Title

Chapter Twelve pH-Free Measurement of Relative Acidities, Including Isotope Effects

Author

Perrin, Charles L

Publication Date

2017

DOI

10.1016/bs.mie.2017.07.012

Peer reviewed

(running title) Measurement of Relative Acidities

pH-Free Measurement of Relative Acidities, Including Isotope Effects

Charles L. Perrin

Department of Chemistry & Biochemistry, University of California—San Diego

La Jolla, CA 92093-0358 USA

cperrin@ucsd.edu

Abstract

A powerful pH-free multicomponent NMR titration method can measure relative acidities, even of closely related compounds, with excellent accuracy. The history of the method is presented, along with details of its implementation and a comparison with earlier NMR titrations using a pH electrode. Many of its areas of applicability are described, especially equilibrium isotope effects. The advantages of the method, some practical considerations, and possible pitfalls are considered.

Introduction to NMR Titration

NMR pH titration was an early NMR method for measurement of acidity constants. It depends on the fact that proton transfer among acid and base forms is very fast. Consequently the observed chemical shift δ of an acid is the average of the chemical shifts δ_A and δ_B of its acid and base forms, but weighted by their respective concentrations, as in Eq. 1. Solving this equation for [B]/[A] and substituting into the Henderson-Hasselbalch equation leads to Eq. 2. Solving Eq. 2 for δ then leads to Eq. 3, expressing the dependence of the chemical shift on pH. This relationship has the appearance of a familiar titration curve, as sketched in Fig. 1. If the limiting chemical shifts are extrapolated to δ_{max} at low pH and δ_{min} at high pH, a linear least-

squares fit of the left-hand side of Eq. 4 vs. pH will give a slope near 1 and an intercept equal to $-pK_a$.

$$\delta = \tag{1}$$

$$pH = pK_a + \log \tag{2}$$

$$\delta = \tag{3}$$

$$\log[(\delta_{max}-\delta)/(\delta-\delta_{min})] = pH - pK_a \tag{4}$$

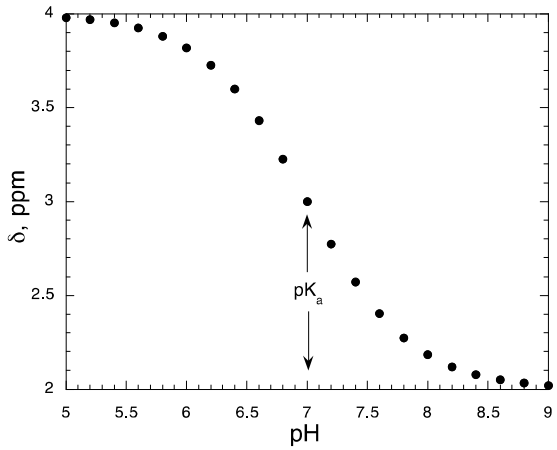


Figure 1. Simulation of Eq. 3.

Multicomponent NMR Titration

The above NMR titration can be extended to more than one substance, because the sensitivity of NMR chemical shifts to environment allows the separate measurement of each substance's chemical shift ~~δ~~. Consequently an NMR titration can be adapted to simultaneously measure the difference in acid dissociation constants (ΔpK_a) of two or more structurally similar compounds ([12]). The NMR titration is capable of high precision and accuracy, and it can be carried

out without the necessity of measuring pH. From the variations of the NMR chemical shifts during the titration of a single solution, the ratio of acidity constants of two or more compounds can be determined. During a titration, the spectral characteristics of the most basic or acidic compound will change at lower proton concentration than those of the other compounds. A linear plot created from the chemical shifts of the neutral and ionic forms of each compound versus the observed chemical shifts after each addition of titrant gives the desired ratio and thus ΔpK_a, the difference between the pK_as of the compounds. This method differs from the closest previous method ([3]), in that it relies explicitly only on chemical shifts and on a linear plot.

One key feature that makes the NMR titration feasible is that proton exchange is rapid on the NMR time scale. Second-order rate constants for thermoneutral exchange are at least $3x10^8$ $M^{-1}s^{-1}$ in water and are reduced only to ~$5x10^6$ $M^{-1}s^{-1}$ for some hindered amines in $CDCl_3$ ([45]). These values mean that peak broadening from insufficiently rapid exchange is negligible even at millimolar concentrations, although this may not be the case for some proteins in solutions lacking sufficient buffer to mediate proton transfer ([67]).

Data Analysis. Certain NMR chemical shifts of an acidic or basic compound undergo a change upon protonation or deprotonation sufficient to permit the NMR determination of the extent of ionization. If a mixture of two compounds, A and B, is titrated with acid, the chemical shifts of the more basic compound will change at lower proton concentration during the titration than those of the less basic one. Likewise, the chemical shifts of the more acidic compound will change at lower concentrations of base. The ratio of acidity constants of the different compounds, called K in Eq. 5, can readily be measured from the variations in chemical shifts, as derived below.

$$K = = \qquad (5)$$

The observed chemical shifts are weighted averages of the chemical shifts of the chemical shifts for the protonated and deprotonated species. The observed chemical shift of base A is given by Eq. 6, where δ_{A° and δ_{AH^+} are the limiting chemical shifts of the neutral and protonated forms, respectively. A similar equation holds for the competing base B.

$$\delta_a = \qquad (6)$$

Algebraic manipulation of these equations leads to Eq. 7, which is nonlinear whenever $K \neq 1$. The desired ratio, K, can be obtained from a nonlinear least-squares fit to Eq. 7. However, further manipulations lead to the linearized form in Eq. 8, relating observed chemical shifts to the desired K. This trivial linearization has been dignified as the Perrin-Fabian method. Then from the observed chemical shifts δ_a and δ_b during the titration of a mixture of compounds, the ratio of acidity constants can be calculated from a plot of $(\delta_b - \delta_{B^\circ})(\delta_{AH^+} - \delta_a)$ versus $(\delta_a - \delta_{A^\circ})(\delta_{BH^+} - \delta_b)$, which ought to be a straight line, with slope K and zero intercept ([8]). Therefore, the beginning chemical shifts of the neutral compounds (δ_{B° and δ_{A°) and the ending chemical shifts of the ions (δ_{BH^+} and δ_{AH^+}) must be known to accurately determine the ratio of acidity constants using this methodology.

$$\delta_a = \delta_{A^\circ} + \qquad (7)$$

$$(\delta_b - \delta_{B^\circ})(\delta_{AH^+} - \delta_a) = K(\delta_a - \delta_{A^\circ})(\delta_{BH^+} - \delta_b) \qquad (8)$$

It must be acknowledged that many laboratories have undertaken to use NMR to measure acidity constants. Consequently there have been various approaches, all rather similar, but each with its own set of parameters and equations. Rather than incorporate one consistent set of

parameters and symbols, this review preserves the original presentations.

A typical experimental procedure (reprinted with permission from Perrin, C. L. & Fabian, M. A. Multicomponent NMR titration for simultaneous measurement of relative pK_as. *Analytical Chemistry, 68*, 2127) Copyright (1996) American Chemical Society.

A stock solution of DCl was prepared from 0.66 g CD_3OD, 0.08 g D_2O, and 0.27 g 35% DCl/D_2O solution. A similar stock solution of NaOD was prepared from 0.66 g CD_3OD, 0.09 g D_2O, and 0.25 g of 40% NaOD/D_2O. For titrations in DMSO-d_6 a stock solution of trifluoroacetic acid (TFA) was prepared from 1.00 g DMSO-d_6 and 0.20 g TFA. A similar stock solution of *t*-BuOK was prepared from 1.00 g DMSO-d_6 and 0.19 g *t*-BuOK.

The NMR samples prepared for each titration contained 1.00 mL solvent, 5 μL tetramethylsilane (TMS) or 1,4-dioxane as internal standard, and 0.05, 0.10 or 0.20 mmol mixture of substances to be compared. An initial ^1H or ^{13}C NMR spectrum was taken of the sample, which is listed as the "0" titration point. Aliquots of 5 μL of the appropriate stock acid or base solution were then continually added until the chemical shifts no longer changed. The ^1H or ^{13}C NMR chemical shifts were recorded after each addition of the stock solution.

Amines titrated in CD_3OD/D_2O required two separate titrations, because a small percent of the amine becomes protonated in the solution. Therefore, two samples were prepared by splitting a solution containing 1.33 g CD_3OD, 0.50 g D_2O, 10 μL TMS, and 0.20 mmol mixture of amines. A 5-μL aliquot of the NaOD stock solution was added to the first sample. A ^1H NMR spectrum was taken of the sample, which is recorded as the "-1" titration point. Further addition of base gave no further change in the chemical shifts. A ^1H NMR spectrum was taken of the second sample, which is recorded as the "0" titration point. Aliquots of 5 μL of the DCl stock solution were then

continually added until the chemical shifts no longer changed, indicating that the endpoint of the titration had been passed. The ^{1}H NMR chemical shifts were recorded after each addition of the stock solution.

NMR spectra were recorded on a Varian Unity-500 spectrometer (499.8 MHz ^{1}H, 125.7 MHz ^{13}C) using an indirect probe. Chemical shifts are referenced to TMS (δ 0.00) for ^{1}H spectra and 1,4-dioxane (δ 66.5) for ^{13}C spectra.

Electrodeless pH titrations

An earlier variant on the pH-free NMR titration was a pH titration, measuring how NMR chemical shifts depend on pH. This has been especially common in biochemical systems, where the first applications were simply to measure pH from the observed chemical shift of various indicators ([9] ,[10], [11], [12]). An example of such a series, which spans much of the acidic pH range, is dichloroacetic acid**,** chloroacetic acid, acetic acid, sodium dihydrogenphosphate, and tris(hydroxymethyl)aminomethane (TRIS) ([13]), but it was noted that extension of the scale to the stronger acids histidine (pK_a 1.83) and dichloroacetic acid (pK_a 0.93) required careful control of ionic strength and extrapolation to the chemical shift of unionized dichloroacetic acid ([14]).

In an early study the acidities of linear and branched carboxylic acids could be evaluated, relative to acetic acid, from the variation with the extent of protonation of the measured ^{13}C NMR shifts of either the carboxyl or α-CH_2 carbons ([15]).

Another challenge is that measurement of pH in strongly basic solutions is also subject to error. Therefore, a series of weakly acidic substances was designed: trimethylammonium ion, tert-butylammonium ion, 4-hydroxypyridinium ion, cytosine, acetone oxime, acetamidinium ion, and methylguanidinium ion. These seven were anchored by sarcosine, whose pK_a was evaluated as

10.15 from a plot of chemical shift vs. measured pH, and then pairwise to less acidic values by NMR titration using a nonlinear fit of the chemical shift of the decreasingly acidic substances vs. the chemical shift of a substance whose pK_a had been evaluated ([16]).

Once such a series of pH indicators became available, with a known dependence of chemical shift on pH, it became possible to carry out an "electrodeless NMR titration" ([17]). Thus the acidity constants of six fluoroquinolone antibacterials could be determined from ^{1}H NMR chemical shift vs. pH, but the pK_a for the most acidic proton is near 0, so this was evaluated by NMR titration in a mixture with dichloroacetic acid, of known pK_a = 1.14 ([18]).

As another example, the pH dependences of ^{13}C chemical shifts for a series of carbamoyl choline homologues with various amino groups were measured ([19]). The data were fit to Eq. 9, where δ_i is the observed chemical shift of the *i*th carbon at a given pH, and δ_i^0 and δ_i^+ are the chemical shifts of the *i*th carbon in non-protonated and protonated amine, respectively. Non-linear regression analysis with simultaneous iteration on all carbons of the molecule (except for the *N*-methyls of the amine) then yielded pK_a.

$$\delta_i = \qquad (9)$$

Among other examples of the use of electrodeless NMR titration are the comparisons of the pK_a's of cis and trans rotamers of *N*-acetyl amino acids ([20]), and of acetylsarcosine, glycylsarcosine, glycylglycylsarcosine glycylproline, glycylhydroxyproline, alanylproline, sarcosine, proline, and hydroxyproline ([21]).

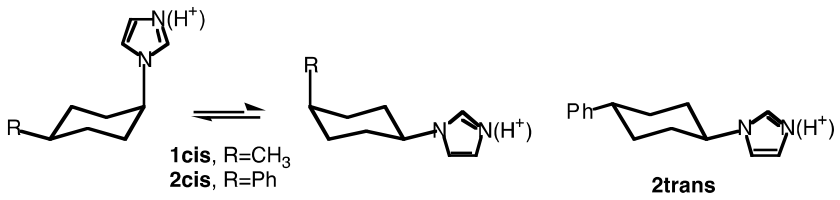
An automated pH–NMR titration set-up, consisting of a Bruker 250-MHz NMR instrument and a potentiometric titration unit, allows pH titrations with simultaneous recording of ^{1}H and ^{31}P NMR spectra at each titration point ([22]). This set-up was applied to protonation of three diazacrown

ethers carrying dangling phosphonate groups. From the fitting of the pH dependences of the chemical shifts of the ^{31}P and ^{1}H signals the protonation constants as well as the chemical shifts of the individual protonated species were obtained. The main advantages of this set-up are that relatively small amounts of substance (0.05 mmol) are needed for a single titration and that the system needs no operator intervention during the entire experiment (about 20 h).

