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Quantitative NMR Spectroscopy Using Coaxial Inserts Containing a Reference Standard: Purity Determinations for Military Nerve Agents

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A novel ^{31}P NMR method for the determination of purity for the military nerve agents sarin, soman, and VX has been developed. In contrast to more conventional quantitative NMR methods, stem coaxial inserts are placed into the sample tube to introduce reference material into the analysis without mixing or reaction with the analyte. All sample preparation is eliminated, and the analysis is completed expeditiously in less than 25 min. The method is highly specific and rugged with respect to operator-induced variability, experimental parameters, and all influences from nuclear magnetic relaxation. Nerve agent purity can be determined with a precision and accuracy typically better than 1%, and impurities can be detected at concentrations as low as 25 μg/mL. The limit of quantitation has been estimated at 85 μg/mL. In terms of precision, accuracy and execution time, the method rivals typical chromatographic methods.

Nuclear magnetic resonance (NMR) spectroscopy is perhaps the most important and powerful technique in chemical analysis and research today. It provides the most detailed and unambiguous information on chemical structure, conformation, dynamics, and function. Although more expensive and less sensitive than typical chromatographic techniques, such as gas chromatography/mass spectometry, NMR spectroscopy is less involved, because it does not require extensive sample preparation and generates results that are easily interpreted. NMR spectroscopy is an accurate quantitative technique when conducted under appropriate conditions; this potential was demonstrated more than 20 years ago.[1−3] Past reports have typically exploited proton[4−7] and ^{13}C NMR spectroscopy;[8−11] however, examples using ^{31}P[12−15] and other nuclei[16−22] are found in the literature. In addition, the accuracy and precision of NMR signal intensity measurements have recently been reviewed.[23] Even more recently, Maniara et al.[24] reported a systematic validation of the quantitative NMR method that demonstrated that it can compete effectively with typical chromatographic methods. Their validation illustrated that when carefully implemented, the purity of major chemical components can be determined with accuracy and precision better than 1%, and impurities comprising 0.1% or less of the sample mass can be quantified. With these results in mind, I started to develop internal standard protocols for the quantitative NMR analysis of chemical warfare agents.

The program for Chemical Agent Standard Analytical Reference Material (CASARM) provides reference material for the monitoring and detection of and analysis for U.S. military chemical agents. At present, the CASARM program provides five military−unique chemical agents at 95% purity or greater. These are either the nerve agents sarin (military agent designation GB), soman (GD), and VX and the vesicants mustard (HD) and Lewisite (L). As would be expected, one of themost critical aspects of the CASARM Program is the certification of agent purity for each lot of reference material.

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A ^{31}P NMR method for determining the purity of CASARM lots of sarin, soman, and VX is presented herein. The method is unique in that it uses stem coaxial inserts to introduce reference material into the analysis without mixing with the nerve agent. This procedure prohibits any reaction between the reference material and either the nerve agent or its impurities, eliminating a potential source of error by leaving the agent in its authentic, unadulterated form during analysis. Further, the requirement of accurately determining the masses of the reference and test materials is eliminated, as is mixing these to homogeneity, thus eliminating additional sources of error. The use of stem coaxial inserts in quantitative NMR spectroscopy is quick and easy to perform. As a result of this approach, the nerve agent and reference material cannot occupy the same volume within the sample tube, almost always a requirement for quantitative NMR spectroscopy. To account for this consequence, the ratio of the insert volume to that of the NMR sample tube, as measured by the spectrometer receiver coils, was accurately determined to serve as a correction factor. When using this correction factor, values for nerve agent purity were found to be exceptionally precise and accurate.

EXPERIMENTAL SECTION

Chemicals, Test and Reference Materials, Solvents, and Supplies. The CASARM Committee, U.S. Army Soldier and Biological Chemical Command (Aberdeen Proving Ground, MD) supplied sarin, soman, and VX for use as test materials (**Warning:** Sarin, soman, and VX are very potent chemical warfare agents and must be handled in a closed system or a fume hood with a minimum air flow velocity of 100 ft/min. The U. S. Army recommends 10% sodium hydroxide to neutralize sarin and soman and 6.8% calcium hypochlorite for VX.). Triphenyl phosphate certified at 99.9% purity was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). Triethyl phosphate purchased from Aldrich (Milwaukee, WI) was used as a quantitative reference standard as well as a ^{31}P chemical shift reference. Methylphosphonic acid and deuterated solvents with deuterium isotope enrichments of 98% or greater were also purchased from Aldrich. O-Isopropyl methylphosphonic acid (~99% pure) was synthesized by and purchased from Radian International (Austin, TX). All 5-mm NMR sample tubes (Product no. 507-PP-7) and stem coaxial inserts (Product no. WGS-5BL) were purchased from Wilmad Glass (Buena, NJ).

