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RESEARCH ARTICLE

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A reliable external calibration method for reaction monitoring with benchtop NMR

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical technique with the ability to acquire both quantitative and structurally insightful data for multiple components in a test sample. This makes NMR spectroscopy a desirable tool to understand, monitor, and optimize chemical transformations. While quantitative NMR (qNMR) approaches relying on internal standards are well-established, using an absolute external calibration scheme is beneficial for reaction monitoring as resonance overlap complications from an added reference material to the sample can be avoided. Particularly, this type of qNMR technique is of interest with benchtop NMR spectrometers as the likelihood of resonance overlap is only enhanced with the lower magnetic field strengths of the used permanent magnets. The included study describes a simple yet robust methodology to determine concentration conversion factors for NMR systems using single- and multi-analyte linear regression models. This approach is leveraged to investigate a pharmaceutically relevant amide coupling batch reaction. An *on-line* stopped-flow (*i.e.*, interrupted-flow or paused-flow) benchtop NMR system was used to monitor both the 1,1′-carbonyldiimidazole (CDI) promoted acid activation and the amide coupling. The results highlight how quantitative measurements in benchtop NMR systems can provide valuable information and enable analysts to make decisions in real time.

KEYWORDS

benchtop NMR, external calibration, quantitative NMR, reaction monitoring

1 | INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy provides structural information about species in the test sample, and its inherent quantitative nature has been understood since the early 1960's.[1,2] Importantly, quantitative information can be obtained with NMR spectroscopy nondestructively, relatively quickly, and with minimal or no sample preparation. Additionally, this information is acquired nonspecifically, allowing quantitative information

to be obtained and assigned to all individual components in the test sample at once.

To many chemists, quantitative NMR (qNMR) is still associated with high-field NMR spectrometers. In recent years, however, the availability of benchtop NMR instruments has enabled the acquisition of structurally rich, quantitative data in settings where chemistry is taking place, such as laboratory fume hoods, pilot plants, and manufacturing facilities.[3,4] While notable limitations with benchtop NMR systems do exist (decrease in both sensitivity and signal dispersion), the benefits include a smaller physical size and a reduced cost (both to purchase and maintain).

As the access to benchtop NMR systems increases the ability of analysts to perform qNMR measurements in a variety of nontraditional settings, special consideration must still be given to two important phenomena: polarization and excitation.[5] Irrespective of the quantitative technique applied, full equilibrium of polarization must be achieved for all nuclei desired to be quantified. Generally, to fulfill this requirement, the sample must be exposed to the external magnetic field of the instrument, and the NMR experiment must have a repetition time (if signal averaged), at least five times the longest spin–lattice relaxation time (T_1) with an applied 90° radio-frequency (rf) pulse. This requirement ensures that over 99% of polarization is recovered prior to the 90° rf pulse. The T_1 of nuclei can be estimated using an inversion-recovery experiment[6,7] or other techniques such as FLIPS (Faster Longitudinal relaxation Investigated by Progressive Saturation).[8] The importance of considering polarization and T_1 values is only underscored with the use of flow NMR systems, where "in-flow" and "out-flow" effects influence the maximum polarization recoverable and necessary repetition time, respectively.[9]

As per the excitation requirement for qNMR, resonances to be compared must be equally excited. In the case of ^{1}H NMR, the small chemical shift range (~12 ppm) generally results in easy satisfaction of this requirement. However, when monitoring nuclei with a larger chemical shift ranges such as ^{19}F, particular attention is due as the pulse width is inversely proportional to the spectral range with equal excitation.[5] This can be mathematically determined using the pulse width or empirically determined by monitoring changes in signal intensity with varying offset values. Importantly, assuming the same pulse width is used; benchtop NMR systems will yield a larger spectral region (in ppm) of equal excitation than high-field NMR instruments.

With the physical requirements for qNMR in mind, many quantitation techniques exist. Using internal calibration,[10] where a calibrant is spiked directly into the sample to be analyzed, is arguably the most applied technique.[11] This allows the integrals of analyte resonances to be compared with the integrals of calibrant resonance to determine quantity. While using an internal calibrant results in only a single NMR spectrum needed for qNMR analysis, it also introduces potential complications in relation to the calibrant's solubility, reactivity, and likelihood of resonance overlap. With a decrease in signal dispersion inherent to data measured with a benchtop NMR spectrometer, the likelihood of overlap between analyte and calibrant resonances only increases, overall decreasing the practicality of such a qNMR technique with this instrumentation.

Additionally, calibration techniques using residual solvent resonances have been applied with ^{1}H NMR and high-field NMR systems.[12–14] While application of this method does not require introduction of an internal calibrant, a residual solvent resonance must be present, observable, and spectrally resolved for quantitation with traditional frequency domain spectral analysis. The decrease in resonance dispersion with ^{1}H benchtop NMR may not allow for this technique to be feasible in many instances. Additionally, solvent resonances are not likely in spectra for nuclei with a degree of signal dispersion (such as ^{19}F and ^{31}P) which increase the practicality of benchtop NMR. Therefore, external referencing qNMR techniques with a benchtop NMR are highly attractive.

External referencing techniques such as ERETIC,[15] ARTSI,[16] or QUANTAS[17] may be applied in some NMR systems, where an artificial reference signal is used to calibrate analyte resonances for quantification. While this method enables qNMR analysis without the introduction of an internal calibrant, some methods possess specific hardware requirements; therefore, some techniques are not applicable/practical with benchtop NMR systems, depending on the hardware of the system itself.

