



EDGEWOOD

CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

ECBC-TR-721

**TRACE LEVEL DETECTION
OF CHEMICAL WEAPONS CONVENTION COMPOUNDS
BY TWO-DIMENSIONAL ^{13}C -NMR SPECTROSCOPY
USING A CRYOGENIC PROBEHEAD AND
 ^1H -DETECTION TECHNIQUES**

Terry J. Henderson

RESEARCH AND TECHNOLOGY DIRECTORATE

David B. Cullinan



SCIENCE APPLICATIONS
INTERNATIONAL CORPORATION
Gunpowder, MD 21010-0068

October 2009

Approved for public release;
distribution is unlimited.



20091023186

ABERDEEN PROVING GROUND, MD 21010-5424

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) XX-10-2009		2. REPORT TYPE Final		3. DATES COVERED (From - To) May 2007 - May 2008	
4. TITLE AND SUBTITLE Trace Level Detection of Chemical Weapons Convention Compounds by Two-Dimensional ¹³ C-NMR Spectroscopy Using a Cryogenic Probehead and ¹ H-Detection Techniques				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Henderson, Terry J. (ECBC); and Cullinan, David B. (SAIC)				5d. PROJECT NUMBER 00P-0082	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) DIR, ECBC, ATTN: RDCB-DRB-C, APG, MD 21010-5424 SAIC, P.O. Box 68, Gunpowder, MD 21010-0068				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-721	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Two-dimensional ¹ H- ¹³ C heteronuclear single quantum correlation (HSQC) and fast-heteronuclear multiple quantum correlation (HMQC) pulse sequences were implemented using a cryogenic probehead for detecting Chemical Weapons Convention compounds in complex mixtures. The ¹ H- ¹³ C correlations of target analytes at ≤25 µg/mL were easily detected in a sample where the ¹ H solvent signal was ~58,000 fold more intense than the analyte ¹ H signals. The problem of overlapping signals typically observed in conventional ¹ H spectroscopy was essentially eliminated, while ¹ H and ¹³ C chemical shift information could be derived quickly and simultaneously from the resulting spectra. The fast-HMQC pulse sequence generated magnitude mode spectra suitable for detailed analysis in ~4.5 h, and can be used to efficiently screen a large number of samples. The HSQC pulse sequence on the other hand, required roughly twice the acquisition time to produce suitable spectra. These spectra, however, were phase sensitive, contained considerably more resolution, and proved to be superior for detecting analyte ¹ H- ¹³ C correlations. Use of the pulse sequences considerably improves the performance of NMR spectroscopy as a complementary technique for the screening, identification, and validation of chemical warfare agents and other small molecule analytes in complex mixtures and environmental samples.					
15. SUBJECT TERMS Chemical Weapons Convention Cryogenic probehead Heteronuclear multiple quantum coherence Trace level detection Nuclear magnetic resonance Heteronuclear single quantum coherence					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UL	18. NUMBER OF PAGES 21	19a. NAME OF RESPONSIBLE PERSON Sandra J. Johnson
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) (410) 436-2914

Blank

PREFACE

The work described in this report was authorized under Project No. 00P-0082. The work was started in May 2007 and completed in May 2008.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release. Registered users should request additional copies from the Defense Technical Information Center; unregistered users should direct such requests to the National Technical Information Service.

Blank

CONTENTS

1.	INTRODUCTION	7
2.	EXPERIMENTAL PROCEDURES	8
2.1	Samples	8
2.2	NMR Spectroscopy	9
3.	RESULTS AND DISCUSSION	10
4.	CONCLUSIONS.....	17
	LITERATURE CITED	19

FIGURES

1.	Compounds Spiked in OPCW Sample	9
2.	500 MHz ^1H Sub-spectra of D_2O -exchanged, Concentrated Spiked Sample and Authentic Reference Standard Samples for I and II	11
3.	^1H - ^{13}C HSQC Sub-spectra of D_2O -exchanged, Concentrated Spiked Sample and Authentic Reference Standard Samples for I and II	13
4.	Multiplicity-edited ^1H - ^{13}C HSQC Sub-spectrum of D_2O -exchanged, Concentrated Spiked Sample and Corresponding ^1H - ^{13}C Fast-HMQC Sub-spectrum	15

TABLE

^{13}C Chemical Shift Values Derived from ^1H - ^{13}C HSQC Spectra	14
--	----

TRACE LEVEL DETECTION
OF CHEMICAL WEAPONS CONVENTION COMPOUNDS
BY TWO-DIMENSIONAL ^{13}C -NMR SPECTROSCOPY
USING A CRYOGENIC PROBEHEAD AND
 ^1H -DETECTION TECHNIQUES

1. INTRODUCTION

The Chemical Weapons Convention (CWC), which banned the development, production, stockpiling, and use of chemical weapons, was first made available to state governments on January 13, 1993. The Convention was ratified by the 65th state in November 1996 and entered into force the following April, thereby creating the Organization for the Prohibition of Chemical Weapons (OPCW).¹ Under the terms of the CWC, each signatory state or country must designate at least one national authority laboratory, which has to participate annually in an international proficiency test overseen by the OPCW. In the tests, participating laboratories must identify target compounds spiked at trace levels in various matrices such as water, soil, rubber, and paint within a 15 day timeframe. Because of its high sensitivity and ability for coupling to chromatographic techniques such as gas or liquid chromatography, mass spectrometry has been the primary analytical technique used in OPCW proficiency tests. Although NMR spectroscopy is less sensitive, more sophisticated NMR techniques have proved valuable in previous proficiency tests.²⁻⁵ Criteria for the NMR identification of target analytes are based on comparisons of sample spectra to reference data,⁶ from either spectral libraries or spectra acquired from an OPCW test sample spiked with an authentic reference compound. Library spectra, therefore, must be recorded under identical conditions of solvent, temperature, concentration, and H^+ concentration in the case of aqueous samples. For the spiking of authentic reference compounds into test samples, the participating laboratory must be able to synthesize all possible compounds within the imposed time limit. These criteria are highly challenging, and NMR spectroscopy has been used most often to contribute supporting information to confirm results obtained from other analytical techniques.

