Expansion of organic reference materials for the analysis of hazardous substances in food and the environment

—Realization of an efficient metrological traceability using the quantitative NMR method —

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Reference materials are indispensable for accurate analysis of hazardous substances in food and the environment. For organic substances, however, the dissemination of reference materials is hopelessly unable to catch up with today's rapidly proliferating analytical needs. To solve this problem, analytical techniques were improved to develop a method in which a single primary reference material could provide accurate quantitative measurements for a wide variety of organic compounds. In pursuit of this goal, we turned our attention to the ^{1}H NMR method. We improved upon the method to allow precise comparisons of signal quantities from protons with different chemical shifts, enabling calibration at an acceptable level of uncertainty for a variety of organic reference materials using a primary reference material for protons. This result opens the prospect of highly efficient metrological traceability, reducing the required number of national reference materials to a minimal level.

Keywords : Chemical metrology, metrological traceability, reference material, nuclear magnetic resonance spectroscopy, primary method of measurement

1 Introduction

Our modern lives are surrounded by chemical compounds, and a wide range of laws and regulations controls these chemical compounds, to ensure safety and to prevent adverse impact on the environment and human health. In recent years, public concern for safety has increased in Japan, prompting an increase in the number of chemical compounds subject to regulation, limitations, and other regulatory controls. For example, in May 2006, the Food Sanitation Law was revised to introduce the "Positive List System[Term 1]" for agricultural chemical residue in foods. With the enforcement of stringent regulations, the number of control subjects expanded from approximately 250 to about 800 kinds of agricultural and other chemical compounds traded domestically and internationally. At the same time, several new Official Methods of Analysis[Term 2] were established to measure the regulated chemical compounds, and as result, the use of advanced analytical equipment capable of conducting multiple simultaneous measurements, such as gas chromatograph/mass spectrometer (GC/MS) and liquid chromatograph/mass spectrometer (LC/MS), increased in food and environmental analyses. In this situation, many laboratories that inspect and test chemical compounds are increasingly employing GC/MS and LC/MS to conduct analyses.

While these analytical equipment are capable of simultaneously measuring multiple components, it is necessary to calibrate the sensitivity of the analytical instrument for each analyte in the samples to ensure the reliability of analytical results. To perform this calibration, reference materials (RMs) that serve as "yardstick" are required for individual analytes. In this type of analysis, the accuracy of inspection and testing results are crucial, and the reliability of the "yardstick" is of paramount importance. The use of certified reference materials (CRMs)[Term 3, [1]] or equivalent RMs is highly recommended in such cases, and therefore various testing and inspection laboratories are working swiftly to acquire the RMs necessary to handle the ever-increasing list of regulated materials.

2 Current problems with RMs

The characterization of RMs by metrologically appropriate procedures is achieved by using measurement methods that offer traceability[Term 4] to SI definitions (in this case, amount-of-substance). Normally, this is a task performed by the national metrology institute[Term 5] of a country, and the RMs produced are known as the national reference materials (primary RMs). Generally, national RMs offer the highest standards of accuracy, and are scrupulously prepared with labor, time, and expense. Normally, they are not transferred directly to the inspection and testing laboratories that perform the actual analysis, because this is not practical due to the quantities and costs involved. Instead, secondary RMs are calibrated based on the national RMs, and working RMs are in turn calibrated using the secondary RMs. In this way, a pyramid structure is constructed, with few higher-order RMs at the top and a larger population of lower-order RMs reproduced below. Order in this proliferation of RMs is enforced through traceability to the original set of accurate "yardstick" or the national RMs. In essence, this concept is similar to the traceability systems where scales are calibrated using a series of weights, and the current RM traceability

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system is not unique to reference materials. However, it is different from the weight system in a number of ways.

Figure 1 is a schematic diagram of the traceability system for the RMs used in the analysis of volatile organic compounds that are used to test the quality of river and tap water in Japan. The national RM is in the form of a single solution incorporating 23 volatile compounds. Traceability to SI is obtained using pure substances for each component that is valuated using the freezing point depression method. The secondary and working RMs also consist of a single solution incorporating 23 volatile organic compounds, but in this case, calibration from upper-order to lower-order standards is conducted separately for each component. Because the lower-order traceability system requires a one-to-one correspondence, the pyramid structure breaks down for these RMs. In other words, the national RM for a given component must be used to calibrate the secondary RM for the same component, and calibration of working RMs for the same component is performed using this secondary RM. Because this is one-to-one calibration of the same chemical compounds, commercially available analytical technologies such as gas chromatography can be used for calibration down to the working RM level while maintaining excellent reliability. This practical system of traceability is used throughout the world.

The drawback of this traceability system is that it requires a wide array of national RMs to match each chemical compound subject to be analyzed. Development of these national RMs is a major bottleneck in the traceability system because it requires enormous time, labor, and expense. The construction of a more efficient traceability system based on an entirely new concept is needed to address the rapidly proliferating demand for RMs prompted by increasingly tight regulation of chemical compounds through the positive list system discussed above.

3 Research target: Construction of an efficient traceability system

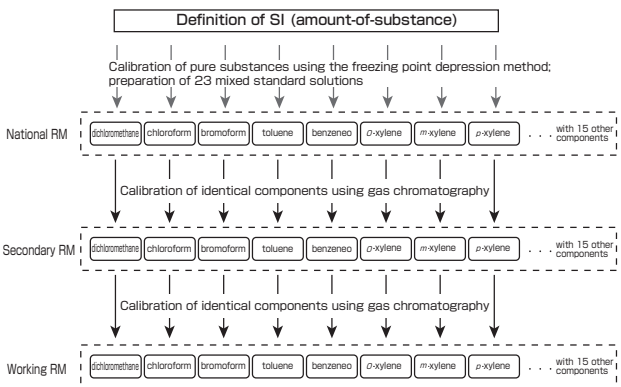

Fig. 1 Traceability system for RMs used in analysis of volatile organic compounds.

To recapitulate the previous chapter, the problem with present traceability system for RMs lies in the dependence on national RMs. Because the system consists of the series of "calibration chains" for the same chemical compound, the system cannot respond promptly to the need for RMs to analyze the growing number of chemical compounds. Although this problem can be solved if minimum types of higher-order RMs could be used to calibrate a wide range of working RMs, this is not possible with current calibration technology, which is designed to calibrate like chemical compound with like chemical compound. An entirely new calibration technology must be developed and introduced: a universal calibration technology capable of analyzing chemical compounds independently of their molecular structure.

