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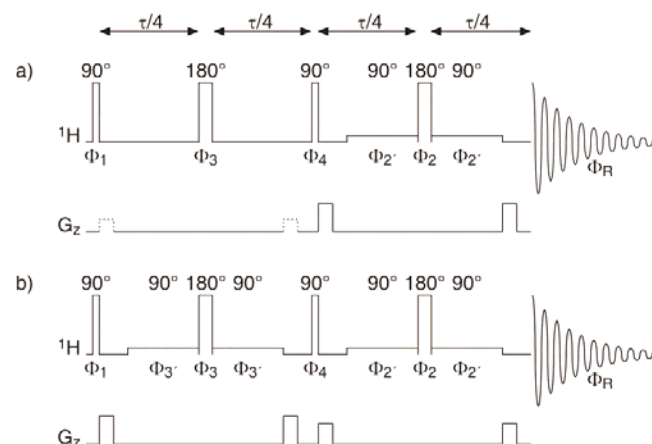
Perfecting WATERGATE: clean proton NMR spectra from aqueous solution**

*Ralph W. Adams, Chloe M. Holroyd, Juan A. Aguilar, Mathias Nilsson, and Gareth A. Morris**

The information about dynamics and structure that can be gained through the use of NMR spectroscopy makes it one of the most powerful tools in the analytical arsenal available to chemists and biologists. Many studies of chemical kinetics,[1] proteins[2] or metabolites[3] rely on samples that contain a protio solvent which introduces a strong signal.[4] Clean and efficient suppression of the solvent signal is a prerequisite for accurate interpretation.[5] Here a simple modification is described that significantly improves the spectra obtainable with one of the most widely-used water suppression techniques.

Many methods are available for signal suppression[6] but WATERGATE[7] (WATER-suppression by GrAdient-Tailored Excitation) is often the sequence of choice as it does not suppress signals from nuclei that exchange with the solvent. In normal use, WATERGATE employs relatively short, and hence not very frequency-selective, radiofrequency pulses, giving a wide excitation minimum around the water signal. This means that about 20% of the spectrum typically experiences some signal reduction, and any signals close to the solvent are obliterated. This is far from ideal: in the study of carbohydrates, for example, anomeric protons give signals very close to the water signal. If WATERGATE is to be used to measure such signals, its suppression band has to be narrowed significantly. This requires more frequency-selective pulses, which in turn require more time. The undesirable consequence is that evolution under the scalar coupling - J modulation - causes the relative signal phases within multiplets to change, distorting the spectrum and severely complicating analysis. In practical spectra the problem is not restricted to simple phase changes; where signals are crowded together, the presence of multiple overlapping signals with different phases can lead to severe signal loss. There is thus a strong incentive to suppress the effects of J modulation, particularly where a narrow water suppression band is required and/or quantitation is important; one area where both of these apply is metabolomics. In this paper we present a simple solution to the unwanted intrusion of J modulation in WATERGATE experiments by using the “perfect echo” (PE).

J modulation in WATERGATE arises because a spin echo is used to refocus evolution under the chemical shift, but does not refocus the effects of scalar coupling. In contrast, for echo times short compared to $1/J$ the perfect echo refocuses both shifts and J couplings.[8] In the perfect echo, an orthogonal 90° pulse placed at the centre of a double spin echo exchanges coherence between spins, reversing the apparent sense of J modulation. The second half of the double spin echo therefore refocuses the modulation caused by the



first. Until recently this effect was believed to be restricted to AX

Figure 1. NMR pulse sequences for a) the prefocussed PE-WATERGATE sequence and b) the PE-ES-WATERGATE sequence. Both sequences are shown with suppression elements that comprise a hard 180° pulse flanked by selective 90° pulses and pulsed field gradients. The coherence transfer pathway can be enforced using the PFGs indicated with the dotted lines. Φ_4 is quadrature to Φ_1.The pulses are phase cycled in the order implied by Φ_x.

spin systems, but it can be shown to hold for arbitrary spin systems if $\tau J << 1$ and the spin system is initially at equilibrium.[9] Replacing a spin–echo with a perfect echo effectively suppresses J modulation in both strongly and weakly coupled spin systems. Some extra T_2 weighting results, but this is generally a small price to pay.

