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GENERAL PAPER

A new traceability scheme for the development of international system-traceable persistent organic pollutant reference materials by quantitative nuclear magnetic resonance

Takeshi Saito · Toshihide Ihara · Masayoshi Koike · Shinichi Kinugasa · Yoshinori Fujimine · Kazutoshi Nose · Tetsuya Hirai

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Abstract Quantitative nuclear magnetic resonance (qNMR) was used for the purity determination of neat compounds of persistent organic pollutants (POPs). qNMR is a unique quantitative method that is not only traceable to the International System of Units (SI), but it also does not require a standard of its own. The purities of the POP compounds determined in this work were traceable to a single certified reference material (CRM), which is extremely attractive for reference material producers. The purities observed by qNMR were equivalent to those observed by gas chromatography with flame ionization detection (GC/FID) or a differential scanning calorimetry (DSC) combined with a thermogravimetric analyzer (TGA). The uncertainties obtained by the qNMR method were comparable to being slightly larger than those observed by DSC.

Keywords Quantitative NMR · Purity determination · Uncertainty · Traceability · POPs

Introduction

Standard solutions of certified reference materials (CRMs) are widely used in environmental applications for many purposes, such the as calibration of an apparatus, assessment of a measurement method, and assigning values to a material. So far, most of the standard solutions provided to the market are supplied as mixtures because of their utility. For observing accurate property values of each component of the mixture, like the concentration of each component in the mixture, it is extremely important to have an accurate determination of the purity of the neat compounds. To date, determination of the purity of neat compounds with an International System of Units (SI)-traceable method is difficult because of limitations of the SI-traceable analytical methods for organic compounds. It is profitable for users if the purity of the neat compound was determined with the SI-traceable method.

Nuclear magnetic resonance (NMR) spectroscopy has been used for the structural determination of organic compounds because the resonance position of the NMR signals has a relation to the functional group of molecules. This feature is unique for a quantitative tool for organic materials, unlike chromatographic techniques, as one can observe separate resonances from more than one compound at a time.

Although accurate absolute intensity measurements are difficult in quantitative nuclear magnetic resonance (qNMR), the area of a signal from an analyte can be measured with respect to the other signal originating from an internal standard (IS). In qNMR, the IS must resonate at a different chemical shift compare to the resonances of the target analyte. This feature makes qNMR extremely attractive for purity determinations; in other words, the requirement of the IS of a qNMR experiment must have a different structure compare to the analyte. Therefore, this makes qNMR possible to need only one standard, or a universal standard [1], for the entire set of determinations.

Uncertainties associated to qNMR have been deeply discussed, such as the offset effect of a pulse, analog to digital conversion of data, the signal-to-noise ratio, and relaxation delay [2, 3], and a combined standard uncertainty associated with qNMR was also proposed [4, 5]. The

T. Saito (✉) · T. Ihara · M. Koike · S. Kinugasa
National Metrology Institute of Japan,
AIST, Tsukuba, Japan
e-mail: takeshi.saito@aist.go.jp

Y. Fujimine · K. Nose · T. Hirai
Otuka Pharmaceutical Co., Ltd., Tokushima, Japan

linearity of NMR signal intensities, robustness, and validation of the experiment were also discussed [6]. Although, nonetheless, no previous papers provided detailed discussion of the signal intensities of individual resonances, only a few papers mentioned the traceability of qNMR [7, 8].

In this paper, we demonstrate a new approach to establish the SI traceability of neat compounds using qNMR. The validity and accuracy of the purity analysis, the uncertainties associated to the analysis and its traceability are described.

