J|A|C|S
ARTICLES
Published on Web 03/14/2003

Quantitative 2D HSQC (Q-HSQC) via Suppression of *J*-Dependence of Polarization Transfer in NMR Spectroscopy: Application to Wood Lignin

Sami Heikkinen,[†] Merja M. Toikka,[‡] Pirkko T. Karhunen,[§] and Ilkka A. Kilpeläinen*[,‡]

Contribution from the NMR laboratory, Structural Biology and Biophysics, Institute of Biotechnology, P.O. Box 65, FIN-00014 University of Helsinki, Finland

Received October 22, 2002; E-mail: ilkka.kilpelainen@helsinki.fi

Abstract: A quantitative method to record ^{1}H-^{13}C correlation NMR spectra (Q-HSQC) is presented. The suppression of $^{1}J_{CH}$-dependence is achieved by modulating the polarization transfer delays of HSQC. In addition, the effect of homonuclear couplings, as well as relaxation during the pulse sequence are discussed. We developed the Q-HSQC approach for the quantitative analysis of wood lignin, a complex polymer where it has been difficult to obtain reliable data on the relative amounts of different structural units. The current method is applicable to a variety of complex mixtures, where normal 1D ^{1}H- and ^{13}C-NMR methods fail.

Introduction

Quantitative NMR analysis of complex organic mixtures is often hampered by overlapping signals. Commonly, normal ^{1}H NMR spectrum is a versatile tool to provide quantitative information for small molecules or simple mixtures of low molecular weight substances. However, as the ^{1}H line widths increase with increasing molecular weight, accurate quantification becomes tedious, as it is essential to know exactly the number of signals, as well as estimates for their line widths, to successfully use deconvolution, or singular value decomposition methods for quantitative analysis. If the sample availability or solubility is not a problem, inverse gated ^{1}H decoupled ^{13}C spectra[1] provide more resolution for quantitative analysis. However, due to the weak sensitivity of the ^{13}C nuclei, and very long relaxation delays needed, the obtainable signal-to-noise (S/N) often remains poor even with very long acquisition times jeopardizing attempts to obtain reliable quantitative data.

We have evaluated the possibilities to use 2D HSQC for quantitative analysis. Many different parameters affect normal 2D methods prohibiting their use for quantification. In conventional ^{1}H-^{13}C HSQC, two polarization transfer delay periods are used to transfer magnetization between ^{1}H and ^{13}C nuclei. Normally, just one value for $^{1}J_{CH}$-coupling is selected to obtain the spectrum, typically optimized for an average $^{1}J_{CH}$-coupling of 145 Hz ($\Delta = 1/(2^{1}J_{CH}) = 3.45$ ms). We simulated the effect of using several Δ-values for each spectrum, and we were able to optimize the Δ-values to give almost uniform response (within $\pm$2%) over the whole natural $^{1}J_{CH}$-coupling range (115−220 Hz). The effects of ^{1}H−^{1}H *J*-couplings, as well as relaxation during the pulse sequence, on correlation peak volumes, are discussed.

We applied the current Quantitative HSQC (Q-HSQC) approach for the quantitative analysis of wood lignin, a heterogeneous polymer, where normal NMR methods for quantification easily fail. We show here that the Q-HSQC gives reliable estimates for the structural units of lignin. The current approach can be easily adapted to various applications of quantification of complex mixtures by NMR.

Experimental Section

General. 4′-hydroxy-3′-methoxyacetophenone (acetovanillone) was purchased from Aldrich. 4-(3-hydroxyprop-1-enyl)-2-methoxyphenol (coniferyl alcohol) was prepared by reduction of the corresponding cinnamic ester.[2,3]

Milled Wood Lignin (MWL). Milled wood lignin (MWL) was isolated from spruce wood (*Picea abies*) by slight modification of the Björkman[4] method, including an ultrasonic dioxane:water (9:1 v/v) extraction step (90 min at 15 °C) after the ball milling. The yield of the MWL was 35% from original (Klason) lignin. 100 mg of the MWL sample was dissolved in 700 μL of d_6-DMSO (Cambridge Isotope Laboratories, CIL).

Internal Standard. The monomeric lignin model compound, 4-(1-hydroxyethyl)-2-methoxyphenol (apocynol) was used as an internal standard. Apocynol was prepared from recrystallized 4′-hydroxy-3′-methoxyacetophenone by reduction with $NaBH_4$ in 56% aqueous ethanol as described by Bailey et al.[5] The product, 4-(1-hydroxyethyl)-2-methoxyphenol was purified by crystallization from ethyl acetate−hexane and ethanol. Purified apocynol (4.2 mg) was added into the MWL sample.

