ABSTRACT: To achieve the measurement reliability of amino acids used as diagnostic markers in clinical fields, establishing a reference measurement system is required, in which certified reference materials (CRMs) are an essential step in the hierarchy of measurement traceability. This study describes the development of dried blood spot (DBS) CRMs for amino acid analysis with complete measurement traceability to the International System of Units (SI). Six essential amino acids—proline, valine, isoleucine, leucine, phenylalanine, and tyrosine—were analyzed using isotope-dilution liquid chromatography–mass spectrometry (ID-MS). For minimizing measurement bias and uncertainty overestimation, whole spots with 50 μL of whole blood were adopted in the certification. The between-spot homogeneities by whole spot sampling were lower than 2.1%. The relative expanded uncertainties of the six amino acids in the developed DBS CRMs were lower than 5.7% at 95% confidence. The certified values are traceable to SI through both gravimetric preparation and the primary method in certification, ID-MS. Comparison among DBS testing laboratories revealed discrepancies between the whole spot and disc sampling methods. The actual sampling volume was accurately estimated by weighing, which revealed the possibility of underestimation in routine DBS testing. The candidate CRMs can support the standardization of DBS testing for amino acids through the qualification and validation of many kinds of measurement procedures to compensate the measurement bias caused by matrix-specific sampling error.

INTRODUCTION

The purpose of the standardization of measurements is to achieve closer comparability of results regardless of the analytical methods and the laboratory where analyses are carried out. Particularly, the implementation of measurement traceability through reference systems provides one of the most important tools that supports the standardization process in laboratory testing. Certified reference materials (CRMs) are reference materials characterized by a metrologically valid procedure for one or more specific properties and provide specified values with associated uncertainties and traceabilities. Among various types of CRMs, matrix CRMs are used to verify measurement procedures and quality control materials, in addition to playing a role as secondary calibrators. Clinical applications require the use of matrix CRMs based on the intended human samples, such as serum, plasma, etc., for the validation and quality assurance of specific measuring systems.

The dried blood spot (DBS) sampling method is an approach to blood sample handling wherein a drop of blood is placed onto an assigned filter paper (or DBS card), after which target compounds are extracted for quantitative determination. This can not only diminish the danger of pathogens but also the limited sample amount required for DBS testing is advantageous for newborns and medically compromised
patients. The method is also straightforward and involves simple storage and transport. In diverse clinical fields such as newborn screening, forensics, doping, and chronic disease surveillance, DBS sampling as a representative microsampling approach is prevalent. Advances in the quality and availability of highly sensitive analytical instrumentation have recently led to increased interest in the use of microvolume samples. In addition, due to the recent pandemic, technical requirements have emerged for the realization of remote diagnosis for ease of both sample collection outside clinical settings and self-sampling at home.

Despite the advantages of DBS sampling in terms of simplicity, low-invasiveness, and cost-effectiveness, it is currently limited to screening only, and unavailable for diagnostic testing because there is no clear criteria for the differences in the physical properties of blood (e.g., hematocrit levels, viscosity) or for sampling bias according to the specifications of the filter paper and its indirect sampling volume. Therefore, efforts have been made to produce and standardize DBS reference materials based on specifications of the related raw materials such as spotting volume, hematocrit levels, homogeneity, etc. Meanwhile, several papers have reported alternative types of microsampling methods to overcome the above weaknesses of traditional DBS testing, particularly in tracing actual sampling volumes. In general, the entire blood spot cannot be used in DBS testing with real clinical samples since it neither shows a perfectly circular form nor a consistent volume in all cases. Consequently, rather than entire spots, portions of blood spots are commonly used as working samples, typically taken with a paper puncher with a nominal diameter of about 3 or 3.2 mm. Sample volumes are then proportionally calculated by the area of the diameter of the puncher. Moreover, preparation details of the paper and spotting volumes in commercially available DBS samples are not clearly standardized and not given to users. From this point of view, such variations and unclear information can cause measurement bias depending on the DBS products. This can lead to redundant testing and decision errors in clinical fields, which are major hurdles for reliability assurance in DBS testing.

