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Development of an Ephedrine In-House Matrix Reference Material and Its Application to Doping Analysis

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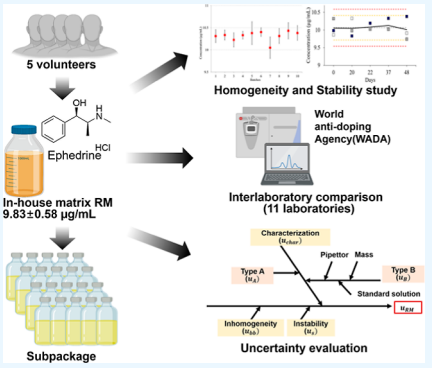
ABSTRACT: Biomatrix-based reference materials (RMs) improve the quality of laboratory test results by better representing actual samples. However, a matrix RM of ephedrine (EP) for threshold substances that require accurate analysis results has not yet been developed. Therefore, this study aimed to develop an in-house matrix RM for EP and subsequently apply it to analytical procedures. During the development of the in-house matrix EP RM, the system underwent homogeneity and stability studies. Additionally, it was subjected to interlaboratory comparison study in 11 laboratories, including 10 World Anti-Doping Agency (WADA)-accredited laboratories and our laboratory. Stability testing revealed no significant changes in the RM characteristics. For homogeneity, 10 random batches out of 200 were analyzed to confirm the uniformity within and between bottles. These results, combined with data from 11 laboratories, ensured retroactive validation. The traceability value of the in-house matrix EP RM was assigned as 9.83 ± 0.57 μg/mL (k = 2) by interlaboratory comparison studies and traceable uncertain evaluation. The feasibility of this method as a single calibration standard was confirmed in two laboratories. This substance is reliable and consistent for quality control during EP quantification, ensuring accurate and trustworthy outcomes. Consequently, this study establishes a framework and guidelines for producing in-house matrix RMs and serves as a reference for generating similar matrix RMs in other contexts.



INTRODUCTION

Ephedrine (EP) is classified as a sympathomimetic amine, which encompasses several compounds such as EP, pseudoephedrine (PEP), methyl EP (MEP), and nor pseudoephedrine (NPEP). These substances are S6 stimulants in the doping prohibited list.[1] EP is acknowledged as a prominent stimulant and can offer unfair benefits in sports by augmenting alertness, reducing fatigue, and reinvigorating cardiovascular functions. This capability has significant advantages across a wide range of sporting disciplines.[2] Although the list of exogenous threshold substances does not include NPEP, distinct threshold concentrations in urine have been established for various other EP derivatives. For instance, EP and MEP, PEP, and NPEP have defined thresholds of 10, 150, and 5 μg/mL, respectively. These thresholds are designed to differentiate between permissible therapeutic usage and prohibited illicit use.[3] When the concentration of stimulants in the urine of athletes exceeds the decision limit, it will be regarded as an adverse analytical finding, unless athletes have been granted a treatment exemption for stimulants. Therefore, there is a crucial necessity for the accurate quantitative analysis of stimulants in athlete urine samples.[4]

Accordingly, many methods have been designed for the accurate analysis of EP, such as gas chromatography with a nitrogen phosphorus detector (GC-NPD),[5] GC mass spectrometry (GC−MS),[6] ion mobility MS (IM-MS),[7] and liquid chromatography MS in tandem (LC−MS/MS).[8] GC−MS is widely used, but urine samples need to be derivatized, which is cumbersome and time-consuming. LC−MS/MS is the most commonly employed approach, allowing the direct injection of diluted urine into the chromatographic system.[9] This dilute-switch method has proven to be uncomplicated, efficient, and rapid. As a result, it reduces sample manipulation and enhanced quantification reliability.[10]

Reference materials (RMs), particularly those representing matrix characteristics, are often real-world samples, such as foods, environmental substances, or clinical materials containing the target analytes.[11] The use of RMs is important in ensuring comparability and traceability between measurements at the national and international levels.[12] In doping tests, quantitative analysis of insulin-like growth factor 1 (IGF-1) involves obtaining the concentration through a calibration curve and then further adjusting it using a single-point

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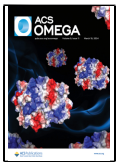

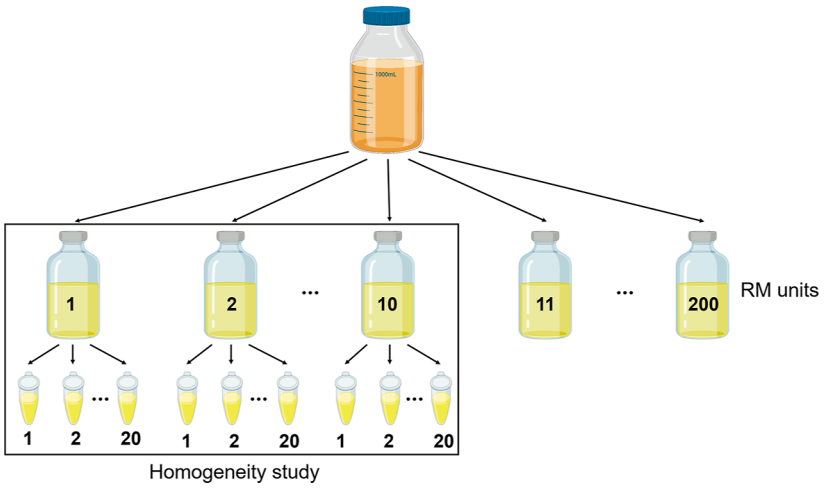

Figure 1. Scheme of matrix RM development. A 1000 mL sample of urine was divided into 200 aliquots. Ten batches were randomly selected from the 200 batches for the homogeneity study, and each batch was divided into 20 subsamples.