Preparation and Purification of Lignin Model Compounds. The *erythro*-β-O-4 model compound was made as

[†] Department of radiology, Helsinki University Central Hospital.
[‡] NMR laboratory, Structural biology and biophysics, Institute of biotechnology, University of Helsinki and ¥Structural chemistry laboratory, University of Oulu.
[§] Laboratory of organic chemistry, Department of chemistry, University of Helsinki.

(1) Schoolery, J. N. *Prog. NMR Spectrosc.* **1977**, *11*, 79.
(2) Freudenberg, K.; Hübner, H. H. *Chem. Ber.* **1952**, *85*, 1181.
(3) Quideau, S.; Ralph, J. *J. Agric. Food Chem.* **1992**, *40*, 1108.
(4) Björkman, A. *Svensk Papperstidn.* **1956**, *60*, 477.
(5) Bailey, C. W.; Dence, C. W. *Tappi* **1969**, *52*, 491.

10.1021/ja029035k CCC: $25.00 **© 2003 American Chemical Society**

previously described.[6] The β-5 and $\beta-\beta$ structures were prepared by oxidation of coniferyl alcohol with HRP/H_2O_2.[7] Oxidation of dehydrodipropylguaiacol by silver oxide in the presence of coniferyl alcohol yielded *trans*-dibenzodioxocin.[8] The β-*O*-4 model compound was purified by recrystallization from ethyl acetate. The β-5 and $\beta-\beta$ dimers were isolated from the oxidation mixture using ISCO HPLC pump, Hibar LiChrospher 100 RP-18, 5 μm, 250 × 50 mm column and Shimadzu SPD-6A UV spectrophotometric detector, detection at 280 nm. Mobile phase was acetonitrile-water 30:70, flow rate 15 mL/min. *trans*-Dibenzodioxocin was purified by silica gel chromatography using acetic acid-diethyl ether-CH_2Cl_2 1:10:100 as eluent. The purity of compounds was determined with HPLC using Waters 717 plus Autosampler, Waters 600 pump, Waters Symmetry C18, 5 μm, 4.6 × 150 mm column and Waters 996 UV spectrophotometric detector with detection at 280 nm. Acetonitrile-water gradient was used as eluent, flow rate 0.8−1 mL/min. According to the HPLC analysis, the purity of lignin model compounds were: β-*O*-4 100%, β-5 97.4%, $\beta-\beta$ 97.4%, and dibenzodioxocin 91.2%.

Model Compound Mixture. The reference mixture of the lignin model compounds was prepared by weighting each compound accurately (25 μmol of each) and dissolving them into 700 μL of d_6-DMSO.

NMR Spectroscopy. All spectra were acquired at 30 °C with Varian Unity INOVA 600 spectrometer (600 MHz ^{1}H frequency) equipped with triple resonance z-gradient probe. Spectral widths of 6600 Hz and 35 000 Hz were used for 1D ^{1}H and ^{13}C spectra, respectively. To obtain quantitative data, the relaxation delay for 1D ^{1}H spectrum was 5 s (90° pulse angle, 11.3 s recycle delay). For 1D inverse-gated ^{13}C spectrum the relaxation delay was set to 10 s (90° pulse angle, 11.3 s recycle delay was sufficient for the protonated carbons that were of interest in the current study). The number of collected points was 25 k and 91 k for ^{1}H and ^{13}C, respectively. The 1D spectra were processed using an exponential weighting function (lb = 0.2 Hz for ^{1}H and lb = 1 Hz for ^{13}C) prior to Fourier transform. The spectral widths for Q-HSQC were 6100 Hz and 15 100 Hz for ^{1}H- and ^{13}C-dimensions, respectively. The number of collected complex points was 2048 for ^{1}H-dimension. A recycle delay of 5.17 s (5 s relaxation delay and 0.17 s acquisition time) was found sufficient to provide quantitative results with the model compound mixture and the same value was used for the MWL sample as well. The number of transients for the Q-HSQC spectra of the model compound mixture was 16, and 256 time increments were recorded in ^{13}C-dimension resulting in an overall experiment time of 12 h. For the MWL sample the number of transients was 64, and 160 time increments were collected in ^{13}C dimension resulting in overall experiment time of 30 h. For Q-HSQC experiments, squared cosine-bell apodization function was applied in both dimensions. Prior to Fourier transform the data matrixes were zero filled up to 1024 points in ^{13}C-dimension. The 1D spectra were processed using standard Varian VNMR 6.1B software, whereas the 2D Q-HSQC spectra were processed with Felix98 software (Molecular Simulations Inc.)

