QNMR for Reference Material Preparation

Subjects: Chemistry, Analytical

Contributor: Kihwan Choi

Quantitative nuclear magnetic resonance (qNMR) has been used as a purity determination method for reference material development, and many related measurement techniques have been designed to acquire accurate and reliable results.

Keywords: qNMR ; purity assay ; reference material ; multidimensional NMR

1. Introduction

The establishment of metrological traceability is important for achieving the comparability of measurement results. In regard to chemical measurements, traceability to the International System of Units (SI) is realized through the use of calibration standards having certified purity values [1]. The purity of primary calibrators has been traditionally determined by the mass balance method, that is, by subtracting the total amount of impurities from 100%, with impurity quantitation performed using techniques such as gas chromatography, liquid chromatography, thermal gravimetric analysis, and Karl Fischer titration. Although the mass balance method is well-established, it cannot be applied to organic substances, which lack chromophores and nonvolatiles. Furthermore, it is very time- and labor-intensive.

Nuclear magnetic resonance (NMR) spectroscopy holds great promise for quantitative analysis, as the NMR peak area is proportional to the number of nuclei. Recently, quantitative NMR (qNMR) has been used for purity analysis as an alternative to the mass balance method, allowing one to determine analyte purity directly by comparison with a standard and thus simplify the overall purity determination procedure. The Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) working group on organic analysis (OAWG) is currently exploring the potential of qNMR through international comparison [2]. Furthermore, qNMR has been adopted as a purity determination method for pharmacopeia. However, to obtain accurate and reliable results, one should further optimize the qNMR sample preparation procedure and measurement parameters.

2. Recent Advances of qNMR

2.1. Heteroatoms

Biological samples often comprise phosphorus-containing analytes, and phosphorylation is one of the most important reactions in biosystems, while fluorine is often present in drugs, pesticides, and perfluorinated polymers. Therefore, ^{31}P and ^{19}F hold great promise as nuclei for qNMR analysis. Owing to the presence of a limited number of ^{31}P and ^{19}F nuclei in most chemicals, ^{31}P and ^{19}F NMR spectra tend to be less complex than ^{1}H spectra, thereby decreasing the possibility of peak overlap and allowing solvent interference to be avoided, since these nuclei are not present in common solvents. Furthermore, ^{31}P and ^{19}F NMR spectra feature better peak separations, as the chemical shift range of ^{31}P and ^{19}F exceeds that of ^{1}H. Moreover, the natural abundance of ^{31}P and ^{19}F equals 100%, while the corresponding sensitivity is comparable to that of ^{1}H NMR. On the other hand, the broad dispersion of chemical shifts observed for ^{31}P and ^{19}F precludes the application of uniform excitation to the entire shift range, which results in the loss of signal intensity at the edge of the range. Therefore, a novel pulse sequence was developed to increase the frequency range [3], and uniform excitation was realized for wide ranges using multiple chirp pulses. The effects of experimental parameters including T_1 relaxation time, excitation bandwidth, and number of transients were also investigated [4].

2.2. Coupling with Chromatography

Unlike to the mass balance method, qNMR is poorly suited for the separation and analysis of impurities with similar structures, as the analyte peaks of analyte may overlap with those of the impurities. Chromatography is a well-established separation technique that allows the risk of overestimation to be reduced by the separation of the analyte from impurities prior to qNMR measurement. Both on-line and off-line coupling were studied. For on-line coupling, NMR measurement conditions may not be identical for the separated analyte and the internal standard (IS), which can be accounted for by

