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Article

The Origin-Adjusted Approach for Reliable Quantification of Endogenous Analytes in Mass Spectrometric Bioanalysis

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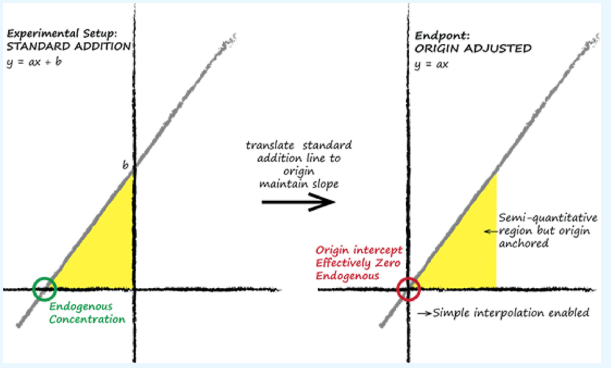
ABSTRACT: The reliably accurate and precise quantification of biomarkers is a priceless objective in the drug development and diagnostic arenas. To employ a technique that brings such reliability and furthermore involves a simpler, faster, and inexpensive regime would only underline the potential importance of the concept and technique. To the existing established approaches for biomarker quantification in bioanalytical LC−MS, surrogate matrix (SUR-M) and surrogate analyte (SUR-A), in this Letter we present an approach that fulfills the aforementioned advantages. The concept builds on the historic method of standard addition (SA), in which one source of biological matrix is spiked with analyte to form a calibration curve. With the SA curve back-calculated, the heart of this procedure is the subsequent adjustment of the intercept to zero, the origin, and using only the slope of the curve for interpolation giving calculated sample concentrations. In SA, the concentration axis intercept indicates the endogenous analyte concentration, and our zeroing of this is equivalent to removing the endogenous level. This key shift of the calculated line to the origin unveils our novel origin-adjusted (OA) approach. It enables use akin to a regular xenobiotic method, with no need to ultimately account for the endogenous analyte level in the control matrix used for calibrants. We present a comparison of OA against the control approach of SUR-M in a representative application for kynurenine and tryptophan in human plasma by LC−MS. A numerical performance analysis performed is demonstrative of equivalence between the two approaches for both analytes.



INTRODUCTION

In the important and fascinating arena of biomarker quantification, there is a dream of finding a way to perform quantification in a simple and reliable fashion, akin to the ease of interpolation through a xenobiotic calibration curve where there are no endogenous levels to deal with, such as in the pharmacokinetic (PK) assay context. To ensure context in this article, the use of calibration curves for quantitative purposes is conducive to accurate data with true concentrations as reference, as opposed to absolute in nature. As things stand, there are two firmly established approaches to overcoming the analytical hurdle of constructing useable calibration curves where an existing endogenous level of analyte is present.[1] These are the SUR-A approach[2−10] and the SUR-M approach[11−18] and both are pivotal on the use of surrogacy in one respect or another, introducing an important compositional change from the quantitative ideal. This aspect pertains to either switching the biological matrix for something chemically treated to strip the analyte from the matrix or even for a solution-based option, both of which equate to a surrogate matrix, and on the other hand, there is the option of switching the analyte for a physicochemically mimicking isotopologue, a stable-labeled analogue, as in the SUR-A approach. Much less popularly, there is also the theoretically ideal scenario reflective of the regular bioanalysis of xenobiotics whereby an unadulterated matrix is spiked with the regular unlabeled analyte reference material. Subsequently, of course, a justifiable means to numerically account for the underlying endogenous concentration must be utilized. One reason why this approach may not have found general favor is likely embedded in the uncertainty involved in the process of removing the calculated endogenous level. The elements of the classical method of SA are at work in this scenario, in order to make this initial determination of endogenous concentration for the matrix used in the calibration sample preparation. In the classical method, several aliquots of a given matrix lot are spiked at different nominal levels, analysis is performed with subsequent linear regression, and the magnitude of the concentration intercept is the endogenous level. In the extension of the classical method to enable interpolation of

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other samples through the back-calculated line, nominal levels of the calibration standards used are adjusted by the endogenous level calculated in the first step, introducing an element of uncertainty to the final results. Perhaps a little confusingly, the name of SA has been assigned to reports of methods where the endogenous level of control matrix has been calculated and then, in the aforementioned extension from true SA, incorporated into the calibrant concentration adjustments to interpolate thus obtain calculated sample concentrations.

