

Reliable, high-quality suppression of NMR signals arising from water and macromolecules: application to bio-fluid analysis

+Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Analysis of metabolites in biofluids using Nuclear Magnetic Resonance often requires the suppression of obscuring signals arising from water and macromolecules. This paper analyses the limitations of the pulse sequence most commonly used to achieve such suppression (presat-CPMG) and proposes new pulse sequences that do not share those limitations. The utility of these improved pulse sequences is demonstrated in a metabolomic study of multiple sclerosis (MS) patients.

Introduction

The NMR analysis of metabolites in biological fluids often requires the suppression of signals from water and macromolecules. For many years, a combination of pre-saturation and the Carr-Purcell-Meiboom-Gill (presat-CPMG) pulse sequence^{1,2} has been used, almost exclusively, for this purpose. At least in part, the presat-CPMG pulse sequence is popular for historical reasons. Pre-saturation is a method that could be implemented when pulsed field gradients (PFGs) were unavailable, while the Carr-Purcell-Meiboom-Gill (CPMG) element (Figure 1a) was, until recently, the best way of implementing a T_2 filter in phase sensitive experiments for the suppression of broad resonances. However, the presat-CPMG pulse sequence suffers from imperfect suppression of the water signal, from large radio-frequency (rf) power demands, and from decreasing performance with increasing magnetic field. This paper analyses these limitations and proposes new pulse sequences that offer excellent suppression of the water signal, allow lower pulsing rates, allow the use of previously inaccessible long T_2 filters, and recover metabolite signals suppressed by the water signal suppression module. The new pulse sequences would be of interest to metabolomics studies.

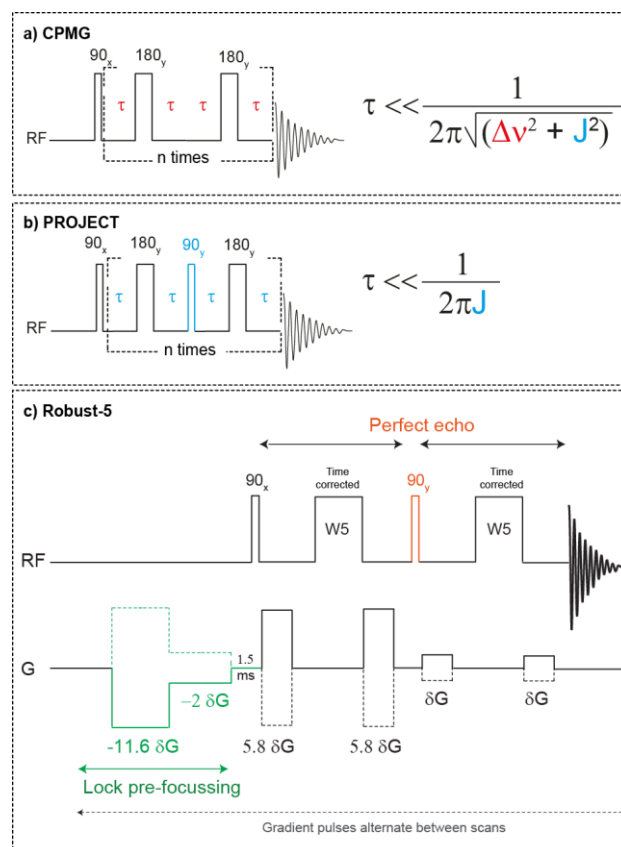


Figure 1. (a) CPMG, (b) PROJECT, and (c) Robust-5 pulse sequences. In (c), eddy current distortions, as well as lock signal destruction, are minimized using lock pre-focussing pulsed field gradients. Distortions caused by J -evolution are minimized using a "perfect echo" module. The polarity of the gradients alternate between transients. $\Delta\nu$ represents the difference in frequency between coupled spins, and J the homonuclear scalar coupling. In the case of CPMG but not PROJECT, τ depends on the magnetic field strength of the spectrometer; see Equations 1-4. W5 is a binomial-like composite pulse that inverts all spins except those in the central notch region of the spectrum which experience a net zero rotation and are hence de-phased by the pulsed field gradients and their signals suppressed.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Limitations of the Carr-Purcell-Meiboom-Gill (CPMG) module

