STRATEGIES AND CHALLENGES IN METHOD DEVELOPMENT AND VALIDATION FOR THE ABSOLUTE QUANTIFICATION OF ENDOGENOUS BIOMARKER METABOLITES USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Metabolomics is a dynamically evolving field, with a major application in identifying biomarkers for drug development and personalized medicine. Numerous metabolomic studies have identified endogenous metabolites that, in principle, are eligible for translation to clinical practice. However, few metabolomic-derived biomarker candidates have been qualified by regulatory bodies for clinical applications. Such interruption in the biomarker qualification process can be largely attributed to various reasons including inappropriate study design and inadequate data to support the clinical utility of the biomarkers. In addition, the lack of robust assays for the routine quantification of candidate biomarkers has been suggested as a potential bottleneck in the biomarker qualification process. In fact, the nature of the endogenous metabolites precludes the application of the current validation guidelines for bioanalytical methods. As a result, there have been individual efforts in modifying existing guidelines and/or developing alternative approaches to facilitate method validation. In this review, three main challenges for method development and validation for endogenous metabolites are discussed, namely matrix effects evaluation, alternative analyte-free matrices, and the choice of internal standards (ISs). Some studies have modified the equations described by the European Medicines Agency for the evaluation of matrix effects. However, alternative strategies were also described; for instance, calibration curves can be generated in solvents and in biological samples and the slopes can be compared through ratios, relative standard deviation, or a modified Stufour suggested approaches while quantifying mainly endogenous metabolitesdent t-test. ISs, on the contrary, are diverse; in which seven different possible types, used in metabolomics-based studies, were identified in the literature. Each type has its advantages and limitations; however, isotope-labeled ISs and ISs created through isotope derivatization show superior performance. Finally, alternative matrices have been described and tested during method development and validation for the quantification of endogenous entities. These alternatives are discussed in detail, highlighting their advantages and shortcomings. The goal of this review is to compare, apprise, and debate current knowledge and practices in order to aid researchers and clinical scientists in developing robust assays needed during the qualification process of candidate metabolite biomarkers.

Keywords: *LC-MS/MS; endogenous biomarkers; metabolomics; validation; internal standards; blank matrix*

I. INTRODUCTION

Biomarkers such as genes, proteins, and metabolites play a pivotal role in the health care system guiding the decisions on disease diagnosis and prognosis as well as therapy management (Lee et al., 2005, 2006; Mamas et al., 2011; Beger & Colatsky, 2012). Indeed, intensive research in biomarker discovery at the preclinical and clinical stages is ongoing to achieve the goal of personalized medicine (Lee et al., 2005, 2006; Mamas et al., 2011; Beger & Colatsky, 2012). Metabolomics assesses all endogenous and exogenous (i.e., from the diet or the environment) metabolites within a biological system (Dettmer, Aronov, & Hammock, 2007; Beger & Colatsky, 2012). It has quickly demonstrated its promising potential in biomarker discovery (Dettmer et al., 2007; Griffiths et al., 2010; Dunn et al., 2011; Mamas et al., 2011). Metabolomics has the ultimate goal of linking metabolites from different biochemical pathways to normal, diseased, and stressed biological states (Dettmer et al., 2007; Mamas et al., 2011; Dunn et al., 2011). Unlike genomics, transcriptomics, or proteomics, metabolomics faces a unique challenge due to the diversity of the physicochemical properties of the metabolites (i.e., hydrophobicity/hydrophilicity, acidity/basicity, stability, and volatility) (Dettmer et al., 2007; Dunn et al., 2011).

Metabolomics uses untargeted and targeted approaches in the process of biomarker discovery (Dettmer et al., 2007; Dunn et al., 2011; Roberts et al., 2012; Mikami et al., 2012; Xiao et al., 2012; Gika et al., 2014). Nuclear magnetic resonance (^{1}H-NMR) or high-resolution mass spectrometers along with the appropriate databases are commonly employed in the early discovery stages of biomarker development where the goal is qualitative and/or semiquantitative measurements (Dettmer et al., 2007; Dunn et al., 2011; Roberts et al., 2012; Mikami et al., 2012; Xiao et al., 2012; Gika et al., 2014). Subsequently, targeted analysis is needed to accurately quantify and validate

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potential biomarker metabolites in the context of the disease state. This is usually achieved using instruments suited for quantitative application, such as triple quadrupole mass spectrometers operated in the multiple reaction monitoring (MRM) mode (Xiao et al., 2012; Roberts et al., 2012; Mikami et al., 2012). Regardless of the mass spectrometry (MS)-based metabolomics approach, chromatographic separation is usually needed to overcome matrix complexity and isobaric interferences (Mikami et al., 2012; Theodoridis et al., 2011; Xiao et al., 2012; Gika et al., 2014). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is one of the most prominent platforms in metabolomics (Dettmer et al., 2007; Mikami et al., 2012; Xiao et al., 2012; Gika et al., 2014). Targeted and untargeted platforms substantially differ in their experimental designs (Dunn et al., 2011; Khamis et al., 2015). In contrast with the targeted platform, metabolomic profiling (untargeted analysis) does not include any prior information regarding the composition of the sample (Dunn et al., 2011; Khamis et al., 2015). Untargeted analysis is of significantly lower accuracy, precision, and selectivity in comparison with the targeted platform. Additionally, untargeted analysis does not employ calibration curves due to technical difficulty attaining linearity, absence of authentic standards, or adequate internal standards (ISs) (Dunn et al., 2011; Khamis et al., 2015). For this reason, it is extremely important to verify the findings from an untargeted method via absolute metabolite quantification to avoid clinical testing of erroneous biomarkers. For instance, Sreekumar et al. (2009) reported the clinical utility of sarcosine as a potential biomarker of prostate cancer metastases through profiling more than 1126 metabolites in tissues, urine, and plasma using linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer. However, sarcosine, later quantified using LC-MS/MS and $^{2}H_{3}$-sarcosine as IS, did not significantly differ between healthy participants, patients with prostate cancer metastases, and patients with increased prostate surface antigen (Struys et al., 2010).

Biomarker discovery experiments can be conducted on any tissue or bodily fluid; however, they are, in particular, widely employed in blood and urine (Snowden, Dahlén and Wheelock 2012; Nobakht M. Gh et al., 2014). Blood reflects one of the earliest time points of catabolic and anabolic states. In contrast, urine represents an averaged picture of the entire body within a specific time period (Álvarez-Sánchez, Priego-Capote, & de Castro 2010). Their integrative use may lead to complementary information (Álvarez-Sánchez et al., 2010). Despite the challenges associated with its high salt content, urine remains an appealing biological fluid due to its high metabolite concentration and noninvasive nature of collection (Adamko et al., 2007; Nobakht M. Gh et al., 2014). Indeed, alterations in the urine metabolome have been investigated in cancer (Wu et al., 2009, Woo et al., 2009), respiratory diseases (Adamko et al., 2012, Adamko et al., 2015), radiation exposure (Tyburski et al., 2009), depression (Zheng et al., 2010), metal toxicity (Lafaye et al., 2003), type 2 diabetes (Menni et al., 2013), and other diseases (Griffin & Nicholls 2006; Beger et al., 2008; Jiang et al., 2011; Wang et al., 2012b). Due to its wide applications, urine-based examples are emphasized in this review in comparison with other biological fluids and tissue extracts. Unlike blood, urine metabolomics require the application of a normalization factor to account for inter- and intra-subject variations in volume (Warrack et al., 2009; Ryan et al., 2011; Wu & Li, 2012; Khamis et al., 2015). While various normalization techniques have been proposed, normalization to creatinine and osmolality remain the most commonly adopted techniques (Warrack et al., 2009; Ryan et al., 2011; Wu & Li, 2012; Khamis et al., 2015). Recently, we have demonstrated the comparable performance of either normalization strategy in the statistical model of a metabolomics dataset of 32 metabolites, acquired via LC-MS/MS validated methods and processed using partial least square discriminant analysis (Khamis et al., 2018b).

Despite the surge in biomarker discovery using metabolomics, few metabolites have been qualified as US-Food and Drug Administration (FDA)-approved clinical biomarkers (Beger and Colatsky 2012). The qualification of a biomarker aims at confirming the consistent association between the marker and the disease. It also expects an elucidation of the physiological, pharmacological, toxicological, or clinical significance of the test results (Hong et al., 2010; Beger & Colatsky, 2012). Several obstacles can hinder the qualification of biomarkers. For instance, bias, small sample size, small effect size, and inappropriate study design can impact the quality of the data that supports the clinical utility of the biomarker. Another potential obstacle is the lack of well-established analytical assays that can provide accurate, precise, and robust quantification data of the biomarker (Lee et al., 2003; Ioannidis, 2005; Lee et al., 2005; Lee et al., 2006; Beger & Colatsky, 2012). Available regulatory guidelines for bioanalytical method validation, such as those issued by the FDA and the European Medicines Agency (EMA) are primarily designated for xenobiotics, that is, active pharmaceutical ingredients, and their metabolites (Lee et al., 2003, 2005, 2006; EMA, 2011; US-FDA, 2018; ICH, 2019). In contrast, the endogenous presence of metabolites in a biological specimen can prevent the direct application of the established guidelines, rendering the validation process challenging and time consuming (Lee et al., 2003; 2005). In fact, absolute quantification of endogenous metabolites is largely impeded by the absence of analyte-free matrices, analytical standards as well as appropriate ISs (Lee et al., 2005). Consequently, there has been confusion on the type and extent of validation needed for the quantification of endogenous metabolites (Swanson 2002; Lee et al., 2003, 2006). A common consensus has been reached that the intended use of the data can guide the level of the analytical validation process (Lee et al., 2003, 2005, 2006; US-FDA, 2018; ICH, 2019). A "Fit-for-purpose" approach, meeting minimum performance characteristics, can be a good starting point for exploratory studies and would inherently require less validation in comparison with methods designed for clinical applications (Lee et al., 2003, 2005, 2006; US-FDA, 2018; ICH, 2019). In 2019, the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), has released draft guidelines for bioanalytical method validation (currently pending for consultation). Although a section was dedicated to endogenous metabolites absolute quantification (ICH, 2019), detailed discussions on challenges and limitations of the proposed approaches are still lacking.

This review discusses selected challenges that can impede the development and validation of LC-MS/MS methods intended for the absolute quantification of endogenous metabolites. It is not intended as a comprehensive guide that details all aspects of LC-MS/MS method validation for endogenous

metabolites. Alternatively, experimental design parameters that can differ significantly from xenobiotics analysis are included. For instance, an overview of the commonly adopted practices for ISs selection is provided. In addition, assessing matrix effects (MEs), in the context of endogenous metabolites, is described in detail. Emphasis is also placed on the challenges in method development and validation resulting from the absence of analyte-free matrices. Past and recent strategies used to overcome these difficulties are discussed. The reader will see that not all these approaches are applicable in every situation and that the innovation of the researcher along with the foundational understanding of the problem is often the solution to the issue.

II. MATRIX EFFECTS

MEs usually result from the coelution of nondetected metabolites, salts, or exogenous interferences introduced into the sample during sample preparation or chromatographic analysis (Annesley 2003; Mei et al., 2003). In addition, detected metabolites can contribute to ion suppression of each other as observed for nucleotides, for example (Fig. 1) (Klawitter et al., 2007). Eleven nucleotides were quantified in rat kidney extract in the single ion monitoring mode using a column switching (LC/LC)-MS technique. In order to confirm the ion suppression effects of nucleotides, the MEs were assessed through the post column infusion of $NADP^+$. In the absence of the matrix, a constant signal of $NADP^+$ was obtained. On the other hand, when the matrix was injected, negative peaks of $NADP^+$ were observed at the retention times of the eluting nucleotides (Fig. 1) (Klawitter et al., 2007).

In addition to sample complexity and the ratio of the metabolite to its surrounding matrix, MEs also vary with the employed ionization source, particularly when comparing the commonly used electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), which can easily be switched within the ionization chamber of modern MS instruments. It is well established that APCI is prone to less MEs in comparison to ESI. Further discussions on the mechanism of charged ion formation in APCI and ESI and its relation to MEs can be found in details in the literature (Annesley 2003; Matuszewski et al., 2003; Gosetti et al., 2010; Trufelli et al., 2011).

A major factor contributing to MEs is the physicochemical properties of the investigated analytes (Annesley, 2003; Gosetti et al., 2010; Trufelli et al., 2011). MEs during ESI-MS analysis was demonstrated using three drugs (caffeine, phenacetin, and a proprietary compound) that were extracted from plasma using four methods (Bonfiglio et al., 1999). Flow injection and post column infusion of the extracts demonstrated that caffeine, the most polar entity, was always the most affected by ion suppression, regardless of the extraction strategy. On the contrary, the most nonpolar compound was identified as the proprietary compound, and it suffered the least ion suppression effects (Bonfiglio et al., 1999). The authors concluded that the chemical nature of the compound may have a more profound effect on the extent of ion-suppression than the method of sample preparation itself.