Modification of the WATERGATE sequence to use a perfect echo rather than a simple spin echo can easily be achieved, for example by placing a spin echo and quadrature 90° pulse in front of a WATERGATE scheme to “prefocus” evolution under the J coupling, Figure 1(a). The WATERGATE part of the sequence then refocuses the J evolution. The WATERGATE block can use any of the frequency-selective elements commonly used, including rectangular soft pulses, binomial-type pulse trains and shaped pulses.

When two cycles of the WATERGATE scheme are used, in a variant of the experiment commonly referred to as “excitation sculpting” (ES),[10] the quadrature 90° pulse can be placed at the centre of the excitation sculpting double spin echo, Figure 1(b). Any solvent signal that has eluded the first WATERGATE element, and been excited by the quadrature pulse, is suppressed by the second WATERGATE element. However, the method only works with individual WATERGATE blocks that do not generate chemical-shift dependent phases: normal excitation sculpting can

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accommodate such sequences, as the second WATERGATE refocuses the phase shifts introduced by the first, but here the need for the extra 90° pulse to be in phase with the transverse magnetization means that phase shifts have to be avoided.

The PE-WATERGATE sequences reduce the limitation on echo time, allowing longer selective pulses to be used, to minimise the bandwidth of the water suppression and/or to optimise the shape of the resultant excitation spectrum. For most samples a total echo time of around 50 ms or less gives good suppression of J modulation. Allowing for field gradient pulses and stabilisation delays, the maximum duration of each of the selective radiofrequency pulses is then about 10 ms.

The benefits of using a perfect echo sequence over standard WATERGATE can be clearly seen in the ^{1}H NMR analysis of a common complex mixture, semi-skimmed milk.[11] Anomeric protons in sugars have NMR signals that typically occur close to the water signal, in milk the lactose anomeric signal occurs at 4.68 ppm at 25°C. To suppress the water signal but retain the anomeric signals requires very narrow bandwidth selective pulses in the WATERGATE blocks.

In the WATERGATE NMR spectrum of milk, Figure 2(a), J modulation is evident throughout, and greatly distorts the region between 2.4 and 2.8 ppm. Perfect echo excitation sculpting suppresses the J modulation, giving the clean absorption mode spectrum of Figure 2(b). When excitation sculpting is applied to improve the solvent suppression, Figure 2(c), the doubling of the J evolution leads to severe signal loss between 2.0 and 2.4 ppm, as overlapping signals evolve into antiphase and hence cancel each other. The addition of the extra 90° pulse to give perfect echo excitation restores absorption mode and full intensity in Figure 2(d).

The shaded profiles in Figure 2 show the calculated excitation spectra for the 10.5 ms rectangular soft pulses used in the WATERGATE blocks in order to retain some excitation of the anomeric signals close to the water. The "wiggles" in the excitation spectra could be suppressed by using shaped selective pulses[12],[13], but only at the expense of a broader excitation null and hence greater loss of anomeric signal. One advantage of using shaped pulses would be to reduce the width of the transition band between suppression and full excitation, since resonances in this region experience rotations between 0 and 180°, compromising the suppression of the effects of J modulation.

The approach described here improves the WATERGATE family of experiments by suppressing J modulation in the resultant spectra. The ability of the WATERGATE method to suppress the solvent signal is not compromised by incorporation with the perfect echo sequence. PE-WATERGATE experiments can be run in a routine fashion with similar settings to a typical WATERGATE. Suppression of J modulation makes PE-WATERGATE spectra easier to analyse and interpret than conventional WATERGATE spectra.