adding the IS to the mobile phase and measuring the analyte and the IS simultaneously . On the other hand, a known amount of IS was injected into a separation column independently from the analyte when isocratic elution was applied. The injected and measured amounts agreed in the LC-NMR experiments [5]. In addition, the chromatograms indicated the separation of the analyte from impurities. Offline hyphenation is simple and straightforward. An IS-fortified sample solution was analyzed by liquid chromatography, and impurity-free fractions containing the analyte and the IS were collected into the same vial, with subsequent purity assessment performed by qNMR [6]. Unlike in the case of on-line LC-NMR analysis, no deuterated solvents were used, and a signal suppression method was applied during NMR measurement. The method developed to correct for the loss of analyte and IS during fraction collection was named as internal standard recovery correction (ISRC)-HPLC-qNMR [7]. Analyte was mixed with IS and injected into an LC column. The analyte- and IS-containing fractions were collected and subjected to qNMR analysis. Additionally, a recovery correction factor was calculated by comparing the analyte: comparing the IS peak area ratios of the initial sample and the collected solution. The initially obtained purities (77.08–171.31%) were corrected to 92.87–93.29% using the ISRC method. This method was further developed for internal standard correction (ISC)-HPLC-qNMR [8]. In this case, the IS was added to the analyte-containing fraction and not to the sample itself before separation. Another solution prepared by the accurate weighing of the non-purified initial sample and the IS was analyzed by LC-UV. When the impurity identified during in LC separation was collected and measured by ^{1}H NMR spectroscopy, the overlap of analyte and impurity peak was confirmed. ISC-HPLC-qNMR was used to correct the purity of the candidate material from 99.7 ± 0.3% to 97.6 ± 0.5%, which well agreed with the reference purity determined by the mass balance method (97.61 ± 0.22%). An extended IS method for ^{1}H qNMR assisted by chromatography (EIC) was developed to perform accurate quantitative analysis without the bias of qNMR due to the overlap with impurity peaks [9]. The relative molar sensitivity (RMS) between the IS and analyte-overlapping impurity was calculated from the chromatography-determined response ratio and the molar ratio obtained by qNMR. Sample solution was analyzed by chromatography, and the impurity: IS molar ratio in this solution was obtained using RMS. Then analyte purity was determined by correcting the analyte peak area using the chromatography result. The purity of 2-chlorophenol containing phenol as impurity was determined to show the validity of the EIC approach.

2.3. Multidimensional NMR

Multidimensional NMR is a promising solution to the problems posed by peak overlap in qNMR, allowing overlapping peaks to be discriminated using the addition of an orthogonal dimension to one-dimensional NMR. However, this approach suffers from a prolonged acquisition time and the dependence of peak volume on various parameters, such as homo-/heteronuclear coupling constants, off-resonance effect, and pulse sequence delay. Several endeavors were made to figure out the drawbacks of multidimensional NMR measurements. Quantitative analysis was performed using heteronuclear single quantum correlation (HSQC). In HSQC experiments, peak volume (V_c) is related to the heteronuclear $^{1}J_{CH}$ coupling constant and the duration of polarization transfer delay (Δ) as $V_c \propto \sin^2(\pi\Delta^{1}J_{CH})$. As the $^{1}J_{CH}$ coupling is specific to the analyte site, the proper setting of Δ values is a challenging task. Heikkinen et al. averaged four HSQC spectra obtained using optimized Δ values [10], applying this approach to the quantitative analysis of lignin isolated from wood. The major drawback of averaging is the increased experiment time. Hence quick, quantitative HSQC (QQ-HSQC) was developed to circumvent this problem [11]. As signals corresponding to shorter and longer delays were obtained in a single scan, the measurement time was identical to that of conventional HSQC. QQ-HSQC was applied to strychnine quantitation in solution, affording results comparable to those of Q-HSQC. Homonuclear J_{HH} coupling was removed by including Carr-Purcell-Meiboom-Gill (CPMG) pulse trains in the polarization transfer period [12]. The effect of resonance offset on the edge of the ^{13}C chemical shift was minimized by replacing 90° rectangle pulses with composite ones having an offset compensation. Adiabatic inversion pulses on the ^{13}C channel were employed during the polarization transfer period to improve the accuracy of quantitative analysis [13]. This quantitative, offset-compensated, CPMG-adjusted HSQC (Q-OCCAHSQC) method was applied to strychnine and human blood plasma samples.