Another external reference qNMR method, which is applicable to all benchtop NMR systems, relies on the acquisition and analysis of NMR spectra for a reference sample containing the calibrant and the test sample containing the analyte to be quantified.[18,19] NMR experiments are commonly collected using the same acquisition parameters (same instrument, number scans, dummy scans, acquisition time, repetition time, spectral width, receiver gain, *etc.*). However, mathematical corrections can be used to quantify components with spectra acquired with differing acquisition parameters,[20] and an external referencing qNMR method (FAINT-NMR) has recently been presented that allows for analysis agnostic of receiver gain and number of scans.[21] Additionally, hardware influences (probe tuning/matching, pulse calibration, *etc.*) on external referencing qNMR techniques have been highlighted in the literature[19] and explored specifically with a benchtop NMR spectrometer.[22]

Fundamentally, this technique leverages a concentration conversion factor (or CCF), determined with the external reference sample and the known number of nuclei responsible for the resonance (or resonances, if applicable). The CCF value correlates absolute integration (per nuclide) for a particular spectral acquisition and processing scheme. Then with the experimental analyte sample, the absolute integration of a spectrally resolved resonance can be used with the conjunction of this CCF to determine the molar concentration of the analyte using Equation (1).

$$Concentration = CCF \times \frac{absolute\ integration}{number\ of\ nuclei} \quad (1)$$

This quantitative technique is attractive for benchtop NMR systems particularly as no additional resonances (from solvent or added internal reference) are needed in the NMR spectrum of interest, other than analyte resonances desired to be quantified. As this technique critically relies on the CCF used for quantitative analysis with the absolute quantitation technique, it is easy to understand how it plays a vital factor in achieving accurate results. The work reported herein highlights a method to determine a more robust CCF with use of single- and multi-analyte linear regression models instead of a single point calibration using the absolute qNMR method. To highlight the applicability of this approach, the CCF values are then applied to quantitatively monitor an industrially relevant model reaction system—CDI (1,1′-carbonyldiimidazole) mediate activation of a carboxylic acid and subsequent nucleophilic capture.

2 | MATERIALS AND METHODS

2.1 | Materials

All materials were obtained from commercial sources and used as received. Acetonitrile (MeCN), CDI, 4-(trifluoromethyl)benzoic acid, 4-fluoroaniline, and benzotrifluoride were sourced from Sigma-Aldrich. Certified reference materials for qNMR (namely, dimethylmalonic acid, maleic acid, methyl 3,5-dinitrobenzoate, and 1,3,5-trimethoxybenzene) were acquired from Millipore-Sigma. Hexadeuterodimethyl sulfoxide (DMSO-d_6) was obtained from Cambridge Isotope Laboratories.

2.2 | Instrumentation, data acquisition, and processing

NMR measurements for reaction monitoring were carried out on a 1.46 T Magritek Spinsolve 60 benchtop NMR system, with operating resonance frequencies of 61.88 and 58.22 MHz for ^{1}H and ^{19}F, respectively. A glass flow cell provided by the instrument manufacturer was used to transfer the test solutions into the benchtop NMR spectrometer without the need for standard NMR tubes.

A prototype *on-line* stopped-flow (*i.e.*, interrupted-flow or paused-flow) reaction monitoring system[23] was utilized to enable periodic static sampling of batch reactors *via* ^{1}H and/or ^{19}F NMR spectroscopy. This system monitors a batch reaction *on-line*, whereas *in-situ* stopped-flow techniques inject and mix reagents directly in the NMR, where the mixture is halted for analysis. In other words, with the *on-line* stopped-flow system, reaction mixture from an ongoing batch reaction is flowed to the NMR, halted in the spectrometer, data are measured, and the reaction mixture is returned to the batch reaction. To allow for this sampling, a Python script, similar to that in the referenced work, was utilized to control a Vapourtec SF-10 peristaltic pump, a Vici six-way valve, and the benchtop NMR spectrometer. For reaction monitoring, measurement of NMR spectra was triggered using the Python script. Further details regarding the reaction monitoring system are available in the supporting information.

NMR measurements were carried out using standard pulse sequences available in the instrument library. Single-pulse 1D ^{1}H NMR experiments were collected using the following acquisition parameters: 90° pulse, 1 scan, no dummy scans, 3.2 s acquisition time, 5,000 Hz (80.8 ppm) spectral width, 5.0 ppm spectral offset, and 42.4 s repetition time. All ^{1}H NMR spectra were processed with an applied 1 Hz exponential apodization, third-order polynomial baseline correction, automatic phase correction (manual corrections applied as needed), and zero-filling to 65,536 data points.

Single-pulse 1D ^{19}F NMR experiments were acquired using the following acquisition parameters: 90° pulse, 1 scan, no dummy scans, 6.5 s acquisition time, 20,000 Hz (343.5 ppm) spectral width, -63.0 ppm spectral offset, and a 20.5 s repetition time. All ^{19}F NMR spectra were processed with an applied 2 Hz exponential apodization, third-order polynomial baseline correction, automatic phase correction (manual corrections applied as needed), and zero-filling to 262,144 data points.

2.3 | Determination of concentration conversion factors (CCF)

To prepare a CCF to be used for ^{1}H qNMR analyses, methyl 3,5-dinitrobenzoate (570.61 mg, 99.7%, TraceCERT) was used to prepare a 0.50 M stock solution by bringing to 5.00 mL with MeCN. After the solution was

flowed into the NMR with a closed-loop flow system, the flow was halted and ^{1}H NMR spectra were measured in triplicate.

After data acquisition, the sample was diluted with additional MeCN to prepare a 0.25 M solution. The solution was mixed well and flushed through the closed-loop flow system. On the sample static in the NMR, ^{1}H NMR spectra were collected in triplicate. This process was repeated upon further diluting the solution to 0.17, 0.10, 0.06, and 0.02 M and measuring ^{1}H NMR in triplicate. Overlapping aromatic resonances ($\delta_H = \sim$ 9.2 ppm, 3 × ^{1}H) were integrated in each spectrum. The absolute integration per nuclide versus concentration was plotted. The inverse of the slope for the line of best fit represented the CCF for this nucleus, set of acquisition parameters, and set of processing parameters.

