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Collaborative Study to Validate Purity Determination by ^{1}H quantitative NMR Spectroscopy by Using Internal Calibration Methodology

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Summary

Nuclear Magnetic Resonance (NMR) spectroscopy has recently been utilized to determine the absolute amounts of organic molecules with metrological traceability since signal intensity is directly proportional to the number of each nucleus in a molecule. The NMR methodology that uses hydrogen nucleus (^{1}H) to quantify chemicals is called quantitative ^{1}H NMR (^{1}H qNMR). The quantitative method using ^{1}H qNMR for determining the purity or content of chemicals has been adopted into some compendial guidelines and official standards. However, there are still few reports in the literature regarding validation of ^{1}H qNMR methodology. Here, we coordinated an international collaborative study to validate a ^{1}H qNMR based on the use of an internal calibration methodology. Thirteen laboratories participated in this study, and the purities of three samples were individually measured using ^{1}H qNMR method. The three samples were all certified via conventional primary methods of measurement, such as butyl *p*-hydroxybenzoate Japanese Pharmacopeia (JP) reference standard certified by mass balance; benzoic acid certified reference material (CRM) certified by coulometric titration; fludioxonil CRM certified by a combination of freezing point depression method and ^{1}H qNMR. For each sample, ^{1}H qNMR experiments were optimized before quantitative analysis. The results showed that the measured values of each sample were equivalent to the corresponding reference labeled value. Furthermore, assessment of these ^{1}H qNMR data using the normalized error, E_n-value, concluded that statistically ^{1}H qNMR has the competence to obtain the same quantification performance and accuracy as the conventional primary methods of measurement.

Keywords: qNMR; Method validation; Collaborative study; Metrological traceability; Measurement uncertainty

1. Introduction

Nuclear Magnetic Resonance (NMR) spectroscopy has played a crucial role in structure elucidation using the information on chemical shift value, ratio of signal area, spin-spin coupling, coupling constant etc. In an NMR spectrum, the area of a signal is proportional to the number of nuclei it represents. Furthermore, the resonance frequency of each hydrogen nucleus in a molecule is shifted according to the difference in chemical environment. Therefore, it is possible to determine the number of nuclei or the number of moles of the analyte in a sample by adding a known amount of a metrologically traceable surrogate material as an internal standard for qNMR to the sample and comparing the signal area between the analyte and qNMR standard. However, in order to obtain an accurate quantitative value, it is necessary to optimize measurement procedure that allows an accurate sample preparation, an accurate ratio of the signal area between the analyte and qNMR standard, etc. In addition, due to the inherently low sensitivity of NMR, quantitation methodology using hydrogen nucleus (proton; ^{1}H) (^{1}H qNMR) has rarely been used except for some purposes. On the other hand, in recent years, ^{1}H qNMR methodology have begun to be investigated, and multiple studies have demonstrated that accurate quantification with metrological traceability can be achieved via ^{1}H qNMR under optimized conditions [1-16] Additionally, ^{1}H qNMR has already been introduced into some compendial guidelines and official standards in such as United States Pharmacopeia (USP), Japanese Pharmacopeia (JP), and Japan's Specifications and Standards for Food Additive as a quantitation method for determining accurate purities or contents of analytical standards. As one outcome of collaborative studies, ^{1}H qNMR was adopted in the Japanese Industrial Standard (JIS) as JIS K 0138 (General rules for quantitative nuclear magnetic resonance spectroscopy) in January 2018. [17] ^{1}H qNMR can efficiently provide accurate quantitative values without identifying and quantifying any impurities in a sample, whereas the mass balance method needs to perform them. [18, 19] Therefore, its widespread application is expected in the fields of pharmaceutical and food analyses. There have been only a few reports describing validation of the ^{1}H qNMR methodology [20-23], however detailed investigations about the performance of ^{1}H qNMR in comparison to other established methodologies are not very common. In the present study, we organized an international collaborative study on ^{1}H

qNMR to evaluate the accuracy of ^{1}H qNMR for purity determination using the internal calibration methodology and assayed three reliable purity-assigned samples whose purities (mass fraction, %) were certified by the conventional primary methods of measurement [24]: mass balance, coulometric titration, and a combination of melting point depression method and ^{1}H qNMR. A total of thirteen laboratories participated in this international collaborative study from all over the world. Among them, eight laboratories were from Japan, two were from U.S., two were from Germany and one was from Italy.

2. Results and discussion

2.1. Experimental design of collaborative study for ^{1}H qNMR

2.1.1. Protocols and purity calculation spread sheets

The coordinating laboratory developed a measurement protocol and a purity calculation spread sheet for each sample that can perform the process of purity determinations with all collaborating laboratories in the same manner. The protocols with the purity calculation spread sheets were allotted to each collaborating laboratory to test complying with the protocols and to calculate results with an uniform purity calculation method. Each collaborating laboratory performed the testing in accordance with this protocol and using this spread sheet. When any collaborating laboratory modified the protocol, all the information was reported to the coordinating laboratory.

2.1.2. Samples, internal standards for ^{1}H qNMR, and deuterated solvents

First of all, a schematic illustration of the sample solution is shown in Fig. 1. This collaborative study aimed to evaluate the accuracy of ^{1}H qNMR when using the internal calibration methodology for purity determination. To avoid the unexpected errors and influences caused by materials used for this collaborative study, a set of samples, internal standards for ^{1}H qNMR, and deuterated solvents suitable for this purpose were designed. The following criteria were used to select the samples: a) The sample should be a substance with highly reliable purity (i.e., a certified value) that can be used as a reference value for the collaborative study. b) The sample should have fully guaranteed within-bottle homogeneity and between-bottle homogeneity. [25, 26] c) The sample should be a solid with minimum hygroscopicity and volatility that is stably capable of being weighed on a balance.

We selected three samples, as followed:

1) butyl *p*-hydroxybenzoate JP reference standard
2) benzoic acid certified reference material (CRM)
3) fludioxonil CRM

These were all certified via conventional primary method of measurement [5)], such as butyl p-hydroxybenzoate JP reference standard certified by mass balance; benzoic acid CRM certified by coulometric titration; fludioxonil CRM certified by a combination of freezing point depression method and ^{1}H qNMR, and had guaranteed within-bottle homogeneity and between-bottle homogeneity. Samples of the same lot number were then allotted to each collaborating laboratory. For the internal standards for ^{1}H qNMR, three CRMs were selected, whose spectrum had a singlet signal that was sufficiently separated from the analyte signals [27)], as well as guaranteed within-bottle homogeneity and between-bottle homogeneity. Internal standards for ^{1}H qNMR of the same lot number were then allotted to each of the collaborating laboratories. Deuterated solvents that can completely dissolve the samples and internal standards for ^{1}H qNMR and sufficiently separate from each signal of the analyte and ^{1}H qNMR standard were selected. [27)]

2.1.3. Sample preparation procedure

A schematic illustration of the measurement procedure is shown in Fig. 2. To minimize the influence of sample preparation, a protocol was developed to ensure uniform sample preparation procedure among all the collaborating laboratories. First, the *minimum weight* listed in USP-NF General Chapter <41> was calculated in accordance with equation (1) (written below). [28, 29)]

$$W_{\min} = \sigma \times 2000 \quad (1)$$

Where $W_{\min}$: *Minimum weight*

σ : Standard deviation calculated with 10 repeated measurements of tare

The mass of the sample and internal standard for ^{1}H qNMR should be twice the *minimum weight* or more. In connection with the above, the protocol also specified that the collaborating laboratories should use an ultra-microbalance (readability: 0.0001 mg) or microbalance (readability: 0.001 mg). The main reason for this requirement was to ensure weighing accuracy. The secondary reason was to reduce the consumption of expensive

deuterated solvents to make the test more economical. By using ultra-micro or microbalances, the amount of the sample and internal standard for ^{1}H qNMR can be reduced while maintaining accuracy in weighing; as a result, the amount of deuterated solvent required to prepare the sample solution was also reduced. The protocol specified that the collaborating laboratories should use a small and lightweight aluminium weighing dish as a tare for weighing. Although the sample and internal standard for ^{1}H qNMR could be weighed directly into the vial on the weighing pan of the balance, they might adhere outside the vial (i.e., on the edge of the vial or the weighing pan of the balance), leading to error of weighing. Additionally, the surface area of ultra-microbalance weighing pan is extremely small, making it difficult to properly place the vial on the weighing pan.

