Journal of Magnetic Resonance 205 (2010) 130–140

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

Chemical-shift referencing and resolution stability in methanol:water gradient LC–NMR

Paul A. Keifer *

Varian, Inc., 3120 Hansen Way D-298, Palo Alto, CA 94304-1030, United States

ARTICLE INFO

Article history:
Received 3 January 2010
Revised 12 April 2010
Available online 18 April 2010

Keywords:
LC–NMR
Referencing
Reference standards
Lineshape
Magnetic susceptibility
Methanol
Water

ABSTRACT

Solvent-gradient LC–NMR can generate wide ranges of solvent conditions during an experiment. This complicates the chemical-shift referencing of the resulting NMR data. This problem and other experimental issues are evaluated here for LC–NMR in methanol:water, using solvent mixtures running from 0% to 100% methanol. It is shown that the use of the methanol methyl signal is superior to the use of the water signal in any form (either the ^{1}H or the ^{2}H signal), either as a secondary reference, as a signal for shimming, or as a lock signal. Also shown are the limitations of the referencing methods and other experimental parameters, and the limitations of the solvent-gradient ramp parameters, primarily as they affect lineshapes.

1. Introduction

We previously evaluated how the use of acetonitrile:water as a mobile phase affects the NMR methodology needed for running solvent-gradient LC–NMR* experiments [1]. (The term "LC–NMR*" is used here to generally refer to all the various hyphenated permutations of the technique [i.e., LC–NMR, LC–NMR–MS, and LC–UV–NMR–MS, etc.] because the conclusions apply equally to all.) It was shown how LC–NMR* has unique NMR chemical-shift referencing issues. It was also shown how the use of acetonitrile:water mixtures in solvent-gradient LC–NMR* influences how a user should lock, shim, and acquire the NMR data. Shown here are the results of a similar evaluation of methanol:water as a mobile phase.

We studied methanol as a mobile phase because it is the second-most heavily used organic solvent in reversed-phase HPLC after acetonitrile. (Methanol and acetonitrile together account for the overwhelming majority of reversed-phase HPLC applications, so it is important to know how to use each of these solvents in LC–NMR*. In addition, methanol may become more important as an HPLC solvent, because acetonitrile's worldwide supply and price pressures continue to cause shortages [2].) The conclusions shown here direct users how to best optimize the acquisition parameters when running LC–NMR* experiments with a methanol:water mobile phase. The differences we found in the data between using methanol and acetonitrile has surprised some LC–NMR* users.

There are several reasons why LC–NMR* cannot easily follow the IUPAC rules for referencing NMR spectra. First, LC–NMR* never uses "dilute TMS in $CDCl_3$" as a solvent. Second, the NMR solvents used in LC–NMR (which are called the "mobile phase" and are primarily determined by the chromatography) are typically *mixtures* of solvents (usually mixtures of water and either CH_3CN or CH_3OH). Third, in solvent-gradient acquisitions, the "solvent" is programmed to change composition continuously during the separation, so wide variations of solvent composition can be encountered within a single experiment. All of these issues make referencing more difficult to rigorously control. We previously explained these issues in detail and showed how these issues (and several others) are manifested when acquiring LC–NMR data with solvent mixtures of acetonitrile and water [1]. Here we show how similar effects can be observed in mixtures of methanol and water.

2. Results and discussion

2.1. The HPLC method

The HPLC method controls the solvent composition as a function of time in LC–NMR* experiments. After the HPLC method makes any change in the solvent composition (which happens immediately at the HPLC pumphead), that change propagates through the HPLC column and tubing into the NMR probe. Whenever the solvent composition within the NMR probe changes, the

* Present Address: Varian, Inc. (Agilent), 2700 Mitchell Drive, Walnut Creek, CA 94598, United States.
E-mail address: PAKeifer@gmail.com

1090-7807/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jmr.2010.04.009

frequencies of all the ^{1}H resonances move, including the solvent resonances.

The mobile phase was a binary mixture of CH_3OH and D_2O, each spiked with a cocktail of additives whose resonances served as monitors of chemical-shift changes (discussed below). The HPLC method is diagrammed in Fig. 1. The diagram is generic in that the duration of the gradient ramp, in both time and solvent volume, was changed in different runs. Although some of the solvent-composition changes are sudden, the change of primary interest is the slow ramp from 0% to 100% CH_3OH located in the middle of the HPLC method.

2.2. Acidification of the mobile phase

It has been previously reported that the two ^{1}H resonances in methanol move relative to each other when methanol is dissolved in different solvents (due to differential hydrogen bonding) [3–5]. (They are also known to move as a function of temperature [6].) We found that these two resonances also move relative to each other as a function of concentration during a 0-to-100% methanol:water solvent gradient in LC–NMR. Wilkins and coworkers reported that when methanol:water is used in LC–NMR, the hydroxyl resonance at ca. 4.8 ppm (which arises from the hydroxyl protons of each solvent undergoing exchange with each other) will be both broadened and split [7] unless catalytic amounts of acid or base are added to the mobile phase [8]. This is illustrated in Fig. 2, which also shows that the extent of this broadening depends heavily upon the composition of the mobile phase, and is largest when the mobile phase is >60% methanol. Fig. 3 shows how the two hydroxyl resonances in an unacidified methanol:water mobile phase

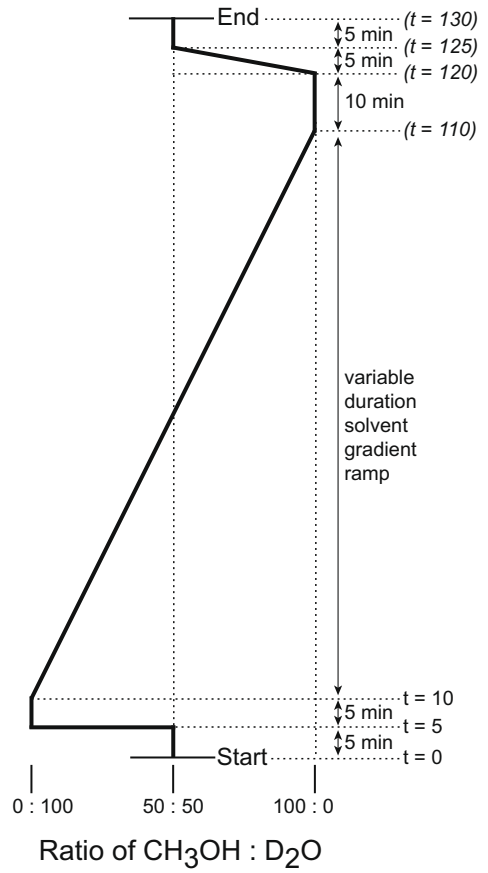

Fig. 1. A generic diagram of the HPLC method used in these studies. This diagram is oriented with time along the vertical axis ($t = 0$ at the bottom) and solvent composition along the horizontal axis, so as to facilitate visual comparisons with the LC–NMR data. The fixed times within the HPLC method are listed along the right-hand edge. The duration of the solvent-gradient ramp was a variable in different experiments, so absolute times after the ramp were not fixed, and the absolute times in parentheses and italics correspond to those used for the default solvent-gradient ramp (which had a 1% solvent-composition change per minute ramp over a 100-min period). Both the CH_3OH and the D_2O (HOD) solvents contained multiple additives that served as chemical-shift markers, as is described in the text.

