 21 steroids in human serum was used to predict analyte concentrations with an original and straightforward strategy, namely the internal calibration (IC) approach. In the initial method, steroid concentrations were obtained thanks to a conventional external calibration (EC) approach, using a surrogate matrix containing isotope-labeled (SIL) analogues as internal standards [41]. Here, the SIL role was considered as a surrogate calibrant, and the analyte MS signal inversely predicted into concentration via the analyte-to-SIL ratio. The calibration function is obtained directly in the study sample and not using an external referential: this methodology is therefore referred as IC [25]. Since the analyte and the SIL have similar physicochemical properties, using the SIL as an internal calibrator allows recovery and matrix effect to be as close as possible to the analyte, correcting for overall analytical variation [19]. By knowing the response factor (RF) and the spiked SIL amount through the selection of one calibrant, the unknown analyte concentration can be easily computed (Eq. (1)).

### 3.1. Validation dataset

The validation samples were prepared at seven and eight concentration levels for free and conjugated steroids, respectively. Concentrations were recalculated using the IC approach (i.e., by a simple analyte-to-SIL ratio and a predetermined RF) and the accuracy profiles were compared with the EC. Trueness and precision were consistent with the EC methodology and, although some steroids had a lower LLOQ, the resulting IC concentration coverage was comprised between 96% and 100% (Table 1). To evaluate the quantitative performance, the  $\beta$ -expectation tolerance interval associated with accuracy profiles was selected. This allows the analyst to make a quick decision on the validity of an analytical method. Tolerance intervals, defined as statistical intervals within which a specified proportion of the population will fall with a certain degree of confidence, have become the gold standard in analytical method validation. More specifically, the  $\beta$ -expectation tolerance interval with a probability of  $\beta = 0.80$ , meaning that on average, 80% of the individual values (results) of the tested population (validation samples) fall within the interval, has been considered to construct a total analytical error profile, namely accuracy profile [48]. A complete comparison of accuracy profiles using epitestosterone as an example of endogenous steroid routinely monitored in the Athlete Biological Passport is shown in Fig. 1 [38]. Overall, for epitestosterone, the quantitative performance between EC and IC is comparable (Fig. 1c and d) and an overestimation is observed when using the relative accuracy profiles (Fig. 1a and b). This has been observed for other steroids in the examined dataset and is discussed below using the example of its isomer, testosterone. All profiles are shown in Fig S1 of the supplementary





**Fig. 1.** Quantitative performance comparison between the IC and the EC for the epitestosterone using the absolute (a and b) and relative (c and d) accuracy profiles. The red line represents the accuracy, the blue densely dashed lines represent the tolerance interval limits and the green loosely dashed lines represent the acceptability limits. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**material.** With the reference EC approach, trueness laid between 88.3% and 112%. With the IC methodology, the trueness ranged between 79% and 115% for all steroids that were in the concentration coverage range. Regarding repeatability and intermediate precision, values ranged between 0.8% and 12.8% for the EC approach. In the same way, these values were comprised between 0.8% and 11.8% for IC.

