

Easy, Precise and Accurate Quantitative NMR

Application Note

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Abstract

Extremely accurate and precise quantitative NMR was demonstrated using the new qNMR tools in VnmrJ 3.1 software and calibration with an external standard. Periodic calibration can deliver accuracy as high as 99.9 % and precision as good as 0.59%, while performing calibration with each study can result in accuracy and precision as high as 100% and 0.35%, respectively.

Introduction

Various techniques can be used to provide quantitative analysis of small-molecule organic compounds. The most common is chromatographic separation followed by detection of the analyte based on spectroscopic detection (e.g., UV/Visible, evaporative light scattering, etc.) or spectrometric detection (e.g., mass spectrometry). While each of these detection methods provides benefits in some aspects of their use, they are all hampered by issues such as response factors or matrix effects. There is no universal chromatography detector.

While used most often for structural analysis, NMR is increasingly being considered as a critical quantitative tool. NMR is most powerful when used quantitatively, as the integrated intensity of a resonance signal is directly proportional to the number of nuclei represented by that signal. In addition, all protons across the spectrum are equally sensitive, so determination of quantitative results does not require the need for compound-specific extinction coefficients or calibrations. NMR has been used to determine concentrations of synthetic and biosynthetic products, fine chemicals, and pharmaceuticals, as well as metabolites, catabolites, and endogenous compounds in biological fluids¹. Quantitative NMR (qNMR) has been shown to be particularly useful in metabolomics, drug discovery and analysis, and natural product analysis².



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Quantitative NMR possesses the required accuracy and precision to become a routine quantitative tool in many analytical laboratories. Chemical referencing with internal standards can provide accurate quantification, but this technique is tedious and contaminates the sample. Several electronic referencing approaches have also been used in an attempt to provide accurate and precise quantitative results, such as ERETIC (Electronic Referencing to access in vivo Concentrations), PIG (Pulse Into Gradient), ARTSI (Amplitude-corrected Referencing Through Signal Injection) and QUANTAS (Quantification by Artificial Signal). However, these methods are limited in scope and/or inherently error-prone³⁻⁷.

Accurate and precise qNMR of small-molecule organic compounds can be attained easily using only absolute integration of an external concentration standard and 1D spectra, given an NMR platform that is highly stable and linear. This Application Note demonstrates the unique capability of the Agilent NMR system to provide easy and accurate absolute quantification for practically any organic chemistry sample. It describes the use of Agilent VnmrJ 3.1 software, the OneNMR probe that is very tolerant to solvent, dielectric, or sample height changes, and the DirectDrive consoles to perform quantification of small-molecule organic compounds. Accuracy (% of actual concentration) of 96.98 to 102.12% and precision (% standard deviation) of 0.59 to 1.07% are attainable with only a one-time determination of calibration. Even higher accuracy and precision are attainable (99.48-100% and 0.35-0.60% respectively) by collecting the calibration data as part of each study to account for minor changes in the laboratory environment.

Experimental

Standards and Reagents

Ibuprofen was obtained from Sigma-Aldrich, and a stock solution was prepared in DMSO-d₆ (dimethylsulfoxide containing 6 deuterium atoms) at a concentration of 200.00 mg/mL, or 969.5 mM. A series of nine samples at concentrations down to 515 μM was prepared from this stock solution by dilution. A blank sample containing just the DMSO-d₆ solvent was also included in the sample set. Caffeine was obtained from Sigma-Aldrich, a 52.11 mM stock solution was prepared in D₂O, and all dilutions of the stock solution were made with D₂O.

Instruments

The ibuprofen quantification experiments were performed on an Agilent 500 MHz DirectDrive NMR spectrometer equipped with a 5 mm OneNMR Probe and a 7600-AS sample changer. All data were collected with active sample temperature regulation at 25 °C. The probe was initially tuned to the calibration standard and left in that configuration for all subsequent measurements.

The caffeine quantification experiments were performed on an Agilent 400 MHz DirectDrive NMR spectrometer equipped with a 5 mm OneNMR Probe and a 7600-AS sample changer. The probe was tuned to the calibration standard before each study.

