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Method Performance and Validation for Quantitative Analysis by ^{1}H and ^{31}P NMR Spectroscopy. Applications to Analytical Standards and Agricultural Chemicals

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Nuclear magnetic resonance (NMR) can be used to provide an independent and intrinsically reliable determination of chemical purity. Unlike chromatography, it is possible to employ a universal reference standard as an internal standard for the majority of chemical products assayed by quantitative NMR (QNMR). This is possible because the NMR response can be made the same for all chemical components, including the internal standard, by optimizing certain instrumental parameters. Experiments were performed to validate the quantitative NMR method described in this paper for the analysis of organic chemicals. Experimental precision, accuracy, specificity, linearity, limits of detection and quantitation, and ruggedness were systematically addressed, and system suitability criteria were established. The level of the major chemical ingredient can be determined with accuracy and precision significantly better than 1%, and impurities may be quantified at the 0.1% level or below. Thus, QNMR rivals chromatography in sensitivity, speed, precision, and accuracy, while avoiding the need for a reference standard for each analyte. Examples are given of ^{1}H and ^{31}P NMR used for quantitative analysis of agricultural chemicals, and a method for characterization of analytical standards is presented.

Nuclear magnetic resonance (NMR) is perhaps the most important, powerful, and widely used form of spectroscopy in both academic and industrial chemical research. It provides the most detailed and unambiguous information on chemical structure, functionality, and dynamics, rivaled only by X-ray diffraction in power but not in speed. However, after more than thirty years of development, NMR remains a largely qualitative technique in the majority of chemical research laboratories. The potential of NMR spectroscopy for the quantitative analysis of organic chemicals was first demonstrated two decades ago.[1−3] Although many original papers and review articles on this subject appeared, the approach has yet to gain widespread acceptance. Analytical chemists and NMR spectroscopists still remain skeptical that quantitative NMR (QNMR) can compete effectively with chromatographic methods due to the perception that NMR measurements are not sufficiently reproducible.

The early publications dealt primarily with ^{13}C NMR spectroscopy as a quantitative tool and addressed different aspects of optimizing conditions for data acquisition and subsequent processing to obtain reliable quantitative results. Recent applications of ^{13}C NMR spectroscopy include polymer chemistry,[4−7] clinical chemistry,[8] and food chemistry studies.[9−13] Several articles dealing with lignin chemistry using quantitative ^{13}C NMR analysis have appeared,[14−18] as well as one on magic angle spinning ^{13}C NMR analysis of crystal polymorphs of an organic chemical in a complex solid matrix.[19]

Reported applications of ^{31}P QNMR include analysis of phospholipids mixtures[20−22] and determination of inorganic phosphorus[23] and organophosphorus compounds.[2,3,24] Quantitative ^{31}P

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NMR has also become an indispensable tool for the lignin chemist.[25−31]

Proton QNMR has been applied to determine "octane number" in gasoline,[32,33] active ingredients in pharmaceutical formulations,[34,35] analysis of organic osmolytes and their metabolites in crude plant tissue extracts,[36] determination of intracellular pH,[37] and in polymer chemistry to determine acrylic unsaturation in ultraviolet light-curable resins.[38] Quantitative ^{1}H NMR has been used to characterize colon tumors,[39] microbial extracts,[40] polyethylene,[41] poly(ethylene glycol)s,[42] starches,[43] peroxidation of lipoproteins,[44] characterization of block copolymers,[45] and quantitation of brain water and cerebral metabolites.[46] Recently, a method for quantitative determination of compounds in continuous-flow LC NMR has been reported.[47] Although ^{1}H, ^{13}C, and ^{31}P QNMR methods are the most frequently applied, QNMR methods employing other nuclei have also been reported.[48−55]

Many of the earlier QNMR measurements showed relative standard deviations as high as 5%, which is unsatisfactory for most analytical purposes, and many did not include an error analysis. The accuracy and precision of NMR intensity measurements has been reviewed in a recent book.[56] In the present work, it is shown that carefully implemented QNMR can compete effectively with chromatographic methods. The level of the major chemical ingredient can be determined with accuracy and precision significantly better than 1%. Impurities at the 0.1% or lower may also be quantified.

In the present paper, we report (for the first time, to the best of our knowledge) a systematic validation of the QNMR method, performed in a manner consistent with the Good Laboratory Practice guidelines of the United States and international government regulatory agencies.[57] The importance of accurate characterization of analytical standards has recently increased in the eyes of these agencies. In the United States, the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA) now require more rigorous characterization of reference standards, together with periodic reanalysis to confirm stability under the conditions of storage. QNMR is of great value in filling this need. This has stimulated the present work where the experimental precision, accuracy, specificity, linearity, limits of detection and quantitation, and ruggedness of QNMR were systematically investigated, and system suitability criteria were defined.