The reader is advised to refer to other extensive reviews and general discussions on MEs in MS analysis, focusing primarily on exogenous compound analysis (Annesley, 2003; Matuszewski et al., 2003; Mei et al., 2003; Taylor, 2005; Niessen et al., 2006; Mei et al., 2003; Gosetti et al., 2010; Trufelli et al., 2011). In the following sections, however, the means for the evaluation of MEs and the minimization strategies for such effects during endogenous metabolites quantification are discussed.

A. Evaluation of MEs for Endogenous Metabolites

Evaluation of MEs is required by the FDA; however, the practical guidelines for such experiment are lacking (US-FDA, 2018). On the contrary, the M10 guidelines released by the ICH

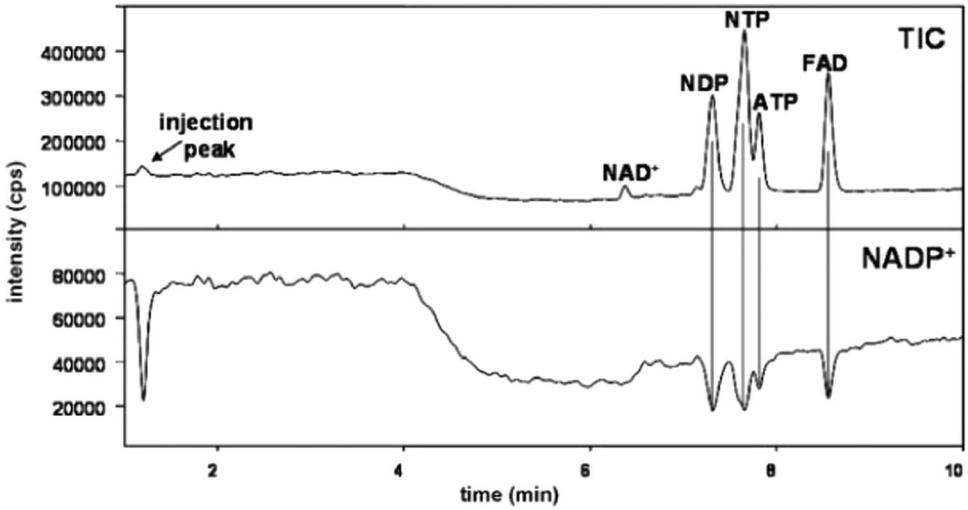

FIGURE 1. TIC of an LC/LC-MS method for the quantification of 11 nucleotides in rat kidney extract (top). Nucleotides have ion suppressive properties, which were investigated through the constant postcolumn infusion of $NADP^+$. In the absence of the extract matrix, a constant signal of $NADP^+$ was obtained (data not shown). The bottom panel shows the suppression of the $NADP^+$ signal through the formation of negative peaks that coincides with the elution of the ion suppressive nucleotides. Reprinted with permission (Klawitter et al., 2007, *Analytical Biochemistry)*. TIC, total ion count

TABLE 1. Internal standards used in the quantification of 18 bile acids in serum (Scherer et al., 2009).

Internal standard	Metabolites	Retention time
D_4-CA	Cholic acid	2.56
D_4-CDCA	Chenodeoxycholic acid	3.51
D_4-DCA	Deoxycholic acid	3.71
D_4-LCA	Lithocholic acid	4.5
	Taurolithocholic acid	4.35
D_4-UDCA	Ursodeoxycholic acid	1.92
	Hyodeoxycholic acid	2.15
	Tauroursodeoxycholicacid	1.83
	Taurohyodeoxycholic acid	2.08
D_4-GCA	Glycocholic acid	2.52
	Glycoursodeoxycholic acid	1.84
	Glycohyodeoxycholic	2.08
	Taurocholic acid	2.48
D_4-GCDCA	Glycochenodeoxycholic acid	3.44
	Glycodeoxycholic acid	3.65
	Glycolithocholic acid	4.41
	Taurochenodeoxycholic acid	3.38
	Taurodeoxycholic acid	3.61

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid.

in 2019 recommend the use of triplicates of low quality control (LQC) and high quality control (HQC) samples prepared from six sources (lots) for MEs evaluation for xenobiotics (ICH, 2019). The accuracy of the measurements should be within ±15% of the nominal values, whereas the precision is demonstrated as relative standard deviation (RSD%) values less than 15% (ICH, 2019). These guidelines can theoretically be extrapolated to endogenous metabolites; however, they require the initial analysis of the six matrix sources for the determination of the metabolites' endogenous levels. As such, substantial time and resources are needed for such additional analysis. In addition, these guidelines do not reveal if the effects of the matrix are consistent among each metabolite and its IS (ICH, 2019).

In contrast, the EMA recommends the use of six different lots of (nonpooled) blank matrices and the evaluation of MEs on LQC and HQC samples (EMA, 2011). The matrix factor (MF) for the metabolite or its IS is calculated according to Equation (1): $MF_{metabolite\ or\ IS}$ = Peak area in blank matrix/Peak area in neat solvent, while the IS-normalized MF is expressed as: $MF_{metabolite}/MF_{IS}$; Equation (2) (EMA, 2011). When the $MF_{analyte}$ is expressed as percentage, the term MEs (ME%) is commonly used (Gosetti et al., 2010). The EMA does not provide any acceptance criteria for MF values; however, the RSD% of the IS-normalized MF should not exceed 15% among the six evaluated lots (EMA, 2011). Neither the EMA nor the ICH guidelines differentiate between the type of the matrix investigated and the number of representative lots of samples (EMA, 2011, ICH, 2019).

The EMA recommendations, while clear, are not directly applicable in method development for endogenous metabolites due to the absence of analyte-free matrices. Accordingly, other approaches or modifications to the above equations have been introduced. The simplest approach has been reported as the generation of MF values for only the ISs. For example, Scherer et al. (2009) developed an LC-MS/MS method for the quantification of 18 endogenous bile acids in serum using seven ISs (Table 1). The ISs were assigned to the metabolites based on their retention time proximity. However, up to 1 min retention time difference was observed between glycolithocholic acid and its IS, d_4-glycochenodeoxycholic acid (Table 1). The MF values of the ISs were found to be suppressed by 25% in serum (Scherer et al., 2009). One major drawback of this approach is the assumption that similar MEs are experienced by the metabolites and their structurally related ISs despite their chromatographic elution at different times (Scherer et al., 2009). In fact, there are reports, demonstrating that an IS can be affected differently by the matrix than the analyte, even when they coelute. Such discrepancies can undermine the quantitative data (Jemal et al., 2003; Sancho et al., 2002). Indeed, such difference in ionization behavior can be completely overlooked if the MEs are only evaluated based on the signals from the ISs as described by Scherer et al. (2009).

Another approach is the correction of endogenous levels prior to data processing by Equations (1) and (2) (Liu et al., 2013; EMA, 2011; Hényková et al., 2016; ICH, 2019). This approach was adopted by Hényková et al. (Hényková et al., 2016) during the analysis of tryptophan and 16 of its related metabolites in serum and cerebrospinal fluid (CSF). Unfortunately, the authors did not clearly specify the means for endogenous levels correction, which is presumably through the subtraction of peak areas of endogenous metabolites from spiked samples. Interestingly, the authors found that the signals of the metabolites in six individuals suffered from extreme matrix suppression (ME% > 15.6%) and enhancement (ME % < 414.4%) effects (Hényková et al., 2016). The RSD% values of the MF were also very high, reaching up to 52% in tryptamine (Hényková et al., 2016). It was only after the

incorporation of 17 isotopically labeled ISs that such effects were corrected and the RSD% values of the IS-normalized MF were reduced to below 15% (Hényková et al., 2016).

Unlike Hényková et al. (2016), other studies have detailed the modifications introduced to the pre-existing EMA equations (Wang et al., 2012a; Liu et al., 2013; Ghassabian et al., 2014). For instance, the evaluation of MEs on 1-methylhistidine and 3-methylhistidine was conducted by dividing six sources of urine into three groups, each prepared in five replicates (Wang et al., 2012a). Group (A) was the "blank urine" samples containing the endogenous levels of the metabolites; group (B) was the "blank urine" samples spiked with standards at 3× the lower limit of quantification (LLOQ); and (C) was neat solvent spiked with standards at 3× LLOQ. All groups were spiked with equal concentration of d_3-3-methylhistidine as IS (Wang et al., 2012a). The ME% for the metabolites were calculated using peak areas according to Equation (3): ME% = [(peak area B − peak area A)/peak area C] × 100. The IS-normalized ME, on the other hand, was calculated as per the EMA guidelines (Equation (2)) (EMA, 2011). This approach, despite being promising had a critical drawback concerning the number of samples need to be prepared. Despite being only assessed at the LQC level, it required a total of 60 matrix containing injections (group A: six sources in five replicates and group B: six sources in five replicates), as well as adequate replicates of equivalent standards in neat solvent (i.e., total of 90 injections). In our perspective, the workload in this experiment can be substantially reduced by decreasing the number of replicates while maintaining the number of different matrix lots to a minimum of six. In such manner, the recommendations of the EMA are still being followed (EMA, 2011).

On the contrary, Lv et al. (2015) described Equation (4) for the assessment of MEs for 14 urinary Alzheimer's disease catecholamine biomarkers, in which ME% = ([(response ratio in spiked blank matrix − response ratio in blank matrix)/response ratio in equivalent standard solution] × 100). Unlike Equation (3), in which the IS was not factored in the calculations, the response ratio in Equation (4) is calculated as: metabolite peak area/IS peak area, and it is equivalent to IS normalization. Despite the simplicity of assessment, this technique does not provide any advantage over the method described by Hényková et al. (2016) in terms of the experimental workload (Lv et al., 2015). The significance of Equation (4) is that it directly generates IS-normalized MF values, from which the RSD% can be calculated (Lv et al., 2015), thus providing further simplification to the current EMA guidelines (EMA, 2011). From our own experience, we, however, recommend the need to evaluate both MF and IS-normalized MF, individually, for the metabolite and its IS, specifically during method development. The information provided by these calculations can critically assess the suitability of the selected ISs, sample cleanup procedure, and chromatographic separation.

The EMA-derived equations are not the only approach for the evaluation of MEs. An alternative method is to compare the slope of the calibration curve for a metabolite standard prepared in a neat solvent versus that prepared in the biological matrix via standard addition (Gosetti et al., 2010). The comparison can be achieved either through a modified Student *t*-test or through the generation of slopes ratio. Stanislaus et al. (2012) quantified 18 acylglcyine (potential biomarkers of inborn errors of metabolism) in human urine through precolumn derivatization with $^{12}C_2/^{13}C_2$-*p*-dimethylaminophenacyl bromide reagent. Calibration curves were generated by spiking derivatized standards into neat solvent as well as into underivatized and derivatized urine to assess MEs. In addition, the approach allowed for the assessment of the suitability of underivatized urine in replacing the derivatized matrix. The slopes were compared using Equation (5): modified student $t\text{-test} = (b_1 - b_2)/S_{b1-b2}$. *Where b is the slope, the subscripts refer to the two regression lines being compared.* S_{b1-b2} *is the standard error of the difference between regression coefficients and it is calculated as* $= [(S^2_{y.x})_p/(\Sigma x^2)_1 + (S^2_{y.x})_p/(\Sigma x^2)_2]^{1/2}$, *where* $(S^2_{y.x})_p$ *is the pooled residual mean square* $=$ *(residual SS)*$_1$ *+ (residual SS)*$_2$*/(residual DF)*$_1$ *+ (residual DF)*$_2$, *SS is the sum of squares and DF is the degrees of freedom.* No significant difference was observed between the underivatized and derivatized urine. Accordingly, the former matrix was deemed appropriate to substitute derivatized urine in the preparation of validation samples (Stanislaus et al., 2012).

Another simpler alternative is comparing the slopes generated in the biological matrix and neat solvent via ratio generation (Flores, Hellín, & Fenoll 2012; Joyce et al., 2016). While a *t*-value can be used to accept or reject the null hypothesis (Stanislaus et al., 2012), a range of acceptable ratio values, at the discretion of the investigators, can be set prior to MEs evaluation. For example, a slope ratio ranging from 0.9 to 1.1 was used to indicate negligible matrix interferences during the quantification of 18 amino acids in urine (Joyce et al., 2016). In contrast, a range of 0.82 to 1.22 was reported by another research team to indicate negligible matrix interferences from urine, feces, and serum (Hou et al., 2016).

