2H SOLCOR: A novel tool for reducing volume variation as a source of error in external standard quantitative NMR

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Abstract
Tube to tube volume difference presents a challenge in obtaining correct external standard quantitative NMR (esqNMR) results. Deuterium (2H) NMR is easily observable, intrinsically quantitative, present in all samples, free of interfering signals, and insensitive to probe tune/match and sample saltiness. These properties make 2H peak integral an ideal parameter in esqNMR for correcting volume differences between the reference standard and analyte. We demonstrate a novel and practical technique abbreviated as “2H SOLCOR” (2H SOLvent CORrected), where the 2H peak integral from the solvent is used as a universal internal standard to correct volume variations in NMR tubes, thereby improving accuracy and precision of esqNMR method. Herein, this simple yet effective technique is described, and practical considerations for successful implementation are presented. 2H SOLCOR can be applied anywhere esqNMR is used, including where precious samples need to be accurately quantified for qualification as an authentic analytical standard.

KEYWORDS
quantitative NMR, 2H deuterium observe, calibration standard, tube volume variation

1 | INTRODUCTION

Internal standard quantitative NMR (isqNMR) has a well-established history of providing precise and accurate results in the hands of a skilled NMR spectroscopist with carefully prepared samples, standards, and thoughtfully designed NMR acquisition and processing parameters. Recently, external standard quantitative NMR (esqNMR) has gained increasing popularity with several distinct advantages as noted in Table 1, specifically in situations where the sample is precious and must be recovered for use in subsequent studies. The advent of highly stable NMR radio frequency hardware, linearized amplifiers, and auto tune and match probes, all of which are now considered the industry standard, have also contributed to the rising popularity of esqNMR. Despite the technological advancements, there has been some reluctance to adopt the external standard method due to several potential sources of error including NMR tube volume variation. To illustrate, Figure 1 provides a depiction of two likely scenarios where tube volume variations between the reference standard and the sample contribute to over/under reporting of sample purity or concentration. The absolute integration (AI) is proportional to the number of nuclei in the active coil of the probe, which equals the product of sample concentration and active volume. To date, most attempts at reducing NMR tube volume variation, as a source of error in esqNMR, have focused
on the use of higher quality, more expensive NMR tubes with well controlled wall thickness and diameter.\cite{4,6,7}

In this manuscript, we present an alternative solution to reduce the problem of NMR tube volume variation by using deuterium NMR (deuteron, or $^2$H), with the resulting peak integral to serve as a proxy for volume. We have called this technique “$^2$H SOLCOR” ($^2$H SOLvent CORrected) where deuterium in NMR solvents, ubiquitously present in solution NMR, is utilized as an internal reference standard (calibrant) to track volume in the external reference standard qNMR analysis. Although various techniques have been employed with residual protonated solvent signals and secondary reference standards serving as isqNMR standard,\cite{1,7-10} we outline the distinct benefits of $^2$H observation, and its role in volume normalization. SOLCOR is a novel hybrid internal and external standard qNMR technique without sample contamination. Given appropriate acquisition and processing parameters, the SOLCOR technique can be used for any NMR active nuclei.

### 2 | EXPERIMENTAL

NMR spectra were collected using quantitative acquisition parameters (Table S3) on a Bruker 400 MHz Ultrashield Magnet with Avance III HD Console, and Prodigy Cryoprobe CPP BBO-H&F 5 mm running Topspin v3.6.2 and ICONNMR (v 5.0.10 build19). All amplifiers had been linearized using Bruker’s Cortab protocols. ICONNMR automation software with the configuration of the tune/match (T/M) settings set to “every experiment,” “tune exact storwobb” for the proton ($^1$H) experiment and the wobb curves for different samples were overlaid and visually inspected for any outliers. Deuterium ($^2$H) experiments were run unlocked, with T/M manually obtained using a representative sample and the setting saved subsequently for all other samples. Spectra were carefully processed and integrated\cite{11,12} (Table S3) using MestReNova v12.0.4 software. Wilmad Lab glass 5mm NMR tubes of varying quality and Bruker 1.7, 3.0, and 5.0 mm

### Table 1

Comparing advantages and disadvantages of isqNMR and esqNMR

| Characteristic          | isqNMR | esqNMR |
|-------------------------|--------|--------|
| Spectral overlap        | ☒      |        |
| Sample recovery         | ☒      | ☑      |
| Reactivity              | ☒      | ☑      |
| Standard preparation burden | ☒ | ☑       |
| NMR tube volume variation | ☑ | ☒       |

Note. A pictorial outlining the differences between isqNMR and esqNMR is provided in Figure S1

Abbreviations: esqNMR, external standard quantitative NMR; isqNMR, internal standard quantitative NMR.

