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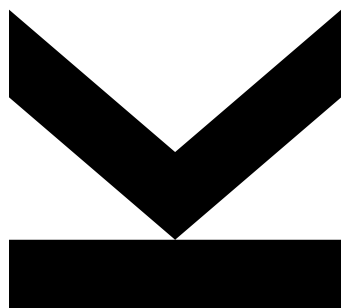
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Nitrogen-15 Labeling and NMR Spectroscopy for the Elucidation of Side Reactions



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Sworn Declaration

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Abstract

^{15}N NMR is a valuable spectroscopic method for qualitative investigations on reaction mechanisms and their side products. Due to the low natural abundance of the ^{15}N isotope sensitivity enhancement by labeling brings along high specificity in tracing the fate of nitrogen containing molecules. This work provides insight into various aspects of the ^{15}N nucleus that have to be considered in ^{15}N NMR. One issue dealt with is the way of referencing of ^{15}N NMR spectra, which has been under debate for some time and requires special consideration when comparing literature data.

The primary objective of this thesis is a study based on ^1H , ^{13}C , ^{19}F and ^{15}N NMR spectroscopy to elucidate reactions occurring in a three component system of small organic molecules. Exclusion of individual components, and applying components in huge excess, as well as investigations of the time course led to the definite disproof of all but one previously suggested reaction hypotheses. The evidence for the remaining hypothesis could be substantiated.

^{15}N NMR ist eine spektroskopische Methode, die vor allem in Studien über Reaktionsmechanismen und Nebenprodukte wertvolle Informationen bietet. Auf Grund der geringen natürlichen Häufigkeit des ^{15}N Isotops kann mittels Anreicherung dessen eine hohe Spezifität im Bezug auf die untersuchten Stickstoff enthaltenden Moleküle erzielt werden. Diese Arbeit bietet einen Einblick in die Besonderheiten des ^{15}N Kerns, welche auf ^{15}N NMR Einfluss nehmen. Unter anderem wird das über längere Zeit uneinheitlich ausgelegte Thema der Referenzierung von ^{15}N NMR Spektren beleuchtet.

Das Hauptaugenmerk dieser Masterarbeit basiert auf einer umfangreichen NMR Studie (^1H , ^{13}C , ^{19}F , ^{15}N), deren Fokus in der Aufklärung der Reaktionen eines Drei-Komponenten-Systems von kleinen organischen Molekülen liegt. Durch gezielten Ausschluss einzelner Komponenten, das Einsetzen der Komponenten in hohen Überschüssen, sowie Untersuchungen des zeitlichen Reaktionsverlaufes konnten, bis auf eine, alle im Voraus aufgestellten Reaktionshypothesen widerlegt und die verbleibende Hypothese weiter gestützt werden.

2. Introduction

2.1. Importance of Nitrogen NMR

Besides hydrogen and carbon, nitrogen atoms represent ubiquitous constituents of natural as well as synthetic molecules, which have suitable NMR-active isotopes. The lone pair of nitrogen causes great chemical variability, which is reflected in the huge chemical shift range of 900 ppm over which nitrogen signals are spread. ¹

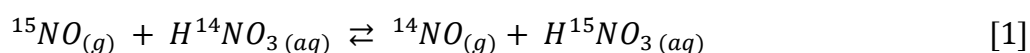
Observations on the most abundant nitrogen isotope, ¹⁴N, a spin 1 nucleus, struggle with quadrupolar relaxation and the resulting peak broadening. This effect can be reduced by taking advantage of diluted solutions in poorly viscous solvents like acetone or ether. ²

The ¹⁵N isotope is a spin ½ nucleus yielding signals of acceptable line width. Due to its very low natural abundance of 0.365% most ¹⁵N-NMR studies require a sensitivity improvement by ¹⁵N labeling. ³

2.2. ¹⁵N Enrichment in the Sample

Although molecules containing different isotopes do neither differ significantly in their electronic structure nor in the potential energy surface they can be discriminated due to the so-called isotope effects. The altered number of neutrons in the nucleus has an impact on the reaction rates, termed kinetic isotope effect, as well as on the equilibrium constants of chemical reactions - the equilibrium isotope effect. Both phenomena are based on a lowered ground-state vibrational energy and consequently on the higher energy input required for disrupting a bond of the heavier isotope. ⁴

One of several options of separating ¹⁵N from ¹⁴N is utilizing a chemical exchange reaction such as the following (Equ.1).