EXPERIMENTAL SECTION

Validation Test Materials. The imidazolinone herbicide imazamox (nicotinic acid, 2-(4-isopropyl-5-oxo-2-imidazolin-2-yl)-5-(methoxymethyl-)) and the soil insecticide terbufos (*S*-[[(1,1-dimethylethyl)thio]methyl]-*O*,*O*-diethylphosphorodithioate) of chemical purity of 99.4 and 93.8%, respectively, are reference standards of American Cyanamid Co. (Princeton, NJ). Benzoic acid was purchased from the National Institute of Standards and Technology (NIST) (Washington, D.C.). NIST certifies this material to be 99.9958% by titration and, as such, is used by American Cyanamid Co. as a primary standard with certified purity of 99.9%. (Tris(hydroxymethyl)aminomethane) (TRIS) was purchased from Aldrich Chemical Co. (Milwaukee, WI). It is a reference standard certified by American Cyanamid with a purity of 99.9%. Malathion (*S*-(1,2-dicarbethoxyethyl)-*O*,*O*-dimethyldithiophosphate), known purity of 98%, and nicotine (1-methyl-2-(3-pyridyl)pyrrolidine), known purity of 99%, were purchased from Chem Service, Inc. (West Chester, PA).

QNMR Internal Reference Materials. Maleic acid of certified purity 99.8% was purchased from Sigma Chemical Co. (St. Louis, MO). Triphenyl phosphate, purity 99.9%, was purchased from NIST.

NMR Chemical Shift Reference Standards and Deuterated Solvents. Three chemical shift reference standards, tetramethylsilane (TMS), from Aldrich Chemical Co., sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) from Merck & Co. for proton, and triethyl phosphate (TEP) from Eastman Kodak Co. for phosphorus NMR experiments were used. Deuterated solvents such as DMSO-d_6, D_2O, acetone-d_6, CD_3CN, $CDCl_3$, and C_6D_6 with

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deuterium isotope enrichments of 99% or better were purchased from different commercial sources including Merck Sharp & Dohme, CIL, and Aldrich.

Instrumentation. Proton NMR experiments were carried out using Bruker AM 500 and AMX 300 NMR spectrometers operating at 500.13 and 299.88 MHz, respectively. Phosphorus NMR experiments were performed on a Bruker AMX 300 NMR spectrometer only, operating at 121.39 MHz. The AMX 500 instrument was equipped with a dedicated 5-mm proton probe, and the AMX 300 with a 5-mm multinuclear "QNP" probe. A Mettler model AT-20 electronic balance with accuracy approaching 1 μg was used for sample and internal standard (IS) weighing.

Proton NMR spectra were recorded at concentrations of 5−100 mM in appropriate solvents using 5-mm NMR tubes. The typical acquisition parameters for proton NMR experiments were as follows: acquisition time 2.72 s, repetition rate 60 s; spectral width 4000−6000 Hz, nutation angle 30°, 16K or 32K data points, and temperature 296 K. The 90° pulse widths were 8.8 μs (^{1}H-dedicated probe, AM 500 spectrometer) and 15.3 and 5.0 μs (QNP probe, AMX 300 spectrometer) for ^{1}H and ^{31}P spectroscopy, respectively. The digital resolution was ∼0.4 Hz.

For phosphorus-31 QNMR, sample concentrations of 5−200 mM in deuterated benzene were used. To observe all components in the sample, a full spectrum was recorded with 128−256 scans using a 200 ppm spectral width, 16K data points, 0.72-s acquisition time, a relaxation delay of 1 s, and a 30° pulse width. The digital resolution was ∼1.4 Hz.

For quantitative NMR, two spectra covering spectral windows of ∼2000 Hz are acquired in separate experiments. In the first experiment, the resonance of the internal standard is offset by −500 Hz from the carrier frequency and the free induction decay (FID) is acquired. In the second experiment, the resonance of the analyte is offset by +500 Hz from the carrier frequency and a second FID is acquired. All other parameters are kept exactly the same in these experiments. The FIDs from the two experiments are then coadded prior to Fourier transformation. Other parameters for this two-step experiment are as follows. Typically, 64−128 transients are collected using 8K data points with spectral width of 2100.84 Hz, a flip angle of 30°, and an acquisition time of 7.8 s. The digital resolution was ∼0.1 Hz. Areas of the peaks were determined by electronic integration of expanded regions around diagnostic resonances. Inverse-gated decoupling was employed using a low-power composite pulse sequence to obtain ^{1}H-decoupled NMR spectra of X-nuclei without signal enhancement by nuclear Overhauser effects (NOE). Prior to Fourier transformation, the FIDs were multiplied by an exponential window function with a line broadening of 0.3 and 1 Hz in ^{1}H and ^{31}P NMR experiments, respectively.

Procedures. The experimental procedure for quantitative NMR analyses is substantially different from the conventional use of NMR. For a major component analysis the minimum experiment comprises 12 replicate quantitative measurements, from which a mean percent purity and its standard deviation are obtained, as described in the Statistical Design section. Data processing included phase correction, which was performed manually for each replicate, and baseline correction over the entire spectral range. In some instances, the baseline was additionally corrected over the integrated regions. The regions of integration were chosen to include the ^{13}C satellites and spinning sidebands, unless stated otherwise. The amount of the sample and IS are adjusted so that approximately equal intensities are obtained in the spectrum. The molecular weight of the analyte, the number of resonating nuclei, and their splitting pattern are taken into account in estimating the weights required. The compatibility of the chosen IS and the analyte was verified prior to the quantitative analysis.