Motivation: Applications to the Reverse Anomeric Effect

Our entry into this topic arose through a consideration of the reverse anomeric effect. The (normal) anomeric effect is the preference of an electronegative substituent for the axial position on a tetrahydrofuran ([23][24][25][26]). The reverse anomeric effect is the preference of a cationic substituent for the equatorial position on a tetrahydropyran. In connection with the claim of a reverse anomeric effect in *N*-xylosylimidazolium ions ([27]), we wanted to compare the steric bulk of a cationic substituent with that of the corresponding neutral one, specifically a comparison of imidazolium with imidazolyl. A measure of the steric bulk of a substituent X is its *A* value, the extra energy required to place that substituent axial, as expressed in Eq. 10. In an attempt to compare the steric bulk of cationic with neutral, the conformational equilibria of cis-*N*-(4-methylcyclohexyl)imidazole (**1cis**, R = CH_3), cis-*N*-(4-phenylcyclohexyl)imidazole (**2cis**, R = Ph), and their conjugate acids were measured by low-temperature NMR ([28]). The *A* value of an imidazolyl group was thereby found to be 2.2 ± 0.1 kcal/mol. The *A* value of an *N*-protonated imidazolyl group was also found to be 2.2 ± 0.1 kcal/mol, which is the same within experimental error.

$$A_X = G^o(\text{axial X}) - G^o(\text{equatorial X}) \qquad (10)$$



To compare the two sizes more precisely, an NMR titration method, applicable to a mixture of isomers, was developed. The ratio of acidity constants of cis- and trans-*N*-(4-phenylcyclohexyl)imidazoles, as expressed in Eq. 11, could be determined from the variation of H1 chemical shifts as a 1:l mixture was titrated with DCl. The observed chemical shift, δ_c, of the *cis* isomer **2cis** is given by Eq. 12, where δ_{CH+} and δ_C are limiting chemical shifts of protonated and unprotonated forms, respectively. A similar equation relates δ_t, δ_{TH+}, and δ_T, the chemical shift of the *trans* isomer, **2trans**, and its limiting chemical shifts. Algebraic manipulation of Eq. 11, Eq. 12, and the equivalent equation for δ_t leads to Eq. 13, which is nonlinear whenever $K \neq 1$. Indeed, a plot of δ_c against δ_t, as shown in Fig. 2, shows slight but systematic upward curvature, indicating that the *trans* isomer is more readily protonated. (To clarify the behavior, the diagonal dotted line corresponds to the hypothetical situation of identical basicity for cis and trans, and the horizontal and vertical dotted lines correspond to the hypothetical extreme where the trans is infinitely more basic than the cis and is protonated first during the titration.)

$$K = = \tag{11}$$

$$\delta_c = \tag{12}$$

$$\delta_c = \delta_C + \tag{13}$$

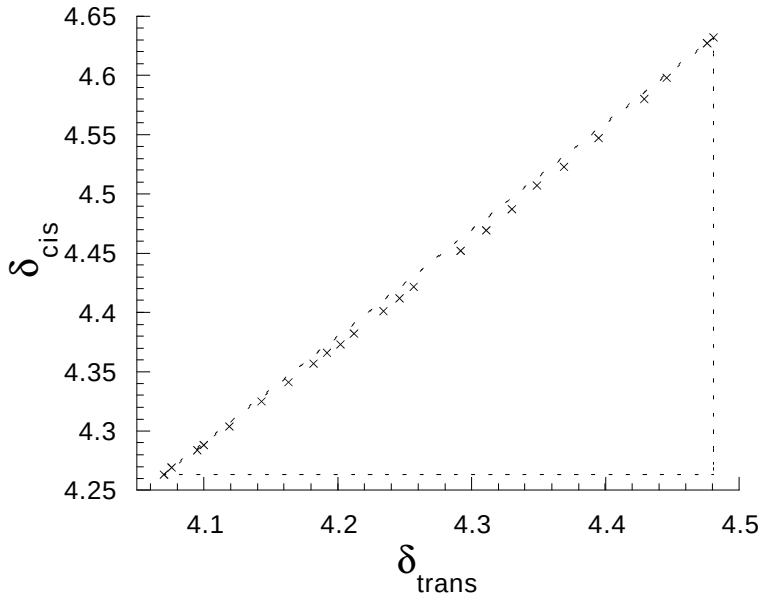


Figure 2. Chemical shifts δ_{cis} and δ_{trans} during titration of a 1:1 mixture of *cis*- and trans- *N*-(4-phenylcyclohexyl)imidazoles (**2**) with DCl in 1:l acetone-d_6/D_2O. (adapted with permission from data in Table 3 of Perrin, C. L., Fabian, M. A., & Armstrong, K. B. Solvation effect on steric bulk of ionic substituents: Imidazolium vs imidazole. *Journal of Organic Chemistry, 59*, 5246-5253) Copyright (1994) American Chemical Society.[29])

Further manipulations lead to the linearized form, Eq. 14**,** relating observed chemical shifts to the desired *K*. In particular, a plot of $(\delta_t - \delta_T)(\delta_{CH^+} - \delta_c)$ vs. $(\delta_c - \delta_C)(\delta_{TH^+} - \delta_t)$ ought to be a straight line, with slope *K* and zero intercept. Fig. 3 shows such a plot. In aqueous acetone the cis isomer was found to be 0.048 ± 0.002 pK units less basic. Through the application of a thermodynamic cycle, this corresponds to $\Delta A = A_{\text{N-imidazolylH+}} - A_{N\text{-imidazolyl}} = 0.089 \pm 0.004$ kcal/mol, with the protonated imidazolyl slightly but detectably larger.

$$(\delta_t - \delta_T)(\delta_{CH^+} - \delta_c) = K(\delta_c - \delta_C)(\delta_{TH^+} - \delta_t) \qquad (14)$$

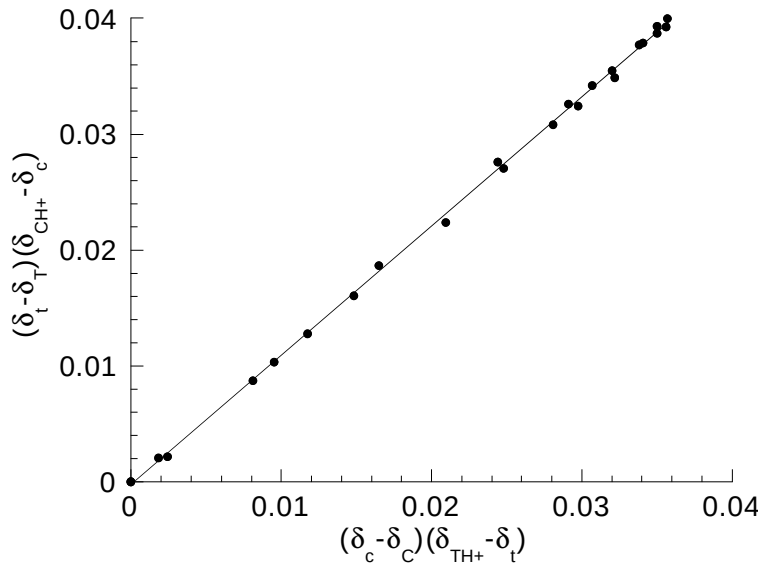
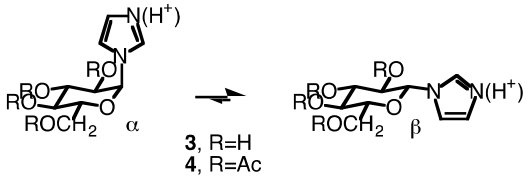


Figure 3. Linearized plot of chemical shifts (Eq. 14) during titration of a 1:1 mixture of *cis*- and trans-*N*-(4-phenylcyclohexyl)imidazoles (**2**) with DCl in 1:l acetone-d_6/D_2O. (reprinted with permission from Perrin, C. L., Fabian, M. A., & Armstrong, K. B. Solvation effect on steric bulk of ionic substituents: Imidazolium vs imidazole. *Journal of Organic Chemistry, 59*, 5246-5253). Copyright (1994) American Chemical Society.[30]

This NMR titration method was used similarly to measure the effects of *N*-protonation on the anomeric equilibria in *N*-(D-glucopyranosy1)imidazole **(3**) and its tetra-*O*-acetyl derivative **4** ([31]). In principle, the magnitude of any reverse anomeric effect could be measured as the increase in the proportion of the β anomer on protonation of an equilibrating mixture of anomers. However, glycosylimidazoles are configurationally stable, so that the two anomers do not equilibrate. Nevertheless, the reverse anomeric effect could be measured instead from the difference in pK_a between α and β anomers, as evaluated from the slope K_a^{α}/K_a^{β} in Eq. 15, analogous to Eq. 14, but where δ_α and δ_β are observed chemical shifts and δ_α^+, δ_α°, δ_β^+, and δ_β° are limiting chemical shifts

of protonated and unprotonated α and β. It was found that *N*-protonation does not shift the equilibrium toward an equatorial imidazolyl, but rather shifts the equilibrium toward axial, which is opposite to what was claimed for the reverse anomeric effect.



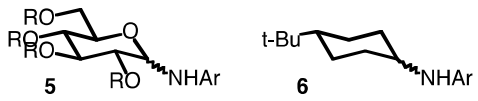
3, R=H
4, R=Ac

$$(\delta_\beta - \delta_\beta^o)(\delta_\alpha^+ - \delta_\alpha) = (K_a^\alpha/K_a^\beta)(\delta_\alpha - \delta_\alpha^o)(\delta_\beta^+ - \delta_\beta) \qquad (15)$$

This study of the anomeric equilibrium was extended to a wider range of *N*-(glycopyranosyl)imidazoles ([32]). The NMR titration data indicate a $\Delta\Delta G^\circ_{\beta\rightarrow\alpha}$ that is almost always negative, corresponding to a greater preference for the axial position of a protonated imidazolyl group than of an unprotonated. This preference counters the small steric effect, arising from hindrance to ionic solvation. These results are exactly opposite to what is expected from the reverse anomeric effect and led to the conclusion that there is no firm evidence for this effect.

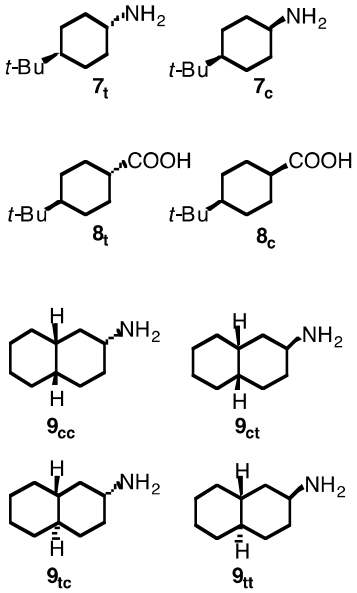
The ability to measure small changes in acidity next permitted the comparison of *N*-protonation-induced shifts of the anomeric equilibrium in a series of *N*-(tetra-*O*-methylgluco-pyranosyl)anilines (**5**, R = CH_3) and of the cis/trans equilibrium in *N*-(4-*tert*-butylcyclohexyl)-anilines (**6**) ([33]). In both series the equilibrium shifts toward equatorial upon *N*-protonation, consistent with steric hindrance to ionic solvation. The shift is smaller for the glucosylanilines than for the cyclohexylanilines, consistent with an enhancement of the normal anomeric effect that counters the steric hindrance and reduces the shift toward the equatorial β anomer. Moreover, the shift of the equilibrium toward equatorial increases slightly but detectably with electron-withdrawing substituents on the aromatic ring, which fine-tune the steric hindrance to ionic solvation. In contrast, the shift of the equilibrium toward equatorial decreases for the

glucosylanilines. This result is consistent with an enhancement of the normal anomeric effect due to a more localized positive charge, rather than with a reverse anomeric effect.