Experimental

Materials

The persistent organic pollutant (POP) samples analyzed in this work were obtained from Cambridge Isotope Laboratories, Inc. (CIL, Andover, MA, USA); the list of compounds are summarized in Table 1. A certified reference material (CRM) used in this work was NIST SRM 350a, benzoic acid, whose purity and uncertainty are (99.9958 ± 0.0027) (mass fraction, 95% confidence interval with 11 degrees of freedom). ERM-AC110a, *p*,*p*′-DDE, whose purity and uncertainty are (99.6 ± 0.4) (mass fraction, 95% confidence interval), was used to check the validation of the qNMR experiment setup. Dimethyl sulfone (DMSO2, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as an IS when SRM 350a was not feasible to use. The NMR solvents used were CD_3CN (Acros Organics, Geel, Belgium), CD_2Cl_2 (Cambridge Isotope Laboratories, Inc., Andover, MA, USA), and acetone-d_6 (Wako), depending on the conditions.

NMR sample preparation

All sample weightings were performed with a Mettler Toledo XP56 Microbalance. An IS and an assay sample of about 3 mg were accurately weighted separately on differential scanning calorimetry (DSC) pans. Three analytical samples were prepared for each purity determination. These were put into a vial. Then, about 1 mL of a NMR solvent was added to the vial, it was shaken well to dissolve both the standard and the assay sample completely. Finally, about 0.8 mL of the solution was transferred to a 5-mm o.d. NMR tube (PS-005, Shigemi Co., Ltd., Tokyo, Japan).

qNMR measurements

All NMR measurements were carried out on a Varian ^{UNITY}INOVA 600A (14.1-T) spectrometer operating at 599.90 MHz equipped a pulsed-field gradient (PFG) unit and a carousel autosampler. The probe used was a Varian's ^{15}N–$^{31}P/^{1}H$–^{19}F PFG dual-broadband probe. The temperature was set at 25 °C within ±0.1 °C. The chemical shift of all spectra was referenced to residual ^{1}H signals in deuterated solvents at 1.95 ppm for CD_3CN, 5.32 ppm for CD_2Cl_2, and 2.03 ppm for acetone-d_6.

A typical parameter set for the qNMR experiments are as follows; 59 970.02 Hz (about 100 ppm) spectral width, $\pi/2$ pulse (11.1 μs), 4 s acquisition time, 60 s relaxation delay, and 32 transients were averaged for obtaining each spectrum. During the acquisition period, the ^{13}C signal was decoupled using the WURST decoupling method [9]. A steady-state pulse was applied before the relaxation delay. Four dummy scans were acquired before the data corrections, no sample spinning was applied. The receiver gain was automatically set by the instrument. Each experiment needed about 40 min to complete. Three qNMR runs were performed with the reproducibility condition. Therefore, a total of nine qNMR experiments were run for one sample, which required a total of about 6 h of qNMR runs for one sample.

All NMR spectra were processed with the ACD/SpecManager software. No window function was applied to a free induction decay signal prior to Fourier transformation

Table 1 Persistent organic pollutant (POP) samples analyzed in this work. The purities of Cambridge Isotope Laboratories' (CIL) test results and their method of analysis that were obtained from the CIL's certificate of analysis are also shown

Sample	Information from certification of analysis	
	Purity (%)	Method
Hexachlorobenzene	100.0	GC/MS
Aldrin	98.8	GC/FID
Dieldrin	98.6	GC/FID
Endrin	99.0, >99.9	GC/FID, HPLC
p,*p*′-DDT	99.4	GC/FID
p,*p*′-DDE	99.7	GC/FID
p,*p*′-DDD	99.6	GC/FID
o,*p*′-DDT	99.7	GC/FID
o,*p*′-DDE	99.6, 99.7	GC/FID, HPLC
o,*p*′-DDD	99.4	GC/FID
Trans-chlordane	99.6	GC/FID
Cis-chlordane	99.5, 99.7	GC/FID, HPLC
Trans-nonachlor	99.5, 99.7	GC/FID, HPLC
Cis-nonachlor	99.7	GC/FID
Oxychlordane	99.7	GC/FID
Heptachlor	99.5	GC/FID
Cis-heptachlor epoxide	98.0	GC/MS
Mirex	99.6	GC/FID
α-HCH	99.1	GC/FID
β-HCH	99.3, 99.1	GC/ECD, GC/MS
γ-HCH	99.9	GC/FID
δ-HCH	99.5	GC/FID

(FT). Baseline correction was applied as in the following procedure. Set 50 points as a threshold for the horizontal spectrum regions that do not contain signals in digital points and a noise factor of 2 was chosen for the vertical threshold. Then, the baseline is constructed by averaging the spectrum curve over these regions. Peak integrations were set to an identical range for each spectrum.

