MAGNETIC RESONANCE IN CHEMISTRY
Magn. Reson. Chem. 2007; **45**: 937–941
Published online in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/mrc.2073

MRC

QQ-HSQC: a quick, quantitative heteronuclear correlation experiment for NMR spectroscopy

Daniel J. Peterson and Nikolaus M. Loening*

Department of Chemistry, Lewis & Clark College, 0615 SW Palatine Hill Road, Portland, OR 97219, USA

Received 25 June 2007; Revised 30 July 2007; Accepted 31 July 2007

Quantitative heteronuclear single quantum coherence (Q-HSQC) is a variant of the HSQC experiment that provides quantitative peak areas. This is accomplished by combining signals acquired using four different INEPT delays. Consequently, the experiment requires four times as many scans as the HSQC experiment to achieve the same resolution in the indirect dimension. We have removed this drawback by modifying the Q-HSQC experiment so that signals corresponding to different INEPT delays are acquired simultaneously from different parts of the sample. This new experiment, which we call Quick, Quantitative HSQC (QQ-HSQC), has the quantitative properties of the Q-HSQC experiment but only requires as many scans as a conventional HSQC experiment. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: 2D NMR; HSQC; quantitation

INTRODUCTION

The results from some NMR experiments, such as conventional one-dimensional spectra for fully-relaxed samples, can be considered to be quantitative. That is, the integral of a peak in the spectrum is directly proportional to the number of spins that contribute to that peak. Consequently, peak integrals from quantitative NMR experiments can be used to identify the relative number of spins from chemically inequivalent positions of a compound and/or to measure the relative concentrations of different components in a sample.

Many complex NMR experiments are not quantitative due to a number of contributing factors that perturb the peak integrals. For example, one reason that heteronuclear correlation experiments (such as HSQC,[1] HMQC,[2] and HetTOCSY[3]) are not quantitative is because the efficiency of the magnetization transfer steps depends on the value of the heteronuclear coupling constant. If a range of heteronuclear coupling constants exists in a sample, as is often the case, then the transfer steps cannot be optimized simultaneously for all spins. Consequently, the peak integrals will vary and the experiment will not be quantitative.

Recently, a variant of the HSQC experiment known as the quantitative HSQC (Q-HQSC) experiment has been developed.[4] This experiment is able to make the efficiency of the transfer steps relatively constant over a range of heteronuclear coupling constants, but at the cost of a four-fold increase in the number of scans per increment relative to conventional HSQC experiments. A more advanced version of the Q-HSQC experiment, referred to as the Q-CAHSQC experiment, has been developed to reduce the influence of resonance offset and homonuclear couplings on the peak integrals.[5] Like the Q-HSQC experiment, the Q-CAHSQC experiment also requires a four-fold increase in the number of scans per increment.

In this paper, we demonstrate the Quick, Quantitative HSQC (QQ-HSQC) experiment. This experiment has the same quantitative attributes as the Q-HSQC and Q-CAHSQC experiments, but requires only the same number of scans per increment as a conventional HSQC experiment. This means that for cases where signal-to-noise ratio is favorable quantitative experiments can be carried out in a quarter of the spectrometer time. The methodology that we employ to achieve this is similar to that used in a number of experiments for measuring diffusion coefficients,[6,7] longitudinal relaxation rates,[8] and homonuclear correlations,[9]. These experiments operate by, in effect, running separate experiments in different parts of the sample at the same time. This methodology is capable of reducing the experiment time by more than an order of magnitude in favorable cases, but usually at the expense of sensitivity. For the case of the QQ-HSQC experiment, we are able to make use of this methodology to reduce the number of scans by a factor of four *without* a decrease in sensitivity per scan relative to the Q-HSQC and Q-CAHSQC experiments.