The method of SA is itself an option for quantifying endogenous material but is reliant on plentiful sample volume to prepare numerous aliquots of different overspiked levels, unfortunately not aligned with the realities of in-life sample collection. Also, in terms of elucidating the results, the element of extrapolation to obtain the resultant concentration gives hesitancy to many a scientist. SA is nonetheless well-known for wonderful inherent usefulness and reliability in determining the endogenous concentration of a given compound native to a biological matrix, proven in comparative tests, and there is interesting work done on proving the precision and reliability.[19,20]

There are reports of the use of SA in biomarker methodologies in both LC−MS[21] and immunoassay platforms[22] and the use of a variation on the technique called addition calibration in chromatographic and atomic absorption techniques.[23−25] In addition calibration, the slope appears to be embraced as a fundamentally important parameter but the approach involves accounting for the endogenous level in the genuine matrix by prior calculation and subsequent spiking.

The importance lying in the slope of SA calibration lines is further realized in recent work where averaging over many replications is performed to give a 'universal slope' for rapid application of SA[26] and then slope and intercept criteria are proposed for selecting usable lots of surrogate or authentic matrixes in LC−MS.[27] This is insightful work that recognizes the value of the slope in such proceedings but shies away from SA in favor of the fully characterizable calibration ranges of the surrogate approaches, focusing on ascertaining appropriate slope and intercept criteria for moving forward with surrogate selection.

To return to the related underlying enticing notion, borne from classical SA, what if an approach could be offered in which no surrogate matrix or surrogate analyte is used and also without a requirement to use SA to calculate endogenous levels and make associated adjustments, thus obviating the need for parallelism and setting the scene akin to simple xenobiotic quantification? Figure 1 shows the ideal nature of the sample makeup, the OA preparation matching the incurred sample while the SUR-M and SUR-A spiked samples have clear differences that necessitate parallelism tests and introduce innate uncertainty.

A line is constructed with effective zero intercept and a slope characteristic and representative of the true concentration−response relationship of the analyte within the method. We propose an adjustment to a line of SA construction, an adjustment that is elegant in simplicity and confidently justified, based on proven linear regression characteristics of bioanalytical LC−MS methods. In the well-known line of regression of y on x, response on concentration, we have the slope a and the response intercept b (eq 1).

$$y = ax + b \tag{1}$$

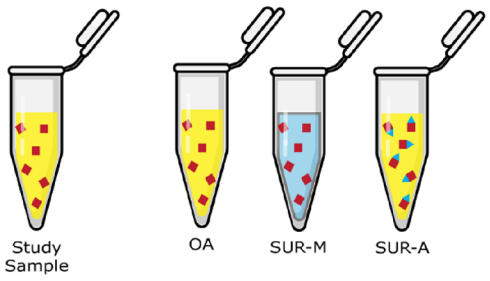

Figure 1. Representation of sample compositions between the approaches. SUR-M has a different composition from study samples and SUR-A uses a different reference material for quantification, while OA is identical to study samples.

