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NMR quantification of trace components in complex matrices by band-selective excitation with adiabatic pulses

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The use of band-selective excitation with adiabatic pulses to rapidly obtain NMR spectra of trace components in the presence of strong signals is described, along with qualitative and quantitative examples from food matrices like olive oil and honey.

Supporting information may be found in the online version of this article.

Keywords: NMR; ^{1}H; selective excitation; adiabatic pulses; complex matrices; trace metabolites; food chemistry

Introduction

In the recent years, the interest in systems where important probe molecules are present in trace amounts alongside more abundant species of relatively poor variability (typically carbohydrates, fats, proteins or water) has been steadily increasing. Some examples in the analysis of these 'complex matrices' are provided by the identification and quantification of metabolites in biofluids[1] (blood, urine) or minor constituents in foodstuff (markers of biological and geographical origin).[2] In this scenario, NMR spectroscopy plays a prominent role owing to its ability to provide both structure elucidation and quantification of chemical species with limited (if any at all) pre-treatment of the matrix.[3] In addition, a number of recent works have combined high-throughput NMR and statistical analysis, for instance, in the characterisation of metabolic profiles deriving from environment-sensitive biosynthetic pathways.[4] This two-step approach relies in general on a 'blindfold' accumulation of as much spectral data as possible, and on the subsequent extraction of the meaningful information achieved by multivariate analysis. Such a method requires a highly sensitive and thorough collection of spectral data, since it is critical to detect all of the possibly unknown signals bearing the largest inter-sample variance.

This task may be hard to accomplish when small informative signals have to be detected together with very strong (but uninteresting) ones of the bulk matrix. In the case of NMR, the ability to record the weaker signals relies on the dynamic range of the analog-to-digital converter (ADC) hardware: in fact, when most of this range is used to digitise the strongest signals, the weak ones may lie close to (or below) detectability. A 16-bit ADC supplied with most modern instruments has a 65 535 : 1 dynamic range (since it can store up to $2^{16} - 1$ integers) implying that the weakest detectable signal must be at least *ca* 15 ppm with respect to the strongest one. Even if this condition is fulfilled, however, such very weak signals will be inaccurately sampled, and poor integrated intensities will result. Twenty-bit ADCs indeed allow a better sampling of the signals and of the noise floor (which enables a recovery of signals even below the quantization threshold); however, relying entirely on such technological advancements is clearly not a general solution to the problem.

Since modern spectrometers are equipped with effective digital filters, it may be tempting to simply cut the uninteresting region out and acquire, after a 90° pulse, just the portion of the spectrum where the small signals lie, letting the digital filters do the rest. Unfortunately, this approach will work only when dynamic-range issues are absent because digital filtering is done after FID acquisition. In fact, when the cut-out region contains strong signals exceeding the ADC range (solvent, matrix, etc.), the spectrometer will convolute the filter window function with a clipped FID, thereby producing an unusable signal (see Fig. S6 of the Supporting Information).

Removal of the strongest signals via selective excitation is, instead, a more general and effective approach. It allows an increase of the receiver gain, which results in improved digitisation of the small amplitude peaks, lower integration errors and, eventually, better quantification of the number of resonant spins. Such a case is epitomised by samples with large residual solvent signals,[5] and many pulse sequences have been devised to reject specific, unwanted resonances. However, all solvent suppression schemes rely on the fact that solvents exhibit one or very few signals, and cannot be applied to a frequency range of arbitrary width.

Thus, there is still a need for pulse schemes capable of exciting a tuneable frequency region: in this context, where the goal is to obtain undistorted sub-spectra suitable for quantitative analysis,[6] technical issues are not conceptually different from those provided by slice-selective excitation in NMR imaging.[7] With these notions

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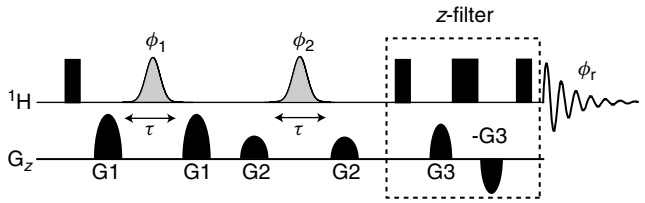

Figure 1. DPFGSE pulse scheme with optional *z*-filter to remove anti-phase magnetisation (see Supporting Information). Adiabatic pulses of duration τ are represented by their amplitude envelope (in grey). Black rectangles represent pulses of 90° (thin) and 180° (thick) flip angles with phase *x*; Exorcycle on ϕ_1 and ϕ_r is nested within a CYCLOPS phase cycling.

in mind, we strove to devise an excitation scheme capable of fulfilling the above requirements.