The two problems presented by the CPMG pulse sequence are that it requires considerable *rf* power to quench J-evolution, and that the amount of power needed increases with the magnetic field strength. The reason for this is that the interval between pulses (τ) in the CPMG module should be considerably shorter than both the inverse of the shift difference ($\Delta\nu$) and the scalar coupling (J) between coupled signals:^{3,4}

$$\tau \ll \frac{1}{2\pi\sqrt{\Delta\nu^2 + J^2}} \quad \text{Eq. 1}$$

For weakly coupled spin systems, Eq. 1 approximates to

$$\tau \ll \frac{1}{2\pi\Delta\nu} \quad \text{Eq. 2}$$

This condition becomes almost impossible to fulfil on spectrometers operating at the high magnetic field strengths typically currently used for the study of biological samples. Many metabolites have coupled protons separated by more than 2.5 ppm, corresponding to more than 2 kHz on a spectrometer operating at 800 MHz. This implies that τ needs to be $\ll 80 \mu\text{s}$ at 800 MHz. These values are very demanding for conventional hardware due to the resulting high radiofrequency power loading. Failure to meet this condition distorts and destroys signals (see below), although, in practice, factors such as pulse imperfections,⁵ and de-phasing of out-of-phase magnetization,^{6,7} contribute to mitigate and conceal these problems. Recently, however, it has been shown that τ values can be extended if “perfect echoes”^{8–10} are added to the CPMG module, as in the PROJECT pulse sequence.¹¹ This approach turns Equations 1 and 2 into Equation 3

$$\tau \ll \frac{1}{2\pi J} \quad \text{Eq. 3}$$

The improvement is of the order of

$$\frac{\tau_{\text{PROJECT}}}{\tau_{\text{CPMG}}} \approx \frac{\Delta\nu}{J} \quad \text{Eq. 4}$$

With J usually less than $\sim 15 \text{ Hz}$, this indicates that for $\Delta\nu > 2 \text{ kHz}$, τ can be ~ 130 times longer at 800 MHz when using PROJECT in place of CPMG. This, on its own, is a good reason to favour PROJECT, as this greatly reduces the power loading on the probe and sample whilst avoiding signal distortions. However, there is another good, but so far unacknowledged,[†] reason to replace CPMG with PROJECT: the performance of PROJECT is largely independent of the magnetic field strength, as Equation 3 shows, since its limiting factor is J and not $\Delta\nu$. In other words, PROJECT is far better suited for use with high field spectrometers than CPMG. Another point that has escaped widespread attention is that the PROJECT pulse sequence can recover signals suppressed by the module that attenuates the water signal. This is a by-product of the periodic exchange of magnetization that underpins PROJECT, as will be shown below. A further advantage of the PROJECT module is that it can be fitted with pulsed field gradients

(PFGs) to exploit diffusion weighting in metabolomic studies,^{12–14} even in the presence of convection.^{15,16}

Limitations of pre-saturation

Pre-saturation is also a popular choice in metabolomic studies,¹⁷ at least in part for historical reasons. It could be implemented on spectrometers lacking PFGs, or in systems where unshielded gradient coils produced badly distorted line-shapes; it also has the advantage that it is fairly tolerant of pulse mis-calibrations. In addition, it can be quite selective, thus minimally affecting adjacent metabolite signals. However, this selectivity comes at a price: the more selective the pre-saturation pulse, the more the water signal is left unsuppressed. This, in turn, lowers the sensitivity of the pulse sequence because the more water signal survives, the more the receiver amplification is determined by the residual water signal, and not by metabolites. Thus, in an attempt to ensure the detection of signals adjacent to the water peak, we effectively decrease the sensitivity for all metabolite peaks. Another problem is that pre-saturation causes an unwanted attenuation of metabolites interacting with macro-structures. Various mechanisms mediate this, with perhaps the most important one being the transfer of saturation from macro-structures coincidentally affected by the saturation pulse to small molecules interacting with them. This problem was identified in the early days of z-spectroscopy-assisted MRI,^{18–20} and is exploited today in Saturation Transfer Difference (STD), a technique that is used to prove the interaction of small molecules with large entities through cross-saturation.^{21,22} Metabolites and drugs that interact with blood proteins are particularly susceptible.^{23–28} The effect is more prevalent when dealing with samples containing cellular components, whole cells, or even tissues, as larger entities facilitate spin diffusion more effectively than smaller ones.

Many techniques can be used as alternatives to pre-saturation,²⁹ but of particular interest are those, such as WATERGATE, that are very efficient for signal attenuation. Whilst a perceived drawback of such pulse sequences is that the suppression band is wider than for narrowband pre-saturation, they have the advantage that the detection of metabolites is enhanced, since the spectrometer receiver gain is not determined by the unsuppressed water signal but by the metabolites signals. Nevertheless, these methods are not without their own problems since they are sensitive to pulse mis-calibrations and may suffer signal distortions arising from a combination of J-evolution and field disturbances caused by the switching of the pulsed field gradients. Recent developments have resolved these problems and in particular, the Robust-5 pulse sequence has been shown to have all the qualities required for metabolomic work:³⁰ the suppression of the water signal is fairly insensitive to pulse mis-calibrations; it tolerates radiation damping; J-evolution distortions are avoided; and, above all, it consistently attenuates the water signal to about 1 mM. To put this in perspective, the concentration of the twelve most abundant metabolites in blood, detectable by NMR range from 5 mM (cholesterol) to