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**TABLE 1** Comparing advantages and disadvantages of isqNMR and esqNMR

**FIGURE 1** Two scenarios of tube volume variation and their implication on accuracy of external standard quantitative NMR (esqNMR) results
sample jet tubes (Bruker part #'s: Z106462, Z112272, Z112273) were used (Table S1). Sigma Aldrich TraceCERT® quantitative NMR maleic acid (MA; product #: 92816) and benzoic acid (product #: 06185) were used as certified reference material. Sigma Aldrich dibucaine hydrochloride (DBC; product #: D0638) and sodium chloride (product #: 746398) as well as Cambridge Isotope labs DMSO-d6, 99.9% (item #: DLM-10-10) were used for sample preparations.

3 | RESULTS AND DISCUSSION

The relationship between three variables (integration, concentration, and volume) is depicted in Figure 2, outlining how knowledge of any two variables allows calculation of the third variable. The relationship between these three variables holds true for any observed nuclei given appropriate acquisition and processing parameters. The traditional 1H esqNMR method has assumed a constant volume from tube to tube allowing the calibration of AI with a certified reference standard and subsequent concentration determinations of samples. For the 2H nuclei, given a constant concentration, the AI effectively serves as a proxy for tube volume. Consideration should be taken to prepare reference standards and samples at similar mg/ml concentrations as this influences the accuracy of volume determinations as detailed in Section 3.4. Finally, sample manipulation or dilution can be effectively corrected using 2H AI as described in Section 3.6.

3.1 | 2H Integral as a proxy for volume

To demonstrate the impact of NMR tube volume variation on absolute proton integration, an experiment was designed composed of three separate NMR tubes types with significant volume differences. A solution of 50-mM MA in DMSO-d6 was transferred into twelve 5-mm NMR tubes. Ten of these tubes were low cost economy grade, one was a medium quality grade, and one was high quality grade (Table S2).

This simple experiment demonstrates that using a thinner wall, higher quality tube allows more spins in the active volume of the NMR probe. Incidentally, vendors’ standard NMR samples are prepared in thin wall high quality tubes for this exact reason, because more sample in the active volume of the probe results in greater signal-to-noise ratios (SNRs) and thus provides greater ease for satisfying the acceptance criteria for SNR.

Seeking a solution to the tube volume variation problem, we hypothesized that deuterium, which is present in each of these twelve tubes at the same concentration, could serve to normalize the tube volume variation. The collection of a 2H spectrum on each of the 12 tubes and the subsequent processing yielded an AI that was directly proportional to the volume of the NMR tube. The

![Figure 2](https://via.placeholder.com/150)

**FIGURE 2** The relationship between absolute integral, concentration, and volume
parameters for the quantitative $^2$H observe experiment are listed in Table S3.

In traditional esqNMR, the concentration of the unknown is obtained by dividing the $^1$H integration of the unknown sample by $^1$H integration of the reference standard and multiplying the result by the concentration of the reference standard (Equation 1). This convention assumes that the tube volume difference between unknown and the reference standard are negligible. This assumption hinges entirely on the quality and consistency of NMR tubes, and any deviations will lead to an over or underestimate of the concentration (purity) of the unknown.

$$C_{1u} = \frac{I_{1u}}{I_{1s}} C_{1s}$$  \hspace{1cm} (1)

In this equation, C is concentration, and I is absolute integral normalized against relevant parameters such as number of scans, receiver gain, and 90-degree pulse length; subscript 1 denotes proton, subscript s denotes standard, and u denotes unknown.

Equations (1)–(14) in the supporting information derive the relationship between $^2$H AI and tube volume with two assumptions. The first assumption is that method precision, both acquisition and processing, is adequate to allow $^2$H integration to serve as a proxy for volume. The second assumption is that the concentration of deuterium in the samples and calibration standards are equal. In cases where the concentration of deuterium in samples and standards is similar but not exactly equal, $^2$H AI serves to approximate the tube volume. These assumptions are experimentally tested in Section 3.