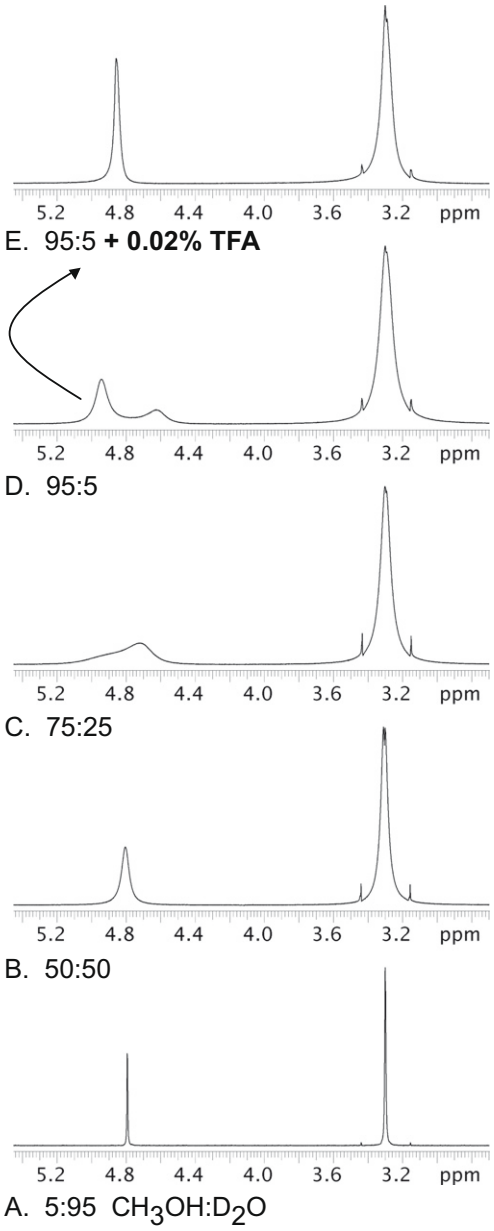

Fig. 2. This figure shows how mobile phases composed of various ratios of methanol:water can have broad hydroxyl resonances (near 4.8 ppm), and how catalytic amounts of acid can sharpen and merge them into a singlet. (The acid increases the exchange rate of the –OH protons between water and methanol.) The CH_3OH:D_2O ratio used to acquire each spectrum was 5:95 (A), 50:50 (B), 25:75 (C), and 95:5 (D) (ranging from bottom to top). The top spectrum (E) was acquired with 95:5 CH_3OH:D_2O containing ca. 0.02% TFA (trifluoro-acetic acid). The acid dramatically sharpens the hydroxyl resonance, as indicated by the arrow between D (no acid) and E (with acid). In all data, the CH_3 resonance of methanol was referenced to 3.3 ppm. The widening of the methyl resonance at 3.3 ppm in the top four spectra is due to radiation damping (as would be expected for samples of this concentration at this magnetic field strength). The vertical scales of each spectrum were normalized, so they are not directly comparable.

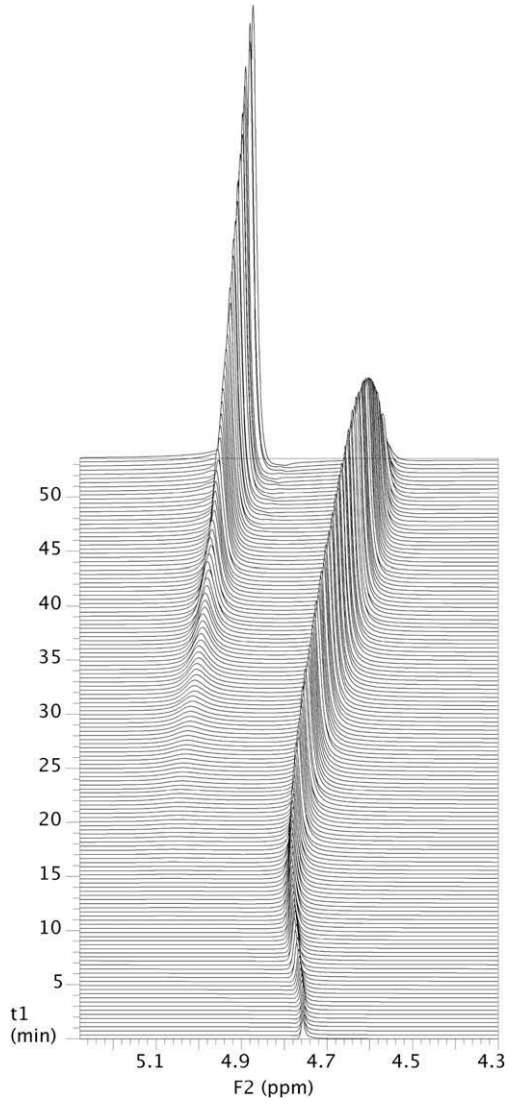

Fig. 3. Solvent gradient data acquired using no acid. This is a frequency expansion of the LC–NMR data acquired on an *unacidified* CH_3OH:D_2O solvent gradient that ran from 5% to 100% CH_3OH over 54 min, shown as a stacked plot. The lack of any acid in the mobile phase causes the resonances from the two hydroxyl groups to remain separate and resolved. Neither of the hydroxyl resonances were suppressed, but the methyl resonance of CH_3OH (located at 3.3 ppm) was suppressed with single-frequency WET. The transmitter was actively maintained on the methyl signal. The HPLC column was removed for this acquisition. (This dataset was not acquired with the HPLC method shown in Fig. 1; it is the only on-flow dataset in this paper that did not.) The resonances move up to 0.52 ppm (at their extremes), which is much more that the 0.1 ppm movement of the hydroxyl resonances in an acidified mobile phase (Fig. 4). Even the methanol hydroxyl group (left-most) moves 0.2–0.25 ppm, which is about twice what the acidified hydroxyl resonance moves.

both get resolved and move around during a 0-to-100% solvent gradient (using the HPLC method shown in Fig. 1). Because these resolved, broad, and moving solvent resonances complicate both solvent suppression and the usefulness of the data, we added TFA (trifluoro-acetic acid; to ca. 0.1% v/v concentration) to all further batches of the mobile phase. This increased the exchange rate of the hydroxyl protons, and caused their NMR signals to always appear as one singlet – in all ratios of methanol:water. All other data shown here were acquired by using acidified mobile phase (with the exception of Figs. 2 and 3, which are specified otherwise). (Note that, although WET can easily suppress broad solvent resonances, using solvent suppression this way renders useless the correspondingly large portion of the spectrum.) (Note also that the use of acidification may not be compatible with the separations needed for a given analysis; this may sometimes make the use of another solvent like acetonitrile a better choice for the separation.)

2.3. Solvent suppression, WET, and Scout-Scan referencing

The solvent resonances were big enough that we suppressed both the methyl and the hydroxyl resonances present in the acidified mixtures of CH_3OH and HOD. (The HOD is formed from the D_2O by chemical exchange from both the hydroxyl of CH_3OH and absorbed H_2O.) Solvent suppression was done with a two-frequency shaped WET [9] pulse in which the transmitter was kept on one resonance and the other resonance was irradiated by the SLP (shifted laminar pulse; phase-ramped pulse) technique [10]. This requires the user to decide which of the two resonances the transmitter should track – either the methyl resonance at 3.3 ppm or the hydroxyl resonance at ca. 4.8 ppm. These two methods are not equivalent, and the choice has an impact upon the resulting data (shown below).

The frequencies used for this solvent suppression need to be re-optimized whenever the solvent resonances move. They move frequently when the composition of the solvent changes frequently, as occurs during a solvent-gradient method. The rate at which the re-optimization needs to happen depends upon both the flow rate and the slope of the solvent gradient (both of which are under user control via the HPLC method). It is also influenced by how much the changes in solvent composition move each resonance (which is not under user control). We used the Scout-Scan technique [1,9,11] to automatically re-optimize these frequencies for every increment (spectrum) of the pseudo-2D data acquired during on-flow LC–NMR* experiments. The Scout-Scan technique first takes a small-tip-angle ^1H spectrum without using solvent suppression, and then analyzes that spectrum both to set the transmitter on the desired resonance and to calculate the resulting frequency offset(s) for the solvent signal(s) to be suppressed. Next, it creates a shaped pulse that excites all these resonances, and then resets the parameters to do a signal-averaged solvent-suppressed ^1H spectrum and starts acquisition. The entire Scout-Scan process takes just a few seconds. The frequency of this process is determined by the number of transients used per spectrum (our default was 16). Also under user control are: the number of resonances searched for, which resonance to keep at a constant chemical shift, and whether to use ^{13}C-satellite suppression.

To get good NMR data you also need to reference the multiple spectra (increments) within the pseudo-2D dataset. In solvent-gradient LC–NMR, this is normally done by actively maintaining one resonance at a constant chemical shift. The Scout-Scan technique does this by actively adjusting the transmitter (the center of the spectrum) for every spectrum onto one of the tall solvent resonances – either the methyl signal at ca. 3.3 ppm or the hydroxyl signal at ca. 4.8 ppm.