Purity determination using qNMR

In an NMR method, the intensity of a given NMR signal after the relaxation delay of T_r is expressed as:

$$\frac{M_z(T_r/T_1)}{M_0} = \frac{1 - e^{-T_r/T_1}}{1 - e^{-T_r/T_1}\cos\beta} \equiv M_t \quad (1)$$

where M_z and M_0 are the steady-state and equilibrium z-magnetizations, respectively, T_1 is a spin-lattice relaxation time for the nucleus, and β is the excitation pulse angle [10]. For the qNMR condition, a long T_r is used for the experiment; the signal intensity M_t for all signals becomes unity. Therefore, when such a condition is chosen, the area intensity of an NMR peak is proportional to the number of the nuclei that represents the peak. The area of the qNMR signal is proportional to the number of equivalent ^{1}H nuclei, N, and the concentration of the solution, C, and the excitation pulse angle:

$$S = NCM_t \sin\beta = N\frac{m}{VM}pM_t \sin\beta \quad (2)$$

where S is the area of the peak, m is the mass of sample weighted, M is the molecular weight, V is the volume of the sample solution, and p is the purity for the sample.

Suppose a mixture of two samples resonates at different chemical shifts. One can obtain signals from a spectrum independently. Under this condition, the purity of an analyte can be calculated from:

$$p_a = \frac{S_a}{S_s}\frac{N_s}{N_a}\frac{M_a}{M_s}\frac{m_s}{m_x}p_s \quad (3)$$

where a, x and s represent the analyte, assay sample and the IS, respectively.

Results and discussion

Selection of qNMR parameters

A typical qNMR spectra observed in this work, shown Fig. 1, is about ten times wider than a typical spectral window for a ^{1}H NMR spectrum. There are two reasons for using such a wide spectral window. The first reason is to make sure all signals of interest fall into a flat response area of an analog filter [3], without suffering from a folding effect of noise outside the region of interest.