While a pre-set range of ratio values is acceptable, the assessment of MEs has also been reported using the RSD% of the slope ratio (Matuszewski et al., 2003). This technique requires preparation of multiple calibration standards (at least three) in both neat solvent and biological fluid for the generation of statistically meaningful mean, standard deviation, and RSD% values. This technique was used for a synthetic representative compound and its structurally related IS, in which a 7-point calibration set was prepared in neat solvent ($n = 5$) as well as in five different lots of plasma (Matuszewski et al., 2003). The main purpose of this work was the investigation of MEs in relation to the ionization source. Using a heated nebulizer as the ion interface, the variability of the slopes in plasma was relatively small (Fig. 2) and insignificantly different from that calculated from neat solvent (RSD%; 3.5% plasma and 1.3% solvent) (Matuszewski et al., 2003). In contrast, when ion spray (ISP) was employed, significant MEs were observed as the RSD% of the slopes varied; 0.9% and 14.9%, in solvent and plasma, respectively (Fig. 2) (Matuszewski et al., 2003). The authors attributed this observation to the higher susceptibility of ISP ionization to MEs in comparison with the heated nebulizer. This conclusion was further confirmed by the reduction of the RSD% values from 14.9% to 2.4%, when the calibration curves were constructed using a single plasma lot (i.e., similar MEs in replicates from the same lot) (Matuszewski et al., 2003).

Overall, each approach has its advantages and drawbacks. ICH guidelines do not assess the individual performance of the metabolite and its IS. Modifications to the EMA equations demonstrate the closest assessment tool to regulatory guidelines

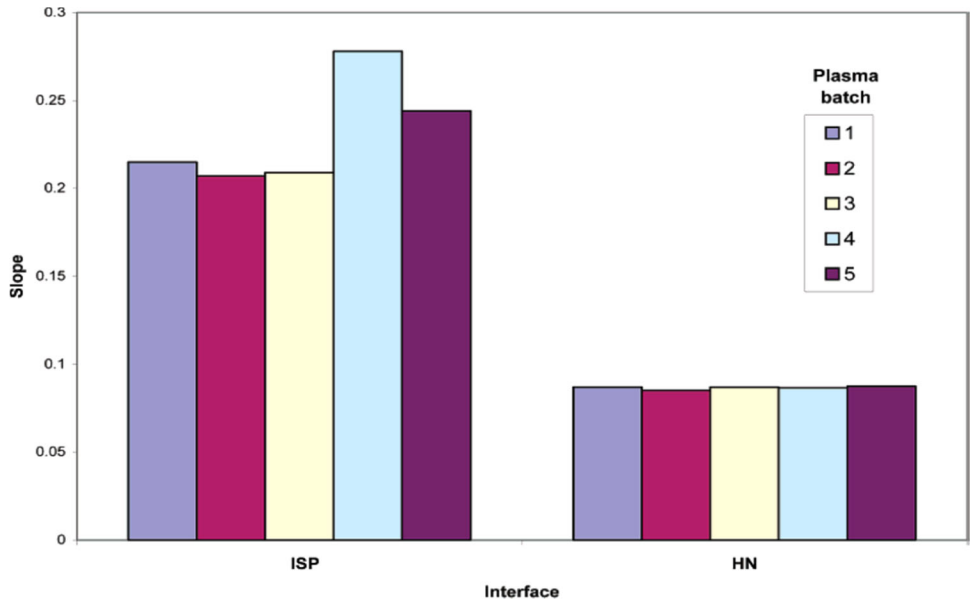

FIGURE 2. Comparison of the slopes of the standard curve of a tetracyclic-based compound prepared in five different lots of plasma and analyzed with high-performance liquid chromatography interfaced to ion spray (ISP) and heated nebulizer (HN). Reprinted with permission (Matuszewski et al., 2003, *Analytical Chemistry*). [Color figure can be viewed at wileyonlinelibrary.com]

and is potentially the simplest strategy. The slope ratio approach, despite its promising potential, is relatively new and not widely applied with no agreed-upon acceptable values. Finally, using RSD% of the slope ratio is more time- and resource-consuming, since multiple calibration sets are needed to be prepared in different lots of the same biological fluid. Researchers are advised to consider all these factors before adopting a method to assess MEs. However, we recommend the method described by Hényková et al. (2016) because it provides detailed information on the effect of the matrix that is essential during method development. In our own work, we adopted this approach as it represents the closest variant to the equations described by the EMA guidelines (Khamis et al., 2018a).

B. Minimization of MEs

In general, MEs are reduced at four main stages: (a) sample preparation, (b) chromatographic separation, (c) MS analysis, or (d) calibration strategy via standard addition (discussed in details in Section IV.B.C) (Annesley 2003; Matuszewski et al., 2003; Gosetti et al., 2010; Trufelli et al., 2011). Table 2 summarizes the different strategies that can be employed at each stage of sample analysis. While all four areas are important, the selection of an appropriate IS is a cornerstone for the control of MEs. Accordingly, this topic will be discussed as a separate section in details. The following sections contain selected studies, that corrected MEs via one of the above four suggested approaches while quantifying mainly endogenous metabolites.

Reducing MEs at the sample preparation stage can be achieved through sample dilution or extraction. However, loss of sensitivity due to dilution is expected and such an approach is used only for metabolites with high concentrations. For example, 39 mycotoxins of diverse polarity from wheat and maize kernels were compared (Sulyok et al., 2006). The MEs in maize extract were more intense than those observed in wheat (e.g., the signal of aflatoxin B_1 was 75% suppressed compared to 4% in maize) (Sulyok et al., 2006). Tenfold dilution of the extracts was attempted with a reduction in the overall MEs, including aflatoxin B_1 (66% signal suppression). This dilution, however, compromised the sensitivity needed for quantification and accordingly 1:1 dilution was finally adopted (Sulyok et al., 2006).

Sample extraction can also reduce MEs. It is, however, hampered by the diversity in the physicochemical properties of the investigated metabolites and/or by the similarity in their physicochemical properties with the coextracted impurities (Sulyok et al., 2006; Trufelli et al., 2011; Hényková et al., 2016). In fact, such challenges can render a selective extraction method unattainable (Ito & Tsukada, 2002; Sulyok et al., 2006; Hényková et al., 2016). For instance, only protein precipitation with methanol was employed for the extraction of 17 tryptophan-related metabolites from CSF and serum (Hényková et al., 2016). The authors acknowledged the lack of extraction selectivity due to the physicochemical diversity of the investigated entities and accordingly, appropriate ISs were included to account for interferences from the matrix (Hényková et al., 2016). In another example, four toxins that are biomarkers of diarrhetic shellfish poisoning were quantified in scallop tissues using LC-MS (Ito & Tsukada 2002). The scallop hepatopancreas was subjected to liquid-liquid extraction and the MS responses of the toxins in the extract were 19%–42% lower than those in neat solutions. Changing the extraction solvent was not successful, especially with the observed vast variation in matrix complexity between samples (Ito & Tsukada 2002). However, unlike the quantification of tryptophan-related metabolites (Hényková et al., 2016), appropriate ISs were not available. As such, the single point standard addition technique was adopted, which requires the injection of the sample with

TABLE 2. Summary of potential matrix effect minimization strategies during endogenous metabolites quantification.

Sample analysis step	Approach to minimize matrix effects	Selected references
Sample preparation	Sample dilution	Sulyok et al. (2006); Trufelli et al. (2011); Hényková et al. (2016)
	Modification of liquid-liquid extraction	
	Application of protein precipitation	
	Modification/application of solid phase extraction	
	Application of ultrafiltration	
	Application of microdialysis	
Chromatographic analysis	Reduction of the injection volume	Sancho et al. (2002); Annesley (2003); Scherer et al. (2009); Trufelli et al. (2011)
	Using a column switching technique	
	Changing the stationary phase technology, e.g. HILIC	
	Modification in the chromatography elution program	
	Modification in mobile phase composition/ additives	
	Reduction in stationary phase particle size, e.g. UPLC	
	Reduction in flow rate, e.g. nano-HPLC	
Mass spectrometric analysis	Changing ionization source to APCI or APPI	Trufelli et al. (2011), Matuszewski et al. (2003)
	Changing ionization mode into negative ion mode	
Calibration strategy	Application of standard addition	Ito and Tsukada (2002); Trufelli et al. (2011)
	Addition of isotope internal standards	

APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; UPLC, ultra high performance liquid chromatography.

and without a known spiked concentration of the standards (Ito & Tsukada 2002).

The next level to correct MEs is through the chromatographic system. LC/LC-MS/MS was used to assess MEs in urine during the quantification of 4-nitrophenol and 3-methyl-4-nitrophenol (Sancho et al., 2002), in which the effluent from the first column (C_{18}) was directed towards a second (alkylamide) column for further separation. Improved MEs (88%–104%) was observed in comparison to the conventional LC-MS/MS platform. The latter technique suffered from extensive ion suppression in the external calibration mode (≥29% for 4-nitrophenol and ≥38% for 3-methyl-4-nitrophenol) and extensive ion enhancement while using d_4-4-nitrophenol as IS for 3-methyl-4-nitrophenol (≤203%). This reduction in MEs is attributed to enhanced method selectivity with a second column of a different analyte-stationary phase interaction (Sancho et al., 2002). While this example discusses exogenous compounds, it highlights the potential utility of LC/LC in overcoming MEs. However, such improvement could be at the expense of analysis time. Reducing the stationary phase particle size offers another valuable modification strategy in the chromatographic system. It results in improved separation of the metabolites of interest with the simultaneous reduction in MEs (Scherer et al., 2009). This technique was adopted during the quantification of 18 bile acids in serum, in which the change in the particle size from 2.5 to 1.8 μm resulted in a twofold reduction of MEs (Scherer et al., 2009). Changes in MS analysis is another strategy to control MEs, and it is applicable to both exogenous and endogenous compounds, in which the ionization source is changed from ESI to APCI (Matuszewski et al., 2003). Further discussions on this topic are beyond the scope of this review.

III. ISs IN TARGETED METABOLOMICS

An IS accounts not only for analyte loss during sample preparation, but also for the errors prompted by the analytical platform that can eventually jeopardize the robustness of quantification (i.e., chromatography or MS-related) (Stokvis et al., 2005; Nilsson & Eklund, 2007; Tan et al., 2009; Hewavitharana, 2011; Xu & Madden, 2012). Accordingly, the use of chemical entities with identical or similar physicochemical properties to ISs becomes inevitable (Nilsson & Eklund, 2007; Hewavitharana 2011; Xu & Madden, 2012). Figure 3 compiles the different types of ISs that can be available for metabolomics application.

A. ISs Selection

1. Stable Isotope Internal Standard (SI-IS)

SI-IS is an isotopic form of the analyte of interest usually bearing deuterium (^{2}H), ^{13}C, or ^{15}N (Stokvis et al., 2005; Harwood & Handelsman, 2009; Berg & Strand, 2011; Hewavitharana, 2011; Xu & Madden, 2012; Bueschl et al., 2013) (Fig. 3). SI-ISs have been extensively used in quantitative metabolomics to correct for MEs that can suppress the ionization of the metabolite by up to 85% (Hényková et al., 2016). Despite their wide use, deuterated ISs may not coelute with their analytes due to differences in lipophilicity (Turowski et al., 2003; Wang et al., 2007; Hewavitharana, 2011). This phenomenon, known as the "deuterium effect" can be detrimental to quantitative analysis. For example, a 0.02 min difference between *S*-carvedilol and its d_5-IS resulted in an inadequate correction of MEs in a specific lot of human plasma (Wang et al., 2007). In contrast, ^{13}C- and ^{12}C-atoms exhibit identical physicochemical properties and hence, similar separation behavior during chromatographic analysis (Berg & Strand, 2011). Deuterium exchange is another drawback and is exclusive to deuterated ISs where the deuterium bound to nitrogen or oxygen atom can be instantaneously interchanged with hydrogen during ionization (Bueschl et al., 2013). The rare exchange of deuterium attached to a carbon atom has been also reported (Chavez-Eng et al., 2002). While there are few commercial sources that provide a wide range of isotopic products (C/D/N Isotopes Inc., Cambridge Isotope Laboroatories, Inc. and Toronto Research Chemicals, Inc.), in-house synthesis is quite common due to the unavailability of the desired product or due to its high cost (Lei et al., 2011).