2.4. The movement of the hydroxyl resonance

Fig. 4 shows the movement of the hydroxyl resonance when the frequency of the methyl resonance is held constant. This dataset was acquired by actively maintaining the transmitter on the methyl signal and referencing it to 3.3 ppm in every increment of the pseudo-2D experiment. Single-frequency solvent suppression (at 3.3 ppm only) was used here to allow the hydroxyl resonance to be seen. This figure shows that the hydroxyl resonance moves as much as 0.1 ppm relative to the methyl resonance during the 0-to-100% solvent gradient. This is much less than the 2.4-ppm movement seen in our previous study for the water resonance relative to acetonitrile in an acetonitrile:water (0–100%) solvent gradient [1]. This striking difference explains some of the differences

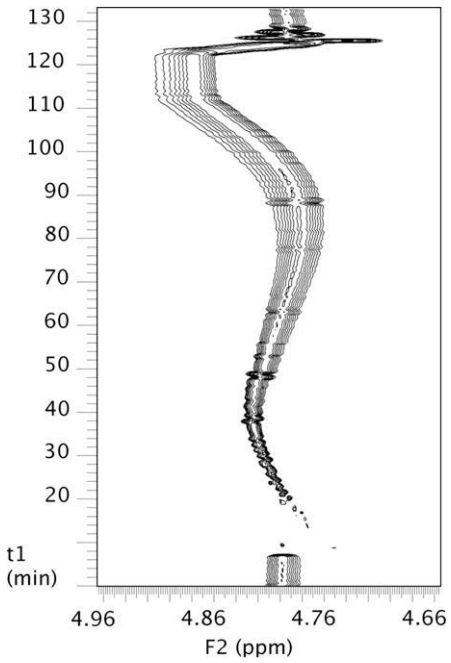

Fig. 4. This figure shows the movement of the water resonance in an acidified methanol:water solvent gradient when the transmitter is actively maintained on the methanol methyl resonance. This data was acquired identically to that shown in Fig. 6, except that only single-frequency solvent suppression was used (on the methanol methyl) to allow us to clearly see the hydroxyl resonance.

in methodology needed between the two solvent systems (as discussed below). (We show below that the frequency of the CH_3OH resonance remains rather constant, and serves as an appropriate secondary reference compound.)

2.5. Relative chemical-shift referencing: CH_3- versus –OH

The movements of the methyl and hydroxyl resonances relative to each other were more carefully evaluated by spiking the mobile phase with a variety of compounds whose relative ^1H chemical-shift movements could be monitored during the 0-to-100% solvent-gradient ramp. The selection of the mobile-phase additives was challenging because they had to be soluble in both solvents (CH_3OH and D_2O), not excessively retained by the chromatographic column, and we wanted sharp ^1H resonances so that we could measure lineshape distortions. The final cocktail contained DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), HMDS (hexamethyldisiloxane), CH_3CN, CH_3OH, sucrose, H_2O, CH_2Cl_2, $CHCl_3$, and sodium formate. Their chemical shifts in 50:50 CH_3OH:D_2O (the starting conditions), as shown in Fig. 5 were (with CH_3OH set to 3.3 ppm): DSS = −0.053 ppm (singlet) and multiplets at 0.6, 1.7, and 2.8 ppm; HMDS = +0.060 ppm (singlet); CH_3CN = 2.015 ppm (singlet); CH_3OH = 3.30 ppm (CH_3 group, singlet; used as the secondary reference signal); sucrose = multiplets ranging from 3.4 to 4.2 ppm, and a doublet at 5.33 ppm (J = 3.9 Hz); HOD = 4.81 ppm; CH_2Cl_2 = 5.4 ppm (singlet); $CHCl_3$ = 7.7 ppm (singlet); and formate = 8.2 ppm (singlet). (The formate resonance exhibits two signals in Fig. 5, with the minor signal appearing at 8.1 ppm. This signal only became apparent after acid was added to the solvents. It appears in all subsequent figures because they were acquired with acidified mobile phase.)

Some of the additives were partly retained on the chromatography column under 100% D_2O conditions. The HPLC method was designed to compensate for this and to improve our ability to monitor every resonance in every additive. The HPLC method was started at 50:50 CH_3OH:D_2O (to equilibrate the system) then changed to 100% D_2O for five minutes (from t = 5 to t = 10 min) before starting the solvent-gradient ramp at t = 10 min (Fig. 1). This allowed reproducible amounts of additives to be placed on the HPLC column during each run, and generated multiple "control" spectra at the beginning of each run where all of the ^1H resonances were visible (for lineshape verification). Although the sudden solvent change from 50:50 to 0:100 CH_3OH:D_2O generated several increments/spectra that were "ugly", this was an acceptable tradeoff to ensure a reproducible method. (The spectra became "ugly" because the sudden large change in solvent moved all of the resonances during one single increment while the NMR spectrometer continued to signal average, and did so with now non-ideal solvent-suppression frequencies. In addition, the lineshape of every resonance was severely broadened by magnetic-susceptibility inhomogeneities in the NMR flow cell created by incomplete mixing of the incoming solvent. This shows that there are limitations to how rapidly the solvent composition should be changed during an LC–NMR* experiment, as is discussed below.) Finally, after the solvent-gradient ramp reached 100% CH_3OH, the HPLC method returned to a 50:50 solvent composition (more gently this time) to allow additional "control" spectra to be acquired at the end of each run. An equivalent HPLC method was used in our previous studies on CH_3CN:D_2O solvent gradients, [1] which allows us to make comparisons between the two solvent systems.

A key decision that a user needs to make when acquiring LC–NMR* data is whether to maintain the transmitter on the methyl resonance of CH_3OH or on the hydroxyl resonance. The additive cocktail and the HPLC method were designed to allow us to evaluate this choice. The results are shown in Figs. 6 and 7. Fig. 6 was acquired by actively maintaining the transmitter on the methanol–methyl resonance for each increment (and referencing it to 3.30 ppm). It shows what a normal LC–NMR dataset should look like (except that no sample was used and the mobile phases were spiked with a cocktail of solutes). Fig. 7A is the same data, but replotted with an unusually compressed time axis to emphasize ^1H chemical-shift movements of the resonances from the cocktail solutes over the entire 0–100% CH_3OH ramp. In contrast, Fig. 7B was acquired by actively maintaining the transmitter on the hydroxyl resonance (and referencing it to 4.81 ppm). (The hydroxyl signal was referenced to 4.81 ppm because this value placed the methanol–methyl resonance in 50:50 CH_3OH:D_2O at 3.30 ppm, which facilitated comparisons between datasets within this study.)

A comparison of Fig. 7A and B shows that the chemical shifts of all of the additives are generally more constant when the methanol–methyl signal is kept on resonance (Figs. 7A and 6), as opposed to when the hydroxyl signal is kept on resonance (Fig. 7B). This shows that holding the hydroxyl resonance constant (with either the ^2H lock or the Scout-Scan method) is undesirable and produces misleading ^1H chemical-shift scales. Although this latter method (keeping the hydroxyl signal on resonance, especially by using a ^2H lock) is easy to run, and is commonly used, it increases chemical shift error and variability. The methanol–methyl signal should be kept on resonance to maintain the most accurate chemical-shift scales. (A similar conclusion was recently found for solvent gradients composed of acetonitrile:water, which generated significantly larger relative chemical-shift movements [1].)

2.6. Referencing on methanol as opposed to other organic solutes

Fig. 7A and B show that the methyl signal of methanol is a more stable chemical-shift reference than the hydroxyl resonance in an acidified mobile phase, but another question is whether a resonance from some other compound might be even more stable. When we made a similar evaluation of an acetonitrile:water mobile phase, we found that the acetonitrile resonance was clearly

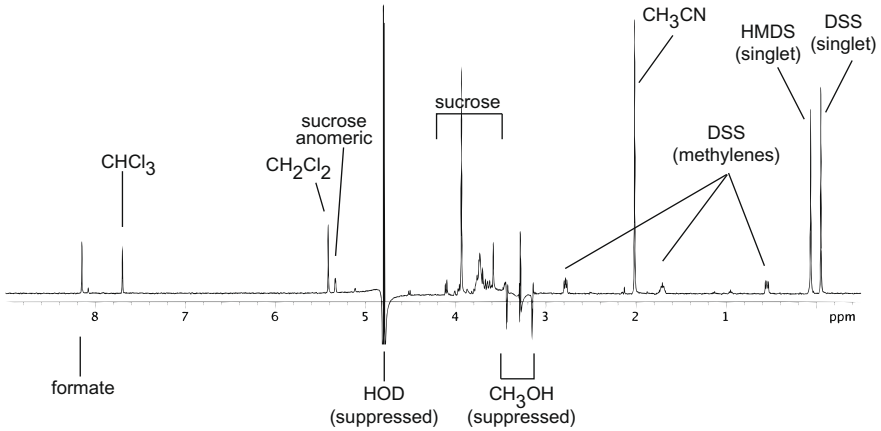

Fig. 5. A ^{1}H spectrum of the mobile phase and its additives. This spectrum was acquired at an equilibrated solvent composition of 50:50 $CH_3OH:D_2O$ using two-frequency WET suppression. The resonances of the additives are labeled. The CH_3OH was used as a secondary reference signal and assigned a value of 3.3 ppm, which places the CH_3CN singlet at 2.015 ppm and the DSS-singlet at −0.053 ppm in this solvent composition. (This spectrum was acquired with 48 transients and no ^{2}H lock, and was processed with no zerofilling, 1.0 Hz linebroadening, and solvent subtraction on the residual methanol methyl signal [ssfilter = 80].)