2.1.4. NMR apparatus and data acquisition parameters

The protocol specified that the collaborating laboratories should follow the measurement conditions described in JP 17th edition and JIS K 0138 [17, 30)] and they optimize the NMR apparatus and data acquisition parameters in accordance with these conditions. First, the NMR apparatus should have a hydrogen (^{1}H) resonance frequency of 300 MHz or higher to ensure satisfactory sensitivity, resolution, and signal separation. The default setting for digital resolution should be no more than 0.25 Hz in order to ensure reliable reproduction of the original analogue data. [31 - 33)] The default spin setting was non-spinning, which prevented spinning side bands from overlapping with the selected analyte signals and qNMR standard signal. [33)] The default setting for the pulse angle was 90°, which provides a better S/N per unit time and higher accumulation efficiency to ensure satisfactory sensitivity. [33)] ^{13}C decoupling was performed by default, which prevented ^{13}C satellite signals from overlapping with the selected analyte signals and ^{1}H qNMR standard signal. [33, 34)] The default delay time was 60 s or more so as to prevent signal absorption saturation. [31, 33)] The default setting for the number of transients was set so that the S/N of each target signal of the analyte and ^{1}H qNMR standard was 1000 or more in order to ensure an accurate signal area.[35)] By default, a digital filter with flat sensitivity over the entire spectral width was used. [33)] Acquisition time is a value that is uniquely determined from the observation spectrum width and the number of data points. By default, these two parameters (observation spectrum width and digital resolution, which is a parameter related

to the number of data points) was set to ensure an acquisition time that was sufficiently long for an accurate signal area to be obtained without truncation artefacts or other types of errors. [33)]

2.1.5. Data processing

The protocol also specified the default settings of data processing for the collaborating laboratories and they optimized the data processing conditions in accordance with the default settings. The collaborating laboratories were instructed to correct the phase of the spectra manually. Although with recent advances in data processing software, satisfactory phase correction can be obtained using automatic phasing processes in many cases, the accuracy of automatic phasing is still inferior to that of manual phasing. [33, 36)] It was also specified that baseline correction should be performed. Normally the baseline of an NMR spectrum has distortions in a way that is not connected to the sample solution. By applying a baseline correction, this can be eliminated, and an accurate signal area can be obtained. [33, 36)]

The information on sample preparation (balance, readability, *minimum weight*, mass of the sample and the internal standard for ^{1}H qNMR), NMR measurement (NMR apparatus and data acquisition parameters), and data processing (data processing software and parameters) for each collaborating laboratory are summarized in Tables 1 and 2. A typical ^{1}H NMR spectrum of each sample is shown in Fig. 3, 4 and 5.

2.2. Method validation

2.2.1. Evaluation of the quantification performance of ^{1}H qNMR by comparison between analytical values and reference values

The analytical results (purity and expanded uncertainty) of each sample provided by each collaborating laboratory can be found in Fig. 6, 7 and 8. For the JP reference standard sample, the solid line indicates purity (100.0 %) and the dotted line indicates acceptable error (±0.5 %) generally recognized in JP reference standard. For the CRM samples, the solid line indicates the certified purity value (mass fraction, %) and the dotted line indicates the expanded uncertainty (k = 2). In the case of benzoic acid CRM, its uncertainty is too small to see the dotted line in the graph magnification. The expanded uncertainty of the measured purity of each collaborating laboratory was evaluated using the combined

standard uncertainty and a coverage factor k = 2. The combined standard uncertainty was evaluated using the following uncertainty component: a) the deviation of the purity determined from three sample preparations, b) the repeatability of the purity determined using one analyte peak and an ^{1}H qNMR standard signal, c) where possible, the deviation among the purity values calculated using different pairs of analyte and ^{1}H qNMR standard signals, d) the uncertainty of the balance used, and e) the uncertainty associated with the purity value of the internal standard for ^{1}H qNMR. For a) - c), the standard uncertainty value for each sample was determined by extracting the variance using analysis of variance (ANOVA) using all the obtained purity values for each sample before they were averaged; in the case of butyl *p*-hydroxybenzoate, 3 test samples × 3 NMR measurements × 4 analyte signals = 36 purities; for benzoic acid, 3 test samples × 3 NMR measurements × 1 analyte signal = 9 purities; for fludioxonil, 3 test samples × 3 NMR measurements × 1 analyte signal = 9 purities. For d), the standard deviation was calculated using the data measured by each collaborating laboratory for the *minimum weight* determination; the standard uncertainty was determined based on the standard deviation. [37] For e), the standard uncertainty was calculated by dividing the expanded uncertainty specified on the certificate of each CRM by the coverage factor (in this case, k=2). Finally, using the laws of propagation of uncertainty, the standard uncertainties a) - e) were combined, to obtain the combined standard uncertainty. [35, 37-40] We evaluated the accuracy of ^{1}H qNMR using internal calibration methodology by comparing the analytical result (purity and expanded uncertainty) from each collaborating laboratory with the reference value of each of the samples. First, as can be seen in Fig. 6, the purities for butyl *p*-hydroxybenzoate determined by all laboratories were within the range of acceptable error and were approximately the same as the reference value. The expanded uncertainty of laboratory 6 was about three times larger than that of the other laboratories. We interpreted that this was because the other laboratories prepared the test samples using at least twice the *minimum weight* value for the analyte and internal standard for ^{1}H qNMR, whereas laboratory 6 prepared the test sample using only the *minimum weight* for the internal standard for ^{1}H qNMR. Also, as shown in Fig. 8, the measured purities for fludioxonil were almost the same as the reference value and within the range of uncertainty for all laboratories except

laboratory 2. The internal standard for ^{1}H qNMR used for fludioxonil was DSS-d_6, which has hygroscopic property. In an ambient environment with a relative humidity between 20 % - 80 %, DSS-d_6 absorbs moisture and then becomes stable as a monohydrate [41], and its certified purity value corresponds to its monohydrate form. Accordingly, the protocol specified that each collaborating laboratory should allow DSS-d_6 to sufficiently absorb moisture before use. We assumed in the case of laboratory 2, the moisture absorption was insufficient, and this insufficient moisture absorption was responsible for the bias in the measured purity values. As shown in Fig. 7 for benzoic acid, as described above, the assigned expanded uncertainty was extremely small; as a result, the measured purity values of all the laboratories fell outside the range of expanded uncertainty. However, the accuracies of the analytical results for benzoic acid were similar to those of butyl *p*-hydroxybenzoate and fludioxonil. Thus, using a measurement protocol optimized for quantitative analysis, ^{1}H qNMR using an internal calibration methodology was confirmed to provide quantification performance and accuracy equivalent to the three conventional primary methods of measurement.

2.2.2. Evaluation of the quantification performance of ^{1}H qNMR using the E_n-value

E_n-value was determined to assess not only the calibration proficiency of each collaborating laboratory, but also the accuracy of ^{1}H qNMR method. The calculation of the E_n-value in this study is shown in equations (2). [42, 43]

$$E_n = \frac{P_{lab} - P_{ref}}{\sqrt{U^2_{lab} + U^2_{ref}}} \qquad (2)$$

Where P_{lab} : Purity (mass fraction, %) measured by each collaborating laboratory

P_{ref} : Purity (mass fraction, %) of the reference value

U_{lab} : Expanded uncertainty of measurement of each collaborating laboratory (k=2)

U_{ref} : Expanded uncertainty of the reference value (k=2)

The value of $-1 \leq E_n \leq 1$ indicates acceptance criteria of comparison between the collaborating laboratory's analytical results and the reference value. The calculated E_n-value is shown in Fig. 9, 10 and 11. In the case of butyl *p*-hydroxybenzoate, the E_n-value of all collaborating laboratories fell within the range of acceptance criteria. In the

case of Benzoic acid, the E_n-value of each collaborating laboratory fell within the range of acceptance criteria except laboratories 1 and 6. We speculated that this was because the expanded uncertainty of the reference value (NIST SRM 350-b) is extremely small. In the case of fludioxonil, the E_n-value of laboratory 2 was out of acceptance criteria. However except laboratory 2, the E_n-value of each collaborating laboratory fell within the range of acceptance criteria. As described above, almost all E_n-values fell within the range of acceptance criteria. Therefore, analytical results of this collaborative study were regarded as satisfactory, according to the recommendation described in ISO/IEC Guide 43-1 [42)], and moreover regarded as the same accuracy as the conventional primary methods of measurement.

