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Selective Quantification by 2D HSQC$_0$ Spectroscopy of Thiocoraline in an Extract from a Sponge-Derived *Verrucosispora* sp.

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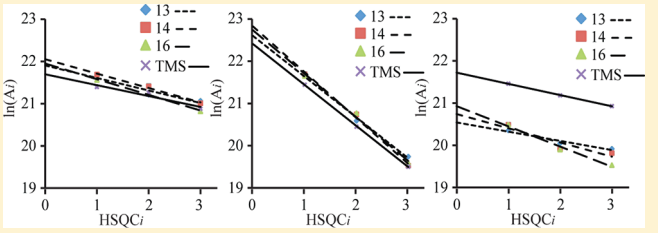
ABSTRACT: We recently developed a 2D ^{1}H−^{13}C HSQC$_0$ approach to quantify individual chemicals in complex mixtures. The HSQC$_0$ approach has been implemented in phase-cycled and gradient-selective versions. As in quantitative 1D NMR, the normalized integrated signal intensities in HSQC$_0$ are proportional to the concentrations of individual chemicals in the mixture. We applied the HSQC$_0$ approach to selectively quantify thiocoraline present at a level of 1% w/w in an extract from a *Verrucosispora* sp. isolated from the sponge *Chondrilla caribensis* f. *caribensis*. We expect that this approach can be used to quantify other natural products of interest in extracts without prior purification.



The field of natural products has seen a constant increase in the application of quantitative NMR methods over the past 40 years.[1] Quantitative NMR methods have been used, among other applications, for biosynthetic studies,[2] quantification of species in complex mixtures,[3] and regulation of reference materials.[4] Quantitative NMR offers advantages over more sensitive MS-based methods in being unbiased and not subject to variability in ionization. Quantitative 1D proton NMR (qHNMR) has become a routine analytical tool because of its universality, sensitivity, precision, and nondestructive nature.[1] Furthermore, the proportionality of the integrated intensities of proton resonances to the overall number of proton spins in the mixture makes qHNMR suitable for quantification of individual compounds in mixtures.[1,5] However, 1D qHNMR has shortcomings for signals that are overlapped,[6] as often is the case with complex mixtures of natural products.

Our recent extension of quantitative NMR to 2D ^{1}H−^{13}C spectroscopy overcomes the overlap problem by taking advantage of peak dispersion in two dimensions. The cross-peaks in standard 2D ^{1}H−^{13}C NMR spectra, such as HSQC (homonuclear single quantum correlation), are not proportional to the concentration as the result of several factors. We have shown that peak intensities that do scale with concentration can be determined by an approach called time-zero HSQC, or HSQC$_0$.[7] We have developed two versions of this experiment: phase-cycled HSQC$_0$[7] and gradient-selective HSQC$_0$.[8] As in 1D qHNMR, the integrated signal intensities in the virtual HSQC$_0$ spectrum are directly proportional to concentrations of individual chemicals in the mixture. With the HSQC$_0$ approach, it is not necessary to relate measured intensities to data from the pure compound at known concentration; concentrations can be determined instead by reference to peak intensities of internal standards added at known concentration, for example, TMS ($(CH_3)_4Si$) for samples in $CDCl_3$, or DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) for samples in aqueous solution. In theory, the HSQC$_0$ approach can be used to quantify all individual chemicals in the mixture without isolation or purification.

We illustrate here the application of the HSQC$_0$ approach to selectively quantify thiocoraline (**1**). Thiocoraline (**1**), which was first isolated in 1997 from the fermentation broth of *Micromonospora marina*,[9,10] has shown potent cytotoxicity to lung, breast, colon, renal, and melanoma cancer cells[10−12] and *in vivo* efficacy against human carcinoma xenografts.[13] For this study, thiocoraline (**1**) was quantified in an extract from a *Verrucosispora* sp. isolated from the sponge *Chondrilla caribensis* f. *caribensis*.[14] The isolation of thiocoraline (**1**) and related analogs from a *Verrucosispora* sp. was recently published.[15]