Another strategy of calculating a time-zero HSQC spectrum named as $HSQC_0$ was proposed by the Markley group [14]. HSQC spectra were acquired with varying repetition numbers, and $HSQC_0$ was calculated by extrapolation assuming that the logarithm of the signal intensity linearly depends on the repetition number. As $HSQC_0$ corresponds to the hypothetical peak volume obtained at an evolution delay of zero, site specificity can be ignored. Compared to the works of the Heikkinen group, transverse relaxation is considered in this method, and a more reliable result can be obtained. When metabolite concentrations were measured in the presence of IS, noise from higher-concentration metabolites hindered the application of $HSQC_0$. Consequently, this technique was improved using gradient-selective HSCQ spectra and the fast maximum likelihood reconstruction approach [15]. Furthermore, two ISs having high and low concentrations were used to increase accuracy and precision. Rai et al. calculated a correction factor based on theoretical considerations and applied it to the quantification of metabolites in urine samples [16]. However, theoretical calculations require information related to

the relaxation process, including coupling constants and relaxation times. Moreover, calibration curves generated for each metabolite were employed for HSQC-based quantitative analysis [17]. Fardus-Reid et al. investigated the bias and uncertainty contribution of measurement parameters [18].

3. Reference Material Development

3.1. Internal Standard

The usage of IS is an essential requirement for purity assessment, with IS values assigned by higher-order methods viewed as critical for the acquisition of SI-traceable purity assessment results. To determine the purity of a wide range of chemicals with various solubilities and chemical shifts, Weber et al. developed 12 CRMs suitable for use as IS in qNMR analysis [19]. The purities of 12 IS candidates were characterized using two benzoic acid CRMs and a potassium hydrogen phthalate (KHP) CRM to maintain SI traceability. The reliability of the obtained values was increased by measuring purities in multiple ways using qNMR. For example, the purity of maleic acid was determined directly using three CRMs as well as using secondary ISs whose purities were determined by maleic acid. The six purity values all agreed within the expanded uncertainty. These candidates were certified, and stability tests were performed to satisfy the ISO guidelines [20]. Westwood et al. developed seven SI-traceable ISs including CRMs certified by NMIs using titrimetric methods [21]. The purities of other compounds were assigned by qNMR using CRMs as ISs to achieve SI traceability. NMR measurement parameters were optimized to obtain accurate results with the relative uncertainties at the level of 0.5%. The relaxation delay time was chosen as 15 times the spin-lattice relaxation delay times and the pulse offset location was recommended to lie within 2 ppm of the midpoint of two quantification signals. If the signal-to-noise ratio was less than 1000, measurement uncertainties were significantly increased. These results were consistent with those of previous studies, and the recommended parameters settings would be suitable for a wide range of qNMR applications. The purity of each IS was assigned by combining values which were obtained using various combinations of ISs and solvents. The properties of ISs including chemical shifts, and solubility in various solvents of internal standards are summarized.

The use of an IS with a purity assigned by a higher-order method is critical to obtain SI-traceable purities. Benzoic acid developed as an organic acidimetric standard by National Institute of Standards and Technology (NIST, standard reference material 350b) were used for this purpose. However, the number of protons does not explicitly represent that of benzoic acid. Recently, NIST developed a benzoic acid CRM (PS1) with a purity that was certified by coulometry and ^{1}H NMR [22]. The purities of 4 ISs for use in qNMR measurements were assessed using NIST SRM 350b benzoic acid and SRM 84k KHP, which had been previously calibrated by acidimetry. Then, purity was determined by combining the results obtained by ^{1}H qNMR using the ISs, and the purity of PS1 was also assessed independently by coulometry. The result of purity assessment by coulometry and ^{1}H NMR were combined to give a value of 999.92 mg/g (-0.00006 to +0.00004). CRM purity was also determined by the mass balance method to confirm the assigned value. Except for water and nonvolatile ash, no structurally related impurities were detected, and the purity of PS1 was determined as 999.9 mg/g (999.83 to 999.97 mg/g with a 95% confidence interval) by the mass balance method.

IS reference materials for ^{31}P qNMR and ^{19}F NMR were also developed. Weber et al. developed two certified reference materials, phosphonoacetic acid and triphenyl phosphate which are soluble in water and organic solvents, respectively [23]. The purity of phosphonoacetic acid was certified by ^{31}P qNMR and ^{1}H qNMR with ammonium dihydrogen phosphate and KHP as ISs, respectively. The two purity values agreed within the expanded uncertainties. As the sensitivity of ^{31}P NMR is lower than that of NMR, the former uncertainty was larger. The purity of triphenyl phosphate was certified using ^{1}H qNMR with NIST benzoic acid as an IS. Signal overlap prohibited direct quantification, and dimethyl terephthalate was therefore employed as a secondary standard. Validation was performed by determining the purity of tris(2-chloroethyl) phosphate using two ISs independently. For use in ^{19}F qNMR, the National Metrology Institute of Japan (NMIJ) distributes two CRMs, BTFMBA and 1,4-bis(trimethylsilyl)-2,3,5,6-tetrafluorobenzene. Rigger et al. developed three ISs for ^{19}F qNMR measurements traceable to NMIJ and NIST CRMs [24].