Instrumentation and NMR Spectroscopy. ^{31}P NMR spectroscopy was conducted using a Bruker Instruments (Billerica, MA) Avance DRX-500 spectrometer operating at 202.46 MHz (11.75 T) or Avance DRX-300 spectrometer operating at 121.49 MHz (7.06 T), both with XWIN NMR version 2.5 software. For these analyses, the DRX-500 spectrometer was fitted with a 5-mm triple-resonance TXI probehead (inverse-detection configuration) with dedicated proton, ^{13}C, and ^{31}P channels. The DRX-300 spectrometer had a dedicated double-resonance 5-mm QNP probehead (inverse-broadband configuration). A Mettler-Toledo, GmbH (Greifensee, Switzerland) model AG-245 electronic analytical balance with an accuracy of 0.01 mg was used for mass determinations.

For quantitative ^{31}P NMR spectroscopy, data sets of 65 536 complex points were recorded onto computer disk from the summation of 16 acquisitions. Data acquisitions were collected with 77 ppm spectral windows and $\pi/2$ pulse widths of either 13.8 (Avance DRX-500) or 7.4 μs (Avance DRX-300). To minimize off-resonance effects, data were acquired with the spectrometer carrier frequency centered between the major component and reference compound signals. Samples were held at 25 ± 0.2 or 32 ± 0.2 °C during acquisition while spinning at 20 Hz. Inverse-gated proton decoupling (decoupling only during data acquisition) with a low-power composite pulse sequence[25] was used to obtain proton-decoupled ^{31}P data sets without signal enhancements from any nuclear Overhauser effect (NOE). Preacquisition delays of 84 s were used at both operating frequencies to ensure complete relaxation of all of the signals.

Recorded free induction decay signals were multiplied by an exponential window function using a line-broadening factor of 5 Hz before Fourier transformation into spectra and manual phase-correction into pure absorption mode; digital resolution was 0.48 Hz. For aqueous samples, chemical shifts were referenced to external 85% phosphoric acid or triethyl phosphate at −0.73[26] or 0.13 ppm, respectively. All other chemical shifts were referenced to 85% phosphoric acid at 0.00 ppm. ^{31}P signal intensities were determined by electronic integration of expanded regions around the signals. Regions of integration were chosen to include any spinning sideband and ^{13}C satellite signals originating from the major signal.

Stem Coaxial Inserts and Experimental Concept. Relative to the more typical uses of NMR spectroscopy, data acquisitions for quantitative NMR experiments must be collected under very specific conditions. The sample to be evaluated must be a homogeneous, single-phase system, and when reference material is added to a test substance, the resulting solution must be mixed to homogeneity. This condition ensures that all components of the test sample occupy the same volume within the NMR sample tube. A second condition is that the major component, all analytes to be quantitated, and any added reference compound each have at least one completely resolved signal in the spectrum. And finally, the intensity for each of these signals must be an absolute representation of the total number of atomic nuclei giving rise to them. This is a direct consequence of the recovery, or relaxation, of the NMR signals. Following a data acquisition, NMR signal intensity does not reappear instantaneously, but does so in an exponential manner over some interval of time. This interval is usually in the range of milliseconds to tens of seconds for liquids at room temperature. To further complicate matters for quantitative NMR spectroscopy, this time interval is usually different for each signal in a spectrum. Enough time must be allowed between successive data acquisitions for the intensity of each signal to pass completely through its exponential recovery to equilibrium.

Because NMR signal intensities represent the total number of their respective nuclei when acquired under quantitative conditions, they also represent the concentration of the corresponding component contained within the NMR sample tube. The concentration of any test substance component C_T can be found directly from the mass of reference compound added to the sample m_R, its formula weight FW_R, and the volume of test substance added to the NMR sample tube V_T simply by measuring the signal intensity of the reference compound I_R and that of the test

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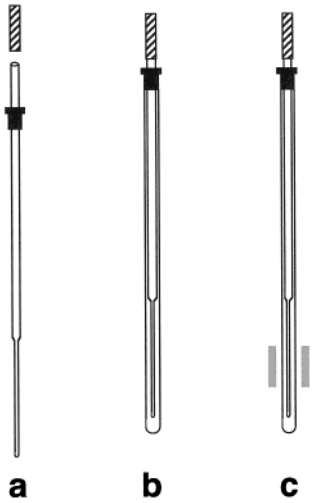

Figure 1. Schematic illustrations of a stem coaxial insert with its plastic cap (striped lines) and NMR sample tube adapter (solid black) in panel a, the stem coaxial insert−NMR sample tube assembly in panel b, and a representation of the assembly with respect to the probehead receiver coils (solid gray) when placed into the cryostat bore shown in panel c.

substance component I_T. This is expressed by the following:

$$C_T = \left[\frac{m_R}{(FW_R V_T)}\right]\left(\frac{I_T}{I_R}\right) \quad (1)$$

Note that I_T/I_R is a ratio and, therefore, is dimensionless. In this example of conventional quantitative NMR spectroscopy, values for m_R and V_T must be measured, generally introducing a small but noticeable error into the value of C_T.