(6) Nakatsubo, F.; Sato, K.; Higuchi, T. *Holzforschung* **1975**, *29*, 165.
(7) Quideau, S.; Ralph, J. *Holzforschung* **1994**, *48*, 12.
(8) Karhunen, P.; Rummakko, P.; Pajunen, A.; Brunow, G. *J. Chem. Soc., Perkin Trans 1* **1996**, 2303.

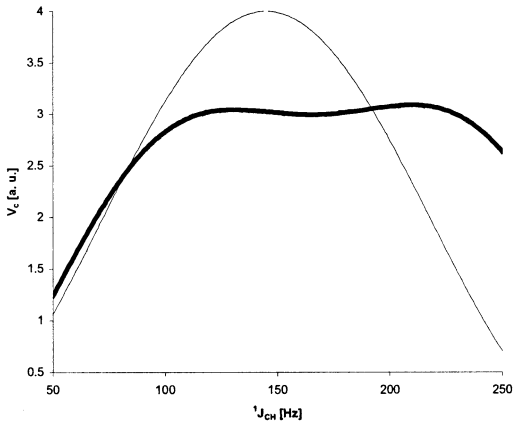

Figure 1. Simulated J-dependence of conventional (thin line) HSQC and Q-HSQC (thick line).

Results

Quantitative Method to Record 2D ^{1}H−^{13}C Correlation NMR Spectrum (Q-HSQC). In conventional HSQC, the duration of polarization transfer delay, $\Delta = 1/(2^1J_{CHtune})$, is typically tuned using some average value of expected $^1J_{CH}$:s in the molecule, since the volume of the HSQC correlation peak is strongly dependent on the relation between the delay Δ and the actual $^1J_{CHtrue}$. This relation is shown in eq 1

$$V_c \propto \sin^2(\pi\Delta^1J_{CHtrue}) \qquad (1)$$

This means, that optimal polarization transfer can only be achieved for one $^1J_{CHtrue}$ value by setting $\Delta = 1/2^1J_{CHtrue}$. This is not usually a problem when HSQC spectrum is used for assignment purpose, as setting the $^1J_{CHtune}$ to some average value typically results in a 2D spectrum where all correlation peaks can be detected. If, in turn, quantitative information is needed, the sine- squared dependence (eq 1) has to be taken into account, because the integrated volume of the correlation peak not only reflects the amount of protons responsible for the correlation peak but also the mismatch between the $^1J_{CHtune}$ and $^1J_{CHtrue}$. This will cause problems if the range of heteronuclear coupling constants is large, as is the case for $^1J_{CH}$ coupling constants in a typical molecule having aliphatic and aromatic carbons, i.e., correlation peaks from ^{1}H−^{13}C-pairs with large deviation of $^1J_{CH}$:s from $^1J_{CHtune}$ will have significantly reduced correlation peak volumes. It is quite obvious that the integration result could be, in principle, corrected if the $^1J_{CH}$-values of the molecule/molecules are known. This, in turn, would necessitate additional $^1J_{CH}$ determination. Another alternative is to design such experiment where the signal intensity (V_c) is uniform over expected range of $^1J_{CH}$:s. This can be done by averaging HSQC spectra recorded with suitably selected Δ-values (Figure 1). The combination of suitable Δ-values can be determined iteratively by minimizing the difference between maximum and minimum correlation peak V_c:s over selected $^1J_{CH}$-range. Already four suitably selected Δ-values average the $^1J_{CH}$-dependence to less than 2% over natural $^1J_{CH}$ range, whereas two values will not suffice and even 16 values will not perform any better.