To prepare a CCF for ^{19}F qNMR analyses, benzotrifluoride (0.700 mL, 99%) was used to prepare a 0.16 M stock solution by bringing to 35.00 mL with MeCN. The solution was pumped into the closed-loop NMR flow system, flow was halted, and ^{19}F NMR spectra were measured in triplicate. The sample was diluted with MeCN to prepare 0.14, 0.12, 0.08, 0.06, and 0.04 M solutions. Each solution was mixed well, flowed through the closed-loop flow system, and analyzed in triplicate.

The trifluoromethyl resonance (δ_F = -61.6 ppm, 3 × ^{19}F) was integrated in each spectrum. The absolute integration per nuclide versus concentration was plotted. The inverse of the slope for the line of best fit represented the CCF for this nucleus, set of acquisition parameters, and set of processing parameters.

2.4 | Synthesis of *N*-(4-fluorophenyl)-4-(trifluoromethyl)benzamide

The benchtop NMR flow system was primed with MeCN and connected to a septum-capped scintillation vial containing 6 mL MeCN and a vent needle. The vial was equipped with a magnetic stir bar and kept at a controlled temperature of 26°C using a Mettler Toledo EasyMax synthesis workstation. Importantly, this was the temperature of the magnet of the benchtop NMR.

With the pump set to off, 445 mg (1.2 eq.) of CDI was charged into the vial as a solid and the resulting solution was stirred at 500 rpm. The Python script was started which turned the pump on with a flow of 2.7 mL/min and facilitated analyte transfer to the benchtop NMR. Altogether, 20 ^{1}H NMR spectra were acquired starting 3 minutes after the CDI solids were dosed. Spectra were measured 3.5 minutes apart with a 10 second premagnetization time prior to initiation of the NMR experiment.

Upon reaching a consistent CDI concentration in solution, as evidenced by an unchanging NMR integration value, the pump was halted, and 442 mg (1 eq.) of 4-(trifluoromethyl)benzoic acid was added to the vial. The activation was monitored *via* benchtop ^{19}F NMR starting 3 minutes after addition of the carboxylic acid with the same ^{19}F NMR data acquisition parameters previously listed but with a spectral offset of -90.0 ppm.

Incomplete activation was observed after acquisition of the first 34 ^{19}F NMR spectra. An additional 127 mg (0.3 eq.) of CDI were added to the vial. Likewise, more CDI (127 mg, 0.3 eq.) was added after the acquisition of the 41st ^{19}F NMR spectrum, resulting in full activation of the carboxylic acid. The process was monitored for 3.5 hours until a total of 60 ^{19}F NMR spectra were acquired.

After full activation of the carboxylic acid, 0.25 mL (1.1 eq.) of 4-fluoroaniline was added to the reaction. The reaction mixture was monitored by ^{19}F NMR. In total, 206 additional ^{19}F NMR spectra were measured with 10 second premagnetization time and a 5 minute delay between them.

3 | RESULTS AND DISCUSSION

3.1 | Preparing a concentration conversion factor (CCF)

A CCF is commonly and easily determined *via* single point analysis. This involves carefully weighing/measuring the standard, bringing to an accurate volume, and transferring to the NMR for analysis. Then, with a predetermined set of acquisition parameters, an NMR spectrum is measured. After processing the reference spectrum in the analyst's designated software package, the resonance(s) of the compound are integrated. To calculate the CCF, the concentration is divided by the absolute integral value per nuclide or average integral per nuclide, if using multiple resonances, as per Equation (2):

$$CCF = \frac{concentration\ of\ reference}{(average)\ absolute\ integration\ per\ nuclide} \quad (2)$$

It is important to note that the CCF is sensitive to acquisition parameters and choice of applied processing—therefore, it is essential to use the same parameters in each of these cases, unless one uses applies an appropriate mathematical correction factor.

Additionally, it is also important to consider how sometimes less obvious hardware attributes (such as tuning, receiver gain, and pulse calibration) of the benchtop NMR spectrometer may influence data measurement and the determined CCF. For the used spectrometer in this

study, the tuning of the probe was factory set, and both the receiver gain and the pulse widths (corresponding to particular pulse angles) were unable to be changed. Therefore, all data were acquired with predefined settings. However, on systems where flexibility exists (probe is actively tuned, receiver gain can be changed, flexibility of pulse widths, *etc.*), a significant level of consideration shall be applied to maintain consistent data measurement.

While use of a single concentration and sample allows for a rapid determination of a CCF, the intrinsic error associated from a single point measurement (and therefore all quantitative measurements to follow) can be decreased by performing replicate measurements and using multiple concentrations of analyte. This both minimizes variability and also allows confirmation of the CCF over a range of different signal-to-noise ratios (SNRs) of an analyte.

Additionally, not only is the resulting CCF more robust when replicates and varied analyte concentrations are used but also the general data work-up and analysis is simple and straightforward. Upon data acquisition and applying appropriate processing, integral values are determined for the resonance (or resonances, if multiple are used) of interest. If the absolute integration per nuclide ("nuclide absolute integration") is plotted against the concentration, the inverse of the slope for the resulting line of best fit represents the CCF.