The quantitative method detailed herein is novel because of its use of stem coaxial inserts to separate the test and reference substances. Roughly the size of NMR sample tubes, the inserts are glass cylinders that rapidly taper down to a much smaller diameter for about one-fifth of their total length, and their end is closed (see Figure 1a). They are designed to be placed into a NMR sample tube and hold the smaller diameter end near the bottom of the tube (Figure 1b). Once in the spectrometer probehead, only the smaller-diameter portion of the insert resides within the receiver coils (Figure 1c). By using these inserts, materials such as reference compounds or deuterated lock solvents can be introduced into the NMR analysis without mixing or reacting with sample components. For quantitative NMR spectroscopy, a stem coaxial insert containing the reference compound is placed into a NMR sample tube containing the test substance. This assembly prevents the test and reference materials from mixing and occurring as a homogeneous solution, or in other words, the specific condition that these materials occupy the same volume is violated. This however, can be accounted for if the volumes occupied by both the test and reference materials, as measured by the spectrometer receiver coils,[27] are accurately established. These are the only relevant volumes for this particular method; the volumes of liquid added to the inserts and sample tubes are therefore, not important. A second caveat of the insert-tube assembly is that C_T cannot be calculated from m_R, because this, too, requires the reference and test substances to exist as a homogeneous liquid. This can be completely circumvented by determining the reference material concentration from its density d_R rather than m_R. By introducing V_R/V_T to correct for the different volumes of the reference and test substances measured by the receiver coils, eq 1 can be recast to describe the case of quantitative NMR spectroscopy using stem coaxial inserts. The equation adopts the following form

$$C_T = \left(\frac{d_R}{FW_R}\right)\left(\frac{I_T}{I_R}\right)\left(\frac{V_R}{V_T}\right) \quad (2)$$

where V_R and V_T are, respectively, the volumes of the coaxial insert and NMR sample tube as measured by the probehead receiver coils. As with I_T/I_R, V_R/V_T is also dimensionless. The equation has no dependence on either m_R or V_T, and any error associated with their measurement is eliminated from the derived value of C_T. Clearly, this is an advantage not possible in conventional quantitative NMR spectroscopy. For the evaluation of test substance purity P, the density of the test substance d_T can be incorporated into eq 2 with its formula mass FW_T to give the following:

$$P(\text{wt\%}) = \left(\frac{d_R}{FW_R}\right)\left(\frac{I_T}{I_R}\right)\left(\frac{V_R}{V_T}\right)\left(\frac{FW_T}{d_T}\right)100\% \quad (3)$$

The expression FW_T/d_T describes the concentration for the test substance, assuming a purity of 100%, and the remaining right-hand side of the equation, the actual measured concentration of this substance.

Because both V_R and V_T are a function of the probehead receiver coils, values for V_R/V_T can only be determined experimentally. In addition, stem coaxial inserts are not manufactured with absolute tolerances, as are NMR sample tubes, and values for V_R/V_T should be measured for each insert to be used for quantitation. In developing this method, these measurements were conducted with two aqueous solutions of methylphosphonic acid, each of identical methylphosphonic acid concentration but very different hydrogen ion concentrations. This is germane, because the phosphorus signal of all phosphonic acids occurs at very different chemical shifts when originating from a protonated or unprotonated molecule (See ref 28 for a detailed discussion of ^{31}P chemical shifts as a function of hydrogen ion concentration.). Protonation and deprotonation occur on a time scale much faster than the NMR time scale, and therefore, the observed ^{31}P chemical shift is an average of both forms of phosphonic acid present in solution. The more the protonated form predominates in solution for instance, the more the chemical shift of the phosphonic acid ^{31}P signal moves toward higher frequency. The amount of the phosphonic acid in a stem coaxial insert and its NMR sample tube can be directly measured by quantitative NMR spectroscopy

(27) The receiver coil not only detects nuclei physically confined to the volume of the sample tube and coaxial insert that it encompasses, but also a small but significant volume of each occurring immediately above and below the coil.