2. Nonisotopic Structural Analogue

Structural analogues or compounds exhibiting similar psychochemical properties to the metabolites of interest (Fig. 3) (Draisci et al., 1998; Lee et al., 2004; Liu et al., 2013) represent an inferior alternative to SI-ISs (Tan et al., 2009). For instance, 14 potential biomarkers of hepatic cancer including spermidine and lysine were quantified in human urine and plasma with the use of only one structural analogue as IS, namely 1, 6-diaminohexane (Liu et al., 2013). All metabolites eluted within 5 min, where the IS demonstrated a centered retention time in between the metabolites, and while the linearity and precision of the method were statistically acceptable, the data for accuracy was not clearly presented (Liu et al., 2013). Similarly, an LC-MS/MS method was developed where isocytosine, eluting at around 2.5 min, was used as an IS for

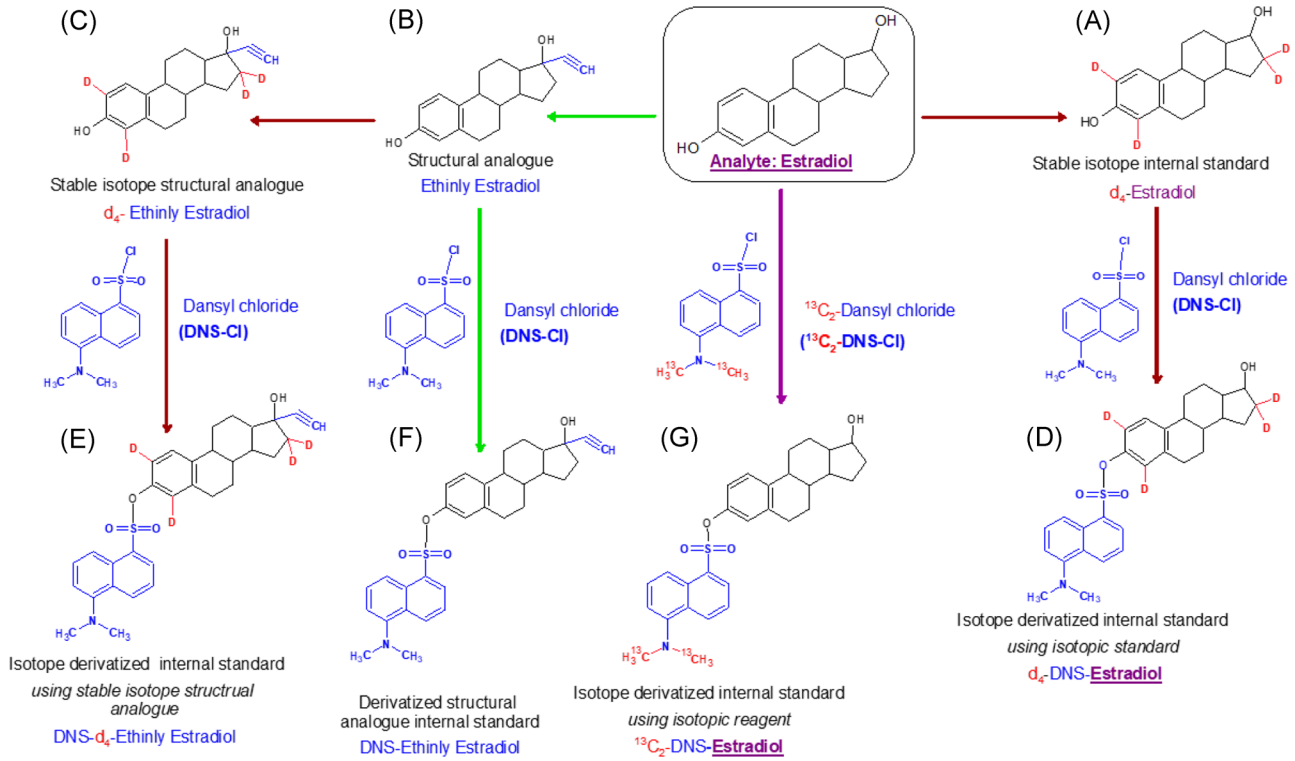

FIGURE 3. ISs in a metabolomic workflow involving endogenous metabolites. Estradiol is used as a representative structure. ISs can be (**A**) an isotopic form of the metabolite; (**B**) a nonisotopic structural analogue; (**C**) isotopic structural analogue; **D**, **F**, and **E** internal standards produced by the derivatization of **A**, **B**, and **C**, respectively; (**G**) the derivatized product of the metabolite of interest. All forms except (**G**), were used for the actual quantification of estradiol or related estrogens (Draisci et al., 1998; Nelson et al., 2004; Xia et al., 2004; Qin et al., 2008; Falk et al., 2008; Harwood & Handelsman 2009). IS, internal standard. [Color figure can be viewed at wileyonlinelibrary.com]

the quantification of 13 potential urinary biomarker nucleosides that eluted over an 18 min time frame (Lee et al., 2004). The nucleosides were injected at an extremely high volume (50 μL) and separated on 150 mm × 2.1 mm ID C_{18} column at a flow rate of 0.2 mL/min (Lee et al., 2004). Despite the large volume of injection and simple sample preparation from urine, the authors reported no MEs. In addition, the linearity range for the majority of the nucleosides extended over a 1,000-fold, but the weighing factor used for calibration was not reported (Lee et al., 2004). Furthermore, no sample stability studies were conducted. Finally, the authors only included the range of values for the calculated RSD% to support high method reproducibility despite the marginally acceptable results (14.92%) (Lee et al., 2004).

From our own experience, structural analogues may be more challenging for the optimization/validation of an analytical method. In our lab, we used two structural analogues to quantify four phytosterols and four tocopherols in canola extract using an LC-APCI-MS/MS method (manuscript in preparation). Ractocol; one of the two ISs, was consistently failing the LQC and LLOQ of two tocopherols due to the fluctuations in its signal. Acceptable LQC and LLOQ values were only obtained after changing the quantification equation from linear to quadratic. In general, we highly recommend extreme caution if structural analogues are selected as ISs during method development. Their usage should preferably be avoided if other isotopically labeled ISs are available.

3. Stable Isotope Structural Analogue (SI Structural Analogue)

In order to overcome cost and availability challenges with multiple SI-ISs, a single SI-IS can be used for the quantification of closely eluting metabolites and can serve as a SI-structural analogue (Fig. 3C) (Harwood & Handelsman, 2009; Klepacki et al., 2015). For example, in the protocol described by Klepacki et al. (2015), 10 potential biomarkers of kidney dysfunction of high polarity and low molecular weight were quantified in urine. Metabolites were divided into five groups, based on their retention times on a hydrophilic interaction liquid chromatography (HILIC) column. An IS was assigned to each group as follows: glucose, oxoglutarate, sorbitol (d_6-glucose), TMAO (d_9-TMAO), creatinine (d_3-creatinine), lactic acid, uric acid, hippuric acid (d_5-hippuric acid) and citrate, and succinate (d_4-succinate). While some metabolites were quantified against their SI-ISs, others, such as lactic acid and uric acid were quantified against structurally irrelevant ISs with similar retention times. Although all metabolites eluted over a 1 min timeframe, the authors did not discuss the rationale towards the selection of the ISs and whether the use of fewer ISs compromised method robustness (Klepacki et al., 2015). However, acceptable accuracy and precision as well as low MEs were demonstrated from the fully validated HILIC-MS/MS method (Klepacki et al., 2015).

Similarly, isotopically labeled glutamine was used in the quantification of a set of six metabolites in urine that are of a biomarker importance in prostate cancer (Jiang et al., 2010). Interestingly, glutamine was not a potential candidate in this study. The authors did not specify the isotopic form of glutamine (^{13}C- or d-containing), nor did they discuss the rationale behind the choice of this IS. Accordingly, it is not clear whether other SI-ISs were not commercially available at the time the study was conducted, or interferences imposed by other SI-ISs dictated the choice of glutamine. Despite the high precision of the method (RSD% less than 15%), the authors adopted a wider acceptance range for accuracy (80%–120%) (Jiang et al., 2010) than the recommended criteria of 85%–115% by regulatory guidelines (EMA, 2011; US-FDA, 2018; ICH, 2019). Despite adopting a wider range, the accuracy of creatinine still failed at the LQC (130%). Furthermore, the LLOQ was not included in the validation of accuracy and precision of the method for all six metabolites and its acceptance criteria in the calibration curve were not also specified, typically 20% as per different regulatory guidelines (EMA, 2011; US-FDA, 2018; ICH, 2019). Errors prompted by retention time differences between isotopically labeled glutamine and the investigated metabolites could be a potential reason for adopting higher acceptance limits for accuracy; however, no justification was provided by the authors (Jiang et al., 2010).

4. Isotope-Derivatized Internal Standards (ID-IS)

Another variant of IS is called ID-IS. An ID-IS is created by a precolumn derivatization reaction that targets a specific functional group within a molecule (e.g., amino, carboxylic, and phenolic groups). In general, chemical derivatization provides various advantages in the analysis of complex samples using LC-MS/MS. Derivatization alters the physicochemical properties of small molecule metabolites resulting in significant improvements in their chromatographic separation and mass-spectrometric analysis. Most importantly, it converts the molecules into derivatized entities of exogenous nature (Guo & Li, 2009; Xu et al., 2011; Toyo'oka 2012; Bruheim et al., 2013).

ID-IS can be created by having isotopic atoms located in the structure of either the IS (Fig. 3D and E) or the derivatizing reagent (Fig. 3F). When isotope-containing ISs are available (i.e., SI-ISs or SI-structural analogues), a nonisotopic derivatizing reagent can be used (Fig. 3D and E) (Shou et al., 2004; Falk et al., 2008; Qin et al., 2008; Wilson et al., 2011; Lee et al., 2012; Tamae et al., 2013). This technique has allowed for the analysis of trace level metabolites in oral fluids for forensic application (Lee et al., 2012). However, its most common application has been in hormone analysis (Shou et al., 2004; Falk et al., 2008; Tamae et al., 2013; Qin et al., 2008; Nelson et al., 2004), allowing simpler sample preparation steps in comparison to other quantitative methods (Adlercreutz et al., 2004). For instance, 15 endogenous estrogen metabolites were reacted with dansyl chloride and 5 ID-IS were created by reacting 5 SI-ISs with the same derivatizing reagent; a validated LC-MS/MS method was successfully developed (Falk et al., 2008). The improved sensitivity allowed for the quantification of the target metabolites in pre- and postmenopausal women and in men using only 0.5 mL urine (Falk et al., 2008). A nonisotopic structural analogue IS can be also combined with nonisotopic derivatizing reagent yielding a nonisotopic product (Fig. 3F); however, this technique is not widely adopted in MS-based studies. Xia et al. (Xia et al., 2004) reported the use of ethinyl estradiol in the quantification of estrone in mouse plasma and brain tissue using LC-MS/MS after dansylation.

The other approach for generating ID-ISs is through the use of an isotopically labeled version of the derivatizing reagent (Fig. 3G) (Guo & Li, 2009; Toyo'oka 2012; Khamis et al., 2015). Through this approach, an IS is produced for every metabolite and a peak pair is observed; thus, providing further confirmation of identity (Toyo'oka, 2012; Guo & Li, 2009; Khamis et al., 2015). This technique has been pioneered by Dr. Li's group at the University of Alberta, Canada. It has been gaining momentum within the metabolomics community with the introduction of isotopic reagents with different chemical reactivity (Guo & Li, 2009; Toyo'oka, 2012; Bruheim et al., 2013; Khamis et al., 2015). For example, dansyl chloride coded by $^{12}C_2/^{13}C_2$-dimethyl amino group was used for the identification and relative quantification of amines (Guo & Li, 2009), phenols (Guo & Li, 2009), and recently alcohols (Zhao, Luo, & Li, 2016). $^{12}C_2/^{13}C_2$-*p*-dimethylaminophenacyl bromide has been used for acids (Stanislaus et al., 2012; Peng & Li, 2013; Awad et al., 2019), while d_7-bromoacetonylquinolinium bromide has been used for thiols (Liu et al., 2014; Liu et al., 2016).

Despite the potential of ID-ISs, their application for absolute quantification of endogenous metabolites is increasing at a slow pace (Stanislaus et al., 2012; Khamis et al., 2017; Awad et al., 2019). Possible reasons include the additional derivatization work load needed during method development and validation, in comparison to nonderivatization (direct) methods, and the vigilance needed to avoid potential metabolite loss during the extra steps of sample handling (Khamis et al., 2017; Khamis et al., 2018c). On the contrary, the analytical improvements introduced by ID-ISs can outweigh the associated workload. For example, they can aid in the unequivocal identification of the metabolite from closely eluting isobaric interferences (Guo & Li, 2009; Khamis et al., 2017). In an LC-MS/MS method for the quantification of dansyl-isoleucine, a potential biomarker of asthma and chronic obstructive pulmonary disease (COPD) (Khamis et al., 2017), the analysis of derivatized urine revealed several chromatographic peaks, two of which were eluted at 0.25 min window from dansyl-isoleucine (Fig. 4A). Both quantifier and qualifier MRM transitions could not distinguish between the isomers (Fig. 4B). In fact, the human metabolome database revealed 10 detected and quantified urine metabolites with a nominal mass of 131 Da that can react with dansyl chloride, similar to isoleucine. Six of these metabolites are positional isomers of isoleucine (Wishart et al., 2009; Wishart et al., 2012), which are expected to closely elute during chromatographic analysis.

