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## Practical Aspects

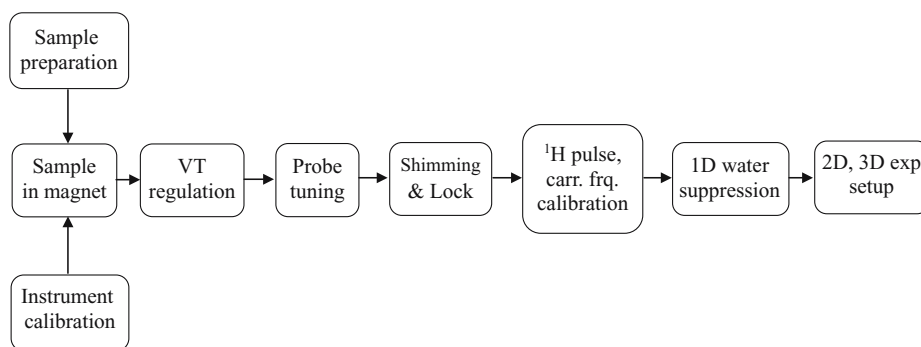
The quality of NMR spectra is critically dependent on numerous factors, among which are the condition of the instrument at the time the data are collected, the homogeneity of the magnetic field, the type of experiments chosen, how the data are acquired and subsequently processed, etc. In this chapter, practical aspects related to the experiments will be discussed in detail.

Key questions to be addressed in the current chapter include:

1. How is a probe tuned correctly?
2. What is a simple and correct way to do shimming?
3. How are transmitter (or observe) hard pulses calibrated?
4. How are decoupler (non-observe) hard pulses calibrated?
5. How are the pulse lengths of decoupling calibrated?
6. What is the procedure to calibrate selective pulses?
7. How are the offset frequencies of the transmitter and decoupler calibrated and how frequently are they calibrated?
8. How can off-resonance pulses ( $^{13}\text{C}'$ ,  $^{13}\text{C}^\alpha$ ,  $^{13}\text{C}^{\alpha,\beta}$ ) be calibrated?
9. How is the temperature calibration of a probe obtained?
10. What is the decoupling efficiency of different decoupling methods?
11. How is pulse field gradient used to remove unwanted magnetization?
12. What are coherence and coherence transfer?
13. What is the coherence transfer pathway?
14. How are they selected by phase cycling or gradient methods?
15. What are the common water suppression techniques?
16. What are the advantages and disadvantages of the techniques?
17. How are the water suppression experiments set up?
18. How is a two-dimensional (or three-, four-dimensional) experiment formed and what is the intrinsic difference between one-dimensional and multidimensional experiments?
19. What are the typical procedures used to set up an experiment?
20. What is the typical procedure for processing data?

### 4.1. TUNING THE PROBE

The general procedure for setting up routine NMR experiments is described in the flow-chart of Figure 4.1. The tuning probe is the initial step before NMR data can be acquired. As discussed in Chapter 1, in order to move the equilibrium magnetization away from the



**Figure 4.1.** Flowchart of a typical procedure for NMR experiment setup. The instrument must be properly calibrated before the experiment can be performed (see text for details). Each of the steps is discussed in the current chapter except sample preparation (previous chapter) and setup of 3D experiments (following chapter).

magnetic field direction, an oscillating magnetic field  $B_1$ , which is generated by an RF pulse during NMR experiments, is needed to interact with the nuclei at the Larmor frequency. In order to efficiently use the RF energy, the probe must be tuned to the carrier frequency (center frequency of the spectrum) for the sample to be measured and the impedance of the probe must be matched to the instrument impedance of  $50\ \Omega$  at resonance to optimize the sensitivity of detecting NMR signals (Chapter 2). Incorrect tuning or matching of the probe will cause an increased  $90^\circ$  pulse length and decreased sensitivity of detection.

Probe tuning and matching are carried out by adjusting the capacitors of the coil circuits in the probe. Probe tuning refers to the adjustment of both the probe resonance frequency to the carrier frequency, and the probe impedance to match  $50\ \Omega$ . A common triple-resonance solution-NMR probe consists of two coils, of which the inner is double-tuned to  $^1\text{H}$  and  $^2\text{H}$  and the outer to  $^{13}\text{C}$  and  $^{15}\text{N}$  for an HCN probe or to  $^{13}\text{C}$  and other nuclei over a wide range for an HCX probe, in which X represents a heteronucleus in the broadband range. Usually, each channel has a pair of adjustable capacitors, one for tuning and the other for matching. Some of the probes have auto-matched  $^{15}\text{N}$  and  $^{13}\text{C}$  channels, in which only tuning capacitors need to be adjusted. There are no adjustable matching capacitors for heteronuclear channels in this kind of probe. A typical procedure for tuning the probe involves turning the rods (or sliding bars) of the two adjustable capacitors and detecting the change of probe resonance frequency and impedance after setting up the carrier frequency for each channel being tuned.

Tuning should always be performed first for the nuclei with the lowest Larmor frequency, then for those with higher resonance frequency because the higher frequency is more sensitive to small changes in capacitance of the probe circuits, which can be caused by tuning neighboring channels. For instance, the  $^{15}\text{N}$  channel is tuned first, then  $^{13}\text{C}$ , and the  $^1\text{H}$  channel is tuned last. In high field spectrometers, the dielectric field of sample solvents is sufficiently high to influence the capacitance of probe circuits, resulting in a shift of probe resonance frequency when changing samples. Therefore, the probe must be tuned every time a sample is placed in the magnet. Since the circuits are temperature dependent, the probe must also be tuned after it reaches the selected temperature when variable temperature (VT) is regulated.

There are two common methods used in probe tuning and matching: detecting the reflection power or detecting the resonance frequency of the probe during tuning and matching.

Both methods are standard capabilities of NMR spectrometers. The reflection power method observes the RF power reflected by the probe and is more frequently used in routine tuning. When the spectrometer is in the tuning mode, RF pulses at the carrier frequency of the nuclei being tuned are delivered to the probe channel. This tuning method aims to minimize the amount of power being reflected by the probe. At the resonance condition with the input RF pulses, the probe circuit holds most of the power it receives. Thus, when a probe is well-tuned, it reflects a minimum portion of the power being delivered to it and uses the RF power more efficiently. Consequently, a  $90^\circ$  pulse length is shorter for a given amount of RF power, compared to a mistuned probe.

The reflection power tuning method uses a directional coupler and a voltage meter to display the portion of the RF power reflected by the probe after an RF pulse with a fixed frequency is sent to the probe. The RF pulses are set to the carrier frequency of the nuclei being tuned. Usually, the repetition rate is short, which is about five 20  $\mu$ s pulses per second. The receiver does not need to be on during probe tuning (use a large number of scans for steady state or dummy scans). Practically, the capacitance of the probe circuits is adjusted by first turning the tuning capacitor (or sliding a bar) in a direction to reduce the amount of reflected RF power, until the reflection reaches a minimum. Keep turning the capacitor in the same direction, which makes the reflected power pass the minimum and increase by just a small amount. The next step is to adjust the matching capacitor in a direction so that the reflected power is back to a new minimum. If the new minimum is smaller than the previous one, keep turning the matching capacitor to pass the minimum by a small amount. If the new minimum is higher than the previous one, start with the adjustment of the tuning capacitor again but in the opposite direction so that a lower minimum can be found. Then, the above procedure is repeated by alternatively adjusting tuning and matching capacitors until the reflection power is best minimized. Usually, the  $^1\text{H}$  channel of the probe can be tuned so well that the reading of power reflected from the probe is zero. Frequently, the reflection power reading for the heteronuclear channel is a little higher but the  $90^\circ$  pulse lengths are still in the range of normal requirement ( $<40 \mu\text{s}$  for  $^{15}\text{N}$ ,  $<15 \mu\text{s}$  for  $^{13}\text{C}$ ).

Tuning using a spectral analyzer and an RF bridge to display the resonance frequency of the probe is another common method, referred as the wobbler method. The majority of NMR spectrometers have the capability to do wobbler tuning as a standard feature (for instance, “qtune” on Varian instruments and “wobb” on Bruker instruments). The basic procedure is the same as the reflection method except that the resonance frequency of the probe is monitored when adjusting the capacitors. The first step is to adjust the tuning capacitor so that the dip on the display moves from one side of the setup frequency to the other, approximately by 50 kHz. Adjusting the matching capacitor then makes the dip pass the setup frequency back to the original side. If the dip of the signal is lower, repeat the above process until it reaches a minimum. If the dip is higher than the initial value, change the direction. The depth of the dip indicates how well the impedance of the probe circuit matches to  $50 \Omega$ . The best tuning position is indicated by the lowest dip of the probe resonance frequency at the setup frequency.

The probe should be tuned every time the sample is changed, as mentioned earlier, especially for those with different solvents or salt concentrations because the dielectric property of the solvents affects the capacitance of probe circuits. However, the tuning of heteronuclear channels is not sensitive to different samples provided the sample does not contain a large amount of salts because the probe coil for heteronuclear channels is the outer coil. Thus, the tuning of  $^{15}\text{N}$  and  $^{13}\text{C}$  channels does not change much from sample to sample. It should also be noted that the correct way to tune the probe is without any filters attached because probe

tuning aims to tune the resonance frequency of the probe. Filters are not integrated parts of the probe circuits and have their own resonance frequencies, which may alter the power fed into them. Therefore, the measurement of the reflected power should be performed directly at the probe output, rather than at any later stage.

## 4.2. SHIMMING AND LOCKING

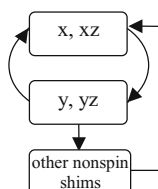
Because superior homogeneity of the magnetic field across the sample volume is necessary in order to obtain a sharp line shape in NMR spectra, the static magnetic field,  $B_0$ , must be perfected by adjusting the current to a series of coils inside the magnet (more details in Chapter 2). The process is called “shimming” and the coils called shimming coils. The number of the coils can be as many as 40 sets, which produce a magnetic field across the sample volume. Manually shimming the magnet is a rather complicated process and very time-consuming. It is almost impossible to manually shim higher order  $z$  shims ( $z$  shims only alter the magnetic field along the  $z$  direction and are also called spinning shims), such as  $z^4$ ,  $z^5$ , or  $z^6$  because they are dependent on each other, meaning that a change in one of them alters the others. Shimming nonspinning shims ( $xy$  shims that only change the field in the  $xy$  plane) is relatively easy because most of them are independent of the others. Luckily, gradient technology has been used in magnet shimming, called gradient shimming, and greatly reduces the amount of work in shimming. Gradient shimming of spinning shims requires a  $z$  gradient capability of the instrument whereas gradient shimming of nonspinning or  $xy$  shims requires  $x$  and  $y$  gradients. Usually, solution NMR instruments do not have  $x$ ,  $y$  pulsed field gradients, and, hence, gradient shimming normally applies only on  $z$  shims.

Under normal operating conditions, all NMR magnets have a constantly fluctuating field strength due to the environment. The magnetic field is stabilized by a mini spectrometer operated usually at the  $^2\text{H}$  frequency, which continuously observes the  $^2\text{H}$  signal frequency through the dedicated lock channel to maintain a stable magnetic field. If the observed  $^2\text{H}$  frequency deviates from the set value, the spectrometer changes the magnetic field strength in the opposite way through a coil inside the magnet to compensate for the fluctuation. This process is called “lock” and is carried out in real time. Four parameters are important to have correct operation of locking: a lock field (also known as  $z_0$  field), which is the  $^2\text{H}$  resonance frequency of the solvent (or the offset of the  $^2\text{H}$  lock frequency); lock power, which controls the  $^2\text{H}$  RF power used to generate the  $^2\text{H}$  signal; lock gain, which is used to amplify the signal received by the lock receiver; and lock phase, which sets the correct dispersion phase of the  $^2\text{H}$  signal used to monitor the deviation of the  $^2\text{H}$  lock frequency caused by the  $B_0$  magnetic field. They should all be optimized for lock. The common procedure is to adjust  $z_0$  first to be on resonance for the  $^2\text{H}$  solvent. After locking on the  $^2\text{H}$ , the lock power is adjusted to the highest possible level without saturating the lock signal. Saturation is the point at which the lock level is decreased when lock power increases. Then, the lock power is slightly reduced to surely avoid saturation (e.g., by 10%). The lock gain is adjusted to bring the lock level to 80%, which is the most sensitive level. Finally, the lock phase is optimized to maximize the lock level.

The lock level is monitored during manual shimming, which is observation of the  $^2\text{H}$  signal. The better the homogeneity of the field, the higher the lock level, provided the lock phase is correctly adjusted. The FID of water may also be used to monitor the quality of shimming. When using the lock level for shimming, the lock phase must be properly adjusted because a poorly phased lock signal distorts the maximum intensity of the signal, which is

used to monitor the homogeneity of the magnetic field. A distorted lock intensity (lock level) may not represent the field homogeneity linearly.

If the shimming is off significantly after changing samples, it is important to first adjust the  $z$  shims while spinning the sample. If  $z$  gradient auto shimming will be used, only  $z_1$ ,  $z_2$ , and  $z_3$  need to be shimmed manually. The usual procedure is to adjust  $z_1$  and  $z_2$  alternatively to obtain a high as possible lock level. Then,  $z_3$  needs to be optimized before  $z_1$  and  $z_2$  are revisited. The above procedure is repeated until the maximum lock level is reached. After initial adjustment on the  $z$  shims, the nonspinning shims must be shimmed without spinning the sample. The procedure usually is in the following order:



where  $x$  and  $xz$  are shimmed first, then  $y$  and  $yz$  to optimize the lock level. The steps are repeated until the lock level does not increase. The rest of the nonspinning shims are checked one by one. If any one of them change the lock level, shims  $x$ ,  $xz$ ,  $y$ , and  $yz$  need to be checked again.

Gradient shimming, which provides rapid automatic optimization of room-temperature shims, is one of the profound applications of pulsed field gradients (PFGs). It not only provides reliable shimming results on high-order  $z$  shims (and nonspinning shims) within a minute, which otherwise would take many hours if shimming manually, but also allows one to shim on nuclei besides  $^2\text{H}$ , such as  $^1\text{H}$ . It is critically useful for samples without  $^2\text{H}$  solvent. For instance, a methanol sample is commonly used for calibration of variable temperature control. Without  $^1\text{H}$  gradient shimming, it is impossible to shim on the sample because it does not contain  $^2\text{H}$  solvent. It is usually done by shimming a deuterated methanol sample with the same volume as the calibration sample. By using  $^1\text{H}$  gradient shimming, one can shim on the actual calibration sample (more in the temperature calibration section). Gradient shimming requires PFG capability (PFG amplifier and probe), which is a standard feature of NMR instruments for biological research, although it can be done by a homospoil gradient through the  $z_1$  room-temperature shim coil. It is a standard practice to always apply  $z$  gradient shimming on all NMR samples. It involves generating a gradient shim map first (an example is shown in Figure 4.2, see color insert after Chapter 8), shimming  $z_1$ – $z_4$  using the map and then  $z_1$ – $z_6$ . The shim map can be repeatedly used for different samples with the same probe provided the sample volume is approximately the same as the sample used to generate the map. Nonspinning shims must be optimized before the shim map is generated. By no means is what has been described above the only way to perform shimming. There are different shimming methods available in different laboratories. However, in our hands, the above procedure has not failed.

“Deuterium lock frequency” and “lock field” are two terms that are sometimes easily confused. The lock frequency is the instrument’s  $^2\text{H}$  frequency at the magnetic field, whereas the lock field—as mentioned earlier—is the  $^2\text{H}$  resonance frequency of the solvent. The function of the  $z_0$  field is two-fold. Different solvents have different  $^2\text{H}$  resonance frequencies. The slightly different  $^2\text{H}$  frequencies are adjusted by changing the  $z_0$  field. The other situation that requires changing  $z_0$  is when the magnetic field drifts. On the other hand, the lock frequency is

unchanged as long as  $z_0$  is adjustable, that is,  $z_0$  is not out of range. Therefore, even if the magnetic field drifts (field strength decreases due to loss of current in the superconductor solenoid of the magnet), a constant field strength is maintained by changing the value of the  $z_0$  field. In other words, the offset frequencies (see below), the chemical shift reference, and other field dependent qualities are unchanged when the instrument lock frequency is unchanged even if the magnetic field strength drifts as the magnet ages, because the drifted field is compensated for through the adjustment of  $z_0$ .

### 4.3. INSTRUMENT CALIBRATIONS

#### 4.3.1. Calibration of Variable Temperature

For each probe, the actual temperature vs the setting value must be calibrated before data are collected for real samples. A typical method for temperature calibration over the range of  $-20$  to  $50^\circ\text{C}$  is to observe the chemical shift difference ( $\Delta\delta$  in ppm) between the two peaks of 100% methanol. The empirical expression in the relationship of the observed  $\Delta\delta$  with temperature is given by:

$$T(^{\circ}\text{C}) = 130.00 - 29.53\Delta\delta - 23.87\Delta\delta^2 \quad (4.1)$$

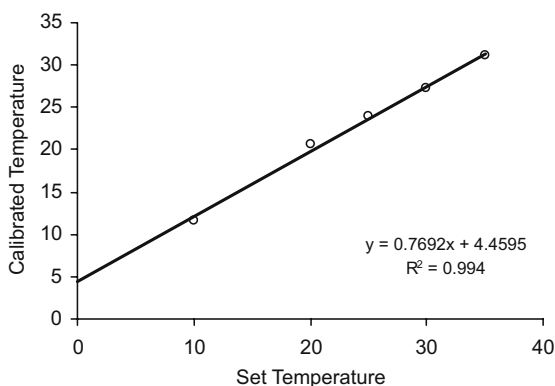
For the calibration over  $50^\circ\text{C}$ , 100% ethylene glycol is used, whose chemical shift difference of the two peaks has a temperature dependence given by:

$$T(^{\circ}\text{C}) = 193.0 - 101.6\Delta\delta \quad (4.2)$$

Spectrometer software may be used to calculate the temperature after  $\Delta\delta$  is measured. Since the accuracy of the measurement is greatly dependent on the line shape of the peaks, the sample should be well-shimmed before the calibration. It is impossible to shim using the  $^2\text{H}$  lock level by the conventional shimming method because the calibration samples do not contain any portion of deuterated solvents. Gradient shimming on  $^1\text{H}$  is the best way to do the  $z$  shimmings. A sample containing about 1 : 1 methanol- $d_4$  in methanol, which has the same volume as the calibration sample, is first used for shimming purposes. After well-shimming the methanol- $d_4$  sample, especially the nonspinning shims, the 100% methanol sample is placed in the probe and  $^1\text{H}$  auto-gradient shimming  $z_1$ – $z_6$  is applied. Now the sample is ready for temperature calibration. Change the temperature to the lowest point to be calibrated. It takes about 10 min for the temperature to reach equilibrium. Collect data with a single transient and calculate the temperature according to the above equation or by using the appropriate command in the instrument's software. Repeat the experiment and average the two calculated values of the calibrated temperature. Figure 4.3 shows a plot of the set temperature vs the calibrated ones. A good VT control unit always has a linear relationship for all probes although the slope for individual probes may be different.