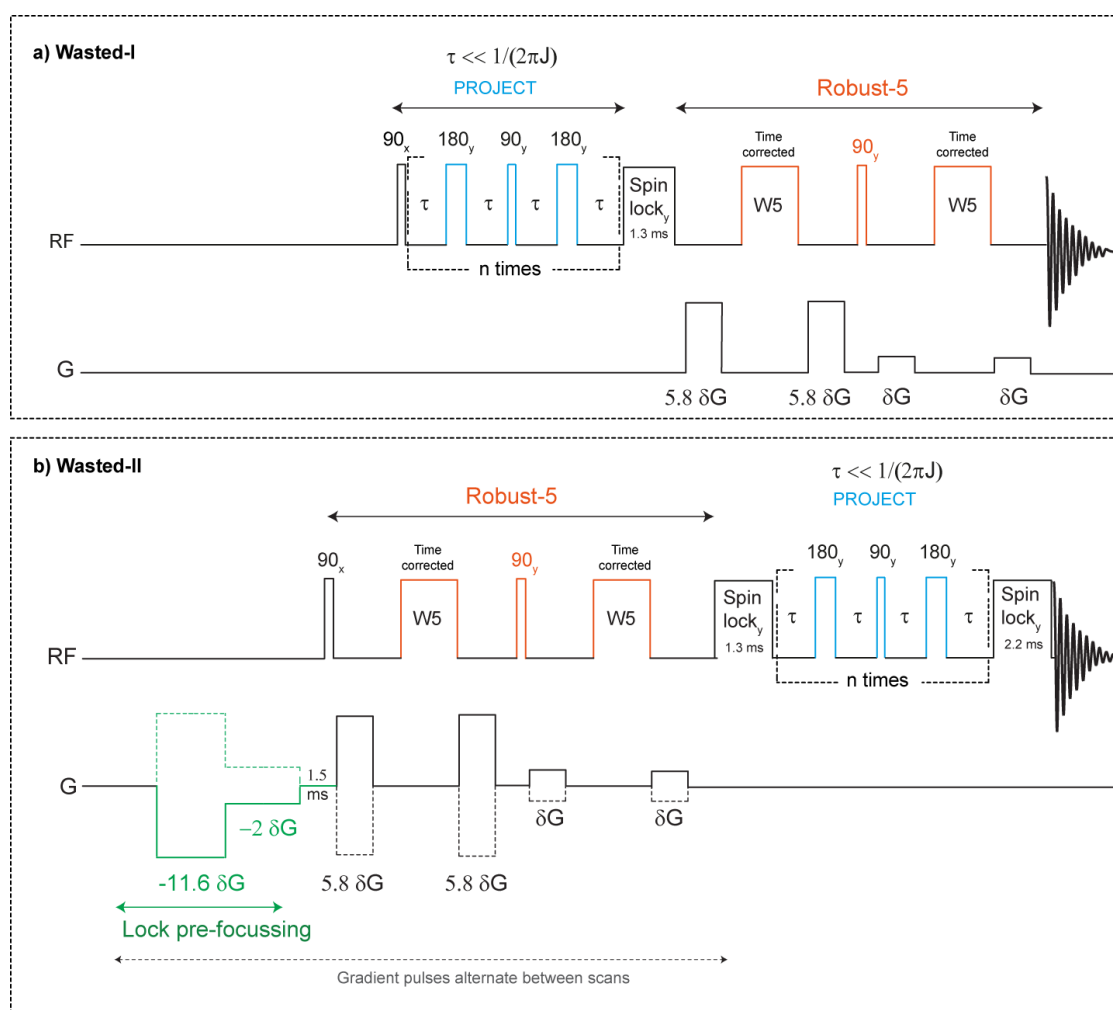


Figure 2. Wasted-I (a) and -II (b) pulse sequences. Wasted-I offers slightly better suppression of the water signal than Wasted-II, but more care has to be taken to ensure adequate gradient stabilization delays. Wasted-II, on the other hand, has the advantage that it recovers signals attenuated by the solvent suppression module. It is also quite insensitive to eddy currents induced by the switching of the pulsed field gradients. Both pulse sequences allow τ values that are up to two orders of magnitude longer than CPMG allows. A spin-lock is used in both pulse sequences to separate the Robust-5 and PROJECT modules. This improves the suppression of the water signal, and in the case of Wasted-I, eliminates dispersive components that are produced if the PROJECT condition is accidentally violated. In the case of Wasted-II a second spin-lock (optional) is introduced for similar reasons. The function of the pre-focussing gradients (Wasted-II) is to minimize lock disturbances as well as the impact of eddy currents. The combination of all these elements makes these pulse sequences very efficient and clean. Clean results are obtained completing the phase cycle (32 transients).