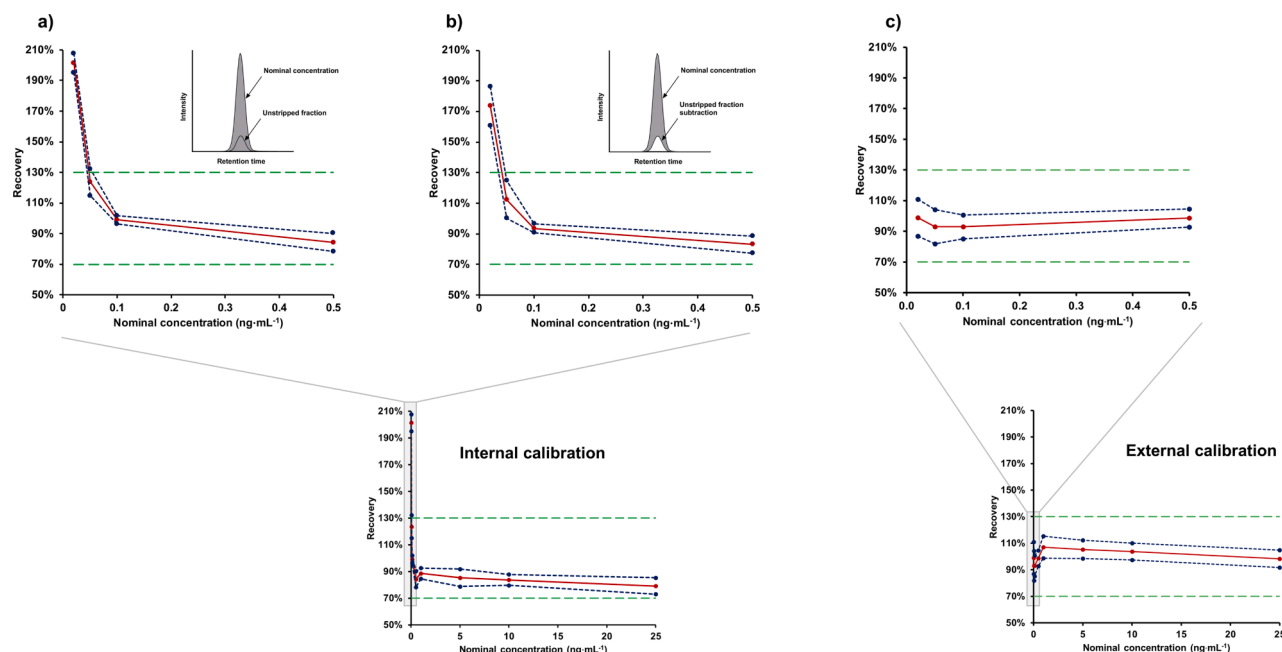
Slight biases were observed at low concentration values for validation samples (i.e., LLOQ) on a limited number of steroids. Different hypotheses may explain these observations: 1) The SIL chemical impurities contribute to the analyte MS/MS signal; 2) The analyte signal is suppressed by SIL's competition in ionization; 3) The RF drifts over validation days; 4) The unstripped analyte fraction in depleted serum is combined with the nominal amount spiked, resulting in higher computed concentration. The first two hypotheses require dedicated studies to be tackled and they are normally performed during the IC methodology development, which was not the case in this study [32]. However, some considerations for the investigated dataset are emphasized below in Section 3.2 with the example of testosterone sulfate. Regarding the RF drift, the coefficient of variation over the three validation days was below 15% for most of the steroids, except for 11-deoxycortisol (18.0%), and etiocholanolone glucuronide (17.9%). Given that the IC methodology mostly relies on the RF to compute an analyte concentration, a small variation on this value can significantly impact the output at low concentrations (i.e., less than 100 pg·mL<sup>-1</sup>). The last hypothesis concerns the mathematical model used to calculate concentrations with the IC approach, i.e., a linear regression equation forced through the origin where the intercept is zero and the slope corresponds to the RF (Eq. (2)). When a stripped serum is used to build calibrants with the EC approach, not all endogenous analytes can be completely removed by charcoal depletion [18]. In this study, calibrants and quality controls were prepared in a single-stripped human serum which can contain residual analyte fractions, especially when steroid concentrations are high. This was observed by inspecting the EC regression intercepts or the analyte signals on the unspiked stripped serum samples. With the conventional EC approach, the concentration computation naturally includes the matrix blank signal (i.e., the intercept) and the nominal spiked concentration is obtained with the regression function. Conversely, in the IC approach, the analyte concentration-response is obtained directly in the study sample and the unstripped fraction contribution from the matrix blank cannot be differentiated from the

nominal spiked concentration. To overcome this limitation, the IC methodology can be used to estimate the matrix blank signal concentration in unspiked stripped serum samples containing SILs, and this contribution can be subtracted from the nominal concentration in validation samples. By removing the unstripped fraction concentration, the bias on the accuracy profile at low concentrations can be markedly reduced, as shown for testosterone in Fig. 2.

