Practical aspects of ^{13}C qNMR

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Webinar link

Introduction

Quantitative NMR is one of the most important tools for the quantification of chemical species in samples. It is most commonly run through the measurement of ^{1}H detected single pulse experiments, but severe spectral overlap makes the quantification of analytes a challenging task. ^{13}C detected NMR alleviates the spectral overlap problem, and with modern instruments achieving higher sensitivity, ^{13}C qNMR becomes a suitable alternative to ^{1}H qNMR. For example, the resolution achieved in ^{13}C data enables the determination of branching in polymers (https://doi.org/10.1016/j.polymer.2019.121965).
Several aspects such as the acquisition of data with low signal-to-noise ratio and the larger bandwidth required to excite the ^{13}C spectrum lead to a number of considerations that must be taken into account when acquiring ^{13}C qNMR. Here we describe some of the practical aspects and consideration to take into account to acquire accurate ^{13}C qNMR data.

Resources

- qNMR portals:
  - https://gfp.people.uic.edu/qnmr/
  - https://www.jeol.co.jp/en/products/nmr/qnmr_index.html
- ^{19}F qNMR: https://doi.org/10.1002/cmr.a.21422
- ^{1}H qNMR:
  - https://doi.org/10.1021%2Fnp200993k
  - https://doi.org/10.1021/acs.analchem.0c02967
- Webinars:
  - Core principles of precise qNMR – Common Pitfalls and Solutions, Ron Crouch: https://connect.acspubs.org/CENWebinar_JEOL_6_24_20
  - Quantitative NMR (qNMR) Applications, Jose Napolitano, Charlotte Corbett, Gennady Khirich: https://www.youtube.com/watch?v=7VYE-W28VYg
  - Introduction to Quantitative NMR —Easy and Reliable Assay, Takanori Komatsu: https://www.jeol.co.jp/en/news/webinar/2021/20210224_movieform_01.html
  - Delta processing part 2: quantitative NMR , Adolfo Botana: https://attendee.gotowebinar.com/register/591245194184783115
  - Principles and application of no-D NMR, Tim Bergeron: https://connect.acspubs.org/CENWebinar_JEOL_4_21_20
  - Quantitative ^{13}C NMR, Adolfo Botana: https://attendee.gotowebinar.com/register/2577550346473705743

Sample preparation

Solvent selection:
- Purity. Impurities may lead to signal overlap and/or interactions
- Signal Overlap. Different solvents and concentrations may change signal overlap
- Volatility. Avoid volatile solvents whenever possible to avoid concentration changes / seal sample
- Conductivity. Use same matrix both for reference and analyte samples to minimize pulse length and sensitivity variations
- Typical solvent considerations: solubility, temperature range, etc.

Reference standard selection:
- Stable and soluble in the solvent
- No signal overlaps with signals of the analyte and solvent
- No interactions with the analyte and solvent
- High and assured purity (CRM)

Relaxation agent:
- It may be useful to consider adding relaxation agents to reduce the relaxation time.

Mass measurement:
- Mass measurements are generally more accurate than volume measurements
- An example of mass measurement procedure:
  - Weigh the tare
  - Weight the tare with the sample
  - Weight a vial
  - Weight the vial with tare with the sample and solvent
- If we set that 2 SD <0.1% (as per USP <41>,<1251>), then the minimum weight can be determined as:

$$W_{min} = \sigma \times 2000$$

W_{min} : *Minimum Weight*
σ : Standard deviation calculated with ten repeated measurements

Measurement example of *Minimum Weight*

Type of balance	*Minimum Weight* (W_{min})
Semi-micro balance (readability: 0.01 mg)	13.9 mg
Micro balance (readability: 0.001 mg)	2.8 mg
Ultra-micro balance (readability: 0.0001 mg)	0.2 mg

(https://labchem-wako.fujifilm.com/europe/category/00622.html)

Sample transfer to NMR tube:
Accuracy of volume transferred less important in internal quantification (concentration of both analyte and internal standard vary in the same way)
Consider:
- liquids viscosity, temperature, interactions with pipette
- air displacement (for aqueous samples) vs positive displacement
- maximum pipette range
- Manual vs electronic pipette
- tip immersion depth
- pipetting rhythm and speed
- immersion angle
- consistent sample dispensing
- tip pre-rinsing
- Lab temperature and hand warming