The slope is characteristic of an analyte within a methodology, indeed technically defining the sensitivity.[10] Additionally, in terms of the intercept, the reality and expectation for any xenobiotic method is that the line essentially intercepts the origin, within a small margin of natural and innate instrumental error, mainly since there is innately no xenobiotic analyte in the control blank matrix. This may be observed for any well-performing and contamination-free xenobiotic method, where the response intercept is far less in magnitude than the slope and characteristically varies between negative and positive in this relatively tiny window centered on the origin. As such, the intercept may intuitively be considered to approach exactly zero as an average over sufficient experimental iterative occasions. Hence, the adjustment we propose and put to the test, which we term 'origin-adjustment', is a simple shift of the back-calculated line, maintaining the slope, to exactly intercept the origin. In other words, adjusting the equation of the line to only involve the slope in the relationship of concentration and response (eq 2)

$$y = ax \tag{2}$$

This is the origin-adjusted (OA) equation.

The process of translation of the line to intersect the origin may be viewed as analogous to the removal of the endogenous concentration. Then, the calibration line, free of endogenous interference, is used for simple interpolation of sample responses to give calculated concentrations, like a regular PK xenobiotic assay scenario. The TOC Graphic shows the process pictorially.

RESULTS AND DISCUSSION

Our example application to show the validity of the new OA approach is based on the important dual analyte assay in the field of biomarkers, tryptophan and kynurenine in plasma which has been reported by SUR-A[5-7] and SUR-M.[17,18] Linking the reasoning for our choice of SUR-M as the control approach for comparative assessment of the OA approach, the reported SUR-A approaches[5-7] all involve the use of response factors (RFs) to account for response differences, hence calibration curve slope differences, between the unlabeled analytes and the surrogate analytes. In instances where this correction is necessary, it is surely deleterious to the reliability of a method or approach. Parallelism is critical, as in uniformity of linear regression characteristics, particularly the slope, to validly represent the genuine analyte's response−concentration characteristics, and this is ideally done without manual intervention.

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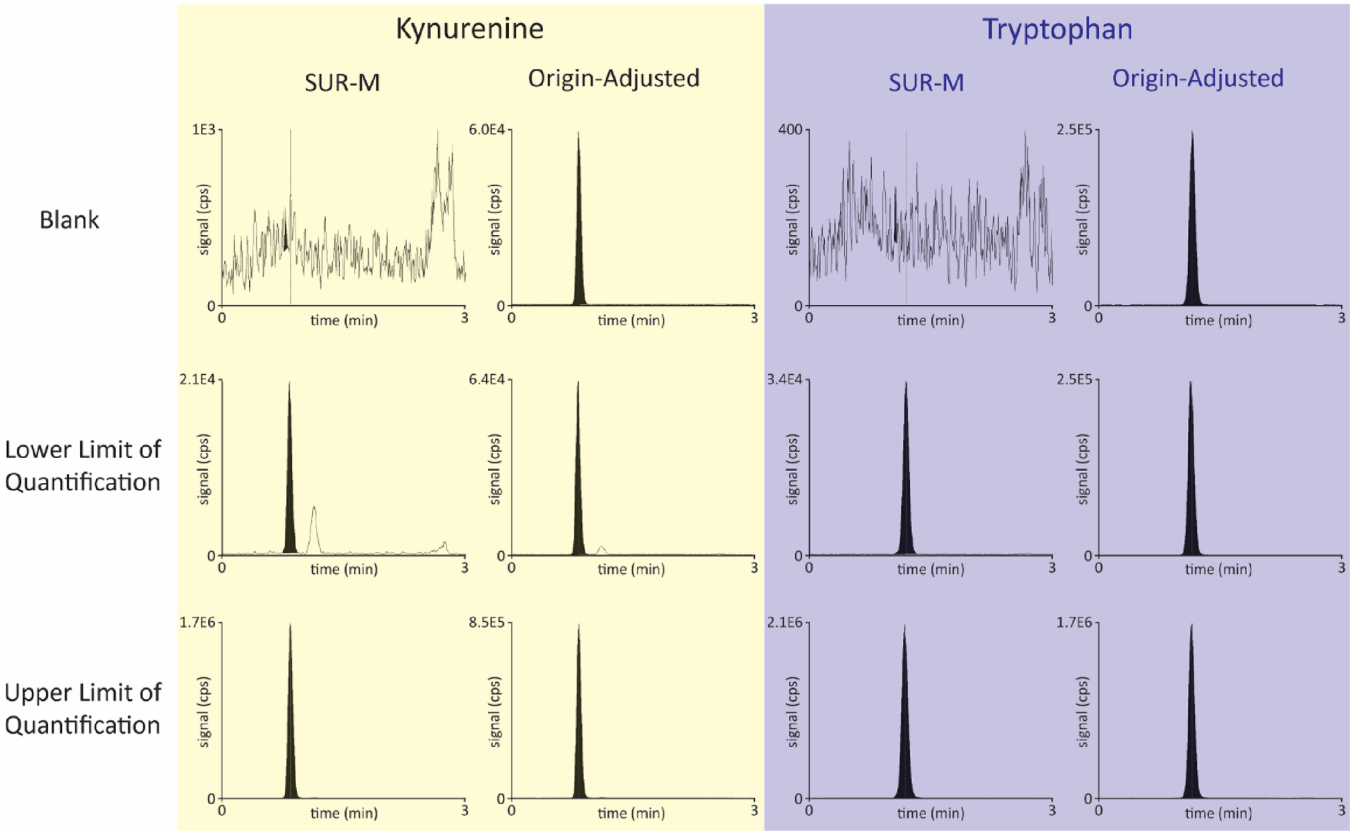