0.35 mM ((R)-3-hydroxybutyric acid). Similar efficient pulse sequences, such as pe-WATERGATE,³¹ could be used provided that the precaution of adding some pre-focusing PFGs is taken, as in Robust-5. Furthermore, it is possible to combine these methods with the PROJECT T_2 filter to produce pulse sequences that recover signals attenuated by the water suppression module.

RESULTS AND DISCUSSION

Putting it all together

There are various ways in which PROJECT and Robust-5 can be combined. From the point of view of efficiency, it is usual to place the solvent suppression module at the end of the pulse sequence so the water signal has little time to recover, (Figure 2a). Other considerations come into play when this is not important. For example, placing the PROJECT module at the end of the pulse sequence, (Figure 2b), potentially allows the

recovery of signals accidentally destroyed by the water suppression block, as discussed below. Indeed, the same mechanism that allows the use of long inter-pulse delays (τ) in PROJECT -- a periodic exchange of magnetization -- allows the restoration of destroyed signals provided the associated protons are coupled to others outside of the suppression band. In addition, placing Robust-5 first has the advantage that eddy currents induced by gradient pulses have plenty of time to dissipate. The ideal combined sequence would provide **WATER Suppression with a Transverse relaxation filter that Eliminates Distortions**. We have given the name Wasted-I to the pulse sequence with PROJECT preceding Robust-5, and Wasted-II to the pulse sequence with the opposite arrangement. In addition, both pulse sequences have been fitted with a short spin-lock pulse between the two modules; this improves solvent signal suppression. In the case of Wasted-II, a second, optional, spin-lock has been placed at the

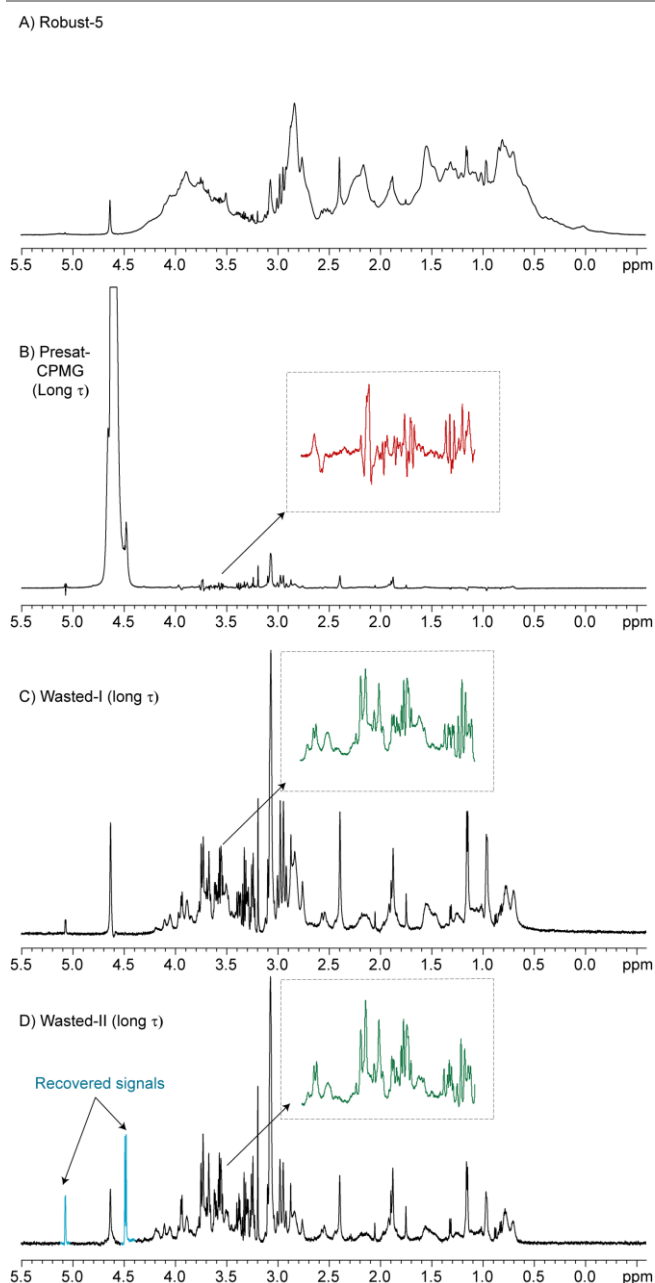


Figure 3. Proton NMR spectra (600 MHz) obtained using Robust-5 (a), Presat-CPMG (b), Wasted-I (c), and Wasted-II (d). All the T_2 filters have been run for 120 ms with $\tau = 5$ ms. Five ms is more than one order of magnitude larger than CPMG can tolerate, thus causing gross signal distortions in spectrum (b). Some signals have been also lost due to either this or to macromolecule-mediated cross-saturation. These problems are absent in Wasted-I and Wasted-II spectra (c and d). The sample was made up by diluting blood plasma by a factor of 10 and adding 200 mg of albumin (see the Experimental section for details).

end of the pulse sequence to eliminate signals with dispersive components that could have been produced by (accidentally) violating Eq. 3. The pre-focusing PFGs used in Robust-5 and Wasted-II are intended to minimize lock disturbances as well as problems with eddy currents. Pre-focussing PFGs have not been used in Wasted-I because they would be separated from the PFGs they aim to balance for a delay that would render

them ineffective. We demonstrate the capabilities of these sequences with two samples; one a diluted plasma sample with added albumin, the second a typical blood sample obtained from a volunteer.