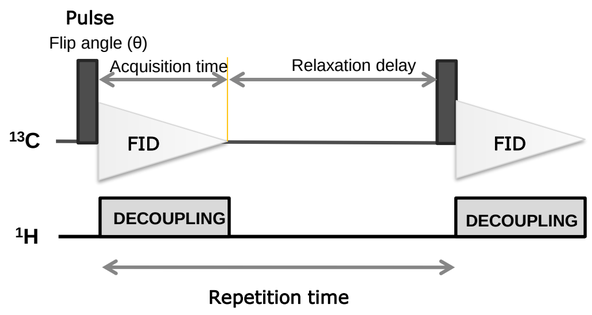
Additional considerations for external quantification:
Keep sample volume the same, as it affects tuning and shimming
Tubes must be built with very low tolerances for external quantification:
- in a tube specified as 4.2± 0.2mm, the effective concentration detected by NMR can vary by 9.75% in different tubes
- in a tube specified as 4.2065 ± 0.0065 mm, the effective concentration detected by NMR can vary by 0.31% in different tubes

Data acquisition

Instrument setup. Evaluate:
- Bias assessment (quantification of analyte with known concentration, typically a CRM)
- Spectral uniformity
- Receiver amplitude linearity
- Actual sample temperature, temperature gradients
- General shimming (lineshape specifications)
- Instrument stability (for external quantification): tuning reproducibility, sensitivity stability (room temperature influence),etc.

Sample setup:
- Temperature equilibration
- Lock: Automatic or no lock for no-D samples
- Shimming: Automatic or custom for difficult samples (microcells, convection, etc.)
- Shimming in no-D samples: set the system to do selective gradient shimming on tallest ^{1}H signal (see no-D webinar)
- Tuning: Automatic
- Pulse width calibration (external quantification): Automatic
  Ask for latest quantification script (Delta 5.3+)

Pulse sequence choice:
- Single pulse with ^{1}H decoupling (inverse gated decoupling)
- Acquisition time should be long enough to digitize the FID, at least until it becomes indistinguishable from the noise (typically 1-2s) (or >5 T_2)



Acquisition time + relaxation delay = repetition time

Pulse width

There are different considerations when choosing the pulse flip angle / pulse width:
- Excitation profile for <1% error (Pulse width dependent)
  - <5.5us @ 400MHz
  - <4.3us @ 500MHz
  - <3.6us @ 600MHz
- Reproducibility for external quantitation (90 degrees pulses have better reproducibility)
- SNR per unit time when using the optimum repetition time for target uncertainty level (https://doi.org/10.1021/ed061p909)
- Signal observed (from equilibrium magnetization) is given by $M_z \sin(\theta)$

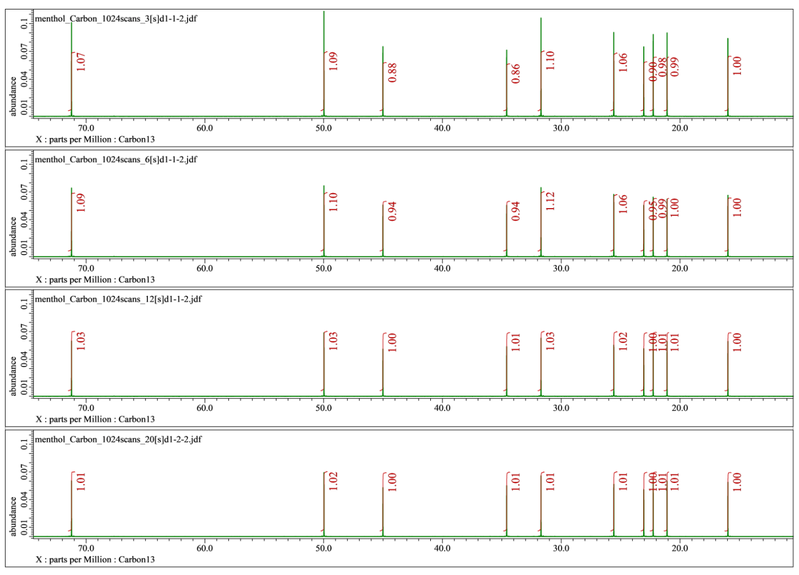
Relaxation delay:
- Magnetization recovers approximately* as per the following equation

$$M_z = M_0[1 - (1 - \cos\vartheta)e^{\frac{-t}{T_1}}]$$

- There is no major gain by increasing the repetition time beyond 5 T_1, without taking great care of other parameters
- Uncertainty as per pulse width and repetition time (in steady state conditions):

