

Systematic Assessment of Matrix Effect, Recovery, and Process Efficiency Using Three Complementary Approaches: Implications for LC-MS/MS Bioanalysis Applied to Glucosylceramides in Human Cerebrospinal Fluid

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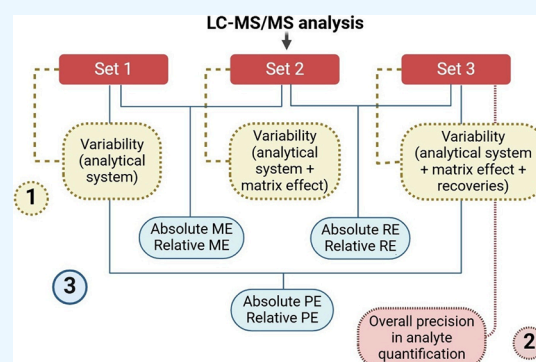
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ABSTRACT: The evaluation of matrix effect, recovery, and process efficiency is essential in the validation of LC-MS/MS bioanalytical methods, as they impact assay accuracy, precision, and sensitivity. However, guidelines on bioanalytical method evaluation are not harmonized and can occasionally be ambiguous. To address this need, the study presents an integration of three different approaches to assess these parameters within a single experiment. The first approach examined the variability of peak areas and standard-to-internal standard (IS) ratios between different matrix lots to assess the influence of the analytical system, relative matrix effects, and recovery on method precision. The second strategy evaluates the influence of the overall process on analyte quantification. The third approach calculates both the absolute and relative values of matrix effect, recovery, and process efficiency, as well as their respective IS-normalized factors, to determine the extent to which the IS compensates for the variability introduced by the matrix and recovery fraction. Applying these strategies to an LC-MS/MS method for quantifying glucosylceramides in cerebrospinal fluid addresses the challenges posed by limited sample volume and endogenous analytes, while providing a comprehensive understanding of the factors that influence method performance and promoting adherence to different guideline recommendations. This study supports the importance of a systematic evaluation of matrix effect, recovery, and process efficiency during method validation. Standardized evaluation methodologies would improve data interpretation, enhance method reliability, and contribute to harmonization in in-house bioanalysis.



1. INTRODUCTION

Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) is a highly valuable analytical tool for the quantification of endogenous and exogenous compounds in clinical laboratories.¹ However, it presents several challenges that can impact the performance of bioanalytical methods. One of the most critical issues is the matrix effect, which impacts assay sensitivity, accuracy, and precision. It is defined as an alteration in the ionization efficiency of the target analyte due to coeluted compounds in the matrix, resulting in either a loss (ion suppression) or an increase (ion enhancement) in signal response.² Matrix effects are influenced by ionization mechanisms, analyte physicochemical properties, fluid composition, pretreatment procedures, and chromatographic conditions.^{3–8} Therefore, the matrix effect should be considered during the evaluation of bioanalytical methods and is included as one of the key parameters in analytical method validation, according to regulatory guidelines.^{9,10}

Other key parameters, which need to be considered to assess method performance, include recovery (the fraction of the

analyte recovered after a chemical procedure), overall process efficiency (reflecting the combined effects of the matrix and recovery), and accuracy and precision (which integrates the calibration curves and indicates the impact of overall method efficiency on analyte quantification).^{11,12}

The main international guidelines provide recommendations for assessing the matrix effect, recovery, and process efficiency using the pre- and postextraction spiking methods (Table 1).^{9,10,13,14} Notably, there is no consensus among such guidelines, the protocols are frequently simplified, focusing primarily on the assessment of the instrumental part, and, in some cases, detailed protocols for conducting such assessments are lacking or may address ambiguous concepts related to

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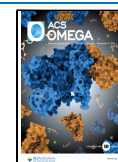


Table 1. Recommendations for Evaluation of Matrix Effects in Different International Guidelines by Pre- and Postextraction Spiking Methods

guideline	matrix lots	levels	recommendations and evaluation protocol	acceptance criteria	comments
EMA 2011 ^{22a}	6	2 conc	evaluation of STD and IS absolute and relative matrix effects: postextraction spiked matrix vs neat solvent IS-normalized matrix factor (MF) should also be evaluated in hemolysed or lipaemic matrix samples	CV <15% for MF; Use of fewer sources/lots may be acceptable in the case of rare matrices.	no evaluation of recovery, no evaluation of process efficiency
FDA 2018 ^{14a}			evaluation of recovery		no protocol of evaluation of matrix effects in chromatographic analysis
ICH M10 2022 ⁹	6	2 conc	evaluation of matrix effect (precision and accuracy)	for each individual matrix sources/lot accuracy <15% of the nominal concentration and precision <15%.	evaluation of recovery in independent experiments.
		3 replic	matrix effect should also be evaluated in relevant patient populations, hemolyzed or lipemic matrix samples	use of fewer sources/lots may be acceptable in the case of rare matrices.	it is not possible to quantify the contribution of matrix effect and recovery to the overall efficiency of the process.
CLSI C62A 2022 ¹⁰	5	7 conc	evaluation of matrix effect: postextraction spiked matrix vs neat solvent absolute matrix effect (%ME) CV of the peak areas	absolute %ME: evaluate the extent of ion suppression (based on TEa limits, expected biological variation...) CV <15% for the peak areas IS-norm %ME: evaluate, along with matrix effect, the fulfillment of the pre-established requirements for sensitivity or specificity	no evaluation of recovery efficiency no evaluation of process efficiency refers to Matuszewski et al. ¹¹ and CLSI C50 ¹³ as best practices
CLSI C50A 2007 ¹³	5		IS-norm %ME evaluation of (a) absolute matrix effect, (b) extraction recovery, and (c) process efficiency: pre- and postextraction spiked matrix and neat solvent sets 1, 2, and 3		refers to Matuszewski et al. ¹¹ and Maurer ²³ as best practices

