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Decoupling 2D NMR in Both Dimensions: Pure Shift NOESY and COSY**

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Spectral assignment is a crucial step in the analysis of chemical structure by NMR, but is often limited by signal overlap. If the multiplet structure caused by spin-spin coupling is suppressed, spectral resolution can be greatly increased, and the process of assignment correspondingly simplified. Collapsing a multiplet to a single "pure shift" peak can be achieved using a variety of methods, as has been demonstrated in 1D NMR, and in one dimension of several different homonuclear 2D experiments.[1-10] Recently it has been shown that applying covariance processing to a TOCSY dataset decoupled in one dimension gives a fully decoupled 2D spectrum.[11] Previous routes to fully decoupled 2D spectra have used pattern recognition postprocessing applied to conventional, fully coupled 2D data.[12-14] The considerably increased resolution in, and simplicity of, fully decoupled 2D spectra makes them particularly attractive for automated structure elucidation.

Here we compare two different decoupling methods, illustrating the very general nature, and potential for resolution gain, of pure shift covariance NMR, by applying different single decoupling methods to the NOESY and *n*QF-COSY experiments, producing doubly pure shift 2D spectra by covariance processing. Using pure shift acquisition for one spectral dimension greatly improves resolution, collapsing multiplet structure to singlets, while the use of covariance processing gives a further gain in interpretability by condensing the correlation information into 2D singlets.

Methods for achieving broadband homonuclear decoupling in 2D NMR include the Zangger-Sterk (ZS) experiment and its adaptations;[1-4] the Pell-Keeler (PK) 45° projection of phase sensitive 2D-*J* data;[5] the "BIRD" (bilinear rotation decoupling) family of sequence elements;[6,7] and the "constant time" (CT) approach.[8-10] All such methods trade sensitivity for resolution, to a greater or lesser extent. The first three methods are appropriate for experiments that generate in-phase cross-peaks, while CT can be used with both in-phase and anti-phase signals. Each has specific advantages; ZS allows a trade-off between sensitivity and decoupling range, BIRD can avoid problems with strong coupling,[6] PK allows J couplings to be measured in a third dimension, and constant time methods have particularly good sensitivity for medium-sized molecules.

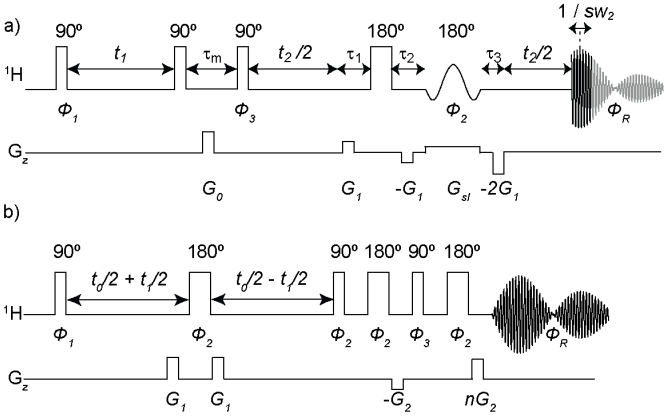

Figure 1. (a) The pure shift NOESY pulse sequence.The delays τ_1, τ_2, τ_3 are chosen so that J-evolution is refocused at the midpoint of the acquisition period, and, δ at its beginning when $t_1 = 0$. (b) The constant time multiple quantum-filtered (CT-*n*QF) COSY pulse sequence; $n = 2$ for double quantum filtration. Phase cycles are summarised in the Supplementary Information.

As an example of the ZS method, a pure shift NOESY technique is shown in Figure 1(a), concatenating a conventional NOESY sequence with a ZS block in t_2 to produce decoupled data in the F_2' dimension. Multiplying the data matrix for the singly decoupled spectrum X by its transpose and taking the square root yields the covariance matrix or correlation spectrum C:

$$\mathbf{C} = (\mathbf{X}\mathbf{X}^{\mathrm{T}})^{1/2}$$

where $^{\mathrm{T}}$ and $^{1/2}$ denote the matrix transpose and matrix square root respectively.[15] Signals in F_2' that share the same modulation as a function of t_1 will show a strong peak in the correlation spectrum, while those with different modulations will not. Both dimensions are now decoupled; the resolution in F_1 is equal to that in F_2', and the number of t_1 increments need only be large enough to prevent the generation of spurious peaks from signal overlap in F_1.