Quality of the water signal suppression

The main reason for suppressing the water signal efficiently is to facilitate the detection of the metabolites of interest that are present typically at (sub)millimolar concentrations in solutions of ~ 50 proteo M water. In the case of the challenging diluted plasma sample, (Figure 3), it is clear that the excellent suppression capabilities of the Robust-5 sequence are preserved by both Wasted I and II, each demonstrating comparable results. Moreover, this suppression is clearly superior to that achievable by the presat-CPMG sequence.

Wasted-II should, in principle, be superior in dealing with radiation damping as it de-phases the water signal at the beginning of the pulse sequence, although we did not find any problem due to radiation damping with either Wasted sequence. Of importance for metabo(l/n)omic studies is the fact that the quality of the Wasted water suppression was maintained when samples were run under automation. This is not always the case for presat-CPMG, especially when very selective pre-saturation pulses are used. Although the performance of presat-CPMG can be improved by increasing the strength of the pre-saturation pulse, this is undesirable if cross-saturation (STD) effects are to be avoided.

Performance of the T_2 filter

The pulse sequences presented here exploit the ability of the PROJECT module to avoid signal distortions, even at echo times (2τ) an order of magnitude longer than those possible by the CPMG module, as illustrated in Figures 3, 4 and 5. In the case of chemical analysis, the appropriate setting of τ for CPMG and PROJECT would be dictated by equations 2 and 3 respectively. Taking the earlier example of a 2.5 ppm shift difference between coupled protons, this demands CPMG τ values to be significantly less than 100 and 80 μ s at 600 and 800 MHz respectively. Minimum safe pulsing intervals are determined by spectrometer hardware and the need to avoid sample *rf* heating (and, for *in-vivo* applications, by the need to avoid injuring the patient) meaning these short inter-pulse intervals are likely to be intolerable or unsustainable, especially for total filter periods of many tens of milliseconds. For Wasted pulse sequences using the PROJECT module, the homonuclear coupling constants dictate τ , most of which are less than 15 Hz. This suggests τ should be below 10 ms and may be set to values that are readily tolerated even for extended filter periods. To illustrate the capabilities of the Wasted pulse sequences with τ values that are much longer than CPMG can tolerate, we acquired spectra using a $\tau = 5$ ms. Figures 3b and 4b show that the presat-CPMG pulse sequence cannot operate correctly with this value at 14.1 Tesla (a 600 MHz spectrometer); most metabolite signals are phase- and