“ICH M10 currently supports the most updated EMA (for EU) and FDA (for USA) guidance on Bioanalytical Method Validation. EMA: European Medicines Agency; FDA: Food and Drug Administration; ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; CLSI: Clinical and Laboratory Standards Institute; conc.: concentration; replic.: replicates; STD: standard; IS: internal standard; TEa: total error allowable.

acceptance criteria. Although they also do address terms related to recovery and process efficiency, methodologies for integrating these concepts into a single experiment are not always provided. This integration would allow for a comprehensive view of the entire process and a deeper understanding of the causes and consequences of the matrix effect. Several studies have integrated these concepts into a unified experiment. Of particular interest are those that include the variability of such effects between independent matrix lots, as well as their normalization by those of the internal standard (IS), since they provide more detailed information about their impact on the overall performance of the method. The studies conducted by Matuszewski et al.^{7,11} constituted a pioneering contribution to this field, with subsequent derivative studies incorporating modifications and providing highly comprehensive insights.^{12,15–21}

Since different studies can provide complementary information, a more comprehensive evaluation should be based on the combination of more than one approach. This would facilitate the identification of the underlying causes of such effects, thereby allowing for their minimization or, at least, to control them. The integration of these strategies into the in-house method validation protocols of the clinical laboratory could facilitate and promote harmonization of the evaluation process, particularly in light of the implementation of the new European regulation for in vitro diagnostic medical devices 2017/746 (IVDR).²⁴

In this study, we aim to provide a systematic assessment of matrix effect, recovery, and process efficiency with three complementary approaches conducted in a single experiment, based on pre- and postextraction spiking methods, in accordance with international guidelines and previous works.^{9–11,15} To demonstrate the applicability of these strategies, we used our liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for quantifying glucosylceramide (GluCer) isoforms in human cerebrospinal fluid (CSF),²⁵ a method with clinical relevance for identifying potential biomarkers in Parkinson's disease. While the analytical requirements, which are crucial for a comprehensive understanding of our method, were previously validated and met the acceptance criteria defined by CLSI C62¹⁰ and ICH⁹ guidelines, the complexity and time-consuming procedures and the significant impact of these parameters on bioanalytical methods highlight the need for a detailed, robust, and systematized approach. Our study addresses this issue, including the protocol design and data analysis to facilitate the interpretation of the results.

2. EXPERIMENTAL SECTION

Human Samples and Ethics. The analysis was performed on CSF samples collected by lumbar puncture from three control individuals included in the VHIP Collection (Vall d'Hebron Initiative for Parkinson). After discarding the first 2 mL, 10–14 mL of CSF was collected in polypropylene tubes following international guidelines,²⁶ centrifuged at 2000g for 10 min at room temperature, and aliquoted into 0.5 mL cryotubes within 2 h. In this study, the volume available per sample was limited to a maximum of 1 mL. Samples were stored at -80°C until analysis. The study was approved by the local Ethics Committee (PR(AG)170-2015 and PR(AG)434-2019), and all participants provided written informed consent.

Chemicals and Reagents. *N*-Hexadecanoyl-glucosylceramide (GluCer C16:0), *N*-octadecanoyl-glucosylceramide

(GluCer C18:0), and *N*-15Z-tetracosenoyl-glucosylceramide (GluCer C24:1), standards (STD) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). *N*-Docosanoyl-D4-glucosylsphingosine (GluCer C22:0-d4) was obtained from Cayman Chemical (Michigan, USA). LC-MS-grade methanol (MeOH) and chloroform (CHCl_3) were purchased from VWR Chemicals (Radnor, Pennsylvania, USA). Formic acid (FA, purity: >99%) was purchased from ITW Reagents (Barcelona, ES). Ammonium formate (amm. form., purity: >99%) and LC-MS grade isopropanol (IPA) were obtained from Sigma-Aldrich (St Louis, Missouri, USA). LC-MS-grade acetonitrile (MeCN) was supplied by Thermo Fisher Scientific (Waltham, Massachusetts, USA). Ultrapure water was obtained using a Milli-Q system (Millipore).

Preparation of Sample Sets. Sample sets were prepared following the approach of Matuszewski et al.¹¹ For the GluCer study presented here, three different lots of CSF matrix obtained from the control group were evaluated, each prepared at two standard concentrations (50 and 100 nM, corresponding to the medium- and high-quality control levels within the validated range of our previously published method²⁵), with a fixed IS concentration (30 nM). Corresponding blank samples for each set and matrix lot were also prepared to subtract endogenous baseline signals. Figure S1 illustrates the preparation of intermediate and working solutions, either standard solutions (WS(STD)), IS solutions (WS(IS)), or mixed solutions containing both (Sol), from which each set was prepared, as follows:

Set 1 was prepared by spiking different volumes of WS(STD) and a fixed IS volume of WS(IS) in a neat solution of mobile phase B (MPB) in triplicate, to obtain the final selected standard concentrations of 50 and 100 nM. This resulted in a total of seven solutions (3×2 different standard concentrations plus 1 blank neat solution of MBP). After mixing, the solutions were transferred to chromatography vials for analysis.