Figures 2a and 2b show respectively the conventional NOESY and singly pure shift NOESY 2D spectra of the antibiotic clarithromycin. The removal of multiplet structure in F_2' makes the interpretation of the busier regions much simpler, while the consolidation of signal intensity means more cross-peaks are visible in Figure 2b. Covariance processing further simplifies the spectrum by removing the multiplet structure in F_1 (see Figure 2c). Considerable effort has historically gone into eliminating through-bond zero-quantum cross-peaks from NOESY spectra;[16] here the problem is bypassed, as the undesired anti-phase terms are collapsed to zero by the decoupling.

This approach is flexible and fairly general; the ZS sequence element may be replaced by the BIRD element if there is strong coupling, or the PK approach may be used to facilitate J coupling measurements. However, none of these is directly compatible with experiments such as COSY that generate antiphase cross-peaks, because decoupling would cause signal cancellation. Using constant time decoupling in F_1 avoids this problem, as in the

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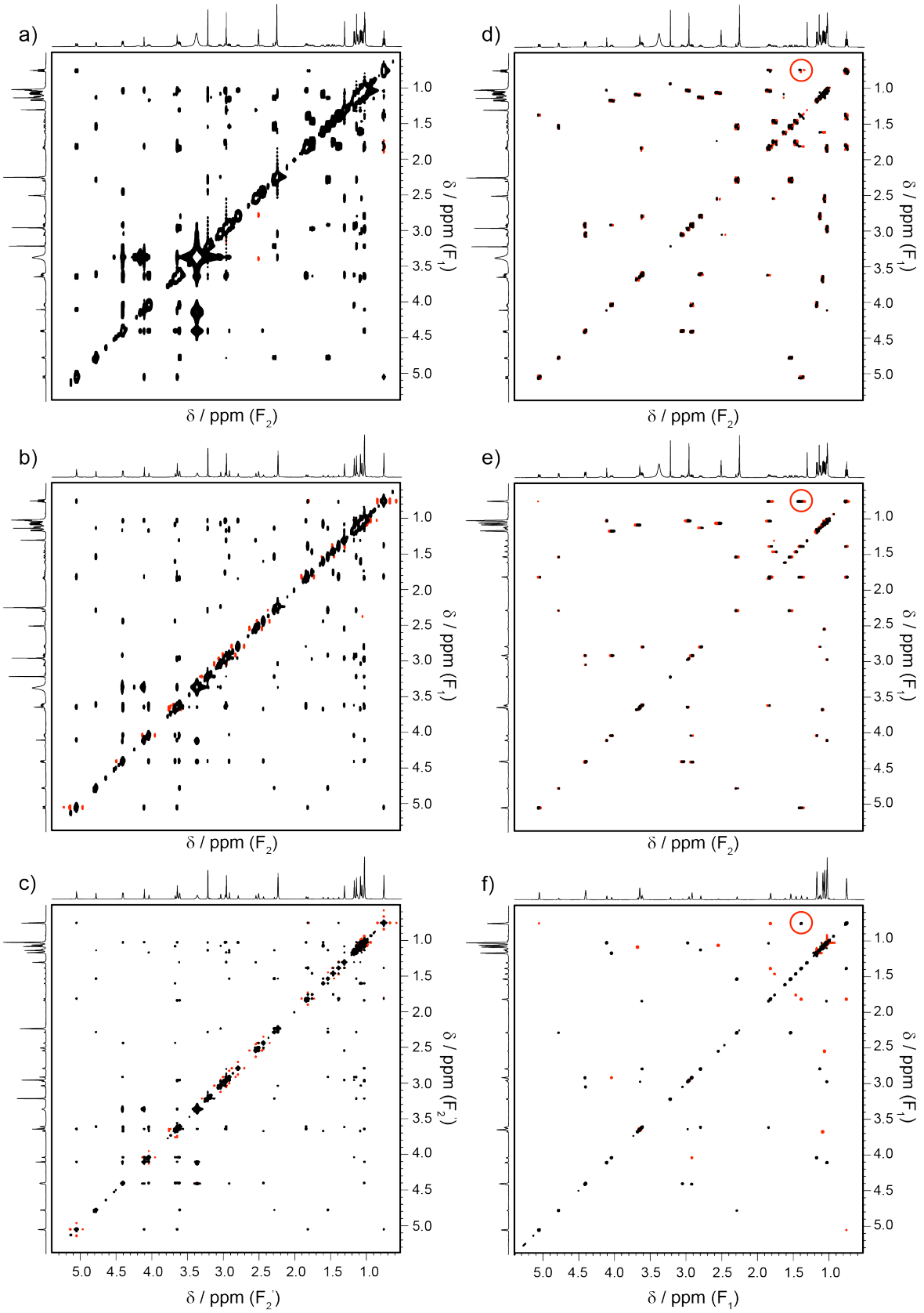