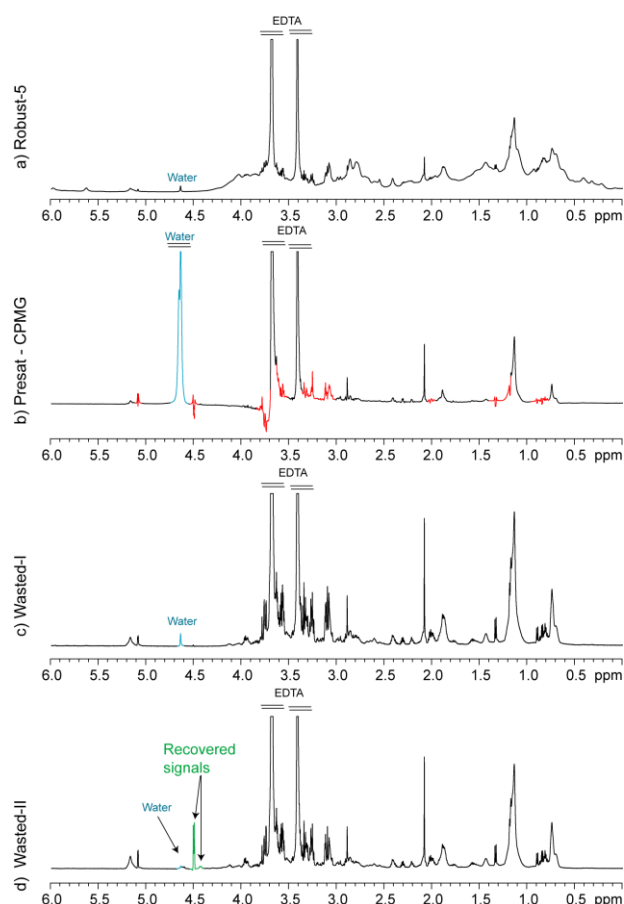


Figure 4. Spectra of a human blood sample. (a) Robust-5. The quality of the water suppression is excellent, but broad signals obscure signals of interest. (b) Presat-CPMG with a T_2 filter run for 80 ms, using $\tau = 5$ ms. Broad signals have been eliminated but most metabolite signals are distorted due to J-modulation; other signals have disappeared, either because the CPMG condition has been violated and/or because macromolecular-mediated pre-saturation has affected them. Furthermore, the resulting water signal prevents the use of an appropriate receiver gain. (c) Wasted-I run under the same conditions. The water signal has been reduced below the level of the metabolites of interest, thus allowing the use of a proper receiver gain. Note the lack of distortions. Note too that some metabolite signals close to the water signal have been suppressed. (d) Wasted-II spectrum run under the same conditions. Note how the lost signals have been recovered.

amplitude-modulated; others are simply lost. In contrast, Wasted pulse sequences produce undistorted spectra whilst maintaining their excellent water suppression characteristics (Figure 4 c and d; Figure 5). This makes these novel pulse sequences well suited to run metabo(l/n)omic studies in state-of-the-art spectrometers where traditional CPMG elements may fail.

In addition to reducing power requirements, the use of larger inter-pulse delays and hence longer T_2 filters, opens new possibilities. So far, the CPMG filter has been used primarily to distinguish between macromolecules with short T_2 values (broad signals) from metabolites with medium or long T_2 . It now becomes possible to differentiate metabolites with far greater selectivity in T_2 or potentially to differentiate macromolecular structures with differing T_2 properties e.g.

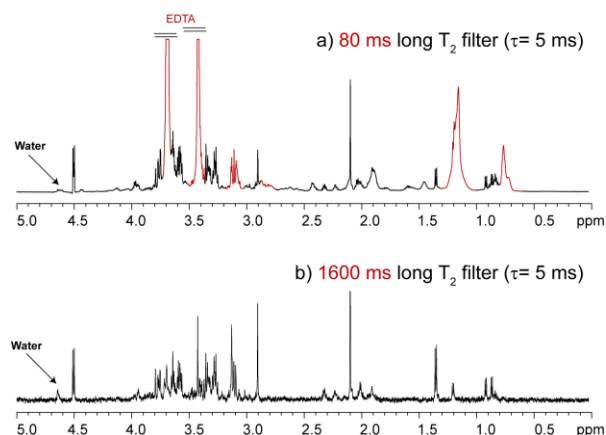


Figure 5. Wasted pulse sequences allow the use of T_2 filters of a length impossible to use achieve with presat-CPMG. This allows the introduction of T_2 encoding to distinguish between different metabolite populations. Compare (b), in which a 1600 ms long filter has been made possible by Wasted-II, with (a) in which an 80 ms filter has been used (also using Wasted-II). Note that the suppression of the water signal is excellent even when using these long filters. The sample is human blood with non-deuterated EDTA.

lipoproteins. The use of very long filter times is demonstrated in Figure 5 where the blood sample of Figure 4 has been further edited by extending the total T_2 filter time from 80 to 1600 ms; this would be impossible to achieve with traditional CPMG pulse sequences. Note that the suppression of the water signal is excellent even when using such long filters. This new possibility should allow variable T_2 weighting as a newly accessible parameter in multivariate analysis of small metabolites; thus, this tool can be used to further analyse bio-fluids beyond the requirement to suppress broad macromolecular signals.

Recovery of signals

A common occurrence when suppressing the water signal is the attenuation of metabolite signals within the suppression band. WATERGATE-based pulse sequences such as the ones presented here are very robust under automation, in part because the suppression notch is wide, which tends to make the problem worse (Figures 3 and 4). In general this is not limiting for metabolomics studies, since information from species that produce signals close to the water signal can often be obtained from other parts of the spectrum. However, it is also possible to keep these signals using Wasted-II, since the exchange of magnetization caused by the perfect echo train restores lost signals by transferring magnetization from coupled partners outside the water suppression band. In this way, PROJECT acts as a multiple relay system.³² To our knowledge, this property of PROJECT has not yet been reported, although it was implicit in the product operator analysis included in the original paper.¹¹ To clarify, we consider two coupled spins I and S with a coupling J. The resonance frequency of spin S is within the suppression notch (i.e. under or close to the water signal) whereas spin I is outside this. The effect of a 90° rf pulse with a phase x on spins I and S is to produce the transverse magnetisation terms $-(I_y + S_y)$. The