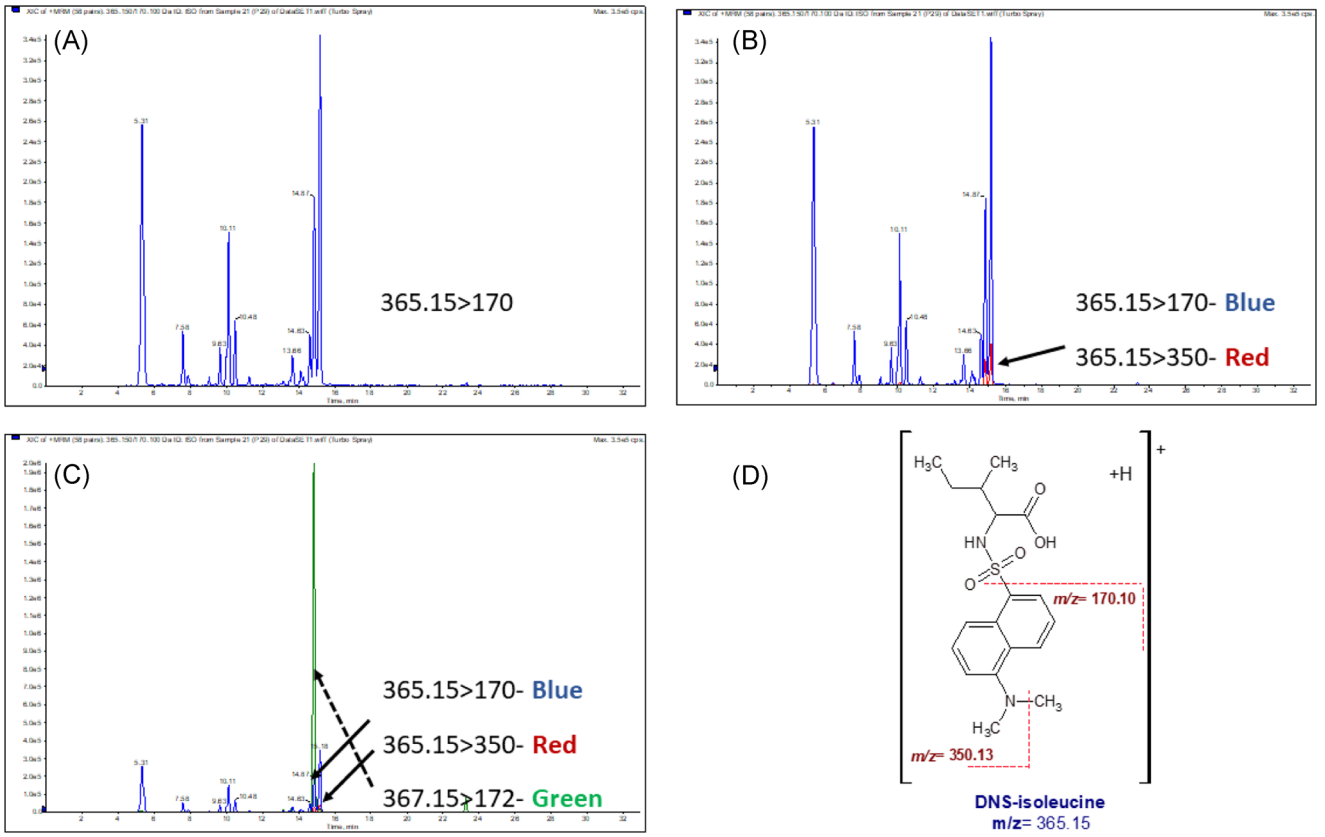

FIGURE 4. Identification of dansylated-isoleucine in asthma patient urine sample in the presence of interfering coderivatized metabolites, where (**A**) is XIC of 365.15 > 170 (quantifier transition); (**B**) is XIC of 365.15 > 170 and 365.15 > 350 (quantifier and qualifier transitions); (**C**) is XIC of 365.15 > 170, 365.15 > 350 (quantifier and qualifier transitions) and 367.15 > 172 ($^{13}C_2$-dansylated-isoleucine as internal standard); and (**D**) is the structure of dansylated-isoleucine and its product ions. Reprinted with permission (Khamis et al., 2017, *Analtyica Chimica Acta*). XIC, extracted ion chromatogram. [Color figure can be viewed at wileyonlinelibrary.com]

However, with the use of $^{13}C_2$-dansyl-isoleucine IS, only 1 peak pair was formed due to the coelution $^{12}C_2/^{13}C_2$-dansyl-isoleucine (Fig. 4C) (Khamis et al., 2017).

B. Challenges and Practices for IS Optimization

1. IS Purity and Signal Stability

The assessment of IS purity is a necessity as isotopic forms are expected to contain traces of the nonisotopic analyte due to similar synthetic routes or reagent impurities (Stokvis et al., 2005; Xu & Madden, 2012). While the error prompted by impure IS is constant, its accepted criterion is not well-defined in the FDA, the EMA or the ICH guidelines (EMA, 2011; US-FDA, 2018; ICH, 2019). The selectivity assessment for the analyte indicates that interference observed in blank matrix at the analyte channel should be less than 20% of the LLOQ (EMA, 2011; US-FDA, 2018; ICH, 2019). Accordingly, the starting practice to evaluate the purity of the IS is to spike the IS into an analyte-free matrix used for sample preparation. The interference observed at the analyte channel should be integrated and compared to the LLOQ (Xu & Madden, 2012). If higher purity of IS is not applicable, the concentration of the IS can be adjusted accordingly to give an interference of less than 20% by reducing its concentration (Xu & Madden, 2012).

A second possible source of interference is related to the inconsistency of the IS signal due to the surrounding matrix. A solid phase extraction (SPE)-LC-MS/MS method was developed and validated for the quantification of piperaquine in plasma, while d_6- piperaquine was used as a SI-IS (Lindegardh et al., 2008). The extraction procedure involved the addition of 19 μL triethylamine and the evaporation of the solvent under a gentle stream of air in a water bath at 70°C (Lindegardh et al., 2008). It was found that triethylamine residue from incomplete evaporation suppressed the ionization of piperaquine and d_6-piperaquine differently, resulting in quantification error of up to 50% (Lindegardh et al., 2008). In order to overcome this problem, the samples were allowed to dry for an additional hour under the same conditions after they were visibly dry. The authors attributed this finding to the slight differences in lipophilicity between the analyte and its IS, resulting in the coelution of the analyte only with traces of the ion suppressive triethyamine residue (Lindegardh et al., 2008). Similarly, in the area of cholesterol drug development, Jemal et al. (2003) reported that d_7-mevalonic acid, the SI-IS for mevalonic acid experienced a different extent of MEs with the change of the urine batch (Jemal et al., 2003). The conclusion was that similar behavior is likely to occur with other ISs in different assays, and therefore, proper attention should be paid to MEs evaluation on both the analytes and their ISs during method development (Jemal et al., 2003).

In a more comprehensive evaluation of the challenges associated with the use of ISs, Tan et al. (2009) discussed 12 different analytical troubleshooting issues, in which the ISs varied unexpectedly in their intensities while being used in various validated bioanalytical methods (Tan et al., 2009). Causes such as procedural, instrumental or human errors were suggested in addition to MEs. It was difficult to provide a clear common conclusion of whether the variations in the intensity of the ISs could dramatically impact quantification or not (Tan et al., 2009). However, the use of ISs that coelute with their analytes (i.e., ^{13}C-analogues) was highly recommended (Tan et al., 2009).

In summary, although the IS's signal instability can be detrimental in LC-MS/MS quantitative metabolomics, the use of SI-IS or ID-IS, specifically ^{13}C-containing, remains the best currently available strategy to correct for MEs or other variations during analysis. Nonetheless, it remains crucial to monitor the signal of the IS during routine sample analysis and identify any red flags (Jemal et al., 2003; Lindegardh et al., 2008; Tan et al., 2009; Xu & Madden, 2012).

2. Concentration of the IS

In general, neither the FDA, the EMA, nor the ICH specify a recommended concentration of the IS in the calibration standards relative to its analyte (EMA, 2011; US-FDA, 2018; ICH, 2019). A common practice is to spike the SI-ISs or ID-ISs at concentrations within the linear range of their respective metabolites (Xu and Madden 2012; Khamis et al., 2017). However, optimization of the IS concentration remains fundamental, especially when quantification errors can be prompted by the ion suppression/enhancement effects induced by the IS or the metabolites. For instance, Liang et al. (2003) demonstrated that the signals from proprietary drugs in pure solvents during ESI and APCI were suppressed up to 88% upon the addition of the ISs. This is detrimental to trace metabolite analysis leading to higher LLOQ and loss of sensitivity (Sojo et al., 2003; Remane et al., 2010). Accordingly, lower concentrations of IS should be endorsed (Xu and Madden 2012). On the contrary, Remane et al. (2010) found that the higher the concentration of antidepressant drugs, the more ion suppression observed in the signals of the SI-ISs. In our own work, we noticed a decrease in the performance of the calibration curves of selected dansylated metabolites with the decrease in the concentration of the $^{13}C_2$-ID-ISs (Figure 5A and B) (Khamis et al., 2017). With only two mass-unit difference, the $^{13}C_2$-dansylated ID-ISs were in the isotopic envelope of their analogous $^{12}C_2$-derivatized metabolites. Accordingly, the influence of the second naturally occurring $^{13}C_2$- isotopic peak of the derivatized metabolite with *m/z* value (+2 mass units) similar to that of the $^{13}C_2$-ID-IS channel was more profound with the decrease in the $^{13}C_2$-ID-IS concentration (Khamis et al., 2017). Therefore, the method was optimized using a concentration of the $^{13}C_2$-ID-ISs at 66.67% of the upper limit of quantification (ULOQ; Fig. 5C) (Khamis et al., 2017).

In fact, it is recommended for an isotopic IS to differ from its analogous metabolite by a minimum of 3 mass-units to avoid cross talk or isotopic overlap during MS analysis (Stokvis et al., 2005; Xu & Madden, 2012). However, due to the limited availability of SI-ISs and isotopic reagents needed for ID-ISs synthesis, this mass difference might not be feasible. In addition, the incorporation of large number of deuterium in isotopic ISs may lead to deuterium effects (Berg & Strand, 2011). Our attempt to increase the mass difference in dansylated ID-ISs using deuterium was not successful. We synthesized d_6-dansyl chloride in our lab and compared it to $^{13}C_2$-dansyl chloride. Unfortunately, significant deuterium effects were observed between $^{12}C_2$-derivatized metabolites and their deuterated counterparts, thus rendering d_6-dansyl chloride unsuitable for quantification (Fig. 6).