Statistical Design. The precision study involves replicated QNMR measurements of four test substances by proton NMR spectroscopy, and two test substances by phosphorus NMR spectroscopy (see the Experimental Section). Each test substance was considered as having a separate experimental design and statistical analysis. For substances analyzed by proton spectroscopy, two analysts performed measurements on each of two spectrometers using three sample weighings with two NMR signal acquisitions on each weight. Each FID was subsequently processed twice. In the statistical analysis, *analysts* and *spectrometers* are crossed factors, with *weighings* nested within *analyst-spectrometer* combinations, *acquisitions* nested within *weighings*, and *processing* nested within *acquisitions*. Thus, the model for the statistical analysis of each test substance is

$$\mathbf{Y_{ijklm}} = \boldsymbol{\mu} + \boldsymbol{\alpha_i} + \boldsymbol{\beta_j} + \boldsymbol{\gamma_{ij}} + \boldsymbol{\bar{\omega}_{k(ij)}} + \boldsymbol{\delta_{l(ijk)}} + \boldsymbol{\epsilon_{m(ijkl)}}$$

where, $\mathbf{Y_{ijklm}}$ is the **m**th *processing* of *data acquisition* **l** of *weighing* **k** conducted by *analyst* **i** on the **j**th *spectrometer*, $\boldsymbol{\mu}$ is the overall mean, $\boldsymbol{\alpha_i}$ is the fixed effect of *analyst* **i**, $\boldsymbol{\beta_j}$ is the fixed effect of *spectrometer* **j**, $\boldsymbol{\gamma_i}$ is the fixed effect of the interaction between the *analyst* **i** and *spectrometer* **j**, $\boldsymbol{\bar{\omega}_{k(ij)}}$ is the random effect of *weighing* **k** conducted by *analyst* **i** on *spectrometer* **j**, $\sim$N (0, σ_w^2) (Note: N (0, σ_w^2) is conventional statistical shorthand for "Normally distributed, mean of zero, and variance components σ^2". $\boldsymbol{\delta_{l(ijk)}}$ is the random effect of *data acquisition* **l** of *weighing* **k** conducted by *analyst* **i** on *spectrometer* **j**, $\sim N(0,\sigma_d^2)$, and $\boldsymbol{\epsilon_{m(ijkl)}}$ is the random effect of *processing* **m** of *data acquisition* **l** of *weighing* **k** conducted by *analyst* **i** on *spectrometer* **j**, $\sim N$ $(0,\sigma_p^2)$.

For phosphorus spectroscopy, two analysts performed assays on a single spectrometer using three weighings each, two data acquisitions per weighing and two data processing per acquisition. *Weighings* are nested within *analysts*, *acquisitions* nested within *weighings*, and *processing* nested within *acquisitions*. The model for the statistical analysis of each test substance is analogous to the one designed for proton test substances, except for the fixed effect of *spectrometer* **j** ($\boldsymbol{\beta_j}$) and the fixed effect of the interaction between the *analyst* **i** and *spectrometer* **j** ($\boldsymbol{\gamma_I}$).

Accuracy. Experimental accuracy is defined as

$$\text{accuracy} = (p^2 + b^2)^{1/2}$$

where p is precision (expressed as relative standard deviation, RSD) and b is the bias (difference between NMR value and the true or "accepted" value from an independent source). The accuracy of the method was established separately for proton and for phosphorus quantitative NMR. For proton NMR spectroscopy, a sample of benzoic acid of known purity 99.9958 ± 0.0027% was assayed vs maleic acid as an IS. For phosphorus NMR spectroscopy, a sample of terbufos insecticide, of known purity 93.8 ±

0.3% was assayed vs triphenyl phosphate as an IS. The total number of 48 individual measurements for proton and 24 for phosphorus were generated as described in the Procedures section and analyzed statistically.

Linearity. The linearity of the method was tested in two experiments by determining the relationship between NMR detector response and sample concentration. In the first experiment, the sample solution was prepared and the signal intensity was measured vs a range of receiver gain (signal amplification factor) settings and, in the second experiment, by measuring the response from serial dilutions of original sample stock solution. The method was tested for linearity separately for proton and phosphorus QNMR. For proton NMR spectroscopy, a sample of imazamox was chosen for illustrative purposes. Seven "linearity solutions" were prepared by serial dilutions of the stock solution (100 mM) and assayed by QNMR. The NMR detector response was tested for linearity by regression analysis of the response data. For phosphorus NMR spectroscopy, a sample of malathion was chosen for illustrative purposes. Five linearity solutions were prepared by serial dilutions of the stock solution (200 mM) and assayed.

Specificity. The specificity of the method was established for each test substance by demonstrating the lack of interference from the internal standard and the solvent. In situations where applicable, the sources of interference from minor components (impurities) and their treatment were documented.