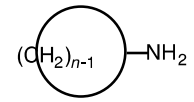


Applications of Other NMR Titrations to Relative Acidities

To demonstrate the versatility of the NMR titration method, the ΔpK_a between the conjugate acids of the two stereoisomers of 4-*t*-butylcyclohexylamine (**7**) was determined in a single ^1H NMR titration experiment in CD_3OD/D_2O and also in DMSO-d_6 ([34]). The ΔpK_a between the two stereoisomers of 4-*t*-butylcyclohexanecarboxylic acid (**8**) was determined in both of these solvents using both ^1H and ^{13}C NMR. To further demonstrate the scope of this method, the ΔpK_as of the conjugate acids of all four (racemic) stereoisomers of 2-decalylamine (**9**) were determined in a single ^1H NMR titration experiment.

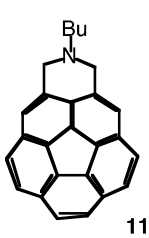


The ^{1}H NMR titration method was applied to measure the basicities of cycloalkylamines (**10**), all relative to isopropylamine ([35]). Cyclopropylamine (*n*=3) and cyclobutylamine (*n*=4) are less basic than cyclopentylamine (*n*=5) and cyclohexylamine (*n*=6), owing to the hybridization of the carbon atoms in the small rings. Medium-ring amines (*n* =7,8,9) are even more basic, with cyclooctylamine (*n*=8) the most basic, again owing to hybridization. Surprisingly, large-ring amines (*n*=12,15,16,21) are slightly less basic than cyclohexylamine, even though they all have the same hybridization. This difference may be due to conformational restrictions in the large-ring ammonium ions. This subtle effect required NMR titration to detect reliably.

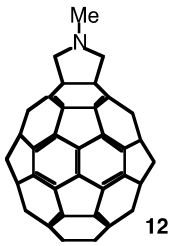


10, *n* = 3-12,15-16,21

To investigate the effect of molecular curvature on basicity, the basicity of a pyrrolidine fused to a fullerene (**11**) was compared to that of a pyrrolidine fused to a coronene (**12**) ([36]).



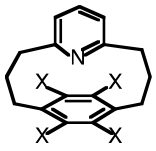
11



12

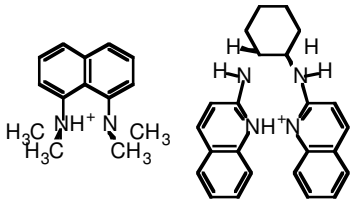
^{1}H NMR titration in DMSO-d_6 was used to probe polar-π interactions in two pyridinocyclophanes (**13**, X = H, F)). It was found that the pyridine is more basic when it faces a benzene ring, compared to when it faces a tetrafluorinated benzene ring, owing to the difference in

electron density, which repels the N lone pair. Surprisingly, 2,6-lutidine, without the cyclophane, is still more basic, even though it lacks the electron density of the benzene ring ([37]).



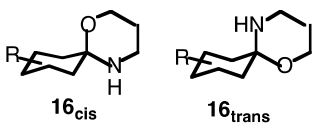
13

NMR titration was used to measure ΔpK for a series of amines in DMSO, including 1,8-bis-dimethylamino naphthalene ("Proton sponge", **14**) and the chiral N,N'-bis(2-quinolinyl-trans-1,2-cyclohexylamine (**15**), which is effective as a chiral Brønsted acid to catalyze an enantioselective reaction ([38]).



14 **15**

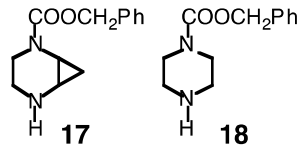
^{1}H NMR titrations were used to measure the relative basicities of cis and trans 1,5-oxazaspiro[5,5]undecanes (**16**, R = methyl or t-butyl) ([39]).



16$_{cis}$ **16$_{trans}$**

The pK_a of *N*-Cbz-2,5-diazabicyclo[4.1.0]heptane (**17**) was determined to be 6.74 ± 0.05

and that of *N*-Cbz piperazine·HCl (**18**) to be 8.09 ± 0.02 ([40]). The latter pK_a is in accord with the reported pK_a of *N*-carbethoxypiperazine and that of *N*-benzoylpiperazine.



The ΔpK_{a1} values for the first ionization of arylphosphonic acids, ArP(=O)(OH)$_2$, relative to ionization of dichloroacetic acid as pH indicator, were determined by electrodeless NMR titration ([41]). The ^{1}H chemical shifts of the aromatic protons of the compounds were fit to Eq. 16, where δL_{obs} and δI_{obs} are the observed chemical shifts of the phosphonic acid and of dichloroacetic acid, δHL and δL are the limiting chemical shifts of the acidic and basic forms of the phosphonic acid, and δHI and δI are the limiting chemical shifts of the acidic and basic forms of the indicator.

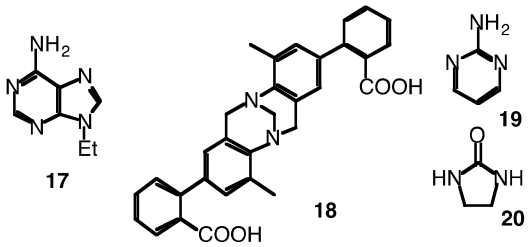
$$\delta L_{obs} = \delta L + \qquad (16)$$

Measurement of Binding Constants

Two detailed guides to measurement of binding constants are available, applicable to both NMR titration and UV-vis ([4243]). Both include much practical advice, including precautions, choice of concentrations, and data analysis.

NMR titration can be used to measure binding constants for formation of binary complexes in multicomponent mixtures. A challenging test is the analysis of the binding of 9-ethyladenine (**17**) to host dicarboxylic acid (**18**) in the presence of 2-aminopyrimidine (**19**) and imidazolidone (**20**) ([44]). Each species is in equilibrium with its dimer and with all binary complexes with the other components. Dimerization constants were evaluated by fitting the NH chemical shifts for each

single component to its concentration dependence. Then binding constants for all 1:1 complexes were evaluated by fitting the NH chemical shifts for each binary mixture. Finally the binding constant for **17** to host **18** was evaluated as $(5.05\pm1.0)\times10^5$ from analysis of chemical shifts in three-component mixtures, where the other binding constants do affect the observed chemical shifts but represent only small corrections.



Binding constants could be measured in toluene-d_8 for the complexes between $(CpSiMe_3)_3M$ and Cp*Ga (Cp = C_5H_5, Cp* = $C_5(CH_3)_5$, M = U or Nd) ([45]). To the solution of $(CpSiMe_3)_3M$ varying amounts of Cp*Ga were added, and X_{cplx}, the mole fraction of the complex, was evaluated as $(\delta_{obs} - \delta_M)/(\delta_{MGa} - \delta_M)$, where δ_{obs}, δ_M, and δ_{MGa} are observed chemical shift and chemical shifts of free and bound $(CpSiMe_3)_3M$, not only at methyl but also at the two different CH of the Cp ring. Next, the average of these three values was converted to the binding constant, $K = [(CpSiMe_3)_3M\text{-}GaCp^*]/[CpSiMe_3)_3M]([Cp^*Ga]_{total}\text{-}[(CpSiMe_3)_3M\text{-}GaCp^*])$. It may be noted that the signals are broadened by the paramagnetic metals, but paramagnetic shifts can be large, and the change of chemical shift on complexation is > 10 ppm at two of the sites, providing high accuracy. Enthalpy and entropy of binding could then be evaluated from the temperature dependence of *K*.

NMR titration is also applicable to relative acidities of Lewis acids. Thus, reaction of azines L (L = pyridine, 3-picoline, 3,5-lutidine, 3-chloropyridine, pyridazine, pyrimidine, pyrazine, 3,5-dimethylpyrazine, and *s*-triazine) with metallocenes $[M(C_5H_4R)_3]$ (M = Ce, U; R = tBu, $SiMe_3$) in

toluene gave the Lewis base adducts $[M(C_5H_4R)_3(L)]$ ([46]). The equilibria between $[M(C_5H_4R)_3]$, L, and $[M(C_5H_4R)_3L)]$ were studied by ^1H NMR spectroscopy, even though the metal complexes are paramagnetic.

Single-point comparisons

Instead of a complete NMR titration and fitting of all the chemical shifts to Eq. 14, it is possible to abbreviate the experiment to a single point near the middle of the titration, along with the endpoints. The slope in Eq. 14 is most sensitive to that point, where the equilibrium is most closely balanced so that the other points only reinforce the slope. Little accuracy is sacrificed by this simplification, because the slope of a line is determined by its extremities.

A single-point NMR titration was used to compare the acidities of *o*- and *p*-aroylaminobenzoic acids ($ArCONHC_6H_4COOH$), in order to evaluate the effect of the intramolecular hydrogen bond between amide NH and carboxyl, which is present only in the ortho isomer ([47]). Titrations were carried out in DMSO by ^1H or ^{13}C NMR of an equimolar mixture of a test acid AH and the tetrabutylammonium salt of B^-, the conjugate base of a standard acid BH (p-bromobenzoic, p-nitrobenzoic, or salicylic) of known acidity constant K_a^{BH}, or else an equimolar mixture of a standard acid BH and the tetrabutylammonium salt of A^-, the conjugate base of the test acid. It should be noted that it is not necessary to mix the two components in exactly equimolar proportions. The resulting chemical shifts of a reporter nucleus (^1H or ^{13}C, not specified further) in each species, along with those chemical shifts in acids AH and BH alone and in conjugate bases A^- and B^- alone, were used to evaluate the position of equilibrium between the two acids and relate K_a^{AH} to the known K_a^{BH}. Equation 17 describes the relationship among those chemical shifts and the ionization constants of the two acids. It was further found that substituent effects on the acidities

could be described well by a Hammett plot. It was also claimed that the best results are obtained when AH and BH are separated by ~2 pK_a units, but this is misleading and is discussed below under Some Practical Considerations.

$$K_a^{AH}(\delta_a - \delta_A)(\delta_{BH} - \delta_b) = K_a^{BH}(\delta_b - \delta_B)(\delta_{AH} - \delta_a) \quad (17)$$

Single-point NMR titrations are particularly convenient for investigating the temperature dependence of a ΔpK_a. Titrations performed at multiple temperatures could be carried out using a single sample ([48]). During each point along the titration the sample was heated to each of five temperatures and allowed to equilibrate within the probe for 15 minutes at each temperature. Temperatures were calibrated using a separate CD_3OD/D_2O sample with an internal ethylene glycol reference.

It may be noted that the binding constants for the complexes between $(CpSiMe_3)_3M$ and Cp*Ga (Cp = C_5H_5, Cp* = $C_5(CH_3)_5$, M = U or Nd) were measured by single-point NMR titrations at 10 ºC intervals between 21.5 and 72.0 ºC or between -33.2 and 19.2 ºC, and then converted to enthalpy and entropy of binding ([49]).

Intramolecular comparisons

There are two different limiting cases of intramolecular comparisons, one where there are two or more functional groups in the molecule with similar acidities, and the other where two or more functional groups differ substantially in acidity. This latter case often applies to the determination of K_1/K_2, the ratio of first and second dissociation constants of a diprotic acid. The equations below are for the case of two functional groups, but the extension to more than two is straightforward mathematically.

Although this NMR titration method was developed initially for intermolecular

comparisons, it is often desirable to measure relative pK_as of similar functional groups in the same molecule. For example, one often wants to assess the role of the local environment on acidity constants of residues in biological macromolecules. A pH titration provides only the macroscopic (overall) pK_as. The ability of NMR to distinguish different nuclei makes it possible to measure microscopic pK_as associated with individual functional groups.

The difference between intermolecular and intramolecular comparisons is that in the former the chemical shift of one functional group is independent of the state of protonation of the other one. To apply the NMR titration to an intramolecular case, it is necessary that this independence also hold, as would be true if the groups are noninteracting. One recommendation is that the differences between the pK_a's must be > 2, or else the different sites must not influence one another ([50]). Alternatively, the dependence of chemical shift on state of protonation may be known, or may be deduced from fitting the data. When these conditions are not fulfilled, the chemical shift is not simply the average of only two forms, and one has to use more sophisticated models and specialized software to determine the pK_a by fitting the experimental data.

Two classic cases studied by measuring chemical shifts vs. pH, *N*,*N*-dimethylpropanediamine ($H_2NCH_2CH_2CH_2N(CH_3)_2$) and histidylhistidine, illustrate the difficulties. In the first case it was assumed that chemical shifts depend only on the state of protonation of the attached nitrogen, and are independent of the state of protonation of the other nitrogen ([51]). This cannot be true for nitrogens that are four bonds away from each other. In the second example, the data were fit so as to estimate how chemical shifts of one imidazole depend on the state of protonation of the other one, but the results were quite inaccurate ([5253]).