Not surprisingly, a single RF pulse with the aforementioned characteristics is difficult to implement.[8] An alternate route to band selectivity makes use of double pulsed field gradient spin echoes (DPFGSEs, Fig. 1) which incorporate band-selective refocusing (rather than excitation) pulses.

DPFGSEs are easy to set up and provide clean excitation profiles free from phase defects, regardless of the refocusing element.[9] In this respect, we tested the performance of an adiabatic inversion pulse[10] to act as the refocusing element.

Figure 2 shows the excitation profile of a DPFGSE incorporating an adiabatic inversion Gaussian pulse[11] of 2 kHz sweep width and 10 ms duration: the experimental profile, obtained by sampling a reference signal at regular offsets, is virtually identical to its theoretical envelope (dashed curve). Even if, in general, this class of pulses is only suitable for inverting the magnetisation (and not to perform plane-rotations), the excitation profile emerging from the DPFGSE exhibits a flat region spanning 1500 Hz and a well-behaved phase, both of which are essential requirements for quantitative applications. Adiabatic pulses also have the advantage of delivering a constant performance provided that the employed RF power is above a limiting value, unlike conventional shaped (i.e. amplitude-modulated) pulses whose efficiency is quite sensitive to power missettings and whose bandwidth is limited by the available peak power. For comparison, Fig. 2 also shows the excitation profile of a DPFGSE incorporating a ReBurp pulse: although the same excitation bandwidth is achieved in a considerably shorter time (2.907 ms), the flat region exhibits rolls that become more pronounced as the RF power deviates from the optimal value. Finally, the signal intensity emerging from a DPFGSE depends on the initial flip angle in a way analogous to that of conventional hard pulses, allowing the use of reduced flip angles to maximise the sensitivity for a given recycle delay.[12]

Results and Discussion

To quantify the absolute concentrations of analytes within a given sample, a reference substance is placed into a coaxial insert, which is then fitted inside a 5-mm NMR sample tube. To calculate the analyte concentration, the ratio between the volume of the coaxial insert (V_c) and the volume of the NMR tube (V_t) must be carefully determined.[13] To this aim, both the coaxial insert and the NMR tube were filled with proper aliquots of a 1 mM acetone-d_6 solution. A small amount of Eu(fod)$_3$ was added to the solution in the external tube so as to shift the acetone signal by approximately 0.1 ppm downfield. As the inner and outer acetone concentrations are identical, the ratio of the two integrated signals gives the V_c/V_t ratio directly. In this way, a value $V_c/V_t = 0.108$ was determined and used thereafter as scale factor.

Quantification of HMF in a standard sample

The ability of the proposed method to provide quantitative information was assessed by running three different DPFGSE experiments where the flip angle of the first pulse was set to 90, 50 and 30° in order to 'dilute' the initial magnetisation. The recycle delay for each experiment was calculated to allow quantitative recovery of the magnetisation (see Supporting Information), which amounts to $5T_1$, $4T_1$ and $3T_1$ for 90, 50 and 30° flip angles, respectively. The sample used was 5-(hydroxymethyl)furfural (HMF) in D_2O, referenced to an external solution of *N*,*N*-dimethylformamide (DMF) placed in a coaxial insert. Each experiment consisted of 128 scans, resulting in acquisition times of 131, 105 and 80 min (see Table 1). The plot of S/N versus flip angle (Fig. 3) demonstrates that the S/N ratio is indeed proportional to the sine of the flip angle, as in the case of a simple pulse-acquire experiment.