The aim of this study is to develop a new calibration technology to realize a system that efficiently secures traceability to the wide range of working RMs without creating national RMs for each chemical compound. Given that the vast majority of chemical compounds subject to strengthened regulation in recent years are organic compounds, we developed a universal calibration technology targeting organic compounds.

4 An analytical method that achieves our objectives: Quantitative NMR

4.1 The required calibration technology

Absolute values for amount-of-substance can be obtained using SI-traceable measurements. This type of measurement is known as the primary method of measurement[Term 6, [2]]. Table 1 shows a list of analytical methods certified as primary methods of measurement, divided into primary direct methods and primary ratio methods. The primary direct methods, also called the absolute measurement methods, are defined as "the methods for measuring the value of an unknown without reference to a standard of the same quantity." Examples of primary direct methods are coulometry[Term 7], gravimetry[Term 8], and the freezing point depression method[Term 9]. Because these analytical methods yield absolute values for amount-of-substance, they are appropriate for valuing the national RMs. However, in general, they tend to be slow and their application are limited to short list of substances, and they are not suitable candidates as universal calibration technology that is the objective of this study. Primary ratio methods, on the other hand, are already in practical use. They are defined as "methods for measuring the value of a ratio of an unknown to a standard of the same quantity; its operation must be completely described by a measurement equation." They include titrimetry[Term 10] and isotope dilution mass spectrometry[Term 11]. Another analytical approach that qualifies as a primary ratio method, though not well established as an analytical technology, is the quantitative nuclear magnetic resonance (quantitative NMR).

A measurement method that can be applied to the calibration of a wide range of working RMs must satisfy the following conditions:
1) It must satisfy market demands regarding uncertainty, while also provide speed and simplicity of use.
2) It must be highly versatile and applicable to a wide variety of chemical compounds (general organic compounds for the purposes of this study).

Quantitative NMR is the most feasible candidate that can satisfy both conditions 1) and 2), although the answer is not yet fully established. Accordingly, in this study, we endeavored to establish quantitative NMR as a universal calibration technology for working RMs in organic compounds.

4.2 Principles of quantitative NMR

NMR is one of the main methods for determining the molecular structure of a chemical compound. It has an extensive track record in unraveling molecular structures, including the analysis of complex molecules such as proteins. Information obtained using NMR, such as chemical shift (the resonance peak position dependent on atomic bonding and the ambient environment) and spin-spin coupling (a split of the peak due to bonded nearby nuclei), provide hints about the chemical species and ambient environment of a molecule. In addition, the area ratio of various peaks, which resonates according to different chemical shifts, generally indicates the ratio of the number of atomic nuclei contributing to the peaks. As Figure 2 shows, the area ratio of ^{1}H NMR signals can easily be used to confirm the relative number of protons for the resonances, which is vital for the qualitative analysis of organic compounds.

Conventionally, this aspect of NMR was used exclusively to determine the chemical structure, solely by expressing the number of protons as a ratio in a molecule. However, the concept can be applied differently. If the molecular structure of an organic compound is already known and assignments of its ^{1}H NMR spectrum has been set, the number of protons contributing to each resonance peak is known, and this information can be applied to the quantitative analysis of chemical compounds. Thus, when the ^{1}H NMR measurement is performed by adding a reference chemical compound to a sample solution separately in an analyte (substance to be analyzed) solution, the spectra of the two chemical compounds overlay each other, as shown in Fig. 3. At this point, if the mass (weight), molecular weight, and purity of the added reference chemical compound (hereinafter, will be called the Primary Standard: PS) are known, the amount-of-substance (number of molecules) corresponding to peak I in Fig. 3 will also be known, and can be used as the criterion for finding the number of molecules in the analyte. To illustrate with a specific example, if the number of protons in PS (I) is the same as the number of protons in analyte (D) (the number is 6 for both), the ratio of the areas for peak I and peak D indicates the relative number of molecules. As such, the relationship can be expressed as follows:

(Peak area I)/(Number of molecules of PS) = (Peak area of D)/(Number of molecules in analyte)

Since the number of molecules in PS is already known, the

Table 1 Types of primary methods of measurement and their characteristics.

Analytical method	Primary direct method			Primary ratio method		
	Coulometry	Gravimetry	Freezing point depression method	Titrimetry	Isotope dilution mass spectrometry	Quantitative NMR
Outline of analytical method	Amount of electricity used in electrysis of specified substances is measured.	Settling quantity of specified substances in solution is measured.	Relationship between fraction melted and temperature around the melting point is measured.	The specified substance is measured using chemical reactions.	Mass spectrometry is performed using a stable isotope.	The ratio of areas of ^{1}H peaks with different chemical shifts is measured.
Main target substance	Metallic elements	Inorganic salts	High purity organic compounds	Acid, base, elements	Trace metals trace organic compounds	Organic compounds
Reference standard	Not required	Not required	Not required	Reference standards based on the principles of titration are required.	Required for each analyte	A reference standard for ^{1}H is required.
Uncertainty (less than 1 %)	○	○	○	○	○	△(Unknown value)
Rapid analysis	×	×	×	×	○	○
General applicability	×	×	×	×	×	○

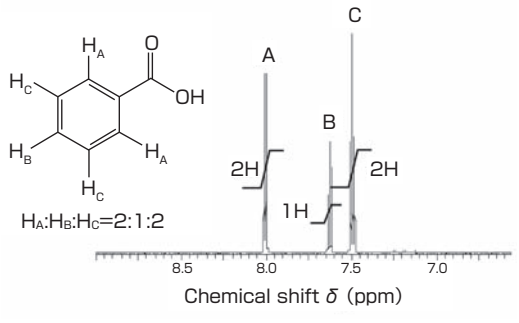

Fig. 2 Qualitative analysis of chemical compounds using ^{1}H NMR.

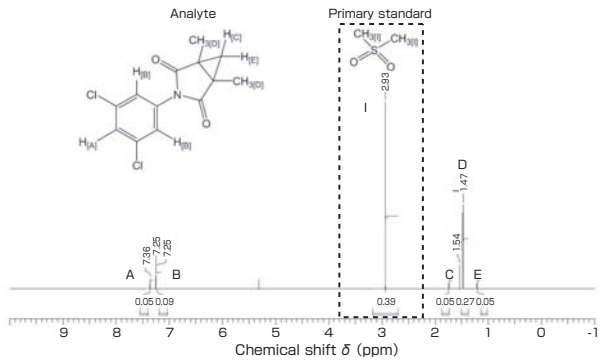

Fig. 3 Quantitative analysis of chemical compounds using ^{1}H NMR.

number of molecules in the analyte can be obtained. The mass (weight) and molecular weight of the target substance can then be used to determine the purity of the analyte[3]. Therefore, quantitative NMR is, in principle, a primary ratio method which can be used to obtain traceable measurement values for the number of protons — that is, amounts of substance in a sample.