3. Conclusions

An international collaborative study involving thirteen laboratories was conducted to validate a method for purity determination using ^{1}H qNMR with internal calibration methodology. According to a protocol optimized for quantitative analysis, each collaborating laboratory measured the purities of three samples which had been certified by three conventional primary methods of measurement. By utilizing a calibrated balance and a metrologically traceable internal standard for ^{1}H qNMR and implementing with a measurement procedure optimized for quantification, ^{1}H qNMR using internal calibration methodology can achieve the same quantification performance and accuracy as conventional primary methods of measurement.

qNMR is a method of quantification that compares moles of nuclei between an analyte and a qNMR standard. It provides not only metrologically traceable and accurate quantification, but also the versatility to be used with a wide range of compounds. Additionally, ^{1}H qNMR using internal calibration methodology was confirmed to be suitable for determining the purity of small organic molecules with high accuracy. In the future, method validation also needs to be performed for impurity analysis with low S/N and the analysis of large molecules and mixtures in which it is difficult to obtain satisfactory signal separation. qNMR will be widely used in fields such as pharmaceuticals and food science and is expected to contribute to ensuring the reliability of analytical

results in future.

4. Experimental

4.1. Samples, internal standards for ^{1}H qNMR, and deuterated solvents

Samples, internal standards for ^{1}H qNMR, and deuterated solvents in the collaborative study were specified, as followed:

1). Test for butyl *p*-hydroxybenzoate:

a) Sample: Butyl *p*-hydroxybenzoate, a JP reference standard whose purity is assigned using mass balance (Control No.: BPB01030101, the Pharmaceutical and Medical Device Regulatory Science Society of Japan, Osaka, Japan).

b) Internal standard for ^{1}H qNMR: 1,4-bis(trimethysilyl)benzene-d_4 (1,4-BTMSB-d_4), an SI-traceable, certified reference material (CRM) that can be used as internal standard for ^{1}H qNMR (Certified value (mass fraction, %): 99.9 ± 0.5, Code No.: 024-17031, Lot No.: TWN2900, FUJIFILM Wako Pure Chemical, Osaka, Japan).

c) Deuterated solvent: Acetone-d_6 , a colorless, transparant, volatile liquid having a purity of 99.0 % or higher (GC), and a deuteration ratio of 99.9 % or higher (Code No.: 010-26682, FUJIFILM Wako Pure Chemical, Osaka, Japan), or equivalent item.

2). Test for benzoic acid:

a) Sample: Benzoic acid, an SI-traceable, CRM certified using coulometric titration as the dried material (Certified value (mass fraction, %)($^{w}C_6H_5COOH$): 99.9978±0.0044, NIST SRM 350-b, National Institute of Standards and Technology, Gaithersburg, USA).

b) Internal standard for ^{1}H qNMR: Dimethyl sulfone, an SI-traceable, CRM that can be used as internal standard for ^{1}H qNMR (Certified value (mass fraction, %): 100.0 ± 0.5, Code No.: 048-33271, Lot No.: TWP1766, FUJIFILM Wako Pure Chemical, Osaka, Japan).

c) Deuterated solvent: Methanol-d_4, a colorless, transparant, volatile liquid having a purity of 99.0 % or higher (GC), and a deuteration ratio of 99.8 % or higher (Code No.: 130-18702, FUJIFILM Wako Pure Chemical, Osaka, Japan), or equivalent

item.

3). Test for fludioxonil:

a) Sample: Fludioxonil, an SI-traceable, CRM certified using combination method of freezing point depression method with Differential Scanning Calorimetry (DSC) and ^{1}H qNMR (Certified value (mass fraction, %): 99.7 ± 0.7, Code No.: 064-06001, Lot No.: ECR1966, FUJIFILM Wako Pure Chemical, Osaka, Japan).

b) Internal standard for ^{1}H qNMR: 3-(trimethysilyl)-1-propane–1,1,2,2,3,3-d_6-sulfonic acid sodium salt (DSS-d_6), an SI-traceable, CRM that can be used as internal standard for ^{1}H qNMR (Certified value (mass fraction, %): 92.4 ± 0.5, Code No.: 044-31671, Lot No.: TWK6177, FUJIFILM Wako Pure Chemical, Osaka, Japan).

c) Deuterated solvent: Dimethylsulfoxide-d_6, a colorless, transparant, liquid having a purity of 99.0 % or higher (GC), and a deuteration ratio of 99.9 % or higher (Code No.: 046-34252, FUJIFILM Wako Pure Chemical, Osaka, Japan), or equivalent item.

4.2. Tools and apparati

Tools and apparati used in the collaborative study were specified to use following 1) – 6).

1). NMR sample tubes: Made from borosilicate glass, with an outer diameter of $4.965^{+0.005}_{-0.014}$ mm and total length of 180 mm to 200 mm. The caps for the NMR sample tubes are made from resin (Code No.: 295-48351, FUJIFILM Wako Pure Chemical, Osaka, Japan), or equivalent item.

2). Sample weighing dishes: Weighing dishes made from aluminum, with a diameter of 8 mm and a capacity of 0.05 mL (Code No.: 013-26351, FUJIFILM Wako Pure Chemical, Osaka, Japan), or equivalent item.

3). Pasteur pipettes: Item that can be used to transfer small quantitites of liquids, with a length of about 22.9 mm (Code No.: Z255688-1PAK, Merck KGaA, Darmstadt, Germany), or equivalent item.

4). Vial: With a capacity of 5 mL to 30 mL.

5). Precision balance: calibrated balace, with readability of 0.0001 mg to 0.001 mg.

6). NMR spectroscopy: Apparatus consisting of a superconducting magnet having a

hydrogen (^{1}H) resonance frequency of 300 MHz or higher. For these ^{1}H qNMR tests, the following conditions were added.

a). The probe must a 5 mm for liquid samples, which can be used for measurements using the NMR sample tubes defined in 4.2.1) above.

b). It is desirable that ^{1}H observations with ^{13}C decoupling are possible during the acquisition.

c). An apparatus that is capable of oversampling with a digital filter during Free Induction Decay (FID) detection.

4.3. Sample preparation procedure

The test sample was prepared under the following conditions: temperature: 20 °C ± 5 °C; relative humidity: 20 % - 60 %. Before the NMR solution was prepared, the sample and the internal standard for ^{1}H qNMR were taken out of refrigerated storage and placed in a desiccator with silica gel as the desiccant, where they were kept at ambient temperature no less than one hour in the room for sample weighing. Next, using a weighing dish and equation (1), we determined the *minimum weight* [28, 29]. Based on the calculated *minimum weight* (weighed mass must be no less than twice the minimum weight), the target mass value for the sample and internal standard for ^{1}H qNMR was set. Furthermore, the target mass value of the sample was set to be approximately five times greater than the mass of internal standard for ^{1}H qNMR.

The applicable masses of the sample and internal standard for ^{1}H qNMR were then weighed out. First, the balance was zeroed, and the weighing dish was weighed (mass A). Next, the weighing dish was then moved from the weighing pan of the balance to the lab bench, and the sample was placed into the weighing dish using a spatula. The balance was zeroed again, and the weighing dish containing the sample was weighed (mass B). The weighing dish containing the sample was then placed into a vial, and the vial was sealed (vial 1). Using mass A and mass B, the net mass of the sample (mass B – mass A) that was placed in vial 1 was obtained. Next, the balance was zeroed, and another clean weighing dish was weighed (mass C). The second weighing dish was moved from the weighing pan of the balance to the lab bench, and a spatula was used to place the internal standard for ^{1}H qNMR onto the weighing dish. The balance was zeroed again, and the weighing dish

containing the internal standard for ^{1}H qNMR was weighed (mass D). The weighing dish containing the internal standard for ^{1}H qNMR was then placed in vial 1, and vial 1 was re-sealed. Using mass C and mass D, the net mass of the internal standard for ^{1}H qNMR (mass D – mass C) that was placed in vial 1 was obtained. The above procedures were repeated three times for each sample to prepare a total of three vials containing the sample and internal standard for ^{1}H qNMR. Next, to prepare the three sample solutions, the designated deuterated solvent was added to vials 1, 2, and 3 using a Pasteur pipette to achieve a sample concentration of about 0.5 % or 1.0 % (w/v) and then dissolved until the solution was clear (order of preparation: sample solutions 1, 2, 3). The prepared sample solutions 1, 2, and 3 were transferred into NMR sample tubes and sealed in sequence (order of preparation: test samples 1, 2, 3).