This result also confirms that by choosing a standard with relaxivities close to those of the analyte the differential attenuation induced by relaxation during the DPFGSE is negligible for quantitation purposes (an estimate of T_1 and T_2 relaxation effects during soft or adiabatic RF pulses is not straightforward and requires a proper mathematical treatment based on the Bloch equations; see e.g. Ref. [14]). As a corollary, in cases where the above condition is met, reduced flip angles can be safely employed within the proposed method to maximise sensitivity.[12]

Application to complex matrices

The advantages of band-selective excitation are showcased by two examples concerning complex food matrices – notably, terpenes in olive oil and minor constituents of honey – both of which highlight marked improvements in the experimental time, detectability and quantitation of the trace components.

The pattern distribution of terpenes is exploited to determine the geographic origin of olive oils.[15] The NMR protocol usually adopted to detect terpenes in oils consists in recording a large number of scans (up to 4000) with a non-selective pulse-acquire sequence, while the same amount of information may be recovered in a shorter time by use of selective excitation of the terpenic region. To demonstrate this claim, both a standard and a DPFGSE experiment (terpenic region, 4.5–5.0 ppm) were run on a sample of olive oil prepared according to Mannina *et al.*[16] (Fig. 4). The quality of the selective spectrum obtained in 34 min (512 scans) is comparable to that of the standard spectrum obtained in 4 h 20 min (4000 scans). Moreover, although the adiabatic and the ReBurp pulses show identical performances in terms of sensitivity, the ReBurp pulse requires a very accurate and time-consuming calibration procedure. In this respect, the adiabatic pulses deliver a clear advantage, particularly when the examined samples include complex matrices of variable composition which would otherwise require a fine tuning of the selective pulses from sample to sample.

The advantages stemming from band selection, however, are not limited to a mere cut down of the experimental times. Rather, cases exist in which weak NMR signals lie well below the ADC minimum threshold. In such an extreme – yet by no means uncommon – situation, extensive averaging of the FID will

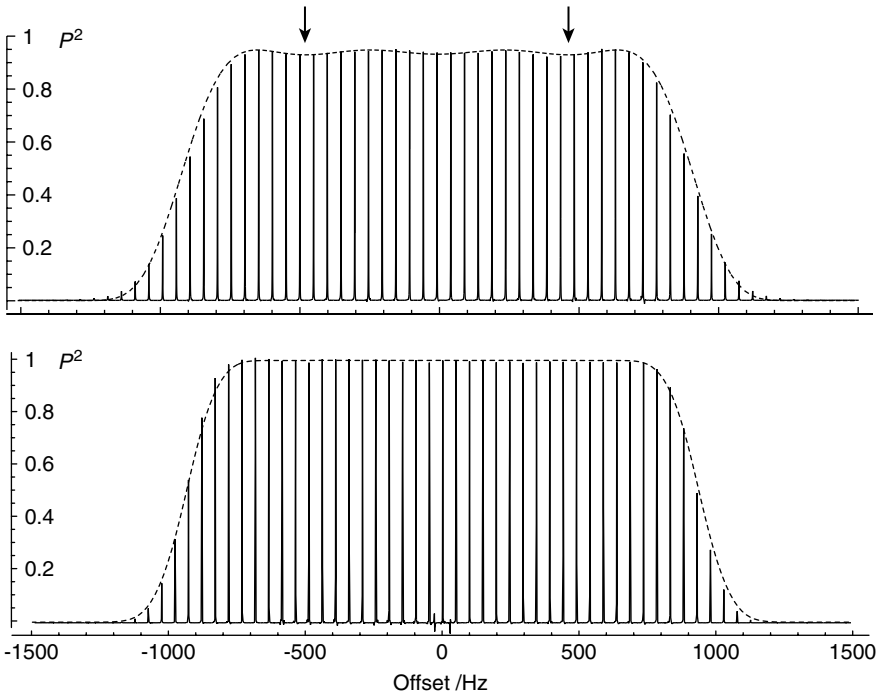

Figure 2. Experimental excitation profiles and their theoretical envelopes (dashed) obtained from a DPFGSE when the refocusing element is a ReBurp pulse (top) or the adiabatic pulse described in the text (bottom). The adiabatic pulse produces a flat bandwidth of about 1500 Hz with transitions of 400 Hz between passband and stopband. Note that, in the case of the ReBurp pulse, a slight miscalibration of the RF power results in a rapid deviation from the theoretical profile; the small dips indicated by the arrows are an intrinsic feature of the ReBurp pulse, and cannot be removed.