In the example in Fig. 3, both the analyte and the PS are pure substances. After weighing the two substances individually, they are dissolved in a deuterated solvent, and quantitative NMR is used to measure the purity of the analyte using the mass ratio of the two substances. Working RMs, in contrast, are often supplied in the form of solution. If supplied at a certain concentration (about 0.1 %), quantitative NMR can be applied by dissolving the working RM in an appropriate deuterated solvent. The concentration of working RM can be found from the number of molecules obtained for the analyte, the mass of sample solution added, and the number of molecules in the analyte.

4.3 Feasibility of quantitative NMR

National metrology institutes in several countries (including AIST), which are members of the Consultative Committee for Amount of Substance (CCQM)[Term 12], have shown interest in the possibility of applying quantitative NMR as a primary ratio method, which was first suggested by Germany's Federal Institute for Material Research and Testing (BAM). In 2001, the Laboratory of the Government Chemist (LGC) in the United Kingdom and BAM served as pilot laboratories to conduct an international comparison[Term 13] for the quantitative analysis of ethanol in aqueous solution, with the participation by 10 institutes in key countries. On this occasion, measurements were conducted on the same sample using conventional analytical approaches such as gas chromatography (GC) as well as quantitative NMR[4]. The sample was precisely produced by LGC, one of the pilot laboratories. The ethanol concentration was 1.072 mg/g ± 0.006 mg/g, but this value was not disclosed to the participants. Also, BAM separately supplied a deuterated water solution of PS (3-trimethylsilyl sodium propionate-d_4) of known concentration to the participating institutions that declared to conduct the quantitative NMR measurement.

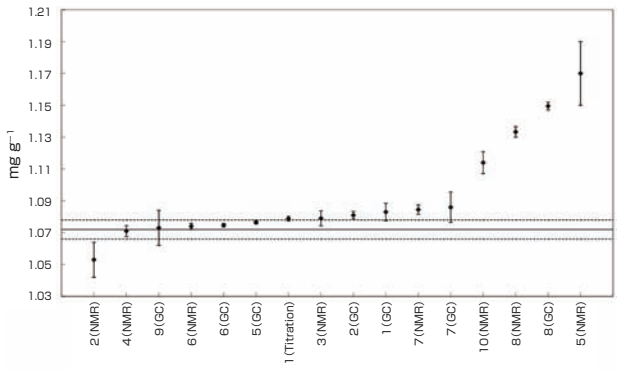

Fig. 4 Results of international comparison on quantitative analysis of ethanol in aqueous solution.

The solid line indicates the preparation value; the dotted line indicates uncertainty for the preparation value. No. 6 is the result for NMIJ/AIST. Participants: BAM (Germany), KRISS (Korea), LGC (UK), LNE (France), NIST (USA), NMi (Netherlands), NMIJ (Japan), NRC (Canada), NRCCRM (China), and VNIM(Russia).

The measurement results were reported individually to the pilot laboratory. Figure 4 is a summary of the results. Each data point represents a reported result. The adjacent error bar is the measurement uncertainty estimated by each participating institution (95 % confidence interval). The uncertainty of the quantitative NMR results from most institutions was in the range that could be described as percentage, and some of the results deviated significantly from the preparation values. In short, it was found that the quantitative NMR lacked accuracy compared to the conventional analytical methods such as GC. From the result of this international comparison, it was determined that the quantitative NMR did not offer sufficient technical accuracy. This view remains essentially unchallenged in the international scientific community today.

At the same time, Fig. 4 shows that the value reported by AIST closely matched the preparation value and its uncertainty was considerably smaller than the quantitative NMR findings of other participating institutions. This is why AIST takes a different stance on quantitative NMR. The uncertainty AIST reported to the pilot laboratory for quantitative NMR in the international comparison is illustrated in Fig. 5. Upon evaluating the relative standard uncertainties of each component, we found that the greatest factor was the uncertainty of the concentration of ^{1}H PS supplied by the pilot laboratory. Because the uncertainty of AIST's quantitative NMR measurement was much smaller, it became clear that a much smaller measurement uncertainty would have resulted if AIST had supplied its own more accurate PS.

It should be emphasized that the quantitative NMR offers a major advance in versatility. Whereas GC and other

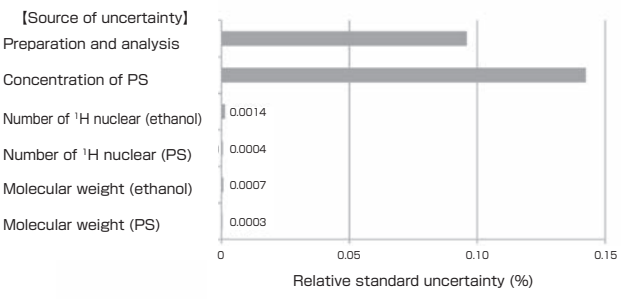

Fig. 5 Uncertainty for ^{1}H NMR in the international comparison on quantitative analysis of ethanol in aqueous solution.

conventional analytical calibration technologies applied in the international comparison can only be used to compare the concentrations of like chemical compounds (PS must be the same chemical compound as the measured substance), quantitative NMR can compare quantities of chemical compounds of different types (that is, PS does not have to be the same type of chemical compound as the measured substance). As such, although quantitative NMR requires at least one substance including ^{1}H, it can be used to measure any organic compound that includes proton, and a wide range of applications can be expected accordingly. The Authors believe that quantitative NMR can be applied in the calibration of working RMs by developing and integrating certain elemental technologies. These are discussed below.

5 Development and integration of elemental technologies to realize the quantitative NMR

5.1 Core elemental technologies

Figure 6 illustrates the elemental technologies developed by the authors, and the combination necessary to realize the potential of quantitative NMR as a universal calibration technology for working RMs. The features required of NMR differ greatly depending on whether the technology is optimized for qualitative analysis or for quantitative analysis, as in our case. With quantitative NMR, the highest priority is to observe the signal in accurate proportion to the number of atomic nuclei in the analysis, rather than improving measurement speed or improving the signal-to-noise ratio (S/N). We therefore revised the conditions for selecting the core elemental technologies.