In this study, we describe the development of candidate DBS CRMs for amino acid (AA) analysis with complete measurement traceability up to the International System of Units (SI). We target six AAs, namely phenylalanine (Phe), tyrosine (Tyr), valine (Val), isoleucine (Ile), leucine (Leu), and proline (Pro), which are essential AAs as well as diagnostic markers of phenylketonuria (PKU), hyperprolinemia, and maple syrup urine disease (MSUD), representative inherited AA metabolic disorders in newborn screening. Isotope dilution mass spectrometry (ID-MS), one of the primary reference measurement procedures, is adopted for AA analysis at the highest metrological level owing to its use of isotopically labeled internal standards (ISTDs) that can compensate nonquantitative recoveries in sample preparation and instrument analysis. The candidate DBS CRMs are characterized, and their homogeneity, stability, and commutability are assessed in accordance with ISO 17034, ISO Guide 35, and ISO 15194.

**EXPERIMENTAL SECTION**

**Chemicals and Reagents.** The purity assessed CRMs of the target AAs (l-Proline, l-Valine, l-Isoleucine, l-Leucine, l-phenylalanine, and l-tyrosine) were obtained from the National Metrology Institute of Japan (NMIJ, Japan). The following isotopic labeled AAs for the ISTDs were obtained from Cambridge Isotopes Laboratory (Andover, MA): l-Proline (U-13C0, 99%; I-15N, 99%; Pro*), l-Valine (U-13C0, 97–99%; I-15N, 97–99%; Val*), l-Isoleucine (U-13C0, 99%; I-15N, 99%; Ile*), l-Leucine (U-13C0, 97–98%; I-15N, 97–98%; Leu*), l-Phenylalanine (U-13C0, 99%; I-15N, 99%; Phe*), and l-Tyrosine (U-13C0, 99%; Tyr*). All other chemicals used in this study are summarized in the Supporting Information.

**Preparation of DBS Samples for CRMs.** Two batches of DBS CRMs with different concentration ranges of AAs were prepared. Fresh whole blood from healthy donors provided for research was obtained from the Korean Red Cross with IRB permissions.

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**Figure 1.** (A) Blood samples with different characters. Whole blood is the raw material for DBS preparation by stabilization and homogenization. A whole spot is a 12-mm diameter circle of dried whole blood with a volume of 50 μL taken from the filter paper (DBS card) by scissors. A disc is approximately a 3-mm diameter circle of dried blood taken from a whole spot by a paper puncher, and the remainder is the remaining part of the whole spot after removing all discs. A maximum of six discs were punched out from specific locations as numbered. (B) Precut mass defined (3 cm × 4 cm) paper for DBS preparation with various sampling volumes from 0 to 100 μL. Three discs were punched out from each DBS sample in the same position, and one disc each was taken from the 0 and 10 μL DBS samples.
Figure 2. Measured values of the six AAs from whole blood, whole spot sampling, and disc sampling. For the latter, two discs from the center of the spot and from the edge of the spot were separately measured. The white bars represent whole blood samples, the black bars are whole spot DBS samples, the gray bars and black-patterned bars are the disc samples in different positions (center and edge), and the light gray-patterned bars are the remainder DBS samples.

approval (KRISS-IRB-2020–1). After transferring to a sterilized bottle, protease inhibition cocktail (Biotool, Cat. no.: B14013) was added according to the instructions to improve the stability of the samples, after which the samples were sufficiently homogenized at 4 °C for 18 h and finally divided into “low” and “high” batches. For the high batch, additional standard mix solution was added within 1% v/v, while the low batch had no further additions. Both batches were additionally homogenized at 4 °C for 2 weeks. In the case of whole blood, an additional purification process was not required, and 50 μL of the raw material was spotted onto filter paper (Whatman 903 level, Macherey-Nagel) using an automatic dispenser (Microlab 600, Hamilton). During the spotting, 10 random aliquots were finely weighed to estimate sampling uncertainty.

The DBS samples were dried completely for 24 h in a clean bench and placed in an aluminum bag with desiccants. The prepared DBS samples were then kept at −70 °C before use. **Measurement Procedure for AA Analysis of DBS Samples.** The measurement procedure using isotope dilution mass spectrometry (ID-MS) was first strictly validated in terms of measurement accuracy and precision using various samples. Four different types of blood samples—whole blood, whole spot, disc, and remainder—were used in this study as shown in Figure 1(A). With a reference of 50 μL for one spot, the target AAs were extracted with a 10-fold volume of distilled water by gently shaking for 30 min at room temperature. The extracted samples were deproteinized in 15% (v/v) S-sulfosalicylic acid solution for purification and then injected into the LC-MS system. Further details of the sample treatments and LC-MS conditions are summarized in the Supporting Information.

To confirm the accuracy and precision, other DBS samples for recovery tests were prepared by gravimetrically fortifying AAs into the raw material to final AA concentrations of 0, 2.5, 25, and 250 μmol/kg.