Figure 1 shows V_c vs. $^1J_{CH}$ curves for both conventional HSQC, where one Δ-value is used ($^1J_{CHtune}$ = 145 Hz) and the corresponding curve for HSQC recorded with four Δ-values (2.94 ms, 2.94, 2.94, and 5.92 ms; resulting from iterative optimization of Δ-values for uniform intensity response over

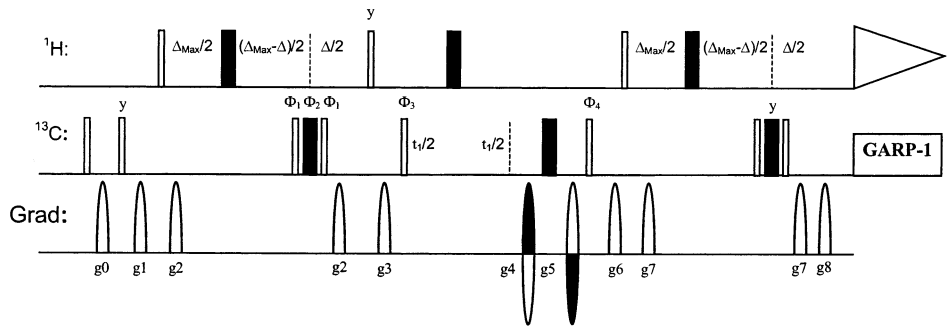

Figure 2. Pulse sequence for $^1J_{CH}$-compensated Q-HSQC.

$^1J_{CH}$ range of 115−190 Hz). Figure 1 clearly shows, that a relatively uniform response of V_c can be achieved over a large range of $^1J_{CH}$:s using four Δ-values only. The resulting spectrum will, however, have lower V_c's (∼25%) as compared to conventional HSQC spectrum when $^1J_{CHtrue}$ is close to $^1J_{CHtune}$ (Figure 1). The overall theoretical variation of the response of Q-HSQC over natural $^1J_{CH}$-range of 115−220 Hz was found to be less than ±2%. The iteration was limited to relatively short Δ-values in order to minimize the relaxation losses and the evolution of homonuclear couplings during the polarization transfer delays. Instead of varying the total length of INEPT-periods, a constant-time version was used. The total length of the period is determined by the largest Δ-value, Δ_{Max}. Polarization transfer corresponding shorter Δ-values is achieved by shifting the ^{13}C 180° pulse with respect to the ^{1}H 180° pulse. The constant time approach ensures that relaxation losses during INEPT-delays are the same for every Δ-value. This also holds for homonuclear coupling evolution. Instead of recording separate HSQC spectra with different Δ-values followed by subsequent addition of 2D-spectra, a better and more straightforward approach is to record one HSQC spectrum in such a way that the Δ-list is cycled through for every single step in the phase cycle.

The pulse sequence for Q-HSQC is presented in Figure 2. In Figure 2 narrow white (wide black) bars correspond to 90° (180°) hard rectangular pulses. Gradient pulses are represented by narrow half-ellipses denoted by g0−g8 (g0 = 26.0 G/cm, 0.5 ms, g1 = 18.2 G/cm, 0.5 ms, g2 = 4.0 G/cm, 0.5 ms, g3 = 22.0 G/cm, 1.0 ms, g4 = 40.0 G/cm, 1ms, g5 = −/+40.0 G/cm, 1.0 ms, g6 = −15.4 G/cm, 1.0 ms, g7 = 16.0 G/cm, 0.5 ms, g8 = 40.0 G/cm, 0.5 ms). All the pulses have x-phase unless otherwise indicated. Δ represents the duration of variable delay (n = number of variable delays, 4 in this work) and Δ_{Max} is the duration of the longest delay Δ. The t_1 represents the incremented delay. Decoupling of ^{13}C during the acquisition was accomplished using the GARP-1 sequence.[9] The employed phase cycle: $\Phi_1 = n(x)$; $\Phi_2 = n(y)$; $\Phi_3 = n(x), n(-x)$; $\Phi_4 = n(x), n(x), n(-x)$; $n(-x)$, receiver = $n(x), n(-x), n(-x), n(x)$, (n = number of variable delays, 4 in this work). The N- and P-type coherence are recorded separately by inverting the sign of gradient g4 and g5. Axial peak displacement is obtained via the States-TPPI method[10] by inverting the phases $\Phi_1-\Phi_3$ and receiver on every second increment.

It is important to notice that the evolution of homonuclear couplings during Δ_{Max} will reduce the correlation peak intensity. The effect of homonuclear couplings can be implemented in eq 1 in a simplified manner by multiplying the $\sin^2(\pi\Delta^1J_{CHtrue})$ term with $\Pi_{i=1}{}^k \cos^2(\pi\Delta_{Max}J_{HHi})$, where k is the number of coupling partners (neglecting possible small contributions from magnetization components incorporating proton zero-quantum coherences, which are not eliminated by purging gradients g3 and g6). The effect of homonuclear proton−proton couplings can be significant if the proton has multiple coupling partners. In such situations the proposed method should be used with caution. One/two 7 Hz coupling/s and Δ_{Max} = 6 ms will reduce the intensity from 1.0 to 0.98/0.97. By adding the effect of −14 Hz geminal coupling will reduce the intensity further to 0.92/0.90. Therefore, it is important to notice that if the spin system under investigation has multiple J_{HH} couplings, their effect on signal intensities should be corrected accordingly.