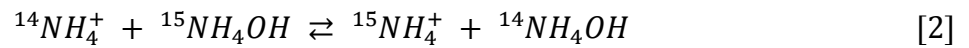


Due to the higher ground-state energy of molecules containing ¹⁴N the lighter isotope tends to be enriched in the gas phase while the heavier isotope accumulates in the liquid phase.

In a sequence of separation columns including a gas back-stream system ¹⁵N can be enriched step by step up to a concentration of 99.8% over a period of two weeks. ⁵

Another way of separation is based on thermal diffusion. An apparatus consisting of a column containing a heated wire inside and a cooling jacket covering the outer surface causes a gas convection stream. Due to the temperature difference two gases, such as NO and NO₂ are separated while the ¹⁵N isotope accumulates in the heavier molecule. ^{6, 7}

An advanced approach of isotope separation is based on ion exchange chromatography. A classical cation exchange column is treated with an aqueous solution of ammonium hydroxide. The heavier ¹⁵N isotope tends to accumulate in the ammonium ion, which is bound to the solid stationary phase. Equation 2 represents the equilibrium reaction on which the separation is based. ⁸



2.3. Nuclear Overhauser Effect

Another aspect worth mentioning when touching on the subject of nitrogen NMR is the Nuclear Overhauser Effect (NOE), which is caused by the fluctuating dipole-dipole interaction of spatially close nuclei. When a spin is saturated its energy levels change in their population. The system tends to restore the equilibrium state by relaxation by single -, double - or zero - quantum transitions. It is the double- and zero-quantum transitions, which result in a transfer of magnetization between the spins, so-called cross-relaxation. In contrast to the case of relaxation via single-quantum transition, whereby the equilibrium state is completely restored, cross-relaxation causes the energy states to be either more or less populated than in the equilibrium state. Consequently, the signal is either increased or reduced in intensity. The NOE is expressed numerically by the NOE enhancement factor, η . It is the ratio between cross-relaxation rate and total relaxation rate of a spin multiplied by the ratio of the interacting spins' gyromagnetic ratios.⁹

$$\eta = \frac{\gamma_S}{\gamma_I} \cdot \frac{w_2 - w_0}{2w_1 + w_2 + w_0} \quad [3]$$

As one can easily deduce from this formula high probability of zero - quantum transition results in a negative NOE enhancement factor whereas the predominating double - quantum transitions push η in the direction of positive values. In contrast to ^1H and ^{13}C , ^{15}N has a negative gyromagnetic ratio. Hence, the NOE enhancement factor of nitrogen is negative.⁹

Whether a system relaxes by zero- or double- quantum transition, in other words whether the NOE is negative or positive is also determined by the molecular size. Larger molecules are slower in their tumbling rates. Linked to that the magnetic field of the dipolar interactions changes slowly, meaning in the Hertz to kiloHertz frequency range – the frequency region that matches with homonuclear zero-quantum transitions. As a consequence in the homonuclear case, large molecules of more than 3000 Da favor cross-relaxation via zero-quantum transitions and their NOEs are negative. In small molecules (below 1000 Da) double-quantum transitions are preferred, because these occur with a megaHertz frequency – comparable with their tumbling rate. In medium weight molecules none of the relaxation pathways is dominant and the total NOE tends to cancel.⁹

Examining the dependence of the various transition probabilities in more detail one first has to focus on the spectral density function $J(\omega)$, which is derived from the rotational correlation function by *Fourier* transformation. The latter describes the probability of finding a molecule at the same angle of rotation as a function of time. It is usually found to be an exponentially decaying function with the time constant τ_c . The molecular tumbling rate is conveniently described as the average angular velocity rad s^{-1} . The Fourier transform of the correlation function yields a frequency dependent function – the spectral density function $J(\omega)$ (Figure 1).¹⁰

$$J(\omega) = 2 \cdot \frac{\tau_c}{1 + \omega^2 \cdot \tau_c} \quad [4]$$

This Lorentzian function describes the power available through the fluctuating magnetic field for inducing relaxation at the angular velocity ω caused by molecular tumbling.¹⁰