A successful ^{1}H NMR method was developed for determining residue-specific acid-dissociation constants for peptides that contain more than one residue of the same acidic or basic amino acid ([54]). In particular, this method was applied to the side-chain -NH_3^+ groups of the four

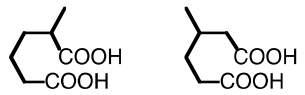
lysine residues in the pentadecapeptide Lys-Asn-Asn-Gln-Lys-Ser-Glu-Pro-Leu-Ile- Gly-Arg-Lys-Lys-Thr-NH_2. The resonances for the $C_\varepsilon H_2$ protons are sensitive to the state of protonation of the adjacent -NH_3^+, but these show an extensive overlap of signals because their environments are so similar. In contrast, the backbone $C_\alpha H$ chemical shifts vary from residue to residue, even for lysines, owing to nearest-neighbor residues and possibly to secondary structure. Therefore, the four lysine signals could be resolved using two-dimensional total correlation spectroscopy (TOCSY) spectra, which show resolved $C_\alpha H$-$C_\varepsilon H_2$ cross peaks. Thus, chemical shift-pH titration data were obtained for each lysine side-chain -NH_3^+ group, measured as a function of pH. Each pK_a could then be evaluated by fitting the observed chemical shifts for each acidic group to Eq. 18, where δ_{HA} and δ_A are fitting parameters describing the chemical shifts of acidic and basic forms, respectively. The pK_as of the Lys1, Lys5, Lys13, and Lys14 side-chain ammonium groups were thus found to be 11.14 ± 0.01, 10.95 ± 0.01, 10.96 ± 0.02, and 11.09 ± 0.02, respectively, showing how it is possible to distinguish even such similar acids. Likewise, the pK_as of the Cys3 and Cys13 thiol groups were determined to be 9.21 ± 0.07 and 8.60 ± 0.06, respectively. Alternatively, the relative acidities of the four lysines could be evaluated with greater accuracy, and without the necessity of measuring pH, directly from the chemical-shift data, which were subjected to a nonlinear least-squares fit to Eq. 19 of Δ, the difference in chemical shifts between the CH signals of two imidazoles. In Eq. 19 $\Delta_A = \delta_a^1 - \delta_A^x$, $\Delta^1 = \delta_A^1 - \delta_{HA}^1$, $\Delta^x = \delta_A^x - \delta_{HA}^x$, $R_{1x} = K_a^1/K_a^x$, and $n = (\delta_{obs}-\delta_A)(\delta_{HA}-\delta_A)$, the fraction of protonation of Lys_1. It may be noted that one of the acid groups is chosen as a reference, designated as "1", and the acidities for other acid groups, designated as "x", are evaluated relative to "1", whereas in Eq. 14 or 17 all acids are treated equivalently. This is a necessary arbitrariness when there are more than two acid groups to be compared, as was also necessary with the four decalylamine stereoisomers (**9**).

$$\delta_{obs} = \qquad (18)$$

$$\Delta = \Delta_A + - n\Delta^1 \qquad (19)$$

In the study of the above pentadecapeptide there is an implicit assumption that protonation of one Lys does not affect the chemical shift of another Lys. Because the $-NH_3^+$ groups are well separated in space, this assumption is valid to a high degree of approximation. Likewise, this method was applied to peptides containing >1 histidyl residue, and ΔpK_a could be evaluated by a nonlinear fit of chemical shifts for the separated histidine signals ([55]).

An instructive example of both intermolecular and intramolecular comparisons was achieved by ^{13}C NMR titration of a mixture of 2-methyl- and 3-methyl- adipic acids (**21**, **22**) and analyzed by Eq. 19 ([56]). There is no problem in relating the acidity of each of the carboxyl groups of **22** to the acidity of the C6 carboxyl of **21**. It is a greater challenge to compare the acidity of the C1 carboxyl of **21** to that of its C6 carboxyl. Although the chemical shift of one carboxyl is not independent of the state of protonation of the other that is separated by five C–C bonds, this long-range variation was found to be more than an order of magnitude smaller than the variation of chemical shift of a carboxyl group with the state of its own protonation. Therefore this intramolecular comparison is valid.



21 **22**

Ratio K_1/K_2

This method can be extended to diacids H_2A in order to evaluate K_1/K_2, the ratio of first and second acidity constants ([57]). If $K_1 >> K_2$ this simplifies to the separate evaluations of two acidity

constants, by using Eq. 17, whereby K_1 or K_2 is compared to K_a^{BH}, the acidity constant of a reference acid. In this context, Eq. 17 becomes Eq. 20 or 21, where the chemical shifts refer to H_2A, HA^-, and $A^=$, and a single-point measurement suffices. If K_1 is not much greater than K_2, then the full equation, Eq. 22, must be used, and two measurements are required, to provide two equations in the two unknowns. The method was successfully applied to fumaric, maleic, malonic, diethylmalonic, phthalic, furan-2,3-dicarboxylic, and bicyclo[2.2.1]-2,5-heptadiene-2,3-dicarboxylic acids.

$$\delta_a - \delta_{HA^-} = (K_a^{BH}/K_1)(\delta_b - \delta_B)(\delta_{H2A} - \delta_a)/(\delta_{BH} - \delta_b) \quad (20)$$

$$\delta_a - \delta_{A=} = (K_a^{BH}/K_2)(\delta_b - \delta_B)(\delta_{HA^-} - \delta_a)/(\delta_{BH} - \delta_b) \quad (21)$$

$$(\delta_a - \delta_{A=}) + (\delta_a - \delta_{HA^-}) + (\delta_a - \delta_{H2A}) = 0 \quad (22)$$

Heavy-Atom Isotope Effects on Acidity

A demanding test of the NMR titration method is its applicability to secondary isotope effects, which is the focus of this volume of *Methods in Enzymology*. Primary isotope effects are those where the bond to the isotope is broken, whereas secondary isotope effects are those where the bond to the isotope remains intact. Therefore secondary isotope effects are quite small, even for deuterium relative to protium, and much smaller for other isotopic substitutions. To measure them, a method of high precision and accuracy is needed.

Imagine an NMR titration applied to a mixture of two acids, HA and HA', each of whose chemical shifts, δ and δ, follows Eq. 1. If $\delta - \delta$, the difference between those two chemical shifts, is designated as Δ, it is then possible to eliminate pH from the equation and express it in terms of n, the number of equivalents of titrant added, leading to Eq. 23, where $\Delta^- = \delta^- - \delta^-$, $\Delta^o = \delta^o - \delta^o$, and $R = K_a/K_a'$ ([58]). Moreover, that n can be evaluated as $(\delta - \delta^-)/(\delta^o - \delta^-)$, from the variation of the

chemical shift of HA during the titration. Then, by using the three variables Δ^-, Δ^o, and R as parameters in a nonlinear fitting routine, it is possible to evaluate the desired R, the ratio of acidity constants.

$$\Delta = \Delta^- + - n(\delta^- - \delta^o) \qquad (23)$$

Eq 23 is general, so that it applies to measurement of isotope effects on acidity. The titration of a mixture of formic acid and $^{18}O_2$-formic acid thus permitted the evaluation of the ^{18}O isotope effect on acidity from the ^{13}C NMR chemical shifts δ and δ of the carboxyl carbons. For one ^{18}O ΔpK_a was found to be 0.00623 ± 0.00005. This same equation was used with ^{31}P chemical shifts to evaluate the ^{18}O isotope effects on the acidities of phosphoric acid and alkyl phosphates ([59]) For four ^{18}O $K_a^{16}/K_a^{18} = 1.019 \pm 0.001$, and proportionally lower for fewer ^{18}O. The ability to accurately measure such small isotope effects is a tribute to the method.

The ^{15}N isotope effect on the first pK_a of glycine was measured from the NMR separations in the carboxyl ^{13}C NMR spectrum ([60]) Although this carbon is more distant from the reactive $-NH_3^+$ than is the CH_2, the CH_2 signal is complicated by spin-spin coupling to the ^{15}N. The equilibrium isotope effect is related to the chemical shifts by Eq. 24, where Δ_d, $= \delta_d^{14} - \delta_p^{15}$, $\Delta^{14} = \delta_d^{14} - \delta_p^{14}$, $\Delta^{15} = \delta_d^{15} - \delta_p^{15}$, $R = K_a^{14}/K_a^{15}$, and $n = (\delta_{obs}-\delta_d)(\delta_p-\delta_d)$. This is analogous to Eq. 19, except that here the term Δ_A is ignored because there is no resolvable isotope effect on the chemical shifts of the acidic and basic forms. It may again be noted that the ^{14}N acid was chosen as the reference, rather than treating both acids equivalently.

$$\Delta = - n\Delta^{14} \qquad (24)$$

Isotope effects were determined by ^{13}C NMR titration for a wide variety of carboxylic acids, including the ^{18}O effect on formic acid, acetic acid, and glycine acidities, the ^{15}N isotope effect on glycine acidity, and the ^{13}C isotope effect on acetic acid and glycine acidities ([61]).

According to DFT calculations, the secondary ^{18}O isotope effect on the acidity of a carboxylic acid is greater than the primary ([62]). This is counterintuitive, because the H atom that is lost is closer to the ^{18}O atom that is responsible for the primary isotope effect. This is a result that could not be verified experimentally, because the proton exchanges too rapidly between the two oxygens.

Secondary Deuterium Isotope Effects on Acidity

It had been found that deuteration increases the basicity of amines, but the effects are small. For benzylamine-α-d_2 the isotope effect, expressed as ΔpK_a (= $-\log_{10}K_a^D + \log_{10}K_a^H$, where K_a is the acidity constant of the conjugate acid), was found by direct titration of the two amines to be 0.054 ± 0.001, which was subsequently revised to 0.032 ± 0.001 ([6364]). These error estimates are measures only of precision, not necessarily of accuracy, and may be overoptimistic if systematic error arises from an impurity in one of the samples. Similarly, ΔpK_a is 0.056 for methylamine-d_3 and 0.12 for dimethylamine-d_6, but surprisingly these were found to be temperature-independent ([65]), as would be consistent with impurities. For trimethylamine-d_9 ΔpK_a is 0.185,[66] well beyond experimental error, but this could be due to steric repulsions, which flatten trimethylamine-h_9.

These cases were puzzling. They differ from the more familiar solvolyses in that there is no rehybridization, neither of the carbon bearing the isotope nor of the nitrogen, which remains nominally sp^3 on deprotonation. The isotope effect was therefore attributed to an inductive effect

arising from an electrostatic interaction between the positive charge on the NH^+ and the dipole moment of the CH or CD bond ([67]). Since dipole moment is charge times distance, and since the average CH bond is longer than CD, owing to anharmonicity, it is not impossible that deuterium could be effectively electron-donating. This sort of inductive effect is consistent with the Born-Oppenheimer Approximation, and it was generally accepted as the source of these isotope effects. Yet both the charge separation in a CH bond, which is quite nonpolar, and the bond-length difference due to anharmonicity, are small. Thus the isotope effect ought to be vanishingly small. Yet they were detected. Since such isotope effects were crucial to a proposed study to assess the symmetry of NHN hydrogen bonds ([6869]), we sought to confirm them.

The NMR titration method permits precise measurement of relative basicities. When successive aliquots of acid are added to a mixture of bases, the acid will preferentially protonate the more basic species, whose NMR chemical shift will move ahead of that of the less basic one. The acidity constants K_a and chemical shifts δ can be related through Eq. 25, where δ^+ or δ^0 is for the protonated or deprotonated form, measured at the beginning or end of the titration. A plot of the quantity on the left vs. $(\delta_1-\delta_1^0)(\delta_2^+-\delta_2)$ should be linear, with zero intercept, and with a slope equal to the ratio of acidity constants.

$$(\delta_1^+ - \delta_1)(\delta_2 - \delta_2^0) = (K_a^1/K_a^2)(\delta_1 - \delta_1^0)(\delta_2^+ - \delta_2) \qquad (25)$$

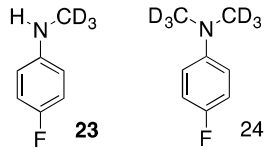
Isotope effects were thereby measured for dimethylamine-d_3, benzylamine-d, and *N,N*-dimethylaniline-d_3 ([70]). Mixing each of these with unlabeled material, or taking a methylamine-$d_{0,1,2,3}$ mixture, produces a ^{1}H NMR spectrum with resolvable signals due to different isotopologues (isomers that differ in the number of isotopic substitutions). Table 1 presents the results, which verify that there really is a secondary deuterium isotope effect on amine basicities, even though there is no rehybridization.