Relaxation effects during the pulse sequence are also an important source of interference for the quantitative analysis with Q-HSQC technique. It is clear that the recycle delay should be sufficient to avoid varying saturation effects due to different T_1:s of ^{13}C-bound protons. Furthermore, the proton T_2-relaxation during the constant-time INEPT-delays affects the signal intensity of the experiment according to eq 2 (in principle, some contribution can also originate from T_1 and T_2 relaxation of proton and carbon during gradients g3−g6, but these effects are expected to be negligible)

$$V_c \propto \exp(-2\Delta_{Max}/T_2) \qquad (2)$$

Thus, provided that the proton T_2's of the different components are of the same order of magnitude, it can be expected that the relaxation effects do not essentially compromize the reliability of the experiment. It should be noted that here we refer to the T_2 relaxation times of the ^{13}C coupled protons. Obviously, for small molecules with long T_2, this effect can be quite safely neglected. The effect of different T_2's is illustrated in Figure 3. In Figure 3 the relative intensities of two correlation peaks are presented as a function of the ratio $T_2(1)/T_2(2)$. $T_2(1)$ and $T_2(2)$ present the shorter and the longer T_2:s of the protons in ^{1}H−^{13}C-pairs producing the HSQC correlation peaks, respectively. The corresponding correlation peak intensities are presented with $V_c(1)$ and $V_c(2)$. Four curves are shown, where $T_2(2)$ is 30 ms (filled diamonds), 75 ms (filled circles), 200 ms (open diamonds), and 500 ms (open circles). The curves are calculated for $2\Delta_{Max}$ = 11.84 ms. For example, curve for $T_2(2)$ (30 ms) shows, that acceptable results ($V_c(1)/V_c(2) > 0.95$) can be still obtained when difference between the two T_2's is ∼10%. Increasing $T_2(2)$'s allow even larger differences. It can be concluded from Figure 3 that even with relatively short T_2 of

(9) Shaka, A. J.; Barker, P. B.; Freeman, R. *J. Magn. Reson.* **1985**, *64*, 547.
(10) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *39*, 163.

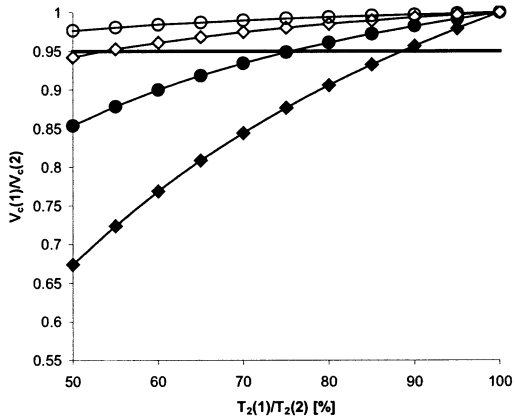

Figure 3. The effect of ^{1}H T_2 differences on the relative correlation peak intensities.

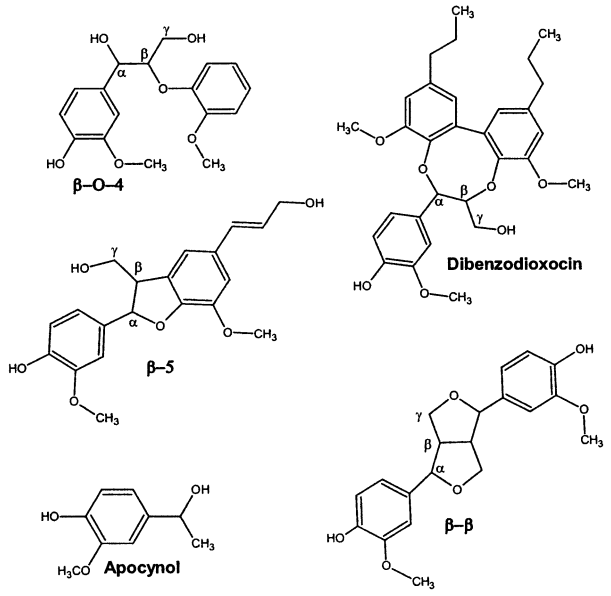

Figure 4. Structures of the lignin model compounds.