Analysis Parameters

The parameters used for each instrument are shown in Table 1.

Table 1. Agilent NMR Spectrometer Conditions

Single Calibration	
Instrument	Agilent Direct Drive 500 MHz NMR with a 5 mm OneNMR™ Probe
Excitation pulse	12.1 μs @ 57 dB; 90° tip angle
Receiver gain	Automatically adjusted for each sample
# of Scans	1
Operating Frequency	499.86 MHz
Acquisition time	3.0 seconds
Fourier Number	256k
Apodization	0.3 Hz exponential line broadening
Multiple Calibrations	
Instrument	Agilent Direct Drive 400 MHz NMR with a 5 mm OneNMR™ Probe
Excitation pulse	11.5 μs @ 59 db; 90 degree tip angle.
Receiver gain	20 dB
# of Scans	1
Operating Frequency	399.92 MHz
Acquisition time	4.0 seconds
Fourier Number	256k
Apodization	0.3 Hz exponential line broadening

Results and Discussion

External Standard Calibration

The Agilent consoles utilize direct signal digitization in the receiver path. This design architecture provides the stability and linearity required to return high quality, absolute quantification data using only an external standard calibration without the need for any internal reference signal.

Calibration can be done using any sample of known concentration in the range of 10 to 100 mM. The sample

should contain a reasonably sharp resonance, or group of resonances, that is well separated from all other signals, and the total number of nuclei represented by the calibrant resonance must be known. A single scan, 1D spectrum is then collected using a 90 degree pulse and the region containing the calibrant resonance is integrated. The value of this integral region is manually set by determining the product of the sample concentration and the number of nuclei, entering that product into the Integral Value field, and clicking on the Set Integral Value button (Figure 1). The

qEstimate command can then be used to populate the probe file with all the parameters required for determination of concentration of an unknown sample.

Once these values are in the probefile, the calibration is complete and sample concentration is accessible by a mouse click. A more detailed description of the calibration process can be found in the Quantification Tools Chapter of the VnmrJ 3.1 Automation User Guide.

Easy integration of the calibration standard

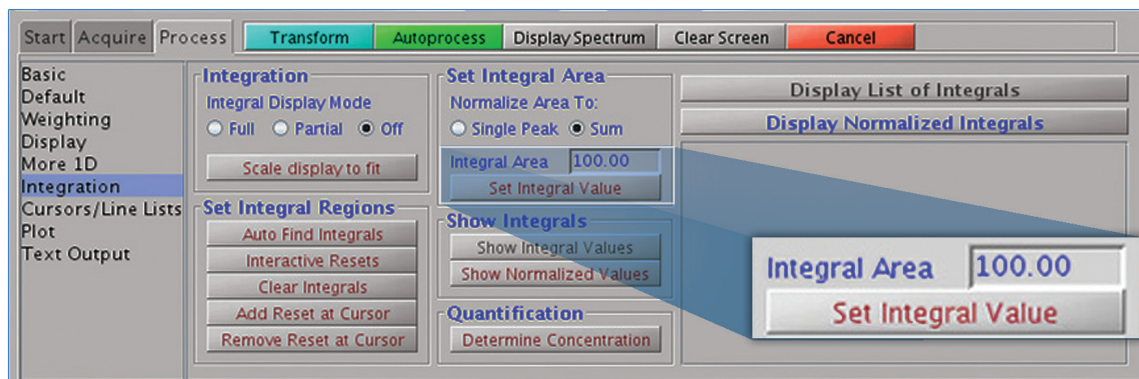


Figure 1. The interface tool for setting integral area can be found on the Integration panel under the Process tab.

Periodic Calibration for Routine qNMR

A minimum of one calibration of the probe is required (or periodic calibration as would be done for 90 degree pulse widths) to use the new qNMR tools to quantify samples with acceptable accuracy and precision. In order to reduce integration errors to below 1%, a signal-to-noise (S/N) of at least 150/1 is required because the error is approximated by the equation $0.25 + (100/SN)^8$. The S/N of the spectra used for calibrations as well as for each analyte must be considered when accuracy and precision are important.

