Why is HMBC superior to LR-HSQC? Influence of homonuclear couplings $J_{HH'}$ on the intensity of long-range correlations

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Abstract Long-Range Heteronuclear Single Quantum Correlation (LR-HSQC) experiments may be applied as an alternative to Heteronuclear Multiple-Bond correlation (HMBC) experiments for detecting long-range correlations, but has never enjoyed popularity for that purpose. To the best of our knowledge, the exact reasons have not yet been fully established. For both experiments it is widely accepted that the evolution of proton-proton homonuclear couplings $J_{HH'}$ during the polarization transfer delays $\Delta$ leads to significant losses, and that the intensity of the observable coherence is zero when $J_{HH'}$ matches the condition $\Delta = 0.5/J_{HH'}$. Here, we analyze the influence of $J_{HH'}$ on the intensity of long-range correlations in HMBC and LR-HSQC spectra. We show that for both experiments long-range correlations will not be canceled because of homonuclear couplings $J_{HH'}$. Our theoretical and experimental results definitely establish and validate the superiority of HMBC-based experiments among the family of heteronuclear long-range correlation experiments: (i) the overall cross peak’s intensity is higher, and (ii) in LR-HSQC experiments the intensity of the long-range cross peaks is additionally influenced in an unwanted way by the magnitude and number of passive homonuclear proton-proton couplings $J_{HH'}$.

Keywords: NMR, $^1$H, $^{13}$C, HMBC, LR-HSQC, homonuclear couplings, cross peak intensity, product operator.
Introduction

Heteronuclear long-range correlation experiments correlate protons and heteronuclei exploiting $^{n}J_{HX}$ long-range couplings. The experiments are essential to connect structural fragments across non-protonated carbons or heteroatoms.$^{[1]-[9]}$ Prior to the introduction of proton-detected NMR methods, experiments such as long-range HETCOR and FLOCK were used for this purpose.$^{[10]}$ Currently, there are a plethora of proton-detected methods available for long-range heteronuclear shift correlation.$^{[10]-[13]}$ The oldest and still, probably, most widely used long-range heteronuclear shift correlation experiment is the basic HMBC experiment described in 1986 by Bax and Summers.$^{[14]}$ This pulse sequence employs only a few RF pulses, making it not only the most sensitive but also very robust in terms of RF inhomogeneity or poorly adjusted pulse lengths.$^{[15]}$ Despite its undeniable strengths, there are several important established issues associated with the basic HMBC experiment:$^{[15], [16]}$ (a) the detected proton magnetization is antiphase with respect to the active carbon which on the one hand prevents the use of carbon broadband decoupling during acquisition and which on the other hand may cancel the cross peaks due to unfavorable signal overlap with very small coupling constants;$^{[17]}$ (b) both multiple quantum coherences and homonuclear couplings ($J_{HH^{'}}$) evolve during the entire $t_1$ evolution period, giving tilted and $J_{HH^{'}}$-split multiplet structures along the $F_1$ dimension; and (c) the final signal intensity is proportional to $\sin(\pi^e J_{XH} \Delta)$ and depends – as outlined below – exclusively on the long-range heteronuclear coupling constant.

Several years after the introduction of the HMBC experiment, numerous variants were introduced. The addition of a delay $\Delta$ for refocusing heteronuclear couplings to the basic HMBC experiment has led to the D-HMBC,$^{[18]}$ which has never enjoyed the popularity of the basic HMBC experiment, although it generates in-phase $^{n}J_{XH}$ correlations and allows the application of $^{13}$C decoupling during the acquisition time. During the period from 1998-2000, several “accordion” optimized long-range correlation experiments, aimed at equalizing the intensities of long-range correlations, were reported.$^{[19]-[22]}$ At the same time, the so-called constant-time (CT-) HMBC experiments with a $t_1$-evolution of fixed length were proposed.$^{[23], [24]}$ These experiments allow $J_{HH^{'}}$ modulations in $t_1$ to be suppressed and provide cross peaks.
that are effectively decoupled in the indirect dimension with respect to $\nu J_{HH'}$ (CT-HMBC-1) and to both $\nu J_{XH}$ and $\nu J_{HH'}$ (CT-HMBC-2), thus significantly improving the resolution and the sensitivity.\cite{23}

In 2001, the sensitivity of the HMBC could be increased by as much as a factor of $\sqrt{2}$ compared with the basic HMBC experiment, by refocusing and detecting two orthogonal in-phase magnetization components.\cite{25-27} This experiment is now known as SE-HMBC. The combination of CT-HMBC, SE-HMBC, an efficient low-pass $J$ filter (for suppressing unwanted $^1 J_{XH}$ artifacts),\cite{28} and of the ASAP building block,\cite{29} that significantly enhances the sensitivity and allows reducing the measurement time, has led to the IMPACT-HMBC experiment, which is probably the most useful and efficient sequence derived from the classical HMBC.\cite{30} Most of these methods have been discussed in a number of reviews and the interested reader is referred to these for a more in-depth treatment of long-range heteronuclear correlation methods.\cite{8, 15, 16, 31-33}

For all these HMBC variants, it was commonly pointed out that possible accidental cancellation of correlations may occur, because the amount of useful magnetization generated in the initial $\nu J_{XH}$-evolution preparation period $\Delta$ is dependent not only on $\nu J_{XH}$, but is additionally modulated by a trigonometric factor $\Sigma \cos(\pi J_{HH_i} \Delta)$, due to the evolution of the homonuclear proton-proton $J_{HH_i}$ couplings.\cite{15, 16, 31, 33} However, cross peaks in HMBC always and exclusively vanish when the long-range coupling evolution delay, $\Delta$, matches the long-range heteronuclear coupling constant, $\Delta = k/\nu J_{XH}$, as recently demonstrated by us.\cite{34} The signal-to-noise ratio (SNR) of long-range correlations in HMBC spectra therefore does not depend upon the proton-proton homonuclear coupling.\cite{34}

As an alternative to HMBC experiments long-range versions of HSQC\cite{35-37} may be applied, but have obviously never enjoyed popularity for that purpose. Yet, HSQC-based techniques appear inherently more efficient, especially because the correlation peak shapes can be improved without a constant-time setting, as no $J_{HH'}$ modulation occurs during $t_1$. However, the $J_{HH'}$ evolution occurs during the long polarization transfer delays of the INEPT, and some of the magnetization will be transferred to homonuclear multiple-quantum coherence.\cite{38, 39} Consequently the intensity of cross-peaks turns out to depend on both $\nu J_{XH}$ and $\nu J_{HH'}$ couplings. Therefore cross-peaks will not only be cancelled with $\Delta = k/\nu J_{XH}$ but their intensity is

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strongly and additionally influenced by the magnitude and number of passive homonuclear proton-proton $J_{HH'}$ couplings.

It is also commonly admitted that with HSQC the intensity of the observable $H_YX_Z$ coherence (if a XHH' spin system is considered) is zero when $J_{HH'}$ matches the condition $\Delta = 0.5/J_{HH'}$.\[^{39}\] We show however that long-range correlations will not be cancelled at this condition and generally do not cancel because of homonuclear couplings $J_{HH'}$. This is as outlined below because for e.g. a CHH' spin system, not only one, but two coherences which partially compensate each other actually contribute to the final signal.

As shown in this manuscript, HMBC-based experiments seem to perform better compared to long-range versions of HSQC, which clarifies why HSQCs have never enjoyed popularity for detecting long-range correlations. The main reason is however not that the observable long-range cross peaks is zero when $\Delta = 0.5/J_{HH'}$, but rather that the intensity of long-range correlations in HMBC spectra are by far less dependent on passive homonuclear proton-proton $J_{HH'}$ couplings, resulting in an improved visibility of cross-peaks and enhanced sensitivity in general compared to HSQC variants.