**Characterization of AAs in DBS CRMs.** Using the established procedure, the mass fractions of the six AAs in the candidate DBS CRMs were characterized based on the quantification of the AAs. Assignments of the certified values and uncertainty were evaluated in accordance with ISO Guide 35.28 At least 10 spots were randomly selected within a batch for measurement and homogeneity tests between spots. Whole spot sampling was adopted in the certification. In addition, within-spot homogeneity was also investigated through the variance of the measured values of four randomly selected discs from 10 randomly selected spots. This serves as an informative value for disc sampling due to its priority in most clinical settings.5,13,19

The mean values of the 10 spots were assigned as the certified values of the six AAs after confirmation of both long- and short-term stability. Measurement uncertainty estimation was performed in accordance with ISO/IEC Guide 98-3, the Guide to the Expression of Uncertainty in Measurement (GUM).24,25

**Stability Assessment of DBS CRMs.** Stability tests should be conducted to set storage, packing, and transport conditions as well as to determine the expiry date.26 Two types of stability were tested: long-term stability in storage conditions, and short-term stability during transportation. The long-term stability of the six AAs in the DBS CRMs in storage conditions (−70 °C) was evaluated up to 1 year until the time of this writing, and will be monitored continuously in the future. The short-term stability was tested over 2 weeks at room temperature and at 5 °C to set the transportation period and conditions.

**Comparative Study.** The candidate CRMs were analyzed by clinical testing laboratories conducting newborn screening (NBS)19 for a commutability assessment. Eight random spots of the CRMs were delivered to two different laboratories in ice packaging. Both laboratories used the disc sampling method with a 3-mm diameter autopuncher. The samples were handled following the same procedure as with real clinical specimens. The conversion factor from mass fraction to mass concentration (mg/L) is (1.05936 ± 0.00055) g/cm³.

**Investigation of Sampling Bias in DBS Testing.** Four different approaches have been devised to track the exact sample volume of the DBSs, three area-based and one mass-based. For the former, the area ratio of one disc to the whole spot was estimated (i) using the nominal value of the punch size (3.0 mm; disposable biopsy punch, Kai Medical), (ii) measuring with a ruler (resolution: 1 mm), and (iii) employing software (Image J, National Institute of Health). For the latter,
the mass ratio of one disc to the whole spot was estimated by weighing (resolution: 1 μg) during the preparation with precut paper and weighing at every step of the DBS sampling (SI Figure S1).

Additional DBS samples were prepared with different spotting volumes ranging from 10 to 100 μL for confirmation of sampling bias with respect to the spotting volumes.

## RESULTS AND DISCUSSION

### Optimization of the Measurement Procedure for AA Analysis of DBS Samples

A typical total ion chromatogram and multiple reaction monitoring scans of an SSA-treated DBS sample are shown in SI Figure S2. The six underivatized AAs (Pro, Val, Ile, Leu, Phe, and Tyr) were clearly separated by gradient elution in a short time (5 min). Since MS analysis is unable to distinguish between the isomeric compounds Ile and Leu, complete separation by LC is critical. Based on previous research, ion-pair chromatography was employed using TFA, a common reagent in LC-MS, as a light ion-pairing reagent. Moreover, AAs are zwitterions, and their ionization state can vary depending on the pH of the solution. The strongly acidic solutions from SSA treatment led to unstable peak shapes, which was successfully resolved with 10 mM AmAc buffer.

The process of DBS sample extraction and pretreatment was attempted in previous experiments, and the selected procedure was verified by a comparison between the measured values of liquid blood and DBS samples; Figure 2 shows the results of the comparison. The results between liquid blood (white bars) and whole spot DBS sampling (black bars) showed no reliable discrepancy, and thus a high extraction efficacy was verified.

The precision and accuracy of the optimized DBS measurement procedure were validated by a recovery test of standard addition. Three different DBS samples containing standard mixtures with different concentrations were measured, and the sample recoveries of the added amounts were calculated by subtraction of the blank values (DBS samples without the addition of standard solution) from the measured values. Comparing the recoveries between the added and found amounts, no significant difference was seen in all concentration ranges (Supporting Information Table S1). These results demonstrate that the optimized procedure is applicable to DBS samples.