Limits of Detection and Quantitation. The limits of detection and quantitation (LOD and LOQ, respectively) are not applicable to major component methods. However, LOD and LOQ are addressed in a discussion of the ability of the quantitative NMR method to detect minor components that may interfere with the detection of the major component. Moreover, the estimation of the LOD and LOQ permit the quantitative NMR method to be applied to formulated products in which the technical-grade active ingredient is diluted with formulation excipient (inert) materials. The LOD and LOQ were established separately for proton and phosphorus quantitative NMR. Imazamox and malathion were used to determine these limits for proton and phosphorus NMR, respectively.

Ruggedness. The ruggedness of the method was tested by varying the number of scans and the relaxation delay for imazamox and then, once these parameters were optimized, the operator-to-operator and instrument-to-instrument variabilities were tested.

System Suitability. The suitability of the analytical system used for the QNMR method was demonstrated by measuring periodically for each instrument the NMR line shape (peak symmetry) and sensitivity (signal-to-noise ratio, S/N), using 10% chloroform in acetone-d_6 and 0.1% ethylbenzene in chloroform-d_1, respectively, for ^{1}H NMR spectra and 0.0485 M triphenyl phosphate in acetone-d_6 for ^{31}P NMR spectra.

RESULTS AND DISCUSSION

The strength of the NMR signal is proportional to the number of nuclei (molecules) present, i.e., the concentration of the compound in a solution, provided that relaxation effects are taken into account. In quantitative NMR analysis, a universal reference standard can be used for the analysis of most materials. This is possible because the NMR response can be made the same for all components. In this report, data are presented to establish the performance characteristics of the quantitative NMR method expressed as precision, accuracy, linearity, specificity, limits of detection and quantitation, ruggedness, and system suitability.

Table 1. Summary of Proton QNMR Data[a]

sample no.	sample id	data				statistics	
		anal.	inst	rep	assay	variance	SD
1	imazamox	1	1	12	99.4654	0.075 76	0.275 24
1	imazamox	1	2	12	99.4493	0.025 67	0.160 21
1	imazamox	2	1	12	99.8459	0.143 48	0.378 78
1	imazamox	2	2	12	99.3960	0.690 76	0.831 12
3	benzoic acid	1	1	12	99.9030	0.037 68	0.194 11
3	benzoic acid	1	2	12	99.9493	0.002 05	0.045 27
3	benzoic acid	2	1	12	99.9884	0.241 42	0.491 34
3	benzoic acid	2	2	12	99.7439	0.093 64	0.306 00
4	TRIS	1	1	12	99.9278	0.007 90	0.088 88
4	TRIS	1	2	12	99.9258	0.012 79	0.113 10
4	TRIS	2	1	12	99.8053	0.194 62	0.441 16
4	TRIS	2	2	12	99.6598	0.109 47	0.330 86
6	nicotine	1	1	12	93.7099	0.147 92	0.384 60
6	nicotine	1	2	12	94.5948	0.651 68	0.807 27
6	nicotine	2	1	12	92.9314	0.272 08	0.521 61
6	nicotine	2	2	12	93.5447	0.466 84	0.683 26

[a] Anal., analyst; inst, instrument; rep, number of replications; assay, purity value; SD, standard deviation.

The results from quantitative analysis for proton test substances are summarized in Table 1. The overall standard deviation (SD) ranged from 0.045 27 to 0.831 12 for the tested substances, indicating excellent reproducibility of the measurements.

Precision. The analysis of variance (ANOVA) method was used to determine the statistical significance of the fixed effects: *analyst*, *spectrometer*, and *analyst-spectrometer* interaction effects by the statistical *F*-test using sample preparation as the error term. These tests were conducted at the 0.05 significance level, and the significant effects were taken to indicate the bias. For the random effects, sample preparation was tested against *acquisitions* and *acquisitions* against *processing* using the *F*-test, again at the 0.05 significance level. The variance components due to sample preparation, σ_w^2, *acquisitions* σ_d^2, and *processing*, σ_p^2, were estimated by the ANOVA method, and any negative estimates were set to zero (by statistical convention). The RSD values, with 47 degrees of freedom, ranged from 0.20 to 0.33.

In Table 2, the components of variance are listed for proton test substances where the significance of each nested factor is given.

For a significance level $\alpha = 0.05$, the variance component is not statistically significant if its *p*-value is equal to or greater than 0.05. The data from the precision study showed that the variance component was significant for sample preparation (weighing, solvent, long-term effects, etc.) for all four proton test substances. In addition, the data show that the variance component was statistically significant for *analyst* only in the case of nicotine. Although the above effects are statistically significant, they are numerically extremely small. The other fixed effects of *analyst* and instrument, and their crossed factors, for the remaining three test substances were not statistically significant (see Table 2).