Prior to the preparation of set 2, generated by postextraction spiking, it was necessary to obtain matrix extracts from each individual sources. To this end, each lot was subjected to the same pretreatment step, corresponding to the method under evaluation, which in our case was based on a liquid–liquid extraction (LLE) procedure described previously.²⁵ Briefly, 80 μL of CSF samples was added to 1.5 mL plastic tubes containing 15 μL of MeCN following a two-step liquid–liquid extraction process with $\text{MeOH}:\text{CHCl}_3$ (1:2, v/v). The resulting organic phases were pooled, dried under N_2 gas, and reconstituted in 250 μL of organic mobile phase (MPB). Several extractions for each lot were required to obtain sufficient extract volume for the complete preparation of set 2. Set 2 was then prepared by adding different volumes of WS(STD) and a fixed volume of WS(IS) to each corresponding extract to obtain the final selected standard concentrations. Additionally, a blank matrix was prepared for each lot by adding MeCN to the corresponding extracts instead of the WS(STD) and WS(IS). A total of nine solutions (3×3) were prepared transferred to chromatographic vials for injection.

For set 3, prepared by pre-extraction spiking, Sol 1 and 2 were added to the same matrix lots as in set 2 prior to extraction, to obtain final sample concentrations of 50 and 100 nM, respectively. A blank matrix was also prepared for each lot by adding MeCN instead of Sol 1 or Sol 2. Therefore, a total of nine samples (3×3) were prepared for extraction in this set.

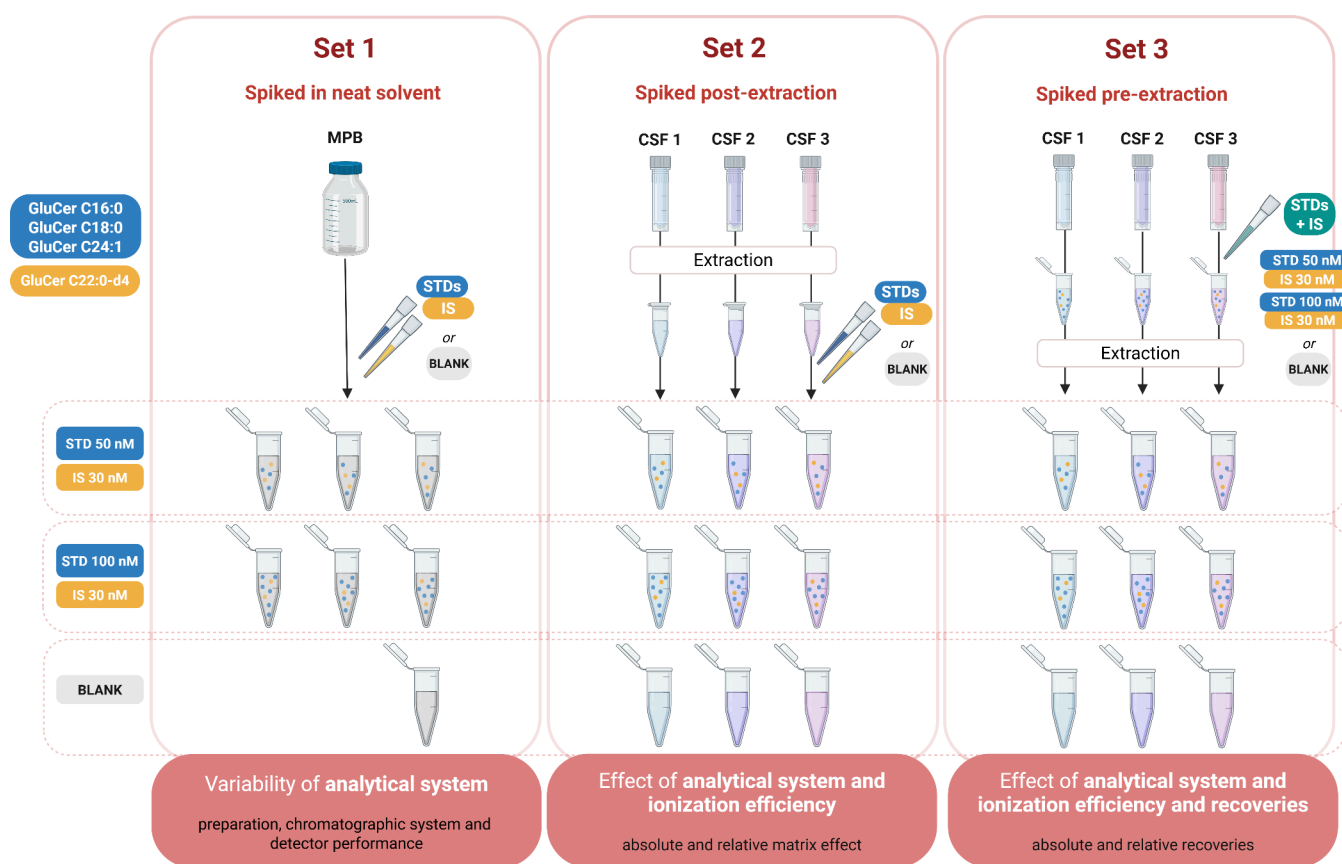


Figure 1. Schematic protocol for the preparation of the three sets of samples and the information that can be obtained from each set. The data obtained in set 1 assess the variability of the entire analytic system (including sample preparation, chromatographic system, and detector performance). Since the main difference between sets 2 and 1 is the sample matrix, the data obtained from set 2 provide information on the combined effect of the analytical system and ionization efficiency. Set 3 evaluates the combined effects of the analytical system, sample matrix, and analyte recoveries on the method performance, as this set includes the addition of the compounds in the matrix prior to extraction. Created in BioRender. Castillo, L. (2025) <https://BioRender.com/ur17de8>.

The extraction procedure was the same as in set 2, and reconstituted extracts were transferred to chromatographic vials for injection.

Figure 1 depicts the schematic protocol for the preparation of the three sample sets and the information obtained from each. The order of the injections is presented in Table S1. The same batch was injected twice to monitor the signal stability over time.