THEORY

The volume of a peak (V) in an HSQC spectrum depends on the efficiency of the magnetization transfer of the two INEPT steps, which in turn depends on the heteronuclear coupling constant (J_{IS}). Consequently, the peak volume varies according to:

$$V \propto \sin^2(\pi \Delta J_{IS}) \quad (1)$$

*Correspondence to: Nikolaus M. Loening, Department of Chemistry, Lewis & Clark College, 0615 SW Palatine Hill Road, Portland, OR 97219, USA. E-mail: loening@lclark.edu

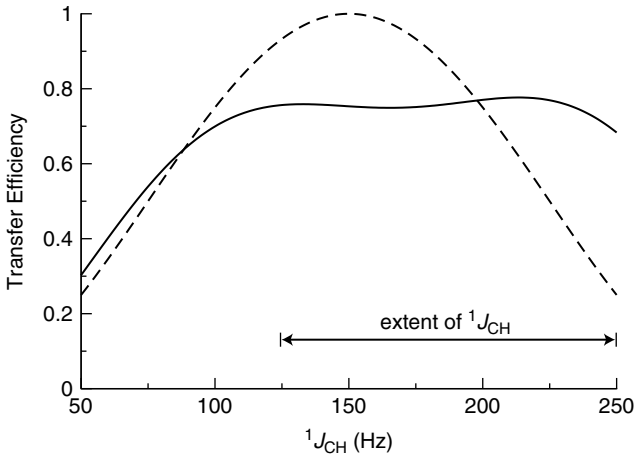

Figure 1. Dependence of the signal intensity on the coupling constant for the HSQC (dashed line) and Q-HSQC (solid line) experiments. The curves assume $\Delta = 3.33$ ms for the HSQC experiment, and $\Delta_{min} = 2.94$ ms and $\Delta_{max} = 5.92$ ms for the Q-HSQC experiment. The double arrow shows the extent of $^1J_{CH}$ found in organic compounds.

where Δ is the length of the INEPT steps. It is possible to maximize the transfer efficiency for a single value of the coupling constant by setting $\Delta = 1/2J_{IS}$. For example, an average value for the one-bond $^{13}C-^1H$ coupling constant ($^1J_{CH}$) is 150 Hz, so the optimal delay time Δ will be 3.33 ms. The transfer efficiency as a function of $^1J_{CH}$ that results from this value of Δ is shown as the dashed curve in Fig. 1. Different coupling values can be maximized by changing Δ, but if there is a range of coupling constants (as is often the case) then it is not possible to simultaneously maximize all of them in a single experiment. This means that the transfer efficiency will differ between peaks in the HSQC spectrum with the consequence that the peak integrals will not be quantitative.

In Q-HSQC,[4] the dependence of the transfer efficiency on J_{IS} is suppressed by summing signals from experiments with different values for the INEPT delays, thereby 'flattening' the transfer efficiency. For $^{13}C-^1H$ HSQC experiments, the best results are achieved by adding together data from experiments with Δ values of 2.94 and 5.92 ms in a 3:1 ratio.[4] The transfer efficiency of the Q-HSQC experiment is indicated by the solid line in Fig. 1. Although the transfer efficiency is reduced compared to an optimized HSQC experiment, it has a relatively constant transfer efficiency of 80% (±2%) in the range of 115 and 220 Hz. In practice, Q-HSQC as well as Q-CAHSQC spectra are acquired by adding three scans with Δ set to 2.94 ms and a fourth scan with $\Delta = 5.92$ ms for every increment in the indirect dimension of the HSQC experiment. This results in a four-fold increase in the number of scans required to complete the experiment relative to a conventional HSQC experiment (if signal-to-noise is not limiting).

For the quick, quantitative HSQC (QQ-HSQC) experiment, we use slice-selective adiabatic sweep pulses so that three-quarters of the active region of the sample experiences the shorter delay ($\Delta_{min} = 2.94$ ms) for the INEPT transfers, while the remaining quarter experiences the longer delay ($\Delta_{max} = 5.92$ ms). This preserves the 3:1 ratio required for the experiment to be quantitative. The sequence shown in Fig. 2 also incorporates XY-8 CPMG pulse trains for part of the transfer periods to make the peak volumes less dependent on small homonuclear couplings.[5]

EXPERIMENTAL

All experiments were conducted on a Bruker Avance 300 spectrometer equipped with a regular geometry broadband, *z*-axis gradient probe or a Bruker DRX 300 spectrometer equipped with an inverse geometry broadband, *z*-axis gradient probe. For testing the experiment a sample of 0.25 M strychnine in deuterated chloroform was used.

Magnetic field gradient strengths of 13.6 and 31.1 G cm^{-1} were used for the 2 ms-long homospoil pulses. The slice-selective gradients were set to 15 G cm^{-1} and were 250 μs-long. The coherence-transfer pathway selection gradients marked G_1 and G_2 were set to 40 and 10.05 G cm^{-1}, respectively; each of these was 2 ms-long.