calibrator with a matrix RM. This process aids in mitigating batch effects within each experiment and diminishing analytical discrepancies across laboratories worldwide.[13,14] Consequently, the use of RMs has become a crucial step in attaining consistency and verifiability across measurements conducted on both national and international scales. However, the supply of RMs capable of fulfilling these criteria remains constrained.[15] However, a matrix RM for the quantification of substances in doping tests, especially those demanding the utmost accuracy, has been conspicuously absent from the scientific landscape. In response to this gap, laboratories often opt to produce in-house matrix RMs. Although there is no formal certification, it can be supplemented through traceability, homogeneity, and stability for their intended applications.[16]

This study aims to develop a matrix-based RM for use for EP. Additionally, we intended to provide comprehensive guidelines for developing a matrix RM to ensure the accurate quantification of substances at the in-house level. We not only assessed the homogeneity and stability of the RM but also verified its traceability and characterization through experiments conducted across 11 laboratories. This indicates the potential utility of the in-house matrix RM as a single-point calibrator. It can be applied in the field of analysis, where precise analytical outcomes are required. Consequently, this study established procedures and considerations for generating in-house-level matrix RMs and can be used as a reference for generating other matrix RMs.

EXPERIMENTAL SECTION

Reagents and Chemicals. EP-hydrochloride, PEP-hydrochloride, and d3-EP were obtained as solutions from Cerilliant Co. (Round Rock, TX, USA). LC−MS-grade acetonitrile (ACN) and methanol were provided by AVANTOR (Radnor Township, PA, USA). Formic acid (FA) was obtained from Wako Pure Chemicals (Osaka, Japan). High-performance liquid chromatography (HPLC)-grade water, 0.1% FA in ACN, and 0.1% FA in water were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

In-House Matrix-Based RM. This study received approval from the Institutional Review Board of the Korea Institute of Science and Technology (KIST-202304-BR-002). Before consent was obtained, all volunteers were provided with verbal and written explanations of the purpose of using the samples in the study and their impact on the research field. To prepare the matrix RM, a 1000 mL urine sample was obtained from five adults. After confirmation of the absence of EP in the urine samples, the samples were spiked with 10 μg/mL EP. The spiked urine samples were divided into 200 aliquots. Figure 1 illustrates the research design. The matrix RM samples, excluding the samples for stability testing, were stored at −80 °C in a deep freezer.

Sample Preparation. A method previously described in ref 9 was optimized to prepare the samples. First, 20 μL of the matrix RM sample was prepared. Subsequently, 20 μL of d3-EP (5 μg/mL) was added to the sample as an internal standard (ISTD) and then diluted by adding 210 μL of loading A buffer (0.1% FA and 2% ACN). After centrifugation at room temperature for 10 min at 16,000*g*, the resulting supernatant was transferred to a vial for subsequent analysis.

LC Method Condition. A UFLC XR series HPLC system (Shimadzu, Japan) equipped with an ACE C18 column (100 × 2.1 mm, 5 μm, Advanced Chromatography Technologies) was used for the LC analysis. The mobile phase consisted of water (A) and ACN (B), both of which contained 0.1% FA as an ion-pair reagent. To enhance the retention of polar EP molecules, a low concentration of organic solvent was utilized in gradient mode. The initial gradient composition (2% B) was maintained for 0.1 min and then gradually increased to 6% B in 0.09 min. Subsequently, it was increased to 11% B over 5.7 min, followed by a linear increase to 95% B over 5.9 min. This composition was maintained for 0.6 min before it was reduced to 2% B over 0.1 min. A postrun equilibration was conducted for 1.4 min. The flow rate was set at 0.4 mL/min, and the column temperature was maintained constant at 35 °C.

MS Method for SRM Mode. A TSQ Ultra Triple Quadrupole MS instrument (Thermo Scientific, Waltham, MA, USA) was used. The positive-ion spray voltage was set to 4500 V, and the auxiliary gas pressure was maintained at 60 arbitrary units (arb). The operating conditions included capillary and vaporizer temperatures of 320 and 340 °C, respectively. The Q1 and Q3 resolutions for the experiment were set to 0.7, and the collision gas pressure was maintained at 1.5 mTorr.

Method Validation. The linearity, selectivity, carryover, limit of quantification (LOQ), intra- and interday precision,

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accuracy, recovery, and matrix effect were included in the validation results according to the WADA and ISO/IEC 17025 guidelines.[17] Samples were prepared at concentrations of 0, 0.5, 1, 5, 8, 10, 12, 15, 20, and 40 μg/mL for linearity. Five replicates were prepared for each concentration. For validation, the calibration curve consisted of eight calibration standards ranging from 50 to 200% of the threshold value, in accordance with the WADA guidelines.[18] The LOQ is the minimum content that can be detected accurately at target concentrations. The signal-to-noise ratio must be >10, and the % CV target concentration must be <10%. Five samples with a 20 μg/mL concentration and five negative urine samples were selected for the selectivity measurements and alternately analyzed. Considering that the main component of urine is water, water was used as a blank sample to assess the carryover. Water was measured after analyzing the highest concentration in the standard curve. To evaluate the matrix effect, the peak area of water was compared with that of the sample. Precision and accuracy were assessed by examining three replicates of each urine sample concentration (1, 2, 8, and 16 μg/mL).

Measurement uncertainty can be applied to quantify the uncertainty of quantitative results and contribute to the improvement of methods by identifying the causes of uncertainty. The contribution of the characterization value (u_{char}), uncertainty due to the calibration curve (u_{CCur}), and method precision ($u_{precision}$) were regarded as the sources of uncertainty.[21] The sources of uncertainty were visualized using the cause and effect diagram, as depicted in Figure S1.