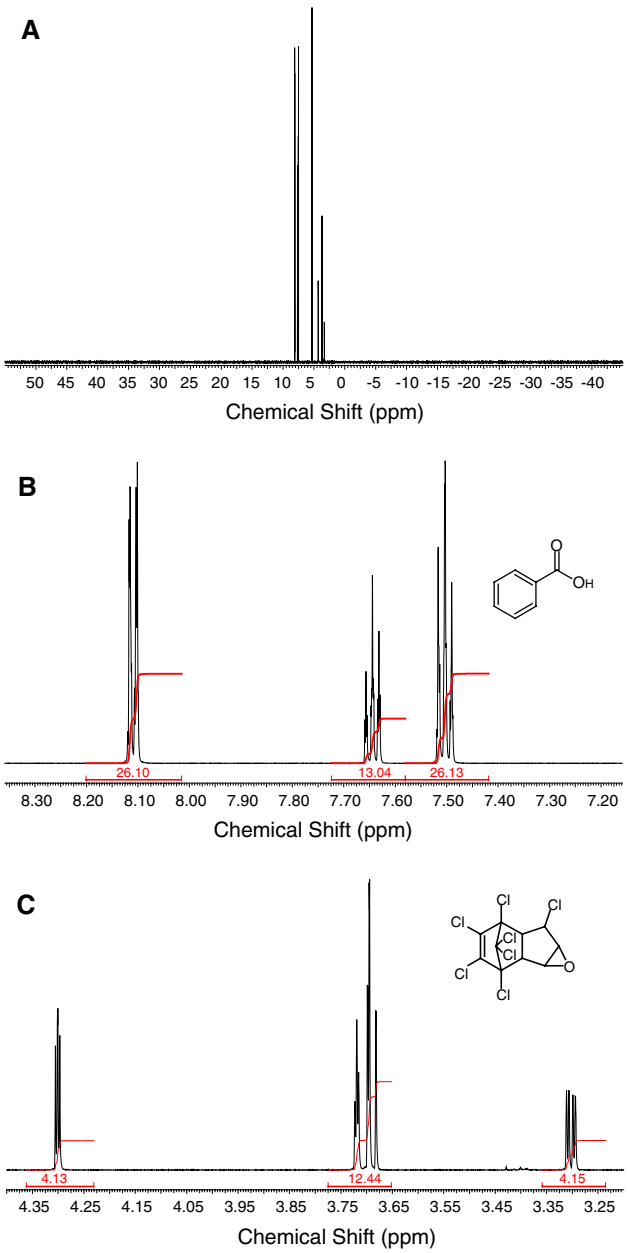

Fig. 1a–c ^{1}H NMR spectrum of a mixture of *cis*-heptachlor epoxide and SRM 350a. **a** The full spectrum observed in this work. **b**, **c** Expansion of the spectrum regions of the internal standard (IS), benzoic acid, and the analyte, and their structures, respectively. In **b** and **c**, the integration curves are also shown. Note that, since ^{13}C is decoupled during the acquisition, no satellite peaks in both sides of the main peaks in **b** and **c** exist. Small resonances of around 3.4 ppm in **c** is the signal originating from the impurity of the assay sample

The second reason relates to the dynamic range of a spectrum. The sampling rate of an NMR signal needs at least twice the frequency of the highest frequency. When oversampling n times faster than the sampling rate required, the dynamic range of the resulting spectrum would experience a gain of $\log_2 n$ [11]. Recent NMR spectrometers combine this technique with digital signal

processing (DSP) to improve the dynamic range of the spectrum. However, a free induction decay signal after the DSP can often have problems during FT with a third-party NMR data processor, so we instead acquired oversampled data and processed them as they were.

A $\pi/2$ pulse was used because minimizing the offset effect of the pulse applied to the sample, as well as obtaining the maximum intensity of the signal. For making a spectrum simple, ^{13}C decoupling was applied during the acquisition period [12]. The relaxation delay was set long enough so as to meet a targeted precision of the data.

It is important to select an IS whose resonance signals do not overlap with those originating from an analyte. Residual ^{1}H resonances from a solvent have to be considered as well.

For obtaining an accurate and precise result, the signal-to-noise ratio of the resulting spectrum is important. Thus, proper settings for the sample concentration and the number of transients is important. However, when the sample is too concentrated, the NMR cannot set the receiver gain properly. This may result in a poor-quality spectrum. With these considerations, Table 2 summarizes typical experimental data obtained from this setting. Note that the relative standard deviations of the individual peak areas for the three runs are about 0.5% at most, which indicates that the stability of the NMR signal and reproducibility condition was good. Additionally, since the qNMR method uses a ratio of the areas obtained from the analyte and the IS, the long-term stability can be canceled.

For assuring a traceability of the analyte, it is good practice to use a CRM as an IS whose purity and uncertainty is stated in the certificate. However, there is no good CRM available for such an application. As a result, qNMR often uses an IS whose purity and uncertainty are not clear. One way to overcome this traceability problem is to set a standard quantified by a CRM whose resonances do not overlap with the analyte. This makes a cumbersome step for the purity determination of an IS by a CRM; it is worthwhile to clear the traceability of the analyte. In this study, DMSO2 was chosen as the IS [1] when SRM 350a was not applicable for analytes.

Validation of the experiment

The validity was checked using the purities of ERM-AC110a. The purity of ERM-AC110a was analyzed by qNMR with DMSO2 as the IS. The purity of the DMSO2 was also determined by qNMR with SRM 350a as the IS. This is an identical approach for the determination of some of the POP compounds whose IS was DMSO2. The purity of the ERM-AC110a obtained by the two independent qNMR steps are equivalent to the certified value; furthermore, the ERM-AC110a is one of the POP compounds determined in this work; the qNMR method was a validated method for the system.