The first elemental technology corrects a signal amplification issue. Generally speaking, NMR signals relax throughout its lifetime called the spin lattice relaxation time (T_1), which is the time taken for the atomic nuclei to settle from their excited state to their ground state. This period varies according to the environment of protons (such as bonding with other atoms). When NMR is performed for qualitative analysis, the sample is irradiated with microwave pulses with short cycle to increase the signal and to improve S/N. In such case, the delay time may be shorter than T_1, where excitation pulse is applied before all protons settled to their ground state. As result, differences in T_1 among the protons of analyte and PS make it impossible to obtain the peak area in correct proportion for the number of protons in each proton. We resolved this problem by measuring the relationship between repetition time and peak area. By taking delay time six times or greater than T_1 for the analyzed protons, it was demonstrated by experiment that 99.9 % or more of original signal intensity could be obtained, providing a stable peak-area ratio[5]. By ensuring that the delay time was sufficiently longer than the longest T_1 for all protons in the analyte, it was possible to obtain accurate peak-area ratio that was unaffected by the T_1 of the protons (though the measurement time increased several times longer than the conventional method).

The second elemental technology also concerns the S/N. Normally, S/N in the NMR signal is further improved by using an audio filter to narrow the bandwidth. However, this filter is not "flat" in sensitivity throughout the bandwidth, but exhibits severe loss of sensitivity at both ends of the filter bandwidth. Depending on the chemical shift, this loss of sensitivity can be in the range of several percents. Greater the chemical shift in the protons observed in the analyte and PS, more difficult it is to obtain an accurate peak-area ratio. To obtain flat sensitivity, we set the audio filter to cover 60 %~70 % of bandwidth and also widened the spectral width for data acquisition to 100 ppm, compared to less than 20 ppm in the conventional setting. This setting allowed the resulting spectrum to remain unaffected by sensitivity loss caused by filter for all chemical shifts. While such filter settings are not practical for ordinary NMR that involves handling of large volume data, we were able to solve several issues by taking an unconventional

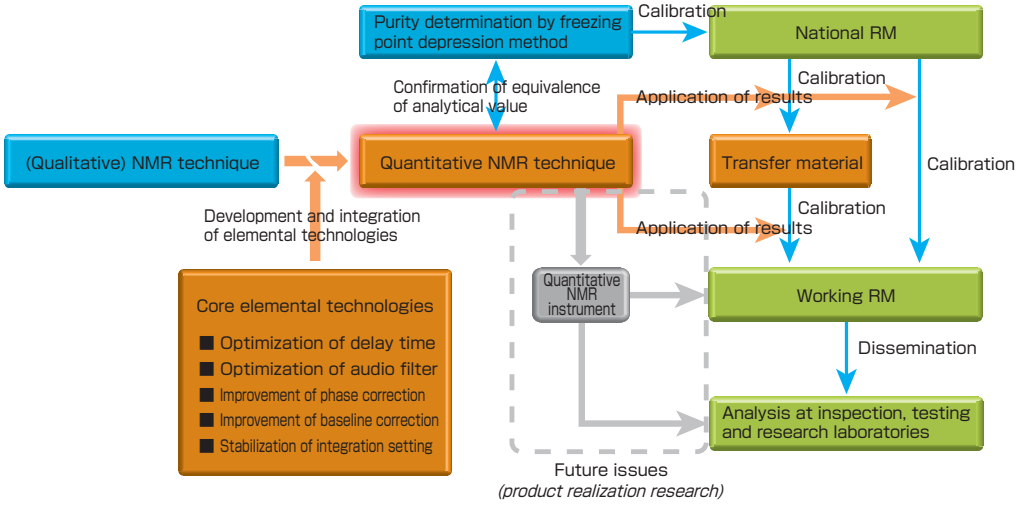

Fig. 6 Development of elemental technologies for the construction of universal calibration technology and the process of integration.

approach with priority on measurement accuracy[5].

In addition to the two elemental technologies described above, the Authors found that to improve the reproducibility of measurement results, phase correction, baseline correction, and peak area integration setting (range) were more important compared to other minor factors.

5.2 Use of transfer materials

Although quantitative NMR requires ^{1}H as the PS, the analyte does not have to be the same substance. The PS (limited to pure substances in this discussion) must satisfy the following conditions:

1) It must have as little impurities as possible, to keep the uncertainty for its purity value small.
2) It must dissolve easily in wide range of solvents, and must be stable in solution.
3) It must have low volatility (sublimability) and absorbency, so its mass (weight) can be measured easily.
4) Its chemical shift must not overlap with that of the target substance.

Although some national RMs satisfy these conditions for PS, many national RMs do not satisfy requirement 2), because a suitable solvent for dissolving both the PS and the analyte has not been found. Also, some national RMs do not satisfy 4), as the PS used depends on the analyte, and different PSs must be used with certain analytes.

The number of national RMs cannot be reduced if different PSs must be prepared according to various analytes. The Authors solved this problem using the calibration methods illustrated in Fig. 7, marshaling the advantages of quantitative NMR. We achieved this by selecting the transfer materials or chemical compounds whose chemical shifts do not overlap with either the PSs or the analytes. In Step 1, the PS (national RM) is used to calibrate the characteristic peak of the transfer material using quantitative NMR. In Step 2, the characteristic peak of the calibrated transfer material

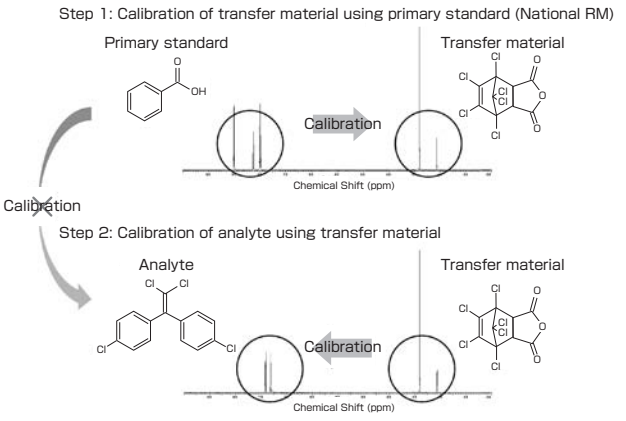

Fig. 7 Use of transfer material in quantitative NMR.

is adopted as the standard for calibration of the analyte. By adopting this two-step calibration method, the number of national RMs, which anchor the traceability system, can be minimized. Moreover, the transfer material does not need to be homogeneous or long-term stable like the RMs, so a wide range of materials is available for selection according to their match with a given analyte. The introduction of transfer materials to quantitative NMR was an important technological development in the process of synthesizing the elemental technologies.