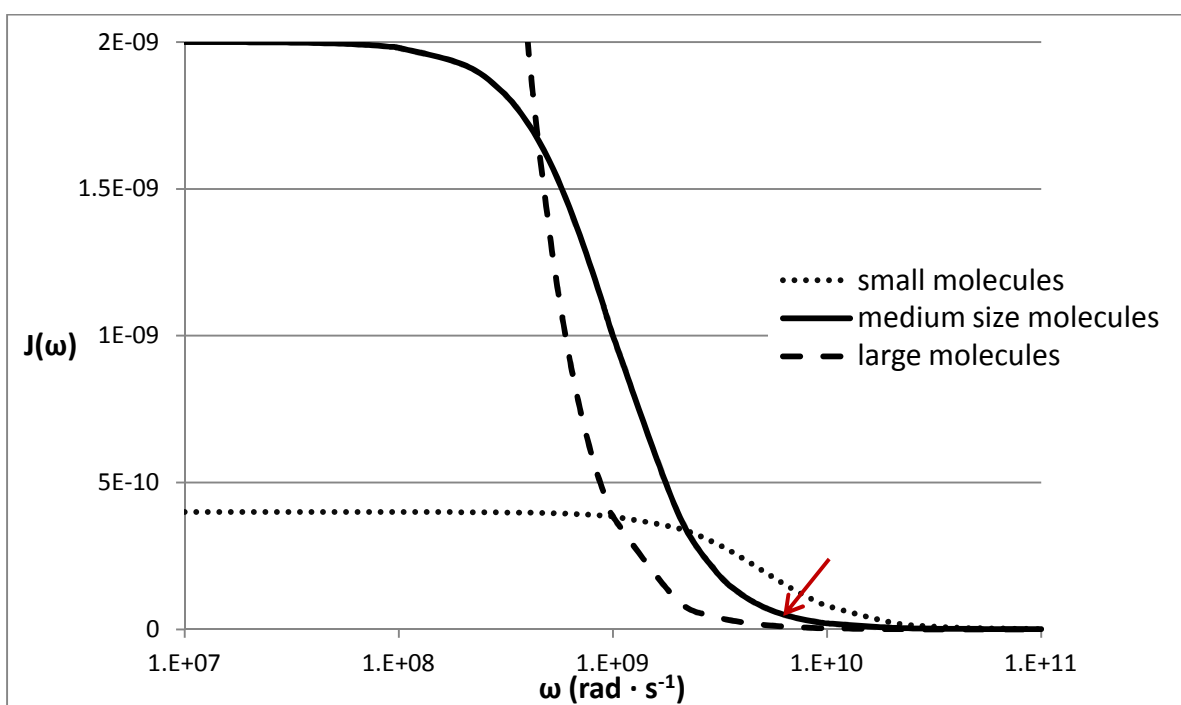


Figure 1: spectral density functions in a doubly logarithmic frame: dotted line $\tau_c = 0.2 \text{ ns}$, solid line $\tau_c = 1 \text{ ns}$, dashed line $\tau_c = 5 \text{ ns}$, On a linear scale the three curves have the same integral.¹⁰

The shape of the curve is a Lorentzian (only the positive frequency side being drawn) parametrized by the value of τ_c . The corresponding angular velocity value $\frac{1}{\tau_c}$ marks the inflection point of the curve and the average isotropic tumbling rate of the molecule.

While a long correlation time of, for example, 5 ns results in a high power level centered on the low frequency region, short correlation time of 0.2 ns leads to a flat function spreading out the power over a broad range of frequencies. However, the integrals of the spectral density functions are the same regardless of τ_c – as they represent the available power at a particular transition frequency. Thus they indicate the effectiveness with which transitions at a particular angular frequency ω_n are induced by the rf-noise caused by the molecular tumbling. The transition frequencies of zero-, single- and double quantum coherences, ω_0 , ω_1 and ω_2 respectively are depending on the Larmor frequencies of the spins involved. E.g. for a homonuclear system of protons in a magnetic field of 11.7 T ω_0 lies between zero and kiloHertz values, ω_1 is approximately 500 MHz ($= 3.1 \cdot 10^9 \text{ rad s}^{-1}$) and ω_2 can be estimated to be 1 GHz ($= 6.3 \cdot 10^9 \text{ rad s}^{-1}$). In case τ_c of the molecule is long, the rapid decline of the function causes the power to be low in the frequency range in which single- and double-quantum transitions occur, so zero- quantum transitions are prevailing. A short τ_c of 0.2 ns causes the power to be low and rather similar at all relevant frequencies.¹⁰