As described previously,[7] a series of HSQC$_i$ spectra can be acquired with one, two, or three repetitions of the basic HSQC building block. The time-zero 2D ^{13}C HSQC spectrum (HSQC$_0$) is then obtained by linear regression extrapolation to zero repetitions (time zero). For gradient-selective HSQC$_i$ spectra,

$$\ln(A_{i,n}) = \ln(2A_{0,n}) + i \times \ln\left(f_{A,n} \cdot \frac{1}{2}\right) \quad (1)$$

and for phase-cycled HSQC$_i$ spectra,

$$\ln(A_{i,n}) = \ln(A_{0,n}) + i \times \ln(f_{A,n}) \quad (2)$$

where $f_{A,n}$ is the amplitude attenuation factor through each HSQC block specific for peak n; $A_{i,n}$ and $A_{0,n}$ are the amplitudes of peak n in HSQC$_i$ spectra and the extrapolated virtual HSQC$_0$ spectra; i is the number of times of repetition of the basic HSQC building block. Although the HSQC$_0$ approach was designed initially for determining the absolute concentrations of individual

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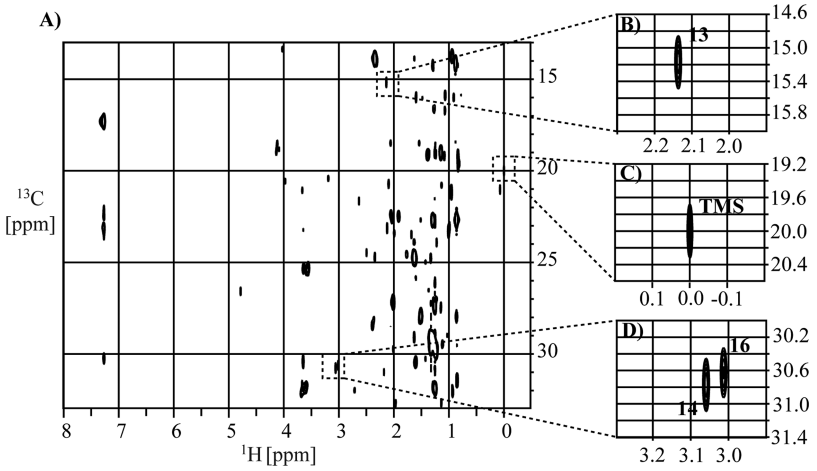


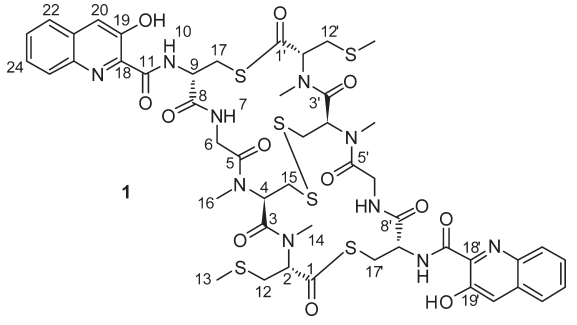
Figure 1. (A) 700 MHz 2D constant-time phase-cycled $HSQC_1$ spectrum of the NMR sample with 8.0 mg of extract and 58.7 μM TMS as concentration reference in $CDCl_3$. The cross-peaks of thiocoraline (**1**) used for the concentration measurement together with that of TMS methyl groups are shown in the expanded panels: (B) 13, 13′ of thiocoraline (**1**), (C) TMS, and (D) 14, 14′ and 16, 16′ of thiocoraline (**1**).