LC-MS/MS Conditions. GluCer isoforms were analyzed by LC-MS/MS using a Nexera X2 HPLC system coupled to an LC-MS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan).²⁵ For the detailed conditions, see the Supporting Information. All data were analyzed using LabSolutions CS Software v5.9 (Shimadzu Corporation, Japan).

Calculations for the Evaluation of the Matrix Effect, Recovery, Process Efficiency, and the Influence of Overall Precision on Analyte Quantification. From the areas obtained for each of the compounds studied, several calculations were performed to evaluate the relationship between the matrix effect, recovery, and overall efficiency of the process in three complementary approaches. The following subsections provide detailed information on the calculations included in each approach. Figure 2 illustrates a flowchart outlining the three distinct approaches and the outcomes derived from each strategy.

First Approach. Coefficients of variation (CVs) were calculated for both absolute peak areas and standard-to-IS area ratios across matrix lots within each set at each concentration. In addition, scatterplots were used to analyze the correlation between standard and IS responses. For each lot, the slopes obtained from the area ratios at the selected concentrations were also calculated, and their variation across matrix lots was expressed as precision (CV), following the approach described by Matuszewski.¹⁵

Second Approach. This approach was adapted from previous work,^{11,12} using the slopes obtained in set 3. Concentrations were calculated using area ratios from one lot and the calibration curve from the lot with the greatest slope difference. The percentage deviation from nominal values at each concentration was then estimated, as addressed in ICH M10.

Third Approach. For each analyte, absolute matrix effect (ME), recovery (RE), and overall process efficiency (PE) were calculated from the mean peak areas for each set.^{11,13} The potential relationship between the ME and RE values for all compounds and concentrations was explored using scatterplots. Matrix effect factor (MEF), recovery effect factor (REF), and process efficiency factor (PEF) were determined by normalizing the ME, RE, and PE values to those obtained in the IS for each standard. Relative matrix effect (rME), relative recovery (rRE), and relative process efficiency (rPE) were