The maximum power level at a transition frequency ω_n is obtained when the condition $\tau_c \approx \frac{1}{\omega_n}$ holds. This is evident from Fig. 1 at τ_c of 1 ns, the position indicated by the arrow.¹⁰ The maximum achievable NOE enhancement factor following Equ. 3 is limited because the ratio of transition frequencies of very small molecules is limited by $\omega_0:\omega_1:\omega_2 = 2:3:12$.^{9,10}

Inserting these values into Equ. 3 one obtains Equ. 5.⁹

$$\eta = \frac{1}{2} \cdot \frac{\gamma_S}{\gamma_I} \quad [5]$$

The lower limit of homonuclear NOE enhancement observed with very large molecules is based on the condition $\omega_1 = \omega_2 = 0$ resulting in Equ. 6.⁹

$$\eta = -\frac{\gamma_S}{\gamma_I} \quad [6]$$

The negative gyromagnetic ratio of ^{15}N has the effect that the NOE enhancement factor is also negative and becomes more and more positive with increasing size of the molecule, or in other words with slowing down of the molecular tumbling rate.

In the particular case of $^{15}\text{N} \{^1\text{H}\}$ experiments Equ. 5 can only be applied at the extreme narrowing limit ($\omega\tau_c \ll 1$) and leads to the following lower NOE enhancement limit represented in Equ. 7. ⁹

$$\eta = \frac{1}{2} \cdot \frac{267.5 \cdot 10^6}{-27.1 \cdot 10^6} = -4.94 \quad [7]$$

In the heteronuclear case the simplification that $\omega_I \approx \omega_S \approx \omega$ does no longer hold. Therefore, to calculate the maximum NOE enhancement Equ. 8 showing the fractional NOE enhancement $f_I\{S\}$ depending on the molecular tumbling rate has to be used in its full complexity. ¹⁰

$$f_I\{S\} = \frac{\gamma_S}{\gamma_I} \frac{\left[\frac{6}{1+(\omega_I+\omega_S)^2 \tau_c^2} - \frac{1}{1+(\omega_I-\omega_S)^2 \tau_c^2} \right]}{\left[\frac{1}{1+(\omega_I-\omega_S)^2 \tau_c^2} + \frac{3}{1+\omega_I^2 \tau_c^2} + \frac{6}{1+(\omega_I+\omega_S)^2 \tau_c^2} \right]} \quad [8]$$

For the upper limit of NOE enhancement ($\omega\tau_c \gg 1$) in ^{15}N -NMR results a value of $f_I\{S\} = -1.22$.

2.3.1. (Inverse)- Gated Decoupling

As described above the NOE enhancement factor can be negative. Consequently, it is counteracting the original positive NMR signals. In order not to reduce the signal intensity, and in unfavorable cases make some signals vanish completely, suppression of the NOE is advantageous for spectra of nuclei with a negative gyromagnetic ratio, as ^{15}N . For suppression of the NOE inverse gated decoupling can be applied. To obtain proton - decoupled spectra the decoupler channel is used to irradiate the protons with a broad - band modulated radio - frequency field. Sufficiently strong irradiation causes fast alternation of the protons' spin states, which effects averaging of the splitting caused by spin interactions, in particular heteronuclear J-coupling. When inverse gated decoupling is applied the decoupler is turned on during the acquisition, but switched off during the relaxation period so the protons are not saturated prior to excitation. Thus, the prerequisite for the NOE to develop is absent.¹¹

By contrast, in the numerous cases where the NOE enhancement is advantageous, but splitting by heteronuclear coupling is desired, gated decoupling is the method of choice. In this case, the decoupler is switched off during recording of the FID. Thus, the protons are saturated during relaxation and the NOE can build up.¹¹

The decisive factor of obtaining a proton decoupled or coupled spectrum is exclusively the decoupler's activity during the acquisition period. If it is switched on the obtained spectra are proton decoupled.¹¹

2.4. Referencing in Nitrogen NMR

In ^1H and ^{13}C NMR it has been agreed upon the use of tetramethyl silane, TMS, as the primary internal standard for referencing.¹² In the field of nitrogen NMR the choice of a reference standard has unfortunately been controversial for some time. Therefore, when data from different periods or from relatively unrelated research fields are compared one inevitably comes across the obstacle of disparate reference substances and methods of their use. While in NMR of organic compounds the reference substances were predominantly chosen to be ammonium nitrate and nitromethane, peptide and protein nitrogen NMR spectra are mainly referenced to (external) liquid ammonia. There was not even an agreement on a reference substance within a specific application field. Adding to this confusion are the use of internal or external standards, opposite directions of the nitrogen δ -scale^{*}, different solvents and temperatures, and often distressingly incomplete documentation of the referencing method used. To get a rough impression of the diversity of nitrogen referencing methods Table II gives a list of reference standards and their applications in research.