**Results and Discussion**

**LR-HSQC experiment**

**Analysis for a CHH' spin system**

We consider first the LR-HSQC experiment (Figure 1),\[^{35, 36}\] also known under the acronym GSQMBC (Gradient-enhanced Single Quantum Multiple Bond Correlation) and initially designed for measuring small heteronuclear coupling constants, not accessible at that time with gradient-enhanced HMBC spectra\[^{40}\] in magnitude mode presentation. In the following and throughout the manuscript, we’ll only discuss CH$_n$ spin systems, but the different conclusions can be generalized to other XH$_n$ spin systems.
Figure 1. Pulse sequence of the LR-HSQC experiment with echo-antiecho gradient selection[36, 39] and without a low-pass filter (pulseprogram hsqcetgplrsp from the Bruker pulse sequence library). Thin bars represent 90° pulses, thick bars 180° pulses. The 180° pulses on the 13C channel are advantageously replaced by broadband adiabatic inversion (first and third) and refocusing (second) pulses, shown as sine pulses. Δ is the long-range coupling evolution delay and is set to an average value 0.5/nJCHav. Delay ζ is set to guarantee proper 1H chemical shift refocusing and is equal to the length of G2 + delay for gradient recovery. The following phase cycling is applied: ϕ1 = x, -x; ϕ2 = x, x, -x, -x; ϕ3 = 4x, 4(-x); ϕrec = x, -x, x, -x, x, -x, x. Phases not shown are applied along the x-axis. Gradient ratios: G1:G2:G3 = 40:20:34 (odd), -40:20:34 (even) for echo-/antiecho detection. The labels a-d denote the four steps of interest in the pulse sequence.

At point a, a product operator evaluation for a CHH' spin system, taking into account that all chemical shifts are refocused, yields the following coherences (H is a proton long-range coupled (nJCH) to carbon C and H' is a proton coupled to H through JHH', and considering nJCH and JHH' ≠ 0). The subscript r will be used subsequently throughout the whole manuscript to emphasize that the proton H is long-range coupled to the carbon. Note that there is no difference if we consider nJCH ≠ 0. The reason is that both spins C and H' are present as z-magnetization during Δ, and therefore the J-coupling between them remains inactive.

\[
H_Z^r (90°) x_H \rightarrow -H_Z^r \rightarrow H_X^r \rightarrow \pi n J_{CH} \Delta H Z C Z (180°) x_H (180°) x_H^r (180°) x_C (90°) y_H (90°) y_H^r \\
- H_Z^r \cos(\pi J_{HH'} \Delta) \cos(\pi n J_{CH} \Delta) - 2H_Z^r C_Z \cos(\pi J_{HH'} \Delta) \sin(\pi n J_{CH} \Delta) \\
- 2H_Z^r H_X^r \sin(\pi J_{HH'} \Delta) \cos(\pi n J_{CH} \Delta) + 4H_Z^r H_X^r C_Z \sin(\pi J_{HH'} \Delta) \sin(\pi n J_{CH} \Delta)
\] (1)
The coherences present immediately after the second 90° ¹H-pulse can be classified as follows: (i) a longitudinal coherence $2H_Z^rC_Z$, (ii) two pure proton single-quantum coherences $H_Y$ and $2H_X^rH'_Z$ and (iii) a multi-quantum coherence $4H_Y^rH_X^rC_Z$.

At this stage of the LR-HSQC sequence a spoil gradient G3 may be applied that dephases all but z-magnetizations and zero-quantum coherences.⁴¹ Note that the pure proton coherences $H_Y$ and $2H_X^rH'_Z$ can be ignored anyway, since on the one side they will not contribute to carbon coherence after the first 90° ¹³C pulse for the subsequent ¹³C-shift evolution period $t_1$, and on the other side they will be destroyed subsequently by the coherence selection gradients G1 and G2 (G1 = ±2, G2 = 1). It’s worth to mention that with the Cartesian operators shown in equation 1, one might assume by mistake that only the longitudinal polarization $2H_Z^rC_Z$ (z-ordered state) survives after the spoil gradient G3. Therefore and to understand the effect of magnetic field gradients the Cartesian operators must be replaced by the respective raising and lowering operators. With the $4H_Y^rH_X^rC_Z$ coherence term correspondingly transformed it is obvious that it represents actually a sum of double (DQ) and zero (ZQ) quantum coherences:⁴²

$$4H_Y^rH_X^rC_Z \rightarrow -i(H_Y^+H_Y'^+ + H_Y^-H_Y'^-) - H_Y^rH_Y'^rC_Z$$  
$$DQ \quad ZQ \quad ZQ \quad DQ$$  

(2)

**G3 turned off**

The 90° pulse applied on the ¹³C channel leads to:

$$-2H_Z^rC_Z \cos(\pi J_{HH'} \Delta) \sin(\pi \nu_j CH \Delta) - i(H_Y^+H_Y'^+ + H_Y^-H_Y'^-) \sin(\pi \nu_j CH \Delta)$$

$$- H_Y^rH_Y'^rC_Z \sin(\pi J_{HH'} \Delta) \sin(\pi \nu_j CH \Delta)$$

$$- H_Y^rH_Y'^rC_Z \sin(\pi J_{HH'} \Delta) \sin(\pi \nu_j CH \Delta)$$

(3)

It can be seen that ¹³C-single quantum coherences (SQ) and ¹H, ¹³C-triple quantum coherences (TQ) are obtained:

$$-2H_Z^rC_Z \cos(\pi J_{HH'} \Delta) \sin(\pi \nu_j CH \Delta) - i(H_Y^+H_Y'^+ + H_Y^-H_Y'^-) \sin(\pi \nu_j CH \Delta)$$

$$- H_Y^rH_Y'^rC_Z \sin(\pi J_{HH'} \Delta) \sin(\pi \nu_j CH \Delta)$$

$$- H_Y^rH_Y'^rC_Z \sin(\pi J_{HH'} \Delta) \sin(\pi \nu_j CH \Delta)$$

(3)
\[ -iH_ZC_+ + iH_ZC. \]

\[ \frac{1}{2}(H_r H'_+ C_+ - H_r H'_- C_+ + H_r H'_+ C_+ - H_r H'_- C_+) \]

\[ (4) \]

The various coherences correspond to different nuclei specific coherence levels \( \text{CL}_k \), e.g. \( H_r H'_+ C_+ \) has coherence levels \( +2(\text{H}) \), \( +1(\text{C}) \) and \( H_r H'_- C_+ \) has coherence levels \( 0(\text{H}), +1(\text{C}) \).

In addition, not only the coherence levels but the corresponding resonance frequencies which in fact depend on the magnetogyric ratios of the involved nuclei have to be taken into account. Therefore and to obtain a detectable signal at the end of a selected coherence pathway, the following condition for the individual gradient strengths \( G_i \) and the correspondingly present (gyromagnetic-weighted) coherence levels \( \text{CL}_i \) across the pulse sequence must be fulfilled:\cite{41}

\[ \sum G_i \cdot \text{CL}_i \cdot \gamma_i = 0 \]

Consequently, the triple quantum coherences \( H_r H'_+ C_+ \), \( H_r H'_- C_+ \), \( H_r H'_+ C_- \), and \( H_r H'_- C_- \) will be dephased by the subsequent coherence selection gradients \( G1 \) and \( G2 \) - with their strengths set to ratios 2:1 and -2:1 respectively - and can be ignored. The remaining \( ^{13}\text{C} \)-single quantum coherences, \( iH_ZC_+ \), \( iH_ZC_- \), \( H_r H'_+ C_+ \), \( H_r H'_- C_- \), \( H_r H'_+ C_- \), and \( H_r H'_- C_+ \), on the other hand, will be finally rephased with these gradient settings and may be detected.