Table 1. Secondary Deuterium Isotope Effects on Amine Basicities

Amine	K_H/K_D	ΔpK_a	$\Delta\Delta G^{0a}$
methylamine-*d*	1.040±0.006	0.017±0.003	23.2±3.4
methylamine-d_2	1.081±0.004	0.034±0.002	23.1±1.1
dimethylamine-d_3	1.144±0.005	0.058±0.002	26.6±0.9
benzylamine-*d*	1.0419±0.0009	0.0178±0.0004	24.4±0.5
N,N-dimethylaniline-d_3	1.1051±0.0018	0.0434±0.0007	19.7±0.3

[a]cal/mol per D.

Isotope effects, K_a^H/K_a^D, on the acidities of *N*-methyl-d_3 (**23**-d_3) and *N*,*N*-dimethyl-d_6-4-fluoroanilinium (**24**-d_6) ions in aqueous dioxane were measured by ^{1}H-decoupled ^{19}F NMR titration, with a resolution-enhancing weighting function ([71]). At a pH far from the pK_a only a small intrinsic shift is observed. Near the pK_a there is a larger isotope shift, reaching a maximum of ~ 1 ppm, as shown for **24** in Fig. 4**.** The downfield shift (deshielding) of the deuterated isotopologue indicates that the labeled aniline is more basic and hence more fully protonated. It may be noted that the intrinsic isotope shift of deuteration is upfield (shielded, negative), so that the signals cross at intermediate pH values. From an analysis of those isotope shifts, K_a^H/K_a^D was found to be 1.117 ± 0.011 for **23** and 1.252 ± 0.011 for **24**.



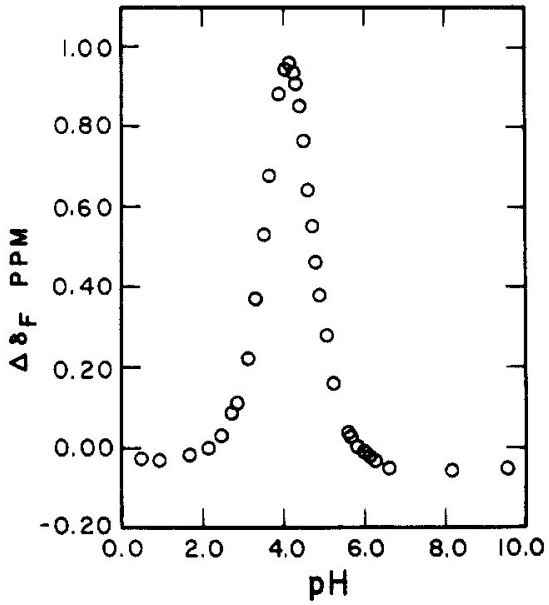


Figure 4. pH dependence of the ^{19}F isotope shift between **24**-d_6 and **24** in 50% aqueous dioxane (reprinted with permission from Forsyth, D. A., & Yang, J.-R. Intrinsic and equilibrium NMR isotope shift evidence for negative hyperconjugation. *Journal of the American Chemical Society, 108,* 2157-2161). Copyright (1986) American Chemical Society.[72]

The method was also applied to secondary deuterium isotope effects on the acidities of a wide variety of carboxylic acids, including formic, acetic, propionic, butyric, caproic, and also glycine and alanine ([73]). The pK_a ratios could be evaluated from the variation with the degree of protonation of the measured ^{13}C NMR chemical-shift differences at atoms close to the site of isotopic substitution in a mixture of isotopologues. The data were fit by nonlinear least squares to Eq. 26, where δ_H and δ_D are the observed chemical shifts of undeuterated and deuterated isotopologues, δ_H° and δ_D° are those chemical shifts in the deprotonated form, δ_H^+ and δ_D^+ are those chemical shifts in the protonated form, $R = K_a^H/K_a^D$, and n is the degree of protonation of the undeuterated material. This is the same equation as Eq. 14, but adapted to deuteration, and again n is evaluated as $(\delta_H^\circ - \delta_H)/(\delta_H^\circ - \delta_H^+)$.

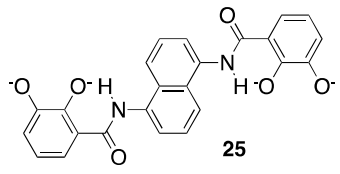
$$\delta_H - \delta_D = \delta_H^o - \delta_D^o - n(\delta_H^o - \delta_H^+) + \quad (26)$$

Trifluoroethanol-d_2 is less acidic than trifluoroethanol, according to ^{13}C NMR pH titration, with a ΔpK_a of 0.056 ([74]).

Additional examples of secondary isotope effects on acidity, some measured by NMR titration, are included in a review ([75]).

Secondary Deuterium Isotope Effects on Binding

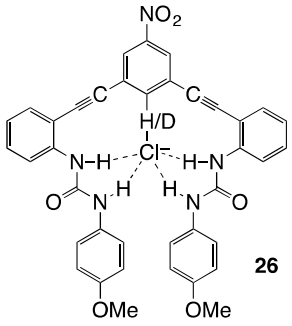
^{31}P NMR titration was used to measure the H/D isotope effects on binding of various deuterium-containing $PhCH_2P(CH_3)_3^+$ isotopologues to the exterior of the supramolecular host tetrahedral $[Ga_4L_6]^{12-}$ (L = 1,5-bis(2,3-dioxybenzamido)naphthalene^{-4}, **25**) containing a strongly bound Et_4N^+ guest ([76]). For the linearized plot the correlation coefficient was an impressive 0.99999. It was found that the binding-constant ratios are $K_{d0}/K_{d7} = 1.0302 \pm 0.0004$ and $K_{d0}/K_{d9} = 1.047 \pm 0.002$, and these two values could be corroborated with $K_{d7}/K_{d9} = 1.017 \pm 0.003$. Similar results, but of greater magnitude, were obtained for the H/D isotope effects on binding of those deuterium-containing $PhCH_2P(CH_3)_3^+$ isotopologues to the interior of the same $[Ga_4L_6]^{12-}$ ([77]).



25

The equilibrium deuterium isotope effect for Cl^- binding to anion receptor (**26**) in DMSO-d_6 was measured by ^{13}C NMR titration ([78]). From the signals of the indicated alkyne in the two

isotopologues the linearized equation gave $K^H/K^D = 1.019 \pm 0.010$, where the value > 1 means that binding is favored with ^1H. This value agrees with a calculated 1.020, whereas the calculated IEs for fragments is inverse (e.g., 0.989 for 2,6-ethynyl-4-nitro), which implies that the contributions from the separate fragments are nonadditive. The precision possible with this competitive titration may be compared with the much lower precision with the direct evaluation of the binding constant in $CHCl_3$, for which $\log K = 4.39 \pm 0.62$.



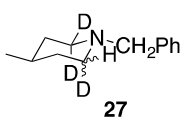
26

Further Studies of Secondary Deuterium Isotope Effects, toward Elucidating their Origin

The data in Table I show that there is indeed a secondary deuterium isotope effect on the basicity of amines. An inductive or electrostatic origin for this isotope effect still seemed unlikely. Therefore we sought to investigate them further to learn their origin.

A key result was the ratio of acidity constants for the isotopomers of 1-benzyl-4-methylpiperidine-d_3 (**27**) ([79]). This was an especially demanding measurement, because isotopomers are isomers (here stereoisomers) that differ only in the position of an isotope, as distinguished from isotopologues, which differ in the number of isotopic substitutions. It was found that the isotopomer with deuterium axial and protium equatorial is more basic, with $K_a^{eq}/K_a^{ax} = 1.060 \pm 0.006$. Therefore the isotope effect can be attributed to delocalization of the nitrogen lone pair into the antiperiplanar C-H bond, which decreases its zero-point energy and causes that isotopomer to resist

protonation more than an antiperiplanar C-D does. Moreover, for 2-methyl-2-azanorbornane-3,3-d_2 (**28**, R = CH_3) and for pyrrolizidine-*d* (**29**) deuteration can exert an isotope effect when synperiplanar to a lone pair, but smaller in magnitude ([80]). These conclusions could be confirmed by computations of the dependence of C-D stretching frequency in DCH_2NH_2 on dihedral angle. In summary, these isotope effects are attributed to a lower zero-point energy of a C-H bond adjacent to an amine nitrogen, arising from delocalization of either a syn or an anti lone pair, and with no detectable angle-independent inductive effect.



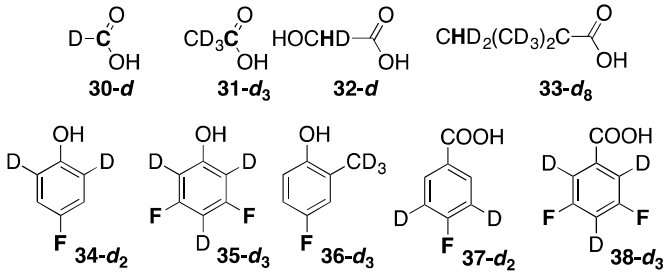
27

28

29

Secondary deuterium isotope effects on acidities of carboxylic acids (**30-33**, **37-38**) and phenols (**34**-**36**) were also measured by ^1H, ^{13}C, and ^{19}F NMR titrations ([81]). Deuteration definitely decreases the acidity of carboxylic acids and phenols, by up to 0.031 in the ΔpK per D. For aliphatic acids the isotope effects decrease as the site of deuteration becomes more distant from the OH, as expected, but a surprising result is that isotope effects in both phenol and benzoic acid do not decrease as the site of deuteration moves from ortho to meta to para. The experimental data are supported by ab initio computations which, however, substantially overestimate the isotope effects, perhaps because of inaccuracies in contributions due to solvation. The isotope effects originate in isotope-sensitive vibrations whose frequencies and zero-point energies are lowered on deprotonation. In the simplest case, formate, the key vibration can be recognized as the C-H stretch, which is weakened by delocalization of the antiperiplanar lone pairs on the oxygens. For the aromatic acids delocalization cannot account for the near constancy of isotope effects from ortho, meta, and para deuteriums, but the observed isotope effects are consistent with calculated

vibrational frequencies and electron densities. Moreover, the ability of the frequency analysis to account for the isotope effects is evidence against an inductive origin.



Secondary deuterium isotope effects on basicities of various deuterated pyridine isotopologues and of 2,6-lutidine-2,6-$(CD_3)_2$ were accurately measured in aqueous solution ([82]). Deuteration at any position of pyridine increases the basicity, but the isotope effect per deuterium is largest for substitution at the 3-position, and smallest for the 2-position that is closest to the site of *N*-protonation, smaller even than for 2-CD_3 substitution. DFT computations at the B3LYP/cc-pVTZ level overestimate the magnitude of the measured isotope effects, but largely reproduce the variability with isotopic position. Because the calculated isotope effects are based on changes in vibrational frequencies on *N*-protonation, the correspondence between calculated and experimental isotope effects implies that they arise from zero-point energies, rather than from inductive effects.

In a remarkable demonstration of the power of the NMR titration method, secondary deuterium isotope effects on the basicities of isotopologues of trimethylamine were measured ([83]). Deuteration definitely increases the basicity, by ~0.021 per D. Figure 5 shows a linear plot from the NMR titration of a mixture of tri(methyl-*d*)ammonium and tri(methyl-d_2)ammonium hydrochlorides with NaOD in D_2O. A remarkable result is that the correlation coefficient is 0.999999. The isotope effect is attributed to the lowering of the CH stretching frequency and zero-point energy by delocalization of the nitrogen lone pair into the C-H antibonding orbital. Because

this depends on the dihedral angle between the lone pair and the C-H, a further consequence is a preference for conformations with H antiperiplanar to the lone pair, and D gauche. This leads to a predicted nonadditivity of isotope effects, which could be confirmed experimentally. Specifically, it was found that the decrease in basicity, per deuterium, increases with the number of deuteriums.