3.2. High Purity Reference Materials

Carbohydrate certified reference materials were developed using ^{1}H qNMR [25]. As carbohydrates lack chromophores, only water and trace metal contents were measured by the mass balance approach. The purities determined by qNMR were lower than those obtained using the mass balance method for five out of six carbohydrates. Certified purities were determined from the weighted average of purities obtained by qNMR and the mass balance method.

NMIJ is currently establishing the traceability scheme of multi-component CRMs by adopting qNMR. For this purpose, the purities of more than 80 pesticides were assessed using a single CRM (1,4-dichloro benzene) [26]. To avoid peak overlap between the CRM and analytes, secondary standards calibrated using the CRM were used for purity determination. For method validation, linear regression analysis and comparison with differential scanning calorimetry (DSC) data were

performed. Calibration services for purity assessment were provided using qNMR validated with DSC and chromatography by NMIJ. The purities of pharmaceutical reference materials were determined using the PUlse Length based CONcentration determination (PULCON) method, and the obtained values were compared with those by the IS method [27]. The PULCON method obviates the need for IS selection and addition, allowing one to simplify and accelerate analysis without losing accuracy.

Josephs et al. assessed the purity of angiotensin I using qNMR [28]. As the sample contained peptide impurities whose peaks could overlapped with those of angiotensin I, the qNMR result was corrected using the data obtained by LC-high resolution mass spectrometry. This method was denoted as qNMR with a correction for structurally related peptide impurities (PICqNMR) and afforded purity that well agreed with the values obtained using the mass balance method, peptide impurity corrected amino acid analysis, and peptide impurity corrected elemental analysis. Melanson et al. developed angiotensin II CRM using qNMR [29]. The purity of candidate materials was determined using the qNMR result corrected by amino acid analysis. Alternatively, the purity obtained by the mass balance method was corrected by subtracting the amount of trifluoroacetate counter ion measured using ^{19}F qNMR.