Assessment of the Homogeneity, Stability Study, and Interlaboratory Test. Homogeneity and stability are crucial properties for the intended use of RMs. Furthermore, the characterization of matrix RMs can be performed by multiple laboratories. ISO Guide 35 provides guidance on the characterization and assessment of homogeneity, stability, and the method for interlaboratory tests.[19]

The combined standard uncertainty (u_c) of the concentration for our in-house matrix RM was estimated by aggregating the contributions from the value assignment (u_{char}), homogeneity (u_{bb}), and uncertainty owing to long-term instability (u_s). Figure 2 shows the sources of uncertainty

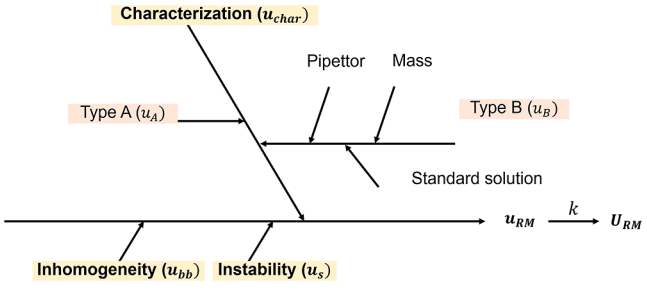

Figure 2. Uncertainty sources during the development of the in-house matrix. The characterization, homogeneity, and long-term instability were regarded as sources of uncertainty.

introduced during development of the in-house matrix RM. Given that the conditions of the short-term stability study (dry ice transport) aligned with the long-term storage conditions, uncertainties arising from transport-related instability were not factored into the analysis.

Homogeneity Study. Of the 200 sample batches, 10 were randomly selected for the between-bottle homogeneity test. Five subsamples from one batch consisting of 20 subsamples were randomly selected for the within-bottle homogeneity test. Each in-house matrix RM was tested in triplicate, and the EP concentrations were measured using LC−MS. The measurements were randomly conducted to minimize the influence of the instrument. Homogeneity was determined using the analysis of variance (ANOVA).

Stability Study. Stability refers to the capacity of the properties of an RM to remain consistent within predefined values for a specific duration. A longer specified time interval indicates better stability of the property value.[20] Both short- and long-term stability tests were conducted to assess the stability of the RM matrix. In the short-term stability test, the stability of the material over a period of 60 days was evaluated while subjecting it to storage conditions of 4, −20, and −80 °C. Each sample was tested in triplicate. In the long-term stability tests, the stability of the material was examined over 6 months under the same storage conditions.

Interlaboratory Comparison. The concentration of the sample was measured using interlaboratory comparisons. The 10 WADA-accredited laboratories performing the doping control analysis and our laboratory participated in this study. Each laboratory received 12 mL of the sample, and three measurements were conducted in five different batches. The measurements were performed using their own protocols and instrument parameters to analyze the EP. Table S1 summarizes the adapted sample preparation method and instruments used.

Data Analysis. Skyline version 22.2.0.255 (University of Washington, USA)[22] was employed to perform the quantitative analysis. The concentrations of the calibrators and samples for the validation, homogeneity, and stability tests were estimated using Skyline software. ANOVA analysis for the homogeneity study was performed on the data as a statistical tool to determine the difference in mean values using MINITAB software (Minitab, version 18.0).

RESULTS AND DISCUSSION

SRM Scan of EP Substances in Pooling Urine. EP was subjected to analysis in selected reaction monitoring (SRM) mode. This mode selectively monitors the transitions specific to the target analyte, ensuring high specificity and minimizing interference from other compounds. Simultaneously, it offers high sensitivity for the detection of compounds in a complex matrix.

EP and PEP are considered cis−trans isomers because of the differences in the positions of their hydroxyl groups. In quantitative experiments for EP compounds, the separation of these isomers is essential but arduous. The compounds were successfully separated not through chromatography but via isocratic or modified gradient elution methods. The SRM transitions for EP and PEP were observed at m/z 166−133, whereas those for d3-EP occurred at m/z 169−117. This separation was accomplished within an 8 min run with EP eluting at 5.21 min and PEP at 5.43 min (Figure 3).

Method Validation. The validation results included linearity, LOQ, selectivity, carryover, intra- and interday precision and accuracy, and matrix effects. The linear reference value (r^2) was 0.9997. A peak of the blank sample was not detected in the selectivity result after analyzing a high EP concentration sample. Carryover was confirmed when the ratio of urine sample peak area to blank peak area was less than 1%. No matrix effects were observed for any of the samples tested in this study. Four concentration-level quality control (QC) samples were measured on 3 separate days using three

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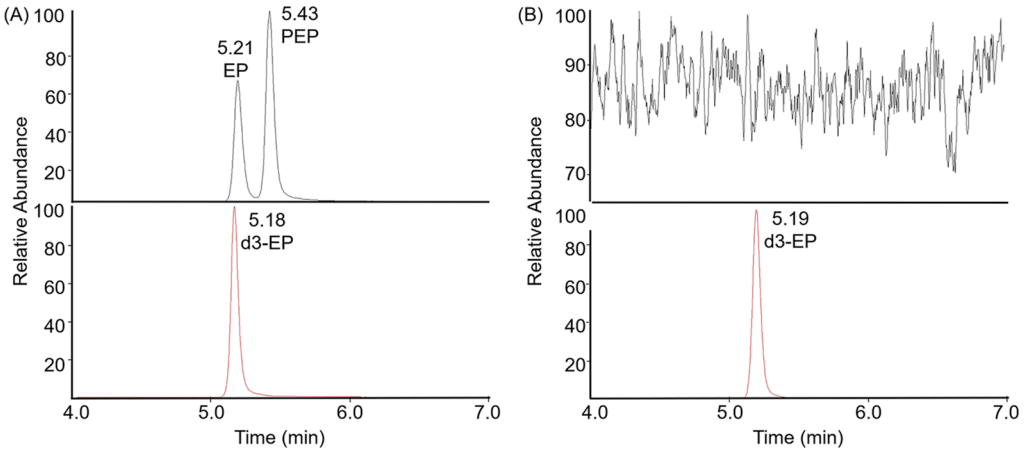