Figure 2. NOESY (a-c) and 2QF-COSY (d-f) spectra of clarithromycin in DMSO-d_6: (a) conventional NOESY; (b) ZS pure shift NOESY; (c) spectrum produced by covariance processing of dataset 2(b); (d) conventional 2QF-COSY spectrum; (e) constant time 2QF-COSY; (f) covariance processing of dataset (e). Note the degree of simplification of (c) and (f) over (a) and (d). An expansion of the cross-peak circled is shown in the supplementary information (SI1).

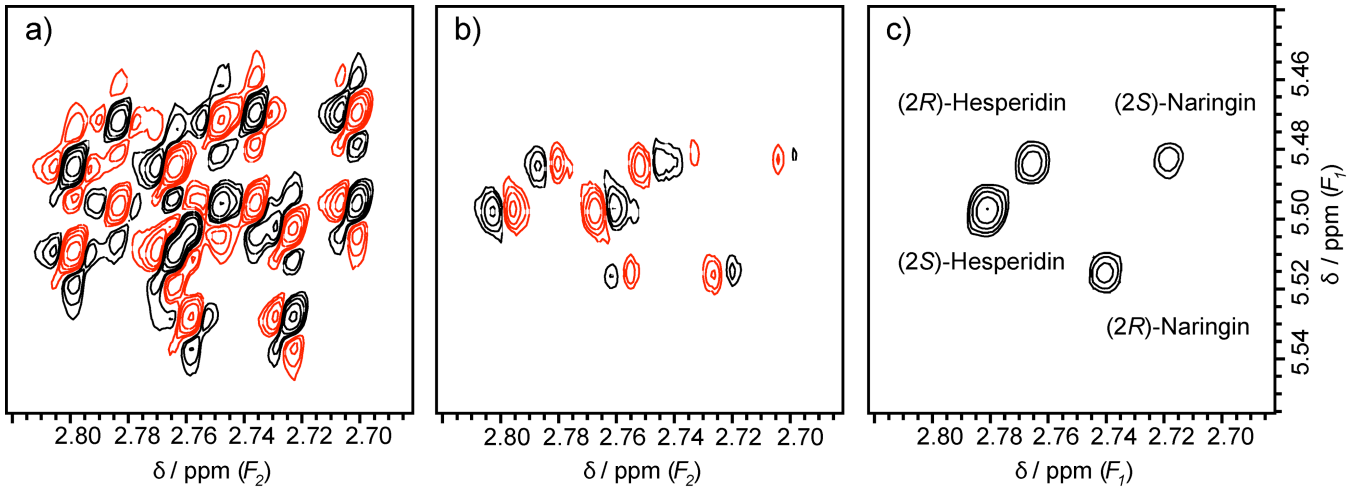
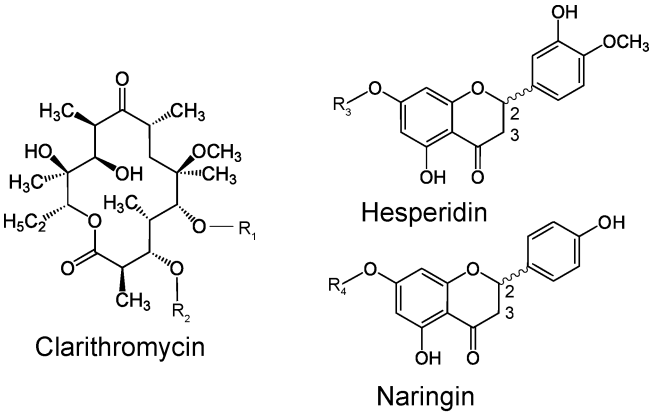