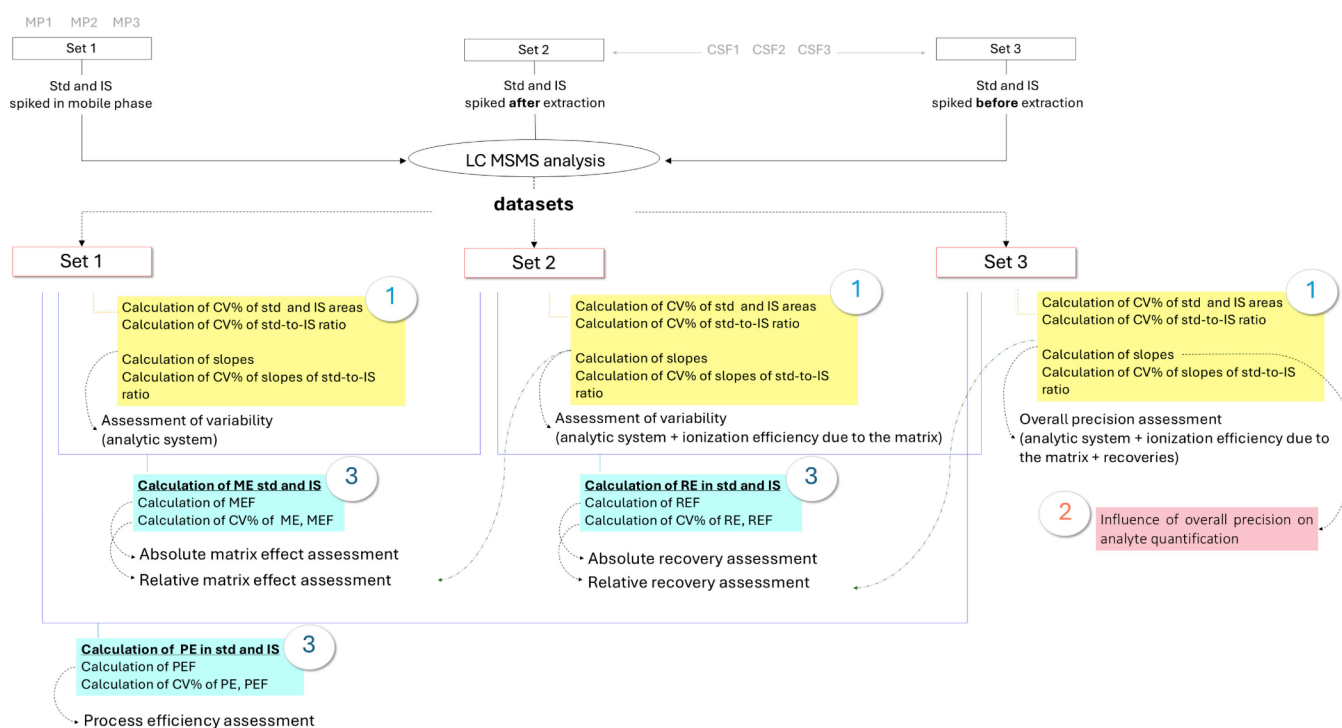


Figure 2. Flowchart containing the three different strategies and the link between the different descriptors. The first strategy involved the evaluation of the factors that influence the overall precision of the method, as well as the impact of IS in compensating for this variation. In set 1, CVs of peak areas reflected overall system variability, including sample preparation, chromatography, and MS performance. Set 2 incorporated relative matrix effect, representing variability in response when the same compound amount is added to each extract. Set 3 included recovery variability between lots and served as an indicator of overall precision. Each set integrated its own contributing sources of variation of the previous one. CVs of standard-to-IS area ratios assessed IS compensatory effect at each stage of the process. The variability between lots of the slopes calculated from the area ratios at each concentration were considered another indicator of the compensating effect of the IS for each factor. The second strategy examined the influence of overall process efficiency on analyte quantification. The third strategy involved the evaluation of the absolute matrix effect (ME), recovery fraction (RE) and overall process efficiency (PE), and their corresponding IS-normalized factors (MEF, REF, and PEF). ME, RE, and PE values represented the effect of the sample matrix on the ionization efficiency of the target compounds, the recovered fraction of the target compounds after various processing and extraction steps of the method, and the combined effect of both on the overall efficiency of the method, respectively. MEF, REF, and PEF indicated the extent to which each process impacts the standard compared to the IS. The variation of these parameters between lots (rME, rRE, and rPE) and the corresponding compensatory performance of the IS (rMEF, rREF, and rPEF) were also estimated.

expressed as coefficients of variation (CV%) by comparing ME, RE, and PE across different matrix lots for each analyte and IS. Similarly, relative MEF (rMEF), REF (rREF), and PEF (rPEF) were also calculated. Detailed equations are provided in the [Supporting Information](#).

Excel Tool. A Microsoft Excel spreadsheet template was created according to the structure of the strategies outlined in the flowchart ([Supporting Information](#)). This template provides the aforementioned calculations and scatterplots in a systematized way, facilitating the interpretation of the data. For scatterplots, Pearson correlation analysis was performed. Acceptance criteria should align with guideline recommendations and/or the clinical decision limits established for each individual test.¹⁰ In our study, method performance was considered acceptable when CV <15%, in accordance with CLSI, indicating effective compensation by the IS throughout the process.

3. RESULTS AND DISCUSSION

The interpretation of our findings must be framed within the current regulatory landscape. While FDA and EMA have historically provided independent recommendations for bioanalytical method validation, both agencies adopted the ICH M10⁹ guideline in 2022 as the current reference. The

ICH M10 addresses matrix effect from a global process efficiency perspective, requiring fewer concentration levels and allowing the use of a limited number of matrix lots, which facilitates implementation in laboratories with constrained resources. However, this strategy does not allow for the identification of the individual contributions of each main factor (matrix effect and recovery) to overall efficiency, thereby hindering the identification of the underlying causes of method performance impairment. In contrast, CLSI guidelines, which are widely recognized in clinical laboratories, provides a comprehensive approach, referencing the work of Matuszewski et al.¹¹ as best practice for the combined evaluation of matrix effect, recovery, and process efficiency. Nevertheless, neither of them provides detailed protocols to promote adherence to guidelines.

An additional consideration is the stage at which matrix effect evaluation is addressed within the validation process, since results that do not meet the acceptance criteria may require returning to earlier stages of method optimization. In this regard, the ICH M10 guideline includes matrix effect assessment as part of overall method validation, alongside other analytical parameters. By contrast, the CLSI C62 guideline addresses it during the preverification step, prior to full method validation.

In this study, we integrated the complementary perspectives of ICH M10,⁹ CLSI C50,¹³ and CLSI C62¹⁰ guidelines, along with other relevant reference studies in the field^{11,15} into a single experimental design and workflow analysis. The limited CSF volume and the structural complexity of endogenous GluCer isoforms provided a practical setting to address experimental design challenges and to evaluate the complementary insights of these approaches.

3.1. First Approach: Comparison of the Variation of Absolute Areas, Area Ratios, and Slopes between Different Matrix Lots. Table S2 indicates the precision of absolute peak areas for standards and IS (Table S2a), and that of the standard-to-IS area ratios across CSF lots in each set (Table S2b). Table S3 depicts the CVs obtained from the slopes in the different CSF lots in sets 1, 2, and 3.

3.1.1. Variation of Absolute Areas: Impact of Ionization Efficiency and Recovery Variability on Method Precision. It is expected that the variability in the absolute areas in set 1 will be lower or equal to that in set 2, and that of the set 2 will be lower or equal to those in set 3, since each set introduces one of three main variation factors (analytical system, matrix, and recoveries), increasing overall variability of the method.¹¹ The CVs obtained from the absolute peak areas in sets 1, 2, and 3 ranged from 5.2 to 13.7%, from 6.4 to 10.4%, and from 14.8 to 34.1%, respectively, for all compounds and concentrations. Our results (Table S2a) show a significantly greater increase in CVs from set 2 to set 3 than from set 1 to set 2, suggesting recovery variability contributes more to overall precision than matrix-induced ionization effects.

3.1.2. Variation of Area Ratios and Slopes: Impact of IS in Compensating for Ionization Efficiency and Recovery Variability. Analysis of Area Ratios. On the other hand, CVs of area ratios should be lower than those of corresponding absolute areas, indicating that absolute area variability does not necessarily indicate a lack of method robustness and that IS compensates for variation in standards in each set. This information would be reflected as a significant positive trend in Pearson correlation analysis (Figure S2). The CVs obtained from the area ratios between the different lots in sets 1, 2, and 3 ranged from 6.2 to 14.8%, from 3.0 to 8.0%, and from 1.3 to 6.1%, respectively. Set 2 showed reduced CVs in area ratios compared to absolute areas for all standards and concentrations, indicating IS correction for combined variability of analytical system and matrix effects. In set 3, the greater CV reduction suggests further compensation for recovery variability. However, an increase in the CVs of the area ratios compared to those of their absolute areas was observed at 50 nM, and some of the CVs obtained in the absolute areas of set 1 exceeded those of set 2. Obtaining large volumes of human CSF is challenging, and experimental protocols had to be adapted to minimize sample volume without affecting matrix composition. For instance, set 3 protocol was modified to use the minimum matrix volume without altering significantly matrix composition. Similarly, set 2 was adapted to use a smaller volume of extract, reducing the amount of CSF required for its preparation. When sufficient matrix volume is available (e.g., plasma), sets should be consistently prepared to avoid introducing variability from pipetting factors.¹² Alternatively, differences in the composition of the extract and mobile phase matrices could result in different interactions with the analytes during the preparation of both sets. It should be considered that the neat solvent itself is a matrix and may have an effect on the calculations.^{12,19} These results were