Figure 3. SRM scan of the EP substances. (A) Separation of the EP and PEP mixture and (B) blank urine; the chromatograms of d3-EP are shown under the chromatograms of EP, PEP, and blank urine.

replicates to determine the inter- and intraday precision and accuracy. Regarding inter- and intraday precision, the % CV values for the EP samples at the four different concentrations were all <10%. The accuracy, defined as the ratio of the measured value to the nominal value (NV) of the calculated QC concentration of each sample, met the acceptance criterion of 15%. Table S2 provides a summary of the results.

Evaluation of the Uncertainty of Method Development. *Evaluation of the Uncertainty Introduced by Characterization.* Type B uncertainty was evaluated in the context of the characterization. The apparatus employed for method validation was identical to that used for the development of the in-house matrix RM. The relative standard uncertainty of characterization was determined using eq 9.

Evaluation of the Uncertainty Introduced by the Calibration Curve. The uncertainty of the optimized method was measured using a 2020 EQAS-01 Sample 05. The External Quality Assessment Scheme (EQAS) distributed by the WADA serves the purpose of obtaining bias estimates and monitoring laboratory capabilities. The EQAS evaluation is carried out blindly, where laboratories are aware that it is provided by WADA but remain unaware of the sample contents. The EQAS concentration is measured based on data submitted by about 30 WADA-accredited laboratories. The interlaboratory EQAS agreed-upon value of 11.1 μg/mL, determined using robust statistical methods, was adopted. We measured the EQAS sample repeatedly for 5 times, and the average value was derived as 11.24. The measurement uncertainty associated with the fitted calibration curve was calculated using the following error propagation formula

$$u_{\mathrm{CCur}} = \frac{S_{y/x}}{b_1}\sqrt{\frac{1}{r_{\mathrm{EQAS}}} + \frac{1}{n} + \frac{(x_{\mathrm{EQAS}} - \overline{x})^2}{S_{xx}}} \tag{1}$$

$$S_{y/x} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \widehat{y_i})^2}{n-2}} \tag{2}$$

where $S_{y/x}$ represents the residual or standard error when regressing y on x; r_{EQAS} represents the number of replicates conducted on the EQAS sample to determine x_{EQAS}; b_1 is the slope of the regression line; n signifies the number of measurements used to generate a calibration curve; x_{EQAS} is the mean concentration of EQAS; and $\overline{x}$ is the mean of the various calibration standards. S_{xx} is calculated as the sum of the squared deviation of x, which is expressed as $\sum^{i=1n}(x_i - \overline{x})^2$.

The relative standard uncertainty of the calibration curve can be determined using eq 3.

$$u_{\mathrm{r}}(\mathrm{CCur}) = \frac{u_{\mathrm{CCur}}}{x_{\mathrm{EQAS}}} \tag{3}$$

Table S3 lists the peak areas of the ISTDs and EP at eight different concentrations. The standard error of the regression was determined using eq 4 and the data values from eq 2 and Table S3.

$$S_{y/x} = \sqrt{\frac{0.024}{8-2}} = 0.063 \tag{4}$$

The uncertainty associated with the calibration curve was 0.19, and the relative measurement uncertainty was calculated to be 0.017.

Evaluation of the Uncertainty Introduced by the Method Precision. Table S4 summarizes the four QC concentrations used to validate the evaluation and uncertainty. The data uncertainty was calculated using eq 5

$$u_{\mathrm{precision}} = \frac{S_{\mathrm{p}}}{\sqrt{r_{\mathrm{cs}}}} \tag{5}$$

$$s_{\mathrm{p}} = \sqrt{\frac{\sum_i (v_i \times S_i^2)}{\sum_i v_i}} \tag{6}$$

where v_i is the degree of freedom of the ith sample, S_i is the standard deviation of the ith sample, and r_{cs} is the number of case sample replicates. The relative standard uncertainty of the method precision was calculated by dividing the standard uncertainty by the NV.

Table S5 details the uncertainty calculations for the method precision for each concentration. x_{EQAS} = 11.24, the closest value (NV = 8) was used for the calculation, and the relative standard uncertainty of precision of the method was 0.0081.

Combined and Expanded Uncertainty. The combined uncertainty (u_{method}) can be determined by combining all individual uncertainty components as represented in eq 7

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$$\frac{u_{\text{method}}}{x_{\text{EQAS}}} = \sqrt{u_r\,(\text{char})^2 + u_r\,(\text{CCur})^2 + u_r\,(\text{precision})^2} \quad (7)$$

Hence

$$u_{\text{method}} = 11.24 \times \sqrt{0.016^2 + 0.017^2 + 0.0081^2}$$

Ultimately, the expanded uncertainty was calculated by multiplying the coverage factor (k) by the combined uncertainty. Using a predetermined coverage factor (k) of 2, corresponding to a 95% confidence level, the expanded uncertainty was determined to be 0.56 μg/mL.