**G3 turned on**

The double-quantum terms of \( 4H_y H'_x C_Z \) in equation (2), \( -i(H_r H'_+ - H_r H'_-)C_Z \) are dephased by the gradient \( G3 \) and can be ignored. The zero-quantum terms, \( -i(H_r H'_+ - H_r H'_-)C_Z \) – as well as the polarization \( 2H_Z C_Z \) - on the other hand, are left
unaffected and continue to evolve. Therefore and including the $2H'ZC$ term the $90^\circ$ pulse applied on the $^{13}$C channel leads to:

$$
-2H'ZC \rightarrow -iH'ZC_+ + iH'ZC_-
$$

$$
$$

which are $^{13}$C-single quantum coherences that continue to evolve and will be selected with the strengths of the gradients G1 and G2 set to ratios 2:1 and -2:1 respectively. It is noteworthy that the outcome of the INEPT block is identical, irrespective of the purge gradient G3, which in fact is applied to ensure that no coherences will be present during the $t_1$-evolution period that did not take part in the INEPT transfer step. \[43\]

The coherences $-iH'z(C_- - C_+)$ evolve during the $t_1$ evolution period and the subsequent $180^\circ$ $^{13}$C-pulse as follows. Note that the two gradients G1 will select either the $C_+$ or the $C_-$ coherence and that we keep only the coherences containing $C_-$:

$$
iH'z(C_- - C_+) \xrightarrow{t_1/2 - (180)^ degrees H', (180)^ degrees H'} \rightarrow 0.5(H',H',C_- - H',H,C_+) e^{i\pi C t_1}
$$

(6)

The coherences $0.5(H',H',C_- - H',H',C_+ - H',H',C_+ + H',H',C_-)$, which will be reduced by the two gradients G1 to $0.5(-H'H', H' + H'H')C_-$ for the reasons mentioned above, evolve during the $t_1$ evolution period and the subsequent $180^\circ$ $^{13}$C-pulse as follows:

$$
-0.5(H'H_- - H'ZC_-) \xrightarrow{t_1/2 - (180)^ degrees H', (180)^ degrees H'} \rightarrow 0.5(H',H')C_-
$$

(7)

The second pair of $90^\circ$ pulses applied on both channels will convert the $H'ZC$ antiphase coherence into observable proton antiphase magnetization $H'C_2$ and will
convert the $H_rH'_zC_z$ and $H_rH'_zC_z$ coherences into observable proton antiphase magnetization $H_rH'_zC_z$ (the effects of the delays $\zeta$ and the second pair of simultaneous $180^\circ \, ^1H$ and $^{13}C$ pulses are neglected):

$$iH_r^zC_z e^{i\pi \Omega_c t_1} \rightarrow (90^\circ)_x C_z (90^\circ)_x H'_r (90^\circ)_x H'_z \rightarrow -0.5iH_r^zC_z e^{i\pi \Omega_c t_1}$$

(8)

$$0.5(H'_rH'_z - H'_rH'_z)C_z e^{i\pi \Omega_c t_1} \rightarrow (90^\circ)_x C_z (90^\circ)_x H'_r (90^\circ)_x H'_z \rightarrow 0.5H_r^zH'_zC_z e^{i\pi \Omega_c t_1}$$

(9)

Thus, at $d$, before acquisition, we have (including the corresponding trigonometric factors):

$$-0.5iH_r^zC_z e^{i\pi \Omega_c t_1} \cos(\pi J_{HH'} \Delta) \sin(\pi \xi_{CH} \Delta)$$

$$+ 0.5H_r^zH'_zC_z e^{i\pi \Omega_c t_1} \sin(\pi J_{HH'} \Delta) \sin(\pi \xi_{CH} \Delta)$$

(10)

The two coherences represent dispersive $y$-magnetization of spin $H_r$ antiphase with respect to spin $C$ and absorptive $x$-magnetization of spin $H_r$ doubly antiphase with respect to spin $H'$ and $C$. Importantly, the presence of cross-peaks with mixed phase can lead to the accidental signal cancellation, especially if the magnitudes of the different coupling constants $\xi_{HH'}$ and $J_{HH'}$ and the linewidths of the individual lines are of the same order. In this respect, the digital resolution in $F_2$ is also important and recording the data with sufficient points is mandatory.$^{[44]}$

It turns out that in LR-HSQC experiments long-range proton-carbon cross-peaks do not cancel when the homonuclear coupling $J_{HH'}$ accidentally matches multiples of twice the long-range coupling evolution delay $\Delta$, i.e. when $\cos(\pi J_{HH'} \Delta) = 0$. To the best of our knowledge, this somewhat surprising characteristic has not been reported so far,$^{[38, 39]}$ but it appears absolutely consistent: According to equation (10) and since the $H_rC_z$ shows a $\cos(\pi \xi_{HH'} \Delta)$- and the $H_rH'_zC_z$ a $\sin(\pi \xi_{HH'} \Delta)$-dependence at least one of the two $\xi_{HH'}$-dependent coherence components will be $>0$ and obviously compensate in part each other when $\Delta$ is
varied. Cancelation of long-range proton-carbon correlations in LR-HSQC spectra only occurs when the delay $\Delta$ unintentionally fulfills the condition $\Delta = k/n J_{CH}$, $k = 1, 2, ...$. The effect of homonuclear coupling $J_{HH'}$ shows up as intermediate drops of the intensity and with a general decrease in sensitivity as corroborated by corresponding simulations (Figure 2).

Figure 2. Simulated spectra obtained for a CHH' spin system, using the 1D $^{13}$C selective version of the LR-HSQC pulse sequence shown in Figure 1 as a function of $\Delta$. For better visualization the spectra are displayed in magnitude mode. (A) $nJ_{CH} = 7$ Hz, $J_{HH'} = 0$, (B) $nJ_{CH} = 7$ Hz, $J_{HH'} = 3$ Hz, (C) $nJ_{CH} = 7$ Hz, $J_{HH'} = 7$ Hz, (D) $nJ_{CH} = 7$ Hz, $J_{HH'} = 11$ Hz, (E) $nJ_{CH} = 7$ Hz, $J_{HH'} = 17$ Hz. Zero intensity in the simulated spectra occurs exclusively with $\Delta = 1/n J_{CH}$. Simulations have been performed with the BRUKER NMRSIM program for MAC (version 5.5.3. 2012).

Yet, as demonstrated by Figure 2, homonuclear couplings $J_{HH'}$ will not additionally zero the peak's intensity when $\Delta$ is varied. However, and depending on the size $J_{HH'}$ of the homonuclear coupling signal intensities may be decreased to
such a degree that long-range proton-carbon cross peaks may be very weak or even absent.

**Analysis for a CHH’H'' spin system**

Similar calculations can be performed for a CHH’H’’ spin system assuming \( n_{J_{CH}}, J_{HH}, J_{HH'} \neq 0, n_{J_{CH'}}, n_{J_{CH''}} = 0 \). This system represents a usual AMQX spin system, where \( A = ^1H \), \( M \) and \( Q = \) remote \( ^1H \), and \( X = ^{13}C \). At a, the following coherences are present (omitting the pure proton coherences that are subsequently dephased by the magnetic field gradients):

\[
\begin{align*}
-2H'ZCZ\cos(\pi J_{HH'}\Delta)\cos(\pi J_{HH''}\Delta)\sin(\pi J_{CH}\Delta) \\
+4H'\gamma H''ZCZ\cos(\pi J_{HH'}\Delta)\sin(\pi J_{HH''}\Delta)\sin(\pi J_{CH}\Delta) \\
+4H'\gamma H'ZCZ\sin(\pi J_{HH'}\Delta)\cos(\pi J_{HH''}\Delta)\sin(\pi J_{CH}\Delta) \\
+8H'ZH'XH''ZCZ\sin(\pi J_{HH'}\Delta)\sin(\pi J_{HH''}\Delta)\sin(\pi J_{CH}\Delta)
\end{align*}
\]

(11)

The last three terms can be rewritten again using the raising and lowering operators as:

\[
\begin{align*}
4H'\gamma H'XZCZ &\rightarrow i(H'_+H'_+ + H'_-H'_-)CZ \\
4H'\gamma H''XZCZ &\rightarrow i(H''_+H''_+ + H''_-H''_-)CZ \\
8H'ZH'XH''ZCZ &\rightarrow 2(H'_+H''_+ + H'_-H''_- + H''_-H''_-)H'ZCZ
\end{align*}
\]

(12)