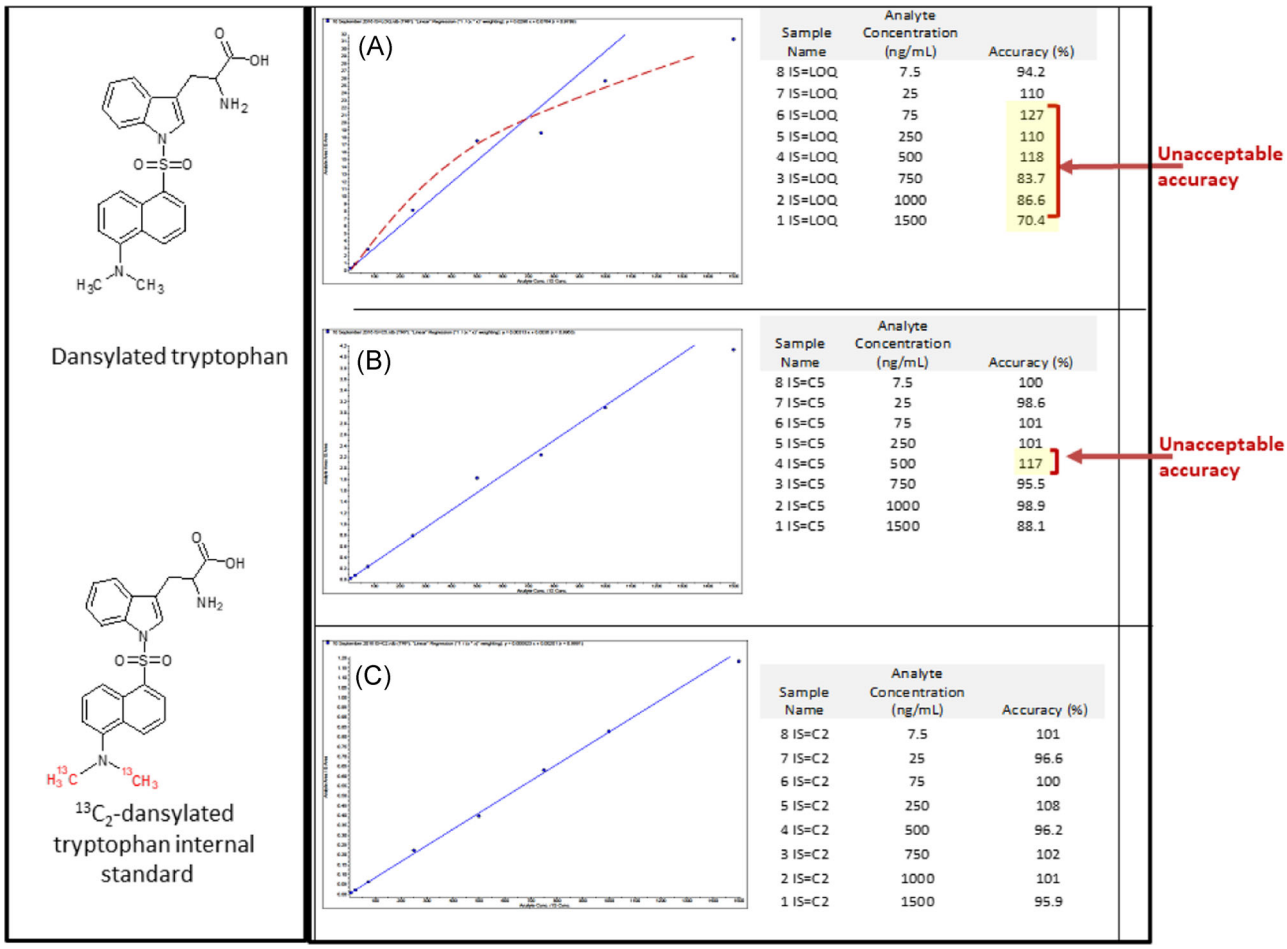

FIGURE 5. Calibration curves of $^{13}C_2$-dansylated-tryptophan constructed using three different concentration levels of spiked $^{13}C_2$-dansylated tryptophan internal standard, in which level (**A**) is at 1.3% of the ULOQ; level (**B**) is at 16.67% of the ULOQ; and level (**C**) is at 66.67% of the ULOQ. Accuracy % of calibration standards demonstrates unacceptable values (highlighted) with the decrease in internal standard concentration (right panel). Reprinted with permission (Khamis et al., 2017, *Analytica Chimica Acta*). ULOQ, upper limit of quantification. [Color figure can be viewed at wileyonlinelibrary.com]

Finally, unlike the common practice, SI-ISs can be spiked at concentrations outside the linear range of their metabolites, if necessary (Khamis et al., 2018a). We developed and validated a HILIC-MS/MS method for the quantification of seven polar metabolites, including glucose, in urine (Khamis et al., 2018a). The formate ion adduct of glucose, monitored in the negative ion mode at *m/z* 225.1 > 179.1, was at least 10-fold more intense than any other transition for glucose. Similar ionization behavior was observed for d_2-glusose IS (Fig. 7A). However, the use of the analogous transition (i.e., *m/z* 227.1 > 181.1) in the urine samples was severely compromised by an unknown endogenous interference at the same MRM channel (Fig. 7B). Unfortunately, an alternative MRM transition for d_2-glucose at *m/z* 227.1 > 121.1 was significantly lower in signal intensity, which was chosen to avoid the observed interference. Due to low intensity signal, d_2-glucose IS was spiked at a concentration twice the ULOQ of glucose to produce quantifiable *m/z* 227.1 > 121.1 signal (Fig. 7C). In conclusion, the above-mentioned examples demonstrate the importance of optimizing the IS concentration during the development phase of the method for endogenous metabolites. The analyst may optimize the IS concentration at half of the ULOQ, lower than half of the ULOQ or even greater than the ULOQ (Xu & Madden, 2012).

IV. BLANK MATRICES FOR METHOD VALIDATION

According to the FDA, the EMA, and the ICH (EMA, 2011; US-FDA, 2018; ICH, 2019), validation should demonstrate the reliability of an analytical method to quantify a particular compound in a specific biological matrix. Therefore, calibration standards and QC samples are ideally prepared by spiking reference standards into an analyte-free matrix identical to that of the study samples (EMA, 2011; US-FDA, 2018; ICH, 2019). However, when endogenous metabolites are being quantified, other approaches should be explored to overcome the scarcity of the blank matrix. In the following sections, the types of matrices that can be used for LC-MS/MS method development and validation in targeted metabolomics are described through selected examples. Figure 8 is a suggestive flowchart that can aid analysts in selecting proper alternative matrices for their target endogenous metabolites.

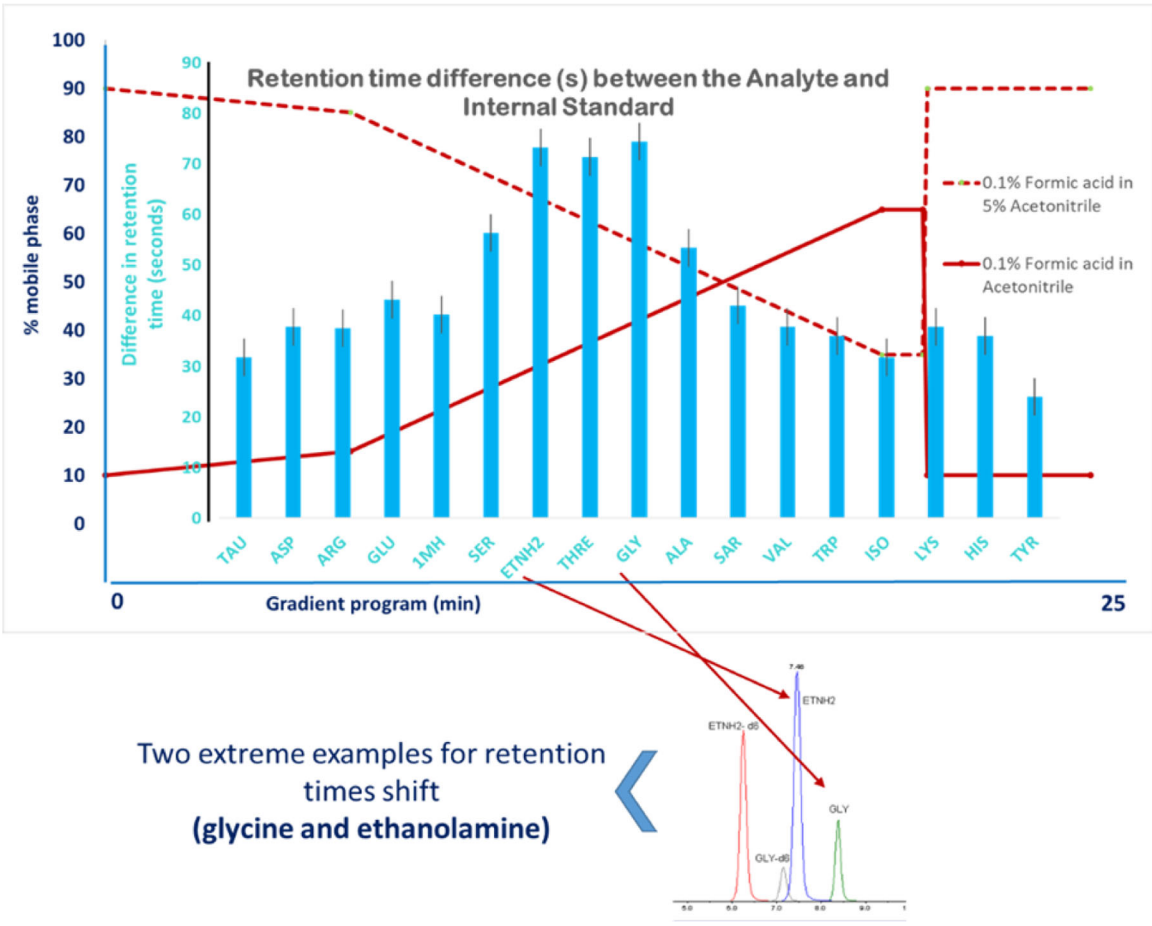

FIGURE 6. Retention time differences (in seconds) between 17 dansylated metabolites and their d_6-dansylated internal standards on C_{18} column (10 cm × 2.1 mm ID, 5 μm) using a gradient binary mobile phase system. Changes of the gradient program is demonstrated in the background against chromatographic run time. Metabolites are arranged according to their elution order using approximate retention time values (Khamis et al., 2017). 1MH, 1-methylhistamine; ALA, alanine; ARG, arginine; ASP, asparagine; $ETNH_2$, ethanolamine; GLU, glutamine; GLY, glycine; HIS, histidine; ISO, isoleucine; LYS, lysine; SAR, sarcosine; SER, serine; TAU, taurine; THRE, threonine; TRP, tryptophan; TYR, tyrosine; VAL, valine. [Color figure can be viewed at wileyonlinelibrary.com]

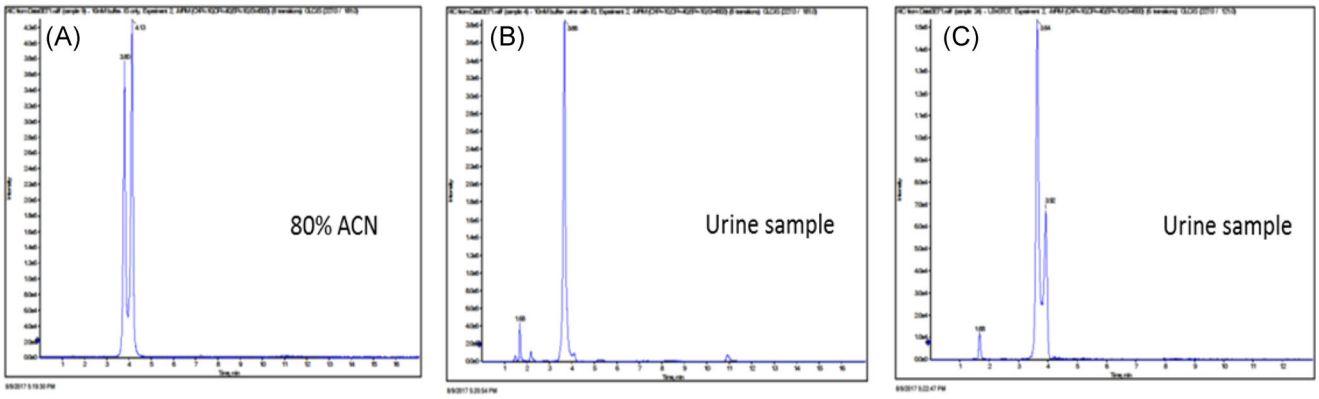

FIGURE 7. Extracted ion chromatogram of (**A**) d_2-glucose internal standard in 80% acetonitrile monitored at m/z 227 > 181.1 and (**B**) the unexpected interference in urine samples also observed at m/z 227 > 181.1 (no IS is added). Changing the quantifier ion transition of d_2-glucose internal standard to m/z 227 > 121.1 (**C**) successfully eliminated the interference previously observed at m/z 227 > 181.1. Reprinted with permission (Khamis et al., 2018a, *Analytica Chimica Acta*). [Color figure can be viewed at wileyonlinelibrary.com]