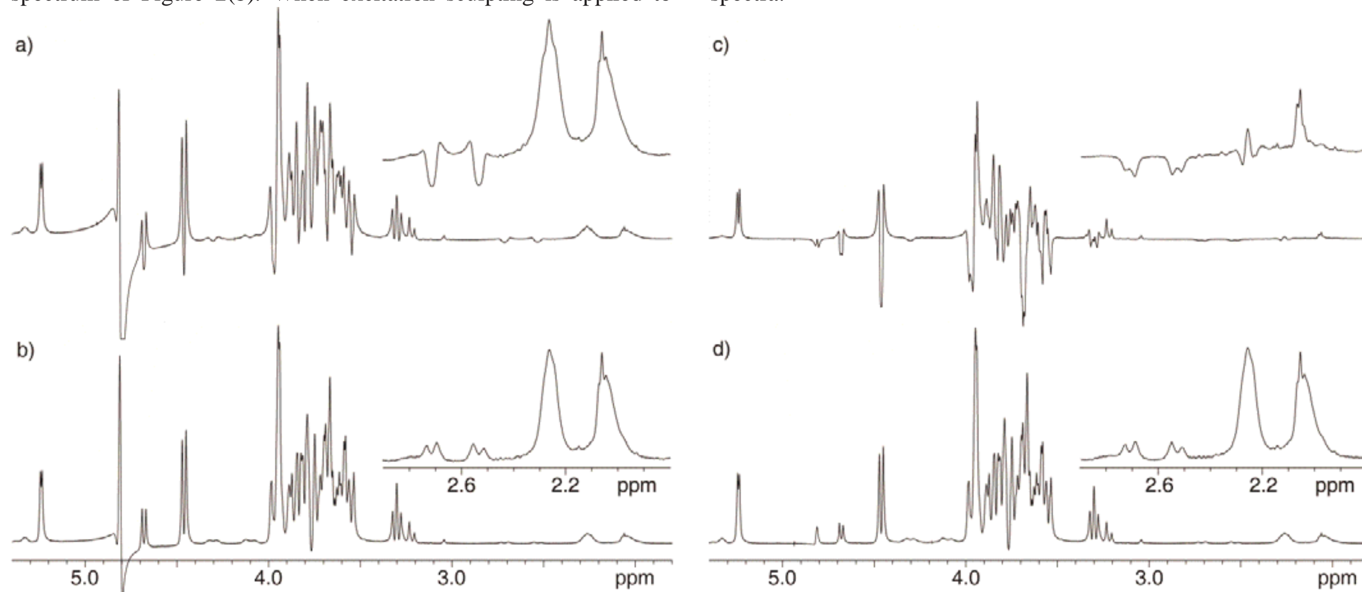

Figure 2. Spectra of semi-skimmed (1.7% fat) milk recorded using a) conventional WATERGATE, b) PE-WATERGATE, c) WATERGATE with excitation sculpting, and d) PE-WATERGATE with excitation sculpting, all using 10.5 ms rectangular 90° selective pulses. Calculated excitation profiles are overlaid.

Experimental Section

Samples were prepared by adding D_2O (Sigma-Aldrich, Poole, UK), 200 µl, and sodium-3-trimethylsilylpropionate (Cambridge Isotope Laboratories, Inc., Andover, MA, USA), c. 1 mg, to “1.7% fat” semi-skimmed milk (J Sainsbury plc, London, UK), 800 µl. Spectra were obtained using a 400 MHz Varian INOVA spectrometer (Agilent Technologies, Santa Clara, CA, USA). 32k complex points were acquired for 32 transients with a recycle delay of 2 s. The phases of the WATERGATE 90° soft pulses of duration 10.5 ms were adjusted for optimum water suppression.[14] Pulsed field gradients of 17 G cm^{-1} (and 6.5 G cm^{-1} in the excitation sculpting experiments), applied for 1 ms followed by a 2 ms stabilisation delay, were used to dephase the water signal. 0.7 Hz line broadening was applied. The pulse sequence, including phase cycle, is included in the Supporting Information.