Because of its high sensitivity in comparison to other nuclei, ^1H spectroscopy typically plays a significant role in the NMR efforts of OPCW proficiency tests. ^1H -NMR spectroscopy, however, suffers from narrow chemical shift dispersion, often leading to overlapping signals in spectra. Liquid chromatography can be coupled to NMR spectrometers for separating target analytes from interfering compounds, or each other, but the resulting sample dilution lowers the already trace analyte concentrations to those well below minimal detection limits.⁷ ^{31}P spectroscopy, on the other hand, has an inherently wide chemical shift dispersion and has been successfully used in several OPCW tests;³⁻⁵ however, OPCW target analytes do not always contain phosphorus nuclei. ^{13}C spectroscopy also has inherently wide chemical shift dispersion, but its very low natural abundance and small magnetogyric ratio afford the technique very poor sensitivity. Much of this sensitivity loss can be recovered by ^1H -detected ^{13}C spectroscopy, but the trace concentrations of target analytes in test samples typically eliminates even this approach as an option. Historically, ^{13}C spectroscopy has been considered an ineffective tool in the analysis of OPCW test samples, even with significant sample concentration.

A number of sophisticated polarization transfer pulse sequences have been recently developed to allow the observation of ^1H nuclei scalar coupled to a particular heteronucleus while effectively suppressing unwanted background signals at the same time. Among these, inverse-detected two-dimensional correlation pulse sequences offer great potential for the detection of small molecule analytes. Particularly for ^1H - ^{13}C correlation spectroscopy, two-dimensional heteronuclear single quantum correlation (HSQC) experiments typically give spectra with good information content and, in some cases, unambiguous identification analytes are possible. The original HSQC experiment⁸ has been refined and modified a number of times over the years, and today, has culminated into a highly sensitive technique.⁹⁻¹¹ Heteronuclear multiple quantum correlation (HMQC) pulse sequences have also been successfully exploited for detecting ^1H - ^{13}C correlations.

Like the HSQC technique, the original HMQC experiment¹² has been modified over the years to increase detection sensitivity. Ross and co-workers¹³ have integrated Ernst angle excitation pulses¹⁴ and very fast repetition rates (time between the first pulse of one acquisition to the first pulse of the next acquisition) into the HMQC pulse sequence to allow very sensitive detection and, therefore, considerably shorter data acquisition times. Their fast-HMQC pulse sequence, for example, allowed the acquisition of a two-dimensional ^1H - ^{15}N correlation spectrum for a 1 mM protein sample in only 37 s,¹³ enabling real-time monitoring of protein-ligand interactions for the first time.

We have overcome the problem of ^{13}C detection for trace concentrations of CWC-related analytes by using a sensitivity-enhanced cryogenic probehead for executing two-dimensional ^1H -detected ^{13}C spectroscopy. Heteronuclear single quantum correlation and fast-HMQC experiments were used to verify the tentative identifications of two target analytes from an OPCW proficiency test sample. In general, the approach represents a tremendous enhancement of signal specificity and detection sensitivity over conventional ^{13}C NMR spectroscopy. To our knowledge, the results described herein are the first ^{13}C data reported for a proficiency test sample in OPCW history.

2. EXPERIMENTAL PROCEDURES

2.1 Samples

A spiked sample was provided by the Republic of China as part of the 21st OPCW Official Proficiency Test. This was an aqueous sample of polyethylene glycol containing three target analytes (Figure 1): 2-(*N,N*-Diisopropylamino) ethanesulfonic acid (**I**) at 5 $\mu\text{g/mL}$, bis(2-Diisopropylaminoethyl) disulfide (**II**) at 10 $\mu\text{g/mL}$, and 2-(*N*-ethyl-*N*-isopropylamino) ethanol (**III**) at 10 $\mu\text{g/mL}$. The sample also contained other low molecular weight compounds that were not identified. Three aliquots, ~ 5 mL each, were separately exchanged three times in 1 mL D_2O using a stream of gaseous nitrogen over the aliquots held at 35 $^\circ\text{C}$ to evaporate the D_2O to near dryness. Each exchanged aliquot was reconstituted in 600 μL D_2O and transferred to a 5 mm NMR sample tube (Catalog no. XR-55-7, Norell, Inc., Landisville, NJ) for NMR spectroscopy. Authentic reference standards of **I** and **II**, also provided by the Republic of China, were individually diluted in D_2O to ~ 200 $\mu\text{g/mL}$ and transferred to individual 5 mm NMR

sample tubes for NMR analysis. All the samples were stored at 4-8 °C and they displayed no signs of degradation after four weeks.

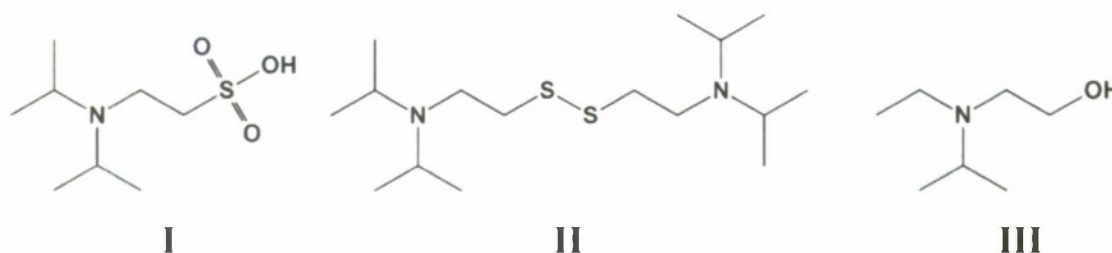


Figure 1. Compounds Spiked in OPCW Sample.