#### 4.3.2. Calibration of Chemical Shift References

Tetramethylsilane (TMS) is the IUPAC standard compound used for  $^1\text{H}$  and  $^{13}\text{C}$  references for samples in organic solution (see Chapter 1). However, its insolubility in aqueous



**Figure 4.3.** Plot of set temperature vs calibrated temperature (°C) using a methanol sample.

solutions makes it a poor reference sample for protein samples. For aqueous samples, DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid) in 90% H<sub>2</sub>O/10% <sup>2</sup>H<sub>2</sub>O is a better standard sample for a <sup>1</sup>H chemical shift reference because of its properties of high water solubility, far-upfield resonance, and insensitivity to pH and temperature. The water resonance is sometimes also used as a secondary (or indirect) <sup>1</sup>H reference. Although it is a convenient standard, the dependences of temperature and pH as well as the relatively broad line shape of water make it a controversial reference. The chemical shift of water at neutral pH is obtained by its temperature dependence in the range of 10–45°C:

$$\delta_{\text{H}_2\text{O}}(\text{ppm}) = 4.76 - (T - 25)0.01 \quad (4.3)$$

in which temperature  $T$  is in °C. Before calibration is performed using any reference sample, the <sup>2</sup>H signal of <sup>2</sup>H<sub>2</sub>O is adjusted to be on-resonance. The chemical shift of DSS is set to zero frequency. For heteronuclei, such as <sup>13</sup>C and <sup>15</sup>N, the best method to obtain chemical shifts is to indirectly reference to DSS <sup>1</sup>H frequency via the frequency ratio  $\Xi$  at 25°C (Table 1.1) rather than using universal reference standards. There is a small temperature dependence  $\Delta\Xi$  in the range of 5–50°C, which can be added to  $\Xi$  according to the relationships:

$$\Delta\Xi^{\text{N}}(T) = (T - 25^\circ\text{C})2.74 \times 10^{-10} \quad (4.4)$$

$$\Delta\Xi^{\text{C}}(T) = (T - 25^\circ\text{C})1.04 \times 10^{-9} \quad (4.5)$$

As the equations indicate, the effect of temperature on the chemical shift for solution samples is extremely small. Therefore, it is generally ignored for most chemical and biological solution samples (Harris *et al.*, 2001). If one prefers to use individual standard samples for a heteronuclear chemical shift reference, the calibration can be done using DSS for <sup>13</sup>C and liquid ammonia for <sup>15</sup>N. The <sup>13</sup>C signal of DSS is directly observed and set to zero frequency. However, it is difficult to observe the <sup>15</sup>N signal of liquid ammonia because the sample is not in a liquid state at laboratory conditions of ambient temperature and pressure. Consequently, <sup>15</sup>N urea is commonly used as a secondary <sup>15</sup>N reference standard, whose chemical shift is referenced to liquid ammonia. One mole concentration of <sup>15</sup>N urea in DMSO gives a <sup>15</sup>N peak at 77.6 ppm relative to liquid ammonia. Although the urea sample is in DMSO, the calibration

**TABLE 4.1**  
Common Pulses Used for 500 and 600 MHz Instruments<sup>a</sup>

Pulses	On/off resonance	500 MHz		600 MHz	
		Pulse length <sup>b</sup>	Est. pwr att <sup>c</sup>	Pulse length <sup>b</sup>	Est. pwr att <sup>c</sup>
<sup>1</sup> H					
<sup>1</sup> H hard 90°	On	7		7	
TOCSY spin lock	On	28	12	28	12
WALTZ dec	On	40	15	40	15
<sup>13</sup> C					
All <sup>13</sup> C 90° hard	On	15		15	
<sup>13</sup> C GARP	On	70	14	70	14
DIPSI-3 spin lock	On	30	6	30	6
C <sup>α</sup> /β 90°, C' null	On	58.4	11–12	48.6	10
C <sup>α</sup> 90°, C' null	On	64.7	12–13	53.9	11
C' 90°, C <sup>α</sup> null	On	64.7	12–13	53.9	11
C' 90, C <sup>α</sup> null	Off	64.7	12–13	53.9	11
C <sup>α</sup> /β 180°, C' null	On	53.0	10–11	44.3	9
C <sup>α</sup> 180°, C' null	On	57.9	11–12	48.3	10
C' 180°, C <sup>α</sup> null <sup>d</sup>	Off	57.9 + 0.8	11–12	48.3 + 0.8	10
C <sup>α</sup> 90° SEDUCE <sup>e</sup>	On	310.5	20	310.5	20
C <sup>α</sup> 90° SEDUCE <sup>e</sup>	Off	310.5	20 ↑ 6	310.5	20 ↑ 6
C' 90° SEDUCE <sup>e</sup>	Off	310.5	20 ↑ 6	310.5	20 ↑ 6
C' 180° SEDUCE <sup>f</sup>	Off	200 + 4	16 ↑ 6	200 + 4	16 ↑ 6
<sup>15</sup> N					
All <sup>15</sup> N hard 90°	On	40		40	
<sup>15</sup> N GARP dec	On	250	15	250	15
<sup>15</sup> N Waltz16 dec	On	200	14	200	14

<sup>a</sup> The pulse lengths listed in the table are used as starting values. Actual pulse lengths and powers should be calibrated accurately for the instruments.

<sup>b</sup> The pulse lengths are calculated according to Equations (4.14) and (4.17) using C<sup>α</sup>/β = 45 ppm, C<sup>α</sup> = 58 ppm, and C' = 177 ppm, and <sup>13</sup>C frequencies of 125.68 and 150.86 MHz.

<sup>c</sup> The power attenuation for the 90° pulse calibration is the decreased power in dB from the power for the hard pulse (increase the power setting value for Bruker, or decrease the power setting value for Varian instruments). "↑ 6" means increase the power by 6dB (decrease the power value by 6 for Bruker instrument). It is estimated relative to a hard 90° of 15 μs, assuming linearity of the amplifier. The reduction of power by 1 dB increases the pulse length by a factor of 1.122.

<sup>d</sup> After the calibration, 0.8 μs is added to the off-resonance 180° pulse length.

<sup>e</sup> The SEDUCE 90° pulses must be divisible by 50 ns and 45°. For off-resonance SEDUCE decoupling pulses: after the calibration, the power is increased by 6 dB (decrease the power setting by 6 for Bruker instrument) because the off-resonance SEDUCE decoupling covers a double bandwidth.

<sup>f</sup> After the calibration, 4 μs is added to the pulse length of the off-resonance 180° SEDUCE and the power is increased by 6 dB (decrease the power setting by 6 for Bruker instrument).

must be done with the lock field on <sup>2</sup>H<sub>2</sub>O resonance. First, the lock field is adjusted to the <sup>2</sup>H<sub>2</sub>O resonance with a water sample in the probe because the real sample is in aqueous solution. Nonspinning shims should be checked before changing to the urea sample. After gradient shimming with z<sub>1</sub>–z<sub>6</sub> gradients, the <sup>15</sup>N spectrum is acquired without changing the lock field. It is crucial to make sure that the lock field is on resonance for <sup>2</sup>H<sub>2</sub>O in order to obtain accurate <sup>15</sup>N calibration.

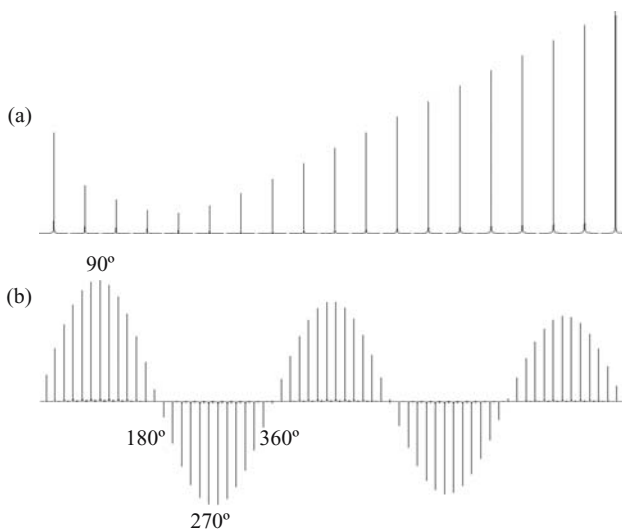


The chemical shift reference is calibrated only periodically unless the magnetic field drift is substantial (which requires changing the lock frequency to correct for loss of magnetic field). How frequently the calibration needs to be performed depends on the stability of the instrument and magnet. Usually it is repeated twice annually if the lock frequency has not been changed.

#### 4.3.3. Calibration of Transmitter Pulse Length

Once probe tuning and shimming have been completed, it is necessary to calibrate the pulse angle produced by a given amount of transmitter RF power, which is achieved by calibrating the  $90^\circ$  pulse length. The  $^1\text{H}$  observe  $90^\circ$  pulse length should be calibrated for every sample. There are many different methods to calibrate the observe pulse length. The easiest one is to calibrate a  $180^\circ$  pulse using a water or solvent signal. Because the maximum intensity of the observed signal by the  $90^\circ$  pulse is relatively insensitive to deviations of the pulse angle around  $90^\circ$ , the null intensity produced by a  $180^\circ$  pulse is commonly used to calibrate the  $90^\circ$  pulse length.

The calibration is performed using a one-pulse sequence with a relaxation delay longer than  $5T_1$  and a single transient. The carrier frequency is set about 100 Hz from the water resonance to avoid interference of the carrier with the water peak. The pulse length is arrayed by  $2\text{ }\mu\text{s}$  for 15 or more FIDs to values sufficiently long to include the estimated  $180^\circ$  pulse length. After the first FID is transformed and phased, all FIDs are then transformed using the same phase correction. The next step is to fine array the range of pulse lengths that give signal intensity near the null value. Because of the inhomogeneity of the  $B_1$  RF field generated at the probe coil, the signal is not completely nulled by a  $180^\circ$  pulse. Instead, a dispersive signal with similar amplitude of positive and negative peaks symmetric about the null position will be observed at the  $180^\circ$  condition (Figure 4.4b). Sometimes, a broad hump at the water resonance



**Figure 4.4.** Calibration of transmitter pulse length and offset frequency. (a) The offset frequency is arrayed with an increment of 1 Hz using the PRESAT sequence. The minimum is obtained when the offset is on water resonance. (b) The pulse length is arrayed and the first minimum corresponds to a  $180^\circ$  pulse length.

appears in the  $180^\circ$  pulse spectrum, which is caused by the signal from the sample outside the probe coil. In the above “array” method, one is able to visualize the relationship of signal intensity with pulse length. The intensity increases with the pulse length and decreases after the pulse angle passes  $90^\circ$ . It passes through a null at  $180^\circ$  and reaches a negative maximum at  $270^\circ$ . With an appropriate power, a  $90^\circ$  pulse should have a pulse length shorter than  $7\mu\text{s}$  (Table 4.1). This calibration can also provide information on RF field homogeneity, which is generally specified by the ratios of signal intensities at  $450^\circ/90^\circ$ , and  $810^\circ/90^\circ$ . However, this takes a longer time to finish.

For a quick calibration, a different method is used, which “targets” the pulse length in the range close to a previously calibrated  $180^\circ$  pulse. Acquire a pair of FIDs with pulse lengths of  $5\mu\text{s}$  and  $14\mu\text{s}$ , and the estimated  $180^\circ$ . After Fourier transformation followed by phase correction, the second FID is transformed with the same phase correction. A positive intensity of the water signal indicates that the pulse angle is less than  $180^\circ$ , whereas a negative intensity is caused by a pulse length greater than  $180^\circ$ . Change the pulse length accordingly to get an evenly distributed dispersive signal obtained using the same phase correction. It is good practice to check the phase correction parameters compared to the first spectrum when the calibrated  $180^\circ$  pulse length is unusually long or short. Similarly, a  $360^\circ$  pulse length can be used to calibrate the  $90^\circ$  pulse length by the above procedure. The  $180^\circ$  pulse should be checked after the  $360^\circ$  is calibrated, to avoid mistaking the  $180^\circ$  as the  $360^\circ$ .

An alternative way to calibrate the  $90^\circ$  pulse is to observe real signals of an aqueous sample rather than the water signal, using a PRESAT sequence (see solvent suppression). Because the concentration of the sample is usually very low, this method needs multiple transients, and thus takes a longer time but gives a more accurate calibration. The normal setup includes setting the saturation frequency on water resonance, the number of transients to 8, steady-state transients (or dummy scans) to 4, presaturation time to 5 s or longer, and acquisition time to 500 ms. Because a full range array will take a considerably longer time, the target method is more practical to utilize. Once the  $180^\circ$  pulse length is determined, the pulse calibration is checked again with a longer presaturation delay to ensure the accuracy of the calibration because signal accumulation with an insufficient delay can cause an incorrect measurement of intensity.

$^1\text{H}$  pulses for spin locking in TOCSY and ROESY and decoupling from  $^{15}\text{N}$  should be applied with a longer pulse length, usually approximately  $25\text{--}30\mu\text{s}$  for TOCSY and  $40\mu\text{s}$  for ROESY and the decoupling (Table 4.1). Therefore, it is necessary to calibrate these pulses with a lower power. As mentioned previously, reduction of the power level by 6 dB results in a pulse length that is twice as long. Therefore, if the pulse length for the hard  $90^\circ$  pulse is approximately  $7\mu\text{s}$ , the power needs to be attenuated by 12 dB for the TOCSY spin lock pulses and by 15 dB for  $40\mu\text{s}$  pulses. The calibration can be done with any aqueous sample by arraying every  $1\mu\text{s}$  in the range near the desired pulse length.

#### 4.3.4. Calibration of Offset Frequencies

The offset frequency ( $\nu_{\text{offset}}$ ) has the following relationship with the carrier frequency  $\nu_{\text{carr}}$ :

$$\nu_{\text{carr}} = \nu_{\text{inst}} + \nu_{\text{offset}} \quad (4.6)$$

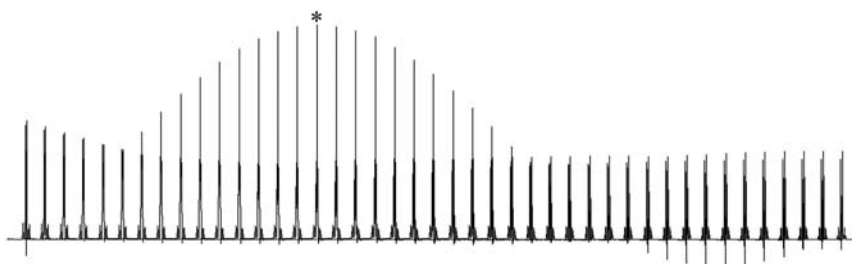
in which  $\nu_{\text{inst}}$  is the base frequency of the instrument for the nucleus, which is the fixed frequency for a specific nucleus. For instance, in a 600 MHz instrument,  $\nu_{\text{inst}}$  for  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  is 599.5200497 MHz, 150.7747596 MHz, and 60.7557335 MHz, respectively.

#### 4.3.4.1. Calibration of Transmitter Offset Frequency

Because common biological samples are in aqueous solution, the transmitter  $\nu_{\text{offset}}$  (e.g., o1 on a Bruker instrument and tof on a Varian instrument) is required to be set on the water resonance for experiments with water suppression. If the transmitter  $\nu_{\text{offset}}$  is off by even 1 Hz, it will significantly affect the result of water suppression experiments. Therefore,  $\nu_{\text{tof}}$  calibration is performed for each individual sample. It is first estimated from the water peak using a one-pulse sequence by setting it on the center of the water peak. It is then arrayed by 0.5 Hz in the range of  $\pm 3$  Hz of the setting value using a PRESAT sequence (see solvent suppression) with 2 transients for steady state and 8 transients per FID. The correct  $\nu_{\text{tof}}$  gives the lowest intensity of the water peak (Figure 4.4a). If the  $^2\text{H}$  signal of  $^2\text{H}_2\text{O}$  is adjusted to be on-resonance prior to  $^2\text{H}$  locking,  $\nu_{\text{tof}}$  should be within the range of a few hertz for different samples at a given temperature. It may have very different values if the lock is not adjusted to be on-resonance prior to the calibration.

#### 4.3.4.2. Calibration of Decoupler Offset Frequency

The correct decoupler offset frequency (e.g., o2, o3 on a Bruker instrument or dof, dof2 on a Varian instrument) needs to be known before pulse calibration for the decoupler channel. A simple one-pulse experiment with continuous wave (CW) heteronuclear decoupling is used for this purpose. The power of the CW decoupling is set very low so that only a narrow frequency range (a few tens of hertz) is decoupled in order to accurately determine the value of  $\nu_{\text{dof}}$ . Arraying the decoupler  $\nu_{\text{offset}}$  in the experiment provides a series of spectra with modulated signal intensity (Figure 4.5). As the  $\nu_{\text{offset}}$  becomes closer to the resonance frequency of the heteronuclear signal, the doublets become closer, resulting in an intensity increase of the peak. The maximum intensity is obtained when the  $\nu_{\text{offset}}$  is on or near resonance. The lower the CW power, the narrower the frequency bandwidth it decouples. The spectra shown in Figure 4.5 were obtained with a CW RF field strength of 70 Hz. The maximum pulse power of a 300 watt heteronuclear amplifier was attenuated by  $-42$  dB, which cuts down the pulse power by a factor of 128 (or  $2^7$ ). (As a reminder, attenuation by  $-6$  dB decreases RF field strength by a factor of 2.) If the bandwidth of the CW decoupling is wider than the increment of the decoupler  $\nu_{\text{offset}}$  array, there will be several spectra with the same maximum intensity as the peak. The average value of the  $\nu_{\text{offset}}$  in the spectra with the same maximum intensity should be taken to get the calibrated decoupler offset frequency.



**Figure 4.5.** Arrayed spectra for calibrating decoupler offset frequency using  $^{15}\text{N}$  urea in DMSO. The decoupler offset frequency is arrayed with increments of 1 Hz as stated in the text. The spectrum labeled with an \* has the offset frequency on urea  $^{15}\text{N}$  resonance.