References

1. Steven Westwood; Tiphaine Choteau; Adeline Daireaux; Ralf Dieter Josephs; Robert Ian Wielgosz; Mass Balance Method for the SI Value Assignment of the Purity of Organic Compounds. *Analytical Chemistry* **2013**, *85*, 3118-3126, 10.1021/ac303329k.
2. Takeshi Saito; Taichi Yamazaki; Masahiko Numata; Development of nuclear magnetic resonance as a tool of quantitative analysis for organic materials. *Metrologia* **2019**, *56*, 054002, 10.1088/1681-7575/ab348d.
3. J. E. Power; Mohammadali Foroozandeh; R. W. Adams; Mathias Nilsson; S. R. Coombes; Andrew Phillips; G. A. Morris; Increasing the quantitative bandwidth of NMR measurements. *Chemical Communications* **2016**, *52*, 2916-2919, 10.1039/C5CC10206E.
4. Allison O. Mattes; David Russell; Evgeny Tishchenko; Yanzhou Liu; Robert H. Cichewicz; Sarah J. Robinson; Application of 19 F quantitative NMR to pharmaceutical analysis. *Concepts in Magnetic Resonance Part A* **2016**, *45A*, e21422, 10.1002/cmr.a.21422.
5. Markus Godejohann; Alfred Preiss; Clemens Mügge; Quantitative Measurements in Continuous-Flow HPLC/NMR. *Analytical Chemistry* **1998**, *70*, 590-595, 10.1021/ac970630s.
6. Ting Huang; Wei Zhang; Xinhua Dai; Na Li; Liang Huang; Can Quan; Hongmei Li; Yi Yang; High performance liquid chromatography-quantitative nuclear magnetic resonance (HPLC-qNMR) with a two-signal suppression method for purity assessment of avermectin B1a. *Analytical Methods* **2016**, *8*, 4482-4486, 10.1039/c6ay00570e.
7. Wei Zhang; Ting Huang; Hongmei Li; Xinhua Dai; Can Quan; Yajuan He; Determination of avermectins by the internal standard recovery correction - high performance liquid chromatography - quantitative Nuclear Magnetic Resonance method. *Talanta* **2017**, *172*, 78-85, 10.1016/j.talanta.2017.04.080.
8. Xiaonan Sun; Wei Zhang; Ting Huang; Yajuan He; Hongmei Li; Ping Su; Yi Yang; Purity determination of pyributicarb by internal standard correction–high-performance liquid chromatography–quantitative nuclear magnetic resonance. *Analytical and Bioanalytical Chemistry* **2020**, *412*, 6983-6993, 10.1007/s00216-020-02832-0.
9. Naoki Saito; Yuko Kitamaki; Satoko Otsuka; Noriko Yamanaka; Yuzo Nishizaki; Naoki Sugimoto; Hisanori Imura; Toshihide Ihara; Extended internal standard method for quantitative 1H NMR assisted by chromatography (EIC) for analyte overlapping impurity on 1H NMR spectra. *Talanta* **2018**, *184*, 484-490, 10.1016/j.talanta.2018.03.003.
10. Sami Heikkinen; Merja M. Toikka; Pirkko T. Karhunen; Ilkka A. Kilpeläinen; Quantitative 2D HSQC (Q-HSQC) via Suppression ofJ-Dependence of Polarization Transfer in NMR Spectroscopy: Application to Wood Lignin. *Journal of the American Chemical Society* **2003**, *125*, 4362-4367, 10.1021/ja029035k.
11. Daniel J. Peterson; Nikolaus M. Loening; QQ-HSQC: a quick, quantitative heteronuclear correlation experiment for NMR spectroscopy. *Magnetic Resonance in Chemistry* **2007**, *45*, 937-941, 10.1002/mrc.2073.
12. Harri Koskela; Ilkka Kilpeläinen; Sami Heikkinen; Some aspects of quantitative 2D NMR. *Journal of Magnetic Resonance* **2005**, *174*, 237-244, 10.1016/j.jmr.2005.02.002.
13. Harri Koskela; Outi Heikkilä; Ilkka Kilpeläinen; Sami Heikkinen; Quantitative two-dimensional HSQC experiment for high magnetic field NMR spectrometers. *Journal of Magnetic Resonance* **2010**, *202*, 24-33, 10.1016/j.jmr.2009.09.021.
14. Kaifeng Hu; William M. Westler; John L. Markley; Simultaneous Quantification and Identification of Individual Chemicals in Metabolite Mixtures by Two-Dimensional Extrapolated Time-Zero1H−13C HSQC (HSQC0). *Journal of the American Chemical Society* **2011**, *133*, 1662-1665, 10.1021/ja1095304.