(28) Gorenstein, D. G. In *Phosphorus-31 NMR Principles and Applications*; Gorenstein, D. G., Ed.; Academic Press: New York, 1984; Chapter 1, pp 7−36.

exploiting this chemical shift phenomenon. For example, placing a coaxial insert containing one of the methylphosphonic acid solutions into a NMR sample tube containing the other will give a ^{31}P spectrum of only two signals, and when acquired under quantitative conditions, the ratio of their integrals will directly reflect the ratio of methylphosphonic acid in the insert relative to the sample tube. Because the two solutions have identical methylphosphonic acid concentrations, the NMR signal intensity ratio will also represent the volume of the insert as detected by the NMR experiment relative to that for the NMR sample tube, or in other words, the value of V_R/V_T.

Precision and Accuracy. As is typically the case for analytical methods, precision and accuracy were determined from replicated measurements. Method precision was evaluated from seven replicated measurements of triphenyl phosphate concentration for a dilute solution in xylene, as well as seven replicated measurements of VX purity. The determined precision is expressed as the percent relative standard deviation (RSD). Experimental accuracy was also evaluated from the seven replicated measurements of triphenyl phosphate concentration. For this method, accuracy, A, is defined as

$$A = \left[p^2 + \left(1 - \frac{n_{NMR}}{n_{IS}}\right)^2\right]^{1/2} \quad (4)$$

where p is the method precision, n_{NMR} the purity derived by NMR spectroscopy and n_{IS} the true purity from an independent source. Note that the quantity $1 - n_{NMR}/n_{IS}$ is the experimental bias.

Specificity, Limits of Detection, and Limits of Quantitation. The specificity of the method was established for the sarin, soman, and VX test materials by demonstrating the absence of interference among each of their respective signals and those of sample impurities and the triethyl phosphate reference. Limits of detection and quantitation are not applicable to a major component method; however, both are addressed in a general discussion of the ability of this method to detect and quantitate low-concentration impurities (see the Results and Discussion section).

Ruggedness. Several aspects of the method were specifically evaluated for ruggedness. The positioning of the coaxial insert−NMR sample tube assembly within the spinner turbine was varied to determine its effects on spectrometer performance. Similar evaluations with incompletely filled NMR sample tubes and stem coaxial inserts were also conducted. Data acquisition was investigated by varying the number of acquisitions constituting a data set, the length of preacquisition delays between successive acquisitions, temperature regulation, and magnetic field strength.

System Suitability. The suitability of the NMR spectrometers for this method was demonstrated by periodically measuring the spectral line shape (signal symmetry) and routinely measuring spectrometer sensitivity (signal-to-noise ratio) using a sample of 48.5 mM triphenyl phosphate in acetone.

RESULTS AND DISCUSSION

In addition to the usual evaluation of precision and accuracy, the measurement of stem coaxial insert volume was a significant component of this method development. Details of all three are discussed accordingly and include all experimental data listed in table form. Specificity, ruggedness, and both the limits of detection and quantitation for the method are addressed in more general terms.

Volume Measurements of Stem Coaxial Inserts. Values of V_R/V_T were evaluated using two methylphosphonic acid solutions. Although nearly identical in methylphosphonic acid concentration, they were vastly different in hydrogen ion concentration and, therefore, observed ^{31}P chemical shift. To prepare methylphosphonic acid solutions with concentrations as close as possible, 100 mL of 0.10 g/mL methylphosphonic acid in water was prepared as a stock solution. Into one 25-mL aliquot, 10 mL of deuterium oxide and ca. 4 mL of 25% sodium hydroxide was added before the entire volume was diluted with water to 50 mL and mixed to homogeneity. The pH of this solution was 12.9, constituting a basic evaluation solution. With the exception of the sodium hydroxide addition, a second 25-mL aliquot of the stock solution was prepared in an identical manner and found to have a pH of 1.1, constituting an acidic evaluation solution. The acidic and basic solutions had ^{31}P methylphosphonic acid signals observed at 30.5 and 21.0 ppm, respectively.

To compare the methylphosphonic acid concentrations of the two solutions, they were analyzed by quantitative ^{31}P NMR spectroscopy against triethyl phosphate placed into a stem coaxial insert. Approximately 300 μL of each solution was separately placed into a NMR sample tube before placing the insert into the tube. Seven replicate quantitative NMR data sets were collected at 202.46 MHz and 32 °C, and the ratio of signal intensity for the methylphosphonic acid ^{31}P signal to that of triethyl phosphate was calculated. A single coaxial insert and NMR sample tube set was used for all of the replicates of both solutions. The mean ratio for the signal intensity of the methylphosphonic acid signal for the acidic solution to that of triethyl phosphate was 0.7685:1 (standard deviation of 0.0020:1); the corresponding ratio for the basic solution was 0.7758:1 (standard deviation of 0.0032:1). The ratios demonstrate that the basic solution was 1.01% more concentrated in methylphosphonic acid than its acidic counterpart, and this difference was used as a correction factor for all insert volume calibrations.