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NMR Solvent Suppression

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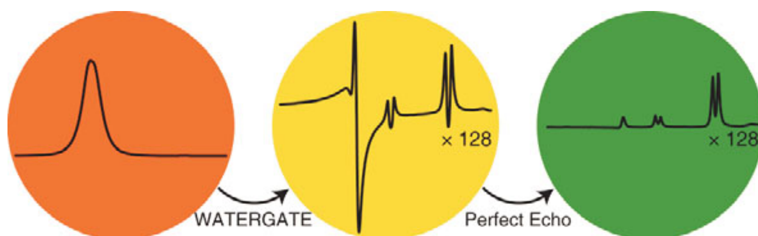
Perfecting WATERGATE: clean proton NMR spectra from aqueous solution



Water suppression, e.g. using WATERGATE, is a necessary component of many NMR experiments using protio solvents such as H_2O. J modulation distorts spectra measured using WATERGATE, making them difficult to interpret and changing relative signal intensities. Upgrading the WATERGATE sequence to use a “perfect echo” allows simultaneous suppression of the solvent signal and refocusing of the J modulation.

Supporting Information for "Perfecting WATERGATE: clean proton NMR spectra from aqueous solution "

```
/*
PEWGES.c
Perfect Echo WATERGATE sequence with soft pulses and optional excitation sculpting.

Ralph W. Adams, Chloe M. Holroyd, Juan A. Aguilar, Mathias Nilsson, and Gareth A. Morris
University of Manchester, Manchester, UK. July 2012
Varian/Agilent pulse sequence code for information only,
The University of Manchester and the authors cannot be held responsible
for any damage or loss resulting from the use of this sequence.

Timing:     |<   t/4   >|<   t/4   >|<   t/4   >|<   t/4   >|
1H:      90x      [S90]180[S90]   {90y}    S90 180 S90       Acquire
PFG:        G'                 G'      G              G

pe flag: {}
es flag: []
G':      0.379*G
S90:     Long duration (c. 10 ms), low power pulse
90/180:  Short duration (c. 8,16 us), high power pulse
t:       Total echo time, tJ << 1 for true perfect echo

gstab       - gradient stabilization delay
gt2         - gradient duration for the solvent suppression echo
gzlvl1      - gradient power for the solvent suppression echo
selpw        - power of the soft pulse
phaseinc - increments between the phases for the transmitter
stepsize - sets the phase of the transmitter to steps of 0.5 deg
xtmrphase    - sets the phase of the transmitter to phaseinc
txphase      - sets the phase of the transmitter to phasetable
selpwr       - power for the soft pulses
selpw        - duration of soft pulses
pe           - flag to use perfect echo
es           - flag to use excitation sculpting
dse          - flag to use a double spin echo, for debugging
*/

#include <standard.h>

pulsesequence()
{
/*Set Variables*/
static int   ph1[2]   = {0,2},
             ph2[32]  = {0,0,0,0,0,0,0,0,1,1,1,1,1,1,1,1,2,2,2,2,2,2,2,2,3,3,3,3,3,3,3,3},
             ph21[32] = {2,2,2,2,2,2,2,2,3,3,3,3,3,3,3,3,0,0,0,0,0,0,0,0,1,1,1,1,1,1,1,1},
             ph3[128] =
{1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1
,1,1,1,1,1,1,1,1,1,1,1,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3
,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3},
             ph4[32]  = {0,0,1,1,2,2,3,3,0,0,1,1,2,2,3,3,0,0,1,1,2,2,3,3,0,0,1,1,2,2,3,3},
             ph5[64]  =
{2,2,3,3,0,0,1,1,2,2,3,3,0,0,1,1,2,2,3,3,0,0,1,1,2,2,3,3,0,0,1,1,0,0,1,1,2,2,3,3,0,0,1,1,2,2,3,3,0,0,1,1,2
,2,3,3,0,0,1,1,2,2,3,3},
             rec[16]  = {0,2,2,0,0,2,2,0,2,0,0,2,2,0,0,2},
             rec2[16] = {0,2,0,2,0,2,0,2,2,0,2,0,2,0,2,0};
double   gstab    = getval("gstab"),
         gt2      = getval("gt2"),
             gzlvl1   = getval("gzlvl1"),
             selpwr   = getval("selpwr"),