Combining Equation 1 with $^2$H SOLCOR correction factor $(I_{2u}/I_{2s})$ yields Equation 2.

$$C_{1u} = \frac{[(I_{1u}/I_{1s})/(I_{2u}/I_{2s})]}{C_{1s}}$$  \hspace{1cm} (2)

Applying the SOLCOR correction to proton integrations of the 12-tube sample set resulted in significantly reduced the relative standard deviation (2.32% to 0.35%) as well as the min/max range (99.1%–105.9% to 99.1%–100.3%). This suggests that SOLCOR could effectively normalize variations in NMR tube volume in this sample set as seen in Figure 3. Additional experiments (Figure S2) using NMR tube of different diameters (5, 3, and 1.7 mm) demonstrate that $^2$H SOLCOR can correct large volume differences. This correction captures both nominal internal diameter difference as well as factors beyond simple geometry, and it is applicable to variations of both axial and radial dimensions in a Shigemi assembly (Figure S5).

None of these results should come as surprise because deuterium is simply another NMR observable nuclei benefiting from the same properties (dynamic range, linear response, etc.) that make proton qNMR so reliable. It is worth noting that deuterium has its own set of properties that need to be addressed to allow for accurate quantitation. Deuterium signals are characteristically broad, due to quadrupole relaxation, but benefit from absence of the usual interfering signals allowing appropriately wide integrations (see Figure S4 for a representative $^2$H spectrum). In contrast to proton 90° pulse width, the impact of salt concentration on deuterium 90° pulse width is negligible, due to its low Q factor (Table S4), eliminating the need for correction or deuterium tune and match.
adjustments. Deuterium signals also exhibit shorter $T_1$ values (Figure S3) than typical proton signals, allowing the experiment to be carried out quickly using a short relaxation delay.\[13\] Additionally, all modern high field NMR spectrometers are equipped with a separate radio frequency channel devoted to the deuterium nuclei for magnetic field lock with a dedicated amplifier. Tuning and matching are often performed automatically. Essentially, a universal internal standard deuterium has always been present in all routine NMR, although its use as quantitation calibrant has been underutilized thus far.

3.2 Evaluating $^1$H and $^2$H absolute integral accuracy and precision

Measurement uncertainty is a fundamental aspect for validating any quantitative assay. Protocols for a quantitative NMR method validation have been thoroughly examined.\[14\] For the determination of the combined acquisition and processing error, acquisition and processing of the same sample solution in the same NMR tube must be replicated and analyzed to obtain the AI range. It is essential that each of these replicate acquisitions should mimic real life samples in that the NMR tube should leave the magnet each time and the operations of sample insertion, locking, tuning/matching, shimming, receiver gain adjust, and acquisition should be repeated for every replicate. Depending on the spectrometer configuration (magnet, console, probe and its tune/match circuitry, RF component such as amplifier, sample changer, lift calibration, shim system, etc.), any one of these operations could add an error to the esqNMR result. The relative error contribution of each of these operations may vary from one configuration to the next. Inconsistencies in sample positioning relative to coil, tune/match efficiency, shimming, pulse power, phase correction, baseline correction, receiver linearization, integration ... etc. may result in error exceeding the expected error in tube volume variation. Without knowledge of the acquisition/processing error,\[11,12\] it is virtually impossible to provide quantitative results with defined uncertainty ranges, let alone successfully correct for tube volume variation. It has been well documented that imperfect tune/match results pose a challenge in obtaining accurate and precise quantitative esqNMR results.\[14,15\] The optimal tune and match settings that result in the lowest integration variation for replicate acquisitions need to be determined for the spectrometer and probe being used as well as the samples being measured. The tune and match of deuterium channel were observed to be much less sensitive to changing sample concentration or salt content, compared with tune/match of proton channel. This can be attributed to the low Q factor of deuterium channel. Even without the implementation of the $^2$H SOLCOR, it is worthwhile determining this error for each nucleus observed. The combined acquisition and processing error range for $^1$H and $^2$H should be an order of magnitude lower than the expected tube volume variation for the $^2$H SOLCOR to work effectively. Figure 4 shows an example where the $^1$H acquisition and processing combined error range is 0.66% and $^2$H acquisition and processing combined error range is 0.42%.