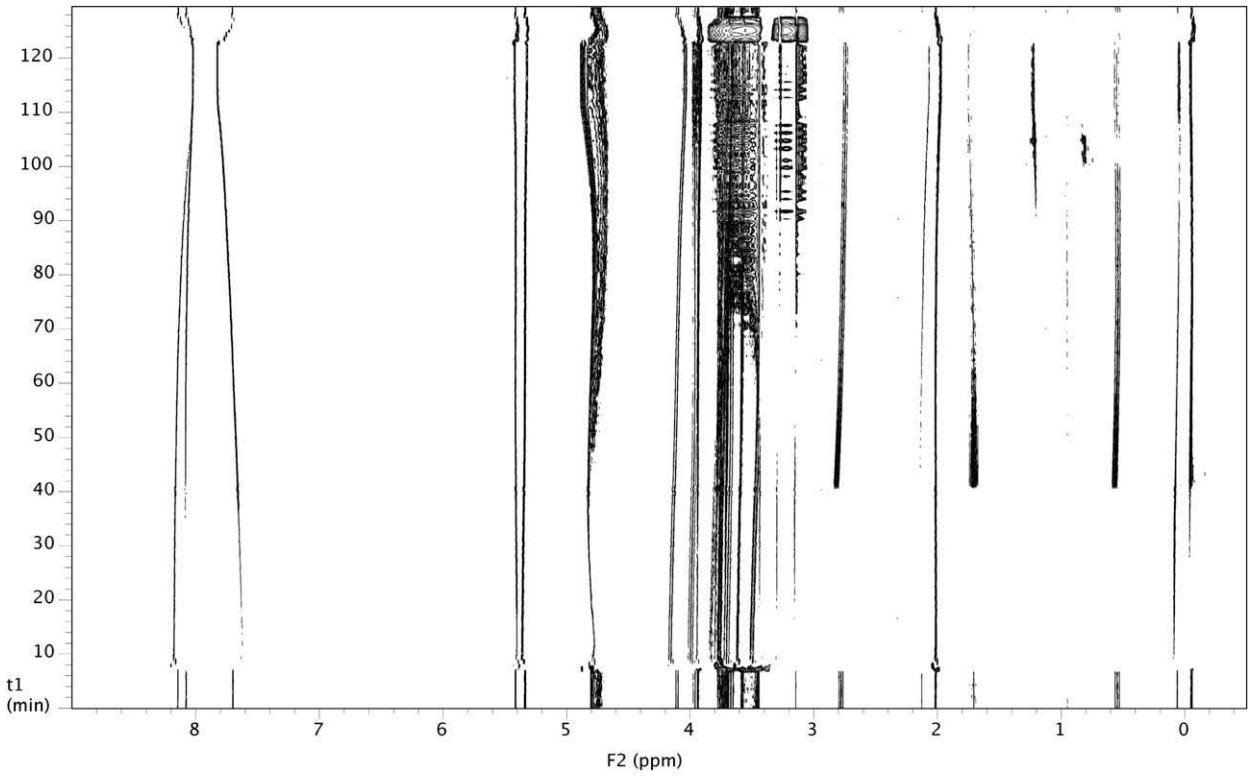

Fig. 6. Two-frequency suppression; methanol–methyl centric. This on-flow LC–NMR data was acquired with two-frequency solvent suppression with the transmitter actively maintained on the methanol–methyl resonance. The mobile phase was a 0-to-100% solvent gradient of acidified methanol:water.

the best choice [1]. The differences shown here with a methanol:water mobile phase are less dramatic, but the data do show that methanol is the best choice. The $CHCl_3$ and formate signals track in opposite directions, each doing so in ways that are unlike any other resonances, which makes them not useful as standards (Fig. 7A). (The minor signal at 8.1 ppm merges with the main formate resonance at high methanol concentrations.) In contrast, the CH_3CN, DSS-singlet, and HMDS signals all track together in the same direction and in approximately the same magnitude, so it is possible that one of those signals more closely represents a more "stable" chemical-shift reference than does the methyl resonance of methanol. The anomeric sucrose and CH_2Cl_2 signals also track the behavior of the methanol–methyl signal somewhat closely, but they do move in opposite ways from each other, with the CH_2Cl_2 diverging somewhat more rapidly.

The most similar-behaving three resonances (the CH_3CN, DSS-singlet, and HMDS-singlet signals) arise from nuclei that are farther from functional groups that could perturb their chemical shifts than is the methyl resonance of methanol. Future work could look into whether LC–NMR* data acquired with methanol:water might benefit from having its chemical shifts corrected to the CH_3CN resonance (as an internal standard). Practical considerations completely preclude the regular use of either HMDS or DSS (because both are retained by reversed-phase chromatography

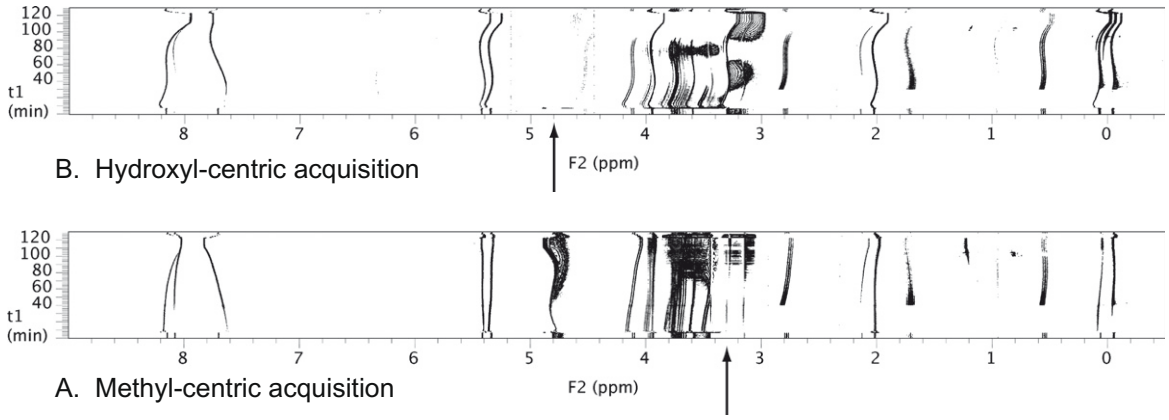

Fig. 7. Relative chemical-shift movements. Figure A (bottom) shows the data from Fig. 6 compressed along "t1" so as to better emphasize the relative chemical-shift movements of the different resonances. Figure B (top) was acquired identically except that during acquisition the transmitter was now actively maintained on the combined hydroxyl resonance (instead of on the methanol–methyl resonance as was used in A). The location of the transmitter in each spectrum is indicated by the vertical arrows. Both spectra were acquired with two-frequency solvent suppression and were acquired without using a ^{2}H lock.

columns, and because both are known to interact with many solutes through weak binding).

In this acidified methanol:water mobile phase, when the transmitter is kept on the methanol–methyl signal, the singlet from CH_3CN moves 0.05 ppm during the 0–100% solvent gradient (Fig. 7A). In contrast, when the transmitter is kept on the *hydroxyl* resonance, the singlet from CH_3CN moves ca. 0.16 ppm (Fig. 7B). This 0.16 ppm must be a combination of the 0.05 ppm movement of the CH_3CN singlet by itself, plus another 0.11 ppm movement of the water resonance during the solvent gradient (as shown in Fig. 4). The hydroxyl resonances in *unacidified* methanol:water (Fig. 3) move much more than they do in *acidified* methanol:water (Fig. 4), so if the transmitter was kept on the hydroxyl resonance in an unacidified mobile phase, we expect that the CH_3CN signal would move much more dramatically (although that experiment was not run).

2.7. The ^{2}H lock

We previously showed that the use of a ^{2}H lock when running steep solvent-gradient ramps in a CH_3CN:D_2O mobile phase can induce linebroadenings in the solute resonances [1]. This is caused by inappropriate chemical-shift tugging by the ^{2}H lock (which occurs within each increment, because the lock is tracking a D_2O/HOD resonance that can move and broaden faster as the solvent composition ramps faster). In contrast, we did not see this problem when using CH_3OH:D_2O – there was no discernable difference in linewidths between acquiring the data with or without a ^{2}H lock (when using the Scout-Scan to 'lock' the spectrum on the methanol methyl resonance). This is despite a careful comparison of the linewidths of multiple resonances in the two cases (especially the DSS and the HMDS resonances; data not shown). This is presumably because the hydroxyl resonance moves less during a 0-to-100% gradient of acidified methanol:water than in a corresponding gradient of acetonitrile:water [1]. However, it still remains a bad idea to use a ^{2}H lock on the D_2O signal if you do not use the Scout-Scan to 'lock' the spectrum on the methanol methyl resonance, for all the same reasons that were shown before [1].