To illustrate the use of a single calibration for routine qNMR, a calibration standard of ibuprofen was prepared at a concentration of 15.17 mM in DMSO-d₆ and placed in a 5 mm NMR tube. In addition, a series of 9 samples at concentrations between 132 mM and 500 μM was also prepared. The concentration of the standard sample and the intensity of the methyl doublet at 1.33 ppm were used as the inputs for the qEstimate calibration (Figure 2).

Once probe calibration was complete, each sample in the concentration series was submitted for acquisition under automation. A single scan ¹H NMR spectrum was collected on each sample after the system performed routine autolock, autoshim, and auto-gain functions. Data were collected on each sample in turn, and the entire series was then repeated until at least 20 replicate data sets were collected for each sample.

The appropriate external standard spectra peaks are integrated in the calibration step

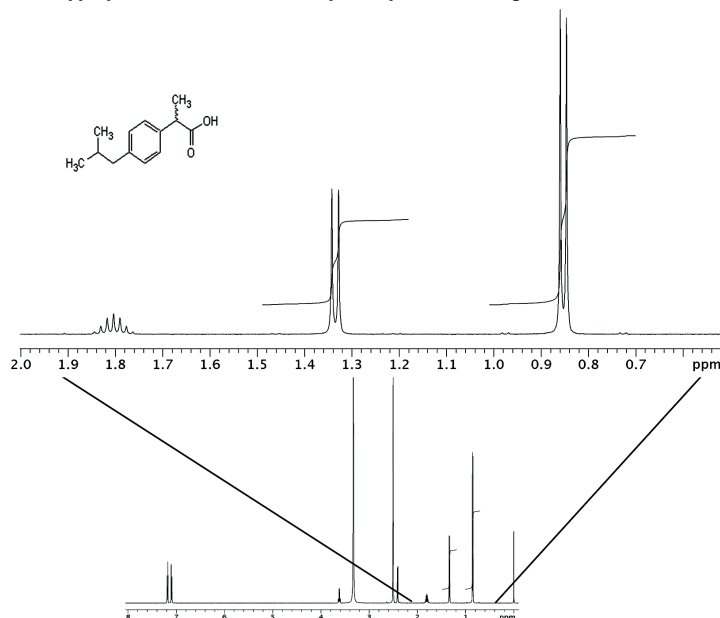


Figure 2. ¹H NMR spectrum of ibuprofen (lower trace) used for calibration and quantification of the ibuprofen concentration series given in Table 2, with the 0.5 to 2 ppm region expanded (upper trace). The methyl doublet appears at 1.33 ppm, and the isobutyl methyl doublet appears at ~0.84 ppm. The structure of ibuprofen is also shown.

Easy software access to concentration determinations

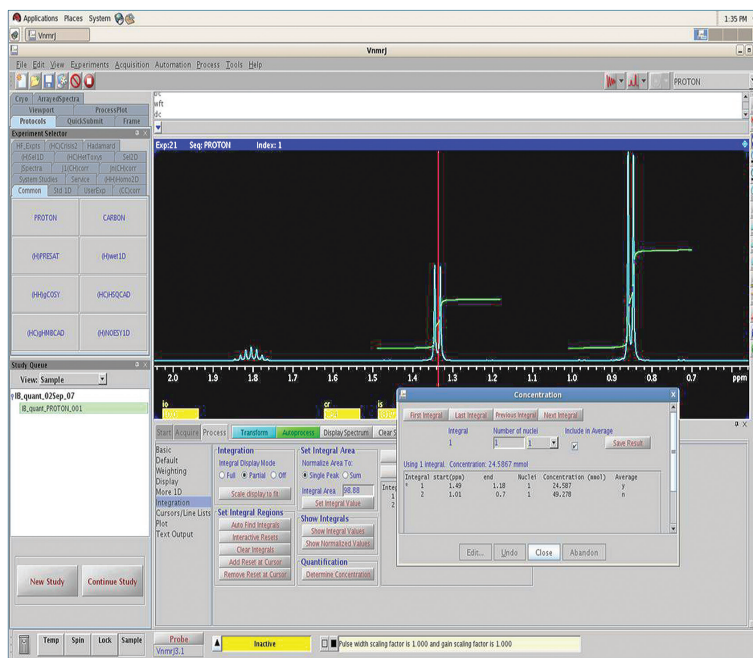


Figure 3. The **Determine Concentration** button is used to initialize the Concentration popup window. This tool allows interactive assignment of multiplicity to each integral region in a spectrum and the choice for which regions to include in the final calculation of concentration.