Uncertainty estimation

According to Eq. 3, the following uncertainty sources are estimated; the rectangular distributions were assumed for the mole fractions of ^{1}H nuclei [13] and molecular masses [14, 15]. The uncertainty associated to the balance and the purity of the IS is taken from the certificates.

It is not straightforward to estimate uncertainties associated to sample preparations and qNMR experiments. Analysis of variance (ANOVA) was introduced to separate uncertainty sources associated to analytical sample preparations and the reproducibility of qNMR measurements [16], thus, Eq. 3 became:

$$p_a = \frac{N_s}{N_a}\frac{M_a}{M_s}p_s g(S_s,\ S_a,\ m_s,\ m_x) \tag{4}$$

The function g was assumed to be as follows:

$$g_{ij} = \mu + \alpha_i + \varepsilon_{ij} \tag{5}$$

where μ is an overall average, and α and ε represent the expectation values for the sample preparations and qNMR experiments, respectively. Table 3 summarizes the ANOVA table for the analysis in a mixture of *cis*-heptachlor epoxide and SRM 350a. As shown, no significant difference between the sample preparation and qNMR measurements was found; when this is the case, the

Table 2 Area integrations of quantitative nuclear magnetic resonance (qNMR) experiments obtained from *cis*-heptachlor epoxide

Area integrated/ppm	Assignments	Number of ^{1}H	Runs			RSD (%)
			1	2	3	
3.235–3.359	Analyte	1	993497216	991357120	988864000	0.234
3.652–3.775	Analyte	3	2978505470	2968608260	2973928700	0.167
4.230–4.362	Analyte	1	990837568	980840640	986059520	0.507
7.419–7.580	Internal standard	2	6076485630	6048919550	6030259710	0.384
7.580–7.724	Internal standard	1	3027910910	3013165310	3009897980	0.318
8.015–8.201	Internal standard	2	6065452540	6043401220	6026716670	0.321

RSD = relative standard deviation of the area integrations for the three runs observed

Table 3 Analysis of variance (ANOVA) table obtained from the mixture of *cis*-heptachlor epoxide and SRM 350a

Source	df	$SS/10^{-6}$	$MS/10^{-7}$	F
Between analytical samples	2	1.590	7.952	2.311
Between qNMR measurements	6	2.065	3.444	
Total	8	3.655		

standard uncertainties obtained from ANOVA were pooled.

According to Eqs. 1 and 2, S is a function of the relaxation delay and excitation pulse width. The relaxation time of 60 s was more than ten times as long as most of the T_1 of resonances used in this work and the uncertainty associated to the relaxation effect was estimated to be less than 0.02%. The uncertainty associated to the excitation pulse and the audio filter effect, according to our previous work, was estimated to be less than 0.18% [3]. When combining the uncertainties of the relaxation delay, the offset effect of the excitation pulse, the pulse duration, and the audio filter, we instead obtained an uncertainty of 0.20%; a rectangular distribution was assumed for this.

Additionally, a signal intensity of M_z is also incorporated to the uncertainty. The relative standard deviation (RSD) of M_z of a sample is a good indication of the qNMR experimental setting, because, in order to meet the requirement of the quantification conditions, all M_z should be identical. This factor might have over contributed to the effects discussed previously; a triangle distribution of the maximum and minimum M_z was chosen for this. However, evaluating this term is more important for finding overlapping signals originating from minor impurities, as well as eliminating the peaks that do not satisfy the quantification conditions.

When ^{13}C was not decoupled, satellite peaks originating from ^{1}H connected to ^{13}C made the spectrum analysis complex. In this work, ^{13}C was decoupled so that the satellite peaks did not interfere with the analysis. However, uncertainties associated to the incomplete ^{13}C decoupling could not be estimated. Integration of the peak area was set to a constant area for one set of analytical samples. Thus, the uncertainty associated to the integration area was set to zero.