5.3 Evaluating the integrated technologies

In sections 5.1 and 5.2, we described how several elemental technologies were integrated to construct a calibration technology using quantitative NMR. Next, we demonstrated the reliability of the technologies by comparing them with long-established techniques. To do this, we first selected several target substances from commercially available, high-purity compounds. Their purity values were determined using the freezing point depression method, a well-established primary direct method that AIST has been using for the valuation of national RMs (see Table 1). Then we measured the same samples with the newly developed quantitative NMR to find the purity value, and checked whether the two values matched in the range of their respective uncertainties.

As the PS for measurements using quantitative NMR, we used benzoic acid (NIST SRM 350a, 99.9958 % ± 0.0027 %), a national RM distributed by the National Institute of Standards and Technology (NIST) of the United States. We performed the two-step calibration process described above using dimethyl sulfone or 1,4-bis-trimethylsilylbenzene-d_4 (1,4-BTMSB-d_4) as the transfer material, as the peak of the chemical shift for several substances overlaps the peak for benzoic acid. To dissolve the PS and the analyte, solvents were selected from a number of deuterated solvents, to minimize skewing of results from the protons of any impurities in the solvent. The solubility and other characteristics of the PS and analyte were also taken into consideration, and a solution with a concentration of about 1000 mg/L was prepared.

The analytical results are summarized in Table 2. Although in many cases the uncertainty was larger for the purity values by quantitative NMR compared to freezing point depression method, the values for the two methods matched within the uncertainty ranges, demonstrating that our calibration technology using quantitative NMR was sufficiently reliable[6]. The uncertainty for quantitative NMR was between 0.3 % and 1.2 % (k=2, 95 % confidence interval). Although this accuracy as a purity measurement technology is somewhat inferior to the freezing point depression method, quantitative NMR can be used to calibrate substances to which the freezing point depression method cannot be applied, including a wide range of organic compounds, and

it satisfies the market demand for the uncertainty levels in working RMs.

6 Issues for further study

We envision a transfer from the current one-to-one traceability system based on separate national RMs for each substance, to one-to-many traceability system in which several substances can be traced to just a few national RMs. So far, we made advancement for the development of universal calibration technology, a core technology applicable to numerous organic compounds. After establishing an ideal scenario for this project, we began by developing elemental technologies, using irradiation pulse delay time and optimization of audio filters. We then demonstrated that these calibration technologies could satisfy market requirements for uncertainty. We learned that the transfer materials could be used to minimize the number of national RMs required as standards for amount-of-substance. Finally, we plotted a roadmap toward an efficient traceability system, as illustrated in Fig. 8.

The system we outlined represents a quantum leap in the efficiency of traceability systems, since it removes the need to maintain one-to-one traceability chain from national RMs to working RMs for individual substances. It is an entirely new approach to RMs, unseen elsewhere in the world. The novelty of this technology, however, means that it is necessary to conduct numerous proving tests and to publish the results. The quantitative NMR technique must be standardized as an analytical method, and new international comparisons will be required at national metrology institutes

Table 2 Purity analysis results for organic compounds using quantitative NMR.

Terget substance	Freezing point depression method		Quantitative NMR				
	Reference value (%)	Uncertainty (%, k=2)	Analytical value (%)	Uncertainty (%, k=2)	Primary standard	Transfer material	Solvent
trans-Nonachlor	99.6	0.2	99.5	0.6	Benzoic acid	-----	Acetone-d_6
cis-Nonachlor	99.8	0.2	99.9	0.5	Benzoic acid	-----	Dichloromethane-d_2
Oxychlordane	99.9	0.1	99.3	0.5	Benzoic acid	-----	Dichloromethane-d_2
Endrin	99.7	0.2	99.2	0.8	Benzoic acid	-----	Dichloromethane-d_2
trans-Chlordane	99.8	0.3	99.5	0.6	Benzoic acid	-----	Dichloromethane-d_2
cis-Chlordane	99.7	0.4	99.1	0.5	Benzoic acid	-----	Dichloromethane-d_2
Trichlorfon (DEP)	99.7	0.3	99.6	0.5	Benzoic acid	-----	Dichloromethane-d_2
Heptachlor	99.7	0.3	99.3	0.3	Benzoic acid	-----	Dichloromethane-d_2
4,4'-DDT	99.6	0.3	99.9	1.2	Benzoic acid	Dimethyl sulfone	Acetonitrile-d_3
4,4'-DDE	99.7	0.3	99.8	0.7	Benzoic acid	Dimethyl sulfone	Acetonitrile-d_3
4,4'-DDD	99.8	0.2	99.9	0.6	Benzoic acid	Dimethyl sulfone	Acetonitrile-d_3
Procymidone	99.9	0.2	99.3	0.5	Benzoic acid	Dimethyl sulfone	Dichloromethane-d_2
Fenobucarb (BPMC)	99.8	0.2	99.8	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
Fenitrothion (MEP)	99.8	0.3	99.6	0.6	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
α-HCH	99.6	0.3	99.2	0.6	Benzoic acid	-----	Dichloromethane-d_2
β-HCH	Inapplicable (thermal decomposition)		99.5	0.3	Benzoic acid	-----	Dichloromethane-d_2
Atrazine	Inapplicable (thermal decomposition)		99.7	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
EPN	Inapplicable (Uncrystallized)		99.4	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
Diazinon	Inapplicable (Uncrystallized)		99.8	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
Malathion	Inapplicable (Uncrystallized)		99.5	0.7	Benzoic acid	-----	Dichloromethane-d_2
Etofenprox	Inapplicable (Uncrystallized)		99.5	0.5	Benzoic acid	Dimethyl sulfone	Dichloromethane-d_2

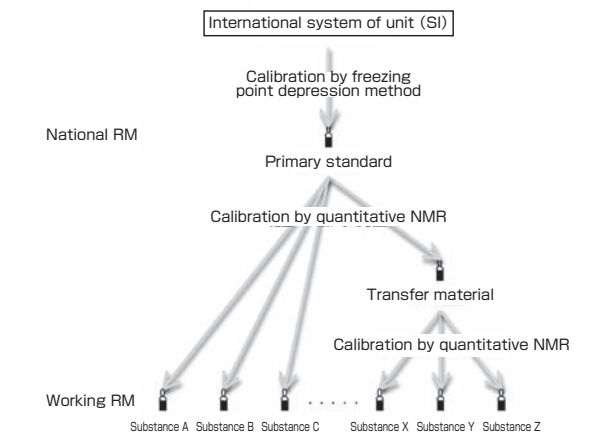

Fig. 8 Efficient traceability system with quantitative NMR.