4.4. NMR measurement

To test samples 1, 2, and 3, nine FIDs were obtained by measuring each sample three times in sequence, i.e. test sample 1 → test sample 2 → test sample 3 → test sample 1 → test sample 2 → test sample 3 → test sample 1 → test sample 2 → test sample 3. NMR measurements were performed by each of the collaborating laboratories using the optimized measurement conditions in accordance with the default settings, as followed:

1). NMR apparatus: Apparatus for measuring nuclear magnetic resonance spectra with a hydrogen (^{1}H) resonance frequency of 300 MHz or higher
2). Target nucleus for measurement: ^{1}H
3). Digital resolution: 0.25 Hz or less
4). Observation spectrum width: 20 ppm or more, and containing the range from -5 ppm to 15 ppm
5). Spinning: No
6). Pulse angle: 90°
7). ^{13}C decoupling: Preferred if available
8). Delay time: Pulse repetition wait time of 60 s or more
9). Number of transients: 8 or more (Signal-to-noise ratio (S/N): 1000 or higher)
10). Dummy scan: 2 or more
11). Measurement temperature: Constant temperature between 20 °C and 30 °C

12). Digital filter: Yes

4.5. Data processing

As is the case with NMR measurement, data processing was performed by each of the collaborating laboratories using the optimized conditions in accordance with the following default settings. Zero-filling was applied twice. The data was then Fourier transformed without applying a window function, and the phase of the obtained spectrum was corrected manually. Each collaborating laboratory performed baseline correction according to their selected algorithm. When the zero-filling was unavailable, the data processing was performed without zero-filling. Similarly, when the baseline correction was unavailable, data processing was performed without baseline correction.

4.6. System suitability test (SST)

Each collaborating laboratory performed a SST in according with the guidelines in the JP 17th edition.[30] In the SST, test sample 3 was repeatedly measured six times using the optimized measurement conditions outlined in "4.4. NMR measurement". The NMR sample tube was ejected from the probe between each measurement.

1). Test for required detectability

The S/N values were confirmed to be 1000 or more for all target signals including qNMR standard signal.

2). System performance

All target signals were confirmed to be free of obvious overlap with signals corresponding to impurities. The ratio of two signal areas per proton of the target signal was confirmed to be within the range 0.99-1.01.

3). System repeatability

When the test is repeated six times with test sample 3, the relative standard deviation (RSD) of the ratio of two signal areas from one pair of a target signal and a qNMR standard signal was confirmed to be not more than 0.5 %.

4.7. Calculations

The purity (mass fraction, %) of each sample was calculated in accordance with equation (3) using each of the nine FIDs acquired for that sample. The average of the nine purity values was used as the purity of that sample. In one acquired FID, if multiple signals

were available for the quantitation of the analyte, the average of all the purities obtained from each of such signals was used as the purity of that sample.

$$P_s = \frac{S_s}{S_i} \times \frac{N_i}{N_s} \times \frac{M_s}{M_i} \times \frac{m_i}{m_s} \times P_i \quad (3)$$

Where P_s : Purity (mass fraction, %) of the sample

P_i : Purity (mass fraction, %) of the internal standard for ^{1}H qNMR

S_s : Signal area of the analyte

S_i : Signal area of the ^{1}H qNMR standard

N_s : Number of resonating hydrogens of the analyte

N_i : Number of resonating hydrogens of the ^{1}H qNMR standard

M_s : Molar mass of the analyte

M_i : Molar mass of the qNMR standard

m_s : Mass of the sample

m_i : Mass of the internal standard for ^{1}H qNMR

4.8. Reporting

Each collaborating laboratory entered information on sample preparation, NMR measurement conditions, data processing conditions, mass values of the sample and the internal standard for ^{1}H qNMR, and signal areas into the purity calculation spread sheet and reported it to the coordinating laboratory (Research period: June 2018 through November 2018).

Acknowledgement

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Conflict of Interest

Toru Miura is employee of FUJIFILM Wako Pure Chemical Corporation. Naoki Sugimoto, Yuzo Nishizaki and Yukihiro Goda are employees of National Institute of Health Sciences. Sitaram Bhavaraju, Yang Liu and Anton Bzhelyansky are employees of United States Pharmacopeial Convention. Taichi Yamazaki is employee of National Metrology Institute of Japan / National Institute of Advanced Industrial Science and Technology. Carlos Amezcua and Joseph Ray were employees of Baxter Healthcare at the time of the study. Elina Zailer and Bernd Diehl are employees of Spectral Service AG. Vito Gallo and Stefano Todisco are employees of Polytechnic University of Bari. Katsuya Ofuji is employee of Chugai Pharma Manufacturing Co., Ltd. Kazuhiro Fujita is employee of SHIONOGI & Co., Ltd. Taro Higano is employee of Taisho Pharmaceutical Co., Ltd. Christian Geletneky, Thomas Hausler and Neeraj Singh are employees of Roche Diagnostics GmbH. Kana Yamamoto and Tsuyoshi Kato are employee of Japan Food Research Laboratories. Ryuichi Sawa is employee of Microbial Chemistry Research Foundation. Ryuichi Watanabe is employee of National Research Institute of Fisheries Science.

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Table 1. Information on sample preparation, NMR measurement and data processing in collaborative study (collaborating laboratory 1 -7)

			collaborating laboratory number						
		apparatus and parameters	1	2	3	4	5	6	7
		balance	Mettler Toledo XPR6U	Mettler Toledo XP26	METTLER TOLEDO	Sartorius MSE2.7s-000-	Mettler Toledo XP2UV	Mettler Toledo XPE26	Mettler Toledo UMX2
		readibility	0.0001 mg	0.001 mg	0.0001 mg	0.0001 mg	0.0001 mg	0.001 mg	0.0001 mg
		minimum weight (max. value)	less than 0.7 mg	less than 2.7 mg	less than 0.7 mg	less than 0.2 mg	less than 0.4 mg	2.7 mg	less than 0.4 mg
		butyl *p* -hydroxybenzoate	≈ 5 mg	≈ 10 mg	≈ 7.5 mg	≈ 5 mg	≈ 5 mg	≈ 30 mg	≈ 5 mg
sample preparation	butyl *p* -hydroxybenzoate	1,4-BTMSB-d_4	≈ 1 mg	≈ 10 mg	≈ 1.5 mg	≈ 1 mg	≈ 1 mg	≈ 3 mg	≈ 1 mg
		acetone-d_6	≈ 1 mL	≈ 3 mL	≈ 1.5 mL	≈ 1 mL	≈ 1 mL	≈ 3 mL	≈ 1 mL
		benzoic acid	≈ 10 mg	≈ 30 mg	≈ 15 mg	≈ 10 mg	≈ 10 mg	≈ 30 mg	≈ 10 mg
	benzoic acid	dimethyl sulfone	≈ 1 mg	≈ 8 mg	≈ 1.5 mg	≈ 1 mg	≈ 1 mg	≈ 3 mg	≈ 1 mg
		methanol-d_4	≈ 1 mL	≈ 3 mL	≈ 1.5 mL	≈ 1 mL	≈ 1 mL	≈ 3 mL	≈ 1 mL
		fludioxonil	≈ 5 mg	≈ 15 mg	≈ 7.5 mg	≈ 5 mg	≈ 5 mg	≈ 30 mg	≈ 7 mg
	fludioxonil	DSS-d_6	≈ 1 mg	≈ 5 mg	≈ 1.5 mg	≈ 1 mg	≈ 1 mg	≈ 3 mg	≈ 1 mg
		dimethyl sulfoxide-d_6	≈ 1 mL	≈ 3 mL	≈ 1.5 mL	≈ 1 mL	≈ 1 mL	≈ 3 mL	≈ 1 mL
		NMR instrument	JEOL Eclipse 300	Bruker Avance III HD	JEOL JNM-ECA600	Varain NMR System 500 DD1	JEOL JNM-ECZ600R	Bruker	JEOL JNM-ECZ400s
		spectrometer frequency	300 MHz	400 MHz / 600 MHz	600 MHz	500 MHz	600 MHz	500 MHz	400 MHz
		spectral width	20 ppm	20 ppm / 21 ppm	20 ppm	40 ppm	20 ppm	24.0187 ppm	20 ppm
		pulse offset	5 ppm	4.697 ppm / 4.9 ppm / 5 ppm	5 ppm	5 ppm	5 ppm	20.13583 ppm	5 ppm
		spinning	No	No	No	No	No	No	No
		digital filter	Yes	Yes	Yes	Yes	Yes	No report	Yes
NMR measurement		pulse angle	90°	90°	90°	90°	90°	30°	90°
		digital resolution	0.2 Hz	less than 0.26 Hz	0.25 Hz	less than 0.25 Hz	0.25 Hz	0.18 Hz	0.25 Hz
		relaxation delay time	60 s	60 s	60 s	60 s	60 s	60 s	60 s
		measurement temperature	22 ˚C - 25 ˚C	25 ˚C	25 ˚C	25 ˚C	25 ˚C	25 ˚C	25 ˚C
		^{13}C decoupling	No	Yes	Yes	Yes	Yes	Yes	Yes
		decoupling sequence	-	GARP4	MPF8	MPF8	MPF8	No report	MPF8
		scan times	24 / 40	8	8/24	8	8	No report	8
		dummy scan times	2	2	2	2	2	4	2
		data processing software	Delta	Topspin 3.2/Topspin 3.5	Delta	VnmrJ	Delta	TopSpin	ACD labs
data processing		window function	No	No	No	No	No	No report	No
		zero filling	Yes	Yes	Yes	No	Yes	No report	Yes
		phase correction	Auto	manual	manual	manual	manual	manual	manual
		baseline correction	Yes	Yes	Yes	Yes	Yes	No report	Yes