Figure 3 shows the window that is displayed when the Determine Concentration tool is accessed via the **Determine Concentration** button on the Integration panel under the Process tab. Each integral area in the spectrum is displayed, and it can be added to the list for calculation of concentration after interactively assigning the appropriate multiplicity. For the investigation of a single sample, manual implementation of this workflow is fast, simple, and easy.

Since the current investigation consisted of more than 180 spectra, an automated processing macro was used to select the regions that correspond to the methyl doublet at ~1.3 ppm and the isobutyl doublet at ~0.8 ppm, scale the integration of those regions based on multiplicity (3 and 6 protons, respectively), calculate the concentration of the sample based on those two signals, and finally write out the results into a comma separated spreadsheet (csv) file. An example of this macro can be found on the spectrometer in the user library (i.e., /vnmr/userlib/mac/lib/qEst2csv). For the purposes of data analysis in this work, each peak was treated as an independent concentration measurement.

The results are shown in Table 2, with less than 1% standard deviation across the entire concentration series. The results demonstrate high linearity ($R^2=0.9995$) across a concentration range of almost 3 orders of magnitude (Figure 4).

Table 2. Accuracy and precision of quantification of an ibuprofen concentration series, using a single-point calibration

Actual Concentration (mM)	Average Measured Concentration (mM)	Standard Deviation (mM)	Accuracy* (% of actual)	Precision (%SD)
0.515	0.531	0.006	96.98%	1.07%
1.03	1.049	0.008	98.13%	0.79%
2.06	2.098	0.012	98.14%	0.59%
4.12	4.171	0.024	98.77%	0.57%
8.24	8.269	0.048	99.65%	0.58%
16.49	16.571	0.103	99.51%	0.62%
32.98	33.014	0.293	99.90%	0.89%
65.97	67.813	0.492	97.21%	0.73%
131.94	129.141	1.091	102.12%	0.84%
Overall			98.46%	0.74%

* Note the expected loss of accuracy with the samples at both very low and high concentrations. Integration errors are expected at low concentration due to the low S/N ratio afforded by the single scan used for acquisition, and radiation damping can potentially affect samples at very high concentration.

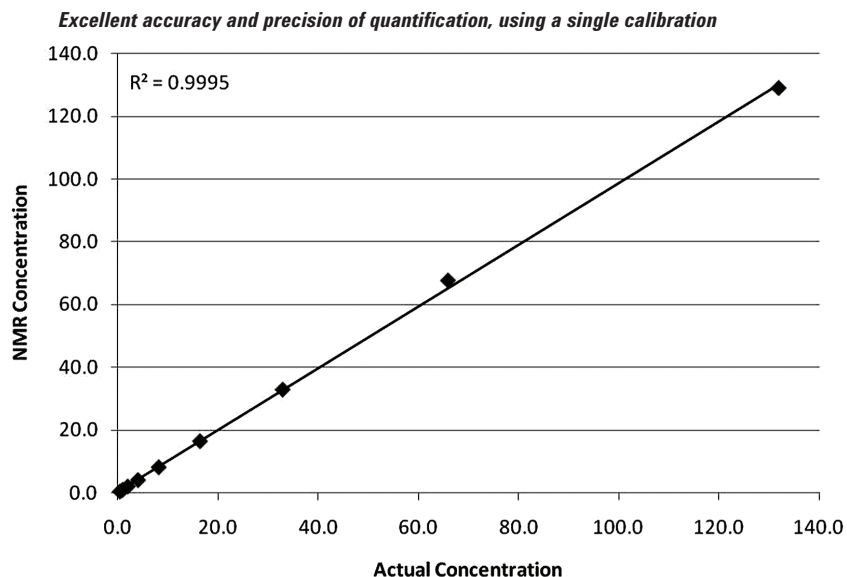


Figure 4. Rapid, easy and accurate determination of ibuprofen concentrations across a dynamic range of more than 3 orders of magnitude, with an R^2 value of 0.9995 for the correlation between actual and measured concentration.