For the utilized benchtop NMR system and set of acquisition/processing parameters, a CCF was determined with the above-described method for both ^{1}H and ^{19}F nuclei using methyl 3,5-dinitrobenzoate (**1**) and benzotrifluoride (**2**), respectively (Figure 1). Importantly, these CCF values were determined with a commercially available Magritek flow cell. As volume of analyte (and therefore number of NMR active spins within active volume) is directly correlated to the recovered signal, it was vital to determine these values under conditions (*i.e.*, NMR tube, flow cell with flow, and flow cell without flow) that are representative of later analyses. As the later reaction monitoring was conducted with an *on-line* stopped-flow NMR system, with reaction mixture analyzed while static in an NMR flow cell, static solutions of both standards used to determine CCF values were analyzed in the same NMR flow cell. Additionally, the CCF values were determined with analytes in the same solvent as later reaction monitoring analyses.

The CCFs for ^{1}H and ^{19}F nuclei and the used system were determined to be 0.0040 M per nuclide absolute integration and 0.0234 M per nuclide absolute integration, respectively. With either nucleus detected, the line of best fit was observed to be linear (R^2 values of 0.9995 and 0.9992 for ^{1}H and ^{19}F, respectively). If one compares the ^{1}H NMR CCF determined with the above method with a CCF determined with just a single point at any one of the concentrations analyzed, on average, a 7.8% difference in CCF was observed. On the other hand, if just a triplicate analysis was used at any of the concentrations analyzed to determine a CCF, an average 7.9% difference resulted. Similar results were additionally observed for ^{19}F NMR CCF determination as, on average, a 6.8% difference was observed if a single point was used and a 6.6% difference for a triplicate analysis at any single concentration. These relative differences are consistent with measured variability between analysts using an external referencing qNMR with a benchtop NMR.[22]

To highlight the robustness of this qNMR technique, ^{1}H NMR spectra were measured with four analytes (maleic acid, methyl 3,5-dinitrobenzoate, dimethylmalonic acid, and trimethoxybenzene) at various concentrations (0.01–0.79 M) in triplicate. The absolute integration per ^{1}H for all analytes and concentrations were plotted, and the resultant graph with line of best fit can be found in Figure 2.

The CCF determined with multiple analytes (0.0043 M per nuclide absolute integration) was in great agreement to the previously determined CCF for the observed nucleus (^{1}H) and benchtop NMR spectrometer. These data, however, underscore robustness of the qNMR

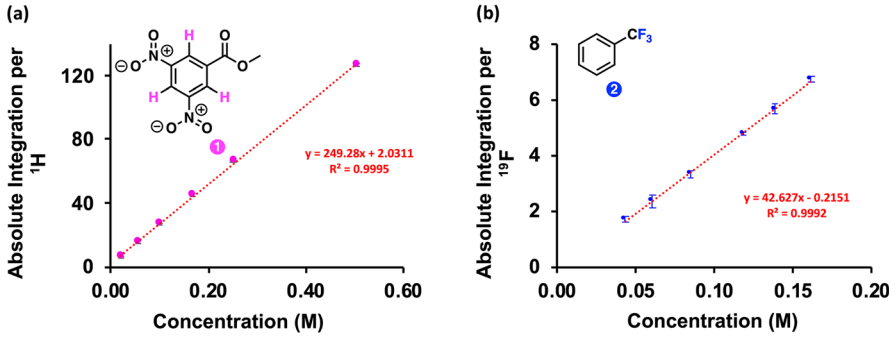

FIGURE 1 Preparation of concentration conversion factors (CCFs) for benchtop nuclear magnetic resonance (NMR) system using Magritek flow cell for (a) ^{1}H NMR using aromatic resonances of methyl 3,5-dinitrobenzoate (**1**, δ_H 9.2 ppm) and (b) ^{19}F NMR using trifluoromethyl resonance of benzotrifluoride (**2**, δ_F -61.6 ppm) in MeCN.

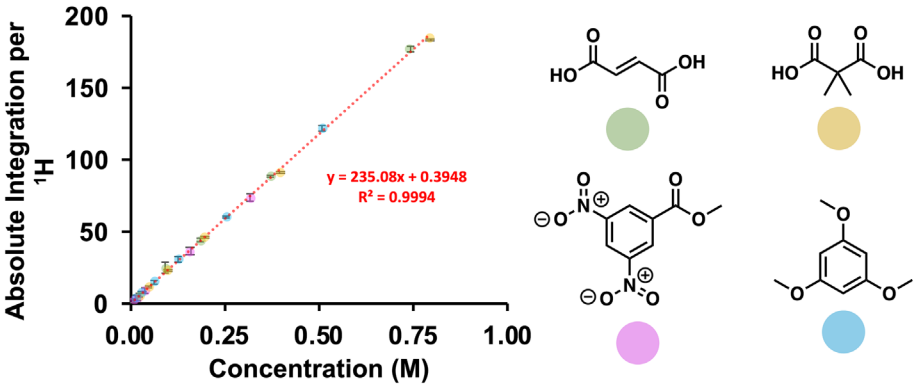

FIGURE 2 Robustness of the qNMR method showcased by great agreement in absolute integration per nuclide versus concentration using multiple analytes: maleic acid (green), methyl 3,5-dinitrobenzoate (pink), dimethylmalonic acid (yellow), and trimethoxybenzene (blue) in DMSO-d_6. Line of best fit determined using data from all analytes.

method not only on the basis of general applicability with a wide range of analytes. For instance, these data were measured with a different solvent (DMSO-d_6) than the initial data (proteo MeCN). Additionally, these data were generated approximately 1 year after the initial CCF data were collected and display only 7.2% difference with the previously determined value. Therefore, these data highlight the long-term stability of a CCF for a given NMR instrument and set of conditions.

