As a new absolute quantitation method for low-molecular compounds, quantitative NMR (qNMR) has emerged. In the Japanese Pharmacopoeia (JP), 15 compounds evaluated by qNMR are listed as reagents used as the HPLC reference standards in the assay of crude drug section of the JP. In a previous study, we revealed that humidity affects purity values of hygroscopic reagents and that (i) humidity control before and during weighing is important for a reproducible preparation and (ii) indication of the absolute amount (not purity value), which is not affected by water content, is important for hygroscopic products determined by qNMR. In this study, typical and optimal conditions that affect the determination of the purity of ginsenoside Rb1 (GRB1), saikosaponin a (SSA), and barbaloin (BB) (i.e., hygroscopic reagents) by qNMR were examined. First, the effect of humidity before and during weighing on the purity of commercial GRB1, with a purity value determined by qNMR, was examined. The results showed the importance aforementioned. The results of SSA, which is relatively unstable in the dissolved state, suggested that the standardization of humidity control before and during weighing for a specific time provides a practical approach for hygroscopic products. In regard to BB, its humidity control for a specific time, only before weighing, is enough for a reproducible purity determination.

Key words quantitative NMR; hygroscopic substance; the Japanese Pharmacopoeia; absolute purity; marker compound; crude drug

Introduction
Quantitative NMR (qNMR) has emerged as a new absolute quantitation method for low-molecular compounds, including reference standards for pharmaceuticals. The Japanese Pharmacopoeia (JP), supplement II to the 16th edition, introduced qNMR as a method for the determination of the purity of reagents used as HPLC reference standards in the assay of crude drugs and Kampo extract formulations.1-4 In supplement II to the JP 17th edition, published in June 2019, the following 15 substances were evaluated by qNMR testing and are listed as the reagents for the assay: (E)-cinnamic acid, geniposide, magnolol, magnoflorine, paenol, rhein, rosmarinic acid, saikosaponin b2, loganin, [6]-gingerol, [6]-shogaol, (E)-ferulic acid, 10-hydroxy-2-(E)-decenoic acid, evodiamine, and sinomenine. The HPLC quantification of 15 markers in 11 crude drugs and 23 Kampo formulae is performed using the reagents as reference standards.1-4 In addition, mangiferin evaluated by qNMR is listed as the reagent for the assay of the Byakkokanjinjito extract, newly listed in the JP18.5

To establish the HPLC reference standards with purity values determined by qNMR in the crude drug section of the JP, there were several problems to be solved. We previously resolved problems related to: 1) the establishment of the reference standards for qNMR, 2) complications due to signals from impurities in the reference standards for qNMR and targeted marker compounds, and 3) peak unity of signals of targeted marker compounds in HPLC.6-9 Furthermore, the stability of the reagents is crucial and we showed that the preparation and distribution of reagents as commercial products with the determined purity values were important for JP users.

Purity evaluation of reagents by qNMR requires accurate weighing. However, the weighing of hygroscopic reagents may be affected by the environment.10 In a previous study,
we examined the hygroscopicity of 21 standard products for crude drug tests in the JP by water sorption-desorption analysis using thermal gravimetric analysis (TGA) equipment. The results showed that ginsenosides and saikosaponins are hygroscopic. It can be predicted that the weights of hygroscopic reagents easily change depending on humidity. Consequently, their purities change. We demonstrated this using saikosaponin b2 (SSB2) in the previous report and that (i) humidity control before and during the weighing is important for a reproducible preparation and (ii) indication of the absolute amount (not purity value), which is not affected by water content, is important for hygroscopic products determined by qNMR. In this study, typical and optimal conditions that affect the determination of the purity of ginsenoside Rb1 (GRB1), saikosaponin a (SSA), and barbaloin (BB) (i.e., hygroscopic reagents) by qNMR were examined.

Results and Discussion

GRB1 We purchased commercially available reagents of GRB1, the purity of which had been determined by qNMR. The reagents were labeled and re-measured using qNMR to obtain the purity after maintenance under different humidity conditions before and during weighing.

First, quantitation signals of GRB1 were examined. The reagent had no interference signal when the 3rd-position signal (around 3.8 ppm) was used as a signal to be integrated (Fig. 1). This signal was used as a quantitation signal to calculate the absolute purity. The labeled purity value of the reagent was 90.84%. After the reagent was adjusted under controlled humidity for 3 h, the purity of the reagent was re-determined by qNMR. The purity of GRB1 under controlled humidity of 26% (Lab. C), 43% (Lab. B), 43% (Lab. A), 60% (Lab. D), and 60% (Lab. E) was 92.58, 88.34, 87.19, 86.12, and 86.65%, respectively (Table 1). The humidity during weighing, determined by a digital hygrometer, was 26, 38, 45, 60, and 60%, respectively. Table 1. Purity (%) of Ginsenoside Rb1 (GRB1) Prepared under Different Humidity Conditions across Five Independent Laboratories

| Laboratory | Average (%) | C | B | A | D | E |
|------------|-------------|---|---|---|---|---|
|            | 92.58       | 88.34 | 87.19 | 86.12 | 86.65 |
| S.D. (%)   | 0.12        | 0.12 | 0.15 | 0.06 | 0.10 |

*Purity (%) of ginsenoside Rb1: 90.84% described in the certificate of analysis determined by qNMR. Standard deviation (S.D.).