Run-time Calibration for Higher Accuracy and Precision

Higher accuracy and precision of quantification can be obtained by performing a calibration prior to each sample study. This helps to compensate for minor changes in stability due to changes in the laboratory environment. Further improvements can be obtained if the same gain value is used for both the calibration standard and the samples of interest. A series of three concentration standards was prepared using caffeine as the analyte to illustrate this higher level of accuracy and precision. The samples were prepared at 50.44 mM, 51.91 mM, and 52.11 mM, yielding a difference in concentration of only 3.3% across the series. Each study of the three samples consisted of an autocalibration of the external standard (54.11 mM caffeine in D₂O), followed by quantification of the three samples. Seven qNMR studies were performed as a series and the NMR console was instructed to resubmit this series every three hours until a total of 630 studies (210 for each concentration) was performed. As shown in Table 3, the overall accuracy of quantification was 99.94%, and the overall precision was 0.51%, assuring the ability to discern the very small differences in the concentrations for the three samples.

A second experiment was performed to assess the affect of sample height in the NMR tube on the quantification results. This was done by removing sample from each tube used in the previous experiment to lower the sample height in each tube to 48, 35, and 30 mm, respectively (Table 4). A total of 35 studies was then performed on each sample with the spectrometer programmed to repeat the series (seven qNMR studies, each study consisting of an autocalibration with the external standard followed by quantification of the three samples) every three hours over a 16 hour period

Table 3. Accuracy and precision of quantification of three caffeine concentrations, using multiple run-time calibrations

Actual Concentration (mM)	Sample Height (mm)	Average Measured Concentration (mM)	Standard Deviation (mM)	Accuracy (% of actual)	Precision
52.11	54	52.16	0.27	99.85%	0.52%
50.44	45	50.70	0.21	99.48%	0.42%
51.91	37	51.99	0.31	99.90%	0.60%
Overall				99.74%	0.51%

Table 4. The effect of sample height on accuracy and precision

Actual Concentration (mM)	Sample Height (mm)	Average Measured Concentration (mM)	Standard Deviation (mM)	Accuracy (% of actual)	Precision
52.11	48	52.13	0.19	99.96%	0.35%
50.44	35	50.44	0.17	100.00%	0.49%
51.91	30	—	—	—	—
Overall				99.98%	0.42%

of time. Using the OneNMR probe, the results confirmed that the sample height can be varied from 35 to 54 mm without degrading either the accuracy or the precision of the result (Table 4). The sample height in Sample 3 (30 mm) was too short to shim routinely and therefore did not produce any useable results.

This second experiment was repeated with samples 1 and 2 using bi-level broadband ¹³C decoupling during acquisition to collapse the ¹³C satellites⁹. A total of 98 studies was performed with each sample, with the spectrometer programmed to repeat each series

(seven qNMR studies, each study consisting of an autocalibration with the external standard followed by quantification of the two samples) every two hours over a 28 hour period. The lower trace in Figure 5 presents the coupled reference spectrum and displays the obvious resolution of the ¹³C satellites from the ¹²C signals. In the upper trace of Figure 5 one can see that the ¹³C satellite peaks have been completely collapsed into the main resonance band. The accuracy and precision of quantification were still very good even with the potential noise/error introduced due to broadband ¹³C decoupling (Table 5).