For each of eight inserts evaluated, two independent sets of four replicate data sets were collected by quantitative ^{31}P NMR spectroscopy, again at 202.46 MHz and 32 °C. Data for the first replicate set were collected with the acidic solution in the NMR sample tubes and the basic solution in the stem coaxial insert, and those for the second replicate set were collected with the two solutions exchanged. All data sets were processed, and for each, the signal intensities of the two methylphosphonic acid signals were determined and expressed as V_R/V_T (see eq 1). These data are compiled in Table 1, where for each coaxial insert, a mean relative volume and standard deviation are listed for both sets of four replicates (data sets I and II) along with the corresponding information for the data sets combined (combined data). Casual observation of the table reveals that the relative volumes of all eight coaxial inserts are remarkably constant; volumes derived from the combined data range from 0.1105 to only 0.1130. Standard deviations ranged between values of 0.0010 and 0.0018 for this combined data set, indicating excellent reproducibility of the measurements. Closer scrutiny suggests a bias toward slightly higher coaxial insert volumes in data set II. For each insert, the

Table 1. Mean Values for the Relative Volumes[a,b] of Stem Coaxial Inserts with Their Standard Deviations

	mean relative included volume (std dev)		
insert	data set I[c]	data set II[d]	combined data (V_R/V_T)
1	0.1125 (0.0011)	0.1135 (0.0010)	0.1130 (0.0010)
2	0.1121 (0.0016)	0.1124 (0.0012)	0.1123 (0.0013)
3	0.1096 (0.0012)	0.1114 (0.0017)	0.1105 (0.0017)
4	0.1103 (0.0008)	0.1124 (0.0004)	0.1114 (0.0013)
5	0.1123 (0.0011)	0.1134 (0.0011)	0.1128 (0.0011)
6	0.1105 (0.0007)	0.1135 (0.0008)	0.1119 (0.0018)
7	0.1114 (0.0009)	0.1127 (0.0008)	0.1121 (0.0011)
8	0.1097 (0.0003)	0.1119 (0.0009)	0.1108 (0.0013)

[a] Included volumes of stem coaxial inserts relative to that of the NMR sample tube (given a value of 1.0000) as measured by the spectrometer receiver coils. [b] Expressions are ratios and, therefore, are dimensionless. [c] Volumes measured by placing the acidic methylphosphonic acid solution in the NMR sample tubes and the basic solution in the stem coaxial inserts. [d] Volumes measured by placing the basic methylphosphonic acid solution in the NMR sample tubes and the acidic solution in the stem coaxial inserts.

Table 2. Triphenyl Phosphate Concentrations[a] Determined by Quantitative ^{31}P NMR Spectroscopy Using Stem Coaxial Inserts Containing a Reference Standard[b]

	replicate	insert	determined concn (mg/mL)
		Data Set I[c]	
	1	1	25.88
	2	1	25.77
	3	1	25.58
	4	1	25.79
	5	1	26.02
	6	1	25.90
	7	1	25.99
mean			25.85
std dev			0.15
		Data Set II[d]	
	1	2	25.87
	2	3	25.61
	3	4	25.73
	4	5	26.02
	5	6	26.03
	6	7	25.72
	7	8	25.75
mean			25.82
std dev			0.16

[a] Triphenyl phosphate prepared at 26.04 mg/mL. [b] Triethyl phosphate internal standard. [c] All replicate measurements used the same stem coaxial insert. [d] Each measurement used a different stem coaxial insert.

reported volumes for data set II are approximately 1% larger than the corresponding values from data set I. Although the reason or significance for this small bias is not clear, combining the data collected from both data sets and weighting them equally (as is the case for the volumes reported for the combined data) should negate this bias. It is for this reason that the values reported for V_R/V_T were calculated from the combined data set.

Precision and Accuracy. Method accuracy was evaluated from the repeated measurement of the triphenyl phosphate concentration for 26.04 mg/mL triphenyl phosphate in xylene, using a triethyl phosphate reference contained in stem coaxial inserts. Two different sets of seven replicate measurements each, referred to as data sets I and II, were conducted by quantitative ^{31}P NMR spectroscopy at 202.46 MHz and 25 °C. All of the replicate measurements comprising data set I used the same coaxial insert, but a different insert was used for each of the replicate measurements in data set II. Triphenyl phosphate concentrations were calculated for each replicate according to eq 2 and are summarized in Table 2 by data set.