As mentioned above, the respective double quantum components of \( 4H'\gamma H'XZCZ \) (\( i(H'_+H'_+ - H'_-H'_-)CZ \)), \( 4H'\gamma H''XZCZ \) (\( i(H''_+H''_+ - H''_-H''_-)CZ \)) and \( 8H'ZH'XH''XZCZ \) (\( H''_+H''_+ + H''_-H''_-)H'ZCZ \) are dephased by the gradient \( G3 \) and can be ignored. The respective zero quantum components, \( (i(H'_+H'_+ - H'_-H'_-)CZ), (i(H''_+H''_+ - H''_-H''_-)CZ) \) and \( 2(H'_+H''_+ + H'_-H''_-)H'ZCZ \), on the other hand, together with the \( 2H'ZCZ \) term, are left unaffected by \( G3 \), continue to evolve and are retained respectively.
The 90° pulse applied on the 13C channel leads to the following coherences (the trigonometric factors are omitted for clarity). Note that – for final detection of the wanted 1H signal - the gradients G1 and G2 (with their strengths set to ratios 2:1 and -2:1) will select C+ or C- respectively. For clarity only the C+ terms are listed:

\[ -iH_Z^r C_+ \]
\[ -0.5(H^r H^r C_+ - H^z H^r C_+) \]
\[ -0.5(H^r H^r C_+ - H^z H^r C_+) \]
\[ i(H^r H^r C_+ + H^z H^r C_+)H_Z^r \]

(13)

After the \( t_1 \) evolution time and the subsequent 180° 13C-pulse the second pair of 90° pulses applied on both channels converts these coherences into observable proton antiphase magnetization \( H.C_Z \), \( HH'z C_Z \), \( HH''z C_Z \) and \( HH'z H''z C_Z \) (the effects of the delays \( \xi \) and of the second pair of simultaneous 180° 1H and 13C pulses are neglected).

\[ -iH_Z^r C_+ t_{1/2} (180)^xH^r(180)^xH'^r - t_{1/2} (180)^xC (90)^yH^r (90)^yH'^r \rightarrow -0.5H_Z^r C_+ e^{i\pi \Omega c t_1} \]

\[ -0.5(H^r H^r C_+ - H^z H^r C_+)^{t_{1/2} (180)^xH^r(180)^xH'^r - t_{1/2} (180)^xC (90)^yH^r (90)^yH'^r} \rightarrow -0.5iH_Z^r C_2 H_Z^r e^{i\pi \Omega c t_1} \]

\[ -0.5(H^r H^r C_+ - H^z H^r C_+)^{t_{1/2} (180)^xH^r(180)^xH'^r - t_{1/2} (180)^xC (90)^yH^r (90)^yH'^r} \rightarrow H_Z^r H_Z^r C_Z e^{i\pi \Omega c t_1} \]

\[ i(H^r H^r C_+ + H^z H^r C_+)H_Z^r \]

(14) \( t_{1-A,1-D} \)

The term \( H^r C_Z \) \( (t_A) \) represents \( x \)-magnetization of spin H antiphase with respect to spin C, the term \( iH^r H^r C_Z \) \( (t_B) \) represents \( y \)-magnetization of spin H which is doubly

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antiphase with respect to spin C and spin H', the term $i\mathbf{H}^\prime \cdot \mathbf{H}'' \mathbf{C}_Z (f_C)$ represents $y$-magnetization of spin H which is doubly antiphase with respect to spin C and spin H'', and the term $\mathbf{H}^\prime \cdot \mathbf{H}'' \mathbf{C}_Z (f_D)$ represents $x$-magnetization of spin H which is triply antiphase with respect to spin C and spins H' and H''. Thus, the magnitude of all four terms not only depends on the evolution of the $^nJ_{CH}$ coupling, but also on the evolution of the homonuclear $J_{HH'}$ and $J_{HH''}$ couplings.

As for a CHH' spin system, additional zeroing of signal intensities caused by $J_{HH'}$ and $J_{HH''}$ does not occur and the accidental cancelation of the long-range proton-carbon correlations due to homonuclear couplings $J_{HH'}$ and $J_{HH''}$ does not exist. Homonuclear coupling however causes several $\Delta$-dependent drops of the intensity accompanied with a general decrease in sensitivity (Figure S2).

Finally, we wish to mention that several versions of the LR-HSQC have been reported to minimize $J_{HH'}$ evolution by adding CPMG elements during the INEPT block (CPMG-INEPT), for the observation of exchange broadened signals in proteins,[45] and to improve the quantitative measurement of long-range coupling constants $^nJ_{CH}$ from complex multiplets patterns.[39, 46, 47] It would be interesting to compare the LR-CAHSQC[39] or CPMG-HSQMBC[46] experiments with the HMBC in terms of sensitivity/number of cross peaks.

**HMBC experiment**

**Analysis for a CHH' spin system**

Compared to the LR-HSQC experiment the outcome for the HMBC-SE pulse sequence[26] (Figure 3) is quite different. The reason is that now at a, before the $t_1$ evolution period, exclusively zero- and double quantum rather than single quantum coherence terms are present, which changes the spin dynamics in the remaining part of the sequence.
Figure 3. Pulse sequence of the slightly modified HMBC-SE experiment with echo-antiecho gradient selection\cite{26,27} and without low-pass \( J \) filter\cite{28} derived from the pulseprogram \textit{hmbcetgpnd} of the Bruker pulseprogram library. Thin bars represent 90\(^\circ\) pulses, thick bars 180\(^\circ\) pulses. The 180\(^\circ\) pulses on the \( ^{13}C \) channel are broadband adiabatic inversion (first) and refocusing (second) pulses, shown as sine pulses. \( \Delta \) is the long-range coupling evolution delay and is set to an average value \( 0.5/\Delta J_{CH} \). Delay \( \zeta \) is set to guarantee no \( ^{13}C \) chemical shift evolution for the first \( t_1 \) value, \( \zeta = (t_1)_0 + p_{180H} \), where \( (t_1)_0 \) is the initial value of \( t_1 \), and \( p_{180H} \) is the duration of the proton 180\(^\circ\) pulse. The following phase cycling is applied: \( \phi_1 = x, -x \); \( \phi_2 = x, x, -x, -x \); \( \phi_3 = 4x, 4(-x) \); \( \phi_{\text{rec}} = x, -x, x, -x, x, -x \). Pulses with no phase indicated are applied along the \( x \)-axis. Gradient ratios: \( G_1:G_2 = 50:30 \) (odd), -30:50 (even) for echo-antiecho detection. The labels \( a-c \) denote the three points of interest in the pulse sequence.

At point \( a \), taking into account that all chemical shifts are refocused, a product operator evaluation for a CHH\(^('*') \) spin system \((\pi J_{CH}, \text{ and } J_{HH} \neq 0, \pi J_{CH} = 0)\) shows that the following coherences are present:

\[
H_Z^x \rightarrow (90^\circ)^x H \rightarrow H_Y^y \rightarrow \pi J_{CH} \Delta H_Z^y \rightarrow (180^\circ)^y H \rightarrow (180^\circ)^y C \rightarrow (90^\circ)^x C \rightarrow 2H_X^x H_Y^y \sin(\pi J_{HH'} \Delta) \cos(\pi J_{CH} \Delta) + 4H_Y^y H_Z^z C_Y \sin(\pi J_{HH'} \Delta) \sin(\pi J_{CH} \Delta)
\]

(15)
As emphasized before for the LR-HSQC experiment, the pure proton coherences $H'_Y$ and $2H'_XH'_Z$ will be dephased by the gradients G1 and G2 and can be ignored for the subsequent analysis. The term $2H'_XC_Y$ (a superposition of double- and zero-quantum coherences, sometimes erroneously assumed to be the only term responsible for the desired HMBC correlation), and $4H'_YH'_ZC_Y$ (also a superposition of double- and zero-quantum coherences which is antiphase with respect to spin $H'$), continue to evolve. In the following, for comparison with the LR-HSQC experiment and for understanding the effect of magnetic field gradients, we'll use again the respective raising and lowering operators.

$$2H'_XC_Y \rightarrow -0.5i(H'_+C_+ - H'_+C_- + H'_-C_+ - H'_-C_-)$$

$$-4H'_YH'_ZC_Y \rightarrow (-H'_+C_+ + H'_+C_- + H'_-C_+ - H'_-C_-)H'_Z$$

(16)