Hyperbolic secant radiofrequency pulses were used for slice selection; these pulses were 250 μs in length and used a maximum field strength of 30 kHz. The hyperbolic secant pulses are applied in pairs so that phase errors from one

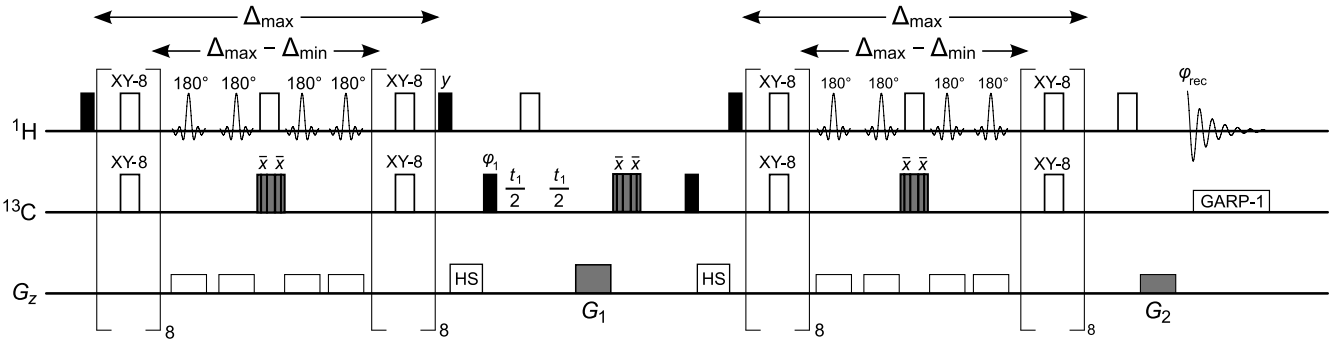

Figure 2. The pulse sequence for the QQ-HSQC experiment. Hard pulses are indicated by rectangles (filled for 90° pulses, hollow for 180° pulses, grey for composite 180° pulses), and slice-selective pulses are indicated by sinc functions. The bracketed blocks indicate XY-8 pulse trains that help reduce the sensitivity of the experiment to homonuclear scalar coupling.[5] The pulse phases are *x* unless otherwise indicated. For the gradient channel, hollow squares indicates gradients used for slice-selection, gray shading indicates gradients used for coherence transfer pathway selection, and ''HS'' indicates gradients used as homospoils.

DOI: 10.1002/mrc

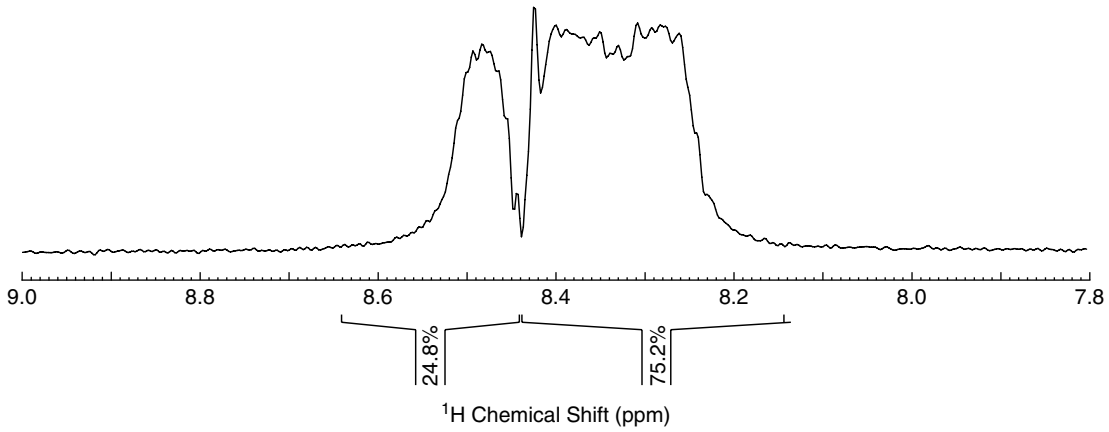

Figure 3. 300 MHz ^{1}H image of a sample of ^{13}C-labelled formaldehyde in deuterated water. The notch in the spectrum is the edge of the slice selected by the adiabatic pulses (which affect the right part of the image). By moving the transmitter offset for the adiabatic pulses, it is possible to calibrate the experiment to achieve the desired 1 : 3 ratio between the unselected and selected regions. To acquire this image, the pulse sequence for the QQ-HSQC experiment was used with the addition of a 0.015 G cm^{-1} gradient during aquisition. Four scans were used with a recycle delay of 10 s, and Δ_{min} and Δ_{max} were set to 2.56 and 7.68 ms, respectively. The image was generated by Fourier transformation of the resulting free induction decay; no line broadening or special manipulations were used.