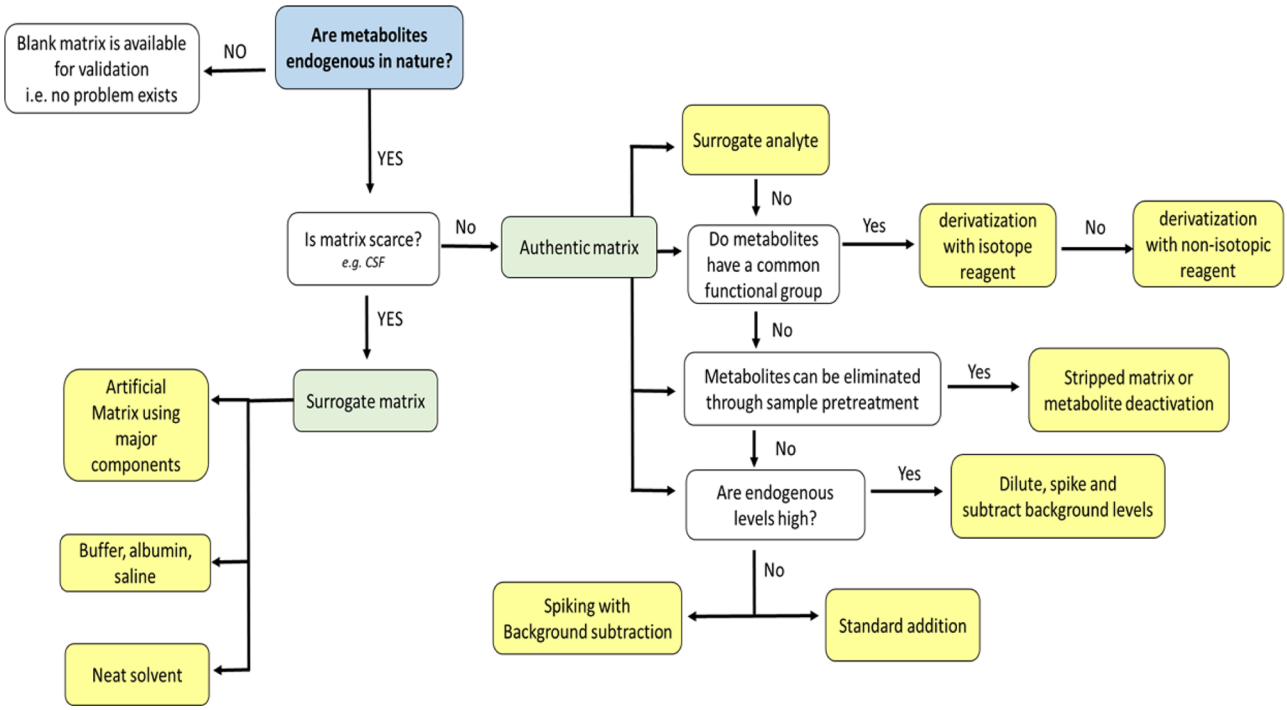

FIGURE 8. Suggested workflow for the selection of an appropriate blank matrix essential in bioanalytical method validation for endogenous metabolites. Surrogate matrix is a synthetic substitute of the biological matrix. Authentic matrix is real biological sample, in which endogenous levels of metabolites have been corrected. Blue-colored box represents the starting point; green-colored boxes indicate the two main type of matrices (surrogate vs. authentic); and yellow boxes represent possible means for utilizing surrogate and authentic matrices. [Color figure can be viewed at wileyonlinelibrary.com]

A. Surrogate Matrices

1. Solvents

The simplest approach for a surrogate matrix involves the use of neat solvents for the preparation of calibration standards and QC samples (Fig. 8) (Hou et al., 2016; Sriboonvorakul et al., 2013; Hewavitharana 2011, ICH, 2019). For instance, 11 microbiota-host endogenous cometabolites were quantified in rat serum, urine, and feces using an ultra high performance liquid chromatography (UPLC)-MS/MS method with polarity switching and two nonisotopic structural analogue ISs (Hou et al., 2016). The authors used the slope ratio method (i.e., slope of calibration curve in biological sample/slope of calibration curve in acetonitrile: water (validation matrix)) to ascertain the absence of problematic MEs (Hou et al., 2016). This matrix lacks any interfering salts or metabolites. It minimally meets the specifications recommended by the FDA, the EMA, and the ICH for the choice of blank matrix (EMA, 2011; US-FDA, 2018; ICH, 2019). In our opinion, this type of matrix should be used only after other alternatives have been explored and excluded.

2. Artificial Matrices

A mixture of the most abundant endogenous metabolites (excluding target metabolites) and salts of a particular biological matrix can be combined into an artificial medium (Fig. 8) (Harwood & Handelsman, 2009; van den Ouweland et al., 2010; Thakare et al., 2016). This is an appealing alternative, especially when the volumes of the biological samples are scarce (Thakare et al., 2016). Different formulas for artificial urine (Sparidans et al., 2006; Jacob, Wilson, & Benowitz, 2007; Thakare et al., 2016), semen (Alvarez et al., 2015; Thakare et al., 2016), vaginal fluid (Alvarez et al., 2015; Thakare et al., 2016), saliva (Özer & Güçer, 2011; Milošev et al., 2013; Thakare et al., 2016), CSF (Oe et al., 2006; Wang et al., 2013; Thakare et al., 2016), and tears (Alexeev et al., 2004; Thakare et al., 2016) can be found in the literature. Commercial sources of artificial matrices are also available. However, based on our best knowledge, the complete list of constituents is usually proprietary and not always revealed by suppliers (ALDON accessed January 1, 2019; AMPLIQON accessed, January 1, 2019; and Pickering-Labs accessed January 1, 2019). This can be problematic since the metabolites of interest can be included in the commercial recipes. For example, 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) is a serum substitute demonstrating considerable endogenous levels of homocysteine, a potential biomarker of vascular diseases. This complicates method development for its quantification in serum (Ghassabian et al., 2014). In this work, rather than correcting endogenous levels by subtraction, the authors used the total content of homocysteine in their calibration standards as the nominal concentration (i.e., the additive amount of endogenous and exogenously spiked concentration) (Ghassabian et al., 2014). QC samples were prepared in two different matrices, namely pooled human serum

(three levels) and 2% BSA in PBS (four levels), ensuring the full validation of the analytical method (Ghassabian et al., 2014). While the preparation of QC samples in two matrices is unconventional, the approach allowed for adequate method validation (Ghassabian et al., 2014).

Artificial matrices may present a challenge in developing robust methods due to the absence of other endogenous interferences that usually exist in the original matrix. For instance, we investigated the use of artificial urine during the quantification of pyruvic acid (a potential biomarker of asthma) using HILIC-MS/MS (Khamis et al., 2018a). An elevated baseline for pyruvic acid was observed in patient urine samples, leading to lower signal-to-noise ratio in comparison to the artificial matrix. Developing the method in the artificial matrix was not reflective of the potential interferences in the biological fluid. As such, this approach was incorrect (Khamis et al., 2018a). Similarly, during the quantification of amyloid β peptide (a potential biomarker of Alzheimer's disease), artificial CSF lacked the amyloid-binding proteins necessary for mimicking human CSF samples (Oe et al., 2006). Rat CSF was used instead for method development and validation (Oe et al., 2006). Artificial matrices may also cause contamination; for example, PBS augmented the ion suppression effects of nucleotides biomarkers and its usage required frequent ion source cleaning (Klawitter et al., 2007). For this reason, Klawitter et al. (2007) developed their method in 6% BSA in isotonic saline for the quantification of 11 nucleotides in rat tissues.

Overall, the challenges with artificial matrices are likely under documented, since published work is mostly biased towards positive results. Ocque et al. (2015) used PBS (pH 7.4) during the validation of a UPLC-MS/MS method for the quantification of trimethylamine N-oxide, choline, and betaine in human plasma and urine (metabolites associated with atherosclerosis). Calibration standards and three levels of QC samples were prepared in PBS. On the other hand, participants plasma and urine samples were extracted using methanolic SI-ISs, and the extracts were further diluted with acetonitrile/methanol (75:25) (Ocque et al., 2015). No rationale was provided on the reason for fully validating the method in PBS while adopting a different sample preparation procedure for the biological specimen (Ocque et al., 2015). On the basis of the most recent recommendations by the ICH, if the surrogate matrix is being used, it should be reserved only to the calibration standards and not the QC samples. However, the analyst should consider the improved signal-to-noise ratio observed mainly in the surrogate matrix and not the real biological matrix, which may result in the inaccurate calculations of the LLOQ (Oe et al., 2006; Khamis et al., 2018a; ICH, 2019).

B. Authentic Matrices

1. Authentic Matrix in QC Samples

Apart from synthetic remedies, the authentic biological matrices can be incorporated into the validation samples, thus mirroring the composition of the study samples (Fig. 8) (Beaudry and Vachon 2006; Joyce et al., 2016; US-FDA, 2018; ICH, 2019). The biological matrix can be incorporated in the QC samples and the additive content can be used as the expected concentration. This approach, in fact, has been recommended by the newest edition of the FDA and ICH guidelines for endogenous compounds (US-FDA, 2018; ICH, 2019). Joyce et al. (2016) prepared their QC samples by spiking metabolite standards into urine pooled from 646 sample during the quantification of 18 amino acids. Calibration standards, on the other hand, were prepared in neat solvents (Joyce et al., 2016). Despite demonstrating acceptable validation data, one shortcoming of this study was the addition of the IS after protein precipitation with acetonitrile. Accordingly, losses during extraction were not accounted for (Joyce et al., 2016). From our experience, this approach may represent a challenge for methods validated for routine applications, in which the endogenous metabolites in pooled samples, used in QCs preparations, may degrade over time. Therefore, we recommend the periodic assessment of the potency of the metabolites in the pooled matrix. A possible alternative to bypass such hurdle is the correction of endogenous levels as demonstrated below.

2. Authentic Matrix in Validation Standards

Authentic biological matrix can be incorporated in both the calibration standards and QC samples (Fig. 8). Endogenous metabolite interference can be corrected through three approaches: (a) subtraction of peak area of endogenous levels (Liu et al., 2013; Li et al., 2016; ICH, 2019), (b) dilution of the biological matrix (Klepacki et al., 2015; ICH, 2019), or (c) endogenous metabolite depletion (Fig. 8) (Li et al., 2003; Oe et al., 2006; van de Merbel 2008; ICH, 2019). An example of the first approach is the work conducted by Li et al. (2016). Pooled plasma samples were spiked with appropriate standards for calibration and QC samples preparation during the quantification of six amino and keto acids. SI-IS of leucine (^{13}C-analogue) and salicylic acid (structural analogue IS) were utilized for quantifying the six metabolites, while employing polarity switching after the first 3.5 min from the positive to the negative ion mode (Li et al., 2016). Peak areas from endogenous levels in the pooled samples (prior to spiking) were subtracted from total peak areas prior to the generation of calibration curves. The method demonstrated acceptable validation data for regression, accuracy, precision, and stability (Li et al., 2016). One drawback of this approach is the relatively lower sensitivity of the developed methods in comparison with the use of surrogate matrix (Thakare et al., 2016). The LLOQ is constrained by the naturally existing levels of the target endogenous metabolites since these levels are factored as a "noise" in the blank matrix during the assessment of method selectivity (Thakare et al., 2016; ICH, 2019). In order to achieve higher sensitivity, low concentrated study samples can be used for the preparation of the pooled matrix (Jemal et al., 2003). However, this approach involves the initial screening of the study samples, which can be time consuming (Jemal et al., 2003).

The second option is diluting the pooled matrix prior to the preparation of the validation samples (Fig. 8) (ICH, 2019). For instance, while quantifying potential biomarkers of kidney dysfunction a 1:5000 dilution step of urine prior to spiking it with metabolite standards for the preparation of validation samples (Klepacki et al., 2015). Patient urine samples, however, were optimized at a dilution of 1:40 (Klepacki et al., 2015). A key challenge with this approach occurs when multiple

metabolites are quantified in the context of varying endogenous levels. This renders a single dilution factor inapplicable (Thakare et al., 2016). In addition, sample dilution may significantly change the MEs experienced between study and validation samples (ICH, 2019).

The third option uses chemical, mechanical, or immunological pretreatment to remove endogenous levels of metabolites from the matrix before validation (Fig. 8) (Li et al., 2003; Oe et al., 2006; ICH, 2019). For example, pooled human plasma samples were incubated at 37°C for 2 hr to remove endogenous thymidine during the validation of an LC-APCI-MS/MS method for its quantification (Li et al., 2003). Stripping authentic biological samples with activated charcoal can remove several metabolites, and has been used for many validated assays (Fig. 8) (Xu et al., 2005; van de Merbel 2008; Thakare et al., 2016). The associated drawback with this approach includes the incomplete removal of endogenous metabolites even from commercial sources that claimed to be analyte-free (Fig. 9) (Oe et al., 2006; van de Merbel 2008). Another challenge is the alteration of the matrix from its original composition and the introduction of exogenous interferences from residual charcoal (Oe et al., 2006; Thakare et al., 2016). Finally, in our opinion, while authentic matrices provide the closest simulation to the biological matrix, their usage should be carefully investigated through the validation of method selectivity and LLOQ. The analyst should assess the benefits introduced by such matrices versus the drawback of a higher LLOQ due to the trace levels of endogenous metabolites treated as noise in the blank matrix.