2.2 NMR Spectroscopy

All NMR spectroscopy was conducted at 11.75 T with an Avance DRX 500 spectrometer (Bruker Biospin Corp., Billerica, MA), using a 5 mm TCI probehead (inverse-detection configuration) with a z-axis gradient coil. The probehead contained cryogenically-cooled ^1H and ^{13}C receiver coils and preamplifier stages to significantly reduce thermal noise and enhance sensitivity. Experiments were conducted at 25 ± 0.2 °C with samples spinning at 20 Hz for one-dimensional experiments only. Data acquisition and processing were controlled with XWIN-NMR (Bruker Biospin Corp.) software on Irix (Silicon Graphics, Inc., Mountain View, CA) computer workstations. ^1H and ^{13}C chemical shift values were referenced to external tetramethylsilane.

One-dimensional ^1H spectroscopy incorporated a presaturation pulse to reduce the intensity of the residual HOD signal. For the concentrated samples, free induction decay data of 65,536 complex points were summations of 64 acquisitions using 90° pulse widths, 15 ppm spectral windows, and 10 s relaxation delays. ^1H spin-lattice relaxation time (T_1) values were estimated with an inversion recovery pulse sequence for the optimization of two-dimensional experiments. Data sets for the authentic reference standards were identical, except for the summation of 16 acquisitions. All data sets were multiplied by an exponential window function with a 0.1 Hz line-broadening factor before Fourier transformation (FT) into spectra and manual phase correction into pure absorption mode.

^1H - ^{13}C HSQC spectroscopy⁸ was conducted using a pulse sequence incorporating the selection of phase coherence with pulsed field gradients,¹⁵⁻¹⁷ sensitivity enhancement via refocusing of orthogonal, in-phase, ^1H magnetization components before detection,¹¹ and an echo-antiecho time proportional phase incrementation scheme¹⁸ for generating phase sensitive spectra. The data set collected for the concentrated samples contained 128 ^1H free induction decays, each with 2048 complex points from 144 acquisitions using 3 s relaxation delays. Spectral windows of 10 ppm were used in the direct ^1H dimension and 220 ppm in the indirect ^{13}C dimension, resulting in a total acquisition time of 16 h 51 min. ^1H - ^{13}C HSQC data sets for the authentic reference standard samples were acquired identically, except for the summation of 64 acquisitions per ^1H free induction decay. A multiplicity-edited ^1H - ^{13}C HSQC¹⁹ data set

collected for one of the concentrated samples contained 128 ^1H free induction decays, each with 2048 complex points from 224 acquisitions and 2 s relaxation delays. Spectral windows of 5 and 100 ppm were used in the ^1H and ^{13}C dimension, respectively, giving a total acquisition time of 19 h 24 min. For all HSQC data sets, the ^1H free induction decay data were multiplied by an exponential window function with a 4 Hz line-broadening factor before FT. The resulting ^{13}C free induction decay data were extended to 256 complex points using a linear prediction algorithm,^{20,21} and were then zero-filled to a full 2048 complex points. These data were multiplied with a sine-squared window function before final FT into a spectrum of 1024 x 1024 real points.

^1H - ^{13}C HMQC spectroscopy¹³ was conducted using the fast-HMQC pulse sequence reported by Koskela and co-workers,² with J_{CH} optimized for 145 Hz and all gradient pulse intensities optimized for the selection of ^1H - ^{13}C zero- and double-quantum phase coherence. This pulse sequence uses an Ernst angle excitation pulse and a repetition time optimized to give the maximum detection sensitivity;^{2,13} for our fast-HMQC experiment, these values were 80° and 204 ms, respectively (see the Results and Discussion section below). The data set contained 256 ^1H free induction decays, each with 2048 real points from 256 acquisitions, and used 10 ppm spectral windows in the ^1H dimension and 200 ppm windows in the ^{13}C dimension. The pulse sequence incorporated 128 dummy scans to give a total acquisition time of 4 h 29 min. The indirect ^{13}C dimension was zero-filled to 1024 points, and both dimensions were multiplied by a sine-squared window function before FT and magnitude calculation into a spectrum of 2048 x 1024 real points.

3. RESULTS AND DISCUSSION

Efforts to identify target analytes in the spiked sample (Figure 1) relied on gas chromatography coupled to mass spectrometry (GC-MS) and NMR spectroscopy. The NMR data were used to augment the interpretation of mass spectra and confirm assignments to specific analytes. Initial NMR examination of one of the concentrated samples generated the ^1H spectrum shown in Figure 2a. Two signals, one from residual HOD (not shown) and another at 3.6 ppm from polyethylene glycol, dominate the spectrum. Other than the two doublet signals between 1.1-1.3 ppm indicative of the methyl protons of *i*-propyl groups, the spectrum is populated with low intensity signals impossible to assign without additional information. Gas chromatography-mass spectrometry analysis of the original spiked sample gave mass spectra most consistent with **I**, **II**, and **III**. Assignments of the *i*-propyl groups, however, were problematic, as it is not possible to distinguish between *n*- and *i*-propyl groups in mass spectra obtained in either standard mode (electron impact or chemical ionization). Consequently, the groups were assigned solely from the NMR spectrum shown in Figure 2a. Gas chromatography-mass spectrometry analysis of the concentrated sample could not detect **III**, indicating that it was lost during sample preparation. Therefore, **I** and **II** were tentatively assigned to the concentrated sample.