### 4.3.5. Calibration of Decoupler Pulse Length

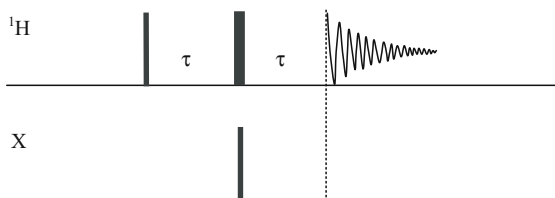
Decoupler pulse length is calibrated periodically because it usually does not change from sample to sample. The calibration is performed indirectly, meaning that the effect of the decoupler pulse on the observed nuclei is calibrated. In principle, any sample enriched with  $^{15}\text{N}$  and/or  $^{13}\text{C}$  can be used for  $^{15}\text{N}$  and  $^{13}\text{C}$  decoupler pulse calibration. Samples used for the calibration include  $^{15}\text{N}$ ,  $^{13}\text{C}$ -NACGly in 90%  $\text{H}_2\text{O}/10\%$   $^2\text{H}_2\text{O}$ , 1 M  $^{15}\text{N}$  Urea in DMSO- $\text{d}_6$  (Dimethyl Sulfoxide- $\text{d}_6$ ), 0.1%  $^{13}\text{C}$  methanol in 99%  $^2\text{H}_2\text{O}/1\%$   $\text{H}_2\text{O}$  (Varian autotest sample), or other samples for indirect calibration. The pulse sequence shown in Figure 4.6 is one of those commonly used to calibrate  $^{15}\text{N}$  and  $^{13}\text{C}$  decoupler pulses. A delay  $\tau$  is set to  $1/(2J_{\text{HX}})$  for calibrating the decoupler pulse of nucleus X.  $^1J_{\text{HX}}$  is measured by the frequency difference between the doublets. It is important to use the correct  $^1\text{H}$   $90^\circ$  pulse length and the decoupler offset frequency must be set on the center frequency of the heteronuclear doublets in order to get an accurate calibration at low decoupler power.

To calibrate the decoupler pulse length, the decoupler offset frequency is set to the calibrated value that is in the center of the coupled doublets. When the angle of the heteronuclear X pulse is zero, a doublet is observed. Similar to the  $^1\text{H}$  pulse calibration, the phase parameters of the first spectrum will be used for the subsequent spectra acquired by arraying the X pulse length. The  $90^\circ$  X pulse will give a null intensity of the doublet. The pulse length at high power is usually calibrated first, and is relatively insensitive to the deviation of decoupler offset frequency. Typical high power  $90^\circ$  pulse lengths for  $^{13}\text{C}$  are approximately  $15\ \mu\text{s}$  whereas those for  $^{15}\text{N}$  can be as long as  $40\ \mu\text{s}$ . When calibrating the high power X pulses, although the maximum allowed  $B_1$  RF field strength (pulse power) can be used, a pulse power which is 3 dB less than the maximum RF power is commonly chosen because it is in the linear range of the amplifier. However, a properly chosen pulse power is that which gives the above short pulse length.

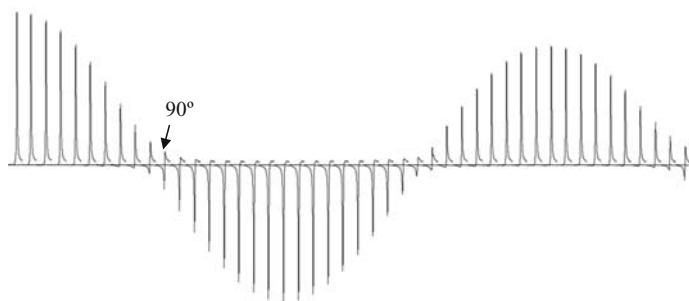
The  $90^\circ$  pulses for heteronuclear decoupling use much less power and, hence, have a longer pulse length. For instance, the desired  $^{13}\text{C}$   $90^\circ$  pulse is  $70\ \mu\text{s}$  for GARP decoupling, and  $300\ \mu\text{s}$  for SEDUCE decoupling, and  $^{15}\text{N}$   $90^\circ$  decoupling pulses are  $200\text{--}250\ \mu\text{s}$  for WALTZ-16 and GARP. Therefore, the decoupling pulses are calibrated with a lower decoupler power. The linearity of the amplifier can be used to estimate the power for the desired pulse length, based on the relationship given by:

$$\text{dB} = 20 \log_{10}(\text{pw}_1/\text{pw}_2) \quad (4.7)$$

in which  $\text{pw}_1$  and  $\text{pw}_2$  are the pulse lengths for the hard  $90^\circ$  pulse and the  $90^\circ$  decoupling pulse, respectively. If pulse power is decreased by one dB, the pulse length is increased by



**Figure 4.6.** Pulse sequence for calibrating the pulse length of the heteronuclear decoupler. The delay  $\tau$  is set to  $1/(2J_{\text{HX}})$ . When the X pulse is  $90^\circ$ , the doublet has minimum intensity.



**Figure 4.7.** Arrayed spectra for decoupler 90° pulse calibration. The 90° pulse gives minimal intensity (reproduced with permission from Kupče, *Meth. Enzymol.* 338, 82, 2001. Copyright © 2001 Elsevier).

a factor of 1.122 linearly:

$$1 \text{ dB} = 10^{1/20} \approx 1.122 \quad (4.8)$$

Therefore, N dB reduction in pulse power lengthens the pulse by a factor of  $(1.122)^N$ . This equation is equivalent to Equation (2.2) in Chapter 2 (instrumentation) because the pulse length is proportional to the  $B_1$  field strength, or the voltage. Specification test data and previous calibration are also reasonable references to estimate the decoupler power setting. For the calibration of low power decoupling pulses, the pulse length is set to the desired value, for example, 70  $\mu\text{s}$  for  $^{13}\text{C}$  or 250  $\mu\text{s}$  for  $^{15}\text{N}$  (Table 4.1). Then the decoupler power is arrayed by 1 dB from lower power to higher in a range of 10 dB (lower power means a small value of the power setting for a Varian spectrometer and a larger value for a Bruker spectrometer). When the power is lower, the selected pulse length is less than that of a 90° pulse, resulting in a positive phase doublet. Phase the first spectrum obtained at the lowest power so that it has a doublet with correct phase. Then all FIDs are transformed using the same phase parameters as the first one. The intensity of the doublet will decrease as the power increases. The power corresponding to the intensity closest to null is used to calibrate the pulse length. The next step is to array the pulse length with the calibrated power in the range of  $70 \pm 10 \mu\text{s}$  by a step of 1  $\mu\text{s}$  for  $^{13}\text{C}$ , or  $250 \pm 50 \mu\text{s}$  by a step of 4  $\mu\text{s}$  for  $^{15}\text{N}$ , to get the 90° pulse length at the calibrated power. When calibrating the pulses, the relaxation delay must be sufficiently long and the decoupler  $\nu_{\text{offset}}$  must be calibrated to be in the center of the doublet. Figure 4.7 shows the spectra obtained with correct delay and  $\nu_{\text{offset}}$ .

#### 4.3.6. Calibration of Decoupler Pulse Length with Off-Resonance Null

In certain triple resonance experiments, it is desirable to excite one region of  $^{13}\text{C}$  resonances but not others. The range of resonances is usually centered at 58 ppm for  $\text{C}^\alpha$ , 177 ppm for carbonyl carbon  $\text{C}'$ , and 45 ppm for  $\text{C}^{\alpha/\beta}$ . In an HNCO experiment (see the following chapter), for instance, the carrier of the  $^{13}\text{C}$  is set on  $\text{C}'$ . A 90° pulse on  $\text{C}'$  with null at  $\text{C}^\alpha$  has a pulse length given by:

$$\tau_{90,\text{null}}(\mu\text{s}) = \frac{0.96825}{\Omega} \times 10^6 \quad (4.9)$$

in which  $\Omega$  is the frequency difference in hertz between the center of  $C'$  and  $C^\alpha$ :

$$\Omega = \nu_{C'} - \nu_{C^\alpha} = (\delta_{C'} - \delta_{C^\alpha})\nu_{\text{carr}} \quad (4.10)$$

in which  $\nu_{C'}$  and  $\nu_{C^\alpha}$  are the center frequencies of  $C'$  and  $C^\alpha$  in hertz, respectively;  $\delta_{C'}$  and  $\delta_{C^\alpha}$  are the spectral centers in ppm; and  $\nu_{\text{carr}}$  is the carrier frequency of the  $^{13}\text{C}$  channel in MHz. Assuming that a 600 MHz spectrometer has a  $\nu_{\text{carr}}$  of 150.86 MHz,  $\Omega = (177 - 58) * 150.86 \approx 17,950$  Hz which gives a pulse length of 53.9  $\mu\text{s}$ .

The effect of a hard  $90^\circ$  pulse on the spins is different when on-resonance and off-resonance. If on-resonance, the  $90^\circ$  pulse length is solely produced by the  $B_1$  field because nuclear spins experience only the  $B_1$  field:

$$\gamma B_1 \text{pw}_{90} = \frac{\pi}{2} \quad (4.11)$$

in which pw is the pulse length, and  $\gamma B_1 = \omega_1$ , which is the frequency of  $B_1$  field. As the resonance frequency moves away from the carrier, the effective field is the vector sum of  $B_1$  and the field along the  $z$  direction with the field strength  $2\pi\Omega/\gamma$  as a function of the frequency difference  $\Omega$  and nuclear gyromagnetic ratio  $\gamma$ :

$$\gamma B_{\text{eff}} = \sqrt{(\gamma B_1)^2 + \left(\gamma \frac{2\pi\Omega}{\gamma}\right)^2} \quad (4.12)$$

The simplest way for an on-resonance  $90^\circ$  pulse to be a null pulse at  $\Omega$  Hz off-resonance is to generate a  $360^\circ$  (or  $2\pi$ ) pulse at the off-resonance frequency. Under this condition, pw must satisfy the following relationship in the same way as the on-resonance pulse does:

$$\sqrt{(\gamma B_1)^2 + (2\pi\Omega)^2} \text{pw}_{90} = 2\pi \quad (4.13)$$

in which  $\gamma B_1 = \pi/(2 \text{pw}_{90})$  according to Equation (4.11) and  $\text{pw}_{90}$  is the  $90^\circ$  pulse length. By substituting  $\gamma B_1$  with  $\pi/(2 \text{pw}_{90})$  and rearranging the equation, the pulse length that gives  $90^\circ$  on-resonance and null ( $360^\circ$ ) off-resonance has a value determined by:

$$\text{pw}_{90} = \frac{\sqrt{15}}{4\Omega} \quad (4.14)$$

For the case when the frequency difference  $\Omega$  between  $C'$  and  $C^\alpha$  is 17950 Hz,  $\text{pw}_{90}$  is 53.9  $\mu\text{s}$ . Similarly, a  $180^\circ$  pulse is generated by the  $B_1$  field on resonance:

$$\gamma B_1 \text{pw}_{180} = \pi \quad (4.15)$$

When the  $180^\circ$  pulse is needed to give a  $360^\circ$  pulse angle at  $\Omega$  Hz off-resonance it must satisfy the following condition:

$$\sqrt{\left(\frac{\pi}{\tau_{180}}\right)^2 + (2\pi\Omega)^2} \text{pw}_{180} = 2\pi \quad (4.16)$$

After the equation is rearranged, the  $\text{pw}_{180}$  can be determined by:

$$\tau_{180} = \frac{\sqrt{3}}{2\Omega} \quad (4.17)$$

With a  $\Omega$  of 17950 Hz,  $\text{pw}_{180}$  is 48.3  $\mu\text{s}$ . In practice, if  $\text{pw}_{180}$  is used for off-resonance excitation, it is set to be 0.8  $\mu\text{s}$  longer than the calibrated value, which is 49.1  $\mu\text{s}$ .

The 90° and 180° hard pulses mentioned above are used to flip the magnetization at the frequencies on-resonance and do not change the magnetization (360° rotation) at the off-resonance region  $\Omega$  Hz away from the carrier. In the cases when perturbation of the off-resonance frequencies is desired, which generates 90° or 180° rotation of the magnetization at off-resonance frequencies without exciting the region at the carrier frequency, shaped hard pulses are used, which are generated using the calibrated  $\text{pw}_{90}$  or  $\text{pw}_{180}$  (see section 4.4). The desired pulse lengths for decoupler off-resonance pulses are calibrated by arraying the pulse power using the pulse sequence for decoupler pulse calibration. In most cases, it is necessary to calibrate fine attenuation of the decoupler power in order to obtain the desired pulse length.

#### 4.4. SELECTIVE EXCITATION WITH NARROW BAND AND OFF-RESONANCE SHAPE PULSES

In many experiments, a certain frequency region must be excited without disturbing the magnetization at other frequencies. Selective pulses that have a narrow excitation range are utilized to selectively excite a group of nuclei, such as protons of water molecules or a kind of carbons of proteins. They are frequently referred to as soft pulses as opposed to hard pulses that have a wide excitation range and cover all the nuclei of the same isotope, such as  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ . Examples of applications of selective pulses include improving solvent suppression, reducing spectral width so as to increase digital resolution in multidimensional experiments, and selectively exciting frequency regions of different spins such as  $\text{C}'$  and  $\text{C}^\alpha$ . A nonselective pulse, or hard pulse, is generated by a high power for a short time. The shorter the hard pulse, the wider the excited frequency range. As an approximation, the bandwidth of a pulse can be considered as the frequency range of its Fourier transformation, and it is inversely proportional to the pulse length. The Fourier transformation of an infinitely long time function gives a delta function in the frequency domain. Therefore, a narrow excitation bandwidth can be achieved by reducing the pulse power and increasing pulse length. Such a selective pulse is utilized in solvent suppression by presaturation to saturate the transition of water spins. However, a desirable selective pulse should have such properties as a relatively short pulse length, a narrow excitation region covering the desired frequency, a uniform excitation band, and linear phase dependence of transverse magnetization on the frequency offset. Amplitude-modulated and/or phase-modulated pulses, called shaped pulses, are used to generate selective pulses with the desired characteristics.

Although the pulse shape cannot be directly determined from the desired excitation profile due to the nonlinearity of the NMR response governed by the Bloch equations, Fourier transformation is a convenient method to design the pulse shape based on the desired excitation profile (Emsley, 1994). The Gaussian function has a unique characteristic that the Fourier transformation of a Gaussian is also a Gaussian. It is for this reason that the Gaussian has been used as a shaped pulse for selective excitation (Bauer *et al.*, 1984). Another example is

a sinc (sine  $x/x$ ) function that has a semi-rectangular frequency response, which is another application of the Fourier similarity theorem. A  $90^\circ$  Gaussian shaped pulse can be used to flip the magnetization by  $90^\circ$  within the excitation bandwidth of the shaped pulse. However, the off-resonance magnetization gradually dephases during the pulse, which produces a significant phase difference between the on- and off-resonance magnetizations. When a Gaussian  $90^\circ$  pulse is used for selective excitation of solvent magnetization, the phase error does not cause any problem because the bandwidth of the pulse is narrow and the solvent magnetization is not desired to be observed. The problem arises if the Gaussian  $90^\circ$  is used to selectively excite a region of particular spin magnetization (e.g.,  $C'$  or  $C^\alpha$ ).

A simple solution to the phase error is to use a  $270^\circ$  Gaussian pulse, which rotates the magnetization by  $270^\circ$  rather than  $90^\circ$  (Emsley and Bodenhausen, 1989). The  $270^\circ$  pulse improves the phase response significantly compared to the  $90^\circ$ . The improvement can be understood as the following. When applying a  $90^\circ$  Gaussian pulse on the  $y$  axis, the on-resonance magnetization is flipped onto the  $x$  axis whereas the magnetization at an offset from the resonance lands near the  $y$  axis, resulting in a nearly  $90^\circ$  phase difference between the magnetizations of on- and off-resonance. On the other hand, if the on-resonance magnetization is rotated by a  $270^\circ$  Gaussian pulse to pass the  $x$  axis, the  $-z$  axis, and reach on the  $-x$  axis, the off-resonance magnetization is, through a different route, flipped to a position very close to the  $-x$  axis. The net result is that both the on- and off-resonance magnetizations are rotated to the  $-x$  axis and phase error is reduced. Thus, a  $270^\circ$  Gaussian pulse produces an approximate in-phase excitation, which is sufficient for common applications in NMR spectroscopy of biological macromolecules. Other popular selective shaped pulses that provide improved in-phase excitations include half Gaussian (Friedrich *et al.*, 1987; Xu *et al.*, 1992), e-BURP-2 (Geen and Freeman, 1991), and  $G^4$  Cascade (a sum of Gaussians; Emsley and Bodenhausen, 1990). The functions require a number of variable parameters to be defined, as many as 12 or more. The parameters can be optimized by software using pulse power and pulse length to produce the desired shaped pulse with the desired excitation bandwidth and offset.

In order for NMR spectrometer hardware to utilize the shaped pulse function, the shaped pulse consists of a series of short rectangular pulses (a few microseconds) with different amplitudes. The duration of the shaped pulse is the sum of the pulse lengths of the element pulses. The shape is approximated by the amplitudes of the element pulses. A typical textfile of a shaped pulse profile lists sequentially the individual width and power for each element pulse.

Like other pulses, the shaped pulse must be calibrated every time the sample is placed in the magnet if it is a transmitter pulse or periodically if it is a decoupler pulse. If the pulse length changes, the shaped pulse needs to be regenerated using the correct pulse length. This means that the transmitter shaped pulse will be regenerated more frequently than we prefer. One way to avoid regeneration of the shaped pulse is to calibrate the pulse power for the fixed pulse length used by the shape function so that the existing shaped pulse function can be used. If the pulse length used in the existing shaped pulse is known, the conventional method is to calibrate the pulse power for the desired duration of the rectangular pulse. If the pulse length used to generate the shaped pulse is unknown, the shaped pulse itself must be used for the calibration.

For calibration of the shaped pulse, the pulse length and pulse power used to generate the shaped pulse profile would be good reference values. For a  $180^\circ$  shaped pulse, the calibration can be performed using a one-pulse sequence in which the shaped pulse is defined as the same shaped pulse profile to be used in the experiment. The correct pulse power for the desired pulse length is determined by arraying the fine power attenuation, which gives a dispersive residual



signal. In general, a  $90^\circ$  shaped pulse cannot be obtained by dividing the calibrated  $180^\circ$  shaped pulse length or by doubling the power. A pulse sequence containing one hard  $90^\circ$  pulse and one shaped pulse (or two identical  $90^\circ$  shaped pulses) with the same phase is used for the  $90^\circ$  shaped pulse calibration. The sequence achieves  $180^\circ$  rotation of the magnetization around the axis. Arraying the pulse power using the same procedure in the  $180^\circ$  pulse calibration is performed to determine the power for the  $90^\circ$  shaped pulse.

## 4.5. COMPOSITE PULSES

RF pulses are used to rotate magnetization at a desired angle, frequency, and duration. For certain applications, pulses are combined together to form a pulse train with or without delay in between pulses to accomplish specific functions. The pulse trains are called composite pulses. The applications of composite pulses range from improving imperfections of single pulses, spin lock for magnetization transfer to off-resonance excitation, and spin decoupling.