We expect that the ^{2}H lock would be much more damaging to stable chemical shifts when used with an *unacidified* methanol:water mobile phase (as compared to *acidified* methanol:water). Fig. 3 shows that the hydroxyl ^{1}H resonances change their chemical shifts much more dramatically when there is no acid in the mobile phase, and the ^{2}H resonances will behave in the same way. Additionally, the unacidified mobile phase also has two deuterated hydroxyl resonances, which would add confusion for a ^{2}H lock. The use of unacidified methanol:water was not further evaluated here, however, because it is impractical to use as a mobile phase for LC–NMR* due to the multiple broad hydroxyl resonances.

2.8. The solvent-gradient-ramp rate

The steeper the solvent-gradient ramp becomes in either time or volume, the more inhomogeneous becomes the solvent mixture in the NMR flow cell, which can in turn cause broadening of the NMR resonances. The solvent inhomogeneity occurs either from the use of ramp rates that are too steep for the NMR flow cell's volume or from incomplete mixing of the contents of the flow cell. (Smaller NMR flow cells tolerate steeper solvent ramps, but they also decrease NMR sensitivity.) Fig. 8 demonstrates this effect by showing the variation in linewidth of a narrow resonance (the CH_3CN in the cocktail) in datasets acquired with 0-to-100% CH_3OH ramps run in 100 min (1%/min), 50 min (2%/min), 25 min (4%/min), and 12.5 min (8%/min), all acquired at a constant flow rate of 1 mL/min. The left-most spectrum has a relatively narrow CH_3CN linewidth throughout the 0-to-100% ramp section, but shows that the resonance gets broader as the solvent-gradient ramp becomes ever steeper (the three right-most spectra). This linebroadening is caused solely by the steepness of the solvent gradient, and cannot be removed by shimming. Equivalent ramp rates in an acetonitrile:water mobile phase produced roughly twice as much linebroadening [1].

Fig. 9 shows that the magnitude of this linebroadening is determined by the steepness of the ramp in both time and volume. The right-most spectrum is a control spectrum of the CH_3CN lineshape during a 1%/mL ramp over 100 min, at 1.0 mL/min (1%/min). The left-most spectrum was acquired with a "steep and fast" gradient ramp of 0–100% at 8%/mL over a period of 12.5 min at a flow rate of 1.0 mL/min (8%/min). The distortion of the lineshape during the 0–100% ramp is clearly visible. The middle spectrum was acquired with a 0–100% ramp of 8%/mL, but by pumping at a rate that was 20-fold slower and over a time period that was 20-fold longer (the flow rate was 0.05 mL/min over 250 min, giving a ramp rate of 0.4%/min). This "steep and slow" spectrum shows that if the flow rate is slow enough, it can compensate for a solvent-gradient ramp that is steep in volume. This effect was also seen with acetonitrile:water solvent gradients [1].

Figs. 8 and 9 together show that NMR lineshapes can degrade significantly if overly steep solvent gradients are used in LC–NMR*. These data show that an upper limit of 2%/mL and 2%/min is

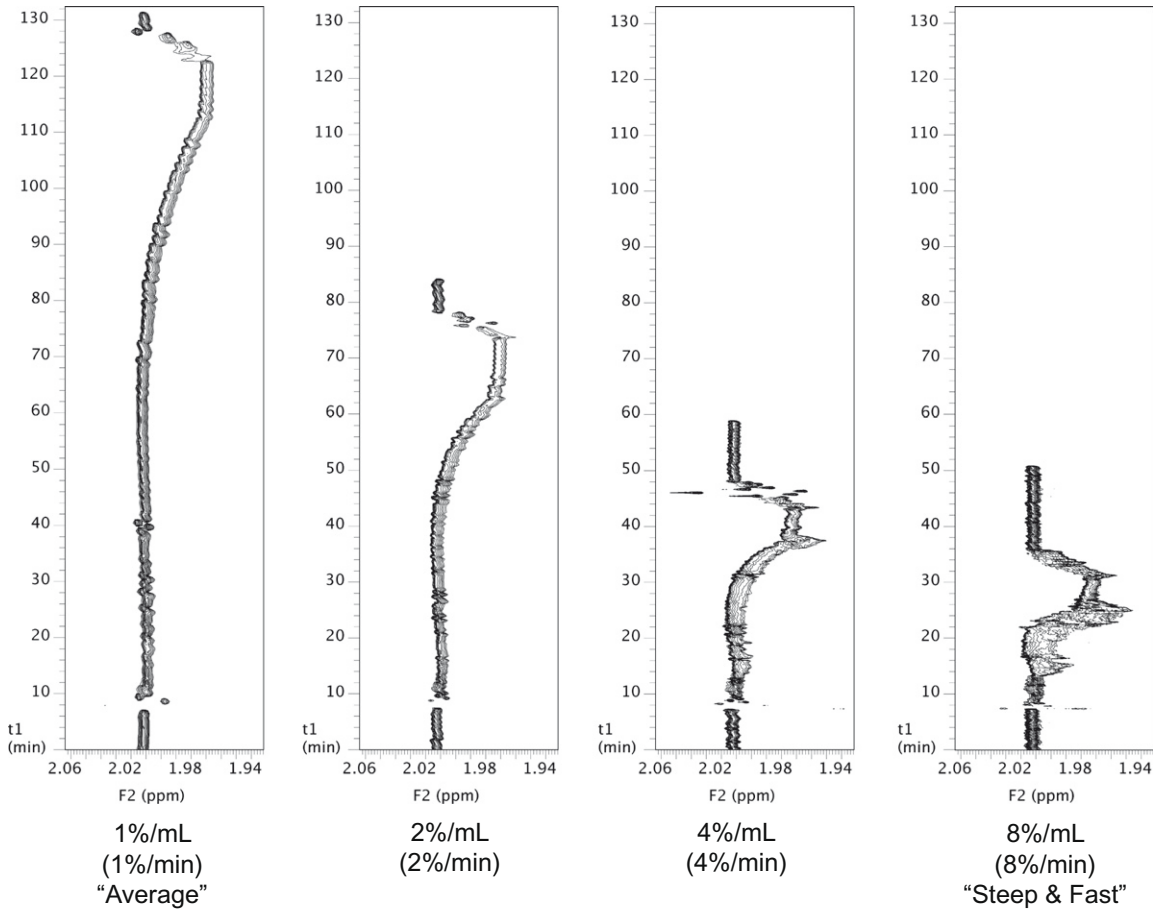

Fig. 8. Different ramp rates. A comparison of the CH_3CN linewidth acquired with 0-to-100% methanol ramps over 100 min (1%/min), 50 min (2%/min), 25 min (4%/min), and 12.5 min (8%/min) (left-to-right). All spectra were acquired at a flow rate of 1 mL/min, and used a ^2H lock. All datasets were plotted with the same horizontal expansion; the different experimental conditions necessitated different vertical scales. Baseline correction was used on all four datasets for spectral clarity. All spectra were acquired identically except for the ramp rate and the number of transients per increment (which was 16, 8, 4, and 2; left-to-right).

reasonable for a 4.6-mm LC column in a 60 μL NMR flow cell when using acidified methanol:water. This is twice the 1% limit that was determined for acetonitrile:water [1]. The upper limit is influenced by the flow rate and the magnetic susceptibilities of the solvents being mixed (both as shown), and by the sizes of the LC column and of the NMR flowcell (data not shown). A mobile-phase-compensation method has been proposed as a way to work around this problem [12].

2.9. Diffusion

The middle spectrum of Fig. 9 was acquired with a ramp that was steep in volume, but run so slowly that the lineshape was acceptably narrow. The lineshape could be narrowed due either to more time for active diffusion within the NMR flow cell (which would actively render the magnetic susceptibility homogeneous over time) or to the lack of "jetting" of fresh solvent into the flow cell (which would avoid the creation of inhomogeneity in the first place). ("Jetting" describes the case where the incoming solvent squirts into the center of the flow cell, rather than enter the flow cell in a steady laminar fashion that could evenly sweep the contents of the flow cell out of the exit tubing. It can potentially occur whenever the flow rate is high and the diameter of the input tubing is small.) To see if active diffusion can contribute, we repeated the "8%/min 8%/mL" experiment, but manually stopped the HPLC pump 22 min into the run, while the NMR spectrometer continued to acquire data. Figs. 10 and 11 show the response of the CH_3CN lineshape during this experiment. Fig. 11 shows that the lineshape recovers from a 23-Hz-wide lump just before the pump was stopped (at 22 min into the run; seen in the bottom trace of Fig. 11), to a 5-Hz-wide lump within 60 s of stopping the pump, to a 3-Hz-wide singlet within 90 s, to a 2.7-Hz-wide singlet within 150 s, and to a 2.2 Hz singlet within 300 s (5 min). The CH_3CN linewidth was 1.54 Hz (±0.14 Hz) at the start of the experiment (in 50:50 $CH_3OH:D_2O$, where it was shimmed). This shows that active diffusion is taking place. (The author's experience is that the elution of concentrated solute peaks into the NMR flow cell often does not show a similar diffusion behavior.) This data does not rule out, however, that jetting could also be involved.