Overall uncertainties are combined as all independent uncertainty factors, although the uncertainties associated to the experimental condition which was evaluated by ANOVA and the peak selection may have some correlation; we had to assume these to be independent because we cannot evaluate the correlation. Figure 2 shows a graphical representation of the uncertainty budget.

Table 4 summarizes the purities, uncertainties, and IS used for the POP materials. In this work, the purities of hexachlorobenzene and mirex could not be obtained because these samples have no ^{1}H atoms in the molecule. Furthermore, the purity of γ-HCH was observed because of a broad signal obtained by NMR from the analyte. In addition to the purities obtained by qNMR, those obtained by the GC/FID, and the DSC combined with the TGA, which can subtract components that might not be directly observed by DCS, were also shown.

Consideration of traceability

According to Eq. 3, the purity obtained by qNMR is a method that compares the number of nuclei, molecular masses, and masses of an analyte and an IS, respectively, and the purity of the IS. The qNMR experiment detects all signals originating from both the analyte and the IS with one detector at a time; no consideration for the time drift of the detector's response is taken. Furthermore, the linearity of the NMR signal has already been shown [6]. The traceability for the purity of the analyte observed by qNMR is defined clearly by the purity of the IS for the reference in SI units.

Due to the nature of the qNMR experiment, it is not possible to use the traceability source of its own or compounds having a similar structure because NMR signals from the analyte and the IS overlapped each other. Additionally, these methods require generating a calibration curve for accurate quantification. Figure 3 shows an

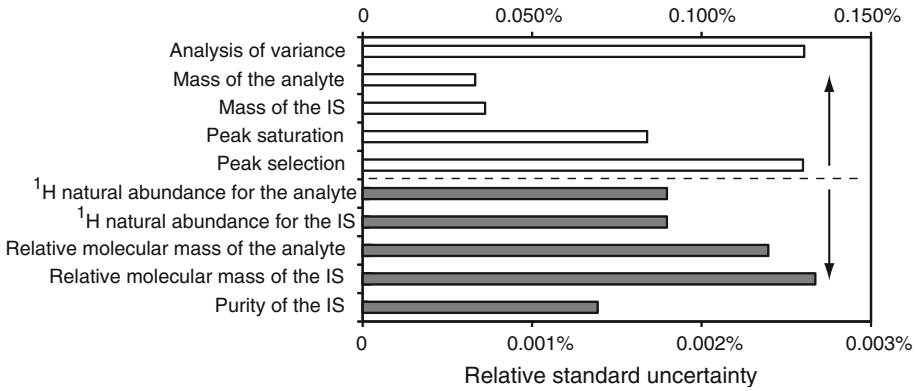

Fig. 2 Uncertainty budget in relative standard uncertainty for the purity determination of *cis*-heptachlor epoxide with SRM 350a. Note that the relative uncertainties associated to the experiments indicated as the *white bars* are 50 times larger than those associated to the other uncertainties, which are shown as the *black bars*

Table 4 Purities and uncertainties for POP samples. The purity of DMSO2 determined by qNMR is also shown. All of the expanded uncertainties are based on $k = 2$. The IS and solvent used for the qNMR determinations are also found from the data analyzed in this work. The purities observed using DSC combined with TGA and GC/FID obtained at our institute is also summarized