Table 1. Manually Integrated Peak Volume from Constant-Time Phase-Cycled $HSQC_i$ of Sample 1

	csC	csH	$HSQC_1$	$HSQC_2$	$HSQC_3$	corr coef	scaling factor	A_0	# of H	normalized A_0	av/stdev	w/w % of thiocoraline
13,13′	15.166	2.137	2.55×10^9	1.63×10^9	1.41×10^9	−0.9592	0.7436	3.26×10^9	6	5.43×10^8	5.81×10^8	
14,14′	30.773	3.059	2.63×10^9	2.00×10^9	1.33×10^9	−0.9936	0.7111	3.78×10^9	6	6.30×10^8	7.69%	1.12%
16,16	30.627	3.013	2.31×10^9	1.69×10^9	1.10×10^9	−0.9959	0.6901	3.41×10^9	6	5.69×10^8	mol of TMS	
TMS	19.999	0	1.96×10^9	1.72×10^9	1.17×10^9	−0.9617	0.7726	2.65×10^9	12	2.21×10^8	2.93584×10^{-8}	

compounds in metabolite mixtures, it also can be used to selectively quantify natural products of interest in an extract.

To evaluate the reproducibility of our measurements, two NMR samples were prepared from extracts from a *Verrucosispora* sp. Sample 1 contained 8.0 mg of the dry extract plus 2.59 μg of TMS (final concentration 58.7 μM), and sample 2 contained 4.0 mg of the dry extract plus 3.63 μg of TMS (final concentration 82.3 μM). Note, the ratios of extract to TMS were intentionally different in the two samples. A 40 μL amount of 4% $Cr(AcAc)_3$ in $CDCl_3$ was added to each sample as a relaxation-enhancing agent, and $CDCl_3$ was then added to a final volume of 500 μL for each sample. Independent $HSQC_i$ data sets were collected for each sample in both constant-time phase-cycled mode and non-constant-time gradient-selective mode.



Cross-peaks assigned[9] to the (13, 13′), (14, 14′), and (16, 16′) methyl groups of thiocoraline (**1**) (Figure 1) were chosen for quantifying thiocoraline (**1**) in the extract because of their characteristic and relatively stronger intensities. Intensities of these peaks, together with that of TMS, were manually integrated as described previously[7] (Tables 1−3).

As shown in Figure 2, the peak intensities of the (13, 13′), (14, 14′), and (16, 16′) methyl groups of thiocoraline (**1**) and that of methyl groups of TMS in $HSQC_0$ were extrapolated by linear regression according to eq 1 or eq 2. As predicted, the slopes of the linear regressions of the gradient-selective data (Figure 2B) are steeper due to the scaling factor of 0.5 (eq 1) than those for the constant-time data (Figure 2A,C; eq 2). The intercepts from the linear regressions that were obtained using the gradient-selective pulse program in Figure 2B are larger because of the signal enhancement due to the linear recombination of the time-domain NMR data recorded in the echo−antiecho mode before Fourier transformation. The extrapolated $HSQC_0$ peak intensities, A_0, are shown in Tables 1−3 along with the values normalized by the corresponding number of attached protons. The averaged normalized peak intensities in $HSQC_0$ from the cross-peaks of the (13, 13′), (14, 14′), and (16, 16′) methyl groups of thiocoraline (**1**) were used to calculate the final quantity of thiocoraline (**1**) in the extract by reference to the TMS reference standard:

$$\text{w/w(\%of Thio)} = \frac{\text{Av Norm } A_0\text{(Thio)}}{\text{Norm } A_0\text{(TMS)}} \text{Mol(TMS) MW(Thio)/Weight(dry extracts)} \quad (3)$$

As shown in Tables 1−3, the quantities of thiocoraline (**1**) in the extract of a *Verrucosispora* sp. were determined to be 1.12%, 1.17%, and 0.90% from the constant-time phase-cycled $HSQC_i$, non-constant-time gradient-selective $HSQC_i$ of sample 1, and constant-time phase-cycled $HSQC_i$ of sample 2, respectively. Thus, the quantity of thiocoraline (**1**) in the extract of a *Verrucosispora* sp. is estimated to be 1.0% (w/w). This calculated