2.10. Shimming

In LC–NMR*, the best lineshapes are always obtained when the data are acquired on the same solvent composition (both in components and ratio) as was used to shim the probe. As the solvent composition used for data acquisition deviates further from that used for shimming, the lineshape will increasingly degrade. The process is completely reversible, which means that the lineshape will recover when the solvent composition is returned to that used for shimming. (In contrast to tube-based probes that must be reshimmed on every sample tube, it is the author's experience that once a flow probe is shimmed, it remains well shimmed for days – unless the solvent composition is changed. This is due to the fixed geometry of both the flow cell and the solvent, and to the lack of a

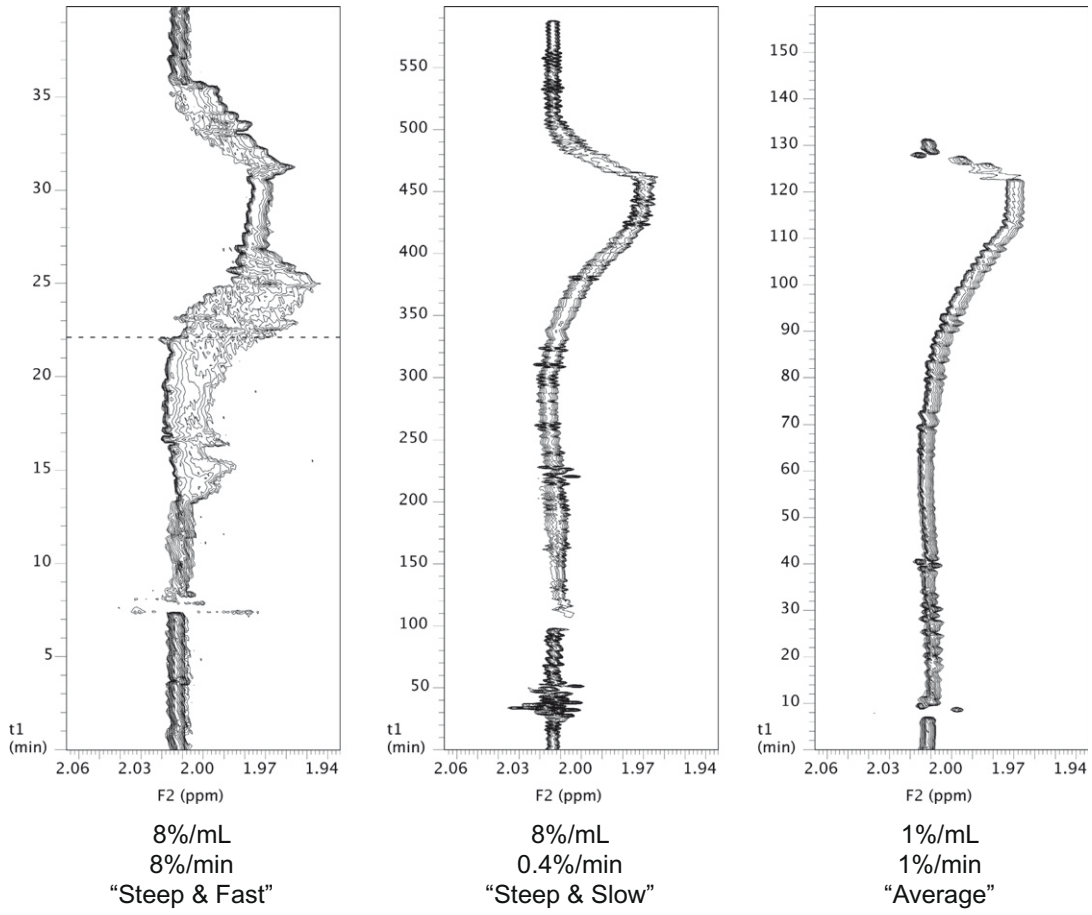

Fig. 9. Different ramp speeds. A comparison of the CH_3CN linewidth acquired with 0-to-100% methanol ramps of three different speeds. The three datasets can be labeled "steep and fast", "steep and slow", and "average" (left-to-right). The 0–100% ramps that were used (left-to-right) occurred over 12.5 min (at 1.0 mL/min, so 8%/mL and 8%/min; "steep and fast"), 250 min (at 0.05 mL/min, so 8%/mL but 0.4%/min; "steep and slow"), and 100 min (at 1.0 mL/min, so 1%/mL and 1%/min; "average"). The middle "steep and slow" spectrum was comparable to the left-hand "steep and fast" spectrum except it was run 20 times slower. The right-hand spectrum is displayed as a control spectrum that shows acceptably good lineshape. The total time of the middle experiment was almost 600 min, including the spectra acquired before and after the 0-to-100% ramp. All datasets were plotted with the same horizontal expansion, but the different experimental conditions necessitated different time-axis expansions and different vertical scales. All spectra were acquired in the same way other than the ramp rate, the flow rate, and the number of transients per increment (which were 2, 40, and 16; left-to-right, respectively). The spectrum on the left has an increased vertical scale as compared to the other two spectra, so as to see the broad signals. (The dotted line on the spectrum on the left is for comparison to Fig. 10.) The spectrum on the right stopped data collection at 131 min; the spectrum on the left continued data acquisition until 45 min (with data after 40 min not shown for clarity). All data in this figure were baseline corrected in F2 for clarity.

meniscus.) This means that, for on-flow solvent-gradient experiments, a user should shim on a solvent composition that is at the mid-point of the solvent-gradient ramp.

This is nicely illustrated by observing that the 2.2-Hz ending linewidth of the CH_3CN singlet shown in Figs. 10 and 11 was not as narrow as the 1.54 Hz measured at the start of the experiment. The data were acquired on a probe that was shimmed on equilibrated 50:50 CH_3OH:D_2O (as were all solvent-gradient runs in this study). The solvent composition at the end of Fig. 10 was no longer 50:50, but when the solvent composition was returned to 50:50 CH_3OH:D_2O (for the start of the next run), the linewidth did recover back to 1.54 Hz.

Fig. 12 shows how the user's choice of initial shimming conditions can affect lineshapes during a run. The bottom spectrum (12A) shows the lineshape of the CH_3CN resonance when the solvent composition was 50:50 CH_3OH:D_2O (acidified) and the probe was shimmed on that solvent composition. The top spectrum (12B) was acquired on the exact same sample, but with the shim values that were obtained when the probe was shimmed on 95:5 CH_3OH:D_2O (acidified). The several hertz of additional linebroadening present in the top spectrum is representative of what will occur in the middle of an acquisition that uses a large solvent-gradient method (i.e., from 5% to 95%) if you shim on the final ending (equilibrium) solvent conditions.

Fig. 13 shows how changes in the solvent composition can degrade the lineshape during a solvent-gradient acquisition, even when the experiment is shimmed "properly". These data were acquired by shimming the probe on 50:50 CH_3OH:D_2O, and then pumping three different solvent compositions into the probe (one-at-a-time). Shown is the linewidth of the CH_3CN resonance when acquired in 5:95 CH_3OH:D_2O (Fig. 13A; bottom), 50:50 CH_3OH:D_2O (Fig. 13B; middle), and 95:5 CH_3OH:D_2O (Fig. 13C; top). The CH_3CN linewidth in the middle spectrum is 1.6 Hz, but it widens at least twofold when the solvent ratios are at their extremes. This shows that, even if a user shims on a solvent composition that is in the middle of the solvent-gradient extremes, several hertz of additional linebroadening will typically occur at the extremes of a 0–100% solvent gradient. (It is also the author's experience that the amount of linebroadening becomes dramatically worse if a user accidentally shims at one solvent extreme and then acquires data at the other solvent extreme; data not shown).