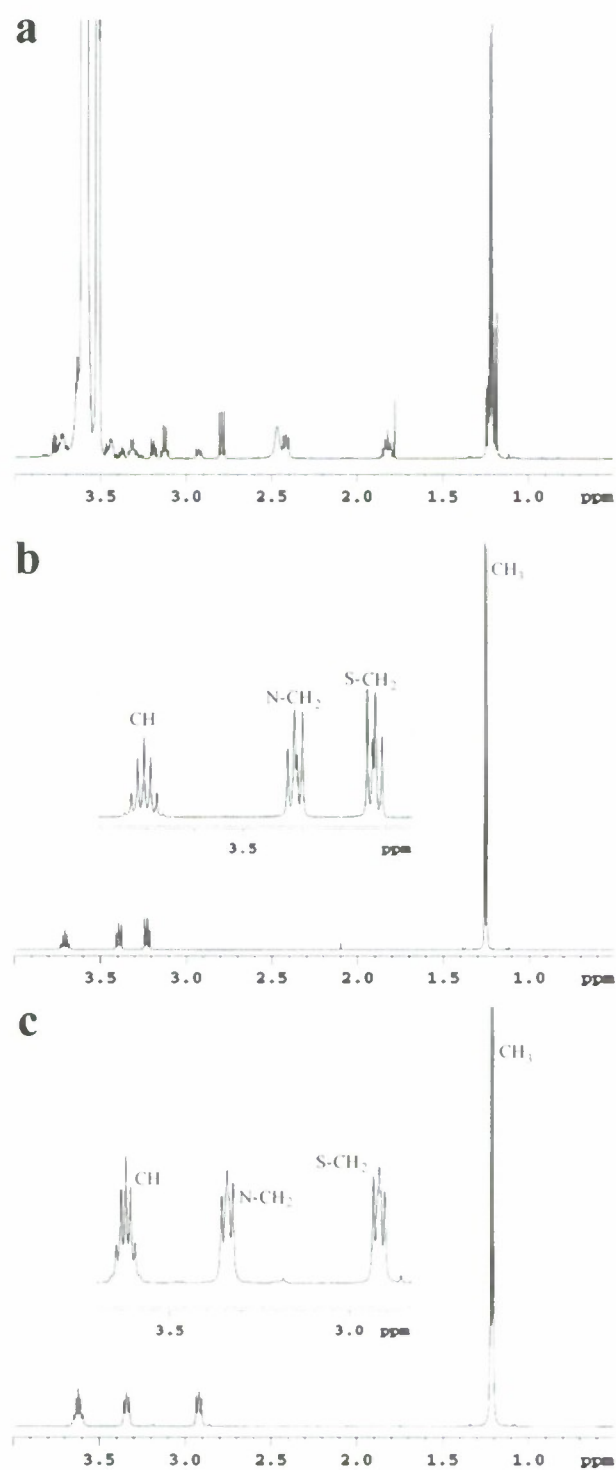


Figure 2. 500 MHz ^1H Sub-spectra of a D_2O -exchanged, Concentrated Spiked Sample (a), and Authentic Reference Standard Samples for **I** (b) and **II** (c).

^1H spectra of the authentic reference standard samples for **I** and **II** are shown in Figures 2b and c, respectively. For our laboratory, the standards were supplied with the spiked sample as we were evaluating this particular proficiency test. Participating laboratories would purchase the compounds commercially, or synthesize them sometime within the 15 day timeframe of the test. Comparison of the reference standard spectra to that for the concentrated sample (Figure 2a) reveals the degree to which signal overlap obscures and masks target analyte signals. None of the methine or methylene signals for **I** and **II** can be identified in the concentrated sample spectrum, even though their exact chemical shift values can be unambiguously derived from the reference standard spectra.

To confirm the assignments of **I** and **II**, ^1H - ^{13}C HSQC spectra were collected for the concentrated as well as the authentic reference standard samples for **I** and **II**. The resulting spectra, presented in Figures 3a, b, and c demonstrate clearly that both **I** and **II** are present in the concentrated samples. For both compounds, each of the four correlations has identical ^1H and ^{13}C chemical shift values in the spectra for the concentrated samples and those for the reference standard compound spectra; these are compelling confirmatory data!

In contrast to their one-dimensional counterpart (Figure 2a), the HSQC spectra of the concentrated samples eliminate nearly all of the signal overlap and allow the simultaneous measurement of both ^1H and ^{13}C chemical shift values from a single spectrum. For example, even in cases where a one-dimensional ^{13}C analysis could detect the signals for the methylene groups of **I** and **II** in the concentrated samples, the overlap between them could never be resolved. The corresponding correlations, however, are clearly resolved in the HSQC sub-spectrum shown in Figure 3a. Additionally, one-dimensional ^1H analyses of a concentrated sample would not resolve the overlap between the N-CH₂ signals of **I** and **II**; but again, their corresponding correlations are completely resolved by our HSQC experiment. While ^1H - ^1H correlation spectroscopy or nuclear Overhauser effect spectroscopy experiments could have also resolved overlapping signals in the ^1H spectrum and provided ^1H chemical shift information in less time, measurement of the ^{13}C chemical shift values would not have been possible. ^{13}C chemical shift values measured from the HSQC spectra are listed in the Table.