ensuing solvent suppression module of Wasted-II will de-phase the signal belonging to spin S. After this point, and for the duration of the first spin echo (2τ), the signal belonging to spin I evolves according to its coupling to spin S, producing in-phase and anti-phase magnetisation ($-I_x\cos(2\pi J_{IS}\tau) + 2I_xS_z\sin(2\pi J_{IS}\tau)$ respectively). The effect of the ensuing perfect echo 90° pulse is to convert the anti-phase term $2I_xS_z$ into $-2I_xS_x$. This amounts to a transfer of magnetization from spin I to spin S. The effect of the following spin echo is to turn this anti-phase term into a detectable spin S signal. The net effect is that part of the magnetisation that started the process as spin I ends as spin S. The recovery of the glucose H1 anomeric proton signals through this process for Wasted-II is highlighted in Figures 3 and 4. The same repopulation process is not possible with Wasted-I as the dephasing of spin S is the last event of the pulse sequence.

Application to metabolomics

To showcase the use of these sequences in metabolomics and demonstrate relevance to a clinical setting, we applied Wasted-II to a multiple sclerosis (MS) patient study. Patients were diagnosed clinically with relapsing-remitting multiple sclerosis (RRMS) if they fulfilled the revised 2010 McDonald criteria.³³ Orthogonal partial least squares discrimination analysis (OPLS-DA) of ^1H NMR data obtained from blood plasma samples was used to investigate differences in the metabolite profiles between secondary-progressive (SPMS) and RRMS patients as previously described.³⁴ This analysis was able to discriminate SPMS from RRMS patients with an accuracy of $83 \pm 2\%$ (Figure 6), and reproduced the separation reported previously on an earlier patient cohort of serum samples.³⁵ In both studies the same discriminatory metabolites were identified in which high- and very-low density lipoproteins and β -hydroxybutyrate were increased while glucose and N-acetylated glycoprotein species were decreased in the RRMS compared to the SPMS cohorts. In direct comparison of methods, an accuracy of $81 \pm 2\%$ was observed for the same sample cohort when using the classical CPMG sequence. Thus, at least in this study, the WASTED sequence provides, as a minimum, data of equal discriminatory capability whilst offering greater flexibility in the choice of filtering parameters.

Conclusions

The Wasted pulse sequences overcome the limitations of the presat-CPMG pulse sequence and facilitate the analysis of samples in which it is necessary to suppress signals arising from water and macro-structures simultaneously. Wasted pulse sequences allow a very efficient detection of metabolites, as shown by the analysis of a plasma sample that was diluted by an order of magnitude. This may open the way

to metabolomic studies in which a small amount of blood, possibly drawn from a finger prick, could be used, especially when highly sensitive probes are employed. Furthermore, Wasted T_2 filters allow inter-pulse delays (τ) that are an order of magnitude larger than those tolerated by CPMG, thus requiring lower total power deposition to operate correctly. Importantly, this ability is fairly field-independent, which is not the case for CPMG, making these pulse sequences ideal for metabolomic studies that make use of state-of-the-art high-field spectrometers. Because of their low power requirements, these pulse sequences open the way to the use of T_2 encoding to distinguish between different metabolite pools or differing macromolecular structures, a possibility of interest to extend multivariate-assisted studies beyond the need to suppress broad signals.

EXPERIMENTAL SECTION

Two samples have been used to test the new pulse sequences. The first sample was designed to be especially difficult: we took 0.1 mL of bovine plasma (Sigma-Aldrich) and diluted it with $\text{H}_2\text{O}/\text{D}_2\text{O}$ 90:10 % (v/v) by a factor of ten. The final volume was 1 mL. We then added 200 mg of bovine albumin to the existing 3-5 mg present in the diluted 1 mL plasma sample so as to mask the small-molecule metabolites of interest. A second sample was prepared taking a blood sample from a volunteer, to which non-deuterated EDTA was added as an anti-coagulant and deuterated water added to a 10% final concentration (v/v).

Wasted pulse sequences were tested using a Varian 600 MHz spectrometer equipped with a probe able to deliver a maximum pulsed field gradient of 62 G cm^{-1} . All spectra were acquired at 25°C . 128 transients were collected, each comprising 65536 complex data points and a spectral width of 10 kHz. The repetition time was 4.8 s, of which 3.3 s comprised the acquisition time. The W5 inter-pulse delay was set to 287 μs , but this should be adjusted for each field (instructions can be found in the Supplementary material). In all cases, rectangular 1 ms pulsed field gradients were used with a strength of $G = 4.8 \text{ G cm}^{-1}$. The gradient stabilization delay was 1 ms. The first pair of lock pre-focusing field gradients used in Wasted-I and Robust-5 were separated from the first radio-frequency pulse by 1.5 ms. In all cases the timing of the W5 element was time corrected as prescribed by Wang et al.³⁶ The same values were used for Robust-5. All pulse sequences can be found in the Supplementary section. In the case of Figure 3, a 120 ms T_2 filter was used. In the case of Figures 4 and 5a, a T_2 filter of 80 ms was used. In the case of Figure 5b, a T_2 filter of 1600 ms was used. In all cases $\tau = 5 \text{ ms}$. The presat-CPMG pulse sequence was run under the same conditions but using a 1.5 s long pre-saturation pulse with $\gamma B_1/2\pi = 26.3 \text{ Hz}$.