Note that in contrast to the LR-HSQC pulse sequence both double-quantum and zero-quantum components of the $2H'_XC_Y$ and $4H'_YH'_ZC_Y$ terms evolve in the subsequent $t_1$-evolution period. For clarity, we consider the evolution of both terms - $0.5i(H'_+C_+ - H'_+C_- + H'_-C_+ - H'_-C_-)$ and $(-H'_+C_+ + H'_+C_- + H'_-C_+ - H'_-C_-)H'_Z$ separately:

$$-0.5i(H'_+C_+ - H'_+C_- + H'_-C_+ - H'_-C_-)$$

The evolution of $-0.5i(H'_+C_+ - H'_+C_- + H'_-C_+ - H'_-C_-)$ during the $t_1$ evolution period and the subsequent $180^\circ$ $^{13}$C pulse provides (the effect of $J_{HH'}$ during $t_1$ is neglected, and the trigonometric factors are omitted for clarity) the following terms. Note that with final quadrature detection of e.g. $H'_-$ only the terms $H'_-C_+$ and $H'_-C_-$ are relevant.

$$-0.5i(H'_-C_+ - H'_-C_-) \frac{t_1/2}{(180)^\circ \times H'_Y, (180)^\circ \times H'_Z} \frac{t_1/2}{(180)^\circ \times C} \xrightarrow{} - 0.5i(H'_-C_+ - H'_-C_-)e^{i\pi\Omega_C t_1}$$

(17)

The final $90^\circ$ $^{13}$C pulse provides observable proton antiphase magnetization $H'_-C_-$. Note that with the two ratios of the gradient strengths for G1 and G2 set to 5:3 (and taking into account the $180^\circ$ $^{13}$C pulse) either the first or the second of these two terms will be transformed (and rephased) to a detectable proton signal.
respectively and that these two, subsequently acquired proton signals are finally combined allowing for quadrature detection in $t_1$.

At $c$ we have therefore:

$$-0.5i(H^+ C_+ - H^- C_-)e^{i\Omega c t_1} \xrightarrow{(90)^{\circ}\times C} 0.5H^+ C_2 e^{i\Omega c t_1} + \text{non-observable DQ coherences}$$

(18)

$$(H^+ C_+ - H^- C_-)H_2 e^{i\Omega c t_1} \xrightarrow{t_1/2-(180)^{\circ}\times H^+ (180)^{\circ}\times H'} \rightarrow (H^+ C_+ - H^- C_-)H'_2 e^{i\Omega c t_1}$$

(19)

The evolution of $(-H^+ C_+ + H^+ C_+ + H^- C_+ - H^- C_-)H'_2$ during the $t_1$ evolution period provides (the effect of $J_{HH'}$ during $t_1$ is neglected, the trigonometric factors are omitted and only the terms $H^+ C_+$ and $H^- C_-$ are relevant):

$$2H^+ C_2 e^{i\Omega c t_1} \xrightarrow{(90)^{\circ}\times C} iH^+ C_2 H'_2 e^{i\Omega c t_1} + \text{non-observable DQ coherences}$$

(20)

Thus, before acquisition, including the trigonometric factors and with the results obtained for the two terms $2H^+ C_2 H'_2$ and $4H^+ C_2 H'_2 C_2$ we have:

$$0.5H^+ C_2 e^{i\Omega c t_1} \cos(\pi J_{HH'} \Delta) \sin(\pi J_{CH} \Delta) + iH^+ C_2 H'_2 e^{i\Omega c t_1} \sin(\pi J_{HH'} \Delta) \sin(\pi J_{CH} \Delta)$$

(21)
The term $0.5H_r'C_z(f_A)$ represents $x$-magnetization of spin $H_r$ antiphase with respect to spin $C$, and the term $iH_r'H_z'C_z(f_B)$ represents $y$-magnetization of spin $H_r$ which is doubly antiphase with respect to spin $C$ and spin $H'$. Note that for F1-band selective HMBC experiments, $t_1$ cannot be considered as short compared to the homonuclear proton-proton coupling constants $J_{HH'}$. In this case, before acquisition, we obtain:

$$
-0.5H_r'C_2 e^{i\pi \Omega_C t_1} \cos(\pi J_{HH'}(\Delta + t_1)) \sin(\pi n J_{CH}\Delta) \\
+ iH_r'H_z'C_2 e^{i\pi \Omega_C t_1} \sin(\pi J_{HH'}(\Delta + t_1)) \sin(\pi n J_{CH}\Delta)
$$

The final signal intensity depends therefore upon $\Delta$ for the heteronuclear coupling $n J_{CH}$ but upon $\Delta + t_1$ for the homonuclear coupling $J_{HH'}$. Nevertheless, the length of $t_1$ has no influence on the intensity of the cross peak, as shown by us and summarized below.

The final expression for the final signal $S_M$ using a magnitude mode processing can be written as:

$$
S_M \sim \sin(\pi n J_{CH}\Delta) \times A_M = \sin(\pi n J_{CH}\Delta) \times B_M
$$

where $A_M = (A_{R(\text{abs})} + A_{I(\text{disp})})^{0.5}$ and $B_M = (B_{R(\text{abs})} + B_{I(\text{disp})})^{0.5}$, $A_{R(\text{abs})}$ and $B_{R(\text{abs})}$ being the absorptive multiplet lines throughout as the real part and $A_{I(\text{disp})}$ and $B_{I(\text{disp})}$ the dispersive multiplet lines throughout as the imaginary part.

Equation 22 demonstrates that there is absolutely no $J_{HH'}$ dependent influence of the initial $\Delta$ delay on the cross-peak intensity (Figure 4). Note also that the evolution of homonuclear couplings $J_{HH'}$ during $t_1$ do not affect cross-peak intensities but are only responsible for corresponding signal modulations in $t_1$ (and corresponding splitting in $F_1$). Cross-peak intensities may however be reduced by short $T_2$ relaxation times.

For the special case where $J_{HH'} = n J_{CH}$, $A_M \neq B_M$, with an overlap of the center lines (Figure 4) the final signal $S_M$ using a magnitude mode processing can be written as:

$$
S_M \sim \sin(\pi n J_{CH}\Delta) \times A_M
$$
In this case, but also with $^{n}J_{CH} \approx ^{n}J_{HH'}$ the intensities of the individual multiplet lines no longer behave uniformly but are influenced by $^{n}J_{HH'}$. However and most importantly, the intensities of all multiplet lines still follow the uniform sine dependence imposed by the long range heteronuclear $^{n}J_{CH}$ coupling constant (Figure 4c).

In summary and as corroborated by equation (22) the intensity of an HMBC cross peak for a H'H'C-three-spin system never vanishes because of an accidental $\Delta/J_{HH'}$, combination, but only when the long-range evolution delay $\Delta$ equals a multiple of the inverse of the heteronuclear coupling constant $^{n}J_{CH}$ (Figure 4).

$$S_M \sim \sin(\pi^{} J_{CH} \Delta) \times \left[ \cos^2(\pi J_{HH'} \Delta) \times A_M^2 + \sin^2(\pi J_{HH'} \Delta) \times B_M^2 \right]^{1/2}$$

(23)

Figure 4. Simulated spectra obtained for a CHH' spin system, using the 1D version of the HMBC-SE pulse sequence shown in Figure 3 as a function of $\Delta$. For clarity the
spectra are displayed in magnitude mode. (A) $^nJ_{CH} = 7$ Hz, $J_{HH'} = 0$, (B) $^nJ_{CH} = 7$ Hz, $J_{HH'} = 3$ Hz, (C) $^nJ_{CH} = 7$ Hz, $J_{HH'} = 7$ Hz, (D) $^nJ_{CH} = 7$ Hz, $J_{HH'} = 11$ Hz, (E) $^nJ_{CH} = 7$ Hz, $J_{HH'} = 17$ Hz. The intensity-zeroes in the simulated spectra occur when $\Delta$ matches the conditions $\Delta = k^nJ_{CH}$, $k = 0, 1, 2, \ldots, n$. Simulations have been performed with the BRUKER NMRSIM program for MAC (version 5.5.3. 2012).