The results of using the $^2$H SOLCOR method to successfully reduce tube volume variation error range from
2.2% to 0.5% in seven separate economy grade NMR tubes are shown in Figure 5. This example is representative of volume variations typically encountered when selecting seven economy grade NMR tubes from the same batch. The precision is adequate to allow $^2$H integration to serve as a proxy for volume.

### 3.3 Comparing esqNMR $^2$H SOLCOR with isqNMR

The next experiment compared isqNMR, which is generally accepted as the qNMR technique with the lowest uncertainty,\(^{[20]}\) to esqNMR and esqNMR with $^2$H
SOLCOR using 42 economy grade NMR tubes. For this experiment, Sigma Aldrich qNMR TraceCERT® MA served as both the isqNMR and esqNMR calibrant. DBC served as the analyte for potency determinations. Stock solutions of MA and DBC in DMSO-d6 were prepared in triplicate. One NMR tube filled with 600-μl stock solution served as the esqNMR reference standard. Three sets of 14 randomly selected economy grade NMR tubes were filled with each of these solutions for a total of 42 NMR tubes. ¹H and ²H qNMR spectra were acquired for each of the 42 samples. The potency of the DBC was calculated on each of 42 samples using three separate methods (isqNMR, esqNMR, and esqNMR with ²H volume correction). A radar plot was created (Figure 6) to demonstrate the effect of NMR tube volume variation on both the accuracy and precision of esqNMR potency.

The isqNMR method resulted in an average potency of 95.3% with a 0.2% relative standard deviation (RSD), whereas the esqNMR method resulted in an average potency of 95.7% with a 1.2% RSD, and finally the ²H SOLCOR esqNMR method resulted in both improved accuracy and precision, with an average potency of 95.2% with a 0.3% RSD. As the radar plot indicates, the ²H SOLCOR esqNMR method effectively corrected the NMR tube variation outliers. At first glance, the improvement in results using ²H SOLCOR esqNMR may seem exceptional compared with the noncorrected esqNMR results but this sample set benefits from the averaging of 42 tubes. In practice, esqNMR assays rarely employ such a large sample set, and the typical selection of a handful of NMR tubes may contain a volume outlier and thus lead to less accurate results in the absence of SOLCOR.

Error analysis (Table 2) of this 42 NMR tube sample set was completed to determine the contributions of errors in sample preparation, acquisition, and processing to the total error. Acquisition error (AE) includes sample insertion, locking, tuning/matching, shimming, receiver gain adjustment, and tube volume associated errors. Relative standard deviation percentage (RSD%) was tabulated for each quantitation method. Note the reduction in AE from 1.14% using esqNMR to 0.29% using ²H SOLCOR that reveals the utility of SOLCOR in reducing

| qNMR method      | Total error of precision (TE) | Sample prep error (SPE) | Acquisition error (AE) | Processing error (PE) |
|------------------|-------------------------------|-------------------------|------------------------|----------------------|
| isqNMR           | 0.25                          | 0.15                    | 0.20                   | 0.05                 |
| esqNMR           | 1.15                          | 0.15                    | 1.14                   | 0.05                 |
| ²H SOLCOR esqNMR | 0.32                          | 0.15                    | 0.29                   | 0.05                 |

Abbreviations: esqNMR, external standard quantitative NMR; isqNMR, internal standard quantitative NMR.

**FIGURE 7** ¹H/²H relative integrations for varying molar concentrations (mM) of dibucaine and maleic acid in DMSO-d6. DBC, dibucaine hydrochloride; MA, maleic acid.
tube volume variation error. This further supports the validity of assumption one of the SOLCOR method.