### Characterization of AAs in DBS CRMs

The property values and between-spot homogeneity of the candidate DBS CRMs were assigned by measuring the mass fraction of the six AAs from 10 spots following the single-shot homogeneity test scheme in ISO Guide 35. The certified values of the AAs were assigned from the mean value of the 10 spots. The RSDs between spots of the six AAs in the two batches were found to be 0.8–1.6% (low) and 0.9–2.1% (high) from whole spot sampling (Table 1). The variations showed a similar range with other CRM batches made with liquid phase matrices such as plasma and serum; therefore, it can be said that the samples were sufficiently homogenized. Table 1 and Supporting Information Figure S3 summarize the measurement results and breakdown of the measurement uncertainties in the two batches with a coverage of 95% confidence. The uncertainty is largely divided into systematic uncertainty from the preparation of the standard samples, and random uncertainty of the sample measurements of batch homogeneity and gravimetrically assessed sampling variation by the aliquots.

The within-spot homogeneity was also investigated for disc sampling; however, the measured values showed significant
differences from the certified values. Figure 2 shows the results of the disc samples, where the results were adjusted based on the sample amount through area calculation. The known whole spot sample volume (50 μL) normally makes a 12-mm diameter spot with an area of 113.04 mm², and the area of one disc with a 3.0-mm diameter is 7.065 mm², equivalent to a volume of 3.125 μL. The results of disc sampling showed around 70% recovery to liquid blood regardless of the position within the spot from which the disc was taken. Table 2 shows the results of 18 discs taken from four spots regardless of position. The RSDs of the measured values were 2.3–3.3% (low) and 2.1–3.9% (high). These could serve as informative values for uncertainty increase by calibration hierarchy in DBS testing.

| AA   | measured value (mean ± SD, n = 18, mg/kg) |
|------|------------------------------------------|
|      | lowa                        | RSDb          | higha                      | RSD           |
| Pro  | 13.0 ± 0.4 3.0%             | 26.8 ± 0.8 2.9% |                           |
| Val  | 16.6 ± 0.4 2.2%             | 42.1 ± 0.9 2.2% |                           |
| Ile  | 6.56 ± 0.14 2.1%            | 40.3 ± 1.1 2.7% |                           |
| Leu  | 12.3 ± 0.3 2.5%             | 55.8 ± 1.3 2.3% |                           |
| Phe  | 7.72 ± 0.16 2.0%            | 45.0 ± 1.0 2.3% |                           |
| Tyr  | 6.46 ± 0.17 2.7%            | 15.8 ± 0.4 2.4% |                           |

aDBS CRM batches named low and high. bRelative standard deviation of measured values from 18 discs.

Stability Assessment of DBS CRMs. Following the certification procedure, long-term stability for storage conditions and short-term stability during transportation were confirmed. Supporting Information Figure S4 summarizes the stability results of the CRMs from four spots for each storage condition. No reasonable trends were detected in either stability test. Uncertainty from instability was able to be ignored, and thus the certified value and uncertainty assigned in Table 1 were used as the final certified value and uncertainty.

Comparative Study. The DBS CRMs were analyzed in a routine clinical setting by two laboratories. As shown in Figure 3(A), the measured values from routine testing using a nominal disc size with a 3-mm diameter were lower than the certified values, and moreover, there was a similar bias with the results in Figure 2. In addition, when we analyzed a commercially available DBS reference material for AAs (AAAC Multilevel DBS in the NeoBase MSMS kit, PerkinElmer) by disc sampling, the results were well harmonized between the laboratories as well as the reference values according to the manufacturer’s claim (Figure 3(B)). Based on these results, we can assume that the DBS-based assays in NBS are harmonized with disc sampling. Indeed, as one of the major application fields of DBS samples, NBS screens out risky groups for confirmation testing for inherited metabolic diseases using venous blood. From this point of view, the current priority in NBS may be high throughput and minimized false-negative tests. Nevertheless, it is no doubt that more accurate results in DBS testing can reduce redundant tests and narrow down the targets for further confirmation tests. Here, we focused on the bias by the sampling method.