It’s useful to compare the observable coherences present before acquisition in both the LR-HSQC and HMBC experiments. For the LR-HSQC experiment, we have (Equation 10):

$$-0.5iH_{-}^rC_Z e^{i\eta C t_1} \cos(\pi J_{HH'} \Delta) \sin(\pi^nJ_{CH} \Delta)$$
$$+ 0.5H_{-}^rH_{Z}^rC_Z e^{i\eta C t_1} \sin(\pi J_{HH'} \Delta) \sin(\pi^nJ_{CH} \Delta)$$

And for the HMBC experiment (Equation 21):

$$0.5H_{-}^rC_Z e^{i\eta C t_1} \cos(\pi J_{HH'} \Delta) \sin(\pi^nJ_{CH} \Delta) + iH_{-}^rH_{Z}^r\cos(\pi J_{HH'} \Delta) \sin(\pi^nJ_{CH} \Delta)$$

Both coherences are identically composed of the same terms, except that they 90° out of phase and especially that the doubly antiphase magnetization is twice as intense in the case of the HMBC. This reflects the fact that in the LR-HSQC experiment, the double-quantum part of $4H_{r}^{r}H_{r}'C_{Z}, i(H_{r}^{r}H_{r}' - H_{r}^{r}H_{r}')C_{Z}$, is eliminated after the INEPT transfer period, and only the zero-quantum part $i(H_{r}^{r}H_{r}' - H_{r}^{r}H_{r}')C_{Z}$ contributes to the final signal, while in the HMBC experiment, both the double-quantum and zero-quantum parts $(H_{r}^{r}C_{Z} + H_{r}'C_{Z} + H_{r}^{r}C_{Z} - H_{r}'C_{Z})H_{Z}$ survive and contribute to the final signal (Figure S4). In other words the $J_{HH'}$ and $\Delta$ dependent intensity variations of the two terms $H_{r}^{r}C_{Z}$ and $H_{r}'C_{Z}$ on $J_{HH'}$ for the initial $\Delta$ delay compensate each other ideally in the HMBC experiment, but only partially in the LR-HSQC experiment. Consequently cross-peak intensities are governed solely by $^nJ_{CH}$ with the HMBC experiment, but are additionally influenced by $J_{HH'}$ with the LR-HSQC experiment. This gives rise to additional $\Delta$ dependent intensity drops and a general decrease in sensitivity for the latter (Figure S4).

Another aspect that might influence the final cross peak’s intensity is the potential different relaxation behavior of the magnetization terms present in both experiments. In the LR-HSQC experiment, SQ relaxation effects are relevant, while in the HMBC, DQ and ZQ effects are applicable. As such, the HMBC experiment
would likely be slightly more sensitive for proton deficient molecules (requiring long \( \Delta \) values) with typically shorter relaxation times.\[48\]

**Analysis for a CHH'H'' spin system**

We can consider the situation of CHH'H'' spin system, with \( ^nJ_{CH}, J_{HH'}, J_{HH''} \neq 0, \ ^nJ_{CHF}, ^nJ_{CH'C} = 0 \). The analysis for such a spin system is very lengthy, and therefore only some intermediate and the final results will be given. At a, four operators are present:

\[
\begin{align*}
-2H'_X C_Y \cos(\pi J_{HH'} \Delta) \cos(\pi J_{HH''} \Delta) \sin(\pi J_{CH'} \Delta) \\
-4H'_Y H'_Z C_Y \cos(\pi J_{HH'} \Delta) \sin(\pi J_{HH''} \Delta) \sin(\pi J_{CH} \Delta) \\
-4H'_X H'_Z C_Y \sin(\pi J_{HH'} \Delta) \cos(\pi J_{HH''} \Delta) \sin(\pi J_{CH} \Delta) \\
+8H'_X H'_Z H''_Z C_Y \sin(\pi J_{HH'} \Delta) \sin(\pi J_{HH''} \Delta) \sin(\pi J_{CH} \Delta)
\end{align*}
\]

(24)

They can be rewritten using the raising and lowering operators as (omitting the trigonometric factors for simplicity):

\[
\begin{align*}
-2H'_X C_Y &\rightarrow 0.5i(H_C - H_H' + H_H'' - H_C') \\
-4H'_Y H'_Z C_Y &\rightarrow (H_C + H_H' - H_H'' + H_C')H'_Z \\
-4H'_X H'_Z C_Y &\rightarrow (H_C + H_H' - H_H'' + H_C')H''_Z \\
8H'_X H'_Z H''_Z C_Y &\rightarrow -2i(H_C' - H_C'' + H_C' + H_C'')H'_Z H''_Z
\end{align*}
\]

(25)

Assuming again that the evolution time \( t_1 \) is short compared to the homonuclear couplings \( J_{HH'} \), i.e. neglecting modulations due to homonuclear couplings in \( t_1 \), we obtain at c (the trigonometric factors are omitted for clarity and only the final coherences containing \( H_c \) are shown):

\[
0.5i(H'_X C_+ - H'_Y C_- + H'_Z C_+ - H'_Z C_-) \\
\xrightarrow{t_1/2-(180)^\circ \times H'_Y, (180)^\circ \times H'-t_1/2-(180)^\circ \times C} \\
\xrightarrow{(90)^\circ \times C} \\
0.5H'_Z C_Z e^{i\pi \omega_C t_1}
\]
\[
\begin{align*}
(H^+_C C_+ - H^+_C C_- - H^+_C C_+ + H^+_C C_-)H'_Z \\
&\quad \xrightarrow{t_1/2-(180)^\circ xH', (180)^\circ xH' - t_1/2-(180)^\circ xC} (90)^\circ xC \\
&\quad \xrightarrow{iH'_Z H'_Z C_2 e^{i\pi \Omega c t_1}}
\end{align*}
\]
\[
\begin{align*}
(H^+_C C_+ - H^+_C C_- - H^+_C C_+ + H^+_C C_-)H''_Z \\
&\quad \xrightarrow{t_1/2-(180)^\circ xH', (180)^\circ xH' - t_1/2-(180)^\circ xC} (90)^\circ xC \\
&\quad \xrightarrow{2H'_Z H'_Z C_2 e^{i\pi \Omega c t_1}}
\end{align*}
\]

\[
\begin{align*}
-2i(H^+_C C_+ - H^+_C C_- - H^+_C C_+ + H^+_C C_-)H'_Z H''_Z \\
&\quad \xrightarrow{t_1/2-(180)^\circ xH', (180)^\circ xH' - t_1/2-(180)^\circ xC} (90)^\circ xC \\
&\quad \xrightarrow{-2H'_Z H'_Z C_2 e^{i\pi \Omega c t_1}}
\end{align*}
\]

+ non-observable multiple-quantum coherences

\[\text{(26) } (f_A-f_D)\]

Note that only these four terms are present and detected, even if all \( J_{hh'} \neq 0 \), but also if the carbon is coupled not only to H but for instance also to H'.

The four terms, but not their intensities (see below), are identical to those obtained for the LR-HSQC experiment: \( x \)-magnetization of spin H antiphase with respect to spin C, \( H^C_2 C_2 \) (\( f_A \)), \( y \)-magnetization of spin H doubly antiphase with respect to spin C and spin H', \( iH'_Z H'_Z C_2 \) (\( f_B \)), \( y \)-magnetization of spin H which is doubly antiphase with respect to spin C and spin H'', \( iH'_Z H' Z C_2 \) (\( f_C \)), and \( x \)-magnetization of spin H which is triply antiphase with respect to spin C and spins H' and H'', \( H'_Z H'_Z H'' Z C_2 \) (\( f_D \)).

Again as aforementioned, the final signal of the magnetization present before acquisition will therefore be calculated as the sums \( S_R \) and \( S_i \) of all absorptive and dispersive components, and with the two sums the signal intensity will be calculated in magnitude mode. It turns out that, also for a CHH'H'' spin system, the intensity of an HMBC cross peak never vanishes because of an accidental \( \Delta \sim J_{hh'} \) combination, but only when the long-range evolution delay \( \Delta \) equals the inverse of the heteronuclear coupling constant \( ^{0}J_{CH} \) (Figure S5).