3.4 | Solute concentration influences $^2$H integration

$^2$H SOLCOR methodology assumes that the concentration of deuterium in each tube is the same. Although this is the case in the experiment outlined in Figure 6, it might not be the case in a typical esqNMR assay. The impact of varying solute (dibucaine and MA) concentration on the deuterium AI was examined in this section. Using 10 high-quality NMR tubes, solutions of 208-mM dibucaine and 208-mM MA stock solution were prepared. Four subsequent serial dilutions were made using the same stock solution of DMSO-d6. Quantitative $^1$H and $^2$H spectra were collected for each of these ten solutions as well as the stock DMSO-d6. Spectra were carefully processed and the linearity of the $^1$H relative integral (relative to stock 200 mM in DMSO-d6) in response to concentration was determined. Additionally, the linearity of the $^2$H normalized integration (relative to stock 100% DMS effectively overcomes the challenges esqNO-d6) in response to concentration was determined. The results of this experiment are plotted as Figure 7. The experimentally observed drop in $^2$H AI from 100% (pure DMSO-d6) to 93.8% (208-mM dibucaine in DMSO-d6) and 98.5% (208-mM MA in DMSO-d6) underscores the importance of preparing the external reference standards and the samples at similar solute concentrations expressed in mg/ml (or mg ml$^{-1}$) rather than similar molar concentrations in mM.

Figure 7 clearly demonstrates the linear ($R^2 = 1$) and unicalibrant characteristics of quantitative $^1$H NMR. The effect of solute concentration on the $^2$H AI is also demonstrated in this plot. The $^2$H integration for equal molar concentration samples of MA and dibucaine seemingly track each other up to 25 mM concentrations yet deviate at higher mM concentrations. Although the deviation at higher concentrations seems counterintuitive, this phenomenon is explained in Figure 8a, illustrating the relationship between $^2$H solvent concentration and $^2$H solute mM concentration. While MA and dibucaine are present at the same molar concentration (same $^1$H integration), the resulting $^2$H integrations are not equal as depicted by two different shades of green with dibucaine excluding more deuterium due to its larger molecular size. The assumption of Equation 2 that $^2$H concentration be the same for standards and unknown samples is not met in Figure 8a. When the unknown samples and standards are prepared at similar mg/ml concentrations, similar $^2$H concentrations result as in Figure 8b (similar green shading for both NMR tubes) and assumption number two of SOLCOR is satisfied thus permitting the $^2$H integration to serve effectively as a proxy for tube volume.

The experimental manifestation of Figure 8b is provided as Figure 9 where the $^2$H integration versus solute

![Figure 8](image-url)
**FIGURE 9** $^1$H/$^2$H integration versus solute concentrations (mg/ml) of dibucaine and maleic acid in DMSO-d$_6$. DBC, dibucaine hydrochloride; MA, maleic acid

**FIGURE 10** Concentration determination using esqNMR with $^2$H SOLCOR and PULCON compensate for tube volume variation and salt concentration error with an accuracy and precision approaching isqNMR. esqNMR, external standard quantitative NMR; isqNMR, internal standard quantitative NMR
concentration (mg/ml) is plotted. When the $^2$H concentrations of MA and dibucaine samples are similar then the slopes of the $^2$H integration vs solute mg/ml concentration are nearly identical for both MA and dibucaine, demonstrating the effectiveness of $^2$H integration to serve as a proxy for tube volume. One should be mindful that differences in $^2$H concentration, resulting from vast differences in solute concentration (mg/ml) between reference standard and sample, may compromise the accuracy of the $^2$H SOLCOR volume correction. This situation may exist in determination of a solution mM concentration where the total solute quantity is unknown.

### 3.5 Combining $^2$H volume correction with PULCON

Confident of the accuracy and utility of the $^2$H SOLCOR esqNMR in correcting tube volume variation but still wary of esqNMR inaccuracies introduced by salty samples, we devised a blind experiment combining SOLCOR and PUlse Length-based CONcentration (PULCON determination corrections)\(^7\) techniques. The full PULCON calculation was not employed here rather PULCON's foundational concept of reciprocity was used to correct integrations for 90° pulse variations. A blinded experiment was devised consisting of 12 samples prepared in random NMR tubes (high-quality and economy grade) and spiked with varying sodium chloride concentrations (0, 49, 214, 728, 834, and 999 mM). Analyst one prepared a stock solution of MA in pure DMSO-d6 and transferred 600 μl to a high-quality NMR tube to serve as the esqNMR reference standard. Analyst one then prepared 12 samples of DBC in the MA in the pure DMSO-d6 stock solution as well as the stock solutions spiked with various concentrations NaCl and transferred 600 μl to high-quality and economy grade NMR tubes. Analyst two, unaware of the tube quality or salt content of the 12 samples, performed concentration determinations using isqNMR, esqNMR, $^2$H SOLCOR, esqNMR with PULCON corrections and esqNMR with $^2$H SOLCOR and PULCON corrections.