In-House Matrix RM Analysis. Individuals who had recently taken medication were excluded from the study to ensure the accuracy of the results. Urine samples obtained from five volunteers were analyzed using LC−MS to determine the presence or absence of interference peaks at the expected retention time for EP. No interfering peaks were observed in the blank urine samples. These urine samples were subsequently used as the matrix and spiked with 10 μg/mL of EP to develop an in-house RM. The dilute-and-shoot method was employed for preparing the sample. This method simplifies sample preparation by directly injecting a diluted sample into the analytical instrument and offers time, labor, and cost savings by eliminating the need for complex extraction and purification steps. Furthermore, it ensures the accuracy and reliability of the results by minimizing the potential for sample loss or contamination, making it highly suitable for high-throughput analysis of multiple samples. These advantages make the dilute-and-shoot method particularly well-suited for doping analysis.[23] This approach was used in this study to ensure consistent and precise results, which are crucial for ensuring the reliability of the doping analysis.[24,25]

Homogeneity Study. The results obtained from the homogeneity study were analyzed using ANOVA (one-way layout). The test statistic (F = 0.95) was below the critical value [$F(9, 40)$], indicating no significant difference among the samples. Figure 4 shows the outcomes of the homogeneity study. The results conclusively established that the sample exhibited a satisfactory level of homogeneity, indicating its suitability as an in-house RM matrix (p-value 0.49).

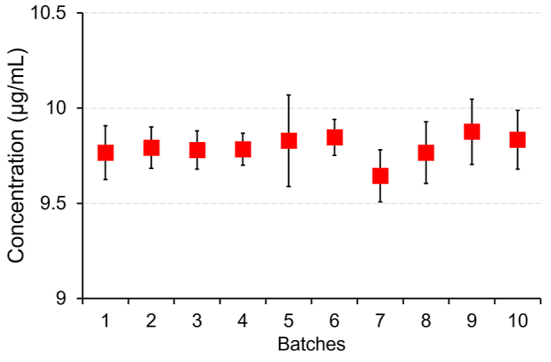

Figure 4. Homogeneity study from ANOVA analysis. The dots represent the mean of five replicates from the batch. Error bars represent the standard errors.

Stability Study. The samples were divided into the minimum packaging units and stored at three predetermined temperatures (4, −20, and −80 °C). The sample concentration was measured over 60 days at days 1, 20, 37, 48, and 60. The results, as presented in Figure 5, indicate that the sample exhibited excellent short-term stability when stored at −80 °C.

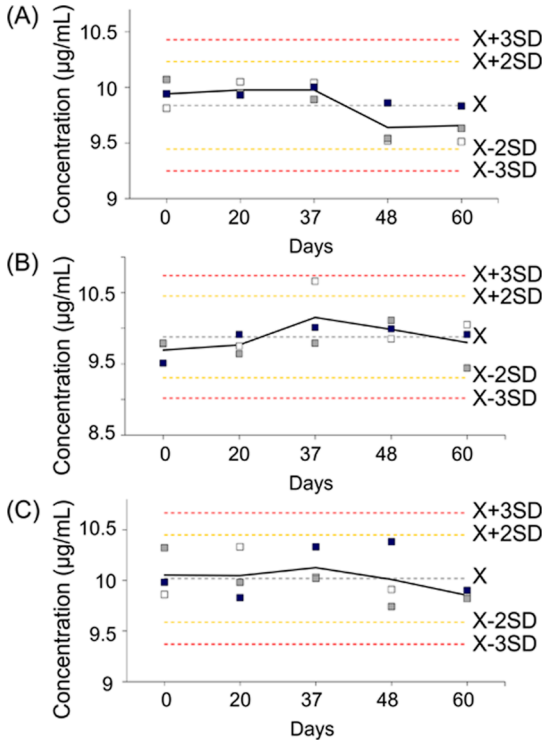

Figure 5. Short-term stability study for 60 days. (A) Result of stability study at 4, (B) −20, and (C) −80 °C. White dots: first result, gray dots: second result, and blue dots: third result. X and SD are the mean concentration and standard deviation obtained at each temperature condition, respectively.

The long-term stability of the sample at −80 °C, the temperature that exhibited the highest stability during the short-term stability, was assessed (Figure 6). Measurements were also performed at 4 and −20 °C. Table S6 details the data for these conditions along with the data for the −80 °C condition.

Interlaboratory Analysis for Traceability. To ensure traceability of the concentration of the in-house-developed matrix RM, analysis results were obtained from 10 independent

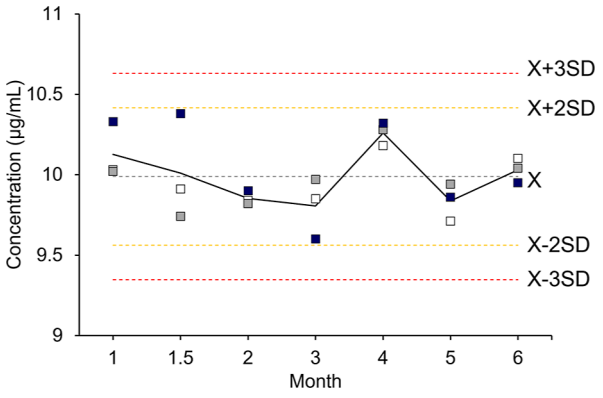

Figure 6. Long-term stability study over 6 months at −80 °C. White dots: first result; gray dots: second result; and blue dots: third result. X and SD are the mean and standard deviation obtained at each temperature condition, respectively.