The overestimation of 15 pg·mL<sup>-1</sup> at the lowest concentration level for testosterone (20 pg·mL<sup>-1</sup>) was explained by the fact that the IC calculation forced the calibration function through the origin (Eq. (2)). When low intensities are measured using the IC approach, the intercept parameter should be accurately estimated to obtain accurate inverse-predicted analyte concentrations [29]. Additionally, the chemical interferences on the analyte MS/MS signal coming from SIL are also a critical point on the concentration determination [27]. Even if providers are manufacturing high quality SILs in terms of chemical purity and isotopic enrichment, it cannot be excluded that cross-signal contribution between analyte and SIL slightly impacted the predicted values. Even if the chemical purity of the SIL is comprised between 98% and 99%, the remaining 1–2% can affect the results at low concentrations, especially for steroids that have very wide reference ranges and therefore linearity (e.g., testosterone and epitestosterone). The concentration of each SIL should be carefully evaluated in advance to limit this contribution, along with its stability over time, which may be related to deuterium scrambling during storage and LC-MS ionization [32,49]. Conversely, the chemical purity has a minor effect on the inverse-predicted concentrations using the EC approach because the SIL contribution is included in the slope intercept value [32,50]. A complete linearity assay over the studied dynamic range generally helps to evaluate when crosstalk between the authentic analyte and the surrogate calibrant, along with ionization competition are occurring [51].

It has to be noted that the investigated dataset was initially designed for an external calibration protocol and not all the experimental parameters were optimized for the IC approach. This includes the RF evaluation, the SILs concentration verification, the SIL spiked concentration in study samples, as well as the crosstalk determination with its corresponding analyte [30–32]. Nevertheless, similar results are obtained in a very simple and straightforward way to compute the observed signals with the IC methodology.

As a second step, a formal comparison based on the results obtained



**Fig. 2.** Testosterone accuracy profiles at low concentration for the IC approach a) without subtracting the unstripped analyte fraction on the depleted serum used and b) by subtracting the estimated residual analyte amount on the stripped serum. The accuracy profile for the EC is also shown (c). The red line represents the accuracy, the blue densely dashed lines represent the tolerance interval limits and the green loosely dashed lines represent the acceptability limits. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the biological study samples was performed. The quantitative performance was discussed to assess advantages and disadvantages between EC and IC.

### 3.2. Female populations study samples

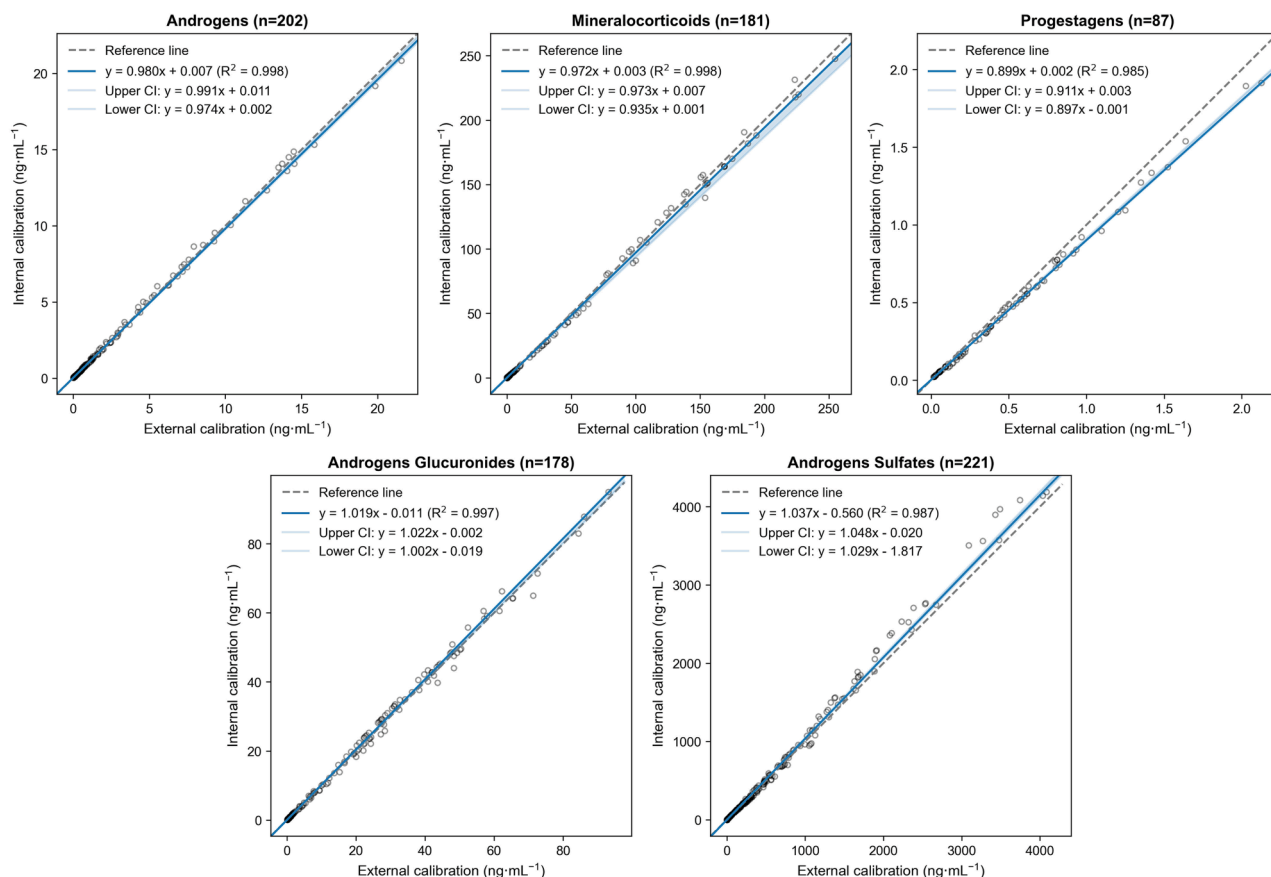
The recovered (or inverse-predicted) concentrations of each steroid in clinical study samples ( $n = 51$ ) were compared with those measured with the EC approach using the Passing-Bablok methodology: regression plots were assessed for each steroid (Fig S2) and the results are summarized in Table 2. Due to the high number of steroids monitored in the LC-MS/MS method, the Passing-Bablok regression plots were clustered