It is further worth noting the linearity of data measured on lower concentration samples (<60 mM). An expansion of the data shown in Figure 2 at this lower concentration range is displayed in the supporting information (SI Figure S4). While relatively high variability was observed with NMR data measured with 10 and 20 mM samples of methyl 3,5-dinitrobenzoate (14.5 and 12.6% RSD, respectively), the robustness of the technique is again displayed as the same CCF is determined if these data points are excluded (SI Figure S4). Therefore, as the CCF is determined using a wide range of analyte concentrations, and in triplicate, a more accurate conversion factor for external calibration qNMR is obtainable with this technique over single point measurements conducted with either one or three replicates.

This method also allows for additional precautions to be applied to further increase the accuracy of the determined CCF for quantitative analyses. For reaction monitoring instances, where analyte concentrations are inherently changing with time, this may include analyzing a range of relevant analyte concentrations for later reactions. Naturally, a concentration regime where only spectra that do not saturate the receiver, considering a particular receiver gain value, shall be used. Additionally, the ionic strength of a sample (and therefore sample's conductivity) can influence an instrument's response. We have previously observed an ~3% decrease in integration or apparent concentration if using an external qNMR method, with a 1.0 M increase in ionic strength with a similar benchtop NMR system.[23]

3.2 | Application to reaction monitoring: CDI-mediated amidation

Amides are an important moiety in numerous active pharmaceutical ingredients.[24] A preferred method for forming amide bonds between a carboxylic acid and an amine is CDI mediated amidation.[25,26] This is an attractive method as CDI is cost efficient, and the byproducts are non-toxic and easily purged. While it is possible to measure the potency (*i.e.*, percent purity) of CDI through established methods, the quality of the solid may vary and the material is moisture sensitive. Therefore, it is often difficult to precisely dose. Benchtop ^{1}H qNMR can be used to measure the concentration of dosed CDI using the external calibration method obviating the use of deuterated solvents and an internal standard. As a model system, CDI (**3**) was used to activate 4-(trifluoromethyl) benzoic acid (**5**). Upon full activation to acyl imidazole (**6**), the activated acid was reacted with 4-fluoroaniline (**7**) to prepare corresponding amide **8**.

Prior to the activation of **5**, CDI (**3**) was charged into the reaction solvent (MeCN) to measure potency and to observe the stability in the presence of residual water (Figure 3). Benchtop ^{1}H NMR provides sufficient signal dispersion to observe CDI in solution quantitatively without the use of an internal standard (using the CCF method highlighted above). These benchtop ^{1}H qNMR data allowed for a 53% potency value to be determined using a spectrally resolved resonance consistent with CDI (δ_H 7.8 ppm) and the system's CCF for the nucleus. The calculation for determining the potency of CDI, along

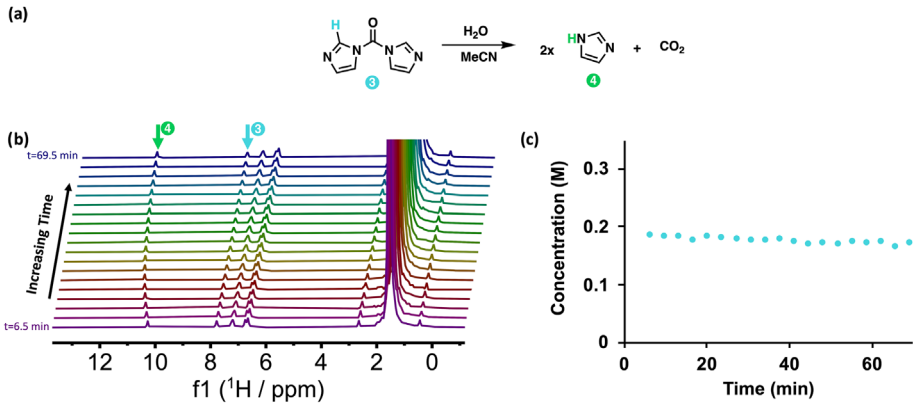

FIGURE 3 (a) Scheme for the hydrolysis of CDI (**3**, 0.19 M, δ_H 7.8 ppm) with residual water to form imidazole **4** (δ_H 11.0 ppm) in MeCN. (b) Representative decimated stacked array of benchtop ^{1}H NMR spectra. (c) Quantitative reaction profile resultant from application of the NMR spectrometer's CCF.