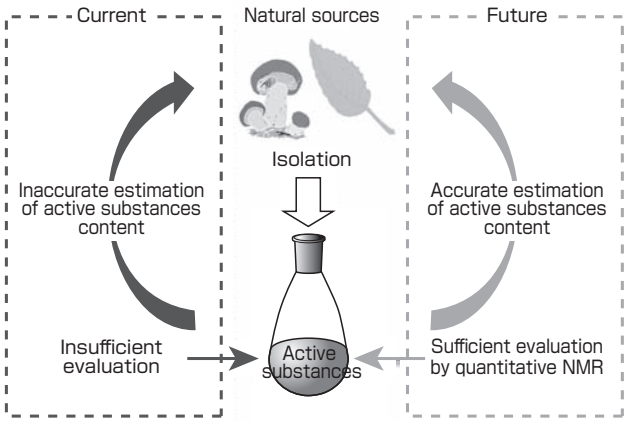

Fig. 9 Quantitative evaluation of active substances in natural sources using quantitative NMR.

around the world. More work must be done before one-to-many traceability can be firmly established.

At the same time, it is necessary to build the infrastructure that allows the industrial community to perform calibration of the wide range of working RMs that are in demand by the society. For this purpose, national RMs that are easy to use with quantitative NMR must be supplied along with sample applications. Automation tools are also necessary, covering all processes from measurement parameter sets using quantitative NMR to data analysis.

7 Future directions

Quantitative NMR has great potential marketability, as the necessary analytical equipment are commercialized (Fig. 6: Future issues). As reasonably priced, easy-to-use equipment, which are optimized for quantitative NMR, become available, and applications for nuclei other than ^{1}H are developed, they will find use not only in calibration technologies for working RMs, but also in quantitative analysis of several organic compounds occurring in numerous fields conducted at a wide variety of proving, testing, and research laboratories.

Many de facto commercial calibration standards are in use today, even though evaluation of their purity or concentration remains inadequate. For example, for active substances in natural sources, such as bioactive constituents and herbal medicines, quantitative analysis often depends on the samples of isolated constituents or the commercially available reagents. Quantitative NMR can offer highly reliable and effective quantitative analysis in such cases (see Fig. 9)[7], where the discovery of appropriate standard would normally be difficult.

Perhaps most exciting of all, an efficient traceability system based on this calibration technology for organic compounds may provide an effective scheme for responding flexibly to today's proliferating demand for RMs. Although core technologies other than quantitative NMR have not yet been demonstrated, universal calibration technologies that can be used similarly in the construction of a rational traceability system may be developed. The Authors hope that this paper will serve as a starting point for the development of such universal calibration technology.

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Finally, Dr. Shinichi Kinugasa (AIST) provided valuable assistance in writing this paper, for which we thank him sincerely.

Terminology

Term 1. Positive List System: Established in 2006 based on a revision of Japan's Food Sanitation Law, this system prohibits the sales of foods that contain agricultural chemical residues above a certain quantity. In cases where the safe (not harmful to human) quantity has been specified (called the residue level), the agricultural chemical must be below that quantity. In case where the safe quantity has not been specified, a uniform limit of 0.01 ppm is applied.

Term 2. Official Method of Analysis: A set of analytical procedures officially published and recognized in accordance with laws governing chemical compounds, to enable comparison of analytical results among different testing laboratories and samples. An official method of analysis must be robust and universally applicable. Examples used in Japan are Japanese Industrial Standard (JIS), Japanese Agricultural Standard (JAS), and Japanese Pharmacopoeia (JP).

Term 3. Certified reference material (CRM): In ISO Guide 35, which provides the international guidelines for RMs, this is defined as "reference material, characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability."

Term 4. Traceability: The characteristic of a measurement result, where the result can be linked to a known reference standard (usually a national standard) through an unbroken chain. In the 3rd version of

the *International Vocabulary of Metrology* (VIM), this term is amended to "metrological traceability" to distinguish from the term used to manage the shipping histories of foods and other goods.

Term 5. National metrology institute: A research institute that sets a country's official measurement standards. In Japan, it is the National Metrology Institute of Japan within the National Institute of Advanced Industrial Science and Technology.

Term 6. Primary method of measurement: The method used to define national RMs. It is defined as follows: "primary method of measurement is a method having the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be expressed in terms of SI units."

Term 7. Coulometry: The method of measuring the amount-of-substance of an analyte from measurements of current and time when electrolysis is applied to a specific substance based on Faraday's Law. It is used in the analyses of inorganic ions of metallic elements as well as of trace amounts of moisture.

Term 8. Gravimetry: An analytical technique in which the quantity of an analyte in a sample is found by separating the analyte from the rest of sample using a reagent that reacts specifically to that component. The resulting mass is used to determine the quantity of the analyte. Generally, mass is found by precipitating the selected component out of the solution, but it can also be found by separating the selected component from the sample as gas, adsorbing the component using an adsorbent, and then calculating the mass from the amount adsorbed.

Term 9. Freezing point depression method: An analytical technique that finds the amount-of-substance fraction of impurities in a sample as a proportion of its amount-of-substance by measuring the temperature and enthalpy of impurities in a sample, as its freezing point decreases. It is generally used to determine the purity of high-purity organic compounds.

Term 10. Titrimetry: This is volumetric measurement in a limited sense. A solution that includes an RM that reacts with the sample is dropped into a sample solution, and the quantity of RM consumed before the equilibrium is reached is measured to find the quantity of the analyte in the solution. Depending on the chemical reaction used, the method includes neutralization (acid-base) titration, oxidation-reduction titration, complex formation titration, or precipitation titration.

Term 11. Isotope dilution mass spectrometry: A method of finding the quantity of an analyte in a sample using substance labeled with a stable isotope. The labeled substance is added to the sample, and the signal ratio of the mass spectrums of the analyte and the labeled substance are obtained. Because the chemical properties of the analyte and the labeled substance are roughly identical, the effect of the process of sample preparations with significant impurities can be cancelled (the signal ratio of the analyte and its labeled substance is maintained). In this technique, the concentration of the labeled substance for the RM must be known in advance.

Term 12. Consultative Committee for Amount of Substance (Comité Consultatif pour la Quantité de Matière: CCQM): One of the consultative committees formed under the aegis of the International Committee of Weights and Measures (Bureau International des Poids et Mésures: BIPM)) that consists of the Meter Convention member institutions. Established in 1993, this consultative committee discusses issues on metrology in chemistry.