by class, as shown in Fig. 3. Satisfactory coefficients of determination ( $R^2 > 0.98$ ) were obtained between IC and EC for most compounds, except for epitestosterone, where computed values were outside the linearity range defined for IC, i.e.,  $75 \text{ pg mL}^{-1}$  (Table 1). All steroids showed a proportional bias between  $-15.0\%$  and  $11.3\%$ , with an average of  $-5.8\%$ . Only testosterone sulfate exhibited a proportional bias of  $-30\%$  (Table 2), probably due to the low biological concentrations to be detected (i.e., less than  $200 \text{ pg mL}^{-1}$ ), as well as the relatively high SIL concentration introduced in the study sample ( $2 \text{ ng mL}^{-1}$ ), which could induce a significant signal suppression of the corresponding analyte [26]. Also, most of the detected compounds had low intercept values, suggesting that the IC methodology provides comparable accuracies to

**Table 2**

Passing-Bablok regression parameters for each steroid quantified in study serum samples using the external calibration versus the internal calibration approach. The dynamic range refers to the IC approach.

#	Analyte	Observations	Systematic difference		-	Proportional differences	
			Intercept	95% CI		Slope	95% CI
1	Testosterone	51	0.02	0.02 - 0.02		0.93	0.90 - 0.93
2	Epitestosterone	51	0.00	0.00 - 0.02		0.99	0.95 - 0.99
3	Androstenedione	12	0.01	0.01 - 0.01		1.04	0.98 - 1.08
4	Dehydroepiandrosterone	47	0.12	0.06 - 0.17		1.00	0.96 - 1.02
5	Dihydrotestosterone	41	0.01	0.01 - 0.01		0.95	0.94 - 0.95
6	Progesterone	41	0.01	0.01 - 0.01		0.89	0.88 - 0.89
7	17 $\alpha$ -Hydroxyprogesterone	46	-0.01	-0.02 - (-0.01)		0.92	0.92 - 0.95
8	Cortisol	51	-2.19	-3.61 - (-1.04)		0.99	0.98 - 1.01
9	Corticosterone	50	-0.02	-0.03 - (-0.02)		0.91	0.91 - 0.91
10	Deoxycorticosterone	31	0.01	0.00 - 0.01		0.87	0.85 - 0.91
11	11-Deoxycortisol	49	-0.001	-0.002 - 0.001		1.05	1.04 - 1.06
12	Testosterone glucuronide	11	0.01	0.01 - 0.02		0.90	0.87 - 0.9
13	Androsterone glucuronide	50	-0.58	-0.92 - (-0.21)		1.00	0.99 - 1.02
14	Etiocholanolone glucuronide	51	-0.01	-0.32 - 0.15		1.06	1.06 - 1.07
15	5 $\beta$ $\alpha$ -Adiol-3-glucuronide	35	-0.001	-0.003 - 0.002		0.95	0.93 - 0.97
16	5 $\beta$ $\alpha$ -Adiol-17-glucuronide	31	-0.03	-0.03 - 0.00		1.11	1.03 - 1.11
17	Testosterone sulfate	18	0.04	0.01 - 0.04		0.70	0.70 - 0.90
18	Androsterone sulfate	50	-7.37	-16.05 - 0.58		1.03	1.01 - 1.05
19	Etiocholanolone sulfate	51	1.33	1.00 - 1.33		1.02	1.02 - 1.03
20	Dehydroepiandrosterone sulfate	51	-10.50	-45.77 - 1.75		1.09	1.09 - 1.13
21	Epiandrosterone sulfate	51	2.67	0.28 - 8.57		0.94	0.91 - 0.96