             selpw    = getval("selpw"),
             phaseinc = getval("phaseinc");
char     pe[MAXSTR],
             es[MAXSTR],
             dse[MAXSTR];
rof1 = getval("rof1"); if(rof1 > 2.0e-6) rof1 = 2.0e-6;
getstr("pe",pe);
getstr("dse",dse);
getstr("es",es);
settable(t1,2,ph1);
settable(t2,32,ph2);
settable(t21,32,ph21);
settable(t3,128,ph3);
```

```
settable(t4,32,ph4);
settable(t5,64,ph5);
settable(t6,16,rec);
settable(t7,16,rec2);
/*Start Sequence*/
status(A);
     delay(d1);
status(B);
     obspower(tpwr);
     rgpulse(pw, t1, rof1, rof1);
     if (es[A] == 'y' ) {
          zgradpulse(gzlvl1*0.379,gt2);
          delay(gstab-rof1-rof1-rof1-rof1);
          if (phaseinc < 0.0) {
               phaseinc = 1440+phaseinc;
          }
          stepsize(0.25,OBSch);
          initval(phaseinc,v1);
          obspower(selpwr);
          xmtrphase(v1);
          txphase(t21);
          rgpulse(selpw,t21,rof1,rof1);
          obspower(tpwr);
          xmtrphase(zero);
          txphase(t2);
          rgpulse(pw*2.0,t2,rof1,rof1);
          obspower(selpwr);
          xmtrphase(v1);
          txphase(t21);
          rgpulse(selpw,t21,rof1,rof1);
          obspower(tpwr);
          xmtrphase(zero);
          zgradpulse(gzlvl1*0.379,gt2);
          delay(gstab);
     }
     if ((pe[A] == 'y'|dse[A] == 'y') && es[A] != 'y'){
          zgradpulse(gzlvl1*0.379,gt2);
          delay(selpw+gstab-rof1-rof1);
          rgpulse(pw*2.0,t2,rof1,rof1);
          zgradpulse(gzlvl1*0.379,gt2);
          delay(selpw+gstab-rof1-rof1);
     }
     if (pe[A] == 'y'){                                   /* Use 90d refocussing pulse */
          rgpulse(pw,t3,rof1,rof1);
     }
     if (pe[A] == 'y'|dse[A] == 'y'|es[A] == 'y'){
          zgradpulse(gzlvl1,gt2);
          delay(gstab-rof1-rof1-rof1-rof1);
          if (phaseinc < 0.0){
               phaseinc = 1440+phaseinc;
          }
          stepsize(0.25,OBSch);
          initval(phaseinc,v1);
          obspower(selpwr);
          xmtrphase(v1);
          txphase(t4);
          rgpulse(selpw,t4,rof1,rof1);
          obspower(tpwr);
          xmtrphase(zero);
          txphase(t5);
          rgpulse(pw*2.0,t5,rof1,rof1);
          obspower(selpwr);
          xmtrphase(v1);
          txphase(t4);
          rgpulse(selpw,t4,rof1,rof1);
          obspower(tpwr);
          xmtrphase(zero);
          delay(gstab/2.0);
          zgradpulse(gzlvl1,gt2);
          delay(gstab/2.0);
          setreceiver(t6);
     }
     if (pe[A] != 'y' && dse[A] != 'y' && es[A] != 'y'){
          zgradpulse(gzlvl1,gt2);
          delay(gstab-rof1-rof1-rof1-rof1);
          if (phaseinc < 0.0){
               phaseinc = 1440+phaseinc;
          }
          stepsize(0.25,OBSch);
```

```
        initval(phaseinc,v1);
        xmtrphase(v1);
        txphase(t21);
        rgpulse(selpw,t21,rof1,rof1);
        obspower(tpwr);
        xmtrphase(zero);
        txphase(t2);
        rgpulse(pw*2.0,t2,rof1,rof1);
        obspower(selpwr);
        xmtrphase(v1);
        txphase(t21);
        rgpulse(selpw,t21,rof1,rof1);
        obspower(tpwr);
        xmtrphase(zero);
        delay(gstab/2.0);
        zgradpulse(gzlvl1,gt2);
        delay(gstab/2.0);
        setreceiver(t7);
    }
status(C);
}
```