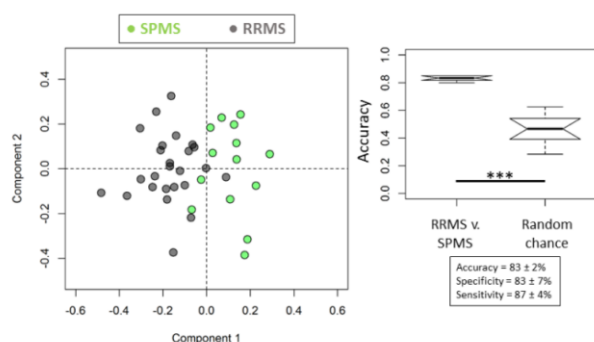


Figure 6. OPLS-DA results from blood plasma Wasted-II ^1H NMR data discriminating SP from RR multiple sclerosis patients. Left, a representative scores plots illustrating separation between SPMS and RRMS plasma spectra in the multivariate models, and right, the accuracy of the cross-validated ensemble of OPLS-DA models is significantly better than random chance. Kolmogorov-Smirnov test p-values <0.001 are represented by ***.

In the MS metabolomics study all patients included in the study were recruited from MS clinics in the John Radcliffe Hospital in Oxford and gave their written consent to participate in the study (Oxford Research Ethics Committee C Ref: 10/H0606/56 and 16/SC/0224). Plasma samples were prepared for NMR analysis by first centrifuging at $100,000 \times g$ for 30 min and diluting 150 μL of supernatant with 450 μL of 75 mM sodium phosphate buffer prepared in D_2O (pH 7.4) as previously described.³⁴ ^1H NMR spectra were collected on a Bruker AVIII 700 spectrometer equipped with a TCI cryoprobe. Wasted-II spectra were acquired with total filter times of 40 ms and PROJECT τ delays of 0.5 ms for comparison with a previous study.³⁵ 32 transients of 64K data points were collected for a spectra width of 21 kHz. The Robust-5 interpulse delay was 98 μs (null at 5.1 kHz offset). Gradient pulses were 1 ms smooth-squared shapes (SMSQ10.1000) with $G = 5.7 \text{ G cm}^{-1}$. For chemometrics, the regions of the spectra between 0.08 – 4.20 ppm and 5.20 – 8.50 ppm were divided into 0.02 ppm width ‘buckets’, Pareto scaled and imported into R software (R foundation for statistical computing, Vienna, Austria) within which orthogonal partial least squares discrimination analysis (OPLS-DA) was performed using the internal ropls package to investigate differences in the metabolite profiles, as previously described.³⁴ Briefly, models were optimized by internal 7-fold cross-validation. The quality of classification was assessed using a 10-fold external cross-validation scheme with 1000 repetitions, correcting for unequal class sizes. This validation scheme involves multiple iterations of splitting the data into training and testing sets, which ensures that any discrimination observed in the models cannot have occurred by chance. We quantified the outcome of the cross-validation by calculating the accuracy, sensitivity, and specificity of each model from the predicted classifications of each external independent test set. It is important to appreciate that the classifier (OPLS-DA) was blinded to each test set when training each model. These values were compared with those of a null distribution (obtained from

randomly permuting the classes) using the two-sided Kolmogorov-Smirnov test (significant if p-value 0.05 or less).

Pulse sequences for Varian/Agilent, Jeol and Bruker can be found in the Supporting Information section for those wishing to evaluate them.

Author Contributions

J. A. Aguilar planned the investigation, wrote, tested and refined the pulse sequences, acquired the data of Figures 3-5, coordinated all authors, and wrote the manuscript. The pulse sequences were also tested by J. Cassani, A. M. Kenwright, T.D.W Claridge, and A. Botana independently of one another. T.D.W Claridge, F. Probert, and J. Palace conducted the metabolomic study. All authors were involved in revising and improving the manuscript.

Conflicts of interest

The authors declare that there are no conflicts.

Acknowledgements

J. A. Aguilar and A. M. Kenwright acknowledge Durham University for its support. We thank the Multiple Sclerosis Society for project funding (FP) and the Science Research Investment Fund (SRIF3) for instrument funding.

Notes and references

‡ JAA (co-author of the original PROJECT paper) recounts that, as far as he remembers, the authors of the original PROJECT paper did not realize at the time of writing the PROJECT paper that a big advantage of PROJECT over CPMG is that its performance is largely field-independent.

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