25 30 35 40 45 50 55 60

Fig. 2. Purity (%) of GRB1 under Different Humidity Conditions across Four Independent Laboratories

The dotted line shows the purity (%) of GRB1 described in the certificate of analysis: 90.84%.

26% (Lab. C), 43% (Lab. B), 43% (Lab. A), 60% (Lab. D), and 60% (Lab. E) was 92.58, 88.34, 87.19, 86.12, and 86.65%, respectively (Table 1). The humidity during weighing, determined by a digital hygrometer, was 26, 38, 45, 60, and 60%, respectively. Figure 2 shows the purity at each humidity level
in Labs A–D. Purity tended to decrease as humidity increased; thus, the purity of the hygroscopic substances determined by qNMR is affected by humidity before and during weighing.

The attached certificate of analysis seemed to be performed in Europe. Our results suggested that the purity of the substances is determined by qNMR after weighing in a place in which the humidity is approximately 30% (Fig. 2). The results also suggested that the absolute purity of hygroscopic reference standards with an indication of purity (or water content) vary if the humidity during the time of preparation of the standard solution for HPLC is different from that at the time of water content determination, as most of the impurity is water.

Next, we examined the weight equilibration time to determine the appropriate duration of humidity control during weight measurement prior to purity analysis (Fig. 3). Under the condition at 25 °C and 60% humidity by TGA equipment, after 3 h (180 min) the rate of weight change per h was less than 0.1% (Fig. 3). Therefore, the humidity control conditions of GRB1 was set as 25 °C and 60% humidity for 3 h or more.

As mentioned in a previous paper, these results indicate that (i) humidity control before and during the weighing of hygroscopic reagents is critical for accurate assessment of purity and (ii) the absolute amount of reagent itself that is not affected by water content should be labeled when the evaluated reagents become commercially available.

SSA The purity of SSA, which is also hygroscopic, was examined. Hosoe et al. reported that the use of the 12th-position signal (around 5.7 ppm), instead of the 11th-position signal (around 5.2 ppm), provide more accurate estimation of purity (quantitative value) without interference peaks for the analysis of SSA (Fig. 4). Thus, we used the 12th-position signal for quantitation to calculate the absolute purity (quantitative value). First, the purity was determined without humidity control before and during weighing in three facilities (Table 2). The results showed a high variation in purity (89.45 ± 2.79%), which indicated that the substance requires humidity control both before and during weighing. SSA is relatively unstable in the dissolved state (data not shown). We concluded that immediate qNMR measurement is essential for accurate quantitation after weighing.

The weight equilibration time of SSA was examined to determine the appropriate duration of humidity control before the weighing of SSA. Under the conditions of 20 °C and 60% humidity by the TGA equipment, the rate of weight change was less than 0.1% per hour after 3 h (180 min) (Fig. 5). Therefore, the humidity control conditions for SSA were set at 3 h or more at 20 °C and 60% humidity.

The subsequent qNMR analysis after the weighing of the reagent with the consideration of the minimum weight (by an ultra-microbalance) under controlled humidity after 4 h of humidity adjustment (at approximately 60% humidity) by a
saturated NaBr solution at 22–23°C in three facilities showed good results with low variation (89.28 ± 0.076%) (Table 3). The humidity around the balance scale, monitored and recorded during weighing with a digital hygrometer in three facilities, was 56–57%.

These results suggested that, by stipulating the humidity control method before and during weighing, the reference standard users of reference standards for HPLC quantification are able to use the labeled purity value previously determined by qNMR in the regent company laboratory, even if the reference standard is hygroscopic.

**BB** The 6th-position signal (around 7.2 ppm) of BB overlapped with the non-deuterated signal of 1,4-BTMSB-d₄ (i.e., the reference standard for qNMR) as a contaminant signal. No interference signal was observed around the 2nd- and 7th-position signals (around 6.8 ppm) and the 4th- and 5th-position signals (around 6.6 ppm), which were used as signals to be integrated (Fig. 6).