### 4.5.1. Composite Excitation Pulses

To compensate for the effects of off-resonance and RF field inhomogeneity, and to produce effective magnetization rotation by a specified angle, numerous composite pulses have been developed. Among them, the composite pulses for  $90^\circ$  and  $180^\circ$  excitation are more frequently utilized in biological NMR spectroscopy. The most commonly used composite pulses consist of a few  $90^\circ$  and/or  $180^\circ$  pulses varying in phase. The pulse sequence  $90^\circ_x 90^\circ_y 90^\circ_{-x} 90^\circ_{-y}$  is a popular version of a composite  $90^\circ$  pulse used as a read out pulse (last pulse in the sequence) in certain NMR experiments with solvent suppression. The composite  $180^\circ$  pulse containing a sequence of  $90^\circ_x 180^\circ_y 90^\circ_x$  is frequently used to improve performance in inversion of  $z$  magnetization in a pulse sequence.

### 4.5.2. Composite Pulses for Isotropic Mixing

The Hartmann–Hahn matching condition (Hartmann and Hahn, 1962) is required to be satisfied during the coherence transfer between spins by isotopic mixing in TOCSY experiments (see TOCSY for detail). When the scalar coupled spins have different frequency offset from the  $B_1$  carrier frequency, the efficiency of the magnetization transfer produced by the continuous RF field is extremely low. The required power of the RF field to cover a frequency range to satisfy the Hartmann–Hahn matching condition would produce a tremendous amount of heat during the period of mixing, which would overheat the probe and damage the NMR samples. Several pulse sequences for Hartmann–Hahn mixing have been developed to achieve effective coherence transfer. MLEV17 (Bax and Davis, 1985a,b) and WALTZ16 (Shaka *et al.*, 1983a,b) are the early versions, which utilize phase-modulated pulses. WALTZ16 combines basic elements of the sequences into supercycles to improve the transfer efficiency. Later, delays were added into the MLEV17 sequence in clean-TOCSY experiments to improve performance. It has been found that the clean-TOCSY mixing scheme produces more heat during the mixing period and can cause overheating problem, which is not suitable for experiments with a long mixing time.

The mixing sequences commonly used in multidimensional NMR spectroscopy are DIPSI sequences that consist of pulses with different pulse angles (Shaka *et al.*, 1988; Rucker and

Shaka, 1989). The DIPSI-2 and DIPSI-3 sequences provide much better efficiency of coherence transfer than phase-modulated sequences, MLEV17 and WALTZ16. For all of the composite pulse sequences, only the  $90^\circ$  pulse length needs to be calibrated. The sequences take the  $90^\circ$  pulse length and convert it into other pulse angles if necessary. A pulse length of the  $90^\circ$  pulse in the range of 25–30  $\mu\text{s}$  is sufficient for the majority of applications to homonuclear magnetization transfer in multidimensional NMR spectroscopy.

### 4.5.3. Composite Pulses for Spin Decoupling

Nuclear scalar coupling can be removed by applying continuous RF irradiation. This technique is commonly used in a one-dimensional  $^1\text{H}$  homonuclear decoupling experiment. However, because of the off-resonance effect, it requires very high power to obtain effective decoupling over the broadband of heteronuclear frequencies using a continuous RF field. The decoupling efficiency of a continuous RF field is described in terms of the scaling factor  $\lambda$  as:

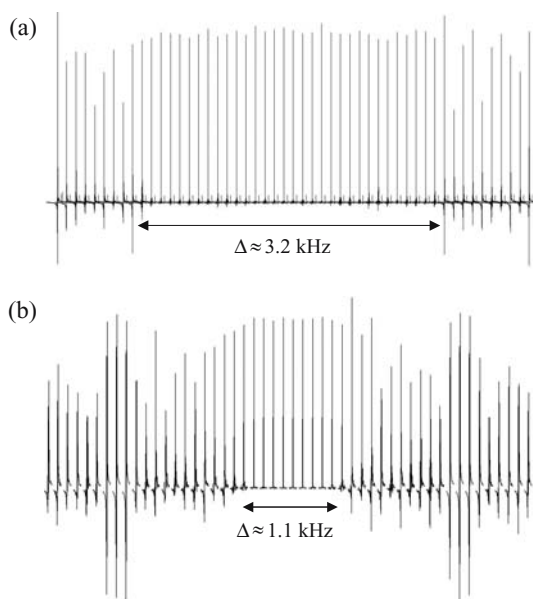
$$\lambda = \frac{2\pi\Omega}{\sqrt{(\gamma B_1)^2 + (2\pi\Omega)^2}} \quad (4.18)$$

in which  $\Omega$  is the offset frequency in Hz from the carrier frequency,  $\gamma B_1$  is the frequency of the  $B_1$  field. The value of  $\lambda = 0$  represents complete decoupling whereas  $\lambda = 1$  provides no decoupling effect. In order to obtain complete decoupling using a continuous wave RF field, the field must be set on resonance, that is,  $\Omega = 0$ . The decoupling power of the CW is required to be higher to decouple a frequency range, which is offset  $\Omega$  from the carrier. Only a small portion of the scalar coupling is decoupled when the offset is about the same amplitude as the applied field ( $\lambda = 0.77$  when  $2\pi\Omega = \gamma B_1$ ).

One of the major applications of composite pulses is to perform spin decoupling. The spin decoupling is achieved by inversion of spin magnetization, based on the assumption of instant flip approximation that the spin magnetization being decoupled is inverted instantly when the frequency of the RF pulse matches its Larmor frequency. To minimize the RF power and increase the decoupling bandwidth, composite pulse sequences including some of the sequences used for isotropic mixing are utilized for heteronuclear decoupling, of which WALTZ16, GARP (Shaka *et al.*, 1985), SEDUCE (Coy and Mueller, 1993), and DIPSI-3 are the most popular decoupling sequences. All of them use supercycles to improve the decoupling efficiency. Often the decoupling efficiency of the sequences is described by the factor of the decoupling bandwidth  $\Delta$  (in units of hertz) to the strength of the applied field:

$$f_d = \frac{2\pi\Delta}{\gamma B_1} = \frac{\Delta}{1/(4\text{pw}_{90})} \quad (4.19)$$

in which  $\text{pw}_{90}$  is the  $90^\circ$  pulse length at the field strength  $1/(4\text{pw}_{90})$  (in hertz) and  $f_d$  is the decoupling efficiency. It has theoretically been demonstrated that GARP increases the decoupling bandwidth by a factor of 2.5 over the field power it uses whereas WALTZ16 gives the same bandwidth as the field power. The decoupling profiles of the sequences observed experimentally are shown in Figure 4.8. In multidimensional NMR spectroscopy, GARP is usually used for  $^{13}\text{C}$  or  $^{15}\text{N}$  broadband decoupling, and SEDUCE for selective decoupling, such as  $\text{C}^\alpha$  decoupling during the experiment whereas WALTZ16 is used for  $^{15}\text{N}$  decoupling.



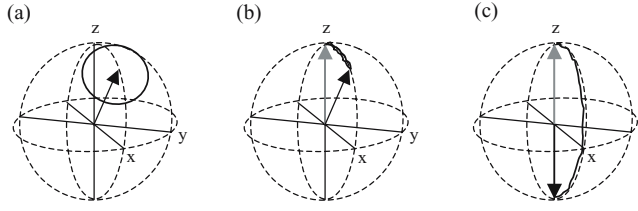
**Figure 4.8.** Bandwidth of (a) GARP and (b) WALTZ16 decoupling. The  $^1\text{H}$ - $^{15}\text{N}$  doublets of  $^{15}\text{N}$  urea in DMSO were observed using 51 decoupler offset frequencies with an increment of 100 Hz. (a) The decoupler  $90^\circ$  pulse length of  $198 \mu\text{s}$  used in the experiments, which gives a  $B_1$  field strength of 1.26 kHz. (b)  $275 \mu\text{s}$  was used for the decoupler  $90^\circ$  pulse length, which corresponds to a  $B_1$  field strength of 0.9 kHz. The observed decoupling bandwidths are indicated below the spectra. WALTZ16 has a decoupling efficiency of 1.2, whereas the observed bandwidth of GARP indicates a decoupling efficiency of 2.5.

#### 4.6. ADIABATIC PULSES

The above composite pulses are regarded as linearly polarized waveforms which are phase-modulated RF pulses. There is another type of RF pulses whose waveforms are described as circularly polarized, referred to as adiabatic pulses. They are much more efficient for inversion of spin magnetization than the phase-modulated pulses. The advantages of adiabatic pulses are their wider bandwidth with a minimum power and their insensitivity to inhomogeneity of the  $B_1$  field. The basic idea behind them is that if the change in the orientation of the effective field with time is sufficiently slow, the tilt angle  $\theta$  of the magnetization with respect to the moving direction of the effective field is very small, with the duration of the pulses shorter than any relaxation process of the system (Abragam, 1961; Kupče, 2001):

$$\frac{1}{T_2^*} \ll \frac{\partial \theta}{\partial t} \ll \omega_{\text{eff}} \quad (4.20)$$

This means that the change in direction of the  $B_1$  field must be sufficiently slower than the rotation of magnetization around the effective field. Initially, the  $B_1$  field is turned on with a frequency far away from the resonance; the effective field  $B_{\text{eff}}$  is virtually parallel to  $B_0$  and the magnetization is along the effective field. The frequency of the  $B_1$  field is then changed to approach the resonance at a rate sufficiently slow so that the magnetization changes its



**Figure 4.9.** Magnetization trajectories when RF pulses are applied (From Kupče, 2001). (a) When the RF pulse is applied suddenly, the magnetization is rotated around  $B_{\text{eff}}$  (arrow). (b) When the RF field is turned on adiabatically, the magnetization follows  $B_{\text{eff}}$ , stays locked along  $B_{\text{eff}}$  for most of the time and then is returned back to equilibrium. (c) When the RF field is swept through the resonance frequency adiabatically, the magnetization follows  $B_{\text{eff}}$  to the  $-z$  axis and becomes inverted.

direction to follow the direction of  $B_{\text{eff}}$  (Figure 4.9). A dimensionless factor  $Q$ , called the adiabaticity factor, has been introduced to quantitatively describe the adiabatic condition (Baum *et al.*, 1985):

$$Q = \frac{\omega_{\text{eff}}}{\dot{\theta}} \quad (4.21)$$

which can be rewritten in terms of pulse field strength  $\omega_1$  and frequency offset  $\Omega$ :

$$Q = \frac{(\omega_1^2 + \Omega^2)^{3/2}}{\omega_1 \dot{\Omega} + \dot{\omega}_1 \Omega} \quad (4.22)$$

in which the dot represents the rate of change. Therefore, the adiabatic condition requires  $Q \gg 1$ .

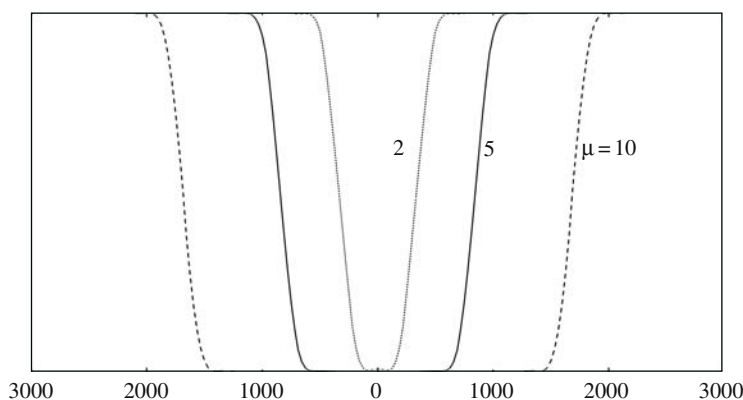
Numerous adiabatic pulses whose amplitudes, phases, or both can be modulated have been developed for application to spin decoupling, magnetization inversion, refocusing, and selective excitation. For instance, WURST (wideband uniform rate and smooth truncation, Kupče and Freeman, 1995a) is an adiabatic pulse generated by adiabatically modulating the amplitude waveform of the  $B_1$  field with the frequency offset swept linearly, according to the relationship:

$$B_1(t) = B_1^{\text{max}} [1 - |\sin(\beta t)|^n] \quad -\frac{\pi}{2} < \beta t < \frac{\pi}{2} \quad (4.23)$$

The higher the value of  $n$ , the higher the bandwidth. WURST-20 has an  $n$  value of 20, and has been used for the inversion of magnetization to yield a uniform profile over a wide frequency range. On the other hand, both the amplitude and frequency sweep can be modulated in a coherent fashion, such as in hyperbolic secant (or sech) pulses (Kupče and Freeman, 1995b; Tannus and Garwood, 1996). The amplitude waveform and frequency sweep of  $B_1$  are modulated in a hyperbolic secant pulse with the forms of:

$$\begin{aligned} B_1(t) &= B_1^{\text{max}} \text{sech}(\beta t) \quad -\frac{\pi}{2} < \beta t < \frac{\pi}{2} \\ \Delta\omega(t) &= -\mu\beta \tanh(\beta t) \end{aligned} \quad (4.24)$$

The excitation profile and bandwidth ( $\Delta\omega_{\text{BW}} = 2\mu\beta$ ) of the pulse is determined by the parameter  $\mu$  in combination with  $\beta$ . As the value of  $\mu$  increases, both the shape and bandwidth



**Figure 4.10.** Inversion profiles of hyperbolic secant pulses with different values of parameter  $\mu$  as indicated. Higher  $\mu$  generates wider bandwidth and flatter profile (reproduced with permission from Norris, *Concepts Magn. Reson.* 14, 89, 2002. Copyright © 2002 Wiley Publishers).

of the excitation are improved significantly (Figure 4.10). The advantage of the adiabatic pulse for spin inversion is that the inversion is insensitive to the  $B_1$  field strength and can have much more tolerance to missetting of the  $B_1$  field strength. Consequently, it is not affected by the inhomogeneity of either the  $B_1$  or the  $B_0$  fields during the experiment. Therefore, adiabatic pulses have found many applications in heteronuclear experiments in which inversion of the heteronuclei is needed, such as HSQC and HMQC (see Chapter 5). They will become more and more popular in biological NMR spectroscopy in the future.

#### 4.7. PULSED FIELD GRADIENTS

In NMR experiments, RF pulses are utilized to rotate the magnetization and create coherence transfers. Different coherence transfer pathways can be produced in the same pulse sequence. In order to extract a specific type of information, certain pathways must be separated from others. Phase cycling is a method used to select the desired coherence transfer pathway, in which the phases of pulses are systematically altered from one FID to another so that the unwanted pathway will be removed by the end of phase cycling at acquisition. The method can be incorporated in a majority of experiments and provides satisfactory results (see section 4.10.3). However, in this method, all types of magnetization (wanted and unwanted) must be collected first and then the unwanted coherence is removed via differentiation of the data. In order to achieve this, the instrument is required to be perfect in stability and dynamic range.

In recent years, pulsed field gradients have been chosen as an alternative method to select a desired coherence transfer pathway in NMR experiments. They provide several advantages over the phase cycling method in that only the desired magnetization is observed during data acquisition in each experiment, resulting in an increase in acquisition dynamic range, reducing artifacts caused by instrument instability, and shortening experimental time. Gradients also provide new methods for solvent suppression, sensitivity enhancement, and diffusion study.

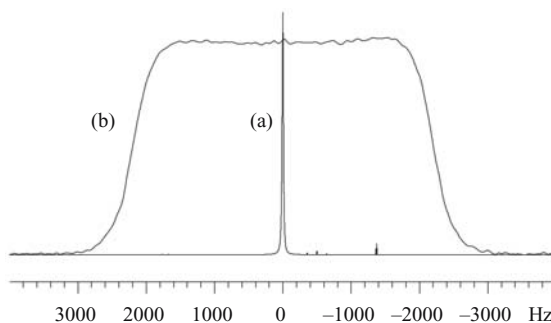
When a gradient varying linearly, for instance on the  $z$  axis, is applied, it causes spatial inhomogeneity of the magnetic field along the axis. The spins in different  $z$  locations

experience different magnetic field strengths, which causes the spin to precess at different frequencies for chemically equivalent spins. This means that the transverse magnetization of the spins will have different precession frequencies for different physical locations along the  $z$  axis in the sample volume. Some will precess faster, and some slower than the resonance frequency. The transverse magnetization of the spins across the sample volume will be decreased with the duration of the gradient irradiation, because a portion of the magnetization will be canceled out due to dephasing of the magnetization. At some time during the gradient irradiation, the transverse magnetization becomes zero. Therefore, the field inhomogeneity created by the gradient eliminates the magnetization on the plane ( $xy$  plane) perpendicular to the gradient axis. The process may be reversed to recover the dephased magnetization by applying the gradient with an opposite direction, or negative to the previous gradient with the same duration. When the gradient reverses the direction of the gradient field, the spins precessing slower under the previous gradient field will now precess faster whereas those that were faster will now precess slower. After the same duration, which caused the magnetization to disappear, the magnetization will regain its original amplitude, ignoring relaxation effects. The former process is called dephasing while the latter is called refocusing.