The results from quantitative analysis for phosphorus test substances are summarized in Table 3. The overall SD ranged

Table 2. Components of Variance for Proton QNMR

sample	component[a]	variance	SD	*p*-value	significant ($p > 0.05$)
imazamox	anal.	fixed		0.4992	no
	inst	fixed		0.3430	no
	anal.*inst	fixed		0.3755	no
	prep(anal.*inst)	0.105 77	0.325 22	0.0453	yes
	ACQ(anal.*inst*prep)	0.056 03	0.236 71		
	proc	0.106 06	0.325 67		
	overall	0.267 86	0.517 55		
benzoic acid	anal.	fixed		0.6919	no
	inst	fixed		0.5161	no
	anal.*inst	fixed		0.3483	no
	prep(anal.*inst)	0.043 11	0.207 63	0.0391	yes
	ACQ(anal.*inst*prep)	0.018 99	0.137 80		
	proc	0.045 08	0.212 32		
	overall	0.107 18	0.327 38		
TRIS	anal.	fixed		0.2371	no
	inst	fixed		0.6405	no
	anal.*inst	fixed		0.6495	no
	prep(anal.*inst)	0.060 82	0.246 62	0.0008	yes
	ACQ(anal.*inst*prep)	0.000 00	0.000 00		
	proc	0.039 49	0.198 72		
	overall	0.100 31	0.316 72		
nicotine	anal.	fixed		0.0298	yes
	inst	fixed		0.0626	no
	anal.*inst	fixed		0.7053	no
	prep(anal.*inst)	0.278 93	0.528 14	0.0106	yes
	ACQ(anal.*inst*prep)	0.131 13	0.362 12		
	proc	0.062 56	0.250 12		
	overall	0.472 62	0.687 47		

[a] Anal., analyst; inst, instrument; prep, sample preparation (weighing, solvent, long-term effects, etc.), ACQ, data acquisition; proc, data processing.

Table 3. Summary of Phosphorus-31 QNMR Data[a]

	anal.	rep	assay	variance	SD
terbufos	1	12	93.9941	0.095 34	0.308 76
terbufos	2	12	94.2668	1.171 20	1.082 22
malathion	1	12	99.6410	0.037 08	0.192 56
malathion	2	12	99.5838	0.438 22	0.661 98

[a] Anal., analyst; rep, number of replicates; assay, assay value; SD, standard deviation.

Table 4. Components of Variance for Phosphorus-31 QNMR

	component[a]	variance	SD	*p*-value	significant ($p > 0.05$)
terbufos	anal.	fixed		0.2992	no
	prep(anal.)	0.059 77	0.244 48	0.0711	no
	ACQ(anal.*prep)	0.231 85	0.481 51	0.1125	no
	proc	0.379 02	0.615 65	0.3729	no
	overall	0.670 64	0.818 93		
malathion	anal.	fixed		0.7460	no
	prep(anal.)	0.000 00	0.000 00	0.5723	no
	ACQ(anal.*prep)	0.122 04	0.349 34	0.0965	no
	proc	0.178 98	0.423 06	0.8548	no
	overall	0.301 02	0.548 65		

[a] See footnote *a* in Table 2.

from 0.192 56 to 1.082 22 for the tested substances, indicating good reproducibility of the measurements.

Analysis of variance was used to determine the statistical significance of the *analyst* effect only by the *F*-test using *weighings* as the error term. The RSD values, with 23 degrees of freedom were 0.65 and 0.42 for terbufos and malathion, respectively. This indicates good precision for these test substances.

The components of variance listed in Table 4 for ^{31}P QNMR test substances showed that none of them was statistically significant since in all cases (including fixed and random effects) the *p*-value was greater than 0.05.

The pooled RSD was calculated to be 0.25 ($n = 192$) and 0.53 ($n = 48$) for the proton and phosphorus-31 QNMR test substances, respectively. These precision values are comparable to those obtained by chromatographic analysis.

Accuracy. The accuracy of the method was calculated as indicated in the Statistical Design section. For the proton QNMR test substance benzoic acid, the purity was 99.9% NMR vs 99.9958% by titration. The QNMR precision was 0.21% ($n = 48$), the bias between the two methods 0.0958%, and the accuracy of the QNMR method was 0.2. Similarly, for the ^{31}P QNMR test substance, terbufos, the purity was determined as 94.1% by QNMR and 93.8% by gas chromatography. The precision of the phosphorus QNMR measurement was 0.65% ($n = 24$), the bias between the two methods was 0.3%, and the accuracy of the QNMR method was 0.7. An independent purity value is not always available from an outside supplier, or it may be of questionable quality, for example, as in the case of the nicotine test substance (see below).

Another very important criterion of accuracy is self-consistency among all integrated signals within a molecule. Since the relative ratio of the integration for the blocks of signals is proportional to the number of protons resonating in the block, this proportionality not only constitutes a very good criterion of integration accuracy but also provides information on the potential presence of impurities in cases where proportionality is not preserved.