**Fig. 3.** Grouped Passing-Bablok regression plots for steroid quantification in serum using external calibration versus internal calibration. Blue lines indicate regression lines, grey dashed lines indicate identity lines, and the confidence bands for regression are delimited in light blue shaded areas.  $R^2$  is the determination coefficient, and CI corresponds to the confidence interval. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

EC, even at low biological concentration levels. Interestingly, the deuterium number on SIL have a limited impact on the quality of the results compared to their concentration and chemical purity. Indeed, lipophilicity increases with the number of deuterium atoms replaced, leading to small differences in chromatographic retention and MS ionization with the corresponding analyte [19]. However, the results obtained with highly deuterated SIL analogs as surrogate calibrants, such as etiocholanolone sulfate-d5 et androstenedione-d7, showed a remarkable agreement ( $R^2 > 0.99$ ) between the EC and IC approach.

Overall, the obtained results from both methodologies were in complete agreement and the measured concentrations using IC similar to those obtained with EC (Table 2). This is in accordance with previously published literature [32,52,53] and what was recently reported by Fanelli *et al.*, suggesting that calibration has a minor impact on overall analytical variability compared with other factors, such as sample preparation, matrix interference evaluation and SIL selection [39,40]. The IC approach can therefore be considered as the most powerful emerging methodological alternative when a matrix free of endogenous analytes is impossible, or difficult to obtain. Also, for multi-analyte quantification, IC presents the advantage of being significantly faster in routine analysis by eliminating the need to introduce an EC curve for each batch. This reduces instrument turnaround, the associated risk of analytical errors in the preparation of multiple calibrants, and the material required for their extraction. Altogether, the IC simplified calibration scheme can provide similar results to EC with an estimated time savings that can be expressed in hours [21]. According to the current level of purity that can be found with commercially available SILs, IC exhibited accurate results at the  $\text{ng}\cdot\text{mL}^{-1}$  and, in the best case, up to  $\text{pg}\cdot\text{mL}^{-1}$  level. Finally, IC is not limited to steroid bioanalysis and the

application is gaining interest for all other metabolites such as endocannabinoids [54], drugs [55,56] or peptides [57,58].

#### 4. Conclusion and perspectives

This work demonstrates that a calibration design conceptualized for a multi-level EC can be used retrospectively to obtain an IC by using the stable isotope-labeled (SIL) analogues as surrogate calibrants. To the best of our knowledge, this is the first study using the IC approach for the simultaneous quantification of an extended pattern of 21 analytes and demonstrating that this strategy is comparable to current practices such as EC procedures. The proposed approach is robust enough to allow the quantification of endogenous compounds with relatively low concentrations in complex biological samples by LC-MS/MS. Compared to the conventional EC approach, the IC requires only few additional steps during method development, such as the experimental investigation of the response factor and the concentration of stable isotope-labeled standard to be introduced in the sample. One potential drawback of using the IC approach is that it may be prone to bias at concentrations close to the LLOQ level, depending on the spiked concentration of the surrogate calibrant (SIL). Indeed, if the SIL concentration is set at a low level within the response function, there may be significant interference from the analyte at higher concentrations due to competitive ion suppression. Thus, the IC may have a limited range of optimal performance compared to traditional EC techniques. However, once the method has been developed, the one-point IC approach is significantly faster in routine, as preparation and analysis of a full set of calibration standards is no longer required, which roughly corresponds to 20% of the total time according to our experience. The IC quantification can reduce the



instrument turnaround time by allowing samples to be analyzed as they arrive, enabling LC-MS random access. Additionally, the possibility to associate different analyte-SIL combinations, when limited SIL are available for the compound of interest, the investigation of alternative surrogate calibrants can be carried out using structural analogs.

### 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

Data will be made available on request.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jchromb.2023.123778>.

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