Table 1. Quantification of HMF in a standard sample as a function of the flip angle (see caption to Fig. 3)[a]

Flip angle (deg)	Duration (min)	HMF concentration (mol/l)[b]
90	131	1.88×10^{-4}
50	105	1.89×10^{-4}
30	80	1.86×10^{-4}

[a] Nominal concentration of HMF: 1.88×10^{-4} mol/l.
[b] The maximum semi-dispersion of the data (1.5%) is well in accordance with ±1% assays attainable by standard quantitative NMR techniques.[6]

retrieve the small signals only if the noise is sampled correctly[17]; otherwise, the weaker signals may be completely missing from the spectrum.

One such example is represented by honey, a complex mixture consisting mainly of moist invert sugar (i.e. hydrolysed saccharose) as well as other carbohydrates, with minor amounts of enzymes, amino and organic acids, minerals, aroma substances and pigments. While sugars alone account for up to 80% of the honey composition and water for most of the remaining 20%, minor constituents like aldehydes are present at concentrations as low as 10 µg/g, thus making this matrix a good test candidate. A sample was prepared by dissolving 0.2 g of honey in 600 µl dimethyl sulfoxide (DMSO)-d_6, and ^{1}H NMR spectra of the aldehydic region were obtained both by a standard pulse-acquire sequence and by selective excitation of a 2.5-kHz spectral band downfield from 7 ppm (Fig. 5).

The selective approach clearly unveils many spectral features that are barely discernible, or even absent, in the standard

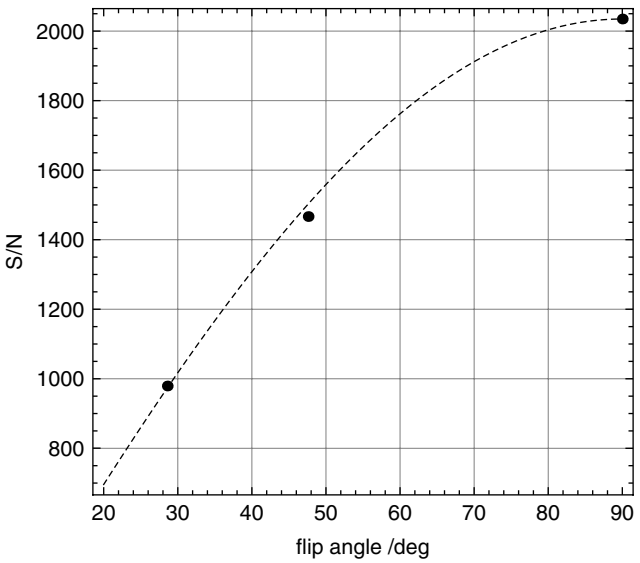

Figure 3. S/N versus flip angle of the first pulse in a selective DPFGSE experiment. The acquisition times for 90°, 50° and 30° flip angles are 131, 105 and 80 min, respectively. Actual flip angles were calculated from the pulse duration with respect to the calibrated length of the 90° pulse. The dashed curve represents the theoretical signal intensity stemming from a simple pulse-acquire experiment. T_1 for the aldehydic proton of 5-(hydroxymethyl)furfural is 12.2 s; T_1 for the aldehydic proton of DMF is 10.1 s.

experiment. We note at this point that the signals of these minor constituents provide undistorted NMR spectra (or sub-spectra) which can be used to identify their structure, where necessary. Of particular interest is the signal at 9.5 ppm, which is attributed