Table 2. Information on sample preparation, NMR measurement and data processing in collaborative study (collaborating laboratory 8 -13)

			collaborating laboratory number					
		apparatus and parameters	8	9	10	11	12	13
		balance	Mettler Toledo XP6U	Mettler Toledo XP6U	Mettler Toledo XP6V	Sartorius CPA2P	Mettler Toledo XPR6U	Sartorius SE2
		readibility	0.0001 mg	0.0001 mg	0.001 mg	0.001 mg	0.0001 mg	0.0001 mg
		minimum weight (max. value)	less than 0.5 mg	less than 0.2 mg	1.0 mg	1.1 mg	less than 0.4 mg	less than 1.1 mg
		butyl *p* -hydroxybenzoate	≈ 5 mg	≈ 15 mg	≈ 15 mg	≈ 20 mg	≈ 10 mg	≈ 5 mg
sample preparation	butyl *p* -hydroxybenzoate	1,4-BTMSB-d_4	≈ 1 mg	≈ 3 mg	≈ 3 mg	≈ 5 mg	≈ 3 mg	≈ 1 mg
		acetone-d_6	≈ 1 mL	≈ 3 mL	≈ 3 mL	≈ 4 mL	≈ 3 mL	≈ 1 mL
		benzoic acid	≈ 10 mg	≈ 20 mg	≈ 15 mg	≈ 20 mg	No report	≈ 10 mg
	benzoic acid	dimethyl sulfone	≈ 1 mg	≈ 2 mg	≈ 3 mg	≈ 2 mg	No report	≈ 1 mg
		methanol-d_4	≈ 1 mL	≈ 2 mL	≈ 3 mL	≈ 2 mL	No report	≈ 1 mL
		fludioxonil	≈ 7.5 mg	≈ 15 mg	≈ 15 mg	≈ 10 mg	≈ 8 mg	≈ 5 mg
	fludioxonil	DSS-d_6	≈ 1.4 mg	≈ 3 mg	≈ 3 mg	≈ 4 mg	≈ 2 mg	≈ 1 mg
		dimethyl sulfoxide-d_6	≈ 1 mL	≈ 3 mL	≈ 3 mL	≈ 2 mL	≈ 2 mL	≈ 1 mL
		NMR instrument	Bruker AVANCE III 800	Varian VNS600	Agilent DD2 600	Bruker Avance 400	Bruker	JEOL JNM-ECA500
		spectrometer frequency	800 MHz	600 MHz	600 MHz	400 MHz	500 MHz	500 MHz
		spectral width	20 ppm	99.2 ppm	20 ppm	20 ppm	20.7 ppm	22 ppm
		pulse offset	5 ppm	3.9 ppm / 4.0 ppm / 5.5 ppm	5 ppm	4.7 ppm	6.2 ppm	5 ppm
		spinning	No	No	No	No	No	No
		digital filter	Yes	Yes	Yes	Yes	Yes	Yes
NMR measurement		pulse angle	90°	90°	90°	90°	90°	90°
		digital resolution	0.25 Hz	0.25 Hz	0.25 Hz	0.061133 Hz	0.13 Hz	0.21 Hz
		relaxation delay time	60 s	60 s	60 s	120 s	60 s	60 s
		measurement temperature	25 ˚C	23 ˚C	27 ˚C	25 ˚C	27 ˚C	26 ˚C / 27 ˚C
		^{13}C decoupling	Yes	Yes	No	No	No	Yes
		decoupling sequence	CHIRP	WURST40	-	-	-	MPF8
		scan times	8	32	8	16	16	8
		dummy scan times	2	2	2	8	2	2
		data processing software	Topspin 3.5 pl7	Mnova 7	VnmrJ 4.2	Topspin 3.0	ACD/Labs 2015 2.7	Delta
data processing		window function	No	No	No	No	Yes(LB:0.15 Hz)	No
		zero filling	Yes	Yes	Yes	No	Yes	Yes
		phase correction	manual	manual	manual	manual	manual	manual
		baseline correction	Yes	Yes	No	Yes	Yes	Yes

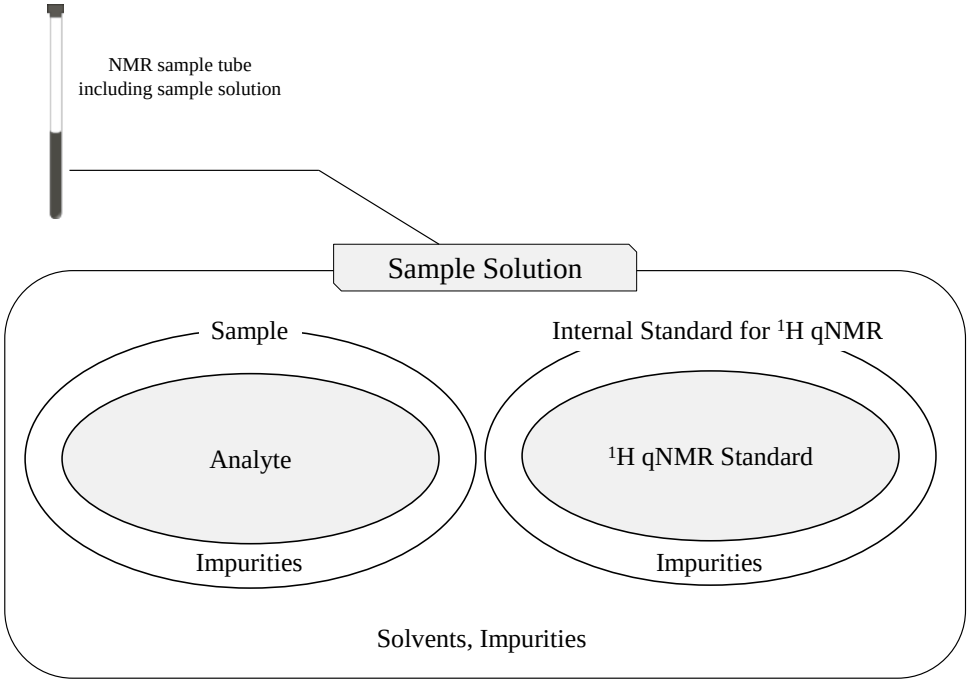

Fig. 1. Schematic illustration of the sample solution

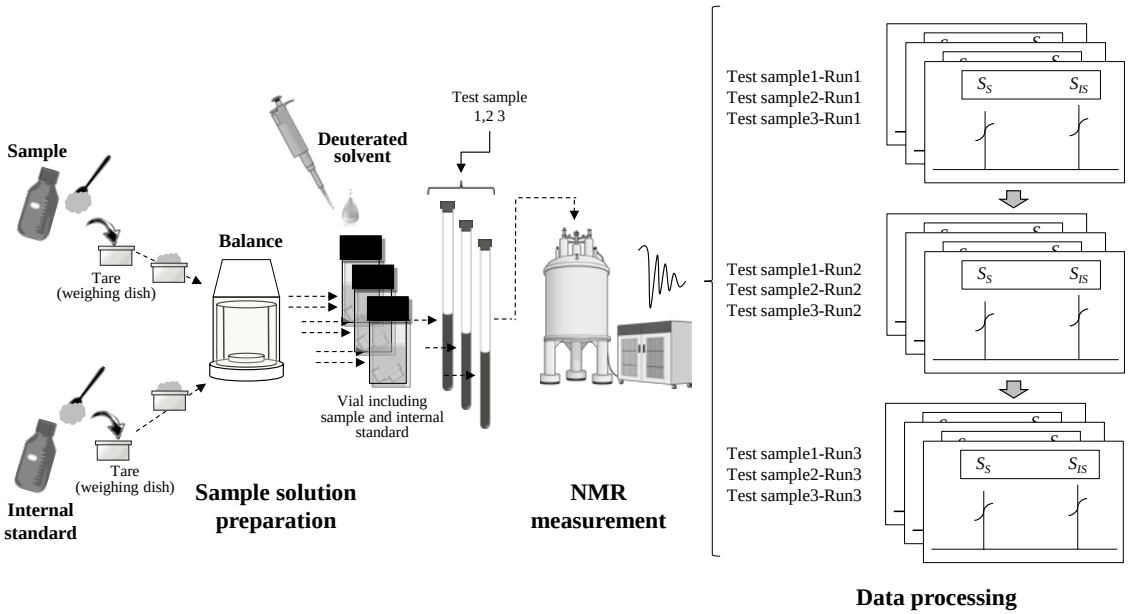

Fig. 2. Schematic illustration of measurement procedure

Test samples were individually prepared and each of them was discontinuously measured with NMR apparatus using the data acquisition parameters optimized in reference to the default settings listed in the protocol.