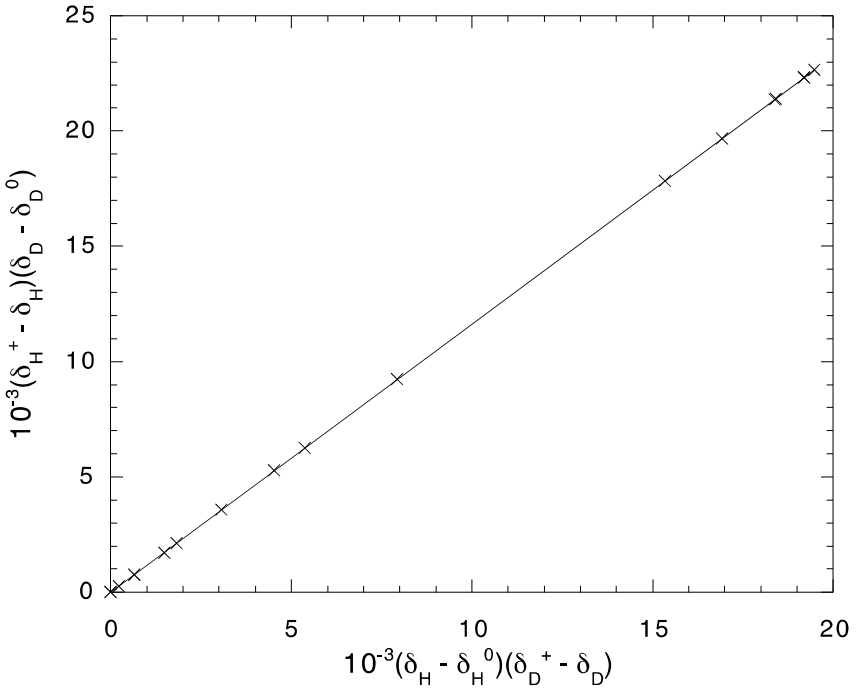


Figure 5. NMR Titration of a mixture of $(CH_2D)_3NH^+$ and $(CH_2D)_3NH^+$. (reprinted with permission from Perrin, C. L., & Dong, Y. (2008). Nonadditivity of secondary deuterium isotope effects on basicity of trimethylamine. *Journal of the American Chemical Society, 130*, 11143-11148). Copyright (2008) American Chemical Society.[84]

Further insight into the origin of these isotope effects comes from their temperature dependence ([85]). The secondary deuterium isotope effects on the acidities of formic-*d* acid, acetic-*d* and acetic-d_2 acids, and 3,5-difluorophenol-2,4,6-d_3 were measured by the NMR titration method, using ^{13}C NMR, ^{1}H NMR, and ^{19}F NMR, respectively. Instead of a complete titration for each

sample, a single-point titration, as described above, used only three titration points, obtained from three samples prepared at room temperature. Two of the points correspond to the fully protonated and fully deprotonated samples, and the third point corresponds to a 50% neutralized sample.

The temperature dependence of the isotope effects could then be analyzed by fitting the data to Eq. 27, where $\Delta\Delta S^{o}$, obtained from the intercept vs. $1/T$, is the entropic contribution to the isotope effect, $(S^{o}_{H-} - S^{o}_{H0}) - (S^{o}_{D-} - S^{o}_{D0})$, and similarly for $\Delta\Delta H^{o}$. The data are presented in Table 2. The data show that these isotope effects lie entirely in the enthalpy, as expected if their origin lies in vibrational frequencies and zero-point energies, whereas inductive effects generally manifest themselves in entropy.

$$\ln () = - + \qquad (27)$$

Table 2. Enthalpy and entropy contributions to secondary deuterium isotope effects on acidities

Acid	$\Delta\Delta H^{\circ}$ (cal/mol)	$\Delta\Delta S^{\circ}$ (cal/mol-K)	$T\Delta\Delta S^{\circ}$, 20ºC
Formic-*d*	-37.4 ± 1.2	0.009 ± 0.004	2.6±1.2
Acetic-*d*	-7.9 ± 0.4	-0.006 ± 0.001	-1.65±0.4
Acetic-d_2	-15.2 ± 0.6	-0.010 ±0.002	-2.9±0.6
3,5-F_2Phenol-d_3	-27.9 ± 1.2	-0.0085 ± 0.004	-2.5±1.2

Advantages of NMR Titration

This method is capable of exquisite precision, since it is based only on chemical-shift measurements. With modern NMR spectrometers, chemical shifts can be measured with remarkable accuracy and precision. In contrast, the classical pH titration requires accurate measurements of both volume and molarity. The data in Table 1 show that ΔpK_a can be measured with an error of as little as ± 0.0004, which is nearly two orders of magnitude better than can commonly be achieved

with a pH electrode.

Since the titration is performed on a mixture of the two bases, under conditions guaranteed identical for both, it avoids systematic error due to possible impurities.

An NMR titration can be performed in any solvent, including ones like dichloromethane or chloroform, where a pH electrode would be useless. The only requirement is that the acidic and basic forms must remain soluble, even if they are ionic.

An important advantage of this approach is that it relies solely on chemical shifts. The concentrations of the components of the mixture are not involved, meaning that sample preparation requires no special care and does not need to produce stoichiometric or precisely known amounts. The same is true for the titrant, which can be added to the sample with a low-precision or uncalibrated addition device.

Although the initial development of NMR titration used ^{1}H NMR, other nuclei are applicable: Certainly ^{13}C, ^{15}N, ^{19}F, and ^{31}P NMR titrations benefit from the broader range of chemical shifts, so that signal overlap is rare and protonation shifts are larger than with ^{1}H NMR. Those chemical shifts are likely to be more sensitive to long-range effects such as protonation on distant groups, but that may be an advantage. It is often feasible to use ^{13}C in natural abundance, but enrichment is probably necessary for ^{15}N. The abundant ^{14}N nucleus is also sensitive to chemical environment, but its nuclear spin I of 1 often leads to signal broadening by nuclear quadrupole effects, which may compromise NMR titration.

NMR titration provides only relative acidities, although these are often more relevant than absolute acidities. Of course, relative acidities can be converted to absolute acidities by anchoring them to an acid of known pK_a, but then the absolute acidities are only as accurate as that known pK_a.

Isotope effects on acidity can be used to guide chromatographic separations on an acid-base

column to separate isotopologues.[86]

Some Practical Considerations

Because NMR chemical shifts of the substrates vary with the solution environment, it is advisable to choose an internal standard for NMR that is as similar to the substrate as possible. Thus an alkane- or arene-sulfonate may be more suitable than tetramethylsilane for titration of a carboxylate, and a quaternary ammonium ion may be more suitable for titration of an amine.

In principle any nucleus that reports on the state of protonation of the molecule is useful. However, the greatest precision is obtained from monitoring those NMR chemical shifts that undergo the largest change. Therefore, those nuclei closest to the site of protonation/deprotonation are best suited for this method. These are also especially favorable, since they are often downfield of (more deshielded than) other signals and are readily discerned in the NMR spectrum.

Because the procedure involves the repeated addition to the sample of small aliquots of acid or base, an NMR titration can be quite time-consuming, especially if it is necessary to remove the sample from the NMR probe, add the aliquot, and mix the solution thoroughly. This is even more time-consuming if it is also necessary to allow the sample to equilibrate to the probe temperature after each addition. To streamline the procedure, it is convenient to add the aliquot directly into the sample in the probe. A device was designed to facilitate this experiment ([87]), and manufacturers offer various systems for flow NMR ([88]), and for monitoring reactions ([89]).

The researcher should decide whether to carry out an NMR titration from low pH to high or from high to low. Because chemical shifts are often sensitive to the medium, and specifically to its ionic strength, it may be advisable to maintain constant ionic strength during the NMR titration. Instead of adding anionic base (such as NaOH) to neutral acid (such as RCOOH, Eq. 28) or cationic acid (such as HCl, Eq. 29), to neutral base (such as an amine), it is possible to first add a slight excess of anionic base or cationic acid to the substrate and then back-titrate with cationic acid or

anionic base, respectively. In the first case the anionic conjugate base of the neutral substrate is gradually replaced during the titration by the anionic conjugate base of the cationic acid (Eq. 30). In the second case the cationic conjugate acid of the neutral substrate is gradually replaced during the titration by the cationic component of the anionic base (Eq. 31). Alternatively, the titration might start with the carboxylate salt or with the amine hydrochloride, if available. It can be seen that this technique maintains constant ionic strength during the titration.

$$OH^- + RCOOH \rightarrow RCO_2^- + H_2O \quad (28)$$

$$H^+ + R_3N \rightarrow R_3NH^+ \quad (29)$$

$$HCl + RCO_2^- \rightarrow RCOOH + Cl^- \quad (30)$$

$$R_3NH^+ + NaOH \rightarrow R_3N + Na^+ + H_2O \quad (31)$$

To take maximum advantage of accurate chemical-shift measurements, it may be necessary to output them in Hz, rather than ppm, because some instruments provide an extra significant figure for the frequencies.

To apply Eq. 8 or the other, equivalent version, it is crucial to have accurate values for the limiting chemical shifts, δ_{A°, δ_{AH^+}, δ_{B°, and δ_{BH^+} of the fully protonated and deprotonated species. Errors resulting from uncertainties in those limits have been presented graphically ([90]). It is not advisable to simply take the chemical shifts at the beginning and end of the titration. Instead the pH of the starting solution should be adjusted by adding a small amount of acid or base so that the protonation or deprotonation of the species of interest starts only after two or three additions of the titrant. Similarly the titration should continue for a few points after the species of interest have been completely protonated or deprotonated. These additional points can then provide an estimate of how

chemical shifts vary with medium, independently of the state of protonation. They may permit an extrapolation to the correct endpoint chemical shifts. Still another possibility is to use the endpoints as additional parameters in the nonlinear fit to Eq. 7.

Although H_2O is the standard solvent for pH measurements, ^1H NMR titrations in H_2O are plagued by the intense water signal, which limits the dynamic range of the spectrum. Therefore such titrations are best carried out in D_2O. This condition has two significant consequences: (1) Signals of the NH groups of amides are often missing because the NH protons exchange into the solvent. (2) A correction of 0.40 must be added to the pH-meter readings in D_2O to get pD values ([91]).

A concentrated solution of titrant should be used, to minimize dilution, which reduces sensitivity and may change the solvent conditions.

Because this NMR titration method depends on a comparison between two acids, one of which serves as a reference, it is advisable to choose the reference wisely and not randomly. Of course, if the comparison is of two isomers (or isotopologues or isotopomers), there is no choice. However, if the reference is of known acidity, it is advisable to choose an acid of similar acidity. This is apparent from consideration of Eq. 23. If the acidities differ widely, one acid is almost completely protonated while the other is almost completely deprotonated, so that one factor on each side is always the difference between two large numbers. In contrast, if the acidities are similar, all factors become of similar magnitude at the midpoint of the titration, which contributes most strongly to the slope. Thus it was misleading advice to claim that the best results are obtained when AH and BH are separated by ~2 pK_a units ([92]).

If signals overlap, the NMR titration method can be applied with two-dimensional NMR. For example, the intramolecular comparison of the relative acidities of the side-chain $-NH_3^+$ groups of the four lysine residues in a pentadecapeptide could be resolved with TOCSY ([93]). Similarly, 2D

^{1}H–^{13}C HSQC (Homonuclear Single-Quantum Correlation Spectroscopy) NMR was used for the titration with DCl of a six-component mixture of carboxylates, with acetic acid as a reference ([94]).

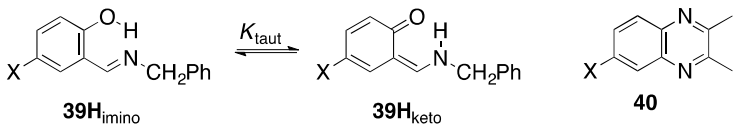
Pitfalls

Because of the need for accurate chemical shifts, in practice it is necessary that the signal of the relevant reporter nucleus show baseline separation from other signals. Overlapping signals are a warning that data will be badly behaved.

The NMR titration method was initially developed for monoprotic acids or bases, and difficulties may arise in connection with intramolecular comparisons, where there are two or more sites of protonation. An example of such a difficulty can be seen in the ^{1}H NMR titration of two 8,8'-biquinolyls, where a linearized plot (Eq. 14) shows a correlation coefficient of only 0.9715, and the points can be seen to follow an ellipse. This was attributed to the complication that one of those substrates can be doubly protonated, but in general the ellipse is a signal that an NMR titration has a problem ([95]). Thus special care was taken in the ^{13}C NMR titration of a mixture of glycine and glycine-d_2 ([96]). In order to determine the deuterium isotope effect on the first deprotonation of the NH_3^+, to produce a zwitterion, the titration was carried out only until the isoelectric point was reached (although it is unclear how that was ascertained). Anyhow, it was found that including an extra point or discarding one from the analysis modifies the result only within the experimental error.

The pK_a's of substituted salicyl *N*-benzylaldimines, **39H**, were determined from pH titration and fitting to Eq. 14 ([97]). It was further claimed that the tautomeric equilibrium constant K_{taut} could be obtained from the slope of a plot of δ_{CH2} vs. δ_{ortho} according to an adaptation of Eq. 14. Unfortunately, this analysis is faulty. While K_a for **39** is $[H^+][\mathbf{39}^-]/([\mathbf{39H}_{imino}]+[\mathbf{39H}_{keto}])$, where

39^{-} is the common anion, so that K_{taut} is the ratio of the separate (microscopic) acidity constants **39H**$_{imino}$ and **39H**$_{keto}$, those separate acidity constants cannot be measured. This differs from the intramolecular comparisons above, where the chemical shift of one reporter nucleus must be independent of the state of protonation of the other. Here both δ_{CH2} and δ_{ortho} follow Eq. 14, governed by the same pK_a, while the position of the tautomeric equilibrium is independent of pH. The information provided by a plot of δ_{CH2} and δ_{ortho} according to Eq. 14 is only of the relative sensitivity of those two chemical shifts to protonation. This same fallacy applies to the attempt to determine by ^{1}H NMR titration the relative basicities of the two nitrogens in 6-substituted 2,3-dimethylquinoxalines (**40**). ([98]).