Table 2. Manually Integrated Peak Volume from Non-Constant-Time Gradient-Selective HSQC$_i$ of Sample 1[a]

	csC	csH	HSQC$_1$	HSQC$_2$	HSQC$_3$	corr coef	scaling factor	A_0	# of H	normalized A_0	av/stdev	w/w % of thiocoraline
13,13′	15.16	2.136	2.64×10^9	8.92×10^8	3.82×10^8	−0.9975	0.3804	6.67×10^9	6	1.11×10^9	1.26×10^9	
14,14′	30.77	3.059	2.70×10^9	1.04×10^9	3.08×10^8	−0.9976	0.3377	8.35×10^9	6	1.39×10^9	11.16%	1.17%
16,16	30.615	3.012	2.58×10^9	1.07×10^9	3.27×10^8	−0.9964	0.3560	7.63×10^9	6	1.27×10^9	mol of TMS	
TMS	19.996	0	2.10×10^9	7.77×10^8	3.01×10^8	−0.9999	0.3786	5.50×10^9	12	4.59×10^8	2.93584×10^{-8}	

[a] NMR sample 1: 58.7 μM TMS (2.59 μg in 500 μL) was added as concentration reference into the NMR sample solution with 8.0 mg of extract. Molecular weights of TMS and thiocoraline are 88.22 and 1157.41, respectively.

Table 3. Manually Integrated Peak Volume from Constant Time Phase-Cycled HSQC$_i$ of Sample 2[a]

	csC	csH	HSQC$_1$	HSQC$_2$	HSQC$_3$	corr. coef	scaling factor	A_0	# of H	normalized A_0	av/stdev	w/w % of thiocoraline
13,13′	15.153	2.139	6.98×10^8	5.13×10^8	4.51×10^8	−0.9731	0.8038	8.43×10^8	6	1.40×10^8	1.73×10^8	
14,14′	30.754	3.059	7.98×10^8	4.44×10^8	4.07×10^8	−0.9193	0.7142	1.03×10^9	6	1.71×10^8	19.15%	0.90%
16,16	30.608	3.011	7.97×10^8	4.42×10^8	3.06×10^8	−0.9912	0.6196	1.24×10^9	6	2.07×10^8	mol of TMS	
TMS	19.997	0	2.11×10^9	1.60×10^9	1.24×10^9	−0.9997	0.7666	2.74×10^9	12	2.29×10^8	4.11471×10^{-8}	

[a] NMR sample 2: 82.3 μM TMS (3.63 μg in 500 μL) was added as concentration reference into the NMR sample solution with 4.0 mg of extract. Molecular weights of TMS and thiocoraline are 88.22 and 1157.41, respectively.

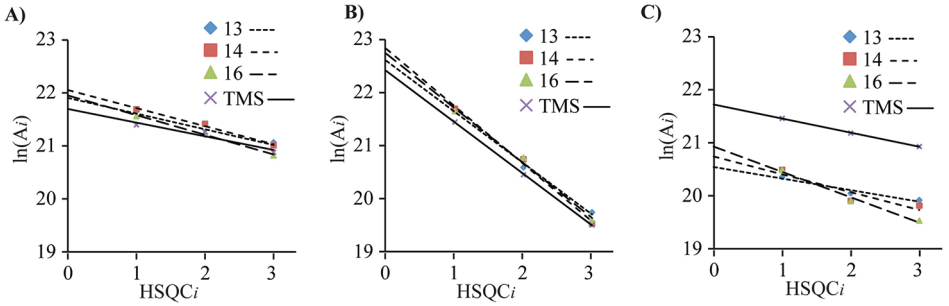


Figure 2. Extrapolations of the peak intensities of 13, 13′ (♦), 14, 14′, (■), and 16, 16′ (▲) methyl groups of thiocoraline and that of the methyl groups of TMS (×) to yield HSQC$_0$ intensities from (A) constant-time phase-cycled HSQC$_i$ of sample 1, (B) non-constant-time gradient-selective HSQC$_i$ of sample 1, and (C) constant-time phase-cycled HSQC$_i$ of sample 2.

concentration equates to ~8.5 mg of thiocoraline (**1**) produced per liter of medium cultured, in agreement with actual quantities of thiocoraline (**1**) isolated in the laboratory.