75 ms, relaxation has little interference for the quantification of Q-HSQC, but relaxation effects increase rapidly along with decreasing T_2's. With the lignin samples we did not correct the obtained values for relaxation, as the measured T_2 values of different structural units were of the same order of magnitude ($\sim$120 $\pm$ 30 ms for the H_α/C_α correlation signals of the lignin preparation). Notably, a quick estimate of the T_2's can be obtained using HSQC sequence incorporating CPMG ^{1}H pulse train. A series of 1D HSQC spectra recorded with different lengths of CPMG period are processed using T_2-determination routine of spectrometer software. In case of low concentration samples also conventional ^{1}H T_2-determination will also suffice.

Quantitative Analysis of Lignin Model Compound Mixture by Q-HSQC. To test the quantitative nature of Q-HSQC, a reference mixture (Figure 4) of small molecule lignin model compounds was prepared. The *β-O-*4, *β-*5, dibenzodioxocin and *β*−*β* structures represent the most prominent aryl propane units in native lignin.[11,12] As the *γ*-protons of *threo* isomer of the *β-O-*4 structure overlap with the *γ*-protons of dibenzodioxocin,

(11) Adler, E. *Wood Sci. Technol*. **1977**, *11*, 169.
(12) Karhunen, P.; Rummakko, P.; Sipilä, J.; Brunow, G.; Kilpeläinen, I. *Tetrahedron Lett*. **1995**, *36*, 169.

Table 1. Comparison of Quantitative ^{1}H, ^{13}C and Q-HSQC Results of the Mixture of Lignin Model Compounds

compd		^{1}H	^{13}C	Q-HSQC	RSD (%) for Q-HSQC
*β-O-*4	α	1.00	1.00	1.00	0
	β	0.96	0.96	0.88	2.2
	γ	*a*	0.91	1.03	0.8
β−5	α	1.09	1.05	0.96	1.3
	β	1.06	1.10	1.09	2.9
	γ	*a*	0.94	1.03	2.1
β-β	α	0.97	0.91	0.89	1.8
	β	0.94	0.80	0.88	0.9
	γ	0.95	0.95	0.95	1.6
dibenzodioxocin	α	1.10	0.91	0.92	1.7
	β	*a*	0.77	0.86	2.6
	γ	0.93	0.93	0.95	2.9
OCH_3	H	1.12[a]		1.06[b]	0.8
	C		1.06		

The reported Q-HSQC values are not corrected for homonuclear ^{1}H−^{1}H couplings, which is reflected especially in the integral values of some of the *β*-correlations (see p 8 for discussion). For clarity, all signals were calculated to correspond the number of carbons in each structure, i.e. the *γ*-signal intensities were divided by 2, and $-OCH_3$ intensities by 27 (sum of all methoxyl protons in the model compound mixture), in the ^{1}H and Q-HSQC results. The values were normalized to the intensity of the α-signal of *β-O-*4 structure (H_α/C_α intensity =1.00). [a] Signals are overlapped with other model compounds. [b] The methoxyl signal is overlapped in ^{13}C dimension of the Q-HSQC spectrum with side products (14%, see experimental), and the reported values are corrected accordingly.

only the *erythro* isomer of *β-O-*4 model compound was chosen for the model compound mixture.

The abundance of the model compounds was determined from the integrals of the side chain α, *β* and *γ*−signals in 1D ^{1}H, quantitative ^{13}C (inverse-gated ^{1}H decoupled) and Q-HSQC spectra. These signals were well separated in quantitative ^{13}C and Q-HSQC spectra, but the *β*−signal of dibenzodioxocin and *γ*−signals of *β*−5 and *β-O-*4 were overlapped in ^{1}H spectrum. The results of the quantification are collected in Table 1. Reproducibility, precision and standard error of the Q-HSQC measurements were determined for the model compound mixture by repeating measurements 4 times. The reproducibility of the measurements was good. The precision of the method was evaluated, the relative standard errors (RSD) for Q-HSQC being less than 3.0% (Table 1). RSD's were calculated for different side chain signals of each model compound. In addition, the detection limit for Q-HSQC was estimated from the MWL sample spectra.