If the  $z$  gradient in a one-pulse experiment is applied during acquisition of signal from a single resonance, the observed FID is a sinc-type function. The pulse sequence is utilized by the gradient shimming method. After Fourier transformation, a spectrum with a flat step shape is obtained (Figure 4.11). The frequency range is symmetric about the resonance frequency in the absence of the gradient (the center of the spectrum) because the origin of the gradient field is in the center of the probe coil. The magnetic field,  $B_g$ , across the sample created by a gradient pulse on the  $z$  axis is a linear function of the position along the  $z$  direction (Keeler *et al.*, 1994):

$$B_g = zG \quad (4.25)$$

in which  $G$  is the gradient field strength in units of Gauss per centimeter,  $G\text{ cm}^{-1}$ , or tesla per meter,  $\text{Tm}^{-1}$ . The convenient and commonly used unit is  $G\text{ cm}^{-1}$  because gradient coils in NMR probes are a few centimeters in length. During the experiments, the field strength of



**Figure 4.11.**  $^1\text{H}$  spectra of water (a) without and (b) with gradients during acquisition. The intensities of the two spectra are not proportional. When the gradient is turned on, water across the sample “sees” different magnetic field strengths due to the linear  $z$  gradient. Therefore, the chemical shifts are different as a function of the  $z$  position of the sample [see equation (4.30)]. The center of the sample has zero frequency shift.

the gradients is defined in terms of digital units such as DAC (digital-to-analog conversion). In order to set the gradient to a specific strength in units of  $\text{G cm}^{-1}$ , the maximum gradient strength corresponding to the maximum DAC unit must be known. For instance, if the maximum gradient strength of a probe is  $70 \text{ G cm}^{-1}$  and the setting range of the gradient is from 32 k DAC to  $-32 \text{ k DAC}$ , 8000 DAC represents a gradient strength of  $17 \text{ G cm}^{-1}$ . The field strength at a given position  $z$  of the sample is described by:

$$B = B_0 + B_g \quad (4.26)$$

Because  $B_0$  will contribute evenly over the sample, we will drop the term and only consider in the rotating frame the effect of the gradient on the sample at different positions. The precession frequency of spins at any specific sample position  $z$  under the interaction of the gradient field is given by:

$$\omega_g = \gamma B_g = \gamma z G \quad (4.27)$$

in which  $\omega_g$  is the precession frequency of the spins in the presence of  $B_g$ . The phase of the precession at the sample position  $z$  after the gradient field  $G$  is applied for a period of time  $t$  is determined by:

$$\phi(z) = \gamma z G t \quad (4.28)$$

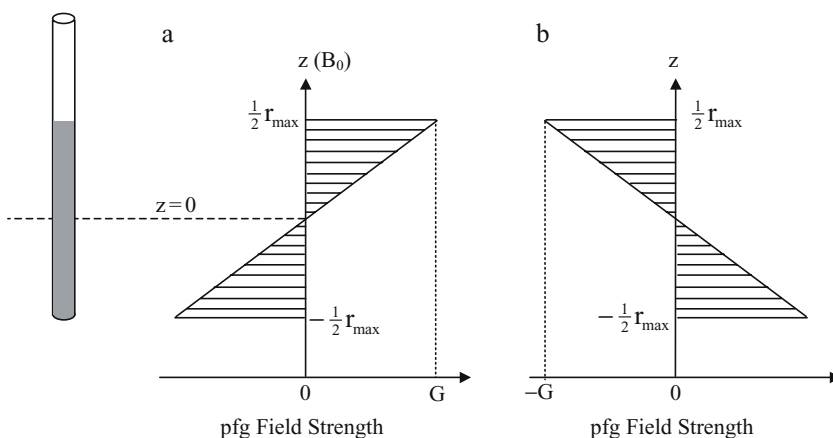
Considering the above example in which a single peak is present in the spectrum,  $x$  magnetization of spins at any specific sample position  $z$  evolves after applying a  $z$  gradient for time  $t$ :

$$M_x(z) \xrightarrow{\gamma z G t} \cos(\gamma z G t) M_x(z) + \sin(\gamma z G t) M_y(z) \quad (4.29)$$

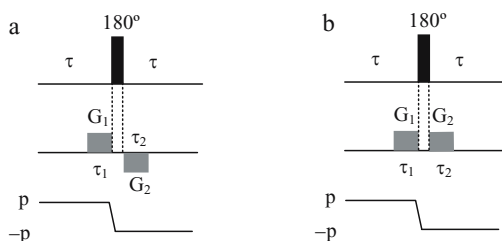
$M_x(z)$  is the transverse  $x$  magnetization at sample position  $z$ , which is dependent on the gradient strength and pulse time  $t$ . The appearing  $x$  magnetization comes from all of the sample slices along the  $z$  direction, and is the average over all sample slices. Because the origin of the gradient field is at the center of the sample, the integration (summation) of the  $M_x(z)$  is from  $-\frac{1}{2}r_{\text{max}}$  to  $\frac{1}{2}r_{\text{max}}$  for a length of  $r_{\text{max}}$  (Figure 4.12):

$$\begin{aligned} M_x &= \frac{1}{r_{\text{max}}} \int_{-\frac{1}{2}r_{\text{max}}}^{\frac{1}{2}r_{\text{max}}} \cos(\gamma z G t) dz = \frac{2 \sin\left(\frac{1}{2}r_{\text{max}} \gamma G t\right)}{r_{\text{max}} \gamma G t} \\ &= \text{sinc}\left(\frac{1}{2}r_{\text{max}} \gamma G t\right) \end{aligned} \quad (4.30)$$

If the gradient pulse is applied during the entire acquisition time, the observed FID is a sinc function as described in Equation (4.30). Fourier transformation of the sinc FID gives a spectrum with a broad range of frequencies caused by the gradient (spectrum b in Figure 4.11). The width of the frequency band depends on the strength of the gradient. Because the origin of the gradient is at the center of the sample, the frequency distribution is symmetric about the resonance frequency as shown in the spectrum.



**Figure 4.12.** The  $z$  gradient field strength across the sample volume. The origin of the gradient field is in the center of the sample (precisely, in the center of the probe coil). The field strength is a linear dependence of the  $z$  positions of the sample volume, and the total length along the  $z$  axis is  $r_{\max}$ . (a) The field strength distribution when a  $G$  gradient is applied. (b) The gradient field strength when a  $-G$  gradient is applied. The sign of the gradients is relative.



**Figure 4.13.** Elimination via gradient pulses of artifacts due to imperfect  $180^\circ$  pulses. (a) Gradient-enhanced spin inversion sequence in which the artifacts are removed by a pair of  $z$  gradient pulses with opposite sign. The gradient pulses sufficiently dephase transverse magnetization while the longitudinal magnetization is not disturbed by the  $z$  gradient pulses. The opposite sign of the second gradient pulse is used to avoid refocusing any transverse magnetization dephased by the first one. (b) Gradient-enhanced spin echo sequence using gradient pulses to select the coherence echo so that the artifacts from the imperfect  $180^\circ$  pulse are removed. The gradient pulses are applied with the same strength and duration ( $G_1 \tau_1 = G_2 \tau_2$ ) to accomplish the selection of the coherence with order of  $-p$ . Any coherence of order other than  $-1$  generated by the  $180^\circ$  pulse is removed after the second gradient pulse.

In multipulse experiments, a  $180^\circ$  pulse is frequently used to invert  $z$  magnetization  $M_z \rightarrow -M_z$  (for instance, a  $180^\circ$  decoupling pulse) or to generate a spin echo, that is, change the sign of the coherence order,  $p \rightarrow -p$  (see section 4.10.3). An imperfect  $180^\circ$  refocusing pulse is a noticeable source of pulse artifacts that can be removed by the phase cycling method EXORCYCLE (Bodenhausen *et al.*, 1977). Gradient pulses can also be used to eliminate artifacts from the  $180^\circ$  refocus pulses. For a  $180^\circ$  inversion pulse, the first gradient pulse is applied before the  $180^\circ$  pulse to dephase the transverse magnetization (Figure 4.13a), whereas the longitudinal magnetization is not influenced by  $z$  gradient pulses. After the  $180^\circ$  pulse, the



second gradient pulse with opposite sign is applied to dephase any transverse magnetization produced by an imperfect  $180^\circ$  excitation. The opposite sign is used to avoid refocusing the transverse magnetization dephased by the first gradient pulse so that it is continuously dephased during the second gradient. To remove artifacts during the inversion of coherence order by an imperfect  $180^\circ$  refocusing pulse, the coherence of order  $p$  is selected by a pair of gradient pulses (Figure 4.13b). The  $180^\circ$  pulse inverts the order of the coherence after the first gradient pulse dephases the coherence. The second gradient pulse is applied to refocus the coherence according to the coherence selection rule:

$$G_1 \tau_1 p + G_2 \tau_2 (-p) = 0 \quad (4.31)$$

$$G_1 \tau_1 = G_2 \tau_2 \quad (4.32)$$

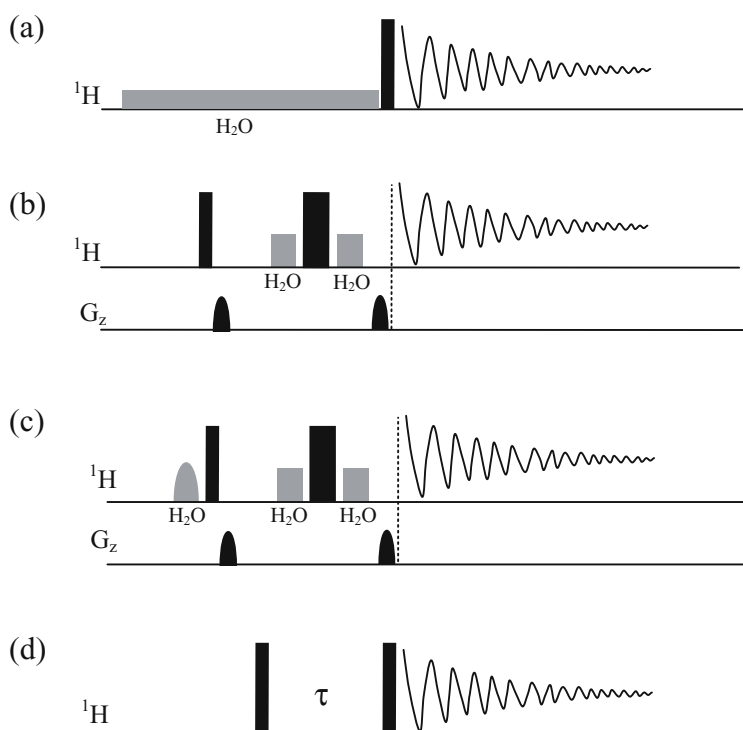
that is, the second gradient pulse is applied with the same strength, duration, and sign as the first one. Any coherence with order other than  $-p$  will sufficiently be removed during the second gradient pulse. For both methods, the artifacts are effectively eliminated by a single transient without any phase cycling. The reduction of phase cycling translates to faster data acquisition or higher digital resolution through collection of more time increments in the indirect dimensions. The selection of coherence transfer pathways by gradients is discussed in more detail in section 4.10.3.

## 4.8. SOLVENT SUPPRESSION

In order to observe amide protons, which contain rich structure information, it is necessary to dissolve protein samples in aqueous buffer solution containing only approximately 5%  $^2\text{H}_2\text{O}$  for  $^2\text{H}$  lock. It is difficult to observe the signals from the solute in the presence of the intense signal from water protons since water protons in 95% aqueous solution with a concentration of approximately 105 M have bulk magnetization approximately  $10^4$  times greater than that from a single proton of a macromolecule. Therefore, the first task to do before acquiring data is to remove the solvent signal by a solvent suppression sequence. In the current section, some commonly used water suppression techniques will be discussed.

### 4.8.1. Presaturation

There are various methods for water suppression, of which presaturation is the most basic and classical technique and is simple to use. A long and low power selective rectangular pulse is applied to saturate the water signal during the relaxation time (or predelay time) in the PRESAT pulse sequence shown in Figure 4.14a. Because the quality of the water saturation experiment depends greatly on the homogeneity of the magnetic field, the quality of shimming will substantially influence the result of water suppression. In the experiment, the frequency of the long pulse is set at the water resonance to saturate the water-proton population. Because the chemical shift of water is near the center of the  $^1\text{H}$  chemical shift range, the carrier frequency is also placed at the water resonance though it can be set to any frequency within the spectrum. The pulse is set to longer than 3 sec for a one-dimensional experiment or to the longest time allowed for two-dimensional experiments (usually 1.0–1.5 sec). The pulse power must be set sufficiently low for important reasons, one of which is because the lower the power, the better



**Figure 4.14.** Water suppression experiments. (a) PRESAT. The water saturation pulse is usually approximately 3 sec. (b) WATERGATE. The  $90^\circ$  water selective pulses in gray can be shaped pulses (e.g., 3 ms sinc shape) or 1.7 ms rectangular pulses. (c) Water-flip-back. The initial  $90^\circ$  water selective pulse is used to bring water magnetization to the  $z$  axis after the hard  $90^\circ$  pulse to avoid radiation damping, and can be a 3 ms sinc or a 7.5 ms eburp1 shaped pulse. The two gradient pulses dephase and then refocus the solute magnetization while the magnetization of residual water is continuously dephased by the gradient pulses because the pulse train formed by the two selective pulses sandwiching the hard  $90^\circ$  pulse keeps the water coherence order unchanged. The gradient strength is approximately  $20 \text{ G cm}^{-1}$ . (d) Jump-return. The delay  $\tau$  is set to  $1/(4\Delta)$  in which  $\Delta$  is the frequency difference between the water resonance and the center frequency of the interested region.

the selectivity of the excitation. A high power will saturate signals in the region close to the water resonance. Additionally, for such a long pulse, the probe could be damaged if the pulse power were not sufficiently low. Usually, the pulse power is set in the range of 100–150 Hz, approximately 2 ms for the  $90^\circ$  pulse. For instance, the correct setting of the power should be attenuated about  $-48 \text{ dB}$  from the power for a hard  $90^\circ$  pulse length of approximately  $7 \mu\text{s}$ , which is typically in the range of  $-59$  to  $-55 \text{ dB}$  lower than the maximum pulse power of a 50 watt  $^1\text{H}$  amplifier (e.g. 4–8 or 55–59 for Varian or Bruker instruments, respectively). A typical setup procedure includes calibration of the  $90^\circ$  pulse, setting the carrier on the water resonance frequency, and adjustment of the receiver gain. The hard pulse is calibrated first using a one-pulse sequence according to the procedure described in the calibration section. The transmitter frequency offset is set to the water peak as described previously. After the receiver gain is adjusted using a single transient, the data is collected with the proper number

of transients for the desired signal-to-noise ratio. Occasionally, a composite  $90^\circ$  pulse is used to replace the  $90^\circ$  pulse so as to minimize the effect of radiation damping (Chapter 2). It has also been noted that solvent signals originating outside the main sample volume (outside the probe coil) degrade solvent suppression.

#### 4.8.2. Watergate

PRESAT is a simple method and is easy to set up. However, it reduces or partially saturates signals from exchangeable protons (amide protons) due to the rapid chemical exchange and spin diffusion between amide protons and saturated water protons and from protons resonating near the water resonance ( $\alpha$  protons), causing partial saturation of  $H^N$  spins and reducing the intensity of amide protons (saturation transfer). The exchange rate of amide protons with water protons linearly increases with the pH value of the solution over a pH range of 3–9 (Connelly *et al.*, 1992). To overcome this drawback, more sophisticated methods have been developed to perform water suppression without saturating the water magnetization.

The WATERGATE sequence (Piotto *et al.*, 1992) shown in Figure 4.14b is a popular technique used for water suppression of aqueous samples. It uses gradient pulses to dephase magnetization of both water and solute, and then to refocus only the signals from the solute and further dephase the water magnetization. After the first hard  $90^\circ$  pulse brings the magnetization of all protons to the transverse plane, a 1 ms gradient pulse with an amplitude of approximately  $20 \text{ G cm}^{-1}$  is applied to dephase the magnetization. The two  $90^\circ$  water selective pulses, which are either shaped or rectangular, are set on the water resonance. The net effect of the hard  $180^\circ$  pulse sandwiched by the two  $90^\circ$  water selective pulses is to keep the coherence order of water magnetization unchanged. Consequently, not only is the water magnetization dephased by the first gradient not refocused by the second, but the second gradient also further dephases the water magnetization. On the other hand, the second gradient refocuses the solute magnetization and produces a gradient echo because the hard  $180^\circ$  pulse changes the coherence order of the solute magnetization. The gradient echo used to dephase the water magnetization can sufficiently suppress water signal intensity compared to the presaturation method. However, it may still reduce the sensitivity of water exchangeable protons due to the partial saturation of water magnetization. Since the water magnetization is along the transverse plane at the time when acquisition starts, it needs much more time to completely relax back to its equilibrium state than do protein resonances due to its long  $T_1$  relaxation time. Thus, the water will not fully relax back during the predelay time and will be partially saturated. The partial saturation will be transferred to the exchangeable protons by saturation transfer, resulting in partial saturation of the  $H^N$  protons as reflected in a reduction of sensitivity for the desired resonances.

#### 4.8.3. Water-Flip-Back

An alternative version of Watergate, called water-flip-back (Grzesiek and Bax, 1993), uses a selective pulse at the water resonance before the Watergate sequence (Figure 4.14c). The combination of the selective  $90^\circ$  and hard  $90^\circ$  brings the water magnetization back to the  $z$  axis before the first gradient pulse in the Watergate sequence. Because of the inhomogeneity of the  $180^\circ$  pulse, some magnetization from residual water is left on the transverse plane. The water-flip-back sequence will suppress the magnetization from the residual water, whereas

the majority of water magnetization stays on the  $z$  axis and is not disturbed to avoid radiation damping (Redfield *et al.*, 1975). Consequently, the sensitivity for the exchangeable amide protons is increased because of the reduction of saturation transfer and because the amplitude of the required gradient pulses is considerably reduced to approximately  $5 \text{ G cm}^{-1}$  for the gradient echo, which also increases the sensitivity of the experiment due to the reduced effect of gradient pulses on the solute magnetization. In order to maximize the suppression of the water magnetization, the selective pulses must be well-calibrated. To simplify the experimental setup, the flip-back selective pulse has a different shape and pulse length than the two Watergate selective pulses. For instance, the first selective  $90^\circ$  pulse is a  $90^\circ$  eburp-1 pulse of 7.5 ms whereas the last two can be either 1.7 ms rectangular  $90^\circ$  pulse or 3 ms sinc  $90^\circ$  pulses. The selective pulses are calibrated using the sequence described in the calibration section and the flip-back selective pulse is retuned in the water-flip-back sequence by adjusting the fine power attenuation of the pulse.

In some experiments, distortion of the baseline occurs due to the fact that a substantial fraction of the FID is lost during the waiting time between the last pulse and acquisition (called the dead time). The time period is needed for the RF power of the high power pulse to go below the observable amplitude of the FID so the receiver is not saturated. A spin-echo sequence can be incorporated into any pulse sequence to prevent the FID from substantial loss during dead time. The idea is to start the acquisition at the top of the first FID echo so that the full FID information can be retained. A typical value for the echo delays is  $500 \mu\text{s}$  in the water suppression sequences whereas the last delay is adjusted within  $500 \pm 50 \mu\text{s}$  to correct any phase errors in the spectrum.

#### 4.8.4. Jump-Return

Although they provide sufficient water suppression, the gradient-based water suppression techniques and spin echo sequence may not produce an observable signal for dilute samples because of partial loss in sensitivity during the experiment. For such samples, a better result can be achieved using the jump-return sequence shown in Figure 4.14d (Plateau and Guéron, 1982). The sequence is rather simple: a two-pulse sequence with two  $90^\circ$  pulses is separated by a delay. Like all other water suppression techniques, the carrier frequency is placed on the water resonance. The time of the delay is dependent on the spectral region of interest, which is determined by the frequency difference between the center of the region and the water resonance according to:

$$\tau = \frac{1}{4(\nu_c - \nu_w)} \quad (4.33)$$

in which  $\nu_c$  is the center frequency of the region of interest and  $\nu_w$  is the water resonance. For example, the signals in the region of 7–10 ppm are expected to be observed. With the delay set to  $111 \mu\text{s}$  for a 600 MHz spectrometer, the resonance at 8.5 ppm will precess  $90^\circ$  out of phase from the water resonance after the delay whereas the water is on resonance. After the second  $90^\circ$  pulse, which has a  $180^\circ$  phase shift from the first  $90^\circ$  pulse, the water is back to the  $z$  axis, whereas resonances in the region are detected with scaled intensity about the center frequency,  $\nu_c$ . Frequently, the second  $90^\circ$  pulse length is required to be optimized to achieve the best solvent suppression.