The quantitative method statistically delivers very accurate results. A bias of 0.7% was found between the mean triphenyl phosphate concentration for data set I and that calculated from its certified purity, giving a method accuracy of 0.9% (RSD of 0.6%). For data set II (RSD also 0.6%), the analogous bias and accuracy were 0.8 and 1.0%, respectively. In addition, there is very little difference between the triphenyl phosphate concentrations resulting from any single calibrated stem coaxial insert. The two data sets have mean triphenyl phosphate concentrations that are almost identical, with very small relative standard deviations, and the two most extreme concentrations reported differ by only 1.7%. This is due in large part to accurate calibration of the stem coaxial inserts. Even more to the point, when considering that the measured concentrations include all errors associated with the preparation of the triphenyl phosphate test solution (which includes drying the solid triphenyl phosphate, determining the exact mass of triphenyl phosphate used to prepare the solution and a determination of the exact volume of the solution), the method accuracy is most likely better than that indicated statistically. It is apparent that very little error attends this particular quantitative method. As previously mentioned (see the Stem Coaxial Inserts and Experimental Concept section), a large advantage for accurately determining test material component concentrations can be expected with this method, because errors associated with mass and volume determinations generally found in conventional quantitative NMR methods are eliminated. This is in agreement with Maniaria et al.,[24] who report that most variance found in quantitative NMR methods arises from sample preparation and not the accompanying NMR spectroscopy.

Method precision was further evaluated with CASARM VX against triethyl phosphate contained in stem coaxial inserts. Seven replicate data sets were collected by quantitative ^{31}P NMR spectroscopy at 161.49 MHz and 25 °C, each using a different coaxial insert. VX purity was calculated for each replicate according to eq 3 (d_T of 1.0083 g/mL at 25 °C for VX[29]) and these data are summarized in Table 3. As evaluated from this data set, method precision (RSD of 0.5%) is almost identical to that found from data sets I and II in the previous table. All data sets then demonstrate excellent reproducibility for measurements using this quantitative method. These precision values are comparable to those from chromatographic analysis. Finally, the mean value for the VX purity is entered into the table with its 95% confidence intervals.

Specificity. Accurate results from quantitative NMR spectroscopy depend on the selection of analyte and internal standard signals free from overlap with each other and any impurity signals, as well as the accurate determination of signal intensities. The inherent broad range for ^{31}P chemical shifts, especially at high magnetic field strengths, allows high spectral resolution and

(29) *Potential Military Chemical/Biological Agents and Compounds*; Army Field Manual Number 3-9; Department of the Army: Washington, DC, **1990**; Chapter 2, pp 14−51.

Table 3. Purity of CASARM VX by Quantitative ^{31}P NMR Spectroscopy Using Stem Coaxial Inserts Containing a Reference Standard[a]

replicate	insert	purity (wt %)
1	1	97.5
2	2	97.5
3	4	96.5
4	5	97.7
5	6	96.9
6	7	96.7
7	8	96.6
mean ± 95% confidence intervals		97.1 ± 1.0

[a] Triethyl phosphate internal standard

Table 4. {^{1}H}^{31}P Signals of the Test Materials and Reference Compound

compd	signal	chem shift[a] (ppm)
Sarin $(CH_3)P(O)(F)$(*O*-*i*-propyl)	doublet (J_{PF} 1037 Hz[c])	29.7
Soman $(CH_3)P(O)(F)$(*O*-pinacolyl)	doublet[d] (J_{PF} 1040 Hz[c]) doublet[d] (J_{PF} 1040 Hz[c])	28.7 29.8
VX $(CH_3)P(O)$(*O*-Et)(SCH_2CH_2N)(*i*-propyl)$_2$	singlet	53.0
triethyl phosphate (*O*-ethyl)$_3$P(O)	singlet	0.1
triphenyl phosphate (aqueous) (*O*-phenyl)$_3$P(O)	singlet	-17.6[b]

[a] Chemical shifts referenced to the signal of 85% phosphoric acid at 0.00 ppm unless otherwise noted. [b] Referenced to the signal of 85% phosphoric acid at −0.73 ppm. [c] ^{31}P−^{19}F scalar coupling constant. [d] Diastereoisomers.

method specificity. This advantage was certainly the rationale in using ^{31}P spectroscopy for this method. It was also found to hold true experimentally, because ^{31}P chemical shifts differed by about 30 ppm or more between the signal of the triethyl phosphate reference and those of the nerve agents (see Table 4). Such resolution minimizes overlapping signals, allowing accurate determinations of signal intensity. It is possible, however, for impurity signals to converge or even superimpose with the major component signal, and this problem must be addressed to ensure accurate purity determinations. Further, the inherent benefit of proton and ^{13}C spectroscopy, where the analyte and internal standard typically give rise to more than one signal to quantitate, is not generally realized in ^{31}P spectroscopy. Purity cannot be calculated from more than one analyte or internal standard signal to afford a higher degree of confidence. For these reasons, the careful treatment of converging or superimposing signals can be of great value in quantitative ^{31}P methods.