Linearity. The relationship between the amount of imazamox (^{1}H QNMR) and malathion (^{31}P QNMR) and the NMR detector

Table 5. Chemical Shift Assignments for the Test Substances and Standards

test substance	functional group	chemical shift[a] (ppm)
imazamox herbicide (nicotinic acid, 2-(4-isopropyl-5-oxo-2-imidazolin-2-yl)-5-(methoxymethyl-))	isopropyl methyls	two singlets, 0.80 and 0.98
	imidazolinone methyls	
	isopropyl CH	singlet, 1.23
	methyl of methoxy	multiplet at ∼1.91
	methylene of methoxy methyl	singlet, 3.36
		singlet ∼4.60
	pyridine hydrogens	doublet at ∼8.15 and 8.74
benzoic acid	phenyl hydrogens	doublet (C-2/C-6), ∼7.94
		multiplet (C-4), ∼7.60
		doublet (C-3/C-5), ∼7.49
nicotine (1-methyl-2-(3-pyridyl)pyrrolidine)	pyridine resonances	
	C-2	singlet, 8.51 vs TMS
	C-6	doublet, 8.46
	C-4	multiplet, 7.72
	C-5	multiplet, 7.35
	pyrrolidine resonances	multiplet, 3.17
		multiplet, 3.11
		multiplet, 2.25
		multiplet, 2.17
		singlet, 2.07
		multiplet, 1.85
		multiplet, 1.78
		multiplet, 1.61
TRIS (tris(hydroxymethyl)aminomethane)	three CH_2 groups	singlet, 3.73 (vs TSP)
Terbufos insecticide (*S*-[[(1,1-dimethylethyl)thio]methyl] *O,O*-diethylphosphorodithioate)	^{31}P	singlet, 93.00
malathion insecticide (*S*-[1,2-dicarbethoxyethyl]-*O,O*-dimethyldithiophosphate)	^{31}P	singlet, 95.72
maleic acid (internal standard)	vinyl protons	Singlet, 6.25
triphenyl phosphate (internal standard)	^{31}P	singlet, −17.06 vs TEP

[a] All proton NMR signals were measured on the δ-scale with reference to TMS or TSP at 0 ppm, and the ^{31}P spectra were referenced to TEP at 0 ppm.

response was determined experimentally. The data were tabulated and specific response (ratio of response/concentration) was calculated. The detector response and the specific response were plotted against sample concentration and coefficients of determination, r^2, were computed. In both cases, the coefficients of determination are ≥0.995 which indicates an excellent detector response linearity within the concentration range studied (see Experimental Section).

Similarly, the relationship between the receiver gain (signal amplification factors) settings, which can be viewed figuratively as "electronic sample dilutions", and signal intensities for each sample concentration has been determined separately for proton and phosphorus NMR. Again, in both cases, a linear detector response was found as indicated by coefficient of determination, r^2, calculated to be ≥0.99.

Specificity. Accurate results from quantitative NMR depend on careful selection of a resonance of the analyte that should be free from overlapping impurity resonances and precise measurement of the NMR peak area. For a higher degree of confidence, the purity based on more than one resonance of the analyte may be computed, when possible. If the resonance of an impurity (minor component) is observed to overlap with that of the analyte, the area of the impurity should be carefully integrated and its area subtracted from the total area of the resonances (which includes the contributions from the major component and the overlapping minor component). This can be done whether the minor is an unknown or a known substance. If the resonance of the minor component overlaps exactly with that of the analyte and it is not observed, then two possible situations can exist. If nothing is known about the minor, it will be included in the total area, thus biasing the purity value high. However, if the overlapping impurity has been identified and the amount is known from the another analytical technique such as HPLC, the NMR purity value may be adjusted to correct for the level of the impurity. Similarly, spinning sidebands and small peaks caused by spin−spin coupling of the protons of the main peaks with ^{13}C (the ^{13}C satellites) can be a source of an error in the integration, thus contributing to potential bias in purity determination. It is, therefore, important for the NMR spectroscopist to recognize them. On the other hand, it is pointed out that the ^{13}C satellite signals at 1.1% (natural) abundance provide an intrinsic internal standard in minor components analysis. Either the sideband/satellite intensities should be included (preferably) for both the analyte and IS in the total peak areas or else they should be neglected in both the analyte and IS. The latter may be necessary if a sideband or satellite overlaps with an impurity peak. The goal is to apply a consistent practice to both the analyte and IS. Specificity is discussed below in more detail for each test substance, and the chemical shifts assignments are given in Table 5.

Imazamox Test Material. The aromatic protons of the pyridine ring were utilized for quantitative analysis, and purity was calculated to be 99.5%.

The minor component profile for imazamox was determined independently by liquid chromatography (HPLC). The level of known organic minor components totaled 0.8% by HPLC, and the ash and water contents totaled 0.4% (0.3% ash and 0.1% water). The purity is determined by subtracting the known impurities

in imidazolinone from 100%, i.e., 100 − 1.2 = 98.8%. Examination of the proton NMR spectrum of imazamox herbicide revealed the presence of signals from its ethyl analogue (nicotinic acid, 2-(4-ethyl-5-oxo-2-imidazolin-2-yl)-5-(methoxymethyl-)). The level of the ethyl analogue was estimated to be 0.2% by NMR and 0.3% by HPLC. However, due to its low concentration and the NMR method's inability to fully resolve this minor component from the major component in the chosen integration region, the calculated QNMR purity of 99.5% includes the contribution from the ethyl analogue (and possibly other unresolved low-level impurities). Therefore, the value for the QNMR purity of the sample is corrected by 0.2% to 99.3%. The QNMR and HPLC determinations, 99.3 and 98.8%, respectively, agree to within the experimental errors of both methods.

