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Development of a Model System of Uncertainty Evaluations for Multiple Measurements by Isotope Dilution Mass Spectrometry: Determination of Folic Acid in Infant Formula†

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A model system has been established for the evaluation of the uncertainty of the value from measurements of multiple subsamples by isotope dilution mass spectrometry (IDMS). In this report, we apply this model system for the evaluation of measurement uncertainty in determination of folic acid in infant formula. Five subsamples were analyzed by IDMS. The mean of the measurement results of the five subsamples was assigned as the final measurement value. The standard deviation (s) of the results from five subsamples was attributable to repeatability of the measurement. The uncertainty components in the IDMS measurement methods were categorized into two groups. Group I includes uncertainty components which give common systematic effects to all subsamples and do not contribute to the variation among multiple measurements (repeatability). Group II includes uncertainty components that give random effects on the measurement results, and are related with the measurement repeatability. These random effects are attributed to s. Therefore, the uncertainty of the final value was calculated by combining the standard deviation of the mean of multiple measurements, $s/\sqrt{n}$ (where $n = 5$), and the measurement uncertainty associated with the uncertainty components that give systematic effects.

Key Words: Uncertainty evaluation, IDMS, Multiple measurements

Introduction

The chemical metrology communities of the world have been paying great attention to traceability to International System (SI) of Units and uncertainty in chemical measurements as these two factors are key to achieve comparability and reliability in chemical measurements.[1-2] The International Organization for Standardization (ISO) has provided an international standard for evaluating and expressing uncertainty across a broad spectrum of measurement through "Guide to the Expression of Uncertainty in Measurement (GUM)" in 1993.[5] The ISO guide has been accepted in all fields of measurements. However, in chemical measurements, it is very difficult to estimate and combine uncertainties following the ISO guide due to the inherent complexity of the measurement procedures which is not common in physical measurements. Eurachem, in collaboration with Cooperation on International Traceability in Analytical Chemistry (CITAC), provided a guide, "Quantifying Uncertainty in Analytical Measurement",[6] describing how the concepts in the ISO guide can be applied in chemical measurements. Though many guides and similar articles appeared with many examples of real world analytical situations, it still lacks of practical application as it is mostly based on the result of *a single* measurement while it is quite common in analytical chemistry to carry out multiple measurements (n) and to report the average value and its uncertainty as results.

The uncertainty of a single measurement can be evaluated by combining all individual uncertainty components involved in measurement processes. Meanwhile, measuring a sample for multiple times is a reliable way of statistically evaluating the measurement "repeatability", which is a new term substituting "method precision",[7] in statistical manner. The measurement repeatability can be theoretically evaluated by combining uncertainty components which can give variation among multiple measurements (random effects).[8] However, the theoretical approach can miss unrecognized uncertainty sources. Therefore, the statistical approach is usually superior to the theoretical one for chemical measurements.

In our previous articles,[8,9] we have designed and logically proved an approach for uncertainty evaluation of multiple measurement results in which the overall uncertainty is determined by combining the uncertainties of the individual results in multiple measurements whether the individual results of n measurements are statistically different or not. In this article, the uncertainty evaluation scheme was applied to measurements with isotope dilution mass spectrometry (IDMS).

Among the several analytical methods, IDMS is the most outstanding method for quantitative analysis of organic compounds in complex matrices, as the method overcomes the difficulty in correction of recovery during the sample preparation and demonstrates high accuracy and repeatability[1,10] of measurement results. Therefore, the IDMS has been chosen as a primary method (reference method) to provide higher-order metrological reference for clinical chemistry, toxicology, food and drug analysis, and environmental analysis.[10-16] A model system used in this article is determination of folic acid in infant formula by an IDMS method.

†This paper is dedicated to Professor Hasuck Kim for his outstanding contribution to electrochemistry and analytical chemistry.
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Experimental

The IDMS used in this experiment was based on liquid chromatography/mass spectrometry and abbreviated to ID-LC/MS. Details of the analytical method was published in our previous article.[16] Brief description of the method is as followings with adding details related with uncertainty evaluation.