## 4.9. NMR DATA PROCESSING

For data analysis, an FID recorded during data acquisition is Fourier transformed to generate a spectrum. Prior to and post FT, the data are manipulated through a series of data processing steps to optimize sensitivity or resolution. A typical data processing includes DC offset correction, application of a solvent suppression filter, linear prediction, apodization, Fourier transformation, phase correction, and spectral baseline correction (if necessary).

### 4.9.1. Drift Correction

An FID collected by quadrature detection contains DC (drift correction) offset error originating from the receiver. A DC offset in the time domain data produces a spike at the carrier frequency after Fourier transformation. The DC correction is automatically applied by NMR data processing programs without the user's attention.

### 4.9.2. Solvent Suppression Filter

Although the water signal is suppressed during experiments, the suppression does not completely eliminate the unwanted water peak and sometimes the residual water signal is sufficiently high to interfere with nearby resonances. Digital filtering the FID will further suppress the water peak. There are two types of popular methods for solvent suppression by digital filtering, namely low frequency suppression and zero frequency suppression. In the low frequency method, a low passband digital filter with the desired bandwidth and resonance frequency on the water frequency is applied to the acquired FID (Marion *et al.*, 1989). The filter only allows the water signal and signals within the bandwidth of the filter to pass through. This filtered FID is subsequently subtracted from the original FID. Fourier transformation of the treated FID gives the water suppressed spectrum. The suppression filtering leaves a flat line in the bandwidth region if the profile of the digital filter fits the water line shape. Three parameters need to be specified for the filtering: the bandwidth in hertz (50–200 Hz), the resonance frequency position offset (in hertz) from the carrier frequency (the center of the spectrum), and the number of coefficients for the digital filter, which define the passband profile of the filter. More coefficients produce a digital filter with a steeper passband (brick wall type).

The zero frequency method first filters the FID using digital filtering the same as in the low frequency method. Then, the filtered FID is fit with a polynomial function using a specified order. The polynomial fit FID is subtracted from the original FID to eliminate the water contribution to the FID. This method only subtracts the water signal residing exactly on resonance. The water peak is missing from the spectrum after Fourier transformation. In addition to the parameters used in low frequency water suppression, the polynomial order needs to be specified, usually around seven. A brick wall type digital filter (with a higher number of coefficients) should be avoided in order to obtain reasonable fitting by the polynomial.

### 4.9.3. Linear Prediction

The first few points of the acquired time domain data are almost always distorted due to the imperfect condition of the hardware, such as tailing of RF pulses. Fourier transformation

of the FID causes problems in the frequency domain such as baseline distortion. In addition, multidimensional data are always recorded with fewer points in the indirectly observed dimensions due to the long experimental time required. Collecting fewer data points in the indirectly observed dimension and more scans in the acquired dimension translates to a higher S/N for a given time. However, Fourier transformation of the truncated time domain data will severely distort the spectrum and reduce both sensitivity and resolution. Linear prediction is utilized to repair both types of FID distortions (Barkhuijsen *et al.*, 1985). The algorithm analyzes the FID using the correct data points of the FID to obtain information on the frequencies present in the FID. Based on the analysis, it extrapolates more data points in the forward direction (forward prediction) to eliminate the truncation problem, or in the backward direction (back prediction) to replace the distorted initial points (in the observe dimension) or to extend a data point (in the indirect dimension). Mirror image linear prediction (Zhu and Bax, 1990) uses the observed positive-time points to extrapolate negative-time points using the relationship of  $f(-t) = f^*(t)$ , in which  $*$  represents complex conjugation, resulting in doubling of the size of the time-domain data points.

For the acquired dimension, it may not be necessary to extend the data because it does not take long time to acquire the desired number of data points. However, backward prediction is frequently used to correct the first few points which are influenced by the power of the last pulse. Forward predictions are always used for indirect dimensions because this provides more data points without actually acquiring the data so as to save experimental time (a more effective way to acquire NMR data). The data points are usually doubled by the prediction (two-fold prediction) because overextended data may introduce artifacts after Fourier transformation. The maximum number of points that can be used is the acquired data points for extending data points in either direction and the total data points minus the number of predicted points (usually the first one or two points) for replacing the distorted points in the backward predication.

#### 4.9.4. Apodization

It is often necessary to manipulate time-domain data (without altering frequencies) to improve the sensitivity or resolution of the spectrum. This can be achieved by the application of weighting functions. The basic idea is that according to the FT similarity theorem, multiplying an FID by a function results in changing the shape of the spectrum, but not the frequency:

$$\begin{aligned} F'(\omega) &= H(\omega)F(\omega) \\ F'(\omega) &= Ft\{h(t)f(t)\} = Ft\{f'(t)\} \end{aligned} \quad (4.34)$$

in which  $h(t)$  is a weighting function (or window function). The process used to change the appearance of the data is called data apodization. Because the FID tends to decay to zero, the S/N ratio (amplitude of FID to noise level) in the tail part of the FID is much lower than that at the beginning. By applying a weighting function, which smooths the tail region of the FID, the noise level can be zeroed out so that the sensitivity of the spectrum can be improved. The signals in the region, if any, will also be reduced to zero. An exponential function is used to serve this purpose:

$$h(t) = e^{-t/a} \quad (4.35)$$

applying to the FID  $f(t)$ :

$$f(t) = e^{-t/T_2} \quad (4.36)$$

results in new time-domain data  $f'(t)$ :

$$f'(t) = h(t)f(t) = (e^{-t/a})(e^{-t/T_2}) = e^{-t/(T_2')} \quad (4.37)$$

in which the apparent  $T_2'$  is given by:

$$\frac{1}{T_2'} = \frac{1}{a} + \frac{1}{T_2} \quad (4.38)$$

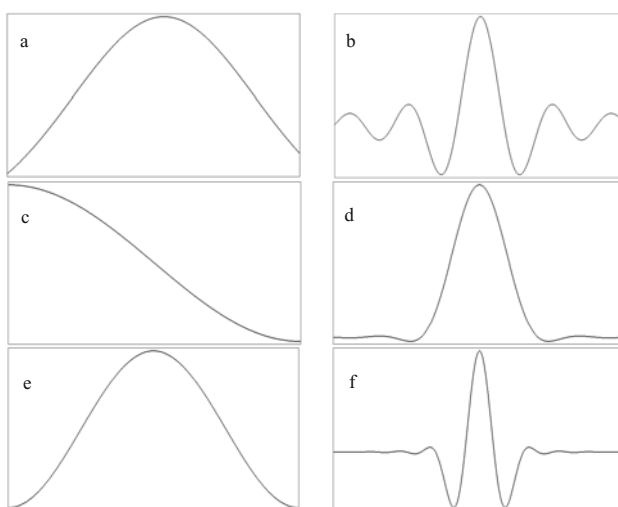
This states that after Fourier transformation the line shape is broadened by the application of the exponential function with the line width increased by a factor of 2 (i.e.,  $T_2' = T_2/2$ ) when  $a = T_2$ . Therefore, the exponential function decreases the resolution of the spectrum but increases sensitivity by removing the noise level at the tail of the FID. On the other hand, the sensitivity enhancement of the exponential function is hence reduced when the line broadening parameter  $a$  becomes larger. Exponential function is usually applied as a matched filter with  $a = T_2$ , which has a decay profile matched to the FID. Alternatively, if the exponential function can be applied with negative  $a$ , the line width should be reduced as the reverse effect to the line broadening. The line shape of the spectral peak can be narrowed by a factor of 2 (i.e.,  $T_2' = 2T_2$ ) when  $a = -2T_2$ . However, as its profile indicates, the function with  $a$  less than zero amplifies the noise level at the end part of the FID and reduces the signal amplitude at the beginning part of the FID, resulting in a significant loss in sensitivity. Resolution enhancement by applying these types of weighting functions will certainly degrade the sensitivity of the spectrum because the functions reduce the amplitude of the FID at the beginning. On the other hand, to increase sensitivity by smoothing the end part of the FID, both the signal and noise levels are reduced and the apparent  $T_2'$  decreased. As a consequence, the resolution of the spectrum is sacrificed. In general, the sensitivity and resolution of an NMR spectrum cannot be improved simultaneously by means of applying a weighting function (or window function). Enhancement in one of the aspects has to be achieved at the expense of the other.

A usual solution to the problem is to combine the negative exponential function with another sensitivity enhancement function, such as a Gaussian function:

$$h(t) = e^{-t/a} e^{-t^2/b} \quad (4.39)$$

Compared to the natural line shape of NMR spectral peaks (Lorentzian line shape) with the same half height width, a Gaussian function has a much sharper line shape. While negative  $a$  gives resolution enhancement,  $b$  (always positive) provides sensitivity enhancement to compensate for the loss of sensitivity caused by  $a$  being less than zero. The optimization of parameters  $a$  and  $b$  of the Lorentz–Gauss function can increase resolution with a minimum loss of sensitivity and transform the Lorentzian line shape to a narrow Gaussian line shape.

The Lorentz–Gauss function has a profile shown in Figure 4.15 with the maximum in the middle of the function. This leads to the application of other functions with a similar profile but less reduction to the beginning of the FID. Among them, the phase shifted sine-bell function



**Figure 4.15.** Common weighting functions for apodization. (a) Lorentz–Gauss function with the parameters  $a = -0.5$ ,  $b = 1.5$ , and (b) its Fourier transformation. (c) Squared  $90^\circ$  shifted sine-bell function and (e) squared sine-bell function, and their Fourier transformations (d) and (f), respectively.

and squared phase shifted sine-bell function are commonly used for multidimensional data to achieve the desired sensitivity or resolution enhancement. Application of weighting functions such as the Lorentz–Gauss, phase shifted sine-bell or squared phase shifted sine-bell function (Figure 4.15) increases the amplitude of the middle part, reduces the beginning, and smooths the tail of the FID, producing an FID with longer decay (flatter FID). The corresponding spectral peak has a narrow line shape and hence the spectral resolution is increased. For one-dimensional data, Lorentz–Gauss is commonly used if resolution needs to be improved; otherwise, an exponential function with  $a = T_2$  is applied. For multidimensional data, an unshifted sine-bell or squared unshifted sine-bell function is utilized in the COSY to gain resolution enhancement, whereas the shifted weighting functions are used for all other two-, three-, and four-dimensional data to improve the sensitivity. A  $90^\circ$ -shifted function provides maximum sensitivity enhancement.

#### 4.9.5. Zero Filling

Data points collected in an FID are necessarily limited to just enough points to cover the FID to avoid collecting noise. However, a spectrum needs more data points to be able to characterize the individual peaks with the correct digital resolution. Digital resolution in the frequency domain is defined as  $SW/f_n$  in which  $SW$  is the spectral window in hertz and  $f_n$  is the number of frequency domain points (a spectrum consists of  $f_n$  data points in the real part and  $f_n$  data points in the imaginary part). Additionally,  $f_n = np/2$ , in which  $np$  is the number of time domain points. By adding more points with zero amplitude to the end of the FID after data are collected (called zero-filling), the digital resolution is improved because  $np$  becomes larger after the zero-filling. The digital resolution without zero-filling is  $SW/(\frac{1}{2} np) = 1/(DW \times np) = 1/at$ , in which  $at$  is acquisition time and  $DW$  is dwell time.



Exchanging the order between apodization and zero-filling (i.e., the data are zero-filled first and then apodization is applied) does not affect the result of the data processing because the amplitudes of the zero-filled data points are still zero after the apodization. However, apodization after the zero-filling takes more computing time. Although this extra computing time is unnoticeable in one- and two-dimensional data processing, it may be substantial during three- and four-dimensional data processing. Therefore, apodization is usually applied before zero-filling.

#### 4.9.6. Phase Correction

Fourier transformation of an FID gives a spectrum, which can be represented by:

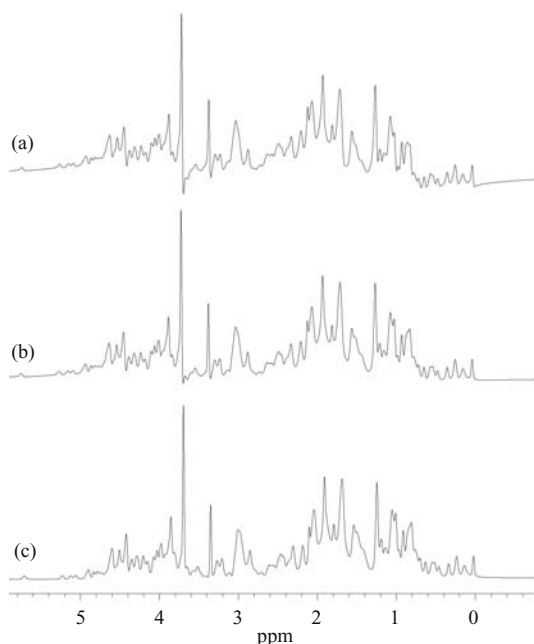
$$F(\omega) = R(\omega) \cos(\theta) + I(\omega) \sin(\theta) \quad (4.40)$$

in which  $R$  and  $I$  are the real and imaginary parts of the spectrum  $F(\omega)$ , respectively, and  $\theta$  is the phase error of the spectrum. Ideally,  $\theta = 0$ , that is, the real part is pure absorption and the imaginary part is pure dispersion. In practice,  $\theta \neq 0$  for most cases, because the FID does not start with the maximum amplitude. There are many factors causing the problem, one of which is that quadrature detection has a phase error and does not acquire the pure cosine and sine components of the FID. This causes an equal amount of phase error on all resonances of the spectrum and is called zero order phase error (Figure 4.16a). The other type is first order phase error typically introduced by the delay between the end of the last pulse and the start of acquisition (dead time, or receiver gating time). This delay is unavoidable because of problem of tailing of the RF pulse. During the dead time, the amplitudes of time domain signals with different frequencies are reduced by different fractions, leading to a frequency-dependent phase error (Figure 4.16b). The total phase error is the linear combination of the two types of phase errors:

$$\theta(\omega) = \phi_0 + (\omega - \omega_0)\phi_1 \quad (4.41)$$

in which  $\phi_0$  and  $\phi_1$  are the zero order and first order phase error, respectively, and can be corrected by the phase correction function of a data processing program.

It should be noted that an experiment can be set up in such a way that the FID is acquired without any phase error (both  $\phi_0$  and  $\phi_1$  are zero). Adjusting the dead time to ensure all signals start at a maximum amplitude eliminates the first order phase error. This can be done by simply optimizing the dead time to the point that the first order phase error is zero, which is usually longer than the value set by the instrument software (rof2 and alfa on a Varian spectrometer, de on a Bruker spectrometer). The drawback is loss in sensitivity due to the longer delay. Alternatively, in a better method, a spin echo sequence is used during the normal delay period, which refocuses all coherence at any desired time so that the FID always starts with the maximum amplitude. The zero order phase error can be corrected by resetting the receiver phase after the first order phase error is eliminated in the above procedure. The adjustment of the receiver phase can be done by the spectrometer software or by adding an adjustable phase parameter to the receiver phase table in the pulse sequence. After the above adjustment of both the zero order and first order phases, the acquired FID does not contain any phase error. Therefore, phase correction after Fourier transformation is not necessary.



**Figure 4.16.** Phase correction of spectrum. (a) The spectrum contains a zero order phase error of  $40^\circ$ . All peaks are distorted by same amount. (b) The spectrum contains a first order phase error of  $40^\circ$ . The degree of distortion of the spectrum is frequency dependent and peaks at downfield are off more than those in the upfield region. (c) Spectrum with correct phase.

The phase error for the indirect dimension can be corrected after the  $t_1$  Fourier transformation (see the following section). However, the first order phase error in the indirect dimension causes problems such as baseline distortion and dispersive folded cross-peaks, which significantly degrade the sensitivity of the spectrum. To avoid these problems, the phases can be set before the acquisition according to the relationships:

$$\begin{aligned}\phi_1 &= -\tau \times \text{SW} \times 360^\circ \\ \phi_0 &= -\frac{1}{2}\phi_1\end{aligned}\quad (4.42)$$

in which SW is the spectral window of the indirect dimension, and  $\tau$  is the sampling delay, which is the sum of all delays and  $180^\circ$  pulses in the evolution time. The  $90^\circ$  pulses that flank the evolution time are also included in the sampling delay in terms of the  $90^\circ$  pulse length multiplied by  $2/\pi$ . The sampling delay  $\tau$  is set to  $\tau = 0$  or  $\tau = \text{DW}/2$  (DW is the dwell time of the indirect dimension. See Table 4.2 for the relationship between SW and DW in the  $t_1$  dimension), resulting in  $\phi_0 = 0^\circ$ ,  $\phi_1 = 0^\circ$  for  $\tau = 0$  and  $\phi_0 = 90^\circ$  and  $\phi_1 = -180^\circ$  for  $\tau = \text{DW}/2$ . The conditions can be met by adjusting the initial value of  $t_1$  as described in the following pulse sequence elements:

- (a) Homonuclear evolution period  $90^\circ$ – $t_1$ – $90^\circ$   
The sampling delay is given by:

$$\tau = t_1(0) + \frac{4 \times \text{pw}_{90}}{\pi} = \frac{\text{DW}}{2} \quad \text{for } \phi_0 = 90^\circ \text{ and } \phi_1 = -180^\circ \quad (4.43)$$

**TABLE 4.2**  
Methods of Quadrature Detection in the Indirect Dimension

Methods	DW	Exp. per incr.	Phase shift	$\tau$	Phase correction	References
States	1/SW	2	90° for same incr. 0° per incr.	DW/2	90°, -180°	States <i>et al.</i> , 1982
TPPI	1/(2 × SW)	1	90° per incr.	DW	90°, 0°	Marion and Wüthrich, 1983
States-TPPI	1/SW	2	90° for same incr. 90° per incr.	DW/2	90°, -180°	Marion <i>et al.</i> , 1989

The initial  $t_1$  value is calculated according to:

$$t_1(0) = \frac{DW}{2} - \frac{4 \times pw_{90}}{\pi} \quad (4.44)$$

in which  $t_1(0)$  is the initial value of  $t_1$ , and  $pw_{90}$  is the 90° pulse length. The effect of the phase variation during the flanking 90° pulses can be removed from the sampling delay in the pulse sequence element 90°- $t_1$ - $\delta$ -180°- $\delta$ -90°, in which  $\delta$  is a fixed delay. Because the 180° pulse is placed symmetrically in the initial evolution period ( $t_1 = 0$ ) and refocuses the effect of the phase variation, the sampling delay is zero. Consequently,  $t_1(0)$  can be set to  $t_1(0) = DW/2$  or  $t_1(0) = 0$ . For  $t_1(0) = 0$ , the first point of the interferogram is usually scaled by a factor of 0.5 prior to Fourier transformation (Otting *et al.*, 1986).