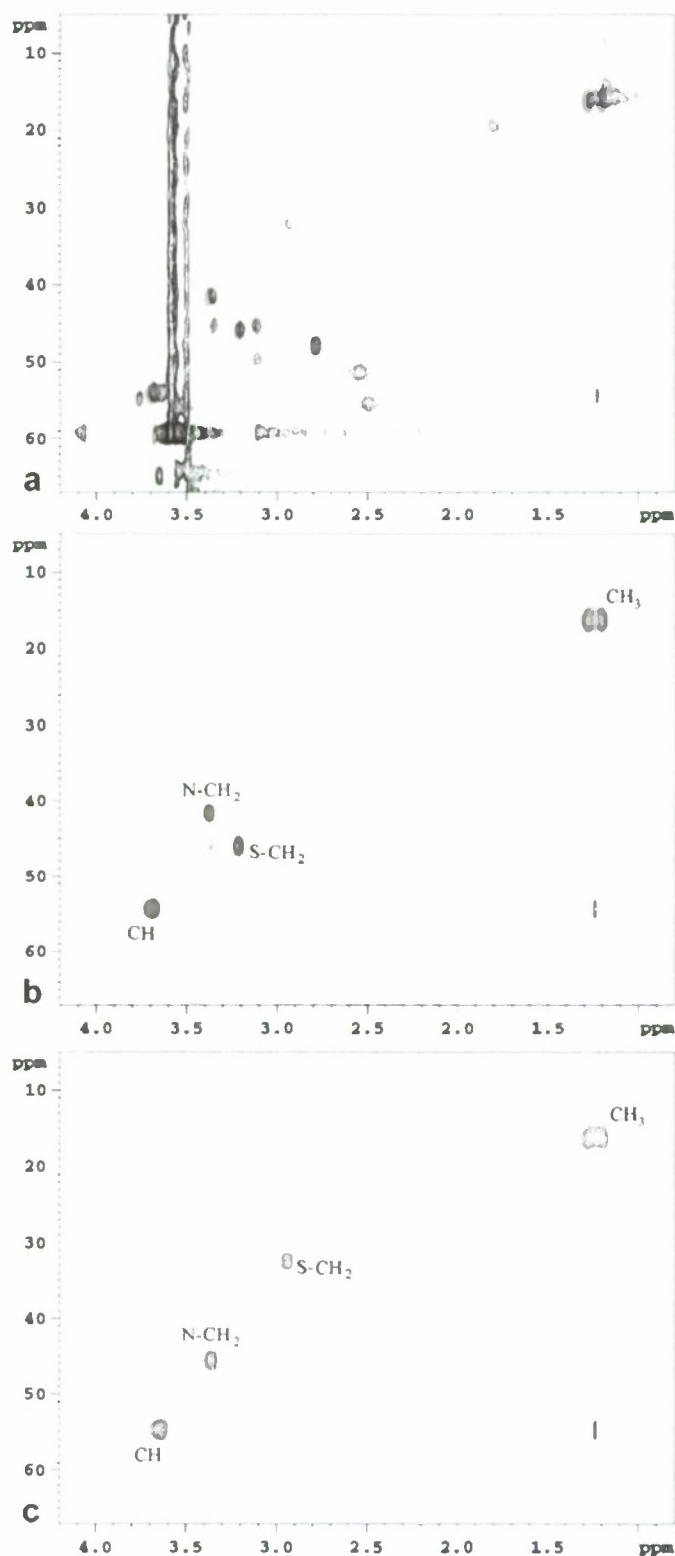


Figure 3. ^1H - ^{13}C HSQC Sub-spectra of D $_2$ O-exchanged, Concentrated Spiked Sample (a), and Authentic Reference Standard Samples for **I** (b) and **II** (c). Correlations from **I** and **II** are red and green, respectively.

Table. ^{13}C Chemical Shift Values Derived from ^1H - ^{13}C HSQC Spectra *

Compound	-NCH ₂ -	-CH ₂ S-	-CH-	-CH ₃
I	42.5	46.9	55.3	17.8
II	45.7	34.9	52.1	17.2

* Chemical shift values are ppm referenced to tetramethylsilane.

The extraordinary sensitivity provided by use of the cryogenic probehead together with ^1H -detection appears essential for detecting ^1H - ^{13}C correlations for **I** and **II** in the concentrated samples. The correlations could not be detected by implementing the HSQC or HMQC pulse sequence with a conventional probehead, and the analyte ^{13}C signals were not detected by one-dimensional spectroscopy using the cryogenic probehead for overnight acquisitions, even when employing polarization transfer²² or nuclear Overhauser effect enhancement for improving sensitivity. We attribute these results to the extreme dynamic range presented by the concentrated sample, which imposes the requirement of a much lower receiver gain for data acquisitions than could be used if the sample contained only the target analytes. The HOD signal in the Fourier transformed ^1H free induction decays of the HSQC data, for example, was ~58,000 fold larger than the methylene and methine signals from **I**. Based on the spiking levels for the original sample, this compound is estimated at ~25 $\mu\text{g/mL}$ in the concentrated samples, with **II** at ~50 $\mu\text{g/mL}$. Correlations for the N-CH₂ group of **I** and the S-CH₂ group of **II** are the least intense of all analyte correlations in the HSQC spectrum. Each has a signal-to-noise ratio of 4:1. Defining the theoretical limit of detection at the usual 3:1 signal-to-noise ratio, a limit of detection at 19 ppm can be estimated from the N-CH₂ correlation of **I**. This most likely represents a conservative estimate as the concentration estimate for **I** in the concentrated samples assumes 100% recovery during the three solvent exchanges.