Figure 2. Representative chromatograms.

Table 1. Percent Differences of QC Samples Interpolated through SUR-M and OA Calibration Lines

kynurenine			tryptophan		
	SUR-M	OA		SUR-M	OA
slope	32.3	34.8	slope	2.20	2.37
intercept	0.166	origin	intercept	0.0404	origin
(n = 6)	% difference		(n = 6)	% difference	
LLOQ QC	13.8		LLOQ QC	8.37	
low QC	−0.338		low QC	−2.33	
mid QC	−6.83		mid QC	−7.00	
ULOQ QC	−7.18		ULOQ QC	−7.25	
mean	−0.137		mean	−2.05	

Our initial phase of testing involved a comparison of SUR-A, SUR-M, and OA sample preparation and analysis for both analytes, using a previously validated SUR-A method for the quantification of two analytes from the kynurenine pathway.[6] Briefly, the scheme involves kynurenine (0.0250 to 2.50 μg/mL) and tryptophan (0.500 to 50.0 μg/mL) quantified in human plasma via HPLC and tandem mass spectrometric detection. The design throughout involved duplicate calibration curves at eight nominal calibrant levels. Chromatography on the stated column and gradient[6] was performed on Shimadzu 20-Series HPLC systems with associated mass spectrometric detection performed on Sciex 5500 instruments in multiple reaction monitoring (MRM) mode. Chromatograms are shown in Figure 2, with the most notable difference being the presence or absence of peaks in the blank sample, aligned with the nature of the approach.

The surrogate analyte was a $^{15}N_2$-labeled isotopologue for kynurenine and the associated IS in all approaches, D4-kynurenine. The surrogate analyte was a $^{15}N_2$-labeled isotopologue for tryptophan and the associated IS in all approaches, D5-tryptophan. The surrogate matrix used was 0.2% (by volume) formic acid (aq). OA calibrants were in a genuine matrix, like for SUR-A, and genuine unlabeled analytes, as per SUR-M, thereby embracing all authenticity available. The results showed that indeed there were correction factors necessary for SUR-A in that the slopes calculated manifestly did not agree with the slopes from the OA and SUR-M approaches. Meanwhile, the slopes from the latter two approaches did correlate convincingly with each other, most notably for kynurenine. The data are shown for both analytes in Table S1. This experiment was repeated for confirmation (data not shown). Therefore, in consideration of the response difference for SUR-A and tying this to the criticality of the

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slope and how it relates to parallelism, it was clear that the only option in this work was to use SUR-M as the comparator approach. This holds the additional ideal of representing the most popular and convenient approach for biomarker quantification.