(b) HMQC element

- (i) With spin decoupling 90°(X)- $\frac{1}{2}t_1$ -180°(<sup>1</sup>H)- $\frac{1}{2}t_1$ -90°(X)

The sampling delay is given by:

$$\tau = t_1(0) + pw_{180(^1H)} + \frac{4 \times pw_{90(X)}}{\pi} = \frac{DW}{2} \quad (4.45)$$

$$t_1(0) = \frac{DW}{2} - pw_{180(^1H)} - \frac{4 \times pw_{90(X)}}{\pi} \quad (4.46)$$

in which  $pw_{180(^1H)}$  and  $pw_{90(X)}$  are the pulse lengths of a <sup>1</sup>H 180° pulse and a heteronuclear 90° pulse, respectively.

- (ii) Without spin decoupling 90°(X) -  $t_1$ -90°(X)

The initial  $t_1(0)$  is set to:

$$t_1(0) = \frac{DW}{2} - \frac{4 \times pw_{90(X)}}{\pi} \quad (4.47)$$

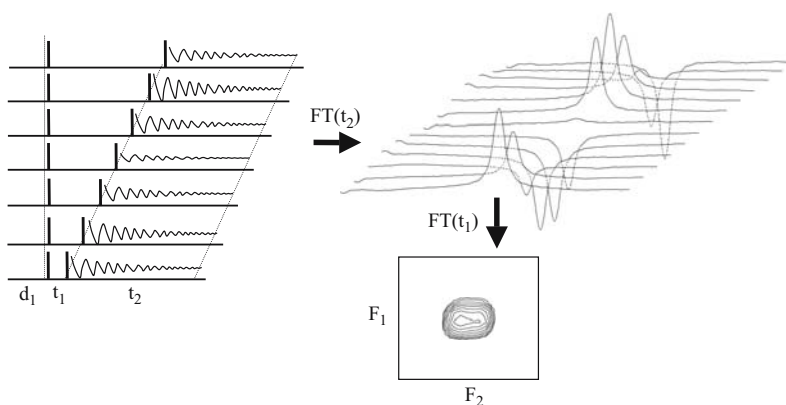
- (iii) Constant time 90°- $\frac{1}{2}t_1$ - $T$ -180°-( $T - \frac{1}{2}t_1$ )-90°

Because the refocusing 180° pulse is placed symmetrically in the initial evolution period ( $t_1 = 0$ ), the sampling delay is zero. As mentioned earlier,  $t_1(0)$  can be set to either  $DW/2$  or zero.

## 4.10. TWO-DIMENSIONAL EXPERIMENTS

### 4.10.1. The Second Dimension

The signals of protons in a protein sample recorded in one-dimensional NMR experiments are not able to be completely assigned due to severe overlapping of the signals. To improve the resolution for spectral assignment, a second frequency dimension is introduced to disperse the signals over two frequency dimensions, forming a two-dimensional NMR spectrum. In a one-dimensional experiment, the FID is acquired after an RF pulse or pulses (called the preparation period). In a two-dimensional (2D) experiment, one additional period called the evolution time, which contains a variable time delay  $t_1$ , is introduced into the experiment between the preparation and acquisition periods (Figure 4.17). The evolution delay increases systematically by the same amount of time for each increment during a two-dimensional experiment from zero to the final value determined by the number of increments. An FID is acquired for one increment with the starting value of the  $t_1$  evolution time. For the next increment, an FID is acquired with an increased  $t_1$  and all other parameters are the same as the previous FID, and so on for the required number of increments. After the experiment is done, all the FIDs are transformed with the same phase parameters. The phase of the signals is modulated during the evolution period as a function of the  $t_1$  evolution time. As a result, the amplitudes of the signals after Fourier transformation vary sinusoidally as a function of the  $t_1$  evolution time, which forms an interferogram similar to an FID when the amplitude of a peak is plotted as a function of  $t_1$  (Figure 4.17). Fourier transformation of the second FID obtained by the  $t_1$  evolution time generates another frequency domain as the transformation of the acquired FIDs. The result of the two Fourier transformations is a two-dimensional NMR spectrum with two frequency axes and an intensity axis on the third dimension that is usually plotted as contours. The second dimension can be frequency for  $^1\text{H}$ , which gives a square spectrum with diagonal peaks, or a heteronucleus, which gives an asymmetric spectrum. 2D experiments may also contain other



**Figure 4.17.** Two-dimensional experiment and two-dimensional spectrum. The evolution time  $t_1$  is increased systematically for each of the FIDs. The FIDs acquired during  $t_2$  are Fourier transformed to obtain a set of 1D spectra. The second Fourier transformation is applied along  $t_1$ , that is, each point of the spectra, resulting in a cross-peak coordinated by both frequencies on  $F_2$  and  $F_1$ . The  $F_2$  dimension has better digital resolution because  $t_2$  is always longer than  $t_1$ . The parameter  $d_1$  is the relaxation delay (or predelay) and  $t_2$  is the acquisition time.

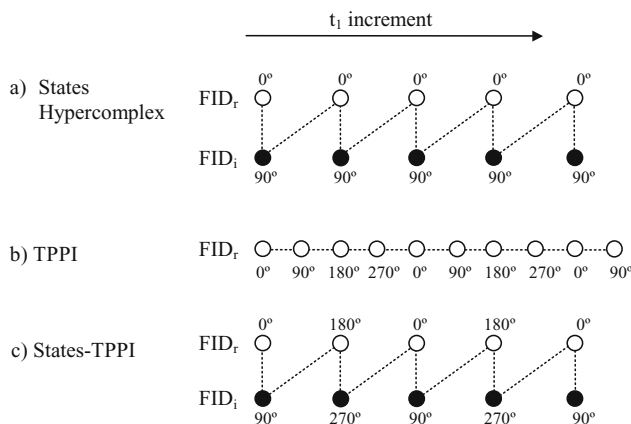
periods in addition to the evolution time, such as a mixing period. However, it is the evolution time that introduces the additional dimension into a spectrum. Similarly, more dimensions can be formed by introducing additional evolution time periods. The widely accepted terminology is that for  $n$ D data,  $t_1, \dots, t_{n-1}$  are evolution delays, and  $t_n$  is the acquisition period, whereas  $F_1, \dots, F_{n-1}$  are the indirect dimensions generated by the corresponding evolution times and  $F_n$  is the direct dimension from the observed FID. For instance, in a three-dimensional spectrum, the  $F_3$  dimension is obtained from the transformation of the observed FID or  $t_3$ , which is the direct dimension, whereas the  $F_1$  and  $F_2$  dimensions are from the evolution times  $t_1$  and  $t_2$ , respectively, and hence the indirect dimensions.

Before setting up a two-dimensional experiment, calibrations of pulses and the water resonance frequency and optimization of the spectral window should first be performed. The spectral window is set to the value optimized in the one-dimensional spectrum. The acquisition is generally set to about 200 ms for homonuclear experiments or 64 ms for heteronuclear experiments with  $^{15}\text{N}$  or  $^{13}\text{C}$  decoupling during acquisition. If each FID is acquired with the number of points  $np$ , and number of increments is  $ni$ , the final size of the data file is  $np \times ni$ . Assuming that each transient of an FID takes  $n$  sec to finish ( $n$  is called the recycle time) and  $nt$  transients are taken for each FID with  $ni$  increments, the total experiment time will be  $nt \times n \times ni$ . A two-dimensional experiment can take as little time as 2–15 minutes to finish (e.g., gCOSY, gHSQC). Instrument software can be used to calculate the total experiment time before hand.

#### 4.10.2. Quadrature Detection in the Indirect Dimension

In order to correct the phase distortion of cross-peaks, quadrature detection in the indirect dimension is necessary to obtain a phase sensitive two-dimensional spectrum. This is achieved by hypercomplex or TPPI methods similar to quadrature detection in the observed dimension (see section 2.4). In the hypercomplex method (also known as the States method; States *et al.*, 1982), two FIDs are recorded for each  $t_1$  increment. In the second FID, the phase of the preparation pulse or pulses (the pulse or pulses prior to the  $t_1$  evolution period) shifts by  $90^\circ$  (Figure 4.18). Two sets of data are obtained: one with  $0^\circ$  phase and the other with  $90^\circ$  phase. After the first Fourier transformation, the  $t_1$  interferogram is constructed by taking the real part of the  $0^\circ$ -phase data (open circles in Figure 4.18) as the real part of the interferogram, and the real part of the  $90^\circ$ -phase data (filled circles) as the imaginary part of the interferogram. Therefore, both parts of the interferogram are from absorptive data. A phase sensitive spectrum is obtained after the interferogram is transformed by complex Fourier transformation. The  $t_1$  increment is set to  $1/(2 \times SW_1)$  in which  $SW_1$  is the spectral window in the  $F_1$  dimension.

In the TPPI method (Figure 4.18b; Marion and Wüthrich, 1983), the phase of the preparation pulse shifts  $90^\circ$  for every  $t_1$  increment. After the first Fourier transformation, the  $t_1$  interferogram is constructed by taking the real part of all FIDs as the real part and the interferogram is transformed by real Fourier transformation, resulting in a phase sensitive spectrum. Similar to the sequential acquisition method, the TPPI method requires twice as fast  $t_1$  increments as in the hypercomplex method. Therefore, the  $t_1$  increment is set to the  $1/SW_1$ . The advantage of TPPI relative to the hypercomplex method is that axial peaks appear at the edge of the  $F_1$  dimension ( $F_1 = 0$ ), whereas axial peaks are located in the center of the two-dimensional spectrum with the States method, which overlap with the signals of interest.



**Figure 4.18.** Quadrature detection methods in the indirect dimension. Each circle represents an FID: the open circles represent the FIDs used for the real part ( $FID_r$ ) of the  $t_1$  interferogram. The filled circles represent the FIDs used for the imaginary part ( $FID_i$ ) of the  $t_1$  interferogram. The values above or below the circles represent the relative phases of the pulse (or pulses) before the  $t_1$  evolution period in a pulse sequence. The dotted lines represent the order of FIDs being acquired. (a) The States or hypercomplex method. For each  $t_1$  increment, two FIDs are acquired. After  $t_2$  Fourier transformation, the real parts of the open-circled FIDs form the real part of the  $t_1$  interferogram and the real parts of the filled FIDs form the imaginary part of the  $t_1$  interferogram. Complex FT is then applied to transform  $t_1$  to the  $F_1$  dimension. (b) TPPI method. The  $t_1$  is incremented for every FID along with a  $90^\circ$  shift of the relative phase of the pulse (or pulses) before the  $t_1$  evolution period. The real parts of the FIDs form the real part of the  $t_1$  interferogram, which is then transformed by a real Fourier transformation. (c) States-TPPI method. The data are acquired in the same way as the States method except the relative phase is increased by  $90^\circ$  after each FID is acquired. As a result, the axial peaks are suppressed by shifting them to the edge of the spectrum. Complex FT is used in both dimensions.

However, TPPI has the disadvantage of undesirable folding properties (Marion and Bax, 1988). To overcome this disadvantage while still suppressing the axial peaks, a combination of the States and TPPI methods was developed (Marion *et al.*, 1989).

The States-TPPI method combines the two methods together as shown in Figure 4.18c. The data are acquired in the same way as the States method except that the phase of the preparation pulse shifts  $90^\circ$  after each FID is acquired. As a result, the axial peaks are suppressed and the spectrum does not have the TPPI folding properties. The  $t_1$  increment is set to  $1/(2 \times SW_1)$ , the same as in the States method. Complex Fourier transformations are used in both dimensions.

#### 4.10.3. Selection of Coherence Transfer Pathways

When quadrature detection is used, only coherence with the order of either  $+1$  or  $-1$  can be observed (single quantum coherence), but not both at the same time. The common choice assumes that the coherence order of  $-1$  is observed in all experiments by quadrature detection. An initial non- $90^\circ$  pulse generates higher order coherence in addition to the single quantum coherence. Therefore, there exist many other coherence transfer pathways along with the desired pathway at the end of a pulse sequence, which produce observable signals during acquisition time. To select the specific coherence transfer pathway and eliminate the

unwanted signals from the other pathways, either phase cycling or gradient pulses are usually utilized.

The change in the phase of the coherence caused by an RF pulse or gradient pulse depends on its coherence order  $p$ . The selection of the coherence pathway is based on this property of the coherence phase. Phase cycling uses changes in the phase of RF pulses that lead to different phase shifts of the coherence. If the phase of an RF pulse is set to  $\phi$ , the phase shift gained by the coherence experiencing a coherence order change  $\Delta p$  by the pulse is given by  $-\phi \Delta p$ . A particular coherence transfer pathway can be selected by phase cycling using  $N$  steps with increments of  $\phi = 360^\circ/N$ . Phase cycling is the process in which the phases of pulses in a pulse sequence are shifted after each acquisition during the signal averaging (detailed treatment of phase cycling methodologies can be found in Bodenhausen *et al.*, 1984; Bain, 1984). The pathway selection by phase cycling requires precise phase shifts of RF pulses and repeated acquisitions with different phase shifts of the pulses. It frequently is not the method of choice. In recent years, coherence selection by gradient pulses is more commonly used in multidimensional NMR experiments to reduce experimental time in addition to other advantages.

The coherence selection by gradient pulses is achieved by applying a gradient pulse to dephase and then refocus a specific coherence while allowing the unwanted coherence to continue dephased. Unlike the phase cycling method, phase changes by gradient pulses only depend on the coherence order at the time the gradient pulse is applied, not the difference of the two coherence orders. The spatially dependent phase induced by a gradient pulse with field strength  $G$  applied on a coherence of order  $p$  for a duration  $\tau$  is given by:

$$\phi = G\tau\gamma p \quad (4.48)$$

in which  $\gamma$  is the gyromagnetic ratio of nuclear isotope. In the case of a heteronuclear system and a shaped gradient, the phase is described by:

$$\phi = sG\tau\gamma p \quad (4.49)$$

in which  $s$  is the shape factor that describes the amplitude profile of the gradient pulse. Considering a pair of simple dephasing–refocusing gradient pulses to select a specific homonuclear coherence transfer pathway from  $p_1$  to  $p_2$ , the dephasing gradient pulse of duration  $\tau_1$  prior to the RF pulse induces a phase of coherence with order  $p$ :

$$\phi_1 = s_1 G_1 \tau_1 p_1 \quad (4.50)$$

Similarly, the refocusing gradient pulse of duration  $\tau_2$  following the pulse generates a phase:

$$\phi_2 = s_2 G_2 \tau_2 p_2 \quad (4.51)$$

The condition for the selected coherence to be refocused at the end of the refocusing gradient pulse is that the overall phase change ( $\phi_1 + \phi_2$ ) after the gradient pulses becomes zero. Because usually the gradient pulses are applied with the same shape in a pulse sequence, the shape factor  $s$  may only be different by the sign of the gradient,  $+$  or  $-$ . For the use of two gradient pulses, the selection condition can be rearranged as:

$$s_1 G_1 \tau_1 p_1 = -s_2 G_2 \tau_2 p_2 \quad (4.52)$$

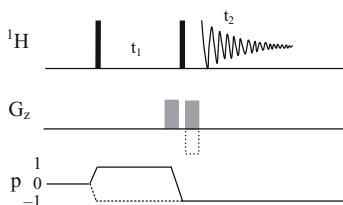
This states that the desired coherence transfer pathway is achieved by adjusting the duration, amplitude, and the sign of the gradient pulses and the unwanted pathways can be removed when the condition is not met for other coherences, provided the amplitude of the gradient pulses is sufficiently high to completely dephase the unwanted coherences. When more than two gradient pulses are used for the coherence selection, the refocusing condition is also the same that the overall phase induced by all gradients for the selected pathway is zero:

$$\sum_i \phi_i = \sum_i s_i G_i \tau_i p_i = 0 \quad (4.53)$$

This condition applies to all combinations of gradients used in the coherence selection. When more than two gradients are used, the combination for dephasing and refocusing can be different. For instance, the coherence can be continuously dephased by many gradients until the final gradient right before acquisition refocuses the desired coherence. Alternatively, the set of gradients can be used to select one part of the preferred pathway whereas others select the other part of the pathway. More examples are discussed in detail for some two-dimensional experiments in the following sections.

#### 4.10.4. COSY

Shown in Figure 4.19 is the gradient version of the two-dimensional **C**orrelation **S**pectroscopy experiment, abbreviated as COSY, which was one of the earliest two-dimensional experiments. It has the simplest two-dimensional pulse sequence consisting of two  $90^\circ$  pulses separated by the evolution time,  $t_1$ . The first  $90^\circ$  pulse rotates the equilibrium magnetization to the transverse plane and generates single-quantum coherence. During the evolution time  $t_1$ , the single-quantum coherence evolves, resulting in  $F_1$  frequency labeling of the detected coherence. The last pulse transfers the magnetizations between spins via the scalar coupling between them. Finally, the correlated coherence is detected as the FID. The coherence selection is achieved by the gradient pulses following each RF pulse.



**Figure 4.19.** COSY pulse sequence with coherence transfer pathway diagram. The general points for coherence selection using a coherence transfer pathway are described as follows. The coherence transfer pathway must start at the equilibrium state in which the magnetization is along the field direction,  $z$  magnetization, represented by a coherence order of  $p = 0$ . The first pulse acting on the equilibrium  $z$  magnetization gives rise to only single quantum coherence  $p = \pm 1$ . Delays without an RF field do not change the coherence order. Finally, the pathway must terminate at  $p = -1$  as quadrature detection is used to observe the complex signals. The solid line in the coherence transfer pathway diagram and the solid gradient represent N-type selection, whereas the dotted coherence pathway and the open gradient represent P-type selection.



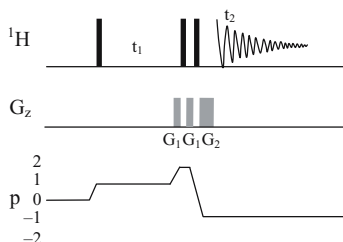
There are two possible pathways for acquiring COSY spectra, as shown in Figure 4.19. A pathway through  $p = -1$  is known as P-type (or anti-echo) in contrast to that through  $p = +1$ , known as N-type (echo). The frequency modulations of the coherence during  $t_1$  and  $t_2$  have the same sign for the P-type pathway, whereas they have an opposite sign for the N-type pathway. The N-type COSY is recorded using the last gradient with the same sign as the first one because the refocusing condition is obtained by  $G_1 p_1 = -G_2 p_2$  in which  $p_1 = 1$  and  $p_2 = -1$ , assuming that both gradient pulses have the same amplitude, shape, and duration. The P-type spectra can be similarly obtained by setting the refocusing gradient pulse with the sign opposite to the first one with the same duration and amplitude. The selection of either the N- or P-type pathway by gradient pulses results in frequency discrimination in the  $F_1$  dimension without using any of the quadrature detection methods. However, the two pathways are not usually selected simultaneously.