As can be seen in Table 1, for the model compound mixture the Q-HSQC method provides as reliable results as 1D ^{1}H and quantitative ^{13}C spectra do, even without any relaxation or homonuclear coupling corrections. It should be noted that the Q-HSQC integrals correspond to intensities of ^{1}H NMR spectra, as the detected nucleus is ^{1}H. Also the total amount of methoxyl signals is in good agreement with the expected amount of substances.

Application of Q-HSQC to Wood Lignin. We applied the Q-HSQC technique to lignin sample isolated from spruce wood. Normally, the quantitative analysis of lignin samples has been accomplished by inverse gated ^{1}H decoupled ^{13}C NMR spectroscopy.[1] However, the quantitative ^{13}C NMR approach has limitations when applied to high molecular weight lignin. Due to severe peak overlap in ^{13}C spectra, quantitative information can be derived from peak clusters representing specific carbon types only. In a typical procedure, lignin samples are derivatized by acetylation. The quantitative information is derived from ^{13}C

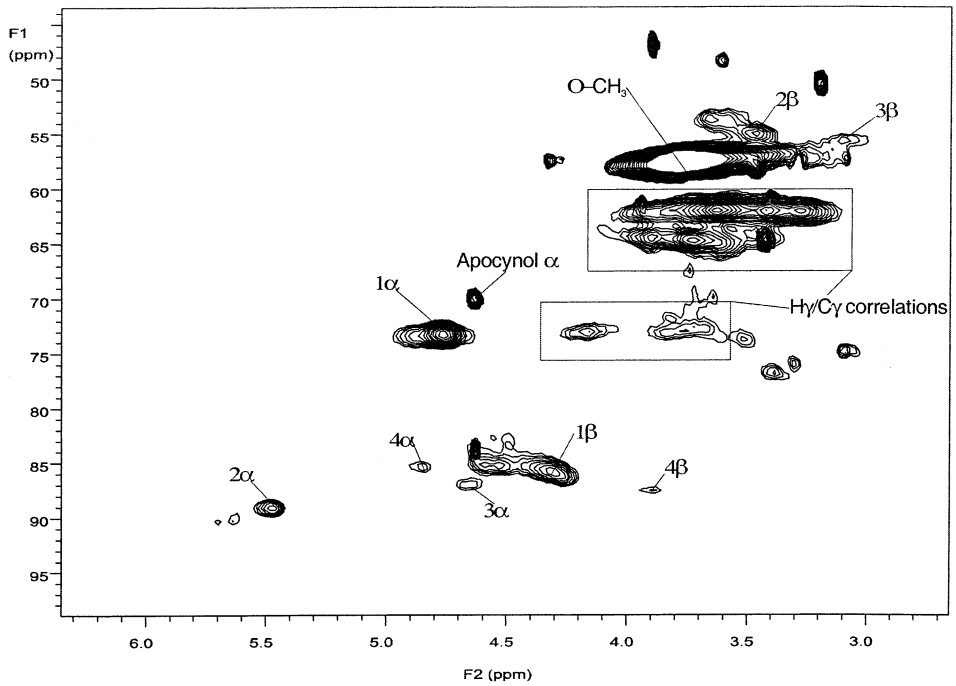

Figure 5. An expansion of the Q-HSQC spectrum of milled wood lignin, MWL (*Picea abies*). The correlation from different structural units were assigned as previously (Figure 6).[19]

spectra by measuring areas of aromatic, aliphatic, methoxyl and acetate signals and expressing them per aromatic or methoxyl area.[13] On the other hand, integration of the aromatic signals provides information about ratios of different lignin types in hardwood, namely syringyl (S) and guaiacyl (G) type lignin.[14,15] In addition, side chain primary and secondary hydroxyl groups and phenolic groups per methoxy groups can be estimated from the intensities of acetyl signals in acetylated lignin samples.[16] The amount of side chain C_α and C_β carbons can be determined with quantitative ^{13}C NMR and DEPT spectroscopy using internal standards. However, the RSD of aliphatic side chain carbons for quantitative ^{13}C NMR spectrum has been reported to be 6% (model compounds) and 8% (lignin).[17] Thus, determination of side chain carbons in different substructures is difficult by quantitative ^{13}C NMR spectroscopy due to poor resolution and low sensitivity. However, the side chain α-correlations of lignin substructures separate well in 2D $^1H-^{13}C$ HSQC spectra of high molecular weight lignin sample. Thus, the Q-HSQC approach provides a powerful tool to analyze the composition of wood lignin.