If the terms present before acquisition in both the LR-HSQC and HMBC experiments are compared (Equations 14&26), it can be seen that the doubly and triply antiphase coherences are twice as intense in the case of the HMBC (Figure S6).
LR-HSQC:

\[
-0.5H^r_C C_2 e^{i\pi\Omega t_1} \cos(\pi J_{HH'}) \cos(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta) \\
- 0.5iH^r_C H'_2 C_2 e^{i\pi\Omega t_1} \cos(\pi J_{HH'}) \sin(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta) \\
- 0.5iH^r_C H''_2 C_2 e^{i\pi\Omega t_1} \sin(\pi J_{HH'}) \cos(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta) \\
+ H^r_C H'_2 H''_2 C_2 e^{i\pi\Omega t_1} \sin(\pi J_{HH'}) \sin(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta)
\]

HMBC:

\[
0.5H^r_C C_2 e^{i\pi\Omega t_1} \cos(\pi J_{HH'}) \cos(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta) \\
+ iH^r_C H'_2 H'_2 e^{i\pi\Omega t_1} \cos(\pi J_{HH'}) \sin(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta) \\
+ iH^r_C H''_2 C_2 e^{i\pi\Omega t_1} \sin(\pi J_{HH'}) \cos(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta) \\
- 2H^r_C H'_2 H''_2 C_2 e^{i\pi\Omega t_1} \sin(\pi J_{HH'}) \sin(\pi J_{HH''} \Delta) \sin(\pi n J_{CH} \Delta)
\]

The consequences of the different behavior of the LR-HSQC and HMBC experiment respectively may be visualized by simulation (Figure S6). Again and similar to the outcome for a H'H'C three-spin system the \( J_{HH'} \) and \( \Delta \) dependent intensity variations of the four terms compensate each other in the HMBC, but only partially in the LR-HSQC experiment.

**Analysis of n-spin systems**

The product operator analysis for an \( n \)-spin system becomes very lengthy, but some general features can still be derived. For a five spin system, operators like \( 2H_x H'_z C_2, 4H_y H'_z C_2, 4H_y H''_z C_2, 8H_x H'_z H''_z C_2, \) and \( 16H'_x H'_z H''_z H'''_z C_2 \) (8 terms in total) will be present and will contribute to final signal intensity. Obviously, the lineshapes are rather complicate to be described, because of the overlap of both several absorption and dispersion components. However, most importantly, and corroborated by simulation (Figure S7) for a model CH'H'H''' spin system, the zero-crossing points for the signal intensity always and exclusively occur when the long-range coupling evolution delay \( \Delta \) matches the inverse of the heteronuclear coupling constant \( J_{CH} \), irrespective of the value of all homonuclear coupling constants \( J_{HH'}, \) \( J_{HH''} \) and \( J_{HH'''} \).
Experimental Results

Ethylbenzene

In Figure 5, sections of 2D HMBC and LR-HSQC spectra of 0.1% ethylbenzene dissolved in CDCl₃ showing long-range correlations associated with the CH₂ and CH₃ resonances are presented. With the initial delay $\Delta$ adjusted to 65.8 ms, and with the value of the homonuclear coupling constant $^{3}J_{CH_{2}CH_{3}} = 7.6$ Hz the condition $\Delta = 0.5/^{3}J_{CH_{2}CH_{3}}$ is fulfilled for which the intensity differences between LR-HSQC and HMBC are expected to be most pronounced. All long-range correlation cross-peaks are expected to be weak in the LR-HSQC- compared to the HMBC-spectrum irrespective of the value of the long-range proton-carbon coupling constant $^{n}J_{CH}$, which is experimentally corroborated (Figure 5).

On the other hand, as expected, the HMBC and LR-HSQC spectra recorded with $\Delta$ adjusted to a long-range coupling constant of 5 Hz exhibit less differences, but the correlations are still more intense in the HMBC spectrum (Figure S8).
Figure 5. Top: Segments of the 7.6 Hz (that exactly matches the homonuclear $^3J_{CH_2CH_3}$ coupling constant between the CH$_2$ and the CH$_3$ protons) adjusted HMBC (left) and LR-HSQC (right) spectra of 0.1% ethylbenzene dissolved in CDCl$_3$ showing $^nJ_{CH}$ long-range correlations associated with the CH$_2$ and CH$_3$ resonances. Both spectra are displayed at the same noise level. Bottom: 1D rows extracted from the HMBC (bottom) and LR-HSQC (top) showing the $^nJ_{CH}$ long-range correlations associated with the C$_{ipso}$ resonance ($\delta = 144.3$ ppm). Both 1D spectra are displayed at the same noise level.

*Strychnine*
The resonances of H8 ($^{2}J_{H8H13} = 10.5$ Hz), and of H20a and H20b ($^{2}J_{H20aH20b} = 14.9$ Hz) of strychnine (Figure 6) exemplify the case of CH'–H' spin systems, while the resonances of H11a and H11b illustrate the case of CH'H'H'' spin systems ($^{2}J_{H11aH11b} = 17.5$ Hz, $^{3}J_{H11aH12} = 8.4$ Hz, $^{3}J_{H11bH12} = 3.2$ Hz) (Figure 6). Series of 1D $^{13}$C-selective HMBC and LR-HSQC spectra centered on the resonance of H8 ($\delta = 3.83$ ppm) and with the selective $^{13}$C-pulse adjusted to the resonance of C12 ($\delta = 77.5$ ppm) are shown as a function of the long-range evolution delay $\Delta$ (Figure 7 & 8). Other examples are shown in the Supplementary information (Figures S9 & S10).
Figure 7. Top: Series of 40 1D $^{13}$C-selective LR-HSQC spectra, obtained using a 1D selective version of the pulse sequence shown in Figure 1, as a function of the long-range evolution delay $\Delta$. The resonance of C12 ($\delta = 77.5$ ppm) was selected using a 2 ms Gaussian pulse. The spectra show the resonance of H8 ($\delta = 3.83$ ppm). The spectra are displayed in magnitude mode. Bottom: Simulated spectra obtained for the C$_{12}$H$_8$H$_{13}$ spin system. The spectra are displayed in magnitude mode. Parameters used for the simulations: $^3J_{C12H8} = 5.8$ Hz, $^3J_{H12H13} = 10.5$ Hz. The relaxation times for H8 have been set to $T_1 = 1$ s, $T_2 = 0.3$ s, and relaxation during both the sequence and acquisition was taken into account. Simulations have been performed with the BRUKER NMRSIM program for WINDOWS (version 5.5.3. 2012).

For the LR-HSQC spectra, it can be seen that simulated and experimental spectra correspond to each other and that especially the intensity drops (at $\Delta = 45$, 145, and 170 ms) match very well (Figure 7).
Figure 8. Top: Series of stacked 1D selective HMBC spectra, obtained using a 1D $^{13}$C-selective version of the pulse sequence shown in Figure 3, as a function of the long-range evolution delay $\Delta$. The resonance of C12 ($\delta = 77.5$ ppm) was selected using a 2 ms Gaussian pulse. The spectra show the resonance of H8 ($\delta = 3.83$ ppm). The spectra are displayed in magnitude mode. Bottom: Simulated spectra obtained for the C$_{12}$H$_8$H$_{13}$ spin system. The spectra are displayed in magnitude mode. Parameters used for the simulations: $^{3}J_{C12H8} = 5.8$ Hz, $^{3}J_{H8H13} = 10.5$ Hz. The relaxation times for H8 and H13 have been set to $T_1 = 1$ s, $T_2 = 0.5$ s (see Supporting Information), and relaxation during both the sequence and acquisition was taken into account. Simulations have been performed with the BRUKER NMRSIM program for WINDOWS (version 5.5.3. 2012).