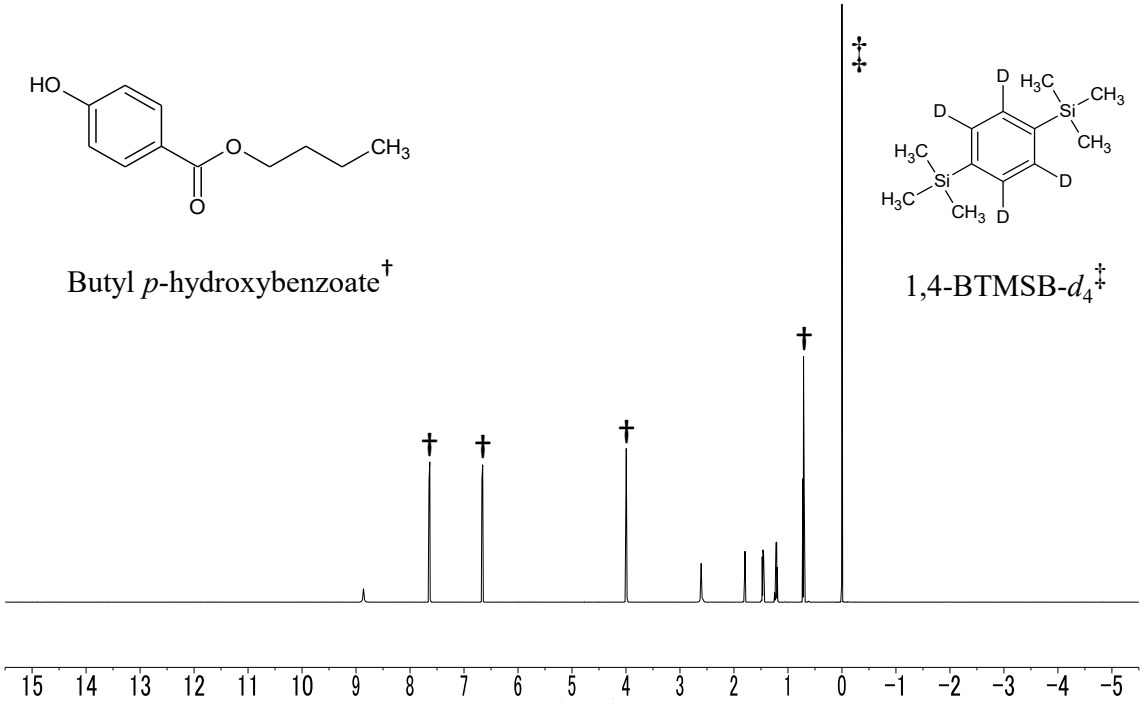

Fig. 3. Typical ^{1}H NMR spectrum of test for butyl *p*-hydroxybenzoate

The test sample was prepared to 0.5 % (w/v) butyl *p*-hydroxybenzoate and 0.1% (w/v) 1,4-BTMSB-d_4 in acetone-d_6. This spectrum was measured with 400 MHz NMR apparatus under data acquisition parameters listed in the protocol. The symbols of dagger and double dagger were attached to only selected analyte signals and qNMR standard signal used for quantification.

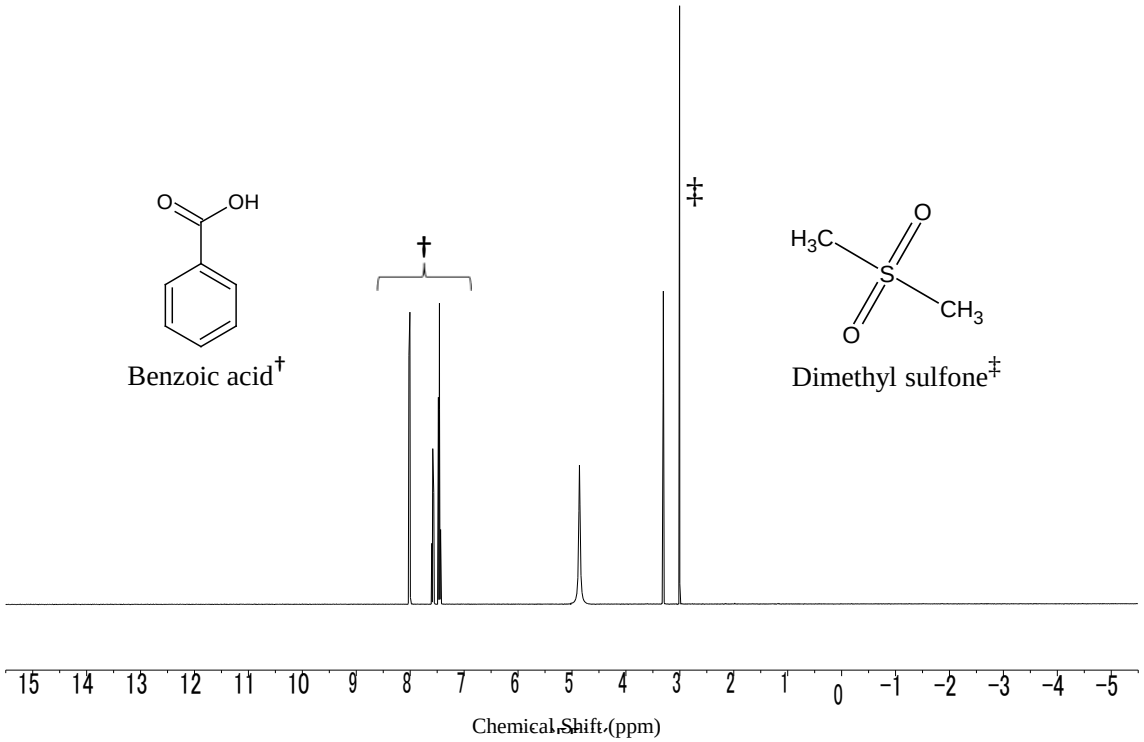

Fig. 4. Typical ^{1}H NMR spectrum of test for benzoic acid

The test sample was prepared to 1.0 % (w/v) benzoic acid and 0.1% (w/v) dimethyl sulfone in methanol-d_4. This spectrum was measured with 400 MHz NMR apparatus under data acquisition parameters listed in the protocol. The symbols of dagger and double dagger were attached to only selected analyte signals and qNMR standard signal used for quantification.