When an impurity signal overlaps with the major component signal, the intensity of the former needs to be carefully determined and subtracted from the total intensity of the converged signals. It may be possible to identify such impurity signals simply by comparing spectra of the test material acquired with and without the reference standard coaxial insert; this, however, will not be possible in many cases. Two situations exist when the signal of an impurity superimposes with that of the major component and cannot be resolved without multidimensional spectroscopy, which is more difficult to quantitate than one-dimensional spectroscopy. When the presence of the impurity is not known, its signal intensity will be included with that of the major component signal, thus biasing the purity toward a higher value. On the other hand, if the identity of the impurity has been verified and its concentration accurately determined from quantitative NMR spectroscopy using a different nucleus or another analytical technique altogether, the NMR-derived purity value may be adjusted to correct for the impurity. It may be worth noting that some recent developments in data set processing, including linear prediction with single value decomposition,[30] detection estimation,[31] filter diagonalization,[32,33] and others[34−37] have focused on deconvoluting overlapping and superimposed signals, but their use is still far from routine. Spinning sidebands, and ^{13}C satellite signals to a much lesser degree, can be similar sources of error in the intensity determination for the major component signal. Because these signals derive from the actual signal itself, they should be included in the corresponding analyte or internal standard signal intensity determination. It may become necessary, however, to neglect sideband or satellite signals when they converge with an impurity signal. The idea is to apply consistent execution for both the analyte and internal standard signals.

Limits of Detection and Quantitation. This is a major component method applied to materials that are not sample-limited, and the concept of limits of detection and quantitation are not applicable in a strict sense. These limits are for example, important in analyses for low-level components present in a technical material, analyses of formulated materials where the technical-grade active ingredient is diluted, or for the analysis of trace components in a material. Quantitative NMR spectroscopy may be applied to such systems by adjusting experimental parameters to attain a signal-to-noise ratio of at least 3:1 for detection and 10:1 for quantitation of any component in a system. Also important to these ends is the optimization of spectrometer receiver gain and magnetic field strength homogeneity together with the stringent regulation of experimental temperature. Because signal line width and height are both directly proportional to spin−spin relaxation (which is related to temperature), signal-to-noise ratios in NMR experiments are directly related to temperature. This is commonly overlooked in analytical chemistry. For this method at 202.46 MHz and 25 °C, *O*-isopropyl methylphosphonic acid (a surrogate for sarin) in water was detected at concentrations as low as 25 μg/mL (signal-to-noise ratio of 3:1), setting this concentration as the limit of detection and the limit of quantitation at roughly 85 μg/mL. Although such limits are only estimates, it must be mentioned that they can be easily improved by simply increasing the number of acquisitions

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constituting a data set.

Ruggedness. Variables potentially arising from an analyst (spectrometer operator) were investigated in terms of method ruggedness. Analyst-to-analyst variability and its impact on quantitative NMR spectroscopy have already been addressed in some detail by Maniaria et al.,[24] who reported no significant analyst influence on purity measurements. In an effort to extend this study, the actual positioning of the NMR sample tube into the spinner turbine and the incomplete filling of the sample tube or stem coaxial insert, variables which can be introduced by an analyst, have been studied in somewhat general terms (data not shown). No quantitative differences were encountered by moving the coaxial insert−NMR sample tube assembly in the spinner turbine up to 4 mm above or 12 mm below the position recommended by the spectrometer manufacturer. Just 12 mm below the recommended position, the sample tube encountered an obstruction within the probehead, and the spinner turbine was not able to seat properly within the cryostat bore. In addition, the same series of experiments was repeated with the coaxial insert and the NMR sample tube only partially filled; both were filled to a point only 3 mm below that where the smaller and larger diameter portions of the coaxial insert converge (see Figure 1). Once again, quantitative differences were not found when moving the coaxial insert−NMR sample tube assembly in the spinner turbine over this same range. It should be reiterated that the analyst-induced variability investigated here was not stringently evaluated. Further, these observations may be spectrometer-dependent. Even with these caveats in mind, the quantitative method is exceptionally rugged with respect to analyst variability.