When doing LC–NMR* in a methanol:water mobile phase, different shimming techniques can yield very different results. Shimming

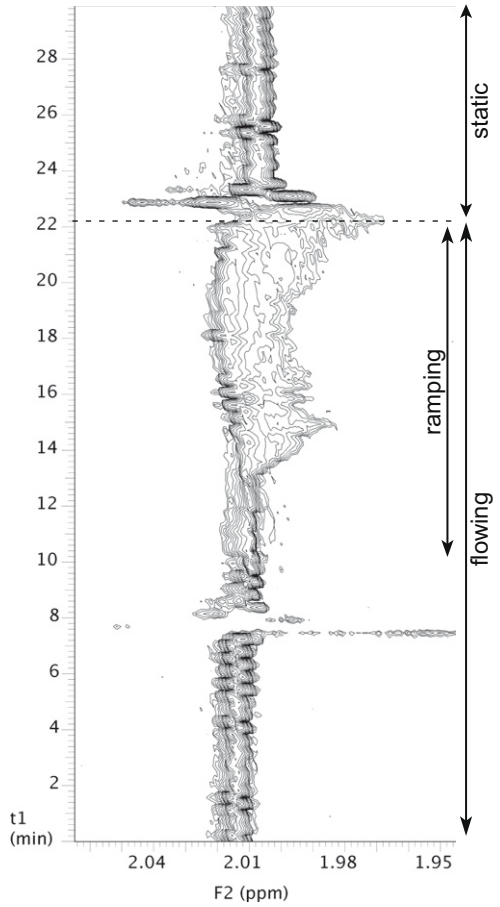

Fig. 10. The effects of static diffusion: contour plot. These data show the linewidth of the CH_3CN resonance during an "8%/min 8%/mL" experiment where the pump was stopped 22 min into the run (at the dotted line), but NMR acquisition continued so as to monitor the effects of diffusion. The spectra at the beginning of the run (from 0 to ca. 7 min along the time axis) were acquired on flowing and equilibrated 50:50 CH_3OH:D_2O, whereas the data acquired after 22 min were acquired on a solvent mixture that was no longer flowing, and was not equilibrated anywhere in the LC–NMR system other than by active diffusion happening within the NMR flow cell. The effect of stopping the pump can be seen by comparing the first 22 min of this spectrum to the first 22 min of the left-most spectrum in Fig. 9 (to the dotted line), which was run with the same conditions except for the stopping of the pump. (This spectrum was acquired with 2 transients per increment.)

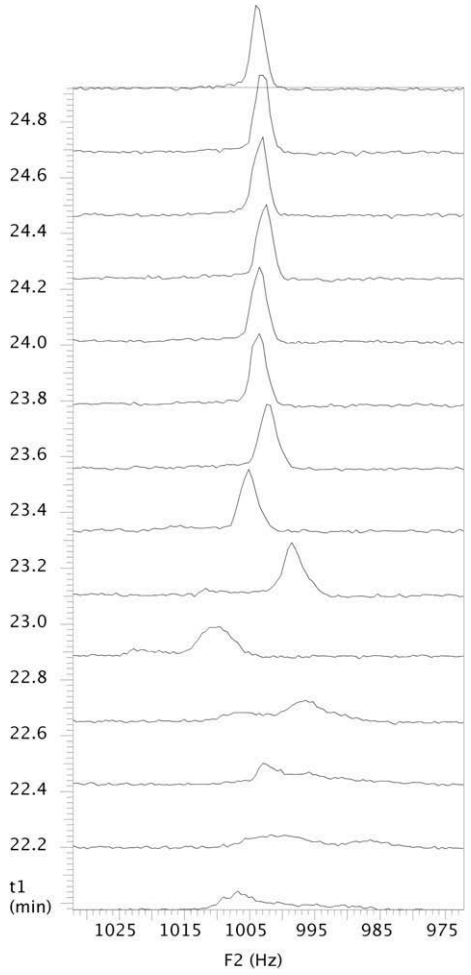

Fig. 11. The effects of static diffusion: stacked plot. An expansion of the data shown in Fig. 10, now displayed as a stacked plot to more easily monitor the linewidths of the CH_3CN resonance. The HPLC pump was manually stopped during the first displayed spectrum. All the remaining increments were acquired while the mobile phase was static. (In contrast to Fig. 10, the F2 axis here is shown in Hz to facilitate linewidth measurements.)

on the ^2H signal – using either the lock signal or a ^2H pulsed-field-gradient (PFG) map – will shim on the hydroxyl resonance, which is notoriously sensitive to broadening from both temperature gradients and solvent-composition gradients. If such broadenings exist, then any form of shimming on the ^2H signal will (undesirably) transfer these broadenings to every other resonance in the ^1H spectrum. This converts a ^1H spectrum with narrow organic resonances and a broad water resonance into an (undesirable) spectrum with broad organic resonances and a narrow water resonance. This happens because temperature gradients are often linearly dependent upon the Z axis, as is the z1 shim, so the temperature-gradient-induced linebroadening can be compensated for by missetting z1.

In contrast, if the ^1H signal is used for PFG shimming (which is dominated by the methyl protons in the CH_3OH:D_2O used here), the narrowest possible ^1H linewidths will be obtained for all of the organic resonances (but this will leave the hydroxyl signal broad, which is fully appropriate). This is also true in ^1H spectral shimming, depending upon which ^1H resonance is monitored. It is true to a lesser extent in ^1H free-induction-decay (FID) shimming, depending upon how much of the FID is made up of an HOD/H_2O signal.

2.11. Acquisition parameters

When using *acetonitrile*:water solvent gradients, we found that the balance between the number of transients and the number of increments can have a noticeable effect on the quality of the NMR data in on-flow solvent-gradient LC–NMR∗ datasets [1]. In contrast, this study did not find this behavior in (acidified) *methanol*:water solvent gradients (data not shown). This is undoubtedly due to the reduced linebroadenings seen with acidified methanol:water solvent gradients (vs. acetonitrile:water).

3. Conclusions

These data show that the maximum solvent-gradient ramp rate for methanol:water is twice as big as that found for acetonitrile:water. They also show that using the water resonance to reference or shim (or sometimes lock) the NMR spectra in LC–NMR∗ experiments is unsatisfactory, especially for on-flow solvent-gradient experiments, despite the fact that this is sometimes still done. This is true regardless of whether the water resonance is used as a

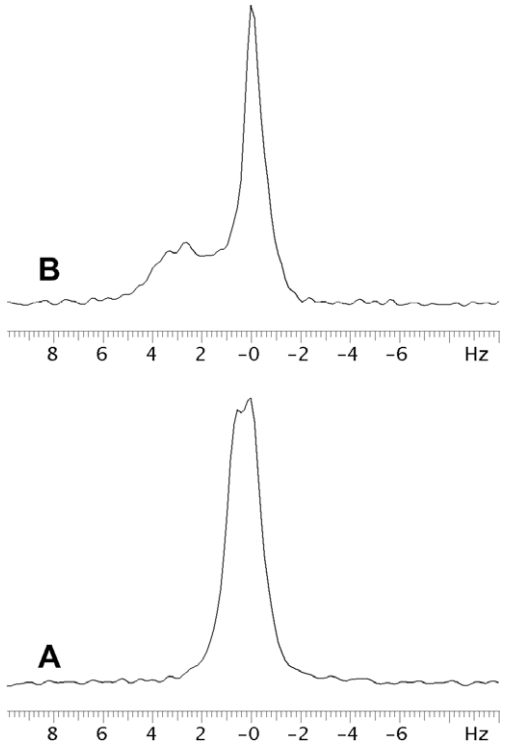

Fig. 12. An illustration of how the initial shimming conditions can affect the lineshape. Both spectra were acquired when the solvent composition was 50:50 $CH_3OH:D_2O$ (acidified) and show the linewidth of the CH_3CN resonance. The bottom spectrum (A) was obtained by using the shim values that were obtained on the same solvent mixture (50:50 $CH_3OH:D_2O$). The top spectrum (B) was obtained by using the shim values that were obtained on 95:5 $CH_3OH:D_2O$. (The linewidth of the bottom spectrum [1.5 Hz] was wider than normal due to contamination built up in the flow cell caused by prior heavy usage of the flow cell for DI-NMR.).