consistent with the trends observed in the scatter plots. Set 1 showed a negative association between the peak areas and those of the IS at 50 nM, and a positive trend at 100 nM. Positive trends were observed at both concentrations for sets 2 and 3, with set 3 showing correlation coefficients (r) ranging from 0.994 to 0.999.

For set 3, CVs of absolute peak areas differed significantly between concentrations (ranging from 14.8 to 18.51% at 50 nM, and from 30.8 to 34.1% at 100 nM), suggesting a concentration-dependent recovery variation across lots, in line with previous findings.²⁷ However, CVs for area ratios were more consistent, suggesting similar variation patterns for standards and IS.

The decreasing trend in CV observed across the sets, with the lowest values observed in set 3, both in peak area ratios (up to 6.1%) (Table S2b) and slopes (up to 12.6%) (Table S3), suggests that the IS was compensating for the variability generated in the overall process, thereby improving the overall method precision. The variability in the area ratios of set 3 reflects within-run precision, including matrix lot differences, often not addressed in guidelines. While these guidelines do encompass the assessment of between-run precision (thus considering other sources of variability, such as different calibrations or environmental conditions), we recommend incorporating this analysis for a more comprehensive evaluation of overall method precision.

Analysis of Slopes. The higher variability observed in the slopes compared to the area ratios at each concentration may be due to the fact that the slopes were estimated using only two concentrations, which may result in limited robustness. Increasing the number of calibration points used to define the standard lines would reduce this variability. Although there is no general consensus regarding the minimum number of concentrations to be included (Table 1), it is important to conduct the study within the targeted concentration range. In this context, the selection of 50 and 100 nM was based on background signals from endogenous analytes, which interfered with curve construction at lower concentrations and prevented the inclusion of additional points within the measurement interval. This limitation had already been reported during the evaluation of the artificial matrix.²⁵ Current guidance for obtaining blank samples for methods involving endogenous compounds is not fully addressed.²⁸ For this reason, other authors have not recommended this approach when a blank matrix is unavailable²⁹ or when spiked standard concentrations are low relative to endogenous compounds.¹² Consequently, although it has been proposed that using CV values of slopes in different lots could serve as a more reliable indicator of relative ME and overall precision,^{15,18} the variability observed in the slope study in our case should be considered a provisional estimate.

3.2. Second Approach: Influence of Overall Process Precision on Analyte Quantification. Table S4 shows that, in set 3, deviations remained below 10% for all standards and concentrations when calculated using the two most divergent slopes, indicating that the increased variability of the slopes compared to the area ratios at each concentration did not significantly affect analyte quantification. However, in our study, both the slope variability and these deviations should be considered approximations, as they are based on two-point calibration curves. When possible, they should be calculated from equations using more standard concentrations, in accordance with the recommendations outlined in CLSI

guidelines¹⁰ and previous studies.¹¹ We previously verified different analytical parameters of this method according to international guidelines, including evaluation of precision, trueness, and equivalence between artificial and native CSF matrices. Differences in slopes observed between this study and those reported²⁵ may be attributable to the lower number of calibration points and the variability introduced by the presence of endogenous analytes.

The calculation of percentage deviation from the nominal concentration is aligned with ICH M10 and previous work.¹¹ However, an alternative strategy that may better reflect the influence of overall precision on analyte quantification involves calculating concentrations using the two calibration curves with the greatest difference in slope between lots, by applying area ratios from each remaining lot to both curves. The percentage deviation between the two resulting concentrations is then determined, instead of comparing them to a nominal value. This approach builds on the concept introduced by Matuszewski,¹⁵ who suggested that the maximum difference between slopes can be considered an indicator of the largest expected variation in analyte concentration across multiple matrix lots, caused by the relative matrix effect (i.e., variations in ionization and recovery efficiencies). Future studies should explore whether differences in intercept, in addition to slope variability, contribute to this deviation across the calibration range.

3.3. Third Approach: Evaluation of ME, RE, PE, and Their Normalized Factors (MEF, REF, and PEF): Comparison of Its Variation between Matrix Lots. The graphical representation of ME, RE, PE, MEF, REF, and PEF is shown in Figure 3, with their mean and CV values summarized in Table S5.

3.3.1. Impact of Absolute Matrix Effect, Recovered Fraction, and Process Efficiency on Method Performance: Implications of Method Development. The IS showed significantly higher ion suppression than in the standards, as reflected in the MEF values >1. The higher ion suppression in the IS may be due to the coelution of other compounds present in the matrix at the same retention time competing for ionization.⁷ Since some of the chromatographic peak areas of the analytes overlap, mutual ion suppression may occur.^{27,30} To compensate or partially reduce these effects, key strategies include preparing calibration standards and quality control samples in the native matrix, optimizing sample pretreatment, improving chromatographic separation, and selecting an appropriate IS.^{29,31,32} In this regard, it is important to conduct an equivalence matrix study when using a surrogate matrix to confirm matrix-matching calibration and ensure a similar behavior to that of native samples. One possibility is to include the surrogate matrix with the other native matrix lots in the same experiment. Additionally, various extraction procedures were tested during method development to maximize recovery while minimizing sample volume, considering laboratory resources. The current procedure was selected as the optimal one to meet such requirements. On the other hand, while international guidelines recommend using stable isotope-labeled internal standards (SIL-IS), ideally ¹³C, ¹⁵N, or ¹⁷O,^{10,13} deuterated isotopes are an alternative when the preferred isotopes are unavailable. Although C18:0-d5 and C17:0 were tested during method development, the resulting areas lacked reproducibility (data not shown). Finally, modifying chromatography could minimize coelution. If IS are not available for each compound, then changes in the