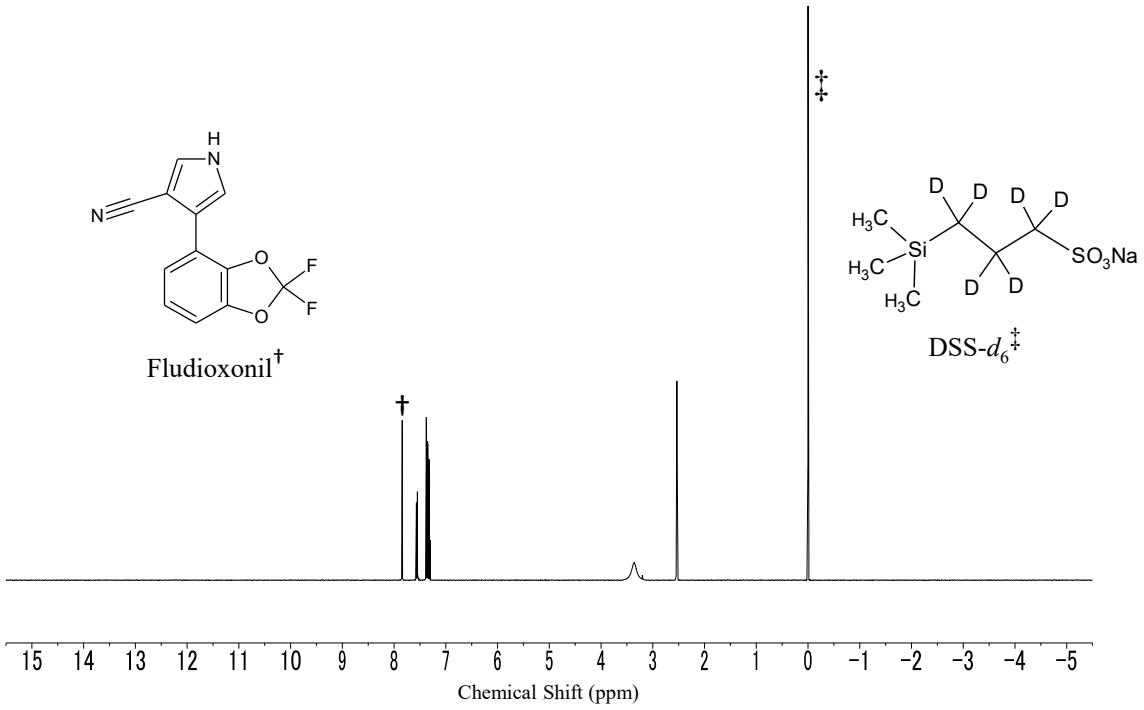

Fig. 5. Typical ^{1}H NMR spectrum of test for fludioxonil

The test sample was prepared to 0.5 % (w/v) fludioxonil and 0.1% (w/v) DSS-d_6 in dimethylsulfoxide-d_6. This spectrum was measured with 400 MHz NMR apparatus under data acquisition parameters listed in the protocol. The symbols of dagger and double dagger were attached to only selected analyte signal and qNMR standard signal used for quantification.

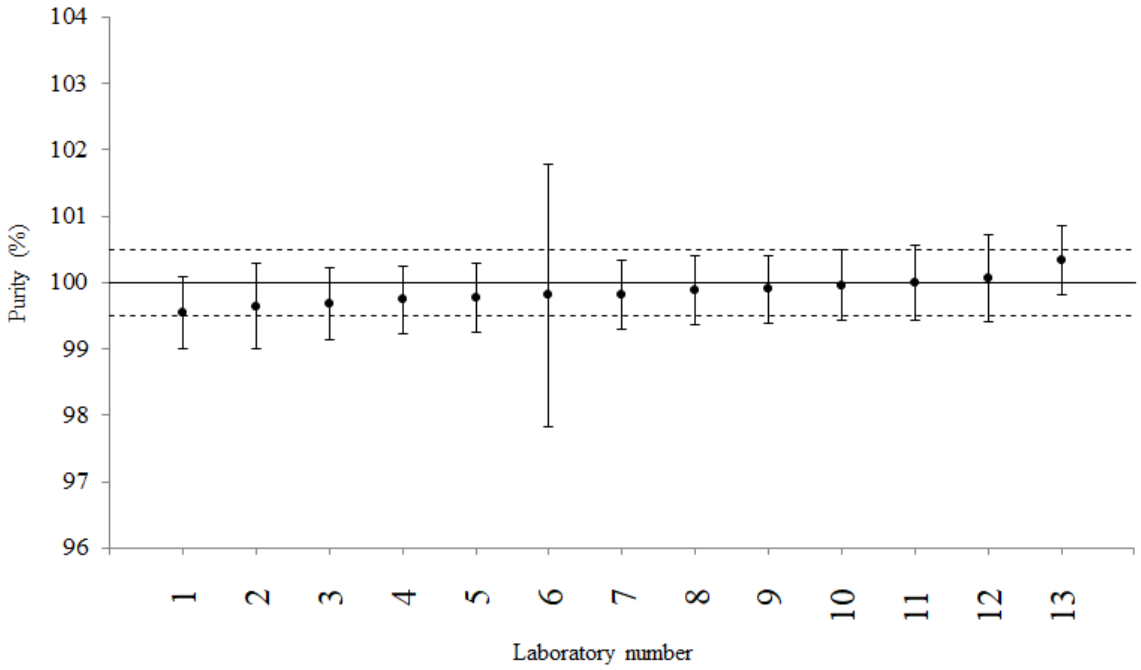

Fig. 6. The result of collaborative study for the purity determination of butyl *p*-hydroxybenzoate

The solid line in the figure is the purity of butyl *p*-hydroxybenzoate Japanese Pharmacopeia reference standard and the dotted line is the acceptable errors of the purity. The filled circle in the figure is the purity of each collaborating laboratory and the error bar is the expanded uncertainty (k=2) of each collaborating laboratory.

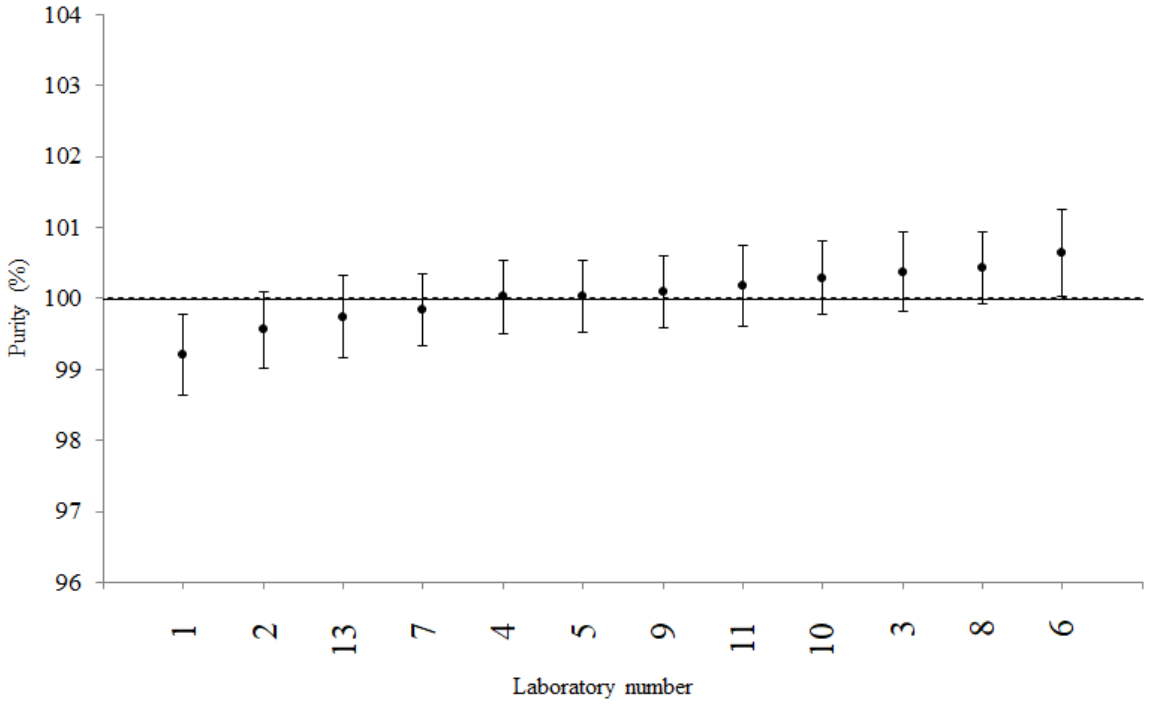

Fig. 7. The result of collaborative study for the purity determination of benzoic acid

The solid line in the figure is the certified value of benzoic acid certified reference material and the dotted line is the expanded uncertainty (k=2) of the certified value.

The filled circle in the figure is the purity of each collaborating laboratory and the error bar is the expanded uncertainty (k=2) of each collaborating laboratory.