Influences specifically from spin lattice relaxation and $\{^1H\}^{31}P$ NOE enhancements on purity values arise when preacquisition delays between successive data acquisitions are not long enough to allow complete signal relaxation. For this particular method, an 84-s preacquisition delay was used, because it exceeds the spin lattice relaxation time of the triethyl phosphate reference more than 10-fold. This holds true at both magnetic field strengths used to develop this method and theoretically is expected to hold true at all intermediate field strengths, as well. Molecules such as nerve agents which contain a methyl group directly bonded to a phosphorus atom typically are observed to have phosphorus spin lattice relaxation times significantly shorter than those, such as triethyl phosphate, which do not. This was the case for all experimental evidence in this method development. Although a preacquisition delay five times the spin lattice relaxation time will allow essentially complete signal recovery from single quantum relaxation pathways, it may not necessarily allow recovery through zero- or double-quantum pathways, a direct consequence of $\{^1H\}^{31}P$ NOE enhancements. Far and away, the largest contribution to complete NOE relaxation stems from the use of proton decoupling only during data acquisition, precluding any chance of generating a steady-state NOE. The 84-s delay may be substantially longer than necessary, but it was incorporated into the method specifically to enhance ruggedness. Experiment confirms this claim; at both field strengths, no detectable differences were found by using a preacquisition delay of 40 s, (less than one-half the full preacquisition delay specified). On the other hand, temperature regulation is very important for NMR spectroscopy in general, because all nuclear magnetic relaxation is intimately related to changes in temperature. In particular for this method, acquisition temperature is expected to be very important, because purity is calculated from density values for 25 °C. The VX purity calculated from one replicate in Table 3 changed from 96.7% when using temperature regulation to 98.3% without it, even though the room temperature, 26.9 °C, was very close to the specified 25.0 °C. When considering that the densities for both VX[38] and triethyl phosphate[39] change about 1×10^{-3} g/mL for each 1 °C change in temperature, such error can be expected. Using this value, a 2.2% error is calculated for determining VX purity at 26.9 °C, giving a final value of 98.9% purity. Finally, the number of data acquisitions constituting a data set was varied to examine the effects of derived purity values. Data sets consisting of only four acquisitions (just one complete phase cycle) were found to have no significant change in the derived purity values when compared to corresponding data sets of the full 16 acquisitions, even though the former had noticeably poorer signal-to-noise ratios. In terms of experimental parameters and the investigated magnetic field strengths, therefore, the developed method is exceptionally rugged.

CONCLUSIONS

A quantitative ^{31}P NMR analytical method using stem coaxial inserts to introduce a reference standard into the analysis has been developed and found to be both suitable and valid for use in purity determinations of military nerve agents. The inserts allow the direct analysis of the authentic, unadulterated nerve agent while preserving the accuracy attained from the use of a reference standard. The method is highly specific, and exceptionally rugged with respect to operator-induced variability, experimental parameters, and influences from nuclear magnetic relaxation. In addition, signal overlap from process-related impurities or residual solvents, although potentially present in all analytical methods, is circumvented to a large degree as a result of the inherently high resolution and spectral width of ^{31}P NMR spectroscopy, especially at high magnetic field strengths. The method requires no sample preparation, only a readily available reference standard. The measurement of mass or volume for the reference material commonly found in quantitative NMR methods is eliminated, as is the associated error. The method precision and accuracy are on the order of 0.5 and 1.0%, respectively, which rivals results from modern chromatographic techniques. The many reports on quantitative NMR spectroscopy appearing in the literature over the years, some of which are referenced herein,[1−24] support the applicability of NMR spectroscopy for quantitative chemical analysis. Of special note are the United States Pharmacopoeia[40] and the British Pharmacopoeia,[41] where the general quantitative NMR methodology is compendial.

Quantitative NMR spectroscopy is extensively used by the U.S. Army Soldier and Biological Chemical Command for purity certification of military chemical agents to be used as standard analytical reference material. This certification is of extreme

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importance, mainly because the material is required throughout the United States for instrument calibrations used in the monitoring, detection, and analysis of military chemical agents. In addition, many costly and complex research programs are dependent upon the proper characterization of these reference materials. They have been typically used in metabolic, toxicology, and ecotoxicology studies, warfare agent decontamination evaluations, the development of warfare agent destruction chemistry, and its associated pilot plant development. Careful and accurate characterization of these military nerve agents as analytical standards, therefore, is essential.

The purity of analytical standards has traditionally been determined by combining a number of analytical techniques, and these most often include gas or liquid chromatography. Because chromatographic detector responses vary widely from compound to compound, chromatographic techniques cannot directly measure the absolute purity of a compound. High purity reference standards, therefore, are required for every chemical compound to be evaluated. The preparation of analytical reference standards is often time-consuming and expensive. This is completely eliminated with this quantitative NMR method, and in addition, only a single reference compound is usually all that is required to determine the purity of many different analytical standards.

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