The combination of $^2$H SOLCOR and PULCON effectively overcomes the challenges esqNMR encounters with different tube volumes and sample salt content as shown in Figure 10. The high salt content samples provide a significant challenge to the esqNMR method that PULCON can effectively correct. One advantage of $^2$H SOLCOR is that we found it unnecessary to apply PULCON corrections to $^2$H integrations as the 90° pulse durations are minimally impacted by salt concentration (Table S3). The traces within this plot track the sample volume and salt content differences. They underscore isqNMR's proven status as the technique with the least uncertainties. isqNMR gives the most accurate and precise results with the least amount of effort, though contamination of the sample in the process may be unacceptable in certain instances. In this experiment, the higher concentrations of NaCl in some samples are diluting the $^2$H concentration to the extent that SOLCOR is slightly overcorrecting the concentration; though this inaccuracy is overshadowed by the larger inaccuracies in $^1$H 90° pulse width by the salty sample.

Another observation from this experiment is that solvent signal integration is influenced by all solutes (dibucaine and NaCl), not just the observable solute of interest (dibucaine). Attempting to use the solvent signal integration, whether $^2$H or residual $^1$H, as a measure of the dibucaine concentration would result in overestimation of the concentration as the nonobservable solute, NaCl, is contributing to the dilution of the solvent DMSO-d6 in this case.

### 3.6 Using $^2$H SOLCOR to track esqNMR sample dilution or manipulation

The ability to accurately measure the dilution of the deuterium in our esqNMR samples in response to sample treatment also lends itself to improved accuracy and flexibility in quantitation. An example of this is where the sample being measured contains a high percentage of water with a broad peak that complicates the integration of nearby signals. Using the internal standard method, the broad water peak can be easily reduced with the addition of D$_2$O that exchanges with water in the sample resulting in an improved baseline, and much more accurate integration. In this case, the dilution factor is common to both the internal standard and the sample thereby eliminating any error caused by dilution.

The addition of D$_2$O to the sample would introduce significant error in esqNMR calculation, as the sample and standard would have different dilution factors causing the analyte to appear to be lower in concentration or potency. Adding D$_2$O to our stock solution used for both the external standard and sample is an option though it excludes the observation of these broad signals, which frequently inform the presence of water in our samples. By collecting an additional $^2$H observe spectrum after sample treatment (adding D$_2$O in this case), we can effectively measure and correct for this dilution and accurately report concentration or potency.

This ability to capture volume manipulations could lead to an easier method determining protein concentration. Determination of protein concentrations using PULCON techniques and Shigemi assemblies has been
explored with the recommendation that volume versus signal strength curves calibration curves be generated to allow volume corrections. The SOLCOR method has been proven capable of correcting large volume differences (Figure S2) and is poised to fill this role for Shigemi assemblies as demonstrated in Figure S5, therefore eliminating the need for such calibration curves. Accomplishing biological sample quantification often requires normalization procedures to overcome the significant complexities in sample preparation, resulting in variable dilution of samples and confounding the ability to compare assay results. The SOLCOR method, using $^2$H or any other solvent signal with NMR active nuclei, is well suited to this situation. Other potential applications of SOLCOR include but are not limited to the following list: integrating parallel medicinal chemistry with in vitro high throughput pharmacology or toxicology test; monitoring reaction by (on or stop) flow NMR; measuring thermodynamic solubility or following kinetic dissolution process; testing drug product content uniformity, or anywhere esqNMR is applied.

### 3.7 $^2$H SOLCOR esqNMR is well suited for analytical reference standard qualification and requalification

One of the distinct advantages of the esqNMR technique is the ability to calculate the concentration, potency or total amount of a sample without sample contamination by internal standard and without the consumption of reference standard material that may be in short supply. Accurate concentration and purity determination are paramount in the qualification of an analytical reference standard. Despite the incapability of achieving detection limits on a par with other techniques (e.g., Liquid Chromatophy-UltraViolet Spectroscopy-Mass Spectrometry or LC-UV-MS), quantitative NMR holds a unique advantage in its ability to characterize, both qualitatively and quantitatively, novel chemical entities with the use of commercially available certified reference standards that are not the analyte of interest. The creation of a qualified reference standard for a novel compound with its total amount accurately quantified, enables subsequent serial dilutions, permitting the establishment of calibration curves, determinations of limits of detection and quantitation required for assay validation and routine testing using other analytical techniques with much higher sensitivity but lower specificity than NMR. The prospect of periodic retest of the reference standard also accentuates the advantage SOLCOR esqNMR provides.