Table 5. The effect of ¹³C decoupling on accuracy and precision

Actual Concentration (mM)	Sample Height (mm)	Average Measured Concentration (mM)	Standard Deviation (mM)	Accuracy (% of actual)	Precision
52.11	54	52.09	0.20	99.96%	0.37%
50.44	35	50.44	0.17	100.00%	0.49%
Overall				99.98%	0.43%

High accuracy and precision of quantification, even with ^{13}C decoupling

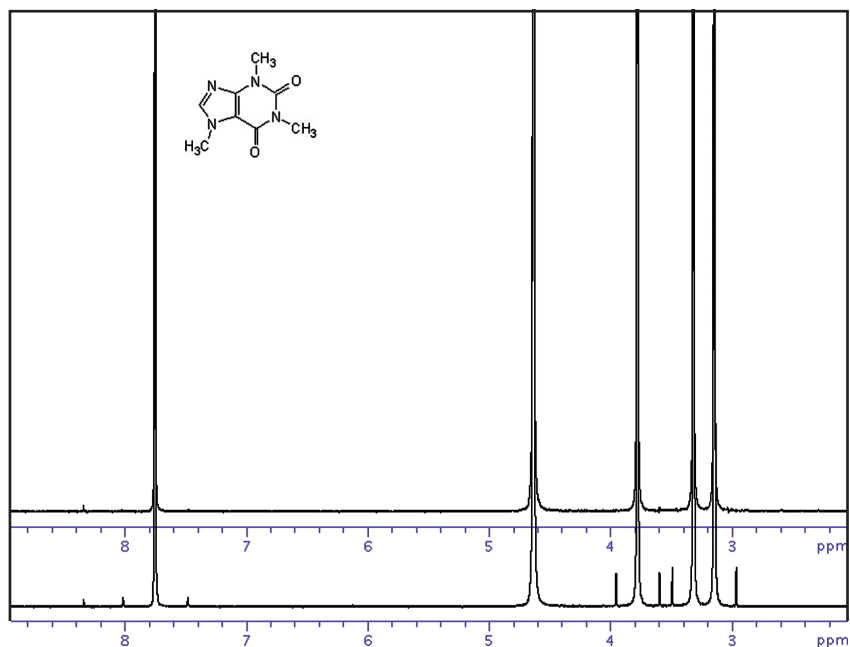


Figure 5. Caffeine sample (structure shown) acquired with (top) or without (bottom) bi-level broadband ^{13}C decoupling of sufficient field to decouple 240 ppm of ^{13}C frequency space. A proton NMR acquisition time of 3.6 seconds was used in both experiments. Decoupling was performed using the BilevelDEC experiment protocol located in Std1D tab of the Experiment Selector.

Conclusion

The NMR experiment itself displays all the properties one might wish for in a quantitative technique: specificity, selectivity, sensitivity, linearity, high dynamic range, unit response factors, and most importantly, universal detection for all proton-bearing organic molecules. The primary drawback to using NMR in quantitative analyses has always been the non-linearity inherent in the commercially available NMR hardware. Correcting for these errors on a sample-to-sample basis is possible, but it requires the addition of internal chemical standards or the incorporation of an external reference signal into the experimental data. Internal standards are tedious to use and bring another layer of error to the measurements, whereas the methods to generate an external reference signal are all very limited in scope and/or inherently error prone themselves.

Agilent NMR systems are unique in that the signal path for the receiver is fully digital, providing an unprecedented linearity of response across a wide range of sample concentrations. The OneNMR Probe is also very tolerant to changes in solvent, dielectric, and sample height. By combining this level of robust hardware with sophisticated software, Agilent has developed an exceptional tool for quantitative analysis. Accurate and precise NMR quantification of samples can be done easily and quickly by absolute integration, using the VnmrJ 3.1 software and a one-time determination of calibration parameters with an external standard of known concentration. Accuracy of 96.98 to 102.12% and precision of 0.59 to 1.07% can be attained with small-molecule organic compounds. Even higher accuracy and precision are attainable (99.48-100% and 0.35-0.60% respectively) by using a run-time calibration with each sample study

to account for minor changes in the laboratory's environmental stability. In fact, even broadband ^{13}C decoupling does not significantly impact the error in these measurements. These levels of accuracy and precision can be attained and reported quickly and easily using the software functions to acquire and store the calibration parameters, set up and schedule the quantification studies, and create custom reports.

Finally, while this Application Note only discusses quantification using proton NMR data, the new qNMR tools are frequency independent. Users can do the same type of calibration using any appropriate nuclei (i.e., ^{31}P , ^{19}F , ^{13}C , etc.) and then implement the same workflows for data collected on those nuclei.

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