In this paper, we demonstrated how to use the newly developed HSQC$_0$ NMR approach to selectively quantify thiocoraline (**1**) in an extract from a *Verrucosispora* sp. isolated from the sponge *C. caribensis* f. *caribensis* without any purification. The HSQC$_0$ approach can be used to quantify a variety of natural products of interest at low concentrations (μM) in complex extracts even if they are difficult to isolate or purify. It can be used to standardize herbal extracts through quantification of the standard ingredients. Furthermore, it can be used in the selection or optimization of media for the production of (isotope labeled) compounds and for optimization of protocols for compound isolation and purification.

EXPERIMENTAL SECTION

Biological Material. Sponge specimens were collected on February 10, 2010, in the Florida Keys (24°39′17.90″, 81°17′51.09″). A voucher specimen for *Chondrilla caribensis* f. *caribensis* (FLK-10-4-24) is housed at the University of Wisconsin−Madison. Strain WMMA107 was isolated from the sponge *C. caribensis* f. *caribensis* (FLK-10-4-24) and was identified as a marine actinomycete, *Verrucosispora* sp. Details of the cultivation, purification, and 16S rDNA sequencing[16,17] of the *Verrucosispora* sp. have been reported.[15]

Fermentation and Extraction. Strain WMMA107 was fermented in 25 × 150 mm culture tubes (4 × 10 mL) in medium ASW-A (20 g soluble starch, 10 g glucose, 10 g peptone, 5 g yeast extract, 5 g $CaCO_3$ per liter of artificial seawater) for one week at 28 °C. A 250 mL baffled flask containing 50 mL of medium ASW-A with Diaion HP20 (4% by weight) was inoculated with 2 mL from the culture tube and shaken at 200 rpm at 28 °C for one week. Filtered HP20 was washed with H_2O and extracted with 50 mL of acetone for 2 h.

NMR Sample Preparation. The dry extract (40.0 mg) was dissolved in 3 mL of $CHCl_3$. Then 600 μL (sample 1) and 300 μL (Sample 2) aliquots were removed and dried (8.0 and 4.0 mg, respectively). Standard solutions of TMS were prepared by dilutions of a stock solution of TMS in $CDCl_3$ (0.147 mM). The NMR samples were prepared by dissolving sample 1 and sample 2 in 500 μL of $CDCl_3$ containing TMS at concentrations of 58.7 and 82.3 μM, respectively. Then 40 μL of 4% $Cr(AcAc)_3$ in $CDCl_3$ was added as a relaxation-enhancing agent to each sample. NMR-grade $CDCl_3$ (99.8% D, Cambridge Isotopes Laboratory) was used for both samples.

NMR Experiments. All NMR data were collected at 25 °C on a Bruker Avance III 700 MHz spectrometer equipped with a 5 mm QCI probe, with radio frequency pulses applied on ^1H and ^{13}C at 2.5 and

23 ppm, respectively. GARP ^{13}C decoupling used a field strength of γB_2 = 2.5 kHz. A total of 2048 × 42 complex data points with spectral width of 16 and 20 ppm, respectively, were collected along the ^{1}H and ^{13}C dimensions, with 64 scans per FID and an interscan delay of 2.6 s (longer than 5 times the longest proton T_1), resulting in a total acquisition time of 4 h 15 min for each HSQC$_i$. Measurement of proton T_1 values was conducted by using a standard inversion recovery pulse sequence (180°−τ−90°). For non-constant-time gradient-selective HSQC$_i$, the strength of the pulsed field gradients applied along the z-axis were g_1 = 80%; g_2 = 20.1%; g_3 = 70%; g_4 = 17.5875%, g_5 = 60%; g_6 = 15.075%; g_{13} = 70%; g_{14} = 85%; g_{15} = 15%; and g_6 = 90% of the maximum of 53 G/cm, all with a duration of 1 ms followed by a gradient recovery period of 200 μs. Quadrature detection in the ^{13}C (t_1) dimension was achieved by echo−antiecho by flipping the polarity of gradient g_1. For constant-time phase-cycled HSQC$_i$, the constant time T was set to 12 ms without applying gradients g_1 to g_6. Quadrature detection in the ^{13}C (t_1) dimension was achieved using States-TPPI applied to the phase ϕ_2. Intensities of relevant peaks are manually integrated as described before,[7] and the data are shown in Tables 1−3.