* Several publications^{1, 2, 13, 14, 15} mentioned in this thesis do not conform to the IUPAC sign convention of 1972 defining that deshielded nuclei resonating at high frequencies (downfield) give more positive chemical shift values (δ) and shielded nuclei result in signals in the more negative region of the ppm scale.¹⁶

Table II: Reference Substances and their Applications in Research. The bold letters represent the nuclei on which's resonance was referenced.

Reference Standard Substance	Study in which the Substance was used as a Reference	Year of Publication of the Study
external $\text{NH}_4^+\text{NO}_3^-$	2	1964
internal $\text{NH}_4^+\text{NO}_3^-$	14	1967
external $\text{NH}_4^+\text{NO}_3^-$	17, 15	1995, 1964
external CH_3NO_2	18, 1	2000, 1981
internal CH_3NO_2	19, 13, 20	1975, 1973, 2006
external NH_4^+Cl^-	21	1974
external liquid NH_3	22, 23	2010, 1998
external HNO_3	24	1971
Internal $\text{Me}_4\text{N}^+\text{I}^-$	25	1971

This section of the thesis provides a comparison of the applicability of the mentioned compounds and promotes the presently IUPAC recommended multi-nuclear referencing method called “*absolute chemical shift referencing*”.

2.4.1. Ammonium and Tetramethylammonium Cations

Besides the criterion of non-interacting with the solvent or components of the analyzed sample, E.-D. Becker²⁵ proposes a resonance at the low frequency end of the spectral range as major requirement for a proper internal reference substance. Linked to that recommendation he mentions the favorability of a uniform direction of chemical shift scales of different nuclei. Since the resonance frequency of TMS, the widely accepted reference compound in proton NMR is set to 0 ppm, higher frequencies are found at a higher chemical shift in the proton NMR spectra. It makes sense to do it in a comparable way for other nuclei including ^{15}N .²⁵

Additionally, it is convenient to keep the scales of the relative chemical shift in ppm and the resonance frequency in Hertz, the actual unit of Fourier transformed spectra, pointing in the same direction. Eligible compounds, which would meet the prerequisite of a low resonance frequency, would be NH_4^+ and related ones carrying four alkyl groups, NR_4^+ .²⁵

Since the ammonium chemical shift is significantly influenced by the environment and especially the pH according to E.-D. Becker the ammonium ion should be abandoned as a nitrogen NMR reference.²⁵

The interaction between reference substance and solvent is minimal for tetrahedral molecules, like TMS, because of the absence of magnetic anisotropy. Considering the tetramethylammonium ion the advantage of a tetrahedral geometry is given. However, in comparison with the uncharged TMS molecule the ionic character makes the tetramethylammonium ion more prone to be influenced by other molecules in solution. E.-D. Becker has conducted a study to evaluate the effect of different anions and solvents on the ^1H and ^{14}N chemical shifts of tetramethylammonium ions. When changing anions he recognized a small variation of 0.05 ppm and 0.2 ppm for ^1H and ^{14}N shifts, respectively, which he classifies as insignificant after considering the comparably broad chemical shift range of the two mentioned nuclei. This view may however not hold when regarding the smaller chemical shift ranges observed for restricted classes of compounds.²⁵

An even more important role is played by the solvent used, since it makes the ^1H chemical shift vary by up to 0.3 ppm and the ^{14}N and ^{15}N shifts deviate up to 2.0 ppm. Although the impact is clearly larger than the 0.1 ppm inaccuracy of the solvent on the TMS signal, E.-D. Becker concludes that tetramethylammonium iodide or related salts differing in the anion are convenient internal reference substances.²⁵

Being aware of the pH sensitivity of the ammonium ion G. Adler and R. L. Lichter as well as J. D. Ray use the resonance of the ammonium ion for external referencing to an acidified solution to ensure full protonation of the ammonium ion and consequently to get reproducible results.^{21, 15}