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Table 1. Inter-Laboratory Comparison Results of Characterization by 11 Laboratories

laboratory	first	second	third	fourth	fifth	mean	z-score
our lab	9.94	9.98	9.89	9.98	9.64	9.89	0.38
A	9.44	9.47	9.38	9.73	9.51	9.51	−0.43
B	9.97	9.90	9.83	9.97	9.90	9.91	0.43
C	10.53	10.73	9.70	9.43	9.73	10.03	0.68
D	9.43	9.33	9.63	9.49	9.05	9.39	−0.68
[a]E	9.98	10.07	10.33	10.01	10.14	10.11	0.85
[b]F	8.82	8.17	8.15	8.69	8.47	8.46	−2.66
G	9.76	10.07	10.20	9.44	9.93	9.88	0.36
H	9.54	10.63	9.72	10.33	[c]8.82	10.05	0.72
I	9.58	10.01	9.99	9.82	[d]	9.85	0.30
J	9.33	9.96	9.93	9.66	9.78	9.73	0.04
			mean ($\bar{\bar{a}}$)			9.83	

[a]Although laboratory E is in the same institution as our laboratory, it uses an independent method and sample preparation procedure registered to ISO 17025 to analyze EP. [b]The z-score of the mean of laboratory F was −2.66, which is interpreted as a questionable performance. Data from laboratory F was excluded. [c]We considered 8.82 as an outlier, as notified by the laboratory H. [d]Laboratory I conducted measurements on four batches. The average was calculated using the four data sets.

WADA-accredited laboratories, as well as from our own lab. The results from each laboratory were assessed using the z-score, with the following typical interpretations: $|z| \leq 2$ indicates satisfactory performance, $2 < |z| < 3$ suggests questionable performance, and $|z| \geq 3$ signifies unsatisfactory performance.[26] Outliers were removed when the z-scores exceeded $|z| > 2$. Laboratory F was excluded from the study due to its unsatisfactory performance based on the z-score result. The arithmetic mean of these values could be considered as the traceability value of the in-house matrix RM, which was determined to be 9.83 μg/mL (Table 1).

Evaluation of the Uncertainty of the In-House Matrix RM Development. *Evaluation of the Uncertainty Introduced by Characterization.* Standard uncertainties in the characteristic values fall into two categories: type A uncertainty is determined using statistical methods, and type B uncertainty is calculated by considering the standard deviation assigned to the measurement and expert judgment. The apparatus used to introduce uncertainty into the characterization results was calibrated by the Metrology Institute. Table S7 specifies the uncertainties associated with the apparatus used. The standard uncertainty evaluation of type A was determined using statistical calculations, considering the standard deviation of the measurement data, number of measurements, and specified confidence level. By integration of the data in Table 1 into eq 8, the type A uncertainty ($u_{\text{type A}}$) for the characterization was determined to be 0.074 μg/mL.

$$u_{\text{A}} = \sqrt{\sum_{i=1}^{m} (\overline{ai} - \bar{\bar{a}})^2 / m(m-1)} \tag{8}$$

where m = 10 represents the number of participating laboratories in the characterization process, $\overline{ai}$ denotes the measurement result of the sample concentration in each laboratory, and $\bar{\bar{a}}$ is the arithmetic mean of $\overline{ai}$. The type B standard uncertainty evaluation relies on scientific analysis and judgment, considering all available information throughout the evaluation process. The type B relative standard uncertainty can be expressed using eq 9 in accordance with the law of propagation of uncertainty.

$$u_{\text{r}}\ (\text{type B}) = \sqrt{[u_{\text{r}}(V)]^2 + [u_{\text{r}}(C)]^2 + [u_{\text{r}}(W)]^2} \tag{9}$$

where u_{r} (V) is 0.016, u_{r} (C) is 0.017, and u_{r} (W) is 1.54×10^{-6}. Therefore, u_{B} is 0.16 μg/mL by multiplying x_{RM}, 9.83. By applying the law of propagation of uncertainty, the combined uncertainty for characterization was calculated as

$$\begin{aligned} u_{\text{char}} &= \sqrt{(u_{\text{A}})^2 + (u_{\text{B}})^2} \\ &= \sqrt{0.074^2 + 0.16^2} \\ &= 0.18 \mu\text{g/mL}. \end{aligned}$$

Evaluation of the Uncertainty Introduced by the Homogeneity Study. The uncertainty arising from the possibility of homogeneity (u_{bb}) was incorporated into the overall uncertainty, which was calculated using eq 10.

$$u_{\text{bb}} = \sqrt{\frac{N(m-1)}{N^2 - \sum_{i=1}^{m} n_i^2}(s_1^2 - s_2^2)} \tag{10}$$

where m = 10 denotes the number of vials selected for measurement, n = 5 represents the number of repeated measurements per vial, N = 50 indicates the total number of measurements, and s_1^2 and s_2^2 correspond to the squared deviations between and within the vials, respectively. Consequently, the uncertainty arising from the homogeneity study (u_{bb}) was 0.12 μg/mL.

Evaluation of the Uncertainty Introduced by the Stability Study. A regression analysis was employed to examine the data points and assess the uncertainty (Table 2). The resulting slope of the regression function (β_1) was determined to be −0.0084, with no statistically significant deviation from zero. This indicates the stability of a_{RM} over 6 months. The uncertainty attributed to the potential instability (u_{s}) was quantified at 0.19 μg/mL, as determined using eq 11.

Table 2. Regression Statistics for the Long-Term Stability Assessment

items	results
slope of the regression function, β_1	−0.0084
intercept of the regression function, β_0	10.02
standard deviation of each data point, s^2	0.028
standard deviation of β_1, $s(\beta 1)$	0.031

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$$u_s = s(\beta_1)\cdot T \quad (11)$$

where $s(\beta_1)$ represents the standard deviation of β_1 and T is the storage time.