Term 13. International comparison (CCQM inter-comparison): Comparison among calibration laboratories to confirm the degree of equivalence in the calibration and measurement capabilities and values assigned to RMs between various national metrology institutes. Normally, this process begins with an international comparison for research purposes, called a pilot study. After the technical groundwork has been established to a certain degree, an official international comparison, called a key comparison, is performed.

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Authors

Toshihide Ihara
Completed the doctoral course in engineering at Tokyo Metropolitan University in 1994. In 1996, he joined the National Institute of Materials and Chemical Research of the Agency of Industrial Science and Technology, where he worked on the development of RMs for volatile organic compounds and other organic compounds. At the National Institute of Standards and Technology in the United States from 2002 to 2003, he pursued development of RMs related to health foods. Moving to BIPM from 2005 to 2006, he worked on purity evaluation techniques for organic compounds. In 2006, he was assigned to his current position at the RMs Systems Division of the National Metrology Institute of Japan of AIST (now the Measurement Standards Systems Division), where he conducts research on standards dissemination systems in the fields of environmental science, foods, and clinical testing. In this paper, he conceived the idea for a new traceability system in chemical metrology and designed specific approaches.

Takeshi Saito
Joined the National Institute of Materials and Chemical Research of the Agency of Industrial Science and Technology, where he engaged in research in advancing Spectral Database (SDBS) for Organic Compounds, which is now publicly available on the Internet. He is now in charge of this project. Since joining the Institute, he has made extensive use of NMR in research. In a contract work with the New Energy and Industrial Technology Development Organization (NEDO), he worked on a platform for measurement at nanoscale, focusing on the measurement of particle diameter in liquid using NMR. Currently he is working to improve the precision and accuracy of quantitative analytical techniques using NMR. He also works on the general application of quantitative analytical methods. In this paper, he constructed the basic technology for quantitative NMR.

Naoki Sugimoto
After completing the doctoral course in Natural Sciences and Technology at Kanazawa University in 1997, he joined the National Institute of Health Sciences (NIHS) in the same year. There, he worked on setting standards for food additives and other substances. From 2005 to 2006, at the Food and Drug Administration (FDA) and Center for Food Safety and Applied Nutrition (CFSAN) in the United States, he developed methods for analyzing food additives, to promote international standardization. Since 2008, he became the chief of 3rd Section, Division of Environmental Chemistry at NIHS, where he currently works on setting criteria and developing guidelines of analytical methods for chemical substances related to water quality. In this paper, he contributed to the application technologies for quantitative NMR and to the proposal of automation tools to make the technology accessible to the general public.

Discussion with Reviewers

1 General evaluation

Comment (Akira Ono)

Today the society is facing difficulties as the development of techniques for accurate analysis of harmful organic compounds in foods and environment cannot keep up with the ever-diversifying demands. The quantitative NMR developed in this research project, along with the new, more efficient traceability system, strike at the heart of this problem. They hold the potential for a revolution in the metrological traceability.

I believe your approach represents the first use of NMR equipment for quantitative analysis that was originally developed for qualitative analysis. What makes this project a particularly outstanding *Type 2 Basic Research* is that you returned to the development of elemental technologies to complete the core technology of quantitative NMR.

Comment (Hisao Ichijo)

Your writing shows clearly how you steadily pursued your program of research and development, by drawing scenarios along the way toward the ambitious goal of switching to a new, more efficient traceability system based on calibration technology.

2 Focus on specific descriptions

Question and comment (Akira Ono)

You advocate a new traceability system using quantitative NMR. Since that alone is a remarkable accomplishment, I think you should describe this system in a more understandable way. Perhaps you could provide a simple description of the freezing point depression method and how it is used to measure the purity of pure substances.

Question and comment (Hisao Ichijo)

Your paper clearly describes the objectives, how they relate to the demands of society, the elements of technology, and so forth. You determined that the quantitative NMR is appropriate (because it can be applied to a wide range of substances within the uncertainty range the market demands), and that the freezing point depression method is inappropriate (because of crystallization problems). I think your paper will be easier to understand if you explain more fully the research processes by which you came to your conclusions (crystallization is difficult, number of applicable substances is limited, and so on).

Answer (Toshihide Ihara)

We rewrote the paper to change the rationale behind the comparison with freezing point depression method and to focus more closely on the technical structure of quantitative NMR. The freezing point depression method is a well-established technique. We described it only to demonstrate the appropriateness of quantitative NMR.

3 Illustration of research scenarios and integration of elemental technologies

Question and comment (Akira Ono)

Please add some figures illustrating your research scenarios for *Type 2 Basic Research* and the integration of elemental technologies, to make your paper more accessible to a general readership.

Answer (Toshihide Ihara)

We added Fig. 6 to illustrate the process of integrating the elemental technologies to construct the universal calibration technology.

4 Selection of primary standards

Question and comment (Akira Ono)

In the purity determinations of organic compounds in Table 2, benzoic acid, a national RM from NIST of the United States, is used as a primary standard for quantitative NMR. Why didn't you use one of the national RMs as high-purity organic compounds available from AIST?

Answer (Toshihide Ihara)

Benzoic acid (NIST SRM 350a), the NIST national RM used in our study, satisfies the conditions 1) to 3) as outlined in section

5.2. We therefore determined that it is the ideal RM among the national RMs currently available for quantitative NMR. Certain national RMs at AIST, such as potassium hydrogen phthalate (NMIJ CRM 3001-a) and 1,4-dichlorobenzene (NMIJ CRM 4039-a), qualify for condition 1), but potassium hydrogen phthalate does not dissolve easily in organic solvents, and therefore, fails to satisfy condition 2) in our view. Similarly, 1,4-dichlorobenzene is highly sublimable and does not meet condition 3). At present, no national RMs have been developed specifically for quantitative NMR. We are currently in the process of developing the AIST national RMs that satisfy condition 4) as well as 1) to 3).

5 Final status of primary standards

Question and comment (Akira Ono)

You assert that, in principle, the ideal outcome of the application of quantitative NMR would be the development of a single primary standard that serves as the national RM for all organic compounds. Realistically, how many national RMs do you expect are required when this future traceability system is completed? Do you have any specific candidates in mind as organic compounds for the national RMs?