Both pathways give rise to phase-twisted line shapes containing the superposition of absorptive and dispersive signals, which is caused by the use of the gradient during the evolution period. A phase sensitive spectrum can be constructed by acquiring both types of data separately and then combining them in such way that the axis-reversed N-type spectrum is added to the P-type one. In the resulting spectrum, the dispersive portion due to the phase twist is cancelled and complete absorptive line shapes are obtained (see quadrature detection in the indirect dimension). The phase sensitive spectrum acquired by this method has a reduction in sensitivity by a factor of  $2^{1/2}$  compared to that by the States, States-TPPI, or TPPI methods due to cancellation of the absorptive and dispersive signals. Alternatively, N- and P-type data can be combined in a different way to obtain a phase sensitive spectrum. Addition of the two data sets generates cosine-modulated data whereas subtraction of the two yields sine-function data. Hypercomplex data are formed by using the constructed cosine and sine portion of the data, which can be processed in the same way as the States method.

Digital resolution in the  $F_1$  dimension also has a great influence on the sensitivity of the COSY spectrum owing to the antiphase character of the cross-peaks. Because the cross-peaks contain scalar coupling, the last  $t_1$  increment must have a  $t_1$  value ( $t_{1,\max}$ ) greater than  $1/(4J)$ , whereas  $t_2$  is set to a value comparable to  $1/(2J)$ . Usually,  $t_{1,\max}$  is chosen in the range of 50–80 ms and  $t_2$  is approximately 200 ms. For example, the data collected with 2048 complex points and 600 increments yields  $t_{1,\max}$  of 50 ms and  $t_2$  of 170 ms for a spectral window of 10 ppm on 600 MHz instruments. Linear predication is frequently used to add sufficient data points for both the  $F_1$  and  $F_2$  dimensions.

#### 4.10.5. DQF COSY

Double quantum filtered (DQF) COSY is sometimes preferred over the COSY experiment because the intense peaks from uncoupled singlets are substantially attenuated and resolution in the region adjacent to the diagonal peaks is dramatically improved. The advantages of DQF COSY also include correction of the phase distortion present in COSY and the absorptive line shapes for both cross-peaks and diagonal peaks. The drawback of DQF COSY is reduced sensitivity, thus requiring longer experimental time. The basic pulse sequence of DQF COSY consists of three  $90^\circ$  pulses (Figure 4.20). The single quantum coherence generated by the  $90^\circ$  pulse evolves during  $t_1$ . After the second  $90^\circ$  pulse transfers the coherence into double quantum coherence, the last  $90^\circ$  pulse produces observable single quantum coherence. Only double quantum coherence will be selected by the gradient pulses. The last gradient pulse



**Figure 4.20.** Double quantum filtered COSY pulse sequence with coherence transfer pathway diagram. The RF pulses are  $90^\circ$  pulses. The double quantum coherence is selected by the second gradient with the coherence order  $p_2 = 2$ . The ratio of the gradients is  $G_2 = 3G_1$ . The N-type coherence pathway is shown in the coherence transfer pathway diagram.

refocuses the double quantum coherence dephased by the first two gradient pulses, leaving other coherence to continue dephased. According to the selection rules, the overall phase created by the gradient pulses must be zero. Assuming that the gradient pulses have the same duration, then

$$p_1G_1 + p_2G_2 + p_3G_3 = 1G_1 + 2G_1 - 1G_2 = 0 \quad (4.54)$$

$$G_2 = 3G_1 \quad (4.55)$$

The spectrum may contain phase errors caused by the application of the gradient pulse during  $t_1$  evolution. Higher order multi-quantum filter experiments can also be implemented using the coherence selection rule. For instance, acquired triple quantum filtered coherence is refocused by the condition:

$$G_1 + 3G_1 - 1G_2 = 0 \quad (4.56)$$

$$G_2 = 4G_1 \quad (4.57)$$

The above selection of double quantum coherence chooses the N-type coherence pathway ( $p = +2$ ) whereas the P-type pathway with  $p = -2$  is not selected by the gradient pulses. As a result, half of the initial magnetization does not contribute to the detected magnetization, causing a loss of signal compared to the data acquired by the phase cycling method. An absorption mode spectrum can be obtained by acquiring P- and N-type data and then combining the data as described above. The States or States-TPPI quadrature detection method can also be employed to obtain the quadrature detection in the  $F_1$  dimension, and thus produce pure absorption spectra.

#### 4.10.6. TOCSY

In a one-dimensional  $^1\text{H}$  spectrum of a protein, the side-chain region (up-field region) is very crowded with severe spectral overlap due to the large number of side-chain protons. In contrast, the chemical shifts of  $\text{H}^\text{N}$  protons are less crowded, especially for small proteins, and the peaks are sometimes well-resolved. It is useful in resonance assignment to transfer the

correlations of side-chain protons to the region of  $H^N$  proton chemical shifts. TOCSY (also known as HOHAHA, Braunschweiler and Ernst, 1983; Bax and Davis, 1985a,b) experiments were developed to obtain, through coherence transfer via  $^3J$  scalar couplings, the relayed correlations between spins within a spin system, a network of mutually coupled spins. COSY experiments provide the information about the correlation between spins, whereas TOCSY transfers the coherence to other coupled spins through the  $^3J$  coupling (through molecular bonds) by isotropic mixing. During the mixing period, the coherence along the  $z$  axis is transferred throughout the spin system under the interaction of scalar coupling, at the same time the multi-quantum coherence is dephased by the inhomogeneity of RF pulses in the isotropic mixing pulse train. Several methods for isotropic mixing have been used in different types of TOCSY experiments (section 4.5.2). Much effort has been spent on the development of mixing pulse sequences (also known as spin lock sequences) which use minimal RF power to produce wide isotropic frequency ranges. DIPSI series mixing sequences are most commonly used now, among others such as Mlev17, Waltz16, and clean-TOCSY.

During the mixing time, the magnetization of amide protons is transferred to other protons within amino acid residues through three-bond scalar coupling. Because there is no through-bond scalar coupling between the inter-residual amide proton  $H^N$  and  $\alpha$  proton  $H^\alpha$ , the magnetization cannot be transferred across the peptide bond. The transfer distance (the number of protons the  $H^N$  magnetization can reach) depends on the efficiency of the spin lock sequence, the length of mixing time, the coupling constants, and the relaxation rate of the molecule. Protons further away from the  $H^N$  proton require a longer mixing time to reach. Ideally, it is preferred to obtain magnetization transfer to every proton within the spin system. However, it is practically difficult to acquire a single TOCSY with a mixing time to optimize magnetization transfer from  $H^N$  to all other protons. The magnitude of transfer to neighboring protons decreases quickly as the mixing time exceeds 30 ms. For remote protons, the magnetization transfer is achieved with the mixing time for as long as 100 ms. The correlations between  $H^N$  and  $H^\alpha$ ,  $H^\beta$  can be observed in a TOCSY with a mixing time of 30 ms whereas a 60 ms or longer mixing time is required to achieve magnetization transfer from  $H^N$  to other side-chain protons. Normally, a TOCSY experiment is first acquired with a mixing time in the range of 30–60 ms to efficiently transfer  $H^N$  magnetization to the neighboring side-chain protons. Then, the experiment is repeated with a longer mixing time to favor the long range magnetization transfer of  $H^N$  magnetization to the end protons of the side chain, but is usually less than 100 ms to avoid magnetization loss caused by relaxation.

TOCSY data are collected in a phase sensitive mode in the  $F_1$  dimension, for example, States–TPPI, with 512 increments which yield 256 complex points in the  $F_1$  dimension. Trim pulses are normally set to 2 ms. The pulse length of the spin lock is dependent on the spectral window of the two-dimensional data. Usually, a  $90^\circ$  spin lock pulse is about 25–30  $\mu$ s, which covers a bandwidth close to 8–10 kHz, whereas a  $90^\circ$  excitation pulse is less than 10  $\mu$ s. It creates a sample heating problem when the  $90^\circ$  spin lock pulse is shorter than 20  $\mu$ s. The problem becomes severe for shorter  $90^\circ$  spin lock pulses in the case of clean TOCSY. A spin lock time in the range of 30–60 ms is required for protein samples, which can only be selected as an integer number of spin lock cycles. A typical set up involves calibrating a  $90^\circ$  pulse at high power, a  $90^\circ$  spin lock pulse at lower power, and optimizing the spectral window. If the data are acquired using less than 200 FIDs, linear prediction should be utilized to double data points for the  $F_1$  dimension. TOCSY data are processed with a square sine-bell function shifted by  $30^\circ$ – $60^\circ$  before Fourier transformation.

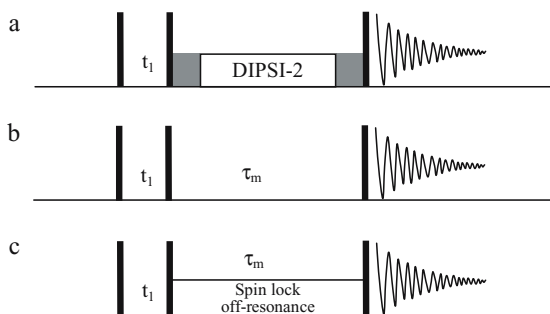
#### 4.10.7. NOESY and ROESY

COSY and TOCSY use scalar coupling (through bond nuclear interaction) to correlate the spins within a spin system. These types of experiments provide orientational information about the molecules via three-bond J coupling in addition to the correlation used in resonance assignment. The through-space nuclear interaction (dipolar interaction) is also used in multidimensional experiments to obtain distance information between spins for structural and dynamic studies of molecules. NOESY (nuclear Overhauser effect spectroscopy) and ROESY (rotating frame Overhauser effect spectroscopy, also known as CAMELSPIN for cross-relaxation appropriate for small molecules emulated by locked spins) experiments utilize the dipolar interaction in the form of cross relaxation to correlate spins that are close in distance (Bothner-By *et al.*, 1984; Bax and Davis, 1985b; Griesinger and Ernest, 1987).

During the mixing time,  $\tau_m$ , of the pulse sequences shown in Figure 4.21b/c, correlations between spins that are close in space occur via cross-relaxation, which give rise to cross-peaks in NOESY and ROESY spectra. In a NOESY experiment, the intensities of the cross-peaks are not only proportional to the cross-relaxation rate but also come from spin diffusion. Although the contributions to the cross-peak intensity from both mechanisms increase as the mixing time  $\tau_m$  increases, the spin diffusion effect can be minimized with a short mixing time. For protein samples, the mixing time is usually chosen in the range of 30–200 ms to avoid the spin diffusion effect. For a quantitative measurement of the internuclear distance, a series of NOESY experiments with different mixing times is collected and an NOE buildup curve is obtained by plotting the cross-peak intensity vs the mixing time for each individual resonance. The NOESY cross-peak intensity (or volume) is proportional to the length of the mixing time:

$$I_a = \xi \frac{\tau_m}{r_{ab}^6} \quad (4.58)$$

in which  $\xi$  is a constant for a given sample at a specific magnetic field strength,  $\tau_m$  is the mixing time, and  $r_{ab}$  is the distance between protons *a* and *b*. The initial linear range of the NOE buildup curve is used to calibrate and calculate the distance based on the cross-peak intensity relative to the intensity from a known distance. The constant  $\xi$  is first obtained by



**Figure 4.21.** Two-dimensional homonuclear pulse sequences. (a) In TOCSY, a DIPSI-2 pulse train is applied with low power during the spin lock period. The trim pulses (gray bars) are usually set to 2 ms. (b)  $\tau_m$  is the mixing time in the NOESY sequence. (c) Spin lock pulses during the mixing time  $\tau_m$  in the ROESY experiment are set to off-resonance to avoid the generation of TOCSY cross-peaks that have an opposite sign to the ROESY cross-peaks. Solid bars represent 90° pulses.

measuring the cross-peak intensity of a NOESY spectrum with a short mixing time for a known internuclear distance:

$$\xi = I_{\text{ref}} \frac{r_{\text{ref}}^6}{\tau_m} \quad (4.59)$$

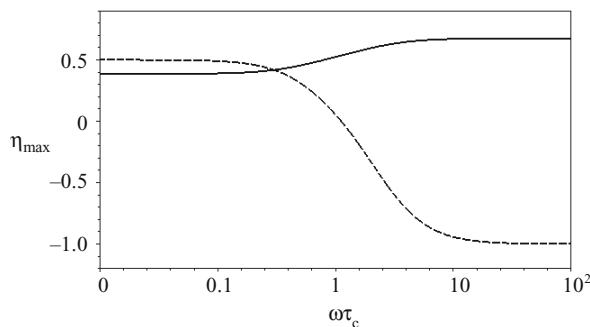
in which  $I_{\text{ref}}$  is the cross-peak intensity of a reference proton to another proton with a fixed and known distance  $r_{\text{ref}}$ .  $\xi$  is used to calculate the distance  $r_{\text{ab}}$  from  $I_a$  for all cross-peaks. Alternatively, the cross-peak intensity  $I_{\text{ref}}$  from  $r_{\text{ref}}$  can be used directly to calculate the distances for other protons using the linear range of the NOE buildup curve:

$$r_{\text{ab}}^6 = \frac{I_{\text{ref}}}{I_a} r_{\text{ref}}^6 \quad (4.60)$$

The intensity ratio of each assigned cross-peak is used to obtain the distance  $r_{\text{ab}}$ .

In addition to the spin diffusion effect, another limitation of the NOESY experiment is that the NOE enhancement is close to zero when  $\omega\tau_c$  is approximately 1 (small and medium size molecules) as indicated in Figure 4.22. To overcome these problems, the ROESY experiment has been developed. The pulse sequence of the ROESY experiment is similar to TOCSY although the cross-peaks of a ROESY spectrum has an opposite phase to those in the TOCSY spectrum. The ROE enhancement is always positive in all regimes, and it is 0.38 for  $\omega\tau_c \ll 1$  and 0.68 for  $\omega\tau_c \gg 1$  (Figure 4.22). The presence of TOCSY peaks in the ROESY spectrum will reduce the intensity of the ROESY cross-peaks. A typical solution is to use an inefficient spin lock scheme with an off-resonance  $B_1$  to mismatch the Hartmann–Hahn matching condition that is required for the TOCSY experiment. Usually, the spin lock field strength for a ROESY experiment is in the order of 1–5 kHz and off-resonance from the carrier frequency by several kilohertz. Such a  $B_1$  field is not sufficient for TOCSY isotropic mixing.

NOESY and ROESY data are collected in a phase sensitive mode in the  $F_1$  dimension, for example, States–TPPI, with 512 increments that yield 256 complex points in the  $F_1$  dimension. A mixing time in the range of 30–200 ms is usually used for NOESY, whereas the mixing time for the ROESY experiment is in the range of the rotating frame relaxation time  $T_{1\rho}$ , which



**Figure 4.22.** NOE/ROE enhancement factor  $\eta_{\text{max}}$  as a function of  $\omega\tau_c$ . The steady-state NOE enhancement (dashed line) is close to zero when  $\omega\tau_c \approx 1$ , equals 0.5 for  $\omega\tau_c \ll 1$  (extreme narrowing limit), and  $-1.0$  for  $\omega\tau_c \gg 1$  (spin diffusion limit). The ROE enhancement (solid line) is always positive in all regimes, and it is 0.38 for  $\omega\tau_c \ll 1$  and 0.68 for  $\omega\tau_c \gg 1$ .

can be longer than 500 ms for small molecules. Spin lock power for the ROESY is usually set to be less than 5 kHz and several kilohertz off-resonance from the carrier frequency. The data are acquired using less than 200 FIDs and linear prediction should be utilized to double data points for the  $F_1$  dimension. The data are processed with a square sine-bell function shifted by 30–60° before Fourier transformation. The cross-peaks of a NOESY spectrum have the same phase as the diagonal peaks, whereas the cross-peaks of a ROESY spectrum have the opposite phase from the diagonal peaks.

## QUESTIONS

- 4.1. For a 14.1 T magnet with a proton resonance frequency of 599.89836472 MHz at 0.0 ppm, what is the  $^{13}\text{C}$   $180^\circ$  pulse length which gives a null at  $\text{C}^\alpha$ ?
- 4.2. For a 14.1 T magnet with a proton resonance frequency of 599.89836472 MHz at 0.0 ppm, what is the  $^{13}\text{C}$   $\text{C}'$  off-resonance  $90^\circ$  pulse length that does not disturb the on-resonance  $\text{C}^\alpha$ ?
- 4.3. When calibrating a shaped pulse (except a rectangular shaped pulse), which of the two pulse parameters (pulse length and pulse power) is usually adjusted? And why?
- 4.4. What pulse length should be used for the GARP decoupling scheme if a 10 kHz decoupling field strength is desired for  $^{13}\text{C}$  decoupling?
- 4.5. What is the difference between the watergate and water-flip-back watergate sequences? Why does a water-flip-back watergate generally yield superior water suppression for aqueous protein samples?
- 4.6. What happens to the spectrum if the receiver gain is set too high and how is the gain set for two- and three-dimensional experiments?
- 4.7. Why is a protein sample never spun when acquiring data (water suppression, two- and three-dimensional data)?
- 4.8. Why must nonspinning shims be optimized first before gradient shimming on  $z$  shims?
- 4.9. Which nucleus do you shim when gradient shimming on a 90%  $\text{H}_2\text{O}$ /10%  $^2\text{H}_2\text{O}$  sample? And explain why.
- 4.10. Why is there a splitting in the  $^2\text{H}$  spectrum of an alignment medium for dipolar measurement?
- 4.11. What is the modification of water-flip-back made to the watergate solvent suppression sequence and for what purpose is the modification?
- 4.12. Why does a weighting function for resolution enhancement almost always decrease the sensitivity of the spectrum?

- 4.13. Why is a non-shifted squared sine-bell function used for COSY data, but an 80–90°-shifted squared sine-bell function used for three-dimensional data?
- 4.14. Why is a 180° pulse used for  $^1\text{H}$  pulse length calibration but a 90° X pulse used for heteronuclear decoupler pulse length calibration?
- 4.15. How many frequency axes do one- and three-dimensional spectra have? Which parameter is present in any two- and three-dimensional experiment but not in any one-dimensional experiment?
- 4.16. What are the typical ranges of gradient strengths used for suppressing water magnetization, perfecting a 180° pulse, and coherence selection?
- 4.17. Assuming that 32000 DAC on an NMR spectrometer corresponds to the gradient strength of  $70 \text{ G cm}^{-1}$ , what is the DAC value for a gradient of  $-30 \text{ G cm}^{-1}$ ?

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