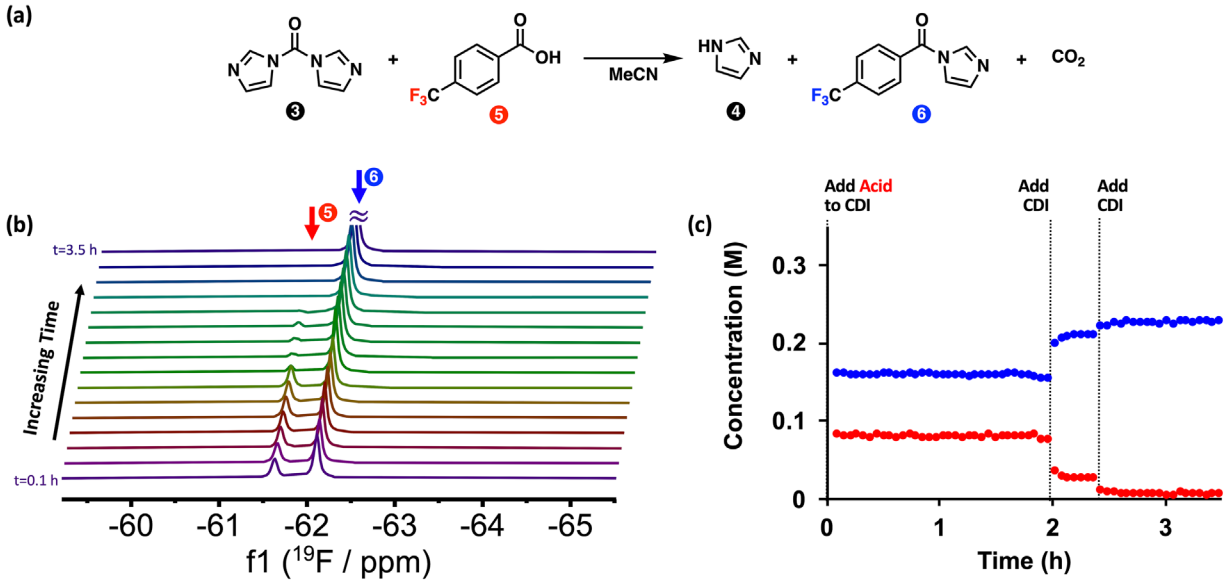

FIGURE 4 (a) Scheme for the activation of carboxylic acid **5** (0.28 M, δ_F -61.6 ppm) with CDI (**3**) to form the corresponding activated acyl imidazole **6** (δ_F -62.1 ppm) in MeCN. (b) Representative decimated stacked array of benchtop ^{19}F NMR spectra. (c) Quantitative reaction profile resultant from application of the NMR spectrometer's CCF.

with real initial concentration (0.19 M) with these experimentally determined values, is included in the SI (SI Section 4b). Additionally, the CDI remained relatively stable over the observed as 0.17 M, 89% of the initial CDI concentration, remained in solution by the end of the 70 minute observation period.

After maintaining a consistent CDI solution concentration, determined by monitoring both the concentrations of CDI and achieving a constant chemical shift for the amine imidazole proton (δ_H 11.0 ppm, a well-established pH indicator[27]), carboxylic acid **5** was dosed into solution to produce the corresponding activated carbonyl imidazole (**6**). The presence of fluorine atoms in both **5** and **6** allowed the activation to be monitored *via* benchtop ^{19}F NMR (Figure 4).

Leveraging ^{19}F benchtop NMR was beneficial as the larger chemical shift range of ^{19}F simplified the resulting spectra (no solvent resonance, less overlap) and therefore simplified the following analysis. Additionally, with the increasing presence of ^{19}F nuclei in active pharmaceutical ingredients,[28] the practicality of benchtop NMR and the applied qNMR method in pharmaceutical settings are further increased. In the case of this model reaction, the two trifluoromethyl resonances of **5** (δ_F -61.6 ppm) and

6 (δ_F -62.1 ppm) could unambiguously be assigned and monitored with time, despite the chemistry occurring on a carbon six bonds away.

A single charge of **5** resulted in incomplete activation to the corresponding acyl imidazole **6**, consistent with the 53% CDI potency determined with the ^{1}H qNMR measurement. Therefore, it was necessary to add more CDI. Importantly, due to the previous CDI potency determination, this could be completed with a single addition of the activating reagent. However, in effort to showcase the real-time chemical information obtainable with this system, this was completed with two additional charges. Noticeably, incomplete activation could be observed after the second charge, prompting the need for a third addition.

While these additions of CDI were determined necessary and physically conducted manually, the opportunity to leverage automation for such processes exists. For example, Bornemann-Pfeiffer *et al.*[29] highlighted an automated chemical synthesis machine which used benchtop NMR as an analysis technique to optimize reaction conditions for Grignard reactions. Additionally, our lab has leveraged an auto-synthesizer with other analytical techniques (*on-line* high-performance liquid chromatography with diode-array detection and *in-situ* Fourier-transform infrared spectroscopy) to successfully execute CDI mediated amidations in a flexible manner.[30] The incorporation of benchtop NMR into an automation platform highlights opportunities to perform such a critical process step, without the need for human intervention.

Nonetheless, adding only the appropriate amount of CDI allows for full conversion of **5** while minimizing additional activating reagent left in solution. The remaining CDI could otherwise contribute to side-product formation upon nucleophile addition, where the nitrogen nucleophile could add to the CDI, forming urea byproducts. Additionally, this could result in an overall decreased yield in the desired amide product as nucleophile is consumed through an unproductive pathway.

After full conversion of the carboxylic acid **5** to the activated acyl imidazole **6**, 4-fluoroaniline (**7**) was added to prepare the corresponding amide **8** (Figure 5). This step was additionally quantitatively monitored by ^{19}F NMR spectroscopy. Upon addition of the nucleophile, great agreement in reaction profiles was observed when tracking the formation of desired amide **8** with either the trifluoromethyl or aryl fluoride ^{19}F NMR resonance. This agreement is apparent by comparing the reaction trends qualitatively (Figure 5c) and the time required to reach 50% conversion (~3 h). Further, the observed second-order rate constant for consumption of both electrophile **7** and nucleophile **8** (3×10^{-4} M^{-1} s^{-1}) was in excellent agreement.

Notably, despite the general agreement between the trends for amide **8**, one can qualitatively decipher a large difference in relative noise between the two trends. Not only is there intrinsically less signal for the aryl fluoride ($1 \times$ ^{19}F per molecule) compared with the trifluoromethyl resonance ($3 \times$ ^{9}F per molecule) but it is additionally coupled to nearby aromatic protons, further dispersing the signal. Nonetheless, the consistent nature of the quantitative results further validates the robustness of the quantitative method, even despite the trifluoromethyl resonance encompassing six times the SNR.