are refocused by the next. These pulses are placed on the ^{1}H channel, rather than the ^{13}C channel, as the greater chemical shift range of ^{13}C nuclei as well as limitations on the bandwidth of the adiabatic pulses and the effective gradient strength make slice selection less quantitative if performed on the ^{13}C channel. All other ^{1}H pulses were applied using a field strength of 30 kHz.

Composite pulses were used on the ^{13}C channel for the 180° pulses. The composite pulses were 58 $\overline{140}$ 344 $\overline{140}$ 38 constant rotation pulses[10]; the numbers represent the desired rotation in degrees and the bars indicate a 180° phase shift. Regular 90° pulses were used on the ^{13}C channel as we found that using composite pulses in their place did not affect the bandwidth over which the experiment was quantitative. A 25-kHz RF field was used for all ^{13}C pulses with the exception of the GARP-1 decoupling,[11] for which a 3.6-kHz field strength was used.

For quantitative work, it is important to ensure adequate spin-lattice (T_1) relaxation of the ^{1}H nuclei between experiments. For the strychnine sample, this necessitated the use of a recycle delay of 10 s. For the QQ-HSQC experiment two scans were acquired for each of 128 increments in the indirect dimension. These settings resulted in a total experiment time of 45 min. For comparison, Q-HSQC and Q-CAHSQC spectra were acquired with the same number of increments but with 8 scans per increment (as required by the two-step phase cycle and the four INEPT delays) resulting in an experiment time of 3.0 hr for each of these experiments.

As the region of the sample affected by the ^{1}H and ^{13}C pulses can differ, it is important to use a 1D version of the QQ-HSQC sequence to calibrate the frequency offset of the slice-selective pulses for each probe. To do this, we use the QQ-HSQC sequence shown in Fig. 2 with the addition of a weak read gradient (around 0.01 G cm^{-1}) applied during acquisition. It is useful to have a strong signal for calibration, so we use a sealed sample of ^{13}C-labelled formaldehyde in deuterated chloroform for this step. The ^{1}H–^{13}C coupling for formaldehyde is 195 Hz, so Δ_{min} and Δ_{max} are set to 2.56 and 7.68 ms, respectively (corresponding to 1/2J and 3/2J). This ensures that the transfer efficiency is 100% for the region affected by the slice-selective pulses as well as the region that is not affected by these pulses. The offset of the hyperbolic secant pulses is then varied until the integral for the part of the image affected by the slice-selected pulses is three times the integral of the region unaffected by these pulses, resulting in an image like the one shown in Fig. 3.

RESULTS AND DISCUSSION

The results for a ^{1}H–^{13}C QQ-HSQC spectrum for strychnine in $CDCl_3$ acquired at 300 MHz for proton are shown in Fig. 4. The integrals of the peaks in this spectrum, as a percentage of the expected peak integral for a completely quantitative experiment, are graphed in Fig. 5. The results show that the relative peak integrals calculated from the QQ-HSQC spectrum match closely the integrals acquired from the Q-HSQC and Q-CAHSQC experiments. The standard deviation between the expected and measured results was 7% for the QQ-HSQC experiment; this is similar to the results from the Q-HSQC and Q-CAHSQC experiments (9 and 8%, respectively).