39H$_{imino}$ **39H**$_{keto}$ **40**

Summary

NMR titration is a valuable technique for measuring relative acidities and basicities with exceptional accuracy. It can be applied to similar substances, including isotopically substituted ones, which thereby provides equilibrium isotope effects. With care it can be applied to diprotic acids and to intramolecular comparisons. It has the advantage that it is not necessary to measure pH, sample volumes, or molarities. It has wide applicability.

Acknowledgment. Preparation of this chapter was supported by NSF Grant CHE11-48992.

1 **References**

()Perrin, C. L., Fabian, M. A., & Armstrong, K. B. (1994). Solvation effect on steric bulk of ionic substituents: Imidazolium vs imidazole. *Journal of Organic Chemistry, 59*, 5246-5253.

2 ()Fabian, M. A., Perrin, C. L., & Sinnott, M. L. (1994). Absence of reverse anomeric effect: Conformational analysis of glucosylimidazolium and glucosylimidazole. *Journal of the American Chemical Society, 116*, 8398-8399.

3 . Rabenstein, D. L. & Mariappan, S. V. S. (1993). Determination of ^{15}N isotope effects on the acid-base equilibria of amino groups in amino acids by ^{13}C NMR. *Journal of Organic Chemistry, 58*, 4487-4489.

4 ()Grunwald, E. & Cocivera, M. (1965). Solvent participation in proton transfer reactions of amines and their conjugate acids. *Discussions of the Faraday Society, 39*, 105-111.

5 ()Perrin, C. L., & Wang, W.-h. (1982). Nitrogen to nitrogen transfer. Significance of large negative entropies of activation. *Journal of the American Chemical Society, 109*, 2325-2326.

6 () Sudmeier, J. L., Evelhoch, J. L., & Jonsson, N. B.-H. (1980). Dependence of NMR lineshape analysis upon chemical rates and mechanisms: Implications for enzyme histidine titrations. *Journal of Magnetic Resonance, 40*, 377-390.

7 () Bachovchin, W. W., & Roberts, J. D. (1978). Nitrogen-15 nuclear magnetic resonance spectroscopy. The state of histidine in the catalytic triad of alpha-lytic protease. Implications for the charge-relay mechanism of peptide-bond cleavage by serine proteases. *Journal of the American Chemical Society, 100*, 8041-8047.

8 () Perrin, C. L., & Fabian, M. A. (1996). Multicomponent NMR titration for simultaneous measurement of relative pK_as. *Analytical Chemistry, 68*, 2127.

9 . Baryshnikova, O. K., Williams, T. C., & Sykes, B. D. (2008). Internal pH indicators for biomolecular NMR. *Journal of Biomolecular NMR,* 41:5–7.

10 . Valcour, A., & Woodworth, R. C. (1986). Internal proton magnetic resonance probes for pH titration of proteins. *Journal of Magnetic Resonance,66*, 536-541.

11 . Tynkkynen, T., Tiainen, M., Soininen, P., & Laatikainen, R. (2009). From proton nuclear magnetic resonance spectra to pH. Assessment of ^{1}H NMR pH indicator compound set for deuterium oxide solutions. *Analytica Chimica Acta, 648*, 105–112.

12 . Szakács, Z., Kraszni,·M., & Noszál, B. (2004). Determination of microscopic acid–base parameters from NMR–pH titrations. *Analytical & Bioanalytical Chemistry, 378*, 1428–1448.

13 . Szakács, Z., Hägele, G., & Tyka, R. (2004). ^{1}H/^{31}P NMR pH indicator series to eliminate the glass electrode in NMR spectroscopic pK_a determinations. *Analytica Chimica Acta*, *522*, 247–258.

14 . Szakács, Z., & Hägele, G. (2004). Accurate determination of low p*K* values by ^{1}H NMR titration. *Talanta, 62* 819–825.

15 . Pehk, T., & Kiirend, E., & Lippmaa, E., & Ragnarsson, U. (1996). General approach to measurements of pKa differences by C NMR spectroscopy. *Journal of the Chemical Society, Perkin Transactions 2*, 2351-2357.

16 . Orgován, G., & Noszál, B. (2011). Electrodeless, accurate pH determination in highly basic media using a new set of ^{1}H NMR pH indicators. *Journal of Pharmaceutical & Biomedical Analysis, 54* 958–964.

17 . Szakács, Z., Hägele, G., & Tyka, R. (2004). ^{1}H/^{31}P NMR pH indicator series to eliminate the glass electrode in NMR spectroscopic pK_a determinations. *Analytica Chimica Acta*, *522*, 247–258.

18 . Rusu, A., Tóth, G., Szöcs, L., Kökösi, J., Kraszni, M., Gyéresi, A., & Noszál, B. (2012). Triprotic site-specific acid–base equilibria and related properties of fluoroquinolone antibacterials. *Journal of Pharmaceutical & Biomedical Analysis, 66* 50– 57.

19 . Jensen, A. A., Mikkelsen, I., Frølund, B., Frydenvang, K., Brehm, L., Jaroszewski, J. W., Brüner-Osborne, H., Falch, E., & Krogsgaard-Larsen, P. (2004). Carbamoylcholine homologs: synthesis and pharmacology at nicotinic acetylcholine receptors, *European Journal of Pharmacology, 497* 125– 137.

20 Kubyshkin, V., Durkin, P., & Budisa, N. (2016). Energetic contribution to both acidity and conformational stability in peptide models. *New Journal of Chemistry, 40*, 5209.

21 . Evans, C. A., & Rabenstein, D. L. (1974). Nuclear magnetic resonance studies of the acid-base chemistry of amino acids and peptides. 11. Dependence of the acidity of the C-Terminal carboxyl group on the conformation of the C-terminal peptide bond. *Journal of the American Chemical Society, 96,* 312-7317.

22 . Peters, M., Siegfried, L., & Kaden, T. A. (1999). A fully automated pH–NMR titration set-up for protonation studies. *Journal of the Chemical Society, Dalton Trans*., 1603–1607.

23 . Kirby, A. J. *The Anomeric and Related Stereoelectronic Effects at Oxygen*, Springer: Berlin, 1983.

24 . Juaristi, E., & Cuevas, G. (1992). Recent studies of the anomeric effect. *Tetrahedron*, *48*, 5019-5087.

25 . Thatcher, G. R. J., Ed., *The Anomeric Effect and Associated Stereoelectronic Effects*, ACS Symposium Series, American Chemical Society: Washington, DC, 1993.

26 . Thibaudeau, C., & Chattopadhyaya, J. *Stereoelectronic Effects in Nucleosides and Nucleotides and Their Structural Implications*, Uppsala University Press, 1999.

27 . Paulsen, H., Gyorgydeak, Z., & Friedmann, M. (1974). Einfluß des anomeren und inversen anomeren effektes auf konformationsgleichgewichte von *N*-substituierten *N*-pentopyranosiden. *Chemisches Berichte*, *107*, 1590-1613.

28 . Perrin, C. L., Fabian, M. A., & Armstrong, K. B. (1994). Solvation effect on steric bulk of ionic substituents: Imidazolium vs imidazole. *Journal of Organic Chemistry, 59*, 5246-5253.

29 . Perrin, C. L., Fabian, M. A., & Armstrong, K. B. (1994). Solvation effect on steric bulk of ionic substituents: Imidazolium vs imidazole. *Journal of Organic Chemistry, 59*, 5246-5253.

30 . Perrin, C. L., Fabian, M. A., & Armstrong, K. B. (1994). Solvation effect on steric bulk of ionic substituents: Imidazolium vs imidazole. *Journal of Organic Chemistry, 59*, 5246-5253.

31 . Fabian, M. A., Perrin, C. L., & Sinnott, M. L. (1994). Absence of Reverse Anomeric Effect: Conformational Analysis of Glucosylimidazolium and Glucosylimidazole. *Journal of the American Chemical Society, 116*, 8398-8399.

32 . Perrin, C. L., Fabian, M. A., Brunckova, J., & Ohta, B. K. (1999). Absence of reverse anomeric effect in glycosylimidazoles. *Journal of the American Chemical Society, 121*, 6911-6918.

33 . Perrin, C. L., & Kuperman, J. (2003). Anomeric effects versus steric hindrance to ionic solvation in protonated glucosylanilines and cyclohexylanilines, *Journal of the American Chemical Society, 125*, 8846-8851

34 () Perrin, C. L., & Fabian, M. A. (1996). Multicomponent NMR titration for simultaneous measurement of relative pK_as. *Analytical Chemistry, 68*, 2127.

35 Perrin, C. L., Fabian, M. A., & Rivero, I. A. (1999). Basicities of cycloalkylamines: Baeyer strain theory revisited. *Tetrahedron, 55*, 5773-5780.

36 . Steffens, R. J., Baldridge, K. K., & Siegel, J. S. (2000). Synthesis and properties of 2,3-dihydro-1h-corannuleno[2,3-*cd*]pyridine (2,3-dihydro-1*h*-dibenzo[1,10:6,7]fluoranthe no[3,4-*cd*]pyridine). derivatives: Heterocyclic *peri*-annellated corannulenes. *Helvetica Chimica Acta*, 83, 2644-2654.

37 . Baldridge, K. K., Cozzi, F., & Siegel, J. S. (2012). Basicity of (2,6-Pyridino)paracyclophanes: Lone pair–π, cation–π, and solvation effects. *Angewandte Chemie International Edition*, 2012, 51, 2903 –2906

38 . Hess, A. S., Yoder, R. A., & Johnston, J. N. (2006). Chiral proton catalysis: pK_a determination for a BAM-HX Brønsted acid. *SYNLETT,* 147–149

39 . Guerrero-Alvarez, J. A., Mas-Ku, W. P., Garcías-Morales, C., & Ariza-Castolo, A. (2010). Relative acidities and structure analysis of *cis* and *trans* isomers of 1,5-oxazaspiro[5.5] undecane derivatives bymultinuclear magnetic resonance. *Magnetic Resonance in Chemistry, 48*, 356–361

40 . Taylor, R. R. R., Twin, H. C., Wen, W. W., Mallot, R. J., Lough, A. J., Gray-Owen, S. D., & Batey, R. A. (2010). Substituted 2,5-diazabicyclo[4.1.0]heptanes and their application as general piperazine surrogates: synthesis and biological activity of a Ciprofloxacin analogue. *Tetrahedron, 66*, 3370–3377.

41 . Keglevich, C., Grün, A., Bölcskei, A., Drahos, L., Kraszni, M., & Balogh, G. T. (2012). Synthesis and proton dissociation properties of arylphosphonates: A microwave-assisted catalytic Arbuzov reaction with aryl bromides. *Heteroatom Chemistry*, 23, 574-582.

42 . Hirose, K. (2001). A practical guide for the determination of binding constants. *Journal of Inclusion Phenomena & Macrocyclic Chemistry*, *39*, 193–209.

43 . Thordarson, P. (2011). Determining association constants from titration experiments in supramolecular chemistry. *Chemical Society Reviews, 40*, 1305–1323.

44 . Wilcox, C. S., Adrian, J. C. Jr., Webb, T. H., & Zawacki, F. J. (1992). Approaches to Quantitative Supramolecular Chemistry. Hydrogen-Bond-Based Molecular Recognition Phenomena and Sigmoidal Behavior in Multicomponent Mixtures. *Journal of the American Chemical Society, 114*, 10189-10197.

45 . Minasian, S. G., Krinsky, J. L., Rinehart, J. D., Copping, R., Tyliszczak, T., Janousch, M., Shuh, D. K., & Arnold, J. (2009). A comparison of 4*f vs* 5*f* metal-metal bonds in $(CpSiMe_3)_3M$-ECp* (M = Nd, U, & E = Al, Ga, & Cp* = C_5Me_5): Synthesis, thermodynamics, magnetism, and electronic structure. *Journal of the American Chemical*

Society, 131, 13767–13783.

46 . Mehdoui, T., Berthet, J.-C., Thuéry, P. & Ephritikhine, M. (2004). Lanthanide(III)/actinide(III) differentiation in coordination of azine molecules to tris(cyclopentadienyl) complexes of cerium and uranium. *Dalton Transactions*, 579–590.