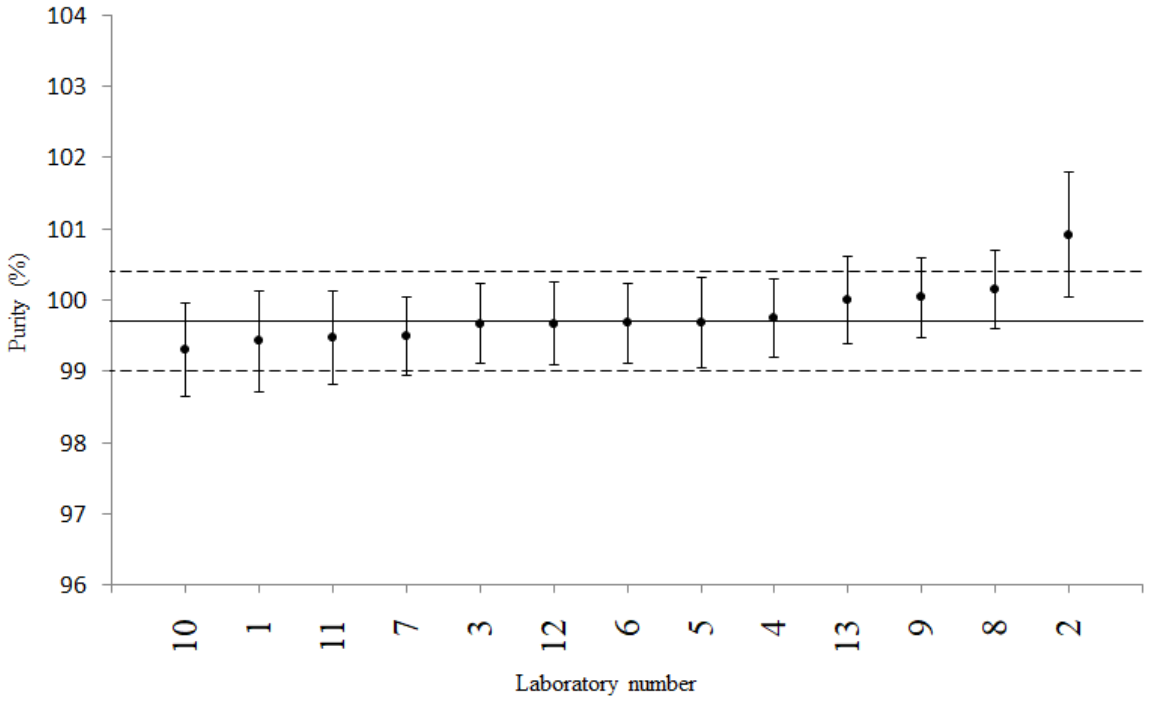

Fig. 8. The result of collaborative study for the purity determination of fludioxonil

The solid line in the figure is the certified value of fludioxonil certified reference material and the dotted line is the expanded uncertainty (k=2) of the certified value.

The filled circle in the figure is the purity of each collaborating laboratory and the error bar is the expanded uncertainty (k=2) of each collaborating laboratory.

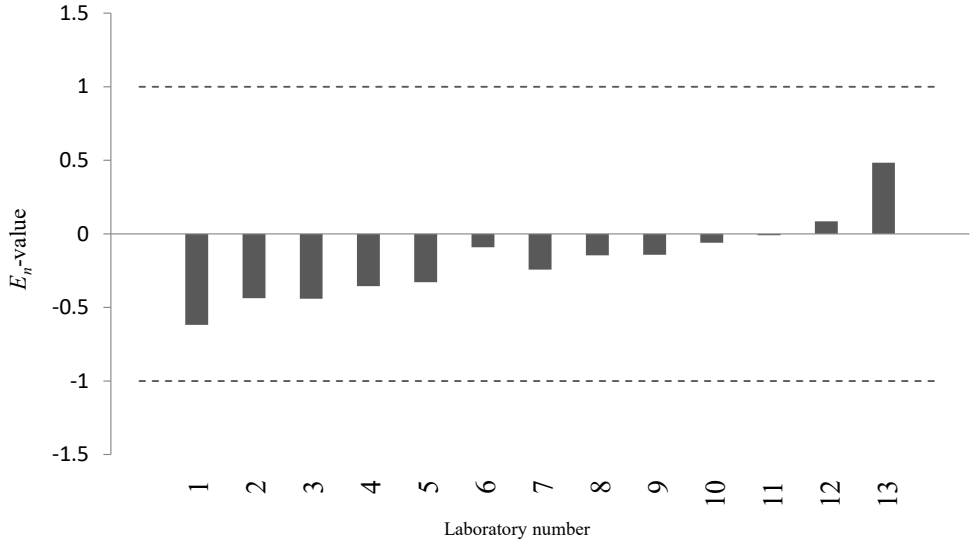

Fig. 9. The *En*-value of collaborative study for the purity determination of butyl *p*-hydroxybenzoate

The two horizontal dotted lines (the value of $-1 \leq E_n \leq 1$) in the figure show the acceptance criteria of *En*-value. The black bar in the figure indicates the *En*-value of each collaborating laboratory.

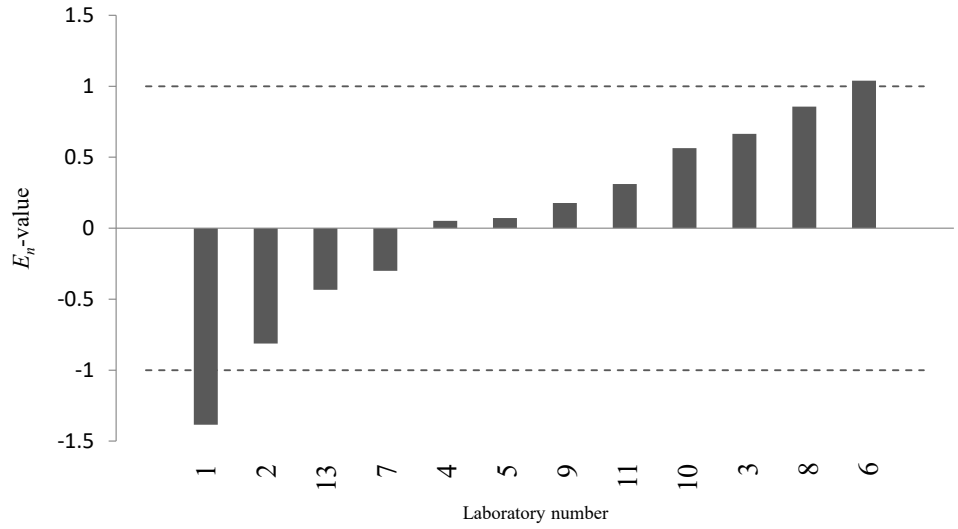

Fig. 10. The *En*-value of collaborative study for the purity determination of benzoic acid
The two horizontal dotted lines (the value of $-1 \leq E_n \leq 1$) in the figure show the acceptance criteria of *En*-value. The black bar in the figure indicates the *En*-value of each collaborating laboratory.

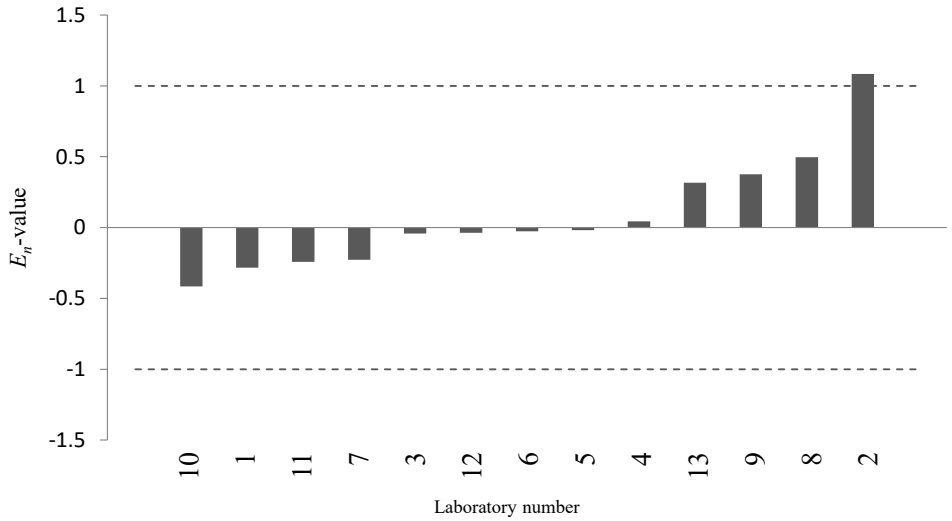

Fig. 11. The *En*-value of collaborative study for the purity determination of fludioxonil
The two horizontal dotted lines (the value of $-1 \leq E_n \leq 1$) in the figure show the acceptance criteria of *En*-value. The black bar in the figure indicates the *En*-value of each collaborating laboratory.