3. Authentic Matrix via Standard Addition

Authentic biological matrix can be employed for method development without endogenous level correction through the standard addition technique (Fig. 8) (van de Merbel 2008; Scherer et al., 2009; Flores et al., 2012; Thakare et al., 2016; ICH, 2019). Standard addition overcomes the difference in composition from one sample to another, and consequently, provides an appropriate correction of MEs (Ito & Tsukada, 2002; Flores et al., 2012; ICH, 2019). In fact, based on the recent recommendations by the ICH, a separate calibration curve should be constructed for each study sample quantified via the standard addition method. Consequently, the larger sample volume and longer time of analysis in comparison to conventional strategies render this approach inapplicable to metabolomics. In addition, full validation, as described by

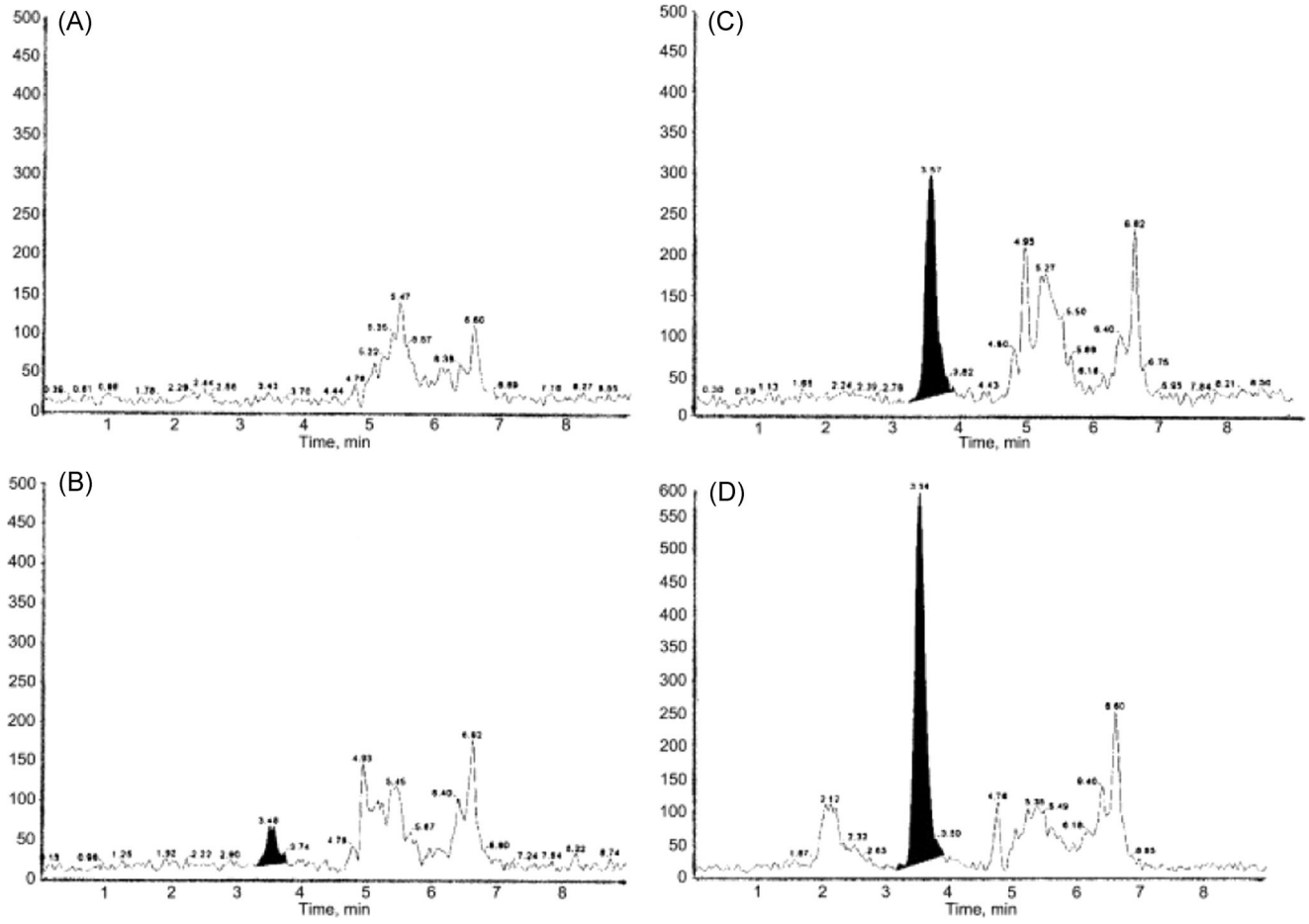

FIGURE 9. XICs showing the incomplete stripping of progesterone from plasma with activated carbon; in which; (**A**) buffer; (**B**) stripped plasma; (**C**) stripped plasma spiked at 20 pg/mL; and (**D**) authentic plasma at a low endogenous concentration (66 pg/mL). Reprinted with permission (van de Merbel 2008, *Trends in Analytical Chemistry*). XIC, extracted ion chromatogram

different regulatory bodies (EMA, 2011; US-FDA, 2018; ICH, 2019), cannot be directly conducted. It also requires the prior establishment of linearity ranges, if single point quantification is pursued (van de Merbel, 2008; Thakare et al., 2016).

4. Authentic Matrix and Surrogate Analyte

In this approach, three different entities are of interest, that is, the target metabolite, its IS, and the surrogate analyte. The response ratio of the surrogate analyte to the IS is extensively used during method development and validation, while the response ratio of the metabolite to its IS is calculated for the clinical data acquisition. The surrogate analyte is an isotopic form of the metabolite spiked at increasing concentration in the authentic matrix for calibration standards preparation, in the presence of the endogenous nonisotopic counterpart. The IS is ideally a second different isotopic form of the metabolite (Li & Cohen, 2003; Penner et al., 2010; Wilson et al., 2011; ICH, 2019). However, other forms of the IS have been used such as d_3-octanyol carnitine as an isotopic structural analogue during the quantification of potential biomarkers of β-oxidation in mice (acetyl and palmitoyl carnitines) (Liu et al., 2008). The calibration curves are then constructed using the peak area ratio of the surrogate analyte and the IS versus the surrogate analyte concentration (Li & Cohen, 2003; Liu et al., 2008; Penner et al., 2010; Wilson et al., 2011). The regression equation is modified by including a response factor (RF) (Equation (6)) (Li & Cohen, 2003). This factor is intended to account for the difference in ionization efficiency between surrogate analytes and their metabolites or to correct for the presence of any isotopic effects (Li & Cohen, 2003).

$$\text{Concentration}_{\text{metabolite}} = [(\text{Area}_{\text{metabolite}}/\text{Area}_{\text{IS}})*\text{RF} - b]/a, \quad \text{Equation (6)}$$

where $\text{RF} = \text{Area}_{\text{surrogate analyte}}/\text{Area}_{\text{metabolite}}$, at equivalent concentration, a is the slope and b is the intercept of the regression line.

Despite the usefulness of the surrogate analyte technique, the lack of appropriate software for quantification remains a challenge. The calibrators and the study samples need to be processed separately as each sample group identifies a different isotopic form of the metabolite as the analyte of interest (Liu et al., 2008). Another challenge was demonstrated by Kindt et al. (2004) during the LC-MS/MS quantification of myo-inositol in rat brain tissue (a potential biomarker of neurological disorders). The surrogate analyte d_6-myo-inositol was unable to provide adequate accuracy and precision data. The authors attributed this to the probable difference in signal-to-noise ratio between the different isotopic forms (Kindt et al., 2004). In summary, we emphasize that the use of the surrogate analyte technique without the inclusion of an IS (i.e., external calibration method) might not provide the level of accuracy expected from the developed assay.

5. Authentic Matrix and ID-IS

As previously described, precolumn derivatization transforms metabolites into new entities that are not endogenously present in the sample (Guo & Li, 2009; Xu et al., 2011; Toyo'oka, 2012; Stanislaus et al., 2012; Bruheim et al., 2013; Khamis et al., 2017). As such, underivatized authentic biological matrices can be used for the preparation of validation samples (Stanislaus et al., 2012; Awad et al., 2019). The authentic biological matrix can be also exposed to all steps of derivatization similar to patient samples while replacing the derivatizing reagent with a solvent (Khamis et al., 2017). In this manner, a matrix can be generated to closely mimic the experimental conditions to which real biological samples are exposed to (i.e., heat, salts, and buffers) (Khamis et al., 2017).

In summary, the optimization of the blank matrix and the IS is integral to the development of robust LC-MS/MS methods in the context of endogenous metabolites. With the absence of adequate guidelines, the analyst may pursue additional experiments to ensure sufficient method validation. For instance, an LC-MS/MS method was developed for the quantification of glycine in CSF (a potential biomarker for psychiatric disorders) (Wilson et al., 2011). ID-IS was generated using dansyl chloride and $^{13}C_2{}^{15}N$-glycine. While the use of the authentic matrix is appropriate, the authors pursued their validation in an artificial CSF. To further validate their approach, the authors demonstrated the comparable results of their method to that of the standard addition technique as well as the surrogate analyte approach using $^{13}C_2$-glycin (Wilson et al., 2011).

V. LC-MS/MS METHOD VALIDATION

Bioanalytical method development for endogenous metabolite quantification aims at optimizing the experimental design and the operating conditions, such as the selection of the appropriate blank matrix and ISs. Following the optimization process, method validation is pursued to assess the applicability/robustness of the method in analyzing the study samples (ICH, 2019). The validation parameters, such as selectivity, linearity, accuracy, and precision and their acceptance criteria are described in details in various regulatory guidelines (EMA, 2011; US-FDA, 2018; ICH, 2019). As such, they are not discussed within this article; however, the major difference from xenobiotics method validation is a result of the absence of analyte-free matrix. In general, the use of authentic matrices should be accompanied by appropriate corrective measures to account for the endogenous levels of the metabolites as described in details in section IV. On the other hand, surrogate matrices circumvent this problem. They may, however, results in false improved signal-to-noise ratio when used for calibration curve construction (Thakare et al., 2016) (Section IV). Furthermore, stability studies should be thoroughly conducted, especially with the individualized behavior of the metabolites in different matrices and under different storage temperatures, storage times, and freeze-thaw cycles (Khamis et al., 2015).

Following the full validation of a bioanalytical method, study samples are analyzed for the absolute quantification of the candidate biomarkers (Tiwari & Tiwari, 2010; ICH, 2019). Moreover, methods should be periodically revalidated, over the life of data collection, in order to ensure the quality of the clinical data being acquired (US-FDA, 2015). Ideally, bioanalytical methods for endogenous metabolite quantification should be cross-validated with a reference method. This is specifically important if the data from different fully validated methods are going to be combined from different studies to support a regulatory decision regarding the efficacy of the biomarkers (Tiwari & Tiwari, 2010; ICH, 2019). In our opinion, however, it is not always possible to cross-validate, given the limited number of fully validated methods that quantify a specific set of metabolites in the same biological

matrix. Finally, the documentation of general and specific standard operating procedures cannot be overemphasized. All relevant information regarding every element of the method validation, revalidation, modification, and operation should be available to ensure the reproducibility of the results among analysts. In addition, this information would eventually constitute a part of the submission file to regulatory authorities (EMA, 2011; US-FDA, 2018; ICH, 2019).

VI. CLOSING REMARKS

Metabolomics research aims at the identification of endogenous and exogenous metabolites that are sufficiently altered in a stressed biological state. The qualification of candidate biomarker metabolites for clinical application requires robust validated assays of high specificity. Herein, we discuss three main challenges for endogenous metabolite quantification, which unlike xenobiotics, are still growing fields of research. While a common consensus has been reached that ^{13}C-containing isotope ISs should be always sought first, a similar consensus for MEs evaluation and blank matrix selection, in the context of endogenous metabolites, is not yet well-established. Each technique has its advantages and disadvantages suggesting a trial and error approach with a final decision based on analytical performance. Finally, the newest edition of the FDA guidelines (May, 2018) has included separate sections for biomarkers and endogenous compounds, reflecting their potentials in improving disease diagnoses and personalized medicine. Similarly, few approaches to quantify endogenous metabolites have been compiled in the most recent ICH guidelines that were drafted in February, 2019.

In conclusion, a rich discussion on LC-MS/MS method development and validation challenges as well as a comprehensive review on the currently available approaches to address these challenges were reviewed. The synthesized knowledge in this paper will aid researchers and clinicians validate identified endogenous biomarkers and translate the knowledge from the discovery stage to clinical practice.

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