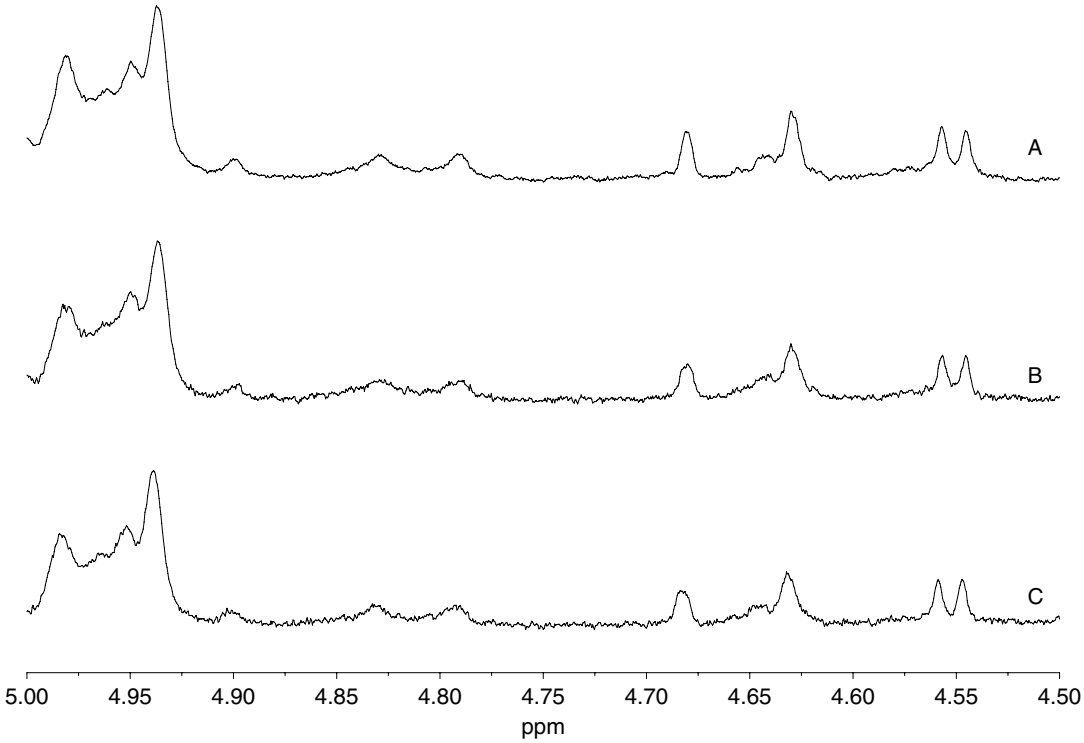

Figure 4. Expanded ^{1}H NMR spectral region of an olive oil sample. (A) standard experiment (4000 scans, 4 h 20 min); (B) DPFGSE with the adiabatic pulse specified in Fig. 1 (512 scans, 34 min, no *z*-filter); (C) DPFGSE with the ReBurp pulse specified in Fig. 1 (512 scans, 34 min, no *z*-filter). The observed signals spanning 4.5–4.7 ppm belong to terpene resonances; S/N ratios of the peak at 4.63 ppm are 27, 20 and 23 for (A), (B) and (C), respectively.