Although results from QQ-HSQC experiments (and the Q-HSQC and Q-CAHSQC experiments) are reasonably quantitative, they are not perfectly accurate. This is due to a combination of factors. First, the bandwidth of the heteronuclear pulses limits the range of chemical shifts for which the results are quantitative. We found that, for the radiofrequency field strengths used for our QQ-HSQC experiments, peaks were quantitative within a bandwidth of 100 ppm for ^{13}C (7.5 kHz); inside this range the variation in peak integrals due to radiofrequency pulses varied by no more than ±2%. This variation was determined by varying the ^{13}C transmitter offset in a series of 1D

DOI: 10.1002/mrc

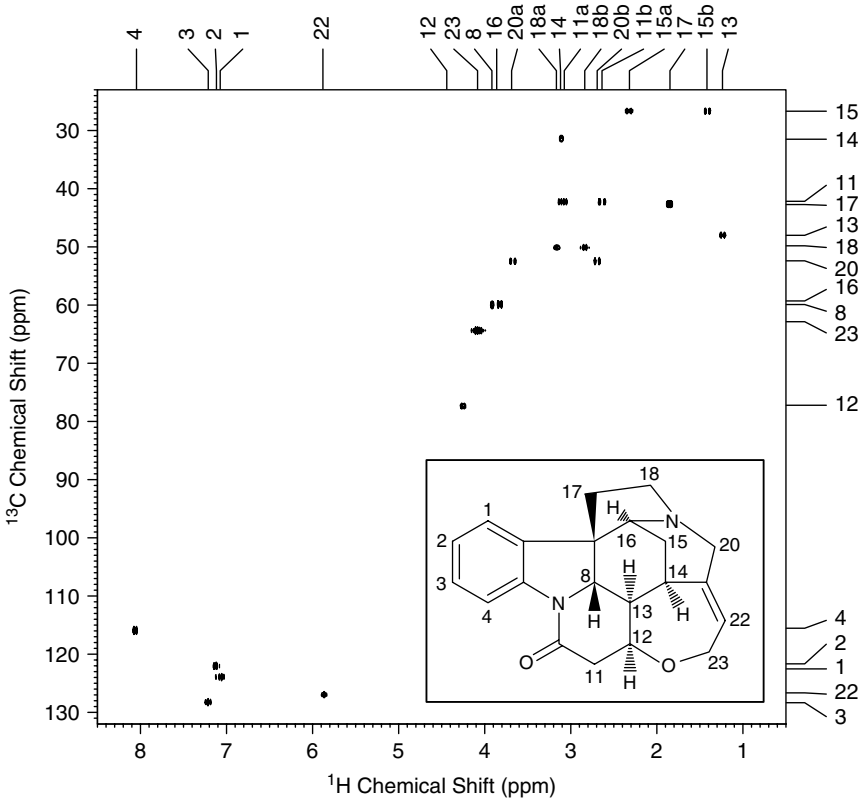

Figure 4. QQ-HSQC spectrum of strychnine acquired at 300 MHz for ^{1}H. Peak assignments are shown in the structure of strychnine in the inset.

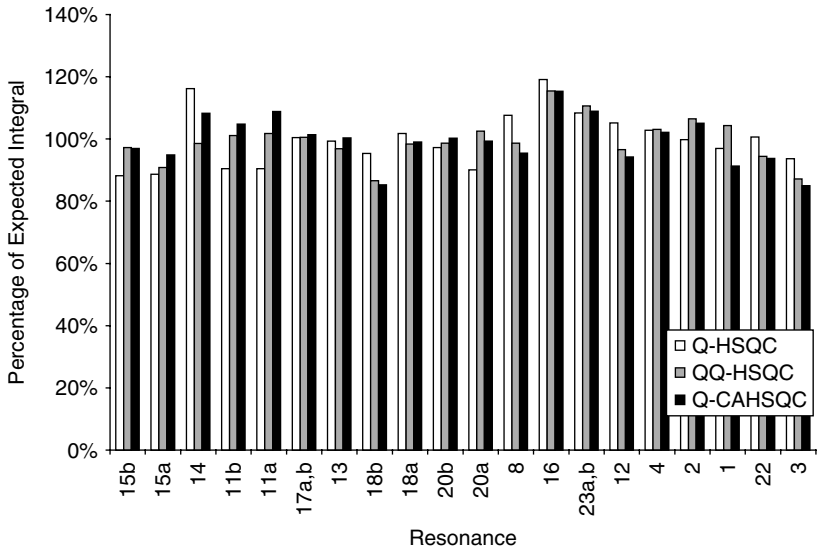

Figure 5. The relative integrals from Q-HSQC, QQ-HSQC, and Q-CAHSQC experiments on a 0.25 M sample of strychnine in deuterated chloroform. The results are shown as the percentage of the integral expected if the experiments were perfectly quantitative; the resonance assignments are given in Fig. 4. The results show that the QQ-HSQC experiment performs similarly to the Q-HSQC and Q-CAHSQC experiments.