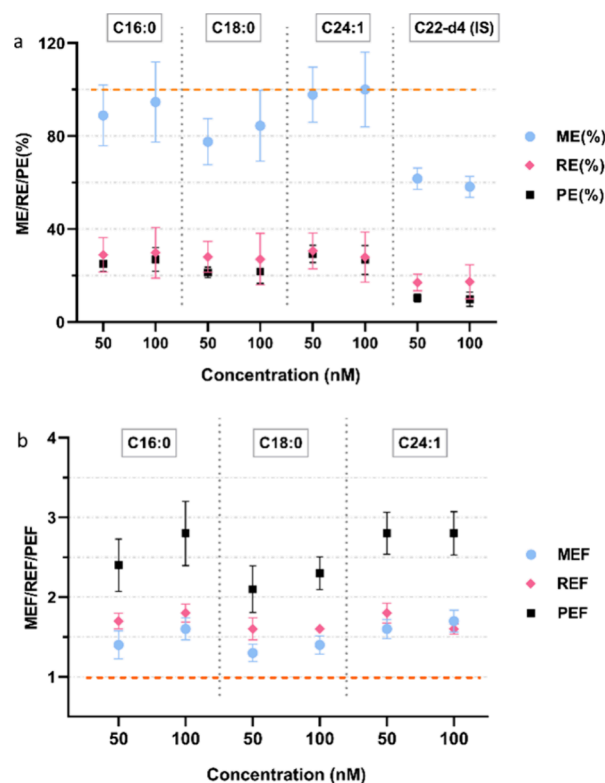


Figure 3. Graphical representation of the ME, RE, and PE values (a) and their normalized-factor by IS (MEF, REF, and PEF) (b) from the third approach. The boxplots (mean + SD) indicate the variability between matrix lots ($n = 3$). The orange dashed line represents the ideal value (100% for absolute values and 1 for normalized factors). The lower ME, RE, and PE of the IS compared to the standards are reflected in the MEF, REF, and PEF >1 for all isoforms and concentrations.

mobile phase composition during gradient chromatography may influence ionization efficiency and introduce potential bias in analyte quantification.

RE and PE values were similar for all standards. Low RE values of the standards may result from losses during extraction, which reduce overall PE and impair the limit of quantification (LoQ).² RE values in our method were similar to those obtained with SPE.³³ The RE of IS was nearly half that obtained for standards, indicating differences in extraction efficiencies. Consequently, the overall PE in the IS was markedly lower, as evidenced by PEF >2 values (Figure 3b and Table S5). Our selected SIL-IS may have chemical structures, physicochemical properties, ionization efficiencies, and retention times that are sufficiently close to, but not identical with, those of the analyte of interest, which may result in variable RE and exposure to ME. Furthermore, deuterated analogues might be unstable, and isotope exchange may occur under extraction conditions.⁷ These differences could explain why the analyte and the IS appear to be affected differently by the overall analytical process. In any case, SIL-IS can help mitigate such effects but may not fully overcome sensitivity loss.³⁴ The higher ion suppression and lower recovery observed in the IS in our case could compromise robustness at low concentrations,¹² especially under nonoptimal instrument conditions. Considering these conditions, the LoQ of our method was set at 5 nM based on precision and accuracy criteria.²⁵

The scatter plots showed a negative trend between ME and RE values for each standard at both concentrations, indicating that lots with higher recovery also had higher matrix effects, and vice versa (Figure S3). This could be explained by increased extraction efficiency, which leads to the coextraction of more interfering compounds that can compete with the analyte during ionization, resulting in higher ME. The correlation observed between ME and RE in the IS was lower, pointing out the differences compared to the standards.

3.3.2. Impact of Variation in Absolute Matrix Effect, Recovered Fraction, and Process Efficiency on Method Precision: Compensatory Capacity of the IS. The parameters discussed above are relevant for understanding method performance but are not definitive indicators of method robustness. A more critical aspect in method evaluation is the study of the variability of these parameters between different matrix lots. The rME and rRE values (Table S5) show variation in the ionic efficiency and recoveries in the standards between different lots (rME: from 12.2 to 18.3%; rRE: from 20.8 to 42.6%), with that of the recoveries being significantly higher. However, when normalized by IS, the corresponding CVs decreased significantly (rMEF: from 7.5 to 12.6%; rREF: from 1.5 to 8.6%), suggesting that the pattern of variation in ionic efficiency and recovery between the different lots appears to be similar in the standards and IS, and therefore, IS compensates for these two factors of variation. Variability also depended on concentration (in our case, rRE and rPE were higher at 100 nM), but this correlation was minimized upon IS normalization. The obtained rPEF of <15% met the acceptance criteria established for this study. This indicates that despite the variability observed, especially in the recoveries, the IS can compensate these factors, enhancing method precision.