Investigation of Disc Sampling Bias in DBS Testing. Actual Sample Volume Assessments. In Figure 2, we focused on the point that all residues showed the same pattern, which may suggest a systematic bias. Interestingly, compared to the whole spot result in Figure 2, the disc results were low while the remainder results were high, with the results compensating each other. The sample volumes used for the results were 3.125 μL for discs and 31.25 μL for the remainder by subtraction of the sample volume of six discs (18.75 μL) from the 50 μL of the whole spot. The lower portion of the disc results and the higher portion of the remainder seemed to be caused by the same factor, namely a calculation error in the sample consumption in the disc. To clearly define the reason for this bias in disc sampling, several approaches were adopted to calculate the sample size accounting for its extremely small size. Figure 4 shows the results of calculating the sample volume from the partial spot method in four different ways. The averages of the calculated sample volumes were 2.09, 2.11, 2.14, and 3.03 μL, respectively, in the four cases. As the measured value of the whole spots was 1, the recoveries differed with respect to the methods for sample size calculation: approximately 66.8%, 62.0%, 78.0%, and 97.1%,
respectively. In particular, the difference between the nominal punch size and the actual punched disc measured with a ruler was not very big, which can be attributed to the resolution of a common millimeter-scale ruler and the human eye. When measuring the diameter with a manual ruler, in the case of a very small disc, micrometer-scale differences cannot be clearly recognized at millimeter resolution. Here, a 0.4 mm (400 μm) bias in the diameter measurement led to a 0.78 μL difference in sample volume, which is almost 25% of the nominal disc sample (3.125 μL). This difference clearly confirms the underestimation in current DBS analysis by disc sampling.

To the best of our knowledge, the present study is the first investigation into the estimation of actual sample volumes. The results support the reason why other types of microsampling methods, such as VAMS and Hemaspot, have claimed better recoveries than traditional DBS sampling. This can also be confirmed from the fact that compensation is possible when the remainder is measured (~130%). In the computer software area calculation, a relatively enhanced recovery could be achieved, but estimation error by shadow or fragments along the cutting-edge could occur in the process of scanning (Supporting Information Figure S5).

From the calculation by the gravimetric method based on the direct dry mass measurement of blood (Supporting Information Figure S1), it was confirmed that the recovery could be significantly improved. Unfortunately, using software or gravimetry in the sampling process is impossible in current routine testing on account of the required time resources and risk of sample contamination. On the other hand, DBS calibrators and/or quality control materials can be handled precisely. Therefore, we can characterize the DBS CRMs with SI traceability by gravimetric preparation. If both the SI-traceable reference material and the patient sample have the same sampling bias in a routine testing procedure, measurement bias can be sufficiently compensated in the field by the reference material.

**Sampling Bias with Different Spotting Volume.** Figure 5 shows the results of different sizes of DBSs to simulate the various DBS calibrators and clinical samples with limited information about the spotting volume. The adsorbed amount
was varied from 10 to 100 μL, and the samples were tracked by gravimetry before and after spotting. The adsorbed amount and the dry mass showed a correlation of 0.9999, and the weights of the discs from any spot taken with the same puncher were the same in all cases. The measured values of the six AAs in the various DBS samples showed an increasing trend with increasing sample volume from 10 to 40 μL, and showed a relatively larger variation at 100 μL than at 40 or 50 μL. The experiment was then repeated with another batch of DBS samples with volumes of 40, 50, 60, and 70 μL. The results showed good stability over the whole range (data not shown). From these results, we found the proper range of spotting volume for common DBS paper: at least 40 μL but not exceeding 100 μL. Based on this concept, it can be assumed that even if the actual spotting volumes differ, there is no significant difference when using the same puncher on samples within the recommended spotting volume. Filter papers for DBS sampling normally print a dashed guideline for spotting; in this study, the circles were almost filled with 40 μL but overfilled with over 70 μL of whole blood. Accordingly, even without detailed volume measurements, the above recommendation can be applied to real clinical settings.15,16,19

■ CONCLUSIONS
In this study, we developed certified reference materials for amino acid analysis in dried blood spot sampling. The target compounds were six AAs that are the diagnostic markers of representative AA metabolic disorders. Two batches of DBS samples were prepared as candidate CRMs. Sample preparation and analytical conditions were optimized. Since a negative bias in disc sampling was found to come from the overestimation of sample size, whole spot sampling was adopted in the certification of the candidate CRMs to minimize uncertainty in measurement. Sample homogeneity was strictly investigated between and within spots throughout the batches for clinical applications, and both long- and short-term stability was assessed.

In terms of such standardization, comparable results regardless of the analytical methods and the laboratory where analyses are carried out can be achieved by the development of reference materials and reference measurement procedures. In this light, we hope that the proposed DBS CRMs with SI traceability can play a role in DBS-based testing. Additionally, it is expected that this will be a base study for the expansion of DBS applications with social and economic benefits such as less-invasive blood collection and ease of sample storage and transportation, as well as reliable sampling outside of hospitals, for example, at home, for the realization of remote diagnosis in the coming generations.

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c01349.

Additional information including Table S1 and Figures S1–S5 (PDF)

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Notes
The authors declare no competing financial interest.

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