QQ-HSQC experiments using a reference sample of ^{13}C-labeled formaldehyde. The 100 ppm bandwidth was more than adequate for the samples that we studied but for higher field spectrometers and/or samples with larger ranges of ^{13}C shifts the limitations on the quantitative bandwidth may be an issue. For the field strengths that we used the main factor that limited the quantitative bandwidth was that of the decoupling sequence. This can be improved by switching to an adiabatic decoupling sequence, in which case the other ^{13}C pulses will begin to limit the quantitative bandwidth (we note that the XY-8 pulse train is partially compensated for offset effects). A few adaptations that can improve the experimental bandwidth in some situations are discussed in Koskela *et al.*[5]. Additional improvements

DOI: 10.1002/mrc

possibly may be achieved by implementing the SCARPER technique recently introduced by Kupče and Freeman,[12] which involves replacing many of the ^{13}C pulses by pairs of adiabatic pulses.

A second factor that affects quantitation is the influence of homonuclear $^{1}H-^{1}H$ couplings. Even with the aid of the XY-8 spin-echo pulse trains,[5] homonuclear couplings will cause variations of the peak integrals and limit the ultimate quantitative accuracy of the technique. A third factor is that large differences in relaxation rates for the various spins in a molecule can reduce the accuracy of the integrals. The small and medium-sized molecules that we have studied have relatively long relaxation times, (*i.e.* greater than 200 ms) so differential relaxation was not a major factor. However, for situations with more rapidly relaxing spins this can be a limiting factor for quantitative experiments.

CONCLUSION

We have demonstrated the QQ-HSQC experiment, which is an improved version of the Q-HSQC experiment that only requires a quarter of the number of scans to complete and retains the same sensitivity per scan. This improvement is achieved through the use of volume-selective adiabatic pulses, which allow different parts of the sample to evolve differently during the INEPT transfer steps. Like the Q-HSQC and Q-CAHSQC experiments, the QQ-HSQC experiment provides quantitative peak integrals. We note, however, that for all three experiments quantitation will at times be limited by finite pulse bandwidth effects, differential relaxation, and homonuclear scalar coupling.

Acknowledgements

We thank the John S. Rogers Science Research Program for summer research support and the Fairchild Foundation for their support of our instrumentation. We also thank David Neuhaus (MRC Laboratory of Molecular Biology, Cambridge, UK) for generously providing instrument time to replicate our measurements and Jonathan Stonehouse (Proctor & Gamble) for his initial interest in this work. N.M.L. thanks the Camille and Henry Dreyfus Foundation for support via a Faculty Start-up Award.

REFERENCES

1. Bodenhausen G, Ruben DJ. *Chem. Phys. Lett.* 1980; **69**: 185.
2. Bax A, Griffey RH, Hawkins BL. *J. Magn. Reson.* 1983; **55**: 301.
3. Ernst M, Griesinger C, Ernst RR. *Mol. Phys.* 1991; **74**: 219.
4. Heikkinen S, Toikka MM, Karhunen PT, Kilpeläinen I. *J. Am. Chem. Soc.* 2003; **125**: 4362.
5. Koskela H, Kilpeläinen I, Heikkinen S. *J. Magn. Reson.* 2005; **174**: 237.
6. Loening NM, Keeler J, Morris GA. *J. Magn. Reson.* 2001; **153**: 103.
7. Thrippleton MJ, Loening NM, Keeler J. *Magn. Reson. Chem.* 2003; **41**: 441.
8. Loening NM, Thrippleton MJ, Keeler J, Griffin RG. *J. Magn. Reson.* 2003; **164**: 321.
9. Frydman L, Scherf T, Lupulescu A. *Proc. Natl. Acad. Sci. U.S.A.* 2002; **99**: 15858.
10. Shaka AJ, Pines A. *J. Magn. Reson.* 1987; **71**: 495.
11. Shaka AJ, Barker PB, Freeman R. *J. Magn. Reson.* 1985; **64**: 547.
12. Kupče E, Freeman R. *J. Magn. Reson.* 2007; **187**: 258.

DOI: 10.1002/mrc