It is noteworthy that CVs calculated for ME, RE, PE, and their normalized factors (rMEF, rREF, and rPEF) differ from those derived from absolute areas and area ratios in the first approach. This discrepancy could be explained by considering that the two approaches do not reflect the same aspects. CVs from absolute areas and ratios relate to the cumulative variability from different factors in sets 1, 2, and 3 (analytical system, matrix, and recoveries, respectively), while CVs of ME, RE, and PE reflect variation in ionization, recovery, and overall efficiency, respectively. In addition, as previously reported,¹⁹ one data set is required for the calculation of CVs of peak area and area ratios (sets 2 and 3, respectively), whereas two data sets are required for the calculation of CVs of ME, MEF (sets 1 and 2), and RE, REF (sets 2 and 3), which may lead to increased variability.

3.4. Alignment with Current Guidelines. The overall strategy, as illustrated in the flowchart (Figure 2), highlights the variety of information that can be obtained for method evaluation. For example, assessing the variation in area ratios at each individual concentration in pre-extraction spiked samples (set 3) in line with Matuszewski et al.,¹¹ or calculating slope variations, as described in a later study by the same author,¹⁵ is crucial for evaluating overall method precision. Additionally, deviations from nominal concentrations may be considered to assess how the overall process precision impacts on analyte quantification, aligned with ICH M10⁹ and Matuszewski et al.¹¹ Alternative strategies, building on prior reasoning,¹⁵ such as comparing two concentrations calculated using lot-specific calibration curves, may provide new insights and offer a more accurate estimation of the influence of relative matrix effects on analyte quantification. The CV of the areas from sets 1 and 2,

and the calculation of ME and RE values, is also important to evaluate the factors contributing to method precision, in accordance with Matuszewski et al.,¹¹ and to assess the impact of ionization efficiency and recovery fraction, respectively. The calculation of ME is recommended by CLSI C50¹³ and previous EMA;²² in contrast, RE estimation is described in ICH M10 and previous FDA,¹⁴ although as an independent experiment. However, method robustness is best understood through the variability of these factors in different matrix lots, particularly those IS-normalized values, which provide information on how effectively the IS compensates for such differences, as reflected in CLSI C62.¹⁰ Collectively, the integrated complementary assessments presented in our study provide guidance on the selection of sample preparation procedures, chromatographic conditions, and ISs.

3.5. Limitations. A gradual signal reduction (average of 15–30%, depending on the compound) was observed upon batch reinjection, impacting on ME, RE, and PE percentage calculations, resulting in a decrease in their values, as previously described.¹¹ In addition, it has been reported that the order of sample injection may affect the calculation of ME variability.²⁰ The small sample size limits statistically significant correlations in the scatterplots; therefore, results should be interpreted in conjunction with the qualitative trend analyses. The reliability of this study could be improved by increasing the number of CSF lots, concentrations, and technical replicates. However, obtaining large volumes of CSF is challenging due to the invasiveness of CSF collection. Future studies should explore additional sample conditions, such as hemolyzed matrices, or test multiple IS concentrations within the measurement range, as compensatory behavior may depend on IS levels.²⁷ This study could also be extended to include commercially available glucose and galactose isoforms and their corresponding IS to obtain a more reliable understanding of the matrix effect, recovery, and process efficiency. Additionally, other studies provide broader strategies for identifying additional sources of analyte loss, by investigating the origins of ME and RE, separating the individual contributions of standards and IS, and offering a detailed analysis of the subtraction of the signal from the blank matrix.^{12,17}

Other studies have evaluated isolated ionization efficiency and recovery variations by minimizing methodological variability, comparing CVs of areas between different matrix lots with those from a single lot analyzed repeatedly.^{15,16}

4. CONCLUSIONS

To our knowledge, this is the first study to combine complementary strategies to assess matrix effect, recovery, and process efficiency within a single experiment, in alignment with CLSI and ICH recommendations, following previous works,^{11,15} demonstrating how each approach contributes to a comprehensive understanding of the factors influencing method performance, and enabling the identification and mitigation of variability at each stage of the process. In addition, a Microsoft Excel spreadsheet has been developed to streamline calculations, systematize the process, and facilitate data interpretation. Our work supports compliance with the rigorous standards for bioanalytical method evaluation in clinical laboratories, as required by the new EU IVDR, while promoting adherence to established guidelines. Moreover, it provides novel insights that contribute to the harmonization of in-house method evaluation. This protocol has broad

applicability in analytical chemistry and offers a practical tool for bioanalysis in both clinical and research laboratories.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.5c05399>.

LC-MS/MS method conditions and equations of approach 3, injection order of the samples in the autosampler, preparation of working solutions, and results obtained from the workflow application (PDF)

Data analysis (XLSX)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

LC, liquid chromatography; MS/MS, tandem mass spectrometry; IS, internal standard; IVDR, in vitro diagnostic regulation; ESI, electrospray ionization; GluCer, glucosylceramide; CSF, cerebrospinal fluid; CLSI, Clinical and Laboratory Standards Institute; EMA, European Medicines Agency; VHIP, Vall d'Hebron Initiative for Parkinson; STD, standard; MeOH, methanol; CHCl₃, chloroform; FA, formic acid; IPA, isopropanol; MeCN, acetonitrile; WS, working solution; MPB, mobile phase B; ME, matrix effect; RE, recovery; PE, process efficiency; MEF, matrix effect factor (standard-to-IS ratio for matrix effect); REF, recovery factor (standard-to-IS ratio for recovery); PEF, process efficiency factor (standard-to-IS ratio for process efficiency); rME, relative matrix effect (interlot variability of ME); rRE, relative recovery (interlot variability of RE); rPE, relative process efficiency (interlot variability of PE); rMEF, relative matrix effect factor (interlot variability of MEF); rREF, relative recovery factor (interlot variability of REF); rPEF, relative process efficiency factor (interlot variability of PEF); SIL-IS, stable isotope-labeled internal standard; LOQ, limit of quantification

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