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REFERENCES

(1) Pauli, G. F.; Jaki, B. U.; Lankin, D. C. *J. Nat. Prod.* **2005**, *68*, 133–149.
(2) Schoen, J.; Modha, A.; Maslow, K.; Novak, M.; Blackburn, B. J. *Int. J. Parasitol.* **1996**, *26*, 713–722.
(3) Rivero-Cruz, B.; Rivero-Cruz, I.; Rodríguez, J. M.; Cerdad-García-Rojas, C. M.; Mata, R. *J. Nat. Prod.* **2006**, *69*, 1172–1176.
(4) Jaki, B.; Sticher, O.; Veit, M.; Frohlich, R.; Pauli, G. F. *J. Nat. Prod.* **2002**, *65*, 517–522.
(5) Akoka, S.; Barantin, L.; Trierweiler, M. *Anal. Chem.* **1999**, *71*, 2554–2557.
(6) Lewis, I. A.; Schommer, S. C.; Hodis, B.; Robb, K. A.; Tonelli, M.; Westler, W. M.; Sussman, M. R.; Markley, J. L. *Anal. Chem.* **2007**, *79*, 9385–9390.
(7) Hu, K. F.; Westler, W. M.; Markley, J. L. *J. Am. Chem. Soc.* **2011**, *133*, 1662–1665.
(8) Hu, K. F.; Ellinger, J. J.; Chylla, R. A.; Markley, J. L. *Anal. Chem.* **2011**submitted.
(9) Pérez Baz, J.; Cañedo, L. M.; Fernández-Puentes, J. L.; Silva Elipe, M. V. *J. Antibiot.* **1997**, *50*, 738–741.
(10) Romero, F.; Espliego, F.; Pérez Baz, J.; García de Quesada, T.; Grávalos, D.; De la Calle, F.; Fernández-Puentes, J. L. *J. Antibiot.* **1997**, *50*, 734–737.
(11) Erba, E.; Bergamaschi, D.; Ronzoni, S.; Faretta, M.; Taverna, S.; Bonfanti, M.; Catapano, C. V.; Faircloth, G.; Jimeno, J.; D'Incalci, M. *Br. J. Cancer* **1999**, *80*, 971–980.
(12) Negri, A.; Marco, E.; García-Hernández, V.; Domingo, A.; Llamas-Saiz, A. L.; Porto-Sandá, S.; Riguera, R.; Laine, W.; David-Cordonnier, M. H.; Bailly, C.; García-Fernández, L. F.; Vaquero, J. J.; Gago, F. *J. Med. Chem.* **2007**, *50*, 3322–3333.
(13) Faircloth, G.; Jimeno, J.; Dincalci, M. *Eur. J. Cancer* **1997**, *33*, 781–781.
(14) Rützler, K.; Duran, S.; Piantoni, C. *Mar. Ecol.* **2007**, *28* (Suppl. 1), 95–111.
(15) Wyche, T. P.; Hou, Y.; Braun, D.; Cohen, H. C.; Xiong, M. P.; Bugni, T. S. *J. Org. Chem.* **2011**, *76*, 6542–6547.
(16) Drancourt, M.; Bollet, C.; Carlioz, A.; Martelin, R.; Gayral, J. P.; Raoult, D. *J. Clin. Microbiol.* **2000**, *38*, 3623–3630.
(17) Zhu, S.; Fushimi, H.; Cai, S. Q.; Komatsu, K. *Planta Med.* **2003**, *69*, 647–653.