2.4.2. Ammonium Nitrate and Nitromethane

During investigations by D. Herbison-Evans and R.E. Richards ammonium nitrate was used as an external reference substance in the form of a 4.5 M solution in 3 N aqueous hydrochloric acid. Compared to the ammonium nitrogen, which in the absence of decoupling yields a quintuplet signal, the nitrogen found within the nitrate ion shows up as a singlet with a much shorter longitudinal relaxation time (T_1). From the point of view of NMR practice the nitrate anion is more suitable. For non-aqueous solvents, nitromethane is the substance of choice as a secondary standard. It results in a broader but much more intense signal than NO_3^- . Using it as an internal standard one must be aware of potential reactions of the hetero-analogous carbonyl compound with the sample.²

According to M. Witanowski and H. Januszewski¹⁴ the great advantage of using the nitrate ion as an internal reference standard in aqueous solutions is that its ^{14}N chemical shift coincides closely with the one of nitromethane.¹⁴

Despite this, in 1969 E. F. Mooney and P. H. Winson reported a disagreement concerning the identity of the chemical shift values of nitromethane and the nitrate ion. Their findings show a deviation of 3 ppm on the ^{14}N shift scale.²⁶

It should be noted here again, that the direction of the chemical shift axes used in both Ref. 14 and Ref. 26 is opposite to the IUPAC convention.

Around 1982 the preference of referencing to an internal standard has changed into a preference of external standards. The crucial argument therefore is the susceptibility of internal standard chemical shifts to medium effects, which is of course eliminated in external referencing. As the primary standard of preference, neat nitromethane became accepted and the convention of referring the plus sign to an increasing magnetic shielding became popular. However, to achieve precise results the effect of the bulk susceptibility difference between the sample and the external reference has to be taken into account. The accuracy of the measurements depends on the filling height of the NMR tube, its position and the shape of the containment, which is included in Equ. 9 by the parameter α . Tables showing the empirically determined correction factors can be found in Ref. ¹. The true shielding difference between sample and reference can be calculated according to the following formula: ¹

$$(\sigma_{sample} - \sigma_{reference})_{true} = (\sigma_{sample} - \sigma_{reference})_{observed} - \left(\frac{3}{4}\pi - \alpha\right) \cdot (\chi_{reference} - \chi_{sample}) \quad [9]$$

Note: This equation can also be used to determine the relative magnetic susceptibilities of two solvents if the same standard is used. The sign of the chemical shift δ is opposite to the sign of σ .

2.4.3. Liquid Ammonia

In 1998 when indirect referencing became popular IUPAC recommended liquid ammonia as a secondary external reference whose ^{15}N resonance should be related to the ^1H resonance of DSS or TMS. This might be the reason why liquid ammonia was routinely used as a reference substance especially in NMR studies on biomolecules.²³

Despite the pH and temperature sensitivity of ammonia R. Marek²² still uses it as a reference standard in 2010. Differently than in the previously mentioned study by M. Witanowski and H. Januszewski¹⁴, R. Marek²² defines chemical shifts as getting more and more positive with deshielding of the nitrogen atoms. The advantage of this style of referencing is its analogy to referencing in ^1H and ^{13}C NMR. Further, the suitability of 1 M urea in $\text{DMSO-}d_6$ as a secondary external standard is mentioned.²²

2.4.4. Absolute Referencing

Today, IUPAC recommends a method of uniform chemical shift scaling regardless of the type of the observed nucleus, relying solely on a primary reference substance, usually TMS. Hence, in the method called “absolute referencing” all chemical shifts of nuclei are related to the resonance frequency of ^1H in TMS measured at the same magnetic field strength.¹²

$$\delta = 100 \cdot \left(\frac{\nu_X^{obs}}{\nu_{TMS}^{obs}} \right) \quad [10]$$

The frequency ratio is essentially the ratio of the magnetogyric ratios of the two nuclei. Using the experimental frequencies on the same instrument ensures that the systematic errors are small (1 ppm error in ν_X^{obs} causes a 0.001 ppb error in the difference to the other shifts).