The HSQC pulse program was chosen principally for its sensitivity enhancement and improved resolution afforded by its phase sensitive data sets. However, other ^1H -detected ^{13}C -spectroscopy techniques are available which can also be used for trace analysis applications. Figure 4a is a sub-spectrum resulting from the use of a multiplicity-edited HSQC pulse sequence for acquiring a data set from one of the concentrated samples. The figure illustrates the additional structural information obtained by using multiplicity-editing; each positive-phased correlation results from a methine or methyl group while negative-phased correlations are the result of methylene groups only. Moreover, not only are the multiplicities of the methylene and methyl groups of **I** and **II** easily discerned, but five methylene groups from interfering compounds are clearly identified as well. The structural information derived from this approach is exceptional, especially when considering analyte concentrations and the extreme dynamic range presented by the sample.

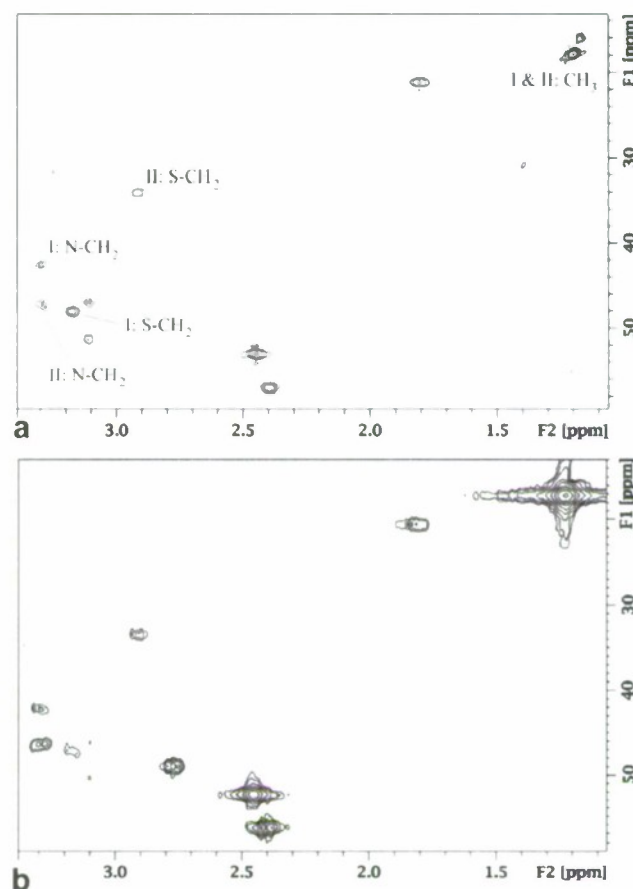


Figure 4. Multiplicity-edited ^1H - ^{13}C HSQC Sub-spectrum of D_2O -exchanged, Concentrated Spiked Sample (a) and Corresponding ^1H - ^{13}C Fast-HMQC Sub-spectrum (b). Negative-phased correlations are red, and positive-phased correlations are black. Correlations for **I** and **II** are designated in the HSQC sub-spectrum.

Finally, we explored the use of Ernst angle excitation pulses incorporated into HMQC pulse sequences, an approach referred to as fast-HMQC spectroscopy,^{13,23} to rapidly detect ^1H - ^{13}C correlations for target analytes. A series of Ernst angles excitation pulses, α_{ernst} , and corresponding repetition rates, T_c , were derived from T_1 values estimated at ~ 130 ms for all **I** and **II** ^1H signals in the concentrated samples using the equation¹³

$$\cos(\alpha_{\text{ernst}}) = \exp(T_c / T_1) \quad (1)$$

We made a deliberate attempt to derive the shortest possible repetition rate, as the sensitivity gain for a fast-HMQC experiment relative to that of a comparable HMQC experiment using 90° excitation pulses increases exponentially with decreasing repetition time.¹³ With $T_1 = 130$ ms, a 204 ms repetition rate was found to give an Ernst angle excitation pulse of 80° . We used these values for our experiment as the repetition rate leaves only 23 ms for execution of the pulse sequence and relaxation delay, essentially the shortest repetition time possible for our

conditions. Using the relationship between sensitivity, S , and signal intensity, I_s , presented by Ross and co-workers¹³

$$S = I_s / (T_c)^{1/2} \quad (2)$$

The theoretical sensitivity gain of this fast-HMQC experiment is 25% higher compared to a similar HMQC experiment using a conventional 90° excitation pulse, yielding a 36% reduction in acquisition time. This sensitivity gain was apparent during data acquisition, where most correlations for **I** and **II** were detected in <1 h, with the methyl group correlations of both compounds detected in <10 min. A sub-spectrum from the fast-HMQC experiment is presented in Figure 4b along with the corresponding multiplicity-edited HSQC sub-spectrum in Figure 4a for direct comparison. Casual inspection of the sub-spectra reveals significantly lower resolution in both dimensions of the fast-HMQC data, a likely consequence of the magnitude mode HMQC spectrum relative to the phase sensitive HSQC spectrum. This is particularly evident for the methyl group correlations for **I** and **II**, which are clearly resolved in the HSQC sub-spectrum but appear to be one large correlation in the fast-HMQC sub-spectrum. Closer examination of the data reveals a correlation in the fast-HMQC sub-spectrum at $\delta_H \sim 2.8$ ppm and $\delta_C \sim 49$ ppm which is not present in the HSQC sub-spectrum. This correlation arises from a compound that appeared in the sample after acquisition of the multiplicity-edited HSQC data set, and is most likely from the degradation of one of the compounds in the sample. Degradation of CWC-related compounds in solution has been observed by us in the past and recently reported by others.²⁴