In line with the results of the product operator treatment shown before the unique zero in both the experimental and simulated spectra for HMBC (Figure 8) occurs for $\Delta \sim 0.17$ s, which corresponds to a long-range coupling constant of $\sim 5.8$ Hz and matches the condition $\Delta = 1/3J_{C12H8}$. It turns out that 1D $^{13}$C-selective HMBC experiments can used for accurately measuring long-range coupling constants of...
any size. The 1D experiment is most advantageously if only a few $^nJ_{CH}$ values for structural elucidation,[49, 50] have to be determined, as described and demonstrated elsewhere.[34, 51]

Parts of HMBC and LR-HSQC spectra of strychnine with two selected cross-peaks of carbons C21 and C6, respectively, recorded for different long-range evolution delays $\Delta$ are shown in Figure 9. The heteronuclear $^3J_{C21H13}$ ($J = 7.4-7.8$ Hz)$^{[52-55]}$ and $^3J_{C6H17}$ ($J = 1.8-1.9$ Hz)$^{[53]}$ cross peaks are indicated with arrows and highlighted with blue and green boxes, respectively. These two cross peaks illustrate the general case of protons coupled to several other protons, which, in the case of the LR-HSQC experiment, is expected to significantly modulate the cross peak’s intensity as a function of the long-range evolution delay $\Delta$. Clearly, the $^3J_{C6H17}$ cross peak (blue boxes) is visible in all five HMBC spectra, with approximately equal intensity. In contrast, this cross peak is very weak in the LR-HSQC spectrum recorded with $\Delta = 29$ ms and weak for $\Delta = 48$ ms. Likewise, the $^3J_{C21H13}$ cross peak (green boxes) is clearly visible in the five HMBC spectra, albeit at a slightly lower intensity for $\Delta = 29$ ms. This cross peak is invisible in the LR-HSQC spectra recorded with $\Delta = 48$ ms and $\Delta = 59$ ms, and remains weak for $\Delta = 29, 71, \text{ and } 91$ ms. In Figure S11, 1D rows extracted from both experiments recorded with $\Delta = 71$ ms exhibiting the long range correlations of C12 at a chemical shift of 77.5 ppm are shown. Clearly, all long-range correlations appear significantly more intense using the HMBC experiment. The average SNR observed for these two cross-peaks in both HMBC and LR-HSQC spectra shown in Fig. 9 is given in the supplementary material (Table 1).
Figure 9. Parts of the HMBC spectra (left), and LR-HSQC spectra (right) of strychnine recorded for different long-range evolution delays $\Delta$. The $^3J_{C21H13}$ and $^3J_{C6H17}$ cross peaks are indicated with arrows and highlighted with blue and green boxes, respectively. All spectra are displayed in magnitude mode and at the same noise level.
LR-HSQC and HMBC-SE experiments are typically acquired and processed in phase sensitive mode. Using this presentation, the LR-HSQC delivers better resolution in F1 compared to the standard HMBC-SE experiment (Figure S12), provided sufficient $t_1$ increments are recorded. In practice, for the same experiment time, it’s therefore conceivable to perform more scans and acquire fewer increments in F1 with the LR-HSQC to deliver the equivalent resolution that one would expect with the standard HMBC data set using fewer scans and more increments. A simple way to avoid the broadening in F1 for the HMBC is to use the CT-HMBC experiments, which provide cross peaks with a resolution similar to that achieved using the LR-HSQC experiment.\cite{23} Also presented in phase sensitive mode, the standard HMBC-SE is more sensitive than the LR-HSQC experiment (Figure S13).

**Conclusion**

It is commonly claimed that the evolution of the homonuclear proton-proton $J_{HH'}$ couplings during the initial delay $\Delta$ of LR-HSQC and HMBC pulse sequences contributes a $\sum \cos(\pi J_{HH'} \Delta)$ trigonometric factor to the intensity of the observed magnetization, which might cause accidental cancellation of cross-peaks in the spectra.

We have shown with our investigation that this still widespread belief is not fully correct. For both experiments, we have demonstrated both theoretically and experimentally that long-range correlation peaks will not be canceled because of homonuclear couplings $J_{HH'}$ but may be weakened at most (LR-HSQC). For a CH'H' spin system, two coherences contribute to the final signal in both experiments: $H_r - C Z$ and $H_r - H' Z C Z$. The different behavior of LR-HSQC and HMBC with respect to homonuclear couplings can be attributed to the following: For LR-HSQC and for a CH'H' three spin system the finally detected $H_r - H' Z C Z$ originates only from one of the components (the single quantum component) of the intermediate coherence $4H_r - H' X C Y$ that survives after the INEPT transfer, while for HMBC the intermediate multiple quantum coherence $4H_r - H' Z C Y$ present after the polarization transfer period is entirely converted back into observable $H_r - H' Z C Z$ coherence before acquisition. As
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a result, the HMBC experiment is inherently more sensitive compared to the LR-HSQC experiment. This is attributed to the fact that for the HMBC– but not for the LR-HSQC-) experiment the $J_{HH}$ and $\Delta$ dependent intensity variations of the two coherences which contribute to the final signal, $H$-$^1C_Z$, containing a $\cos(\pi^nJ_{HH'}\Delta)$ term and $H'$-$^1C_Z$ containing a $\sin(\pi^nJ_{HH'}\Delta)$ term compensate each other when $\Delta$ is varied. This outcome can be generalized to $n$-spin systems as demonstrated for a CH'H'' spin system.

In both experiments, the cross peak’s intensity always and exclusively vanishes for the condition $\Delta = k/nJ_{CH}$, irrespective of the magnitude and numbers of additional passive homonuclear $^nJ_{HH'}$ couplings. However, for the LR-HSQC experiment, homonuclear couplings $J_{HH'}$ cause severe drops of the long-range correlation’s intensity at certain $^nJ_{HH'}$ dependent values of $\Delta$, while the cross peak’s intensity in HMBC spectra follows a simple $\sin(\pi^nJ_{CH}\Delta)\exp(-\Delta/T_{2eff})$ dependence.

HMBC-based experiments are therefore the best choice among the family of heteronuclear long-range correlation experiments because: (i) the overall cross peak’s intensity is generally higher, and (ii) in LR-HSQC experiments the intensity of the long-range cross peaks is additionally influenced in an unwanted way by the magnitude and number of passive homonuclear proton-proton couplings $J_{HH'}$. Therefore, depending on the choice of the long-range coupling delay $\Delta$, important correlations might be missing with LR-HSQC.

Experimentally, it turned out that a refocused HSQC experiment optimized for long-range couplings, the LR-HSQMBC experiment, performs much better than the D-HMBC, not only for very long-range responses, but also for the total number of $^nJ_{CH}$ correlations. For strychnine, the LR-HSQMBC provided a total of 68 very long-range correlations that reached further than 3 bonds ($>^3J_{CH}$). There were 14 more very long-range responses compared with the 2 Hz HMBC, and 24 more very long-range responses than were observed in the D-HMBC data. The LR-HSQMBC also revealed a total of 160 $^nJ_{CH}$ correlations, while 142 correlations were observed using the 2 Hz HMBC, and only 115 correlations in the D-HMBC data. Here, it should be mentioned that many of the very long-range correlations that can be observed in an LR-HSQMBC spectrum may not be observed at all when less than 512 $t_1$ increments are acquired in the F1 dimension. In many instances, $^5J_{CH}$ and $^6J_{CH}$ correlations aren’t observed until 640 or even 768 $t_1$ increments have been
acquired.\cite{17,56} We are currently investigating theoretically and experimentally both experiments to explore these experimental evidences.

References


Why is HMBC superior to LR-HSQC? Influence of homonuclear couplings \( J_{HH'} \) on the intensity of long-range correlations

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HMBC or LR-HSQC? HMBC is superior to LR-HSQC for detecting long-range heteronuclear correlations, because: (i) the cross peak’s intensity is higher, and (ii) in LR-HSQC the intensity of the cross peaks is additionally influenced in an unwanted way by the magnitude and number of passive homonuclear proton-proton couplings \( J_{HH'} \). Notably, for both experiments, long-range correlations do not cancel because of homonuclear couplings \( J_{HH'} \).