Benzoic Acid and TRIS Test Materials. No impurity resonances were observed in both test materials. There was excellent agreement between the COAs and the QNMR results which were 99.9 (QNMR) and 99.99% (NIST COA) for benzoic acid and 99.8 (QNMR) and 99.9% (Cyanamid COA) for TRIS.

Nicotine Test Material. Several impurity resonances were detected in the proton NMR spectrum of nicotine. The Certificate of Analysis from the supplier claimed its purity to be ∼99%. Proton NMR data clearly indicate that the sample quality is much lower than claimed. In fact, the purity of this sample as determined by ^{1}H NMR is 93.7% with RSD = 0.27 (n = 48). Due to severe resonance crowding in the region of interest, the diagnostic resonances were integrated exclusive of the carbon satellites and were additionally corrected for overlapping minor components by subtracting their intensities from the total integral. Chromatography data indicate that the sample is ∼96.7% pure (GC area percent estimation) and contains at least fifteen minor components. Although there is a 3% gap between the QNMR and GC area percent determinations, both methods of analysis clearly indicate a lower purity than claimed by the supplier of this sample.

Terbufos Test Material. As many as 15 low-level minor components are detected in the ^{31}P NMR spectrum. No interfering impurities in the regions of integration for the major component and IS were observed. There was excellent agreement between the QNMR and gas chromatography purity determinations, which were 94.1 and 93.8%, respectively.

Malathion Test Material. No minor components are detected in the spectrum of this test material. There was agreement within two standard deviations between the QNMR determination and the purity given in the vendor's Certificate of Analysis which were 99.6 and 98.0%, respectively.

Limits of Detection and Quantitation. The QNMR method is primarily a major component method applied to materials that are not sample-limited, for which the concept of LOD and LOQ are not applicable. However, in chemical analysis, LOD and LOQ are normally determined only for the analysis of low-level components in a technical material, for the analysis of formulated materials where the technical-grade active ingredient is diluted with excipients, or for the analysis of trace components such as pesticide residues in soil. QNMR may be applied for the analysis of such systems by adjusting the experimental conditions to achieve a S/N ≥ 3:1 for detection or a S/N ≥ 10:1 for quantitation of a component in the system.

Table 6. LOD/LOQ Determination at the Maximum Allowable Receiver Gain (RG) for Proton QNMR

				minor component[a]	
concn (mM)	purity (wt %)	RSD (%)	S/N	LOD (mM)	LOQ (mM)
5	104.8	7.08	100	0.15	0.50
10	99.1	3.32	176	0.17	0.57
20	99.0	0.61	362	0.17	0.55
40	99.5	0.16	670	0.18	0.60
60	99.3	0.31	917	0.20	0.65
80	99.4	0.24	1182	0.20	0.68
100	99.3	0.23	1336	0.22	0.75

[a] LOD = 3C/S/N; LOQ = 10C/S/N (defined for minor components only). All concentrations above 20 mM meet the criteria of the method for the analysis of the major component.

To determine the experimental limit of quantitation for the major component, as defined above, the data from the linearity experiments were reprocessed in order to obtain the purity values. For each concentration, the NMR experiment performed at the optimum receiver gain was chosen and processed three times to obtain the mean purity value and RSD.

The ^{1}H NMR data for the imidazolinone herbicide, imazamox, are shown in Table 6.

The data in Table 6 clearly indicate that concentrations below 10 mM cannot be relied upon since the purity value at concentrations 5 and 10 mM does not fall within an acceptable standard deviation (preferably 2% RSD or less), and the S/N are below the minimum of 200:1 specified for this method. It is reasonable to conclude that only concentrations higher than ∼20 mM should be used to obtain reliable quantitative results. The LOQ for the major component is established to be ∼20 mM under the standard experimental conditions of the QNMR method.

The QNMR method gives typical S/N of ∼350:1 for ^{1}H and ∼600:1 for ^{31}P NMR, which are normally achieved for quantitation under the standard conditions specified for the method (i.e., NS = 64, RD = 60 s, and a 30° spin nutation) as practiced in our laboratory. Referring to Table 6, a 20 mM solution with a S/N of 362:1 for the major component will have an LOD of 20 × 3/362 = 0.17 mM or 0.83% for a minor component, assuming that it has the same spin multiplicity and molecular weight. Similarly, for a 100 mM solution with a S/N of 1336:1, the LOD would be 100 × 3/1336 = 0.22 mM or 0.22%. The LOQs are calculated similarly and are given in Table 6. Note that a lower LOD and LOQ may be obtained by increasing the number of scans (in NMR, the S/N is proportional to the square root of the number of scans).