Uncertainty in %	Repetition time for 90 deg (T_1 times)	Repetition time for 60 deg (T_1 times)	Repetition time for 30 deg (T_1 times)
10	2.3	1.6	~0.7
5	3.0	2.3	1.0
1	4.6	3.9	2.6
0.5	5.3	4.6	3.3
0.1	6.9	6.2	4.9

*https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Quantitative_NMR_(Larive_and_Korir)/02%3A_Practical_Aspects_of_Q-NMR/2.05%3A_Effects_of_Tip_Angle_in_Quantitative_NMR_Experiments
See https://doi.org/10.1021/ed061p909 for a more accurate description



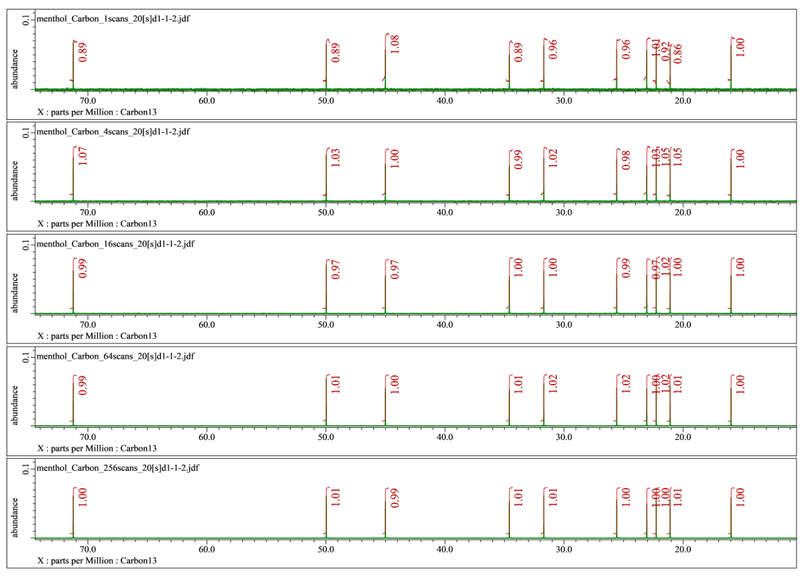
Spectra acquired for 30% menthol with relaxation delays of 3,6,12 and 20 s, and 2s acquisition time. The T1 values for menthol peaks range from 1.7 to 2.3 s

Scans:
- Dependent on uncertainty level target.
- Achieving enough SNR to achieve uncertainty criteria can be the most challenging aspect of ^{13}C qNMR
- Depending on the application we can settle with an SNR around 50, but ideally SNR should reach 150. There is no major gain by increasing the SNR beyond this, without taking great care of other parameters
- As a rule of thumb, if no signals are discernible after 4 scans, acquiring a quantitative spectrum is generally not feasible with that sample concentration/probe.
- The following tables are an example of how the SNR scales with the number of scans:

SNR	Uncertainty in %
30	10
60	3
150	1
400	0.5
1200	0.1

SNR and uncertainty as per http://dx.doi.org/10.13140/RG.2.1.1244.3689

SNR	Scans	SNR	Scans
10	4	2	4
40	64	8	64
160	1024	32	1024
640	16384	128	16384



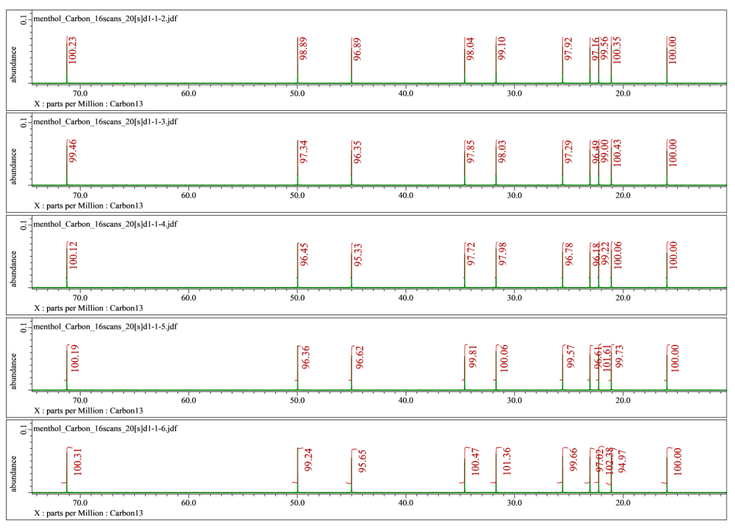
Spectra acquired for 30% menthol with 1, 4, 16, 64 and 256 scans. After 0.3Hz line broadening (>2s acquisition time) the SNR for the rightmost signal with 1ppm RMS window were 70, 139, 256, 492, 1006 respectively