Figure 3. Expansions of the cross-peaks between proton 2 and the more shielded geminal proton 3 in (a) 3QF-COSY and (b) CT-3QF-COSY spectra obtained by conventional 2DFT, and (c) CT-3QF-COSY covariance spectrum, for a mixture of (*2R*)-hesperidin, (*2S*)-hesperidin, (*2R*)-naringin and (*2S*)-naringin in DMSO-d_6. Full spectra and further expansions are shown in the supplementary information (SI2), as are traces illustrating the approximate factor of 2 difference in signal-to-noise ratio between (a) and (b).



Scheme 1. Clarithromycin (R_1 = cladinose, R_2 = desosamine), hesperidin (2R and 2S; R_3 = rutinoside) and naringin (2R and 2S; R_4 = neohesperidoside).

constant time *n*QF-COSY experiment of Figure 1b, in which the constant time t_0 is chosen to optimise coherence transfer for signals of interest. The result is an F_1 pure shift experiment (Figure 2e), which can be combined with covariance processing in the same manner as above to yield the fully decoupled 2D spectrum of Figure 2f. Figure SI1 illustrates the power of the technique by comparing cross-peak patterns in a standard 2QF-COSY spectrum with the 2D-covariance CT-2QF-COSY version: 24 peaks collapse into a singlet. One advantage of using an F_1 pure shift method is that the dimension in which the covariance calculation is carried out, t_2 or F_2, is well-sampled and hence covariance artefacts caused by signal overlap are minimised.

The particular stimulus to the development of these techniques was the difficulty encountered in assigning the spectra of the flavonoids hesperidin and naringin, found naturally in citrus fruits. These compounds are normally encountered as mixtures of the diastereomeric forms *2S* and *2R*, in proportions determined by the degree of ripeness of the fruit, and as a result show highly overlapped spectra. Such overlap is common in mixtures of chemically cognate species, e.g. natural product extracts, and is particularly difficult to deal with. Because isomers, and in particular diastereomers, frequently show very similar chemical shifts in regions that share the same stereochemistry, peaks tend to overlap in both 1D and 2D spectra.

As a stringent test of the pure shift methodology, triple quantum COSY spectra of a mixture of hesperidin and naringin were measured. Figure 3 compares the cross-peaks between proton 2 and the more shielded geminal proton 3 in the conventional 3QF-COSY, pure shift 3QF-CT-COSY, and covariance processed pure shift 3QF-CT-COSY spectra. The increase in resolution and interpretability afforded by the pure shift experiment and covariance processing respectively is clear. It would be extremely difficult to interpret Figure 3a. In principle all the requisite information for assignment is accessible in Figure 3b, which consists of four 2D multiplets which are antiphase doublets of doublets in F_2 and singlets in F_1, but the structure of the set of four correlations is made immediately obvious by Figure 3c.

The appropriate choice of decoupling technique will depend on the problem at hand. The CT approach has an inherent sensitivity advantage over BIRD, ZS and PK methods, as it does not rely on observing only a subset of spins. However, both phases and amplitudes of peaks depend on the spin system and on the constant time t_0. Hence where relative cross-peak signs are important (e.g. in NOESY), BIRD, ZS or PK methods are preferable. For relatively sensitive experiments such as COSY, acquiring spectra with multiple t_0 values can circumvent the problem. A further problem with CT-*n*QF-COSY that is specific to covariance processing stems from the difference in structure between cross- and diagonal peaks, which can cause peaks to attenuate, or (rarely) even disappear, in the covariance spectrum. This can readily be detected by comparison with absolute value covariance or $m \neq n$ quantum filtered data, or by comparing spectra acquired with different t_0 values.