The analytical facets of the main body of the work included, for both analytes, an initial numerical comparison of the slopes generated from six separate analytical occasions, and then for the latter analytical batch in the sequence, the two sets of QC sample concentrations interpolated through the SUR-M line and the OA line are compared. The QC samples were all prepared in the SUR-M manner and at a replication of $n = 6$ for each level.

Table S2 shows the data resultant from the multibatch slope comparisons, where the slope, as described, is the critical component for reliable calculation of sample concentrations. For both analytes, the percent differences are demonstrative of equivalence between SUR-M and OA. In the case of kynurenine, the difference never exceeds a magnitude of 13.2%, and the overall average difference over the six analytical occasions is −6.05%. In the case of tryptophan, the data are even more convincing. The difference never exceeds a magnitude of 8.56%, and the overall average difference over the six analytical occasions is −1.63%. These numbers speak for themselves in the context of typical bioanalytical acceptance criteria for percent difference, firmly established at within ±15%.

Table 1 shows the data for the slope and interpolated QC sample comparisons. In a manner aligned with the slope test (*vide supra*), the results of this test also strongly indicate that OA, for both analytes, is equivalent to SUR-M. For each analyte, the QC samples, prepared in the SUR-M manner, have peak area ratio responses interpolated through the SUR-M line complete with the intercept. At the heart of this test lies the sample responses being interpolated through the OA line, with the slope generated from its own back-calculation and the intercept simply set to zero, the origin-adjustment. For kynurenine, the difference never exceeds a magnitude of 13.8%, which is seen at the lower limit of quantification (LLOQ), and the overall average difference over the six analytical nominal concentrations is −0.137%. For tryptophan, the difference never exceeds a magnitude of 8.37%, which again is seen at the LLOQ, and the overall average difference over the six analytical nominal concentrations is −2.05%. The LLOQ showing the most difference in both cases is likely an artifact of the closest proximity to the origin and where the imprecision is highest for the extrapolative approach.

It may readily be observed that there exists a semi-quantitative region in the calibrations, as denoted by the yellow area within Figure 1. This region is below the point of lowest calibrant peak area, hence it lacks complete characterization in the same way that SA extrapolates from the origin to the intercept. The region extends to zero, the origin. Any question marks are offset quite elegantly, however, by the nature of the origin in the scheme. In the way it is used, within the very definition of the approach, the origin acts as an anchor point. Therein, the line anchored to the origin scythes through the undefined region between itself and the lowest calibrant peak area responses, and it similarly scythes through the imprecision encountered with the lower concentration calibrants. Indeed, it presents the alternative term of origin-anchored approach, in a more colloquial sense. Precision is poorest at the lowest concentrations, and this is directly linked to the nature of spiking the calibrants atop an existing endogenous level, 'overspiking', in conjunction with the heteroscedasticity of the concentration−response relationship. The inherent response variability observed for a given nominal concentration calibrant sample is in reality that associated with the endogenous in addition to the overspiked nominal, more variability than in a PK assay calibration and which will worsen with increasing underlying endogenous. It will also affect the lower concentration calibrants more than higher. It is important to realize however that in addition to the anchor confronting this calibrant effect, this added imprecision would not be associated with real study samples as they will not have been overspiked prior to analysis; thus, their response variability will not deviate from that of their own innate levels.

In further commentary about the semi-quantitative region, we can readily acknowledge that biomarker levels can vary in either direction, naturally, but must also be considered unlikely to take a drastic drop toward zero in the context of the characteristic window of native concentration variability. Furthermore, having sample levels impinge on the semi-quantitative region is unlikely to affect a clinical outcome. It may also be borne in mind that, in this region, if an empirical degree of concentration−response characterization is ever called for, it can be done in a solution or surrogate scenario. Then, it is also worth acknowledging that nonlinearity or curvature effects in LC−MS typically will not be manifest in lower concentration regions but rather at higher concentrations. Imprecision may be encountered, synonymous with matrix effect, which may sometimes seem like curvature but in reality is not. Again, the origin anchor helps in this regard.