The values chosen for the Ernst angle excitation pulse and repetition rate in our fast-HMQC experiments served well for rapidly detecting ^1H - ^{13}C correlations of CWC-relevant analytes with ^1H T_1 values at ~ 130 ms. These T_1 values, however, are significantly shorter than those expected for low molecular weight analytes in solution at 11.75 T and our 25 °C analysis temperature, and are probably the result of effects from one or more components in the original spiked sample (e.g., paramagnetic nuclei). ^1H T_1 values for the authentic reference standard samples were estimated to be between 0.6-1.2 s at this magnetic field strength and temperature. These are much closer to values anticipated for low molecular weight analytes in solution. Both the Ernst angle excitation pulse and repetition rate need to be recalculated for acquiring fast-HMQC data from samples containing analytes with these, or longer T_1 values. Under such conditions, the highest sensitivity gain deriving from the use of Ernst angle excitation pulses relative to 90° excitation pulses would be realized by using a very short repetition rate similar to that in our fast-HMQC experiments, and recalculating the required Ernst angle excitation pulse. In practice, the duty cycle of the spectrometer's power amplifier limits the repetition rate of experiments and, therefore, the sensitivity gain from using fast-HMQC experiments relative to conventional HMQC experiments as well.

Relative to conventional one-dimensional ^{13}C spectroscopy, both two-dimensional techniques represent a tremendous advance in signal sensitivity and specificity, the ability of a technique to completely resolve an analyte signal from all impurity signals and artifacts. While the broad chemical shift dispersion inherent to the ^{13}C nucleus does benefit specificity for one-dimensional ^{13}C spectroscopy, this does not compare to the additional specificity gain afforded by the second dimension in the HSQC and HMQC spectra. This is especially the case for the

phase-sensitive HSQC technique, which provides the best means for identifying analyte signals obscured by impurity signals typically observed in OPCW samples. Alternatively, because chemical shift values are dependent upon H^+ concentration for some CWC-relevant compounds in water, changes in H^+ concentration may also be used to resolve analyte signals obscured by impurity signals. Fast-HMQC spectroscopy, on the other hand, clearly represents the best approach for optimizing signal sensitivity. This is reflected by the very short data acquisition times used to collect the fast-HMQC data sets. Our fast-HMQC data set for Figure 4b was collected in <33% of the time used to acquire the HSQC data set for Figure 3a and <25% of the time used to acquire the multiplicity-edited HSQC data set for Figure 4b. In the fast-HMQC spectrum, correlations for the N-CH₂ group of **I** and the S-CH₂ group of **II**, the least intense analyte correlations in the spectrum, both have a signal-to-noise ratio of ~5:1. Because signal-to-noise ratio is directly proportional to the square root of the number of data acquisitions collected, a fast-HMQC data set giving a spectrum with a 3:1 signal-to-noise ratio for the correlations (their theoretical detection limit) can be collected in about half of the time as that used for the spectrum in Figure 4b (about 2 h 5 min). All of our HSQC data sets, however, contained more acquisitions than necessary for generating useful spectra. We collected some useful HSQC data sets in <8 h (not shown), which is almost twice the time used to acquire the fast-HMQC data set. Although the resolution and signal-to-noise ratio were noticeably lower in these HSQC spectra in comparison to those shown in Figures 3a and 4a, correlations for **I** and **II** were still discernable above background noise. We typically collected all HSQC data sets overnight, leaving working hours available for sample preparation, one-dimensional data collection, and data interpretation. An ideal approach for OPCW proficiency tests, therefore, might be to use fast-HMQC experiments during working hours to rapidly screen samples for target analytes before using overnight HSQC acquisitions to collect high quality data sets.

4. CONCLUSIONS

We have demonstrated the feasibility of executing two-dimensional 1H - ^{13}C heteronuclear single quantum correlation (HSQC) and fast- heteronuclear multiple quantum correlation (HMQC) techniques with a sensitivity-enhanced cryogenic probehead to characterize Chemical Weapons Convention (CWC)-related analytes at trace concentrations in complex mixtures. The techniques eliminated nearly all of the signal overlap often encountered in conventional 1H spectroscopy, and allowed the simultaneous extraction of both 1H and ^{13}C chemical shift information, much of which was not possible using one-dimensional techniques alone. While the fast-HMQC pulse sequences generated magnitude mode spectra suitable for detailed analysis in ~4.5 h, the HSQC pulse sequences required roughly twice the data acquisition time to produce suitable spectra. These spectra, however, were phase sensitive and therefore contained considerably more resolution in both the 1H and ^{13}C dimensions. Multiplicity-edited HSQC experiments provided additional structural information valuable in the identification of target analytes. The extraordinary sensitivity and selectivity afforded by these techniques considerably improves the performance of NMR spectroscopy as a complementary technique for the identification and confirmation of small molecule analytes present in environmental samples. Our approach of using fast-HMQC experiments for screening samples

for potential target analytes and HSQC experiments for acquiring high quality data sets can be easily adapted to various applications to analyze complex mixtures, especially when conventional one-dimensional NMR methods fail to produce spectra with well-resolved analyte signals.