Expanded Uncertainty Evaluation of the Traceability Value of the In-House Matrix RM. Various uncertainties may arise during the development of an in-house matrix RM, which can be categorized into homogeneity, stability, and characteristic values. The combined uncertainty (u_{RM}) was determined by combining these three types of uncertainties using the root-sum square. eq 12 given below was used to calculate the combined uncertainty, which was 0.28 μg/mL. Using a determined coverage factor (k) of 2, which corresponds to a 95% confidence level, the expanded uncertainty was calculated to be 0.57 μg/mL.

$$u_{RM} = \sqrt{{u_{char}}^2 + {u_{bb}}^2 + {u_s}^2} \quad (12)$$

$$U_{RM} = u_{RM} \times k \quad (13)$$

Consequently, the assigned traceability value for the in-house matrix RM of EP was 9.83 ± 0.57 μg/mL, as established through interlaboratory comparative studies and uncertainty assessments.

Application to Doping Analysis. Deviations may have occurred in the experimental results because the instruments and pretreatment methods used in each laboratory differed. To reduce variability, researchers have employed various calibration methods to enhance precision and interpret arbitrary signal intensities. External calibration materials are used to calibrate interlaboratory results. The calibration materials from a single laboratory result in less variation than the materials produced in individual laboratories. The matrix RM developed in this study could serve as a single-point calibrator, and its quantitative accuracy was evaluated.

Three concentrations of EQAS (2016 EQAS-03 sample 13, 2020 EQAS-01 sample 05, and 2019 EQAS-01 sample 05) provided by our laboratory and laboratory E were compared. As shown in Table 3, laboratory E exhibited higher numerical values across all data sets. The reasons that the two laboratories show different patterns can be attributed to various factors. For instance, differences in the experimental conditions, equipment accuracy, sample processing methods, or variations among experimenters could have influenced the outcomes. It is speculated that these diverse factors contributed to the formation of distinct accuracy improvement patterns in each laboratory.

Table 3. Accuracy Comparison before and after the Application of the Matrix RM as a Single-Point Calibrator

lab	2016 EQAS-03 sample 13		2020 EQAS-01 sample 05		2019 EQAS-01 sample 05	
	7.4 μg/mL		11.1 μg/mL		12.3 μg/mL	
	w/o RM (%)	with RM (%)	w/o RM (%)	with RM (%)	w/o RM (%)	with RM (%)
our lab	95.27	98.90	96.49	98.92	97.64	98.86
lab E	106.38	102.22	104.86	102.19	105.33	102.30

Although the accuracy exhibited different patterns, the variation in the concentration with the matrix RM as a single-point calibrator was lower than that without the calibrator. The consistency with the nominal concentration value of the EQAS also improved. The accuracy, which is the percentage difference from the observed concentration nominated by WADA, was also improved in both the laboratories and the three samples. Improved imprecision across laboratories has profound implications for doping analysis. This study demonstrates that the developed RM matrix can reduce the deviation between antidoping laboratories worldwide. Furthermore, the results of interlaboratory testing were established, ensuring its applicability in international laboratories specializing in EP analysis.

CONCLUSIONS

Matrix RMs ideally represent the composition of the studied real-world samples. Therefore, matrix RMs play a pivotal role in validating the entire analytical method and evaluating the accuracy and comparability of the results across various laboratories over time. However, the development of matrix RMs poses many challenges. Even if matrix RMs are developed at the laboratory level, these studies are susceptible to high research costs and slow processes. Furthermore, a comparison of results between different laboratories also presents a limitation to the study. Additionally, matrix RMs for substances requiring precise quantification in urine have not yet been developed. Therefore, this study aimed to develop a matrix RM for EP and provide insights into the matrix RM production process, effectively serving as a guideline for future research in this field.

In this study, an in-house matrix RM for EP was developed. The homogeneity and stability of the in-house matrix RM were confirmed, and traceability was ensured through analyses performed in 11 different laboratories. Additionally, the results of a comparison between two laboratories demonstrate that the in-house matrix RM can effectively serve as a single calibrator, reducing the variation in results between laboratories in the analysis of threshold substances in doping tests. Through various processes and validation steps, we successfully produced 1000 mL of an in-house RM matrix for EP as a result of this research. We intend to share this in-house matrix RM with the research community in need of accurate analysis. Furthermore, this study provides valuable insights into the matrix RM production process and can serve as a practical guide for future research in this field.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08316.

Uncertainty of method for quantitative analysis; sample preparation method and instrument used by each laboratory for interlaboratory comparison; result of validation; peak area ratio of seven calibration curve points, linear regression coefficients, and the sum of squares of regression for the calibration curve data; data for four concentration levels for three different days with three replicates; uncertainty of the method precision calculation for four concentration levels; long-term stability study; and uncertainty of the relevant reagents and apparatuses (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Heuberger, J. A.; Cohen, A. F. Review of WADA prohibited substances: limited evidence for performance-enhancing effects. *Sports Med.* **2019**, *49* (4), 525−539.

(2) Docherty, J. Pharmacology of stimulants prohibited by the World Anti-Doping Agency (WADA). *Br. J. Pharmacol.* **2008**, *154* (3), 606−622.

(3) World Anti-Doping Agency. *WADA Technical Document - TD2021DL version 1.0*, 2022.

(4) Deventer, K.; Pozo, O.; Verstraete, A.; Van Eenoo, P. Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology. *TrAC, Trends Anal. Chem.* **2014**, *55*, 1−13.

(5) Van Eenoo, P.; Delbeke, F. T.; Roels, K.; De Backer, P. Simultaneous quantitation of ephedrines in urine by gas chromatography-nitrogen-phosphorus detection for doping control purposes. *J. Chromatogr. B: Biomed. Sci. Appl.* **2001**, *760* (2), 255−261.