2.4.5. Referencing in Biomolecular NMR

In NMR of proteins and nucleic acids it is recommended to take the ^1H resonance of DSS as the primary reference. The reason for this is that the ^1H frequency of DSS resonates almost at the same frequency as the one of TMS, but DSS is highly water soluble. Nevertheless, it is recommended to indicate frequency ratios obtained by absolute referencing to DSS clearly by δ^{DSS} .²⁸

2.4.6. Exemplary Application of Referencing in ^{15}N NMR

Substitution Method: Nitromethane as External Reference Substance

The referencing was done by recording a ^{15}N spectrum of a reference sample consisting of 500 μL CH_3NO_2 and 160 μL CDCl_3 followed by replacement of the reference sample tube by the sample tube and recording ^{15}N spectra thereof.

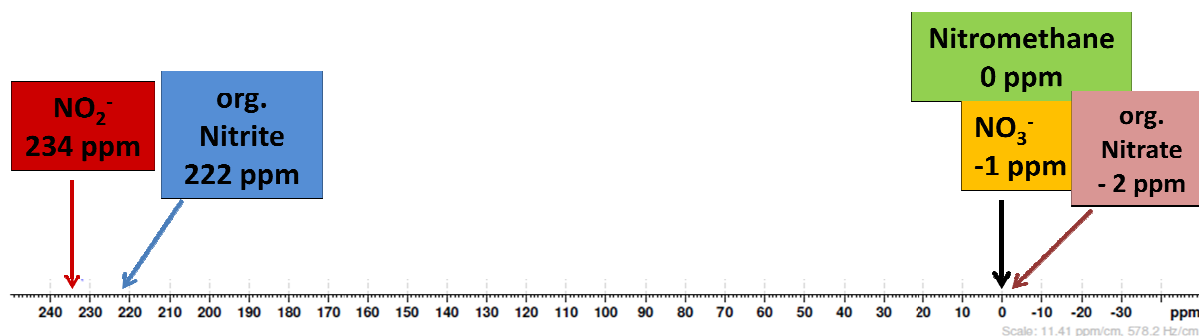


Figure 2: Observed ^{15}N chemical shifts relative to external nitromethane

Absolute Referencing

Following values were read out from the corresponding spectra obtained on the 500 MHz spectrometer.

Table III: Parameters read out from the instrumentation

BF1 (^1H)	500.1300000 MHz
BF1 (^{15}N)	50.6777330 MHz
SF (^1H)	500.1299981 MHz

$$\varepsilon_N = 100 \cdot \frac{BF1(^{15}\text{N})}{BF1(^1\text{H})}$$

$$\varepsilon_N = 100 \cdot \frac{50.6777330 \text{ MHz}}{500.1300000 \text{ MHz}} = 10.13291204 \%$$

$$SF(^{15}\text{N}) = \frac{10.13291204}{100} \cdot 500.1299981 \text{ MHz} = 50.67773279 \text{ MHz}$$

$$SR_N = SF(^{15}\text{N}) - BF1(^{15}\text{N}) = 50.67773279 \text{ MHz} - 50.6777330 \text{ MHz} = -0.21 \text{ Hz}$$

After calculation the SR (^{15}N) value was set for selected nitrogen spectra of the experimental section below and the chemical shift values shown graphically in Figure 3 were read out.

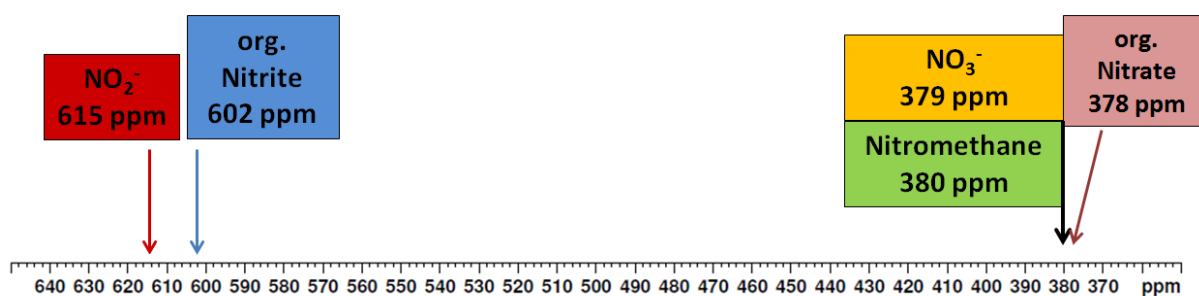


Figure 3: Observed ^{15}N chemical shift values; absolute referencing