As the results for these two prototype experiments show, collapsing all multiplet structure in cross- and diagonal peaks to singlets greatly improves the accessible information content of spectra. Almost all common 2D NMR experiments can be adapted to use such decoupling methods. The generality of pure shift methods should be of considerable value in manual and automated structure determination alike. The choice of method will, as noted above, depend on the sample concentration and instrument sensitivity available. Where there is sufficient signal-to-noise ratio, either conventional or covariance[17,18] heteronuclear correlation methods are other possibilities, while in extreme cases using covariance processing to combine heteronuclear and pure shift homonuclear correlation would be particularly powerful.

Experimental Section

The NOESY and pure shift NOESY data of Figures 2a and 2b were acquired on a 500 MHz Varian VNMRS spectrometer using a 75 mM sample of clarithromycin in DMSO-d_6. For both phase-sensitive NOESY datasets, 2 scans of 16384 complex data points were acquired for each of 128 t_1 increments, with spectral widths of 3500 Hz in both frequency dimensions. For the pure shift NOESY, 20 increments in t_2 were used with a spectral width of 3500 Hz. The experiment times were 0.5 h and 10.75 h respectively. After assembly of the pure shift data using an in-house VNMRJ 2.2C macro, the data were imported to TopSpin 3.0 and adjusted to 4096 × 4096 points (zero filling in F_1, truncating in F_2) for covariance processing. The phase sensitive 2QF-COSY datasets of the same sample were acquired on a Bruker Avance II+ spectrometer with a spectral width of 2750 Hz, 1024 t_1 increments, a constant time t_0 of 0.38s and one scan per increment in 42 min. Covariance processing was performed in TopSpin 2.1 as above. The 3QF-COSY experiments of Figure 3 were performed on an equimolar sample of 53 mM hesperidin and naringin in DMSO-d_6 using the same Bruker spectrometer, with a spectral width of 1976 Hz, 1024 t_1 increments, a constant time t_0 of 0.38 s and two scans per increment in 127 min. Covariance processing was performed as above but with 4096 × 2048 points.

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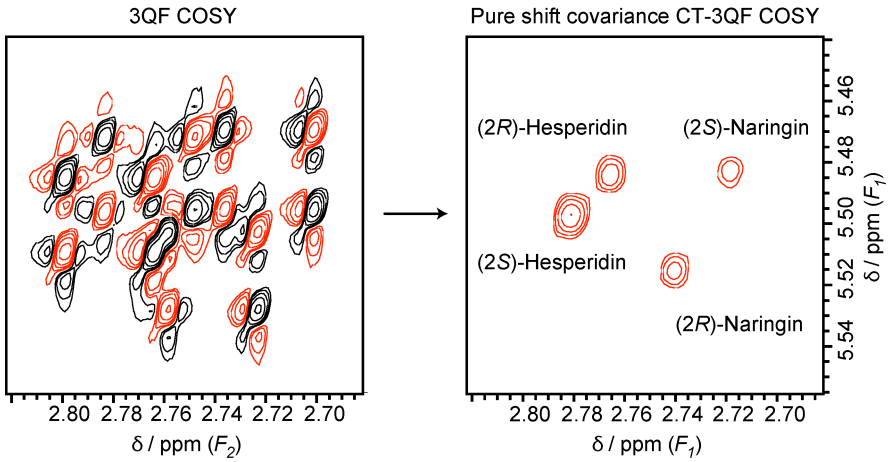

The problem of increasing resolving power in 2D NMR is attacked by collapsing 2D peaks with multiplet structure into 2D singlets. This is achieved by combining 2D experiments with pure shift techniques and covariance processing. The method is illustrated with fully decoupled phase-sensitive pure shift NOESY and *n*QF-COSY experiments, and should be of value in both manual and automated structure determination.