47 . Emenike, B. U., Liu, A. T., Naveo, E. P., & Roberts, J. D. (2013). Substituent effects on energetics of peptide-carboxylate hydrogen bonds as studied by ^{1}H NMR spectroscopy: implications for enzyme catalysis. *Journal of Organic Chemistry, 78*, 11765-11771.

48 () Perrin, C. L., & Fabian, M. A. (1996). Multicomponent NMR titration for simultaneous measurement of relative pK_as. *Analytical Chemistry, 68*, 2127.

49 . Minasian, S. G., Krinsky, J. L., Rinehart, J. D., Copping, R., Tyliszczak, T., Janousch, M., Shuh, D. K., & Arnold, J. (2009). A comparison of 4*f vs* 5*f* metal-metal bonds in $(CpSiMe_3)_3M$-ECp* (M = Nd, U, & E = Al, Ga, & Cp* = C_5Me_5): Synthesis, thermodynamics, magnetism, and electronic structure. *Journal of the American Chemical Society, 131*, 13767–13783.

50 . Shivapurkar, R., & Jeannerat, D. (2011). Determination of the relative pKa's of mixtures of organic acids using NMR titration experiments based on aliased ^{1}H–^{13}C HSQC spectra. *Analytical Methods*, *3*, 1316.

51 Onasch, O. F., Schwartz, H. M., Aikens, D. A., & Bunce, S. C. (1991). The microscopic protonation constants of *N*,*N*-dimethyl-1,3-propanediamine: An NMR titration experiment. *Journal of Chemical Education*, *68*, 791.

52 Shrager, R. I., Cohen, J. S., Heller, S. R., Sachs, D. H., & Schechter, A. N. (1972). Nuclear magnetic resonance titration curves of histidine ring protons. *Biochemistry*, *11*, 541.

53 Bradbury, J. H., & Sheraga, H. A. (1966). Structural studies of ribonuclease. XXIV. The application of nuclear magnetic resonance spectroscopy to distinguish between the histidine

residues of ribonuclease1. *Journal of the American Chemical Society, 88*, 4240.

54 . Rabenstein, D. L., Hari, S. P., & Kaerner, A. (1997). Determination of acid dissociation constants of peptide side-chain functional groups by two-dimensional NMR. *Analytical Chemistry, 69*, 4310-4316.

55 . Kállay, C., Ösz, K., Dávid, A., Valastyán, Z., Malandrinos, G., Hadjiliadis, N., & Sóvágó, I. (2007). Zinc(II) binding ability of tri-, tetra- and penta-peptides containing two or three histidyl residues. *Dalton Transactions,* 4040–4047.

56 . Pehk, T., Kiirend, E., Lippmaa, E., & Ragnarsson, U. (1996). General approach to measurements of pKa differences by C NMR spectroscopy. *Journal of the Chemical Society, Perkin Transactions 2*, 2351-2357.

57 . Choi, P. J., Petterson, K. A., & Roberts, J. D. (2002). Ionization equilibria of dicarboxylic acids in dimethylsulfoxide as studied by NMR. *Journal of Physical Organic Chemistry, 15*, 278.

58 . Ellison, S. L. R., & Robinson, M. J. T. (1963). A precise N.M.R. method for measuring isotope effects on acid-base equilibria: Effects of solvent, solvent isotopic composition, and ionic strength on hydrogen and oxygen isotope effects in formic acid. *Journal of the Chemical Society, Chemical Commun*icatons, 745-746.

59 . Knight, W. B., Weiss, P. M., & Cleland, W. W. (1986). Determination of equilibrium ^{18}O isotope effects on the deprotonation of phosphate and phosphate esters and the anomeric effect on deprotonation of glucose-6-phosphate. *Journal of the American Chemical Society, 108*, 2759-2761.

60 . Rabenstein, D. L. & Mariappan, S. V. S. (1993). Determination of ^{15}N isotope effects on the acid-base equilibria of amino groups in amino acids by ^{13}C NMR. *Journal of Organic Chemistry, 58*, 4487-4489.

61 . Pehk, T., Kiirend, E., Lippmaa, E., Ragnarsson, U., & Grehn, L. (1997). Determination of isotope effects on acid–base equilibria by ^{13}C NMR spectroscopy. *Journal of the Chemical Society, Perkin Transactions 2*, 445-450.

62 Perrin, C. L., & Burke, K. D. (2015). Can a secondary isotope effect be larger than a primary? *Journal of Physical Chemistry A*, *119*, 5009–5018.

63 () Halevi, E. A., Nussim, M., & Ron, A. (1963). Secondary hydrogen isotope effects. Part I. Strengths of α-deuterated carboxylic acids and amines. *Journal of the Chemical Society*, 866.

64 () Bary, Y., Gilboa, H., & Halevi, E. A. (1979). Secondary hydrogen isotope effects. Corrigendum and addendum. *Journal of the Chemical Society, Perkin Transactions, 2*, 938.

65 () Van der Linde, W., & Robertson, R. E.(1964). K_D/K_H for base ionization of mono- and dimethylamine. *Journal of the American Chemical Society, 86*, 4505.

66 () Northcott, D., & Robertson, R. E. (1969). Secondary deuterium isotope effect in the ionization of trimethylamine-d_9 in water. *Journal of Physical Chemistry, 73*, 1559.

67 () Halevi, E. A. (1963). Secondary isotope effects. *Progress in Physical Organic Chemistry*, *1*, 109.

68 () Perrin, C. L., & Ohta, B. K. (2001). Symmetry of N-H-N hydrogen bonds in 1,8-bis(dimethylamino)naphthalene·H^+ and 2,7-dimethoxy-1,8-bis(dimethylamino)naphthalene·H^+. *Journal of the American Chemical Society, 123*, 6520.

69 () Perrin, C. L., & Ohta, B. K. (2003). Symmetry of NHN hydrogen bonds in solution. *Journal of Molecular Structure*, *644*, 1.

70 Perrin, C. L., Ohta, B. K., & Kuperman, J. (2003). β-Deuterium isotope effects on amine basicity, 'inductive' and stereochemical. *Journal of the American Chemical Society, 125*, 15008-15009.

71 Forsyth, D. A., & Yang, J.-R. (1986). Intrinsic and equilibrium NMR isotope shift evidence for negative hyperconjugation. *J. Am. Chem. Soc. 108,* 2157-2161.

72 Forsyth, D. A., & Yang, J.-R. (1986). Intrinsic and equilibrium NMR isotope shift evidence for negative hyperconjugation. *Journal of the American Chemical Society 108,* 2157-2161.

73 . Pehk, T., Kiirend, E., Lippmaa, E., Ragnarsson, U., & Grehn, L. (1997). Determination of isotope effects on acid–base equilibria by ^{13}C NMR spectroscopy, *Journal of the Chemical Society, Perkin Transactions 2*, 445-450.

74 Gawlita, E., Lantz, M., Paneth, P., Bell, A. F., Tonge, P. J., & Anderson, V. E. (2000). H-Bonding in alcohols is reflected in the $C_{\alpha}H$ bond strength: variation of C–D vibrational frequency and fractionation factor. *Journal of the American Chemical Society, 122*, 11660–9.

75 Perrin, C. L. (2010). Secondary equilibrium isotope effects on acidity. *Advances in Physical Organic Chem*istry*,* Chapter 3, J. P. Richard, Ed. *44*, 123-171.

76 . Mugridge, J. S., Bergman, R. G., & Raymond, K. N. (2010). High-precision measurement of isotope effects on noncovalent host-guest interactions. *Journal of the American Chemical Society, 132*, 1182–1183.

77 . Mugridge, J. S., Bergman, R. G., & Raymond, K. N. (2012). Equilibrium isotope effects on noncovalent interactions in a supramolecular host-guest system. *Journal of the American Chemical Society, 134*, 2057-2066.

78 . Tresca, B. W., Brueckner, A. C., Haley, M. M., Cheong, P. H.-Y., & Johnson, D. W. (2017). Computational and experimental evidence of emergent equilibrium isotope effects in anion receptor complexes. *Journal of the American Chemical Society, 139*, 3962-3965.

79 Perrin, C. L., Ohta, B. K., & Kuperman, J. (2003). β-Deuterium isotope effects on amine basicity, "inductive" and stereochemical. *Journal of the American Chemical Society, 125*, 15008-15009.

80 Perrin, C. L., Ohta, B. K., Kuperman, J., Liberman, J., Erdélyi, M. (2005). Stereochemistry of β-deuterium isotope effects on amine basicity. *Journal of the American Chemical Society, 127*, 9641-9647.

81 Perrin, C. L., & Dong, Y. (2007). Secondary deuterium isotope effects on the acidity of carboxylic acids and phenols. *Journal of the American Chemical Society, 129*, 4490-4497.

82 Perrin, C. L., & Karri, P. (2010). Position-specific secondary deuterium isotope effects on basicity of pyridine. *Journal of the American Chemical Society, 132*, 12145–12149.

83 Perrin, C. L., & Dong, Y. (2008). Nonadditivity of secondary deuterium isotope effects on basicity of trimethylamine. *Journal of the American Chemical Society, 130*, 11143-11148.

84 Perrin, C. L., & Dong, Y. (2008). Nonadditivity of secondary deuterium isotope effects on basicity of trimethylamine. *Journal of the American Chemical Society, 130*, 11143-11148.

85 Perrin, C. L., & Flach, A. (2011). No contribution of an inductive effect to secondary deuterium isotope effects on acidity. *Angewandte Chemie International Edition 50*, 7674–7676.

86 . Tanaka, N. et al. (1989). Separation of nitrogen and oxygen isotopes by liquid chromatography. *Nature*, *341*, 727-728.

87 . Perrin, C. L., & Rivero, I. A. (1999). Apparatus for direct addition of reagents into a nuclear magnetic resonance (NMR) sample in the NMR probe. *Review of Scientific Instruments*, *70*, 2173-2174.

88 Hentschel, P., Holtin, K., Steinhauser, L. & Albert, K.(2012). Monitoring the on-line titration of enantiomeric omeprazole employing continuous-flow capillary microcoil ^{1}H NMR spectroscopy. *Chirality*, *24*, 1074–1076 .

89 . Gomez, M. V., & de la Hoz, A. (2017). NMR reaction monitoring in flow synthesis. *Beilstein Journal of Organic Chemistry, 13,* 285–300.

90 . Shivapurkar, R., & Jeannerat, D. (2011). Determination of the relative p*K*a's of mixtures of organic acids using NMR titration experiments based on aliased ^{1}H–^{13}C HSQC spectra.

Analytical Methods, *3*, 1316.

91 . Glasoe, P. K, & Long, F. A. (1960). Use of glass electrodes to measure acidities in deuterium oxide. *Journal of Physical Chemistry, 64*, 188–190.

92 . Emenike, B. U., Liu, A. T., Naveo, E. P., & Roberts, J. D. (2013). Substituent effects on energetics of peptide-carboxylate hydrogen bonds as studied by ^{1}H NMR spectroscopy: Implications for enzyme catalysis. *Journal of Organic Chemistry, 78*, 11765-11771.

93 . Rabenstein, D. L., Hari, S. P., & Kaerner, A. (1997). Determination of acid dissociation constants of peptide side-chain functional groups by two-dimensional NMR. *Analytical Chemistry, 69*, 4310-4316.

94 . Shivapurkar, R., & Jeannerat, D. (2011). Determination of the relative p*K*a's of mixtures of organic acids using NMR titration experiments based on aliased ^{1}H–^{13}C HSQC spectra. *Analytical Methods*, *3*, 1316.

95 . Blakemore, P., Milicevic, S. D., Perera, H., Shvarev, A., & Zakharov, L. N. (2008). Determination of pK_a values for diether derivatives of 7,7'-dihydroxy-8,8'-biquinolyl: dependence of basicity on interannular dihedral angle. *SYNTHESIS* , 2271–2277

96 . Romero, J., Reyes, A., & Wist, J. (2010). Secondary deuterium isotope effects on the acidity of glycine. *Spectrochimica Acta A*, *77*, 845–848.

97 . Ortegón-Reyna, D., Garcías-Morales, C., Padilla-Martínez, I., García-Báez, E., Aríza-Castolo, A., Peraza-Campos, A., & Martínez-Martínez, F. (2014). NMR structural study of the prototropic equilibrium in solution of schiff bases as model compounds. *Molecules, 19*, 459-481.

98 . Peralta-Cruz, J., Díaz-Fernández, M., Avila-Castro, A., Ortegón-Reyna, D., & Ariza-Castolo, A. (2016). An experimental and theoretical study of intramolecular regioselective oxidations of 6-substituted 2,3-dimethylquinoxaline derivatives. *New Journal of Chemistry*, *40*, 5501-5515.