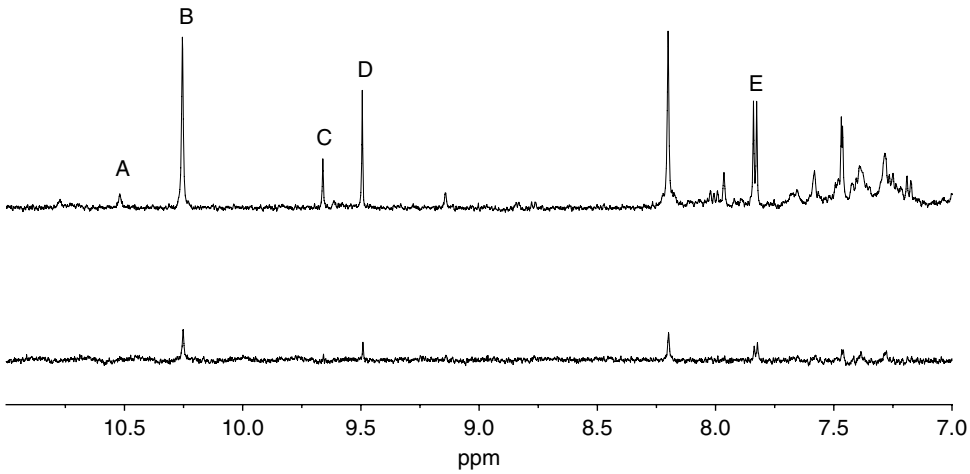

Figure 5. Expanded ^{1}H NMR spectral region of a honey sample. Top: selective experiment (512 scans, 45 min, no *z*-filter), bottom: standard experiment (4096 scans, 7 h 22 min). The two spectra are scaled so as to match the noise amplitudes. D: 5-(hydroxymethyl)furfural (HMF). Other signals are tentatively assigned as, C: furfural, A, B, E: *o*- and *p*-methoxybenzaldehyde.

to the aldehydic proton of HMF. This substance is produced by thermal degradation of sugars, and its concentration in honey is legally restricted[18] because high levels of HMF suggest bad processing practice or adulteration. Thus, by using an external standard (chloroform in DMSO) arranged into a coaxial insert, we attempted the quantification of HMF in a honey of certified origin. Two samples of the same honey dissolved either in DMSO or D_2O were prepared, revealing HMF levels of 14.5 and 15.0 μg/g (ppm), respectively (well below the legal limit of 40 μg/g). For comparison, the standard high performance liquid chromatography (HPLC) method[19] provided an HMF content of 15.0 ± 0.2 μg/g.

Conclusions

In conclusion, the band-selective excitation scheme presented herein allows one to obtain NMR spectra with a high S/N ratio within narrow spectral regions, regardless of the presence of very strong signals from the matrix, whereby small peaks can be quantitatively integrated. The implementation is simple and the pulse sequence is virtually insensitive to pulse miscalibration. Hence, applications of this methodology to many other similar cases, including 2D spectroscopy,[20] are easy to envision even as an alternative to existing analytical techniques, which, despite

their established usage, lack the amount of information delivered by NMR, most notably the capability to provide molecular structure elucidation.

Experimental

DMF (99.9%), HMF (99%), chloroform (99.9%), $Eu(fod)_3$ (99%) were purchased from Sigma and used as supplied. Deuterated solvents ($CDCl_3$, D_2O, DMSO-d_6) (>98% D) were purchased from Eurisotop. Coaxial insert and NMR tubes were obtained from Wilmad-LabGlass.

NMR spectra were acquired at 298 K on a Bruker Avance DMX 600 spectrometer equipped with a 5-mm TXI *xyz*-gradient inverse probe and a HRD16 ADC delivering a total 18-bit resolution (16 bits hardware with two additional bits by oversampling). The duration of the adiabatic Gaussian pulses of 2.0 and 2.5 kHz sweep width was 10 and 8 ms, respectively. All gradient pulses were followed by a 100-μs recovery delay. The DPFGSE pulse program was created according to Ref. 9 (see Supporting Information for details).

In the case of the olive oil sample, an adiabatic inversion Gaussian pulse of 2 kHz sweep width does also refocus some strong resonances arising from glycerol and alkene protons. To attenuate these signals, the adiabatic pulse was followed by a biselective soft 180° pulse, the aim of which is to let the unwanted signals experience a total 360° (and hence ineffective) rotation. A ReBurp shape was adopted for the soft pulse because of its sharp transitions between the passband and the stopband, while biselectivity was achieved by means of cosine modulation.

In the case of the honey sample, an adiabatic inversion Gaussian pulse of 2.5 kHz sweep width was employed as the refocusing element.

If two or more *J*-coupled spins are refocused by the adiabatic pulses, anti-phase magnetisation will likely evolve during the DPFGSE. Since anti-phase magnetisation generates peaks of opposite phases, it will decrease the overall integrated signal intensity, although by a factor that can be estimated. Lineshape effects of anti-phase magnetisation can be eliminated by means of trim pulses or *z*-filters[21]: in our case, a *z*-filter featuring a bipolar gradient pulse pair with a 'nulling' 180° pulse is advantageous by close analogy to its use in DPFGSE-NOE spectroscopy.[22] See Supporting Information for details.

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Supporting information

Supporting information may be found in the online version of this article.

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