Sample	NMR				DSC–TGA		GC/FID
	Internal standard	Solvent	Purity (kg/kg)	Expanded uncertainty (kg/kg)	Purity (kg/kg)	Expanded uncertainty (kg/kg)	Purity (relative peak area)
Hexachlorobenzene	–	–	–	–	0.999	0.002	1.000
Aldrin	SRM 350a	CD_2Cl_2	0.987	0.005	0.998	0.001	0.988
Dieldrin	SRM 350a	CD_2Cl_2	0.978	0.010	0.998	0.003	0.990
Endrin	SRM 350a	CD_2Cl_2	0.992	0.008	0.997	0.002	0.977
p,*p*′-DDT	DMSO2	CD_3CN	0.999	0.012	0.996	0.003	0.995
p,*p*′-DDE	DMSO2	CD_3CN	0.998	0.007	0.997	0.003	0.996
p,*p*′-DDD	DMSO2	CD_3CN	0.999	0.006	0.998	0.002	0.996
o,*p*′-DDT	DMSO2	CD_3CN	0.999	0.005	0.995	0.005	0.997
o,*p*′-DDE	DMSO2	CD_2Cl_2	1.000	0.006	0.997	0.004	0.997
o,*p*′-DDD	DMSO2	CD_3CN	1.000	0.008	0.997	0.004	0.996
Trans-chlordane	SRM 350a	CD_2Cl_2	0.995	0.006	0.998	0.003	0.996
Cis-chlordane	SRM 350a	Acetone-d_6	0.991	0.005	0.997	0.004	0.997
Trans-nonachlor	SRM 350a	Acetone-d_6	0.995	0.006	0.996	0.002	0.991
Cis-nonachlor	SRM 350a	CD_2Cl_2	0.999	0.005	0.998	0.002	0.998
Oxychlordane	SRM 350a	CD_2Cl_2	0.993	0.005	0.999	0.001	0.997
Heptachlor	SRM 350a	CD_2Cl_2	0.993	0.003	0.997	0.003	0.993
Cis-heptachlor epoxide	SRM 350a	CD_2Cl_2	0.975	0.004	0.990	0.013	0.972
Mirex	–	–	–	–	0.999	0.002	0.998
α-HCH	SRM 350a	CD_2Cl_2	0.992	0.006	0.996	0.003	0.991
β-HCH	SRM 350a	CD_2Cl_2	0.995	0.003	–	–	0.991
γ-HCH	–	–	–	–	0.998	0.002	0.998
δ-HCH	SRM 350a	CD_2Cl_2	0.990	0.007	0.999	0.002	0.992
DMS	SRM 350a	CD_2Cl_2	1.000	0.003	–	–	–

illustrative description of the traceability scheme of qNMR for this work. As indicated, structures having similar functional groups are not used as the IS. This makes it an extremely attractive technique because it is a quantitative technique whose traceable source is a different material.

Conclusions

In this paper, we present a precise and accurate purity determination technique with a new International System of Units (SI)-traceable scheme using quantitative nuclear magnetic resonance (qNMR). Since qNMR does not require complicated pretreatments for the sample preparation, it is also an easy method.

The qNMR method can detect signals spread by chemical shift, much like an ordinary NMR spectrum used more for the determination of the structure of molecules. The signals originating from two or more molecules in a solution can be detected simultaneously. Therefore, when these signals do not interfere or overlap each other, the magnitudes of these signals are directly compared. Because of this requirement of the internal standard (IS), qNMR requires a reference material whose structure is different from the analyte. As a result, the qNMR method can transfer the property value of an IS to the others, unlike other analytical techniques.

qNMR does not require the construction of a calibration curve independently. This makes the calibration process much easier than the chromatographic methods. A qNMR experiment is a non-destructive method and the signal-to-noise ratio can be improved by signal averaging; results from repeatable and reproducible conditions of an identical sample can be compared in many ways when it is necessary. In many cases, multiple peaks are observed in a spectrum from one compound; validation of a quantitative condition can be checked within a spectrum by comparing the peak areas of a molecule.