Sometimes it is required to measure the concentration of a chemical at a low level in an inert matrix by QNMR. It is assumed that the matrix does not interfere with the desired NMR signal. The behavior of the QNMR experiment for such systems may be predicted from the data in Table 6 for proton QNMR. For example, since the proton NMR signal for a 20 mM solution was observed with the receiver gain (RG) set to 1, one can also observe the same "signal plus noise" level for a 0.02 mM solution (i.e., 1000 times diluted) if RG is set to 1024 (i.e., amplified ∼1000 times, since the intensity of the signal plus noise is proportional to RG). Under these conditions, the LOD at a S/N of 3:1 will be obtained when the number of scans is increased from 64 to 4400, since (362/1000)((4400/64)$^{1/2}$) = 3. Similarly the LOQ at S/N = 10

will be obtained with 49 000 scans, since (362/1000)((49000/64)$^{1/2}$) = 10. Therefore, the LOD of the ^{1}H NMR signal of this neat chemical diluted in an inert matrix is 0.02 mM under the standard NMR conditions with the appropriate increase in NS. In this way, the LOD and LOQ for actual experimental conditions may be predicted.

The discussion for phosphorus QNMR is similar to that for proton QNMR. The experimental data for ^{31}P NMR indicate that the concentrations below 25 mM cannot be used for reliable quantitative analysis since the purity values at 5 and 25 mM are not obtained with an RSD < 2.0%. It is concluded that concentrations higher than 50 mM should be used for ^{31}P NMR quantitative analysis. Therefore, the LOQ is established to be ~50 mM under the standard QNMR conditions.

Statistical Effect of Operators and Instruments (Ruggedness). To check the operator-to-operator (i.e., analyst-to-analyst) and instrument-to-instrument variabilities on the purity values obtained by the QNMR method, the precision data were analyzed by ANOVA (cf. Tables 1 and 3). In Table 1, the mean purity values for the proton NMR test samples obtained by different analysts and on different instruments are listed. The results of both analysts on both instruments are acceptable as the standard deviations are <1.0%. In Table 3, the mean purity values for the phosphorus NMR test samples obtained by different analysts but on only one instrument are listed. In the case of phosphorus NMR analysis, the results are also acceptable as the relative standard deviations are <1.1%. Therefore, it may be concluded that the purity values are not significantly dependent upon the analyst or the instrument for both ^{1}H and ^{31}P NMR analyses.

Characterization of Analytical Standards. NMR spectroscopy is extensively used in our laboratory for the certification of analytical standards. This application is of special importance since many complex and expensive research programs are dependent upon the proper characterization of such reference materials. Reference materials are used in metabolism, residue, toxicology, and ecotoxicology studies, manufacturing process development and quality control, formulations research, and analytical programs to develop data for registration of products with domestic and international government regulatory agencies. Thus, careful and accurate characterization of analytical standards is essential.

The purity of a standard is traditionally determined by combining a number of analytical techniques, including most prevalently gas or liquid chromatography. Chromatography cannot directly measure the absolute purity of a compound as chromatographic detector responses vary widely from chemical to chemical. Because of this, a high-purity reference standard is required for every chemical component of interest. The preparation of primary analytical standards is both time-consuming and expensive.

In our laboratories, primary analytical standards are characterized by QNMR, elemental analysis, and the procedure of "100% minus known impurities". The known impurities are determined by a variety of methods such as gas and liquid chromatography with various detectors including mass spectrometry, ash (residue after combustion), inductively coupled plasma spectrophotometry (for metals), Karl Fischer titration (for water content), and other methods as appropriate. Potential disagreement among methods usually is found to originate from impurities that are unresolved or undetected in one or more of the various methods applied. However, in most cases, the purity obtained by QNMR and other methods agree within normal experimental error.

CONCLUSIONS

Quantitative NMR spectroscopy was found to be suitable and valid for the characterization of analytical standards and other chemical materials used in regulated industries such as the pharmaceutical and agricultural chemicals businesses. The major potential pitfalls of the method were identified and discussed. The QNMR method is considered universal and highly specific. Nevertheless, the analyst must be aware that signal overlap from process-related impurities or residual solvents may lead to errors in quantitation. Such problems are potentially present in all analytical methods, and it is generally advisable to compare results from at least two independent analytical technologies. The numerous literature references cited herein fully support the applicability NMR for quantitative chemical analysis. Especially noteworthy are references to the United States Pharmacopoeia[58] and the British Pharmacopoeia,[59] where the general QNMR methodology is compendial. The experimental precision and accuracy of QNMR are of the order of 0.5%, which rivals results obtained via modern chromatographic techniques. The method is shown to be rugged with no significant effect of *analyst*, instrument, magnetic field strength, or experimental parameters. It requires minimal sample preparation, only a few analytical reagents, and a readily available internal standard. While the method is linear over a wide range, the analyst must ensure that the minimum acceptable sample concentration (20 mM for ^{1}H QNMR, and 50 mM for ^{31}P QNMR) is met to achieve adequate signal-to-noise ratio and experimental precision. For the characterization of analytical standards, we require the results of QNMR and other methods to agree to within a maximum range of 0.5%. Otherwise, further work is performed to resolve any discrepancies.

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