The abundance of main structural units in milled wood lignin (MWL) was determined from Q-HSQC spectra (Figure 5). The MWL sample was measured without prior derivatization. Quantitative information of *β*−*O*-4, *β*−5, dibenzodioxocin and *β*−*β* structures was derived from signal intensities of their H_α/C_α correlations to avoid the interference from homonuclear $^1H-^1H$ couplings (see p 8). Apocynol (Figure 1) was used as internal standard, as the $^1H/^{13}C$ correlation signals of apocynol do not overlap with any of the known structures of lignin side chains. In addition, it is easy to prepare and purify without any special

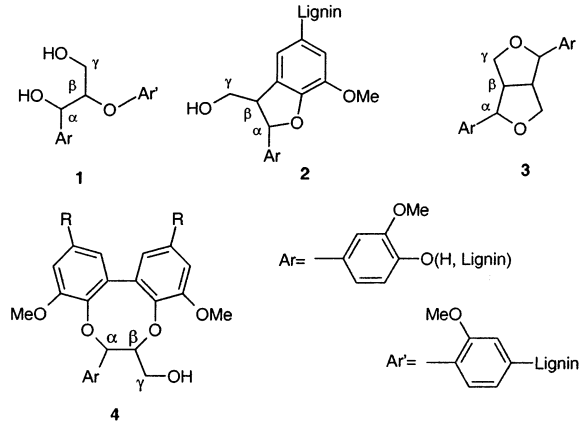

Figure 6. The main structural units of lignin.

equipment. The H_α/C_α-correlation of apocynol appears at 4.72/68.2 ppm in HSQC spectrum. This correlation is well positioned to the lignin side chain area without overlapping any of the lignin or residual carbohydrate signals (Figure 5). The detection limit of Q-HSQC was estimated from apocynol signal using for the lowest detectable signal-to-noise ratio value of 4. The calculated detection limit for apocynol was around 0.3 mg/mL. It should be noted here that it is not easy to estimate 2D spectra integrals by visually looking the contour level maps, as the whole correlation peak volume is to be integrated. The contour levels only reflect the height of the signal, not the volume. Especially in lignin samples some signals are very "broad" due to the heterogenity of the samples.

According to Q-HSQC, the relative amounts of structural units in the MWL sample were 1.50 (*β*−*O*-4):0.31 (*β*−5):0.23 (dibenzodioxocin):0.09 (*β*−*β*). Here, a relative number of 1.00 was given for the apocynol internal standard (0.025 mmol), and the amounts of the different structural units were calculated from

(13) Landucci, L. *Holzforschung* **1985**, *39*, 355.
(14) Lapierre, C.; Monties, B. *Holzforschung* **1985**, *39*, 367.
(15) Obst, J. R.; Landucci, L. L. *Holzforschung* **1986**, *40*, Suppl. 87.
(16) Robert, D.; Chen, C.-L. *Holzforschung* **1989**, *43*, 323.
(17) Xia, Z.; Akim, L. G.; Argyropoulos, D. S. *J. Agric. Food Chem.* **2001**, *49*, 3573.

the intensities of their H_α/C_α correlations. Our results are somewhat different from previously suggested figures.[18] However, it should be noted that we have analyzed only one MWL sample in the current study (originating from one wood individual), and that MWL always represents only a fraction of lignin in wood.

We are currently proceeding with the absolute quantification of all the structural units of lignin. However, these results will be published separately, as for the absolute quantification it is essential that the absolute amount of sample, as well as its lignin content are accurately known, which is out of the scope of the current paper.

Conclusions

We have shown here that it is possible to record quantitative $^1H-^{13}C$-correlation NMR spectra by modulating the polarization transfer delays of HSQC. Also, the effects of relaxation and homonuclear couplings can be taken into account. We applied the current approach to wood lignin, where it has been difficult to obtain reliable information over the different structural units. The current approach provides information on all of the structural units of lignin, and is not, like many of the alternative methods, based on assumptions of specific reactions. The current approach can be easily adapted to various applications to analyze complex mixtures, especially when normal one-dimensional (^1H or ^{13}C) NMR methods fail.[19]

Acknowledgment. Financial support from the National Technology Agency TEKES and Academy of Finland is gratefully acknowledged.

JA029035K

(18) Adler, E. *Wood Sci. Technol*. **1977**, *11*, 169.

(19) Ämmälahti, E.; Brunow, G.; Bardet, M.; Robert, D.; Kilpeläinen, I. *J. Agric. Food Chem*. **1999**, *46*, 5113.