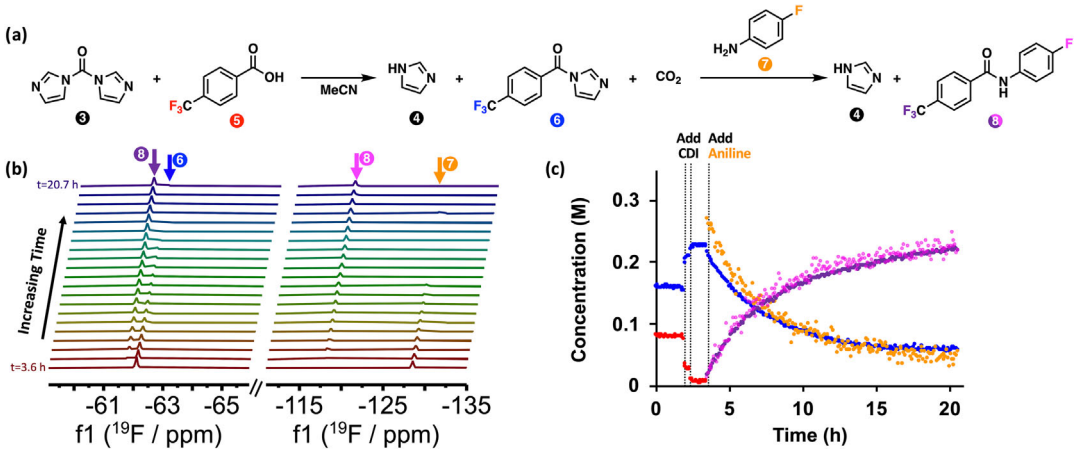

FIGURE 5 (a) Full reaction scheme for the activation of carboxylic acid **5** (0.28 M, δ_F -61.6 ppm) with CDI (**3**) to form acyl imidazole (**6**, δ_F -62.1 ppm) that was captured with 4-fluoroaniline (**7**, δ_F -128.5 ppm) to form the corresponding amide **8** (δ_F -61.6 ppm and -117.9 ppm) in MeCN. (b) Decimated stacked array of benchtop ^{19}F NMR spectra of the nucleophilic capture. (c) Quantitative reaction profile resultant from application of the NMR spectrometer's CCF.

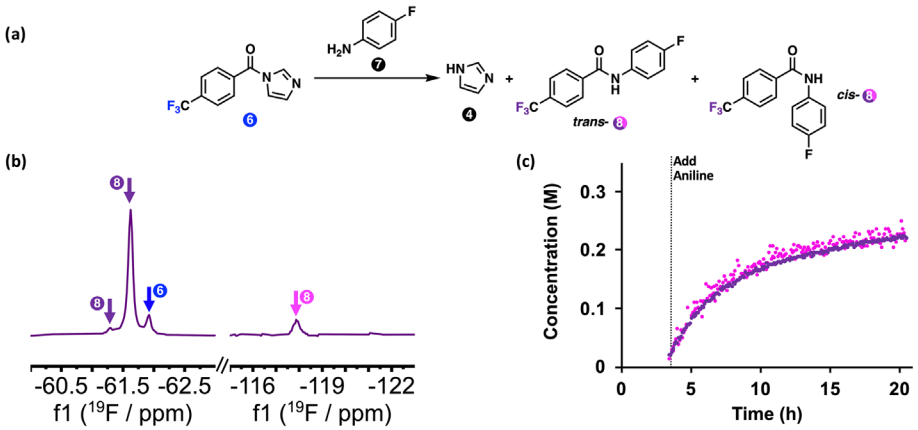

FIGURE 6 (a) Scheme for the nucleophilic capture of prepared acyl imidazole (**6**) with 4-fluoroaniline (**7**) to form the corresponding amide **8** in MeCN. (b) Annotated spectral regions of final benchtop ^{19}F NMR spectrum, with trifluoromethyl resonances observable for both *cis*- and *trans*- amide conformations of **8**. (c) Quantitative reaction profiles; agreement between trifluoromethyl and aryl fluoride resonances observed only when trifluoromethyl resonances of both conformations are considered.

It should be further noted that in this final step, excess aniline **7** was initially added relative to acyl imidazole **6**. This was to ensure that sufficient nucleophile remained in the case that **7** reacted with CO_2 in solution (side product from CDI activation) to form the corresponding formate by-product or if the reaction solution possessed any remaining unreacted CDI in solution. However, both unreacted aniline and acyl imidazole were present in solution upon the end of the observation period. We hypothesize that the pH of the reaction could have changed through the reaction course, allowing for the reaction to stall. As this model reaction was conducted without the addition of base, the pH could decrease, increasing the population of protonated aniline **7** and decreasing the nucleophilicity.

Nonetheless, the power of benchtop ^{19}F NMR is further displayed with the observation of two trifluoromethyl resonances for amide **8**, consistent with both *cis*- and *trans*- amide conformers (Figure 6). This was initially noted when comparing reaction trends of the trifluoromethyl resonance of the major conformer and the aryl fluoride resonance of **8**, where unexpected disagreement was observed. Then, when the integral region of the minor trifluoromethyl resonance was additionally incorporated, great agreement between the trends resulted.

As no temperature control of the sample is possible with the benchtop NMR used in this study, variable temperature (VT) ^{19}F NMR data were measured on a high-field system (at 565 MHz). The VT NMR data were further consistent with the presence of two conformers, as coalescence of the two resonances with heat was observed (See SI Section 4d).

4 | CONCLUSION

The influx in commercially available benchtop NMR spectrometers has allowed for NMR analyses to be conducted in spaces otherwise inaccessible with high-field NMR instruments. This is particularly of interest for reaction monitoring, as these instruments allow for the ability to quantitatively monitor a reaction under native reaction settings/conditions. This is further enhanced with the use of an external reference method for qNMR measurements, as an additional species is not introduced into the reaction mixture as a quantitative standard. The work reported herein describes a method resulting in more robust qNMR analyses using the external referencing technique.