Answer (Toshihide Ihara)

In this study, our priority was to minimize the number of national RMs required, thus reducing development time and expense. That is why we proposed the use of transfer materials in the multi-stage calibration process. Benzoic acid has served as the primary standard for all organic compounds we have measured so far. This success gives us confidence that a traceability system based on a single national RM can be constructed for all organic compounds for which ^{1}H NMR measurement can be performed.

On the other hand, such a traceability system has its disadvantages. Multi-stage calibration is time-consuming and increases uncertainty. If the accuracy or swiftness of analysis becomes more important for users, it is necessary to develop multiple national RMs with different polarities and chemical shifts. We are looking at ways of restricting calibration to single stage. To handle organic compounds that do not have protons, it is necessary to develop quantitative NMR for other nuclei, such as phosphorus and fluorine, along with the corresponding national RMs.

6 Preparation and use of transfer materials

Question and comment (Akira Ono)

I ask about how the transfer materials are used. When this new, efficient traceability system is completed in the future, will AIST produce, store, and disseminate these transfer materials as needed? Or can the reagent manufacturers that produce working RMs make the transfer material when needed, and dispose it when they are done?

Answer (Toshihide Ihara)

In our paper, we envisioned the transfer material to be prepared by the developers or suppliers of the working RMs (RM producers) according to their objectives. To ensure appropriate evaluations, the transfer materials will not be prepared for each batch, but the RM producers will be responsible for producing and storing them for a certain period.

Also, as described in chapter 7, if quantitative NMR becomes widely used as a quantitative analytical method for organic compounds, prepared transfer materials can be used. Moreover, AIST or RM producers may supply easy-to-use transfer materials as RMs.

7 Comparison of quantitative NMR and freezing point depression method

Question and comment (Akira Ono)

My question concerns the analytical results in Table 2. In the freezing point depression method, uncertainty for purity determinations rarely exceeds the upper limit of 100 %, whereas in many cases using quantitative NMR, the upper limit for analytical result exceeds 100 %. Such results are unreasonable. Since the freezing point depression method directly measures impurities in pure substance, the upper limit for analytical result over 100 % is rare. Using quantitative NMR, on the other hand, measurement of the main components is performed when the concentration of the pure substance is diluted to about 1000 mg/L. Isn't this one reason why the upper limit for analytical result can rise above 100 %? Isn't this the case where quantitative NMR is fine for measuring components in a solution but is inappropriate for measuring the purity of pure substances? If so, quantitative NMR seems to be most promising for *Product Realization Research* surrounded by the dotted line in Fig. 6. I'd like to hear the authors' views on this.

Answer (Toshihide Ihara)

Although the factors contributing to the uncertainty of quantitative NMR are not separated in Fig. 5 between preparation uncertainty and measurement uncertainty, preparation uncertainty is not relatively small. Thus, when applied to purity determination, quantitative NMR is undeniably inferior to the freezing point depression method in terms of uncertainty for preparation of solutions, and purity determination higher than the upper limit for analytical result exceeding 100 % is obtained as a result, as you pointed out (however, this does not indicate any bias in the purity determinations).

The freezing point depression method cannot be applied to measure concentrations of components in solution, but there are many examples where the characteristics of quantitative NMR can be applied, as you also pointed out. Because many organic solvents contain hydrogen, we must find ways of reducing these effects so NMR can be applied to protons. In *Product Realization Research*, including the development of quantitative NMR equipment, solving the issue of protons in solution and enabling measurement of concentrations of components in solution are keys to establishing the use of quantitative NMR.

8 Other candidates for universal calibration technologies

Question and comment (Akira Ono)

In chapter 7, "Future Directions," you raised the possibility that universal calibration technologies other than quantitative NMR may be found in the future. Are there any candidate calibration technologies at this time?

Answer (Toshihide Ihara)

In section 4.1, we stated that a universal calibration technology should theoretically be an analytical method qualified as a primary ratio method (measures the value of a ratio of an unknown to a standard of the same quantity; its operation must be completely described by a measurement equation).

Although not yet established as an analytical technique, one candidate the Authors are examining is a combination of chromatography and atomic emission spectrometry. In this process, the analytes are separated from the sample by chromatography. Then each analyte is introduced into high-temperature plasma and atomized into constituent carbon, hydrogen, oxygen, and other atoms. These atoms can then be measured to find the emission of spectrally separated (for example) carbon atoms. By adding a primary standard containing a known quantity of carbon to the sample, the quantity can be combined with the emission of carbon to find the quantity of analyte, as the primary standard itself is also atomized. The point here is that the efficiency of atomization is not dependent on the molecular species. Currently, the combination of gas chromatography and helium-plasma atomic emission spectrometry can obtain uncertainty of 5 % (95 % confidence interval). Further

improvements are needed for the commercialization of this technique.

9 Reason for using deuterated solvents

Question and comment (Akira Ono)

You noted in section 4.2 that you used a deuterated solvent. Can you explain why you used deuterated solvents for quantitative NMR? Should we infer that using ^{1}H (proton) solvents disable quantitative NMR?

Answer (Toshihide Ihara)

In our study, ^{1}H was used as the measurement nucleus. When solvents contain ^{1}H or protonated solvents are used, the ^{1}H signals from the solvents become much stronger than those from the compounds intended to be observed. As a result, the dynamic range of an instrument may prevent the accurate measurement of the analyte signal. Deuterated solvents are used to minimize the ^{1}H from the solvents to resolve this problem. This is, in general, not just for quantitative NMR, but is also for conventional ^{1}H NMR measurements.

On the other hand, in the international comparison of ethanol, aqueous solution was used, and the solvent in this case was protonated water (H_2O) rather than deuterated water (D_2O). Therefore, the problem of dynamic range may occur. In such case, the resonance frequency of the solvent (water) signal is irradiated selectively with low power radio frequency pulse to saturate this signal. This saturation pulse is immediately followed by a normal pulse. This approach, called the pre-saturation method, cancels the interference of a strong H_2O peak. Although power applied to this saturation pulse is low, peaks resonating at nearby frequencies are influenced by the pulse. This may compromise the accuracy of the analytical value obtained in this method. In other cases, irradiation strength, duration, and other factors must be set correctly to obtain the accurate analytical values. For these reasons, it is simple and safe to use a deuterated solvent.

Additionally, to maintain the stability of the magnetic field, resonance frequency of the deuterium signal from the solvent is monitored to adjust the strength of the magnetic field from time to time to maintain constancy of the signal frequency. This process is called a “deuterium lock.” Since NMR measurements, including quantitative NMR, tend to take relatively long time, deuterium lock is indispensable to obtain spectra of high resolution. If the sample solvent is not deuterated, deuterated solvent must be added.