(6) Sardela, V. F.; Sardela, P. D. O.; Pereira, H. M. G.; Aquino Neto, F. R. Consequences of the formation of 3,4-dimethyl-5-phenyl-1,3-oxazolidine on the analysis of ephedrines in urine by gas chromatography and a new method for confirmation as N-trifluoroacetyl-O-t-butyldimethylsilyl ether derivatives. *J. Chromatogr. A* **2011**, *1218* (9), 1266−1272.

(7) Cumeras, R.; Figueras, E.; Davis, C. E.; Baumbach, J. I.; Gràcia, I. Review on Ion Mobility Spectrometry. Part 1: current instrumentation. *Analyst* **2015**, *140* (5), 1376−1390.

(8) Deventer, K.; Pozo, O. J.; Van Eenoo, P.; Delbeke, F. T. Development and validation of an LC-MS/MS method for the quantification of ephedrines in urine. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877* (4), 369−374.

(9) Zhu, T.; Jin, Y.; Zhao, J.; Jing, J.; Tian, T.; Shan, Y.; Xu, X.; Wang, Y. Qualitative and quantitative analysis of ephedrine stimulants in urine by ultra-performance liquid chromatography-tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2022**, *36* (4), No. e9229.

(10) Deventer, K.; Pozo, O. J.; Verstraete, A. G.; Van Eenoo, P. Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology. *TrAC, Trends Anal. Chem.* **2014**, *55*, 1−13.

(11) Walker, R.; Lumley, I. Pitfalls in terminology and use of reference materials. *TrAC, Trends Anal. Chem.* **1999**, *18* (9−10), 594−616.

(12) Jedziniak, P.; Szprengier-Juszkiewicz, T.; Olejnik, M. In-house reference materials: 5-hydroxyflunixin and meloxicam in cow milk—preparation and evaluation. *Anal. Chim. Acta* **2009**, *637* (1−2), 346−350.

(13) Cox, H. D.; Lopes, F.; Woldemariam, G. A.; Becker, J. O.; Parkin, M. C.; Thomas, A.; Butch, A. W.; Cowan, D. A.; Thevis, M.; Bowers, L. D.; Hoofnagle, A. N. Interlaboratory agreement of insulin-like growth factor 1 concentrations measured by mass spectrometry. *Clin. Chem.* **2014**, *60* (3), 541−548.

(14) Seo, Y.; Park, J.; Kim, M.; Sung, C.; Kwon, O. S.; Lee, H. J.; Min, H. Optimization, validation, and comparison of a rapid method for the quantification of insulin-like growth factor 1 in serum using liquid chromatography-high-resolution mass spectrometry. *Drug Test. Anal.* **2021**, *13* (2), 451−459.

(15) Jedziniak, P.; Szprengier-Juszkiewicz, T.; Olejnik, M. In-house reference materials: 5-hydroxyflunixin and meloxicam in cow milk-preparation and evaluation. *Anal. Chim. Acta* **2009**, *637* (1−2), 346−350.

(16) Emons, H. The 'RM family'—Identification of all of its members. *Accredit. Qual. Assur.* **2006**, *10*, 690−691.

(17) Camino-Sánchez, F.; Zafra-Gómez, A.; Oliver-Rodríguez, B.; Ballesteros, O.; Navalón, A.; Crovetto, G.; Vílchez, J. UNE-EN ISO/IEC 17025:2005-accredited method for the determination of pesticide residues in fruit and vegetable samples by LC-MS/MS. *Food Addit. Contam.: Part A* **2010**, *27* (11), 1532−1544.

(18) World Anti-Doping Agency. *International Standard for Laboratories*, 2016.

(19) Klu, J. K.; Officer, J. A.; Park, A.; Mudie, R.; NicDaeid, N. Measurement uncertainty in quantifying delta-9-tetrahydrocannabinol (THC) in blood using SPE and LC/MS/MS. *Forensic Sci. Int.* **2021**, *322*, 110744.

(20) Dong, H.; Wang, G.; Feng, K.; Wu, X.; Fan, Y.; Zhang, W.; Ma, M.; Gu, N.; Zhang, Y. Reference material of Prussian blue nanozymes for their peroxidase-like activity. *Analyst* **2022**, *147* (24), 5633−5642.

(21) Liu, J.; Zhu, W.; Sun, H.; Song, D.; Xiao, P.; Xu, B.; Li, H. Development of a primary reference material of natural C-reactive protein: verification of its natural pentameric structure and certification by two isotope dilution mass spectrometry. *Anal. Methods* **2021**, *13* (5), 626−635.

(22) MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **2010**, *26* (7), 966−968.

(23) Guddat, S.; Solymos, E.; Orlovius, A.; Thomas, A.; Sigmund, G.; Geyer, H.; Thevis, M.; Schänzer, W. High-throughput screening for various classes of doping agents using a new 'dilute-and-shoot' liquid chromatography-tandem mass spectrometry multi-target approach. *Drug Test. Anal.* **2011**, *3* (11−12), 836−850.

(24) Wang, Y.; Yan, K.; Wang, Z.; He, G.; Chang, W. Quantification of Ephedrine Substances in Human Urine by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. Sci.* **2022**, *61* (1), 32−37.
(25) Zhao, X.; Yuan, Y.; Wei, H.; Fei, Q.; Luan, Z.; Wang, X.; Xu, Y.; Lu, J. Identification and characterization of higenamine metabolites in human urine by quadrupole-orbitrap LC-MS/MS for doping control. *J. Pharm. Biomed. Anal.* **2022**, *214*, 114732.
(26) Valente, A.; Sanches-Silva, A.; Albuquerque, T. G.; Costa, H. S. Development of an orange juice in-house reference material and its application to guarantee the quality of vitamin C determination in fruits, juices and fruit pulps. *Food Chem.* **2014**, *154*, 71−77.

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