One author has first-hand experience of a critical metabolite reference standard being exhausted during yearly requalification using traditional destructive analytical techniques (LC-UV-MS, Karl Fischer water determinations). The unavailability of this metabolite disrupted a toxicology study and delayed the project progression. While it might be practical to minimize esqNMR tube volume variation error through the selection of high-quality NMR tubes from the same manufactured lot for the initial qualification of a reference standard, a periodic retest of the reference standard will likely be performed using a different manufactured lot of NMR tubes and hence introducing additional uncertainty with tube volume variation. We have encountered situations where the SOLCOR esqNMR method, used in combination with traditional 1D/2D structure confirmation experiments afforded a process that was quick, reliable, nondestructive with minimal sample handling. This enabled and therefore created a reference standard qualification for downstream analyses by LC-MS with its inherent lower limit of detection/quantification. SOLCOR esqNMR can

### Table 3 Revised advantages and disadvantages of isqNMR, esqNMR, and $^2$H SOLCOR esqNMR

| Characteristic                              | isqNMR | esqNMR | $^2$H SOLCOR esqNMR |
|---------------------------------------------|--------|--------|---------------------|
| Spectral overlap                            | ☒      | ☒      | ☑                   |
| Sample recovery                             | ☒      | ☐      | ☑                   |
| Reactivity                                  | ☒      | ☐      | ☑                   |
| Standard preparation burden                 | ☒      | ☐      | ☑                   |
| NMR tube volume variation                   | ☑      | ☒      | ☑                   |
| Acquisition/Processing error determination  | Must be determined | Must be determined | Must be determined |
| (measurement uncertainty)                   |        |        |                     |
| Salty samples                               | ☑      | ☑      | ☐                   |
| Reference standard retest                   | ☒      | ☒      | ☑                   |
| Sample manipulations                        | ☑      | ☒      | ☑                   |

Abbreviations: esqNMR, external standard quantitative NMR; isqNMR, internal standard quantitative NMR.
assist in creating qualified primary analytical reference standard, whenever novel chemical entities are involved, including drug metabolites[19], unknown drugs of abuse, highly toxic natural product isolates, performance-enhancing drug screening, labile or photoreactive chemical intermediates.

4 | CONCLUSIONS

Deuterium is easily observable, present in all samples, free of interfering signals, insensitive to probe mistune or mismatch, and sample saltiness. These features make $^2$H SOLCOR an ideal esqNMR tool for volume correction whenever NMR tube consistency is uncertain. Revisiting the advantages and the disadvantages of the isqNMR and esqNMR methods from Table 1, we have added a new column to summarize the advantages that $^2$H SOLCOR provides in Table 3. We have also added new rows underlining the need to determine acquisition and processing error, as well as highlighting SOLCOR’s ability to track sample handling and periodic retesting of reference standards.

The $^2$H SOLCOR method easily delivers reliable, accurate, and precise esqNMR results. Newer hardware configuration with multiple receivers[20] may allow parallel acquisition of both $^1$H and $^2$H, thus provide continuous volume monitoring and correction factor automatically utilizing SOLCOR. Even for a spectroscopist who judiciously selects high quality NMR tubes from the same batch to carry out the esqNMR, $^2$H SOLCOR can still track tube volume variations, as well as sample manipulations either dilution or concentration, and corrects for these factors retroactively when required. For the analyst who performs esqNMR in a high throughput setting, the prospect of using disposable NMR tubes and employing $^2$H SOLCOR provides significant cost reductions. For the analytical scientist accountable for creating and periodically retesting qualified reference standards for use in orthogonal analytical techniques (HPLC, LC/UV/MS, etc.) the $^2$H SOLCOR esqNMR method provides unrivaled utility.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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