Determining a CCF with multiple concentrations of analyte, and in triplicate at a given concentration, allows for a more robust response factor to be achieved for a particular NMR instrument, set of acquisition parameters, and volume of spins. Additionally, the method is widely accessible as the CCF is simply the slope of the best linear model fit when plotting the concentration of analyte versus the (average) absolute integration per nuclide.

Application of this qNMR technique was demonstrated by monitoring a chemical transformation highly relevant to pharmaceutical settings. Combining benchtop NMR and qNMR allows critical process knowledge such as reagent potency and reaction rates to be determined with minimal experimentation. Overall, this model system displays the practicality of this qNMR technique, and benchtop NMR, to a pharmaceutical environment as critical process relevant information can readily be obtained.

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CONFLICT OF INTEREST STATEMENT

The authors declare the following potential conflicts of interests with respect to the research, authorship, and publication of this article: Authors affiliated with Genentech Inc. were employees of the company when the work was performed and may own company stock.

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REFERENCES

[1] D. P. Hollis, *Anal. Chem.* **1963**, *35*, 1682.
[2] J. L. Jungnickel, J. W. Forbes, *Anal. Chem.* **1963**, *35*, 938.
[3] P. Giraudeau, F.-X. Felpin, *React. Chem. Eng.* **2018**, *3*, 399.
[4] W. G. Lee, M. T. Zell, T. Ouchi, M. J. Milton, *Magn. Reson. Chem.* **2020**, *58*, 1193.
[5] A. O. Mattes, D. Russell, E. Tishchenko, Y. Liu, R. H. Cichewicz, S. J. Robinson, *Concepts Magn. Reson. Part a* **2016**, *45*, e21422.
[6] R. L. Vold, J. S. Waugh, M. P. Klein, D. E. Phelps, *J. Chem. Phys.* **1968**, *48*, 3831.
[7] R. Freeman, H. D. W. Hill, *J. Chem. Phys.* **1969**, *51*, 3140.
[8] R. Wei, C. L. Dickson, D. Uhrín, G. C. Lloyd-Jones, *J. Org. Chem.* **2021**, *86*, 9023.
[9] A. M. R. Hall, J. C. Chouler, A. Codina, P. T. Gierth, J. P. Lowe, U. Hintermair, *Catal. Sci. Technol.* **2016**, *6*, 8406.
[10] F. Malz, H. Jancke, *J. Pharm. Biomed. Anal.* **2005**, *38*, 813.
[11] G. F. Pauli, S.-N. Chen, C. Simmler, D. C. Lankin, T. Gödecke, B. U. Jaki, J. B. Friesen, J. B. McAlpine, J. G. Napolitano, *J. Med. Chem.* **2014**, *57*, 9220.
[12] H. Mo, D. Raftery, *Anal. Chem.* **2008**, *80*, 9835.
[13] G. K. Pierens, A. R. Carroll, R. A. Davis, M. E. Palframan, R. J. Quinn, *J. Nat. Prod.* **2008**, *71*, 810.
[14] R. Muhamadejev, R. Melngaile, P. Paegle, I. Zibarte, M. Petrova, K. Jaudzems, J. Veliks, *J. Org. Chem.* **2021**, *86*, 3890.
[15] S. Akoka, L. Barantin, M. Trierweiler, *Anal. Chem.* **1999**, *71*, 2554.
[16] K. Mehr, B. John, D. Russell, D. Avizonis, *Anal. Chem.* **2008**, *80*, 8320.
[17] R. D. Farrant, J. C. Hollerton, S. M. Lynn, S. Provera, P. J. Sidebottom, R. J. Upton, *Magn. Reson. Chem.* **2010**, *48*, 753.
[18] C. Sterling, R. Crouch, D. J. Russell, A. I. Calderón, *Phytochem. Anal.* **2013**, *24*, 631.
[19] I. W. Burton, M. A. Quilliam, J. A. Walter, *Anal. Chem.* **2005**, *77*, 3123.
[20] H. Mo, J. S. Harwood, D. Raftery, *Magn. Reson. Chem.* **2010**, *48*, 235.
[21] P. I. Rubim de Santana, J. S. F. Diz de Almeida, T. C. C. França, J. Junker, *J. Anal. Methods Chem.* **2022**, *2022*, 7490691.
[22] Y. Lee, Y. Matviychuk, D. J. Holland, *J. Magn. Reson.* **2020**, *320*, 106826.
[23] T. Maschmeyer, L. P. E. Yunker, J. E. Hein, *React. Chem. Eng.* **2022**, *7*, 1061.
[24] M. T. Sabatini, L. T. Boulton, H. F. Sneddon, T. D. Sheppard, *Nat. Catal.* **2019**, *2*, 10.
[25] R. Paul, G. W. Anderson, *J. Am. Chem. Soc.* **1960**, *82*, 4596.
[26] C. A. G. N. Montalbetti, V. Falque, *Tetrahedron* **2005**, *61*, 10827.
[27] O. K. Baryshnikova, T. C. Williams, B. D. Sykes, *J. Biomol. NMR* **2008**, *41*, 5.
[28] Y. Zhou, J. Wang, Z. Gu, S. Wang, W. Zhu, J. Luis Aceña, V. A. Soloshonok, K. Izawa, H. Liu, *Chem. Rev.* **2016**, *116*, 442.
[29] M. Bornemann-Pfeiffer, J. Wolf, K. Meyer, S. Kern, D. Angelone, A. Leonov, L. Cronin, F. Emmerling, *Angew. Chem., Int. Ed.* **2021**, *60*, 23202.
[30] J. Liu, Y. Sato, F. Yang, A. J. Kukor, J. E. Hein, *Chem.-Methods* **2022**, *2*, e202200009.

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