The weight equilibration time of BB was examined using TGA equipment to determine the appropriate duration of the humidity control before weighing (Fig. 7). The 160 min observation suggested that the time for BB should be set at 2 h or more at 25°C and 60% humidity. As TGA analysis revealed that BB is less hygroscopic than SSA, we tried to weigh BB with or without humidity control after maintenance for more than 2 h under the 60% humidity by a saturated NaBr solution. The subsequent qNMR analysis after the weighing of the reagent with consideration of the minimum weight with or without humidity control suggested that humidity control during weighing does not affect purity (Table 4). As there was almost no difference in quantitative values, equilibration time (more than 2 h) for humidity control of BB was necessary before weighing, although a relatively quick weighing time (less than 30 min) should be considered.

**Hygroscopic Substances and Absolute Purities Determined by qNMR** We examined an optimal and reproducible sample preparative approach on hygroscopic compounds, GRB1, SSA, and BB to qNMR. The results showed the importance of humidity control before and/or during weighing. This study used a saturated salt solution for humidity control. Since laboratory users normally do not want to set a saturated salt solution in a balance box, a more convenient method would be preferable. To solve this problem, a constant temperature and humidity box with a built-in balance has been recently developed as a humidity control system. The box can be used in future studies to determine the purity of hygroscopic substances.

**Experiment**

**Facilities** Six investigators from six laboratories (A–F) separately performed an experiment in all operations. The experiment of GRB1 was performed in five laboratories (A–E).

**Samples**

**Reference Standards for qNMR and Solvents** In this study, 1,4-BTMSB-d₄ (1,4-bis(trimethylsilyl)benzene-d₄, MW = 226.50), a certified reference material (NMIJ CRM), was purchased from FUJIFILM Wako Pure Chemi-

| Laboratory | A   | B   | E   | Average of three labs |
|------------|-----|-----|-----|-----------------------|
| Average (%)| 89.29 | 89.36 | 89.20 | 89.28                 |
| S.D. (%)   | 0.20 | 0.10 | 0.16 | 0.076                 |
Table 4. Purity (%) of Barbaloin Prepared across Six Independent Laboratories

| Position | Laboratory | Humidity control during weighing | Humidity non-control during weighing |
|----------|------------|----------------------------------|-------------------------------------|
|          |            | A      | D     | E      | B      | C      | F      |
| 2,7-H    | Average    | 91.73  | 92.97 | 92.16  | 91.73  | 93.26  | 92.03  |
|          | S.D.       | 0.15   | 0.26  | 0.08   | 0.11   | 0.08   | 0.15   |
| 4,5-H    | Average    | 91.85  | 93.07 | 92.30  | 92.18  | 93.12  | 92.38  |
|          | S.D.       | 0.39   | 0.09  | 0.04   | 0.09   | 0.07   | 0.29   |
| Average (%) of 2,7-H and 4,5-H | 91.79 | 93.02 | 92.23 | 91.95 | 93.19 | 92.20 |
| S.D. (%) |            | 0.63   |       |        |        |        |        |

Approximately 5 mg of each reagent and about 1 mg of reference standard for qNMR, which were precisely weighed and placed in the same vial together for each tare, were dissolved in NMR solvent (0.6–1 mL). In the sample solution, this solution (0.6 mL) was sealed in an NMR sample tube.

Humidity Control Conditions

Approximately 5 mg of each reagent and about 1 mg of reference standard for qNMR, which were precisely weighed and placed in the same vial together for each tare, were dissolved in NMR solvent (0.6–1 mL). The purity of the reagents was calculated using the following formula based on a previous study \(^{12-15}\).

\[
P_{\text{sample}} = \left[ \frac{I_{\text{sample}}}{I_{\text{std}}} \times \frac{H_{\text{std}}}{H_{\text{sample}}} \times \frac{W_{\text{std}}}{W_{\text{sample}}} \times \frac{M_{\text{sample}}}{M_{\text{std}}} \right] \times P_{\text{std}}
\]

\(I = \) signal area, \(H = \) number of protons, \(W = \) weight, \(M = \) molecular weight, \(P = \) purity (\%), std, sample = reference standard for qNMR and sample.
The following numbers were used for the calculation: number of protons of methyl groups in 1,4-BTMSB-d₄ (reference standard for qNMR), 18; molecular weight of 1,4-BTMSB-d₄, 226.50; molecular weight of GRB1, 1109.29; molecular weight of SSA, 780.98; and molecular weight of BB, 418.39.

**TGA Equipment** TGA Q5000SA (TA Instruments) was used as the thermogravimetric analyzer. The conditions for the determination were as follows: purge gas, nitrogen; feed rate in a humidity chamber, 200 mL/min; balance, 10 mL/min; sample weight, 1.6–5.0 mg; sample pan, metalized quartz sample pan; temperature in weight equilibration determination, 20 or 25 °C; relative humidity, 60%.

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**Conflict of Interest** The authors declare no conflict of interest.

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