Further to the anchor notion, the work done in ref 27 actually touches on a key dynamic of the possibility of using the SA slope in interpolation. However, there is no recognition of the anchor utility of the origin in addressing the semi-quantitative region, which is shied away from, and the use of the origin seems uncertain and tentative in this context. The thrust of the work is in advocating surrogate matrix approaches using carefully established slope and intercept criteria.

It is also noteworthy that any previous work that has involved SA in any application could be 'mined' to verify the approach retrospectively. The slopes of these existing calibration data could be harnessed for interpolation of study samples, while the intercept is set to the origin. The data would, in theory, match the data emergent from the more laborious subtractive accounting for the calculated endogenous. The authors invite the readership to investigate as such any prior work in their archives, which is of the SA design.

To touch again on the broader perspective, it remains true that the validity of the origin intercept and furthermore the results obtained through OA are as innately reliable as the endogenous concentrations calculated through classical SA where the intercept is utilized to this end, via extrapolation. The origin anchor is as valid as the reliability of classical SA-calculated concentrations, known to be reliable despite the element of extrapolation. In a well-designed calibration scheme with carefully placed nominal levels and sufficient replicates, the characterization of the calibration line afforded from above the endogenous level is sufficient for reliability. The non-zero blank, unspiked authentic matrix, may also be used in the regression for added characterization, although we did not in this work.

Finally, to dwell briefly on the way in which the OA scheme characterizes the calibration line and how this affects the

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important bioanalytical procedure of dilution, it must be first emphasized that dilution, on the rare occasion that it would need to be used in a biomarker quantitative context, would need to be with a non-authentic matrix in order to be analyte-free. This itself obstructs the underlying reliability of the approach but under the circumstances should be established as useable with a single parallelism experiment between the authentic matrix and the composition resultant after the proposed dilution. Such dilutions would also introduce a possibility of a differential-type matrix effect involving the compositional change, and the aforementioned single parallelism experiment would suffice, provided there are enough replicates for definition.

CONCLUSIONS

A novel multiadvantageous approach, referred to as origin-adjusted, for the determination of biomarkers in bioanalytical LC−MS has been presented. The approach firmly grasps the ideals of using a genuine unadulterated matrix together with genuine unlabeled analyte reference materials, obviating the need to prove parallelism in this context. The manner in which the endogenous level is removed from an OA calibration line, which is initially constructed as per standard addition, is to simply remove the intercept from the calculated regression equation and use only the slope. In essence, translating the line to intercept the origin while maintaining the slope thereby eliminating the endogenous level from calibrants, and interpolation of study samples' responses can then take place through the OA line, giving reliable calculated concentrations. Furthermore, in the same way that the concentration-intercept in standard addition is a reliable measurement of the endogenous, the origin becomes a reliable anchor point in OA, addressing concern over the semi-quantitative region of the graph below the peak area response of the lowest concentration calibrants. This semi-quantitative region is one possible perceived limitation, as described, where the established surrogate approaches show full curve definition. Interpolated concentrations may sometimes show differences between approaches, especially if the endogenous level in the OA control matrix is high, altogether requiring careful consideration for decision making and outcomes. The OA approach may nonetheless be embraced in its elegance, simplicity, speed, affordability, and reliability. An approach that could even innately bring the perception and performance expectations of biomarker assays closer to those of PK assays.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c06850.

Table S1: initial test results comparing slopes from SUR-A, SUR-M, and OA; Table S2: slope percent differences between OA and SUR-M (PDF)

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Notes

The authors declare no competing financial interest.

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