LITERATURE CITED

1. *Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and their Destruction*, signed in Paris in January, 1993. Printed and distributed by the Provisional Technical Secretariat for the Preparatory Commission for the Organization for the Prohibition of Chemical Weapons. Depositary of the Convention is the Secretary-General of the United Nations.
2. Koskela, H.; Grigoriu, N.; Vanninen, P. Screening and Identification of Organophosphorus Compounds Related to the Chemical Weapons Convention with 1D and 2D NMR Spectroscopy. *Anal. Chem.* **2006**, *78*, 3715-3722.
3. Meier, U.C. Application of Nonselective 1D ^1H - ^{31}P Inverse NMR Spectroscopy to the Screening of Solutions for the Presence of Organophosphorus Compounds Related to the Chemical Weapons Convention. *Anal. Chem.* **2004**, *76*, 392-398.
4. Albaret, C.; Lœillet, D.; Augé, P.; Fortier, P-L. Application of Two-Dimensional ^1H - ^{31}P Inverse NMR Spectroscopy to the Detection of Trace Amounts of Organophosphorus Compounds Related to the Chemical Weapons Convention. *Anal. Chem.* **1997**, *69*, 2694-2700.
5. Mesiälaakso, M.; Tolppa, E.-L. Detection of Trace Amounts of Chemical Warfare Agents and Related Compounds in Rubber, Paint, and Soil by ^1H and $^{31}\text{P}\{^1\text{H}\}$ NMR Spectroscopy. *Anal. Chem.* **1996**, *68*, 2313-2318.
6. *Recommended Operating Procedures for Sampling and Analysis in the Verification of Chemical Disarmament*; The Ministry of Foreign Affairs of Finland: Helsinki, 1994.
7. Bayer, E.; Albert, K.; Nieder, M.; Grom, E. On-Line Coupling of High-Performance Liquid Chromatography and Nuclear Magnetic Resonance. *J. Chromatogr.* **1979**, *186*, 497-507.
8. Bodenhausen, G.; Ruben, D.J. Natural Abundance Nitrogen-15 NMR by Enhanced Heteronuclear Spectroscopy. *Chem. Phys. Lett.* **1980**, *69*, 185-189.
9. Schleucher, J.; Schwendinger, M.; Sattler, M.; Schmidt, P.; Schedletsky, O.; Glaser, S.J.; Sørensen, O.W.; Griesinger, C. A General Enhancement Scheme in Heteronuclear NMR Employing Pulsed Field Gradients. *J. Biomol. NMR* **1994**, *4*, 301-306.
10. Kay, L.E.; Keifer, P.; Saarinen, T. Pure Absorption Gradient Enhanced Heteronuclear Single Quantum Correlation Spectroscopy with Improved Sensitivity. *J. Am. Chem. Soc.* **1992**, *114*, 10663-10665.

11. Palmer, A.G., III; Cavanaugh, J.; Wright, P.E.; Rance, M. Sensitivity Improvement in Proton-Detected Two-Dimensional Heteronuclear Correlation NMR Spectroscopy. *J. Magn. Reson.* **1991**, *93*, 151-170.
12. Müller, L. Sensitivity Enhanced Detection of Weak Nuclei Using Heteronuclear Multiple Quantum Coherence. *J. Am. Chem. Soc.* **1979**, *101*, 4481-4484.
13. Ross, A.; Salzmann, M.; Senn, H. Fast-HMQC Using Ernst Angle Pulses: An Efficient Tool for Screening of Ligand Binding to Target Proteins. *J. Biomol. NMR* **1997**, *10*, 389-396.
14. Ernst, R.R. *Advances in Magnetic Resonance*; Vol. 2; Waugh J.S., Ed.; Academic Press: New York, 1966.
15. Keeler, J.; Clowes, R.T.; Davis, A.L.; Laue, E.D. Pulsed-Field Gradients: Theory and Practice. *Methods Enzymol.* **1994**, *239*, 145-207.
16. Davis, A.L.; Laue, E.D.; Keeler, J. Absorption-Mode Two-Dimensional NMR Spectra Recorded Using Pulsed-Field Gradients. *J. Magn. Reson.* **1991**, *94*, 637-644.
17. Bax, A.; DeJong, P.G.; Mehlkopf, A.F.; Smidt, J. Separation of Different Orders of NMR Multiple-Quantum Transitions by the Use of Pulsed Field Gradients. *Chem. Phys. Lett.* **1980**, *69*, 567-570.
18. Davis, A.L.; Keeler, J.; Laue, E.D.; Moskau, D. Experiments for Recording Pure-Adsorption Heteronuclear Correlation Spectra Using Pulsed Field Gradients. *J. Magn. Reson.* **1992**, *98*, 207-216.
19. Willker, W.; Leibfritz, D.; Kerssebaum, R.; Bermel, W. Gradient Selection in Inverse Heteronuclear Correlation Spectroscopy. *Magn. Reson. Chem.* **1993**, *31*, 287-292.
20. Olejniczak, E.T.; Eaton, H.L. Extrapolation of Time Domain Data with Linear Prediction Increases Resolution and Sensitivity. *J. Magn. Reson.* **1990**, *87*, 628-632.
21. Zeng, Y.; Tang, J.; Bush, C.A.; Norris, J.R. Enhanced Spectral Resolution in 2D NMR Signal Analysis Using Linear Prediction Extrapolation and Apodization. *J. Magn. Reson.* **1989**, *83*, 473-483.
22. Henderson, T.J. Sensitivity-Enhanced Quantitative ^{13}C NMR Spectroscopy via Cancellation of $^1J_{\text{CH}}$ Dependence in DEPT Polarization Transfers. *J. Am. Chem. Soc.* **2004**, *126*, 3682-3683.
23. Bax, A.; Giffey, R.H.; Hawkins, B.L. Sensitivity-Enhanced Correlation of Nitrogen-15 and Proton Chemical Shifts in Natural Abundance Samples via Multiple Quantum Coherence. *J. Am. Chem. Soc.* **1983**, *105*, 7188-7190.

24. Koskela, H.; Rapinoja, M.-J.; Kuitunen, M.-J.; Vannien, P. Determination of Trace Amounts of Chemical Warfare Agent Degradation Products in Decontamination Solutions with NMR Spectroscopy. *Anal. Chem.* **2007**, 79, 9098-9106.