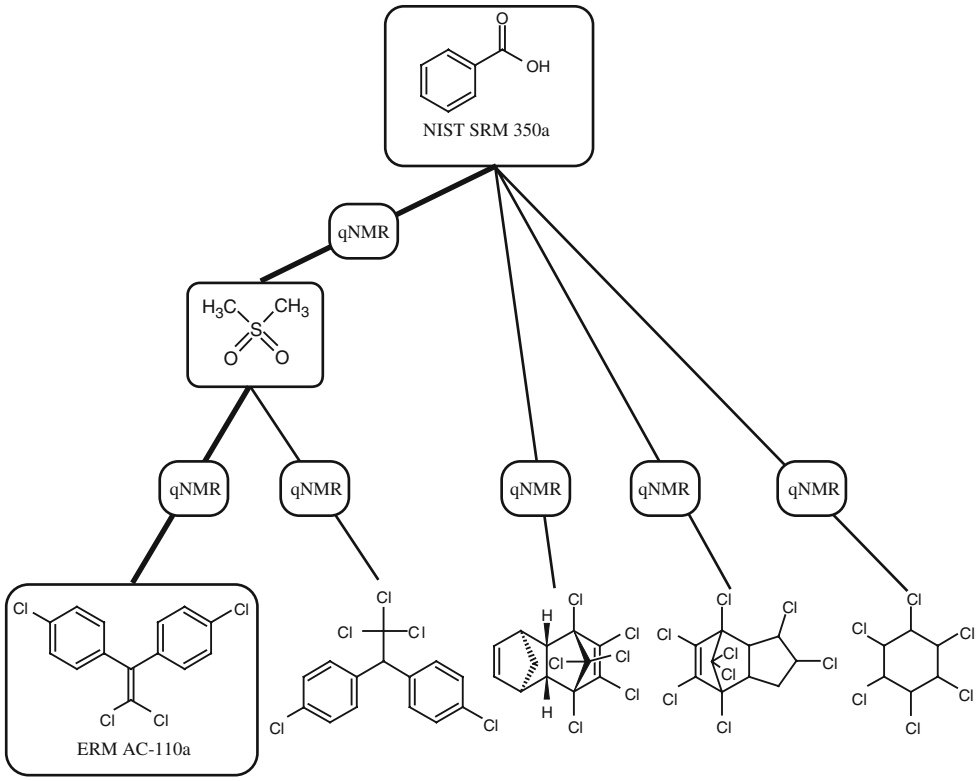

Fig. 3 Illustrative description of the traceability scheme for this work. The purities of all compounds determined in this work were traceable to one compound. ERM-AC110a was used for the validation of this scheme. Note that the compounds that have no ^{1}H atoms were not applicable; the peak shapes for one of the γ-hexachloro cyclohexanes was too broad to observe an accurate purity value

Traceability to the SI can be achieved by a mole-to-mole comparison between the IS and the analyte in one NMR measurement, this can make available a new traceability scheme for the production of SI-traceable reference materials; only a limited number of CRMs, which are used as the IS of qNMR, is required for the production of a variety of CRMs that are demanded by market, such as pesticides and food additives. The production of these CRMs can be accelerated. However, there are no such CRMs specifically prepared for use in qNMR. Thus, a good CRM for qNMR is urgent.

Setting up the qNMR experimental parameters requires several important points; a long relaxation delay; to keep all signals in the flat region of audio filter or use a wide spectral width for the experiment; to achieve a good signal-to-noise ratio for accurate and precise results; to chose a good IS as well as a good solvent. In this sense, a good CRM for qNMR is required.

The purity of all persistent organic pollutant (POP) materials determined in this work were obtained using only two ISs, SRM 350a and DMSO2. Since the purity of DMSO2 was determined with SRM 350a prior to its use as an IS, all purity values in this work are traceable to SRM 350a.

Purities obtained by qNMR are equivalent to those observed by other techniques. The expanded uncertainties associated with the qNMR measurements in this work are almost less than 1% of the purity. The technique is feasible for purity determination. Uncertainties associated with the sample preparation and the deviation of M_z are the largest uncertainty sources.

When there are no ^{1}H atoms in a molecule, this method is not applicable. However, the qNMR method can be expanded to other nuclei, such as ^{19}F and ^{31}P. Additionally, it is difficult to obtain precise and accurate area integration when the NMR peaks to be determined are broad.

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