Decoupling:
- The ^{1}H decoupling is an important source of error. NOE effects are negligible with no decoupling during the long relaxation delay.
- The decoupling modulation used affects both the effective bandwidth and artifacts. WALTZ (WALTZ65) generally provides spectra with low level of artifacts. WALTZ65 is the default decoupling in JEOL Delta (named WALTZ)
- Other decoupling schemes such as adiabatic decoupling (https://doi.org/10.1016/j.jmr.2006.11.007) and bilevel decoupling (https://doi.org/10.1021/acs.analchem.0c03753), may be considered, particularly in systems at higher field.
- Default decoupling pulse width may be insufficient to achieve 1% uncertainty:
  - Decoupling pulse width sufficient to decouple the proton bandwidth with less than 1% error are approximately 85us @ 400MHz, 67us @ 500MHz, 55us @ 600MHz
  - Higher power decoupling may lead to heating, which can lead to an unstable lock and line broadening. This effect is probe and sample dependent. If sample heating is substantial lower power decoupling should be used. If lock becomes unstable , the data should be acquired with the lock hold function,
  - Ensure that the decoupling attenuation irr_atn_dec is at least 18[dB] higher that the power level for the square pulse for Proton in the probe tool (or as per the limit set by the NMR manufacturer). Otherwise probe may be damaged. Beware heating effects in salty samples. In case of doubt reach out to the NMR manufacturer.

Data processing and analysis

Processing
- No clear evidence of improvement by using window functions
  - However, the use of window functions can help homogenize the linewidth of different peaks, facilitating the integration across peaks with different T_2.
- For cold probes, FID prediction (blip_cld, automatically applied in Delta 6) may be needed, typically two predicted points are sufficient to obtain flat baselines
- Double the number of points to use all the information contained in the FID (2 times zerofill in Delta definition). There is no clear evidence on advantages on further zero-filling
- Baseline noise is typically considerable
  - The default JEOL Delta integration does a local baseline correction, where the baseline is considered to be at the edges of the integral. This leads to substantial error when integrating spectra with low SNR
  - Reset the slope and offset when integrating spectra with low SNR.
  - The default Delta behavior can be changed in menu Options>Preferences, Geometry tab: Adjust Integral Slope/Offset
  - Alternatively increase the number of points averaged to more than 11. The default behavior can be changed in menu Options>Preferences, Geometry tab: Integral Averaging Points. This is less accurate than zero slope and offset
  - Use the simplest possible baseline correction (such as polynomial order 1 or 3)

Integration
- Increasing integral regions improves trueness, but it decreases precision due to the integrating more noise region.
- For lower SNR data, integral regions should be smaller because “While an increase in the integration range results in improved accuracy, the precision decreases because the variance of the integral of white Gaussian noise is a Wiener process and thus increases linearly with range.” (https://doi.org/10.1021/acs.analchem.1c00407).
- Smaller integral region : higher precision, lower trueness for each integral
- Note that the absolute value of an integral is not important, we compare the integral of the analyte of interest with the reference integral
- Both analyte peak and reference peak must therefore have similar linewidths to improve accuracy. If peaks have different linewidths, consider using different integral widths or more line broadening.
- About 1% of the signal is split due to ^{13}C-^{13}C coupling, which may typically range between 15 to 20Hz. Care should be taken to either include these splittings (preferable in case of isotopic ratio variations) or exclude them for all integrals



Spectra acquired for 30% menthol with 16 scans, and integrated with integrals widths of 5, 10, 20, 50 and 100 Hz respectively. Integrals can be largely different with 50 and 100 Hz widths.