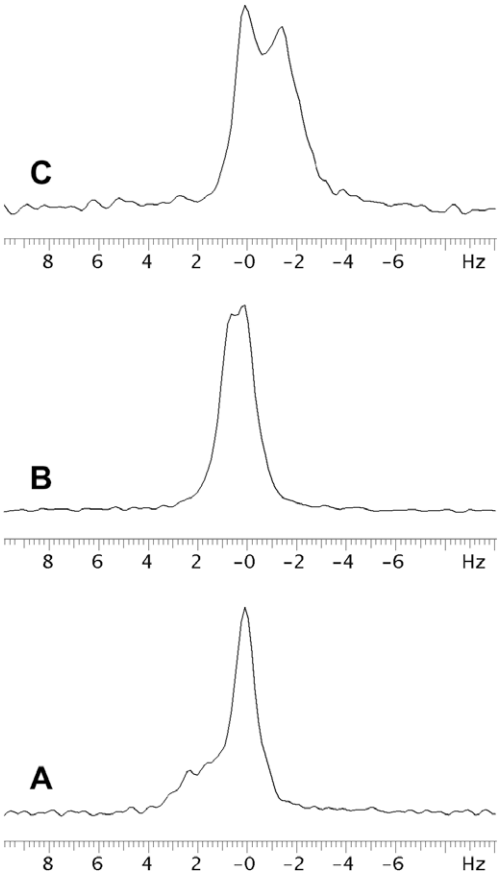

Fig. 13. An illustration of how the solvent composition influences lineshape during a solvent-gradient acquisition. All three spectra were acquired with the same shim values – which were obtained by shimming on 50:50 $CH_3OH:D_2O$ – and show the linewidth of the CH_3CN resonance. The bottom spectrum (A) was acquired on 5:95 $CH_3OH:D_2O$; the middle reference spectrum (B) was acquired on 50:50 $CH_3OH:D_2O$, and the top spectrum (C) was acquired on 95:5 $CH_3OH:D_2O$. (The linewidth of the middle spectrum – 1.5 Hz – was wider than normal due to contamination built up in the flow cell caused by prior heavy usage of the flow cell for DI-NMR.) (Although all three spectra were acquired sequentially, for this figure the peak amplitudes were normalized and the peaks were re-referenced to zero.)

^2H lock (for D_2O) or as an internal standard (for H_2O or HOD). Using the ^1H methyl resonance of CH_3OH as a secondary reference and as a signal for shimming (and effectively as a lock signal) is shown here to have many advantages and be a better choice. When comparing CH_3OH to other available compounds as secondary chemical-shift reference signals, it appears to be a reasonable choice.

These conclusions apply to any experiments that use LC–NMR or related techniques (such as LC–NMR–MS, LC–PDA–NMR–MS, LC–MS–NMR–CD, CapLC–NMR, LC–SPE–NMR. [13]; hence the use here of the term LC–NMR*). They certainly apply to LC–NMR* experiments that use solvent gradients and are acquired "on-flow". Some of these conclusions also apply to *isocratic* on-flow experiments, and some apply to solvent-gradient *stopped-flow* experiments. Some of these conclusions also apply to other flow-NMR methods such as flow-injection-analysis NMR (FIA-NMR) [14] and direct-injection NMR (DI-NMR) techniques [11] such as VAST or BEST. Some also apply to conventional (i.e., 5-mm) tube-based experiments where the samples either have temperature gradients or use solvent mixtures that may not be sufficiently well mixed.

4. Experimental

All NMR spectra were acquired on a Varian INOVA 500-MHz NMR spectrometer running VNMR software. It was equipped with an H{C,N} IFC flow probe with an active volume of 60 μL (115 μL total volume) maintained at 20 °C. All NMR data were acquired with the following conditions unless otherwise indicated: 2.048 s acquisition time (at), 0.001 s recovery delay (d1), 8000 Hz spectral width, 32,768 complex points (np), 2 steady-state scans, 16 scans (nt), no digital signal processing, no ^2H lock, two-frequency solvent suppression with the transmitter on the methyl resonance of CH_3OH, and 21.5-ms seduce pulses for WET. Unless otherwise indicated, all NMR data were processed without zerofilling, weighting functions, solvent-subtraction notch filters (ssfilter), or baseline correction, so as to best evaluate the raw data.

The chromatography was performed with a Varian 9012 pump, a 9050 UV detector, an LC–NMR Analyte Collector, and a Varian ResElut 5-μm C18 HPLC column (150 × 4.6 mm, #105420). The default HPLC method was [(time) action]: (0.0) 50:50 $CH_3OH:D_2O$; (5.00) 50:50 $CH_3OH:D_2O$; (5.01) 0:100 $CH_3OH:D_2O$; (10.0) 0:100 $CH_3OH:D_2O$; (110.0) 100:0 $CH_3OH:D_2O$; (120.0) 100:0 $CH_3OH:D_2O$; (125.0) 50:50 $CH_3OH:D_2O$; (130.0) End, as is graphically shown in Fig. 1. Experiments were then run using different flow rates (the default was 1.0 mL/min) and different ramp durations/rates (the default was 1%/min = 1%/mL). (Users should be cautioned that the use of a 100% aqueous mobile phase even temporarily, as is shown here, can damage some C-18 columns.)

The solvents used were methanol (CH_3OH; EM Science Omnisolv Glass Distilled; #MX0480–1) and D_2O (99.9 atom%D, Isotec #151882). No significant measures were used to keep the (hygroscopic) D_2O free of absorbed water during use, so it contained a measurable amount of HOD. Both solvents were spiked with a cocktail of compounds that were dissolved in 50:50 $CH_3OH:D_2O$, so that they

each contained (listed in chemical-shift order; amounts added and final concentrations listed in brackets): DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate hydrate) [36.6 mg/L, 0.155 mM]; HMDS (hexamethyldisiloxane) [104.2 μL/L, 0.490 mM]; CH_3CN [25 μL/L, 0.479 mM]; sucrose [101.9 mg/L, 0.298 mM]; CH_2Cl_2 [37.5 μL/L, 0.63 mM]; $CHCl_3$ [75 μL/L, 0.91 mM]; sodium formate [41.8 mg/L, 0.615 mM]; and TFA (trifluoro-acetic acid) [1 mL/L, 13.4 mM]. As the spiking cocktail was dissolved in 50:50 CH_3OH:D_2O, each solvent also contained CH_3OH [5 mL/L, ~123 mM] and D_2O [5 mL/L, ~275 mM].

PEEK tubing was used in the LC–NMR system. Readers should be cautioned that PEEK tubing can absorb methanol that can later be leached back out of the tubing. This effect can cause methanol contamination for long periods of time after the solvent system is switched to another solvent system (or deuterated methanol).

Acknowledgments

I would like to thank Steve Smallcombe, Karen Salomon, Igor Goljer, Wolf Hiller, and Varian Associates for their help in making these studies possible.

References

[1] P.A. Keifer, Chemical-shift referencing and resolution stability in gradient LC–NMR (acetonitrile:water), J. Magn. Reson. 199 (2009) 75–87.
[2] E. Rojas, M.O. Guerrero-Perez, M.A. Banares, Direct ammoxidation of ethane: an approach to tackle the worldwide shortage of acetonitrile, Catal. Commun. 10 (2009) 1555–1557.
[3] M.A. Wendt, J. Meiler, F. Weinhold, T.C. Farrar, Solvent and concentration dependence of the hydroxyl chemical shift of methanol, Mol. Phys. 93 (1998) 145–151.
[4] E.D. Becker, NMR studies of hydrogen bonding in alcohols and phenol, J. Chem. Phys. 31 (1959) 269–270.
[5] E.D. Becker, U. Liddel, J.N. Shoolery, Nuclear magnetic resonance studies of hydrogen bonding in ethanol, J. Mol. Spectrosc. 2 (1958) 1–8.
[6] R.E. Hoffman, Standardization of chemical shifts of TMS and solvent signals in NMR solvents, Magn. Reson. Chem. 44 (2006) 606–616.
[7] C. Dalvit, G. Shapiro, J.M. Bohlen, T. Parella, Technical aspects of an efficient multiple solvent suppression pulse sequence, Magn. Reson. Chem. 37 (1999) 7–14.
[8] D.A. Laude Jr., C.L. Wilkins, Reverse-phase high-performance liquid chromatography/nuclear magnetic resonance spectrometry in protonated solvents, Anal. Chem. 59 (1987) 546–551.
[9] S.H. Smallcombe, S.L. Patt, P.A. Keifer, WET solvent suppression and its applications to LC NMR and high-resolution NMR spectroscopy, J. Magn. Reson. A 117 (1995) 295–303.
[10] S.L. Patt, Single- and multiple-frequency-shifted laminar pulses, J. Magn. Reson. 96 (1992) 94–102.
[11] P.A. Keifer, S.H. Smallcombe, E.H. Williams, K.E. Salomon, G. Mendez, J.L. Belletire, C.D. Moore, Direct-injection NMR (DI-NMR): a flow NMR technique for the analysis of combinatorial chemistry libraries, J. Comb. Chem. 2 (2000) 151–171.
[12] D.A. Jayawickrama, A.M. Wolters, J.V. Sweedler, Mobile phase compensation to improve NMR spectral properties during solvent gradients, Analyst 128 (2003) 421–426.
[13] P.A. Keifer, Flow techniques in NMR spectroscopy, Annu. Rep. NMR Spectrosc. 62 (2007) 1–47.
[14] P.A. Keifer, Flow injection analysis NMR (FIA-NMR) – a novel flow NMR technique that complements LC–NMR and direct injection NMR (DI-NMR), Magn. Reson. Chem. 41 (2003) 509–516.