15. Kaifeng Hu; James J. Ellinger; Roger A. Chylla; John L. Markley; Measurement of Absolute Concentrations of Individual Compounds in Metabolite Mixtures by Gradient-Selective Time-Zero1H–13C HSQC with Two Concentration References and Fast Maximum Likelihood Reconstruction Analysis. *Analytical Chemistry* **2011**, *83*, 9352-9360, 10.1021/ac201948f.
16. Ratan Kumar Rai; Pratima Tripathi; Neeraj Sinha; Quantification of Metabolites from Two-Dimensional Nuclear Magnetic Resonance Spectroscopy: Application to Human Urine Samples. *Analytical Chemistry* **2009**, *81*, 10232-10238, 10.1021/ac902405z.
17. Wolfram Gronwald; Matthias S. Klein; Hannelore Kaspar; Stephan R. Fagerer; Nadine Nürnberger; Katja Dettmer; Thomas Bertsch; Peter J. Oefner; Urinary Metabolite Quantification Employing 2D NMR Spectroscopy. *Analytical Chemistry* **2008**, *80*, 9288-9297, 10.1021/ac801627c.
18. Fahmina Fardus-Reid; Adam Le Gresley; John Warren; Validating heteronuclear 2D quantitative NMR. *Analytical Methods* **2016**, *8*, 2013-2019, 10.1039/C6AY00111D.
19. Michael Weber; Christine Hellriegel; Alex Rück; Robert Sauermoser; Jürg Wüthrich; Using high-performance quantitative NMR (HP-qNMR®) for certifying traceable and highly accurate purity values of organic reference materials with uncertainties <0.1 %. *Accreditation and Quality Assurance* **2013**, *18*, 91-98, 10.1007/s00769-012-0944-9.
20. Michael Weber; Christine Hellriegel; Alexander Rueck; Juerg Wuethrich; Peter Jenks; Using high-performance 1H NMR (HP-qNMR®) for the certification of organic reference materials under accreditation guidelines—Describing the overall process with focus on homogeneity and stability assessment. *Journal of Pharmaceutical and Biomedical Analysis* **2014**, *93*, 102-110, 10.1016/j.jpba.2013.09.007.
21. Steven William Westwood; Taichi Yamazaki; Ting Huang; Bruno Carius Garrido; Ilker Un; Wei Zhang; Gustavo Martos; Norbert Stoppacher; Takeshi Saito; Robert Wielgosz; et al. Development and validation of a suite of standards for the purity assignment of organic compounds by quantitative NMR spectroscopy. *Metrologia* **2019**, *56*, 064001, 10.1088/1681-7575/ab45cb.
22. Michael A. Nelson; Jason F. Waters; Blaza Toman; Brian E Lang; A. Rück; Kathrin Breitruck; Markus Obkircher; Tony W indust; Katrice A. Lippa; A New Realization of SI for Organic Chemical Measurement: NIST PS1 Primary Standard for Quantitative NMR (Benzoic Acid). *Analytical Chemistry* **2018**, *90*, 10510-10517, 10.1021/acs.analchem.8b02575.
23. Michael Weber; Christine Hellriegel; Alexander Rueck; Juerg Wuethrich; Peter Jenks; Markus Obkircher; Method development in quantitative NMR towards metrologically traceable organic certified reference materials used as (31)P qNMR standards.. *Analytical and Bioanalytical Chemistry* **2014**, *407*, 3115-23, 10.1007/s00216-014-8306-6.
24. Romana Rigger; Alexander Rück; Christine Hellriegel; Robert Sauermoser; Fabienne Morf; KathrinBreitruck Breitruck; Markus Obkircher; Certified Reference Material for Use in 1H, 31P, and 19F Quantitative NMR, Ensuring Traceability to the International System of Units. *Journal of AOAC International* **2017**, *100*, 1365-1375, 10.5740/jaoacint.17-0093.
25. Can Quan; Establishment of the purity values of carbohydrate certified reference materials using quantitative nuclear magnetic resonance and mass balance approach. *Food Chemistry* **2014**, *153*, 378-386, 10.1016/j.foodchem.2013.12.086.
26. Takeshi Saito; Toshihide Ihara; Tooru Miura; Yuuko Yamada; Koichi Chiba; Efficient production of reference materials of hazardous organics using smart calibration by nuclear magnetic resonance. *Accreditation and Quality Assurance* **2011**, *16*, 421-428, 10.1007/s00769-011-0798-6.
27. Yulia B. Monakhova; Matthias Kohl-Himmelseher; Thomas Kuballa; Dirk W. Lachenmeier; Determination of the purity of pharmaceutical reference materials by 1 H NMR using the standardless PULCON methodology. *Journal of Pharmaceutical and Biomedical Analysis* **2014**, *100*, 381-386, 10.1016/j.jpba.2014.08.024.
28. Ralf D. Josephs; Norbert Stoppacher; Adeline Daireaux; Tiphaine Choteau; Katrice A. Lippa; Karen W. Phinney; Steven Westwood; Robert I. Wielgosz; State-of-the-art and trends for the SI traceable value assignment of the purity of peptides using the model compound angiotensin I. *TrAC Trends in Analytical Chemistry* **2018**, *101*, 108-119, 10.1016/j.trac.2017.09.026.
29. Jeremy E. Melanson; Marie-Pier Thibeault; Bradley B. Stocks; Donald M. Leek; Garnet McRae; Juris Meija; Purity assignment for peptide certified reference materials by combining qNMR and LC-MS/MS amino acid analysis results: application to angiotensin II. *Analytical and Bioanalytical Chemistry* **2018**, *410*, 6719-6731, 10.1007/s00216-018-1272-7.