Materials. Folic acid used as a reference material was obtained from LGC Promochem (Teddington, UK). The purity, (90.6 ± 1.4)%, of the reference material was assayed in our laboratory. $^{13}C_5$-folic acid, which has ^{13}C atoms substituted at the five carbons on the glutamic acid portion, was obtained from Eprova AG (Schaffhausen, Switzerland). Infant formula used in this study was obtained from National Institute of Standards and Technology (NIST), USA, as a part of an interlaboratory comparison organized by Consultative Committee for Amount of Substance - Metrology in Chemistry (CCQM).

Calibration standards. Four folic acid calibration standard solutions at 5 mg/kg level were prepared independently by a gravimetric method, dissolving a weighed amount of the reference material into a weighed amount of solvent [acetonitrile 26% + methanol 14% + water 60%] containing 10 mmol/L 2-mercaptoethanol. A $^{13}C_5$-folic acid standard solution of 5 mg/kg level was prepared with the same way and used as an internal standard solution. Two isotope ratio (1:1) standard solutions from each of the four replicates were prepared by gravimetrically mixing the weighed portions of the corresponding folic acid standard solution and the $^{13}C_5$-folic acid standard solution. Total of eight isotope ratio standards were cross-checked by LC/MS to ensure the absence of significant error and to test self-consistency. Based on the test results, one calibration standard solution and an isotope ratio standard solution from it were chosen for the calibration of sample analysis.

Sample preparation and cleanup. 1 g of sample was taken and spiked with an appropriate amount of the $^{13}C_5$-folic acid standard solution to match the isotope ratio to around 1:1. The exact masses of the sample and the spike solution added into the vial were calculated from weight of the vial before and after adding them into it. The subsample was then subject to sample clean-up processes, as previously described.[16] Total of five subsamples were prepared.

LC/MS conditions. The instrumentation consisted of a liquid chromatography (Agilent Technology 1100) with an automatic liquid sample injector, and triple quadrupole mass spectrometer (Quantum Ultra, Thermo Finnigan) with electrospray ionization. The mass spectrometer was operated on a selected reaction monitoring (SRM) mode for detecting the collisionally induced dissociation (CID) channels of *m*/*z* 442 → *m*/*z* 295 and *m*/*z* 447 → *m*/*z* 295, which are the neutral glutamyl loss of the $[M+H]^+$ ions of folic acid and $^{13}C_5$-folic acid, respectively.

Measurement protocol. Extracts of the five subsamples were analyzed by LC/MS in comparison with the isotope ratio standard. A single LC/MS run of the isotope ratio standard was followed by a single LC/MS run of each of sample extracts. The single run cycle was repeated for five times.

Calculations. The master equation for the IDMS measurements of an analyte by one point calibration (with isotope-ratio matching) can be written as

$$C_{\text{sample}} = \frac{M_{\text{is-sol,spiked}} \cdot AR_{\text{sample}} \cdot M_{\text{s-sol,std. mix.}} \cdot C_{\text{s-sol}}}{M_{\text{sample}} \cdot AR_{\text{std. mix.}} \cdot M_{\text{is-sol,std.mix.}}} \quad (1)$$

where,

C_{sample} is the concentration of folic acid in the sample;
$C_{\text{s-sol}}$ is the concentration of the calibration standard solution;
M_{sample} is the mass of the sample taken for analysis;
$M_{\text{is-sol, spiked}}$ is the mass of the $^{13}C_5$-folic acid standard solution added to the sample aliquot;
$M_{\text{is-sol, std. mix.}}$ is the mass of the $^{13}C_5$-folic acid standard solution added to the isotope ratio standard solution;
$M_{\text{s-sol, std. mix.}}$ is the mass of the calibration standard solution added to the isotope ratio standard solution;
AR_{sample} is the area ratio of folic acid/$^{13}C_5$-folic acid for sample extract, observed by LC/MS;
$AR_{\text{std. mix.}}$ is the area ratio of folic acid/$^{13}C_5$-folic acid for the isotope ratio standard solution, observed by LC/MS.

Results and Discussion

When multiple measurements are carried out for a single sample, the mean (C_{mean}) and its uncertainty are usually assigned to the final measurement results. The mean value is involved with two uncertainty components: the uncertainty in the measurement of each single subsample ($u_{\text{s.p.}}$) and the uncertainty associated with the standard deviation (s) of results among multiple subsamples.[8,9] The standard deviation (s) is corresponding to the statistically evaluated measurement repeatability when homogeneous sample is used. However, $u_{\text{s.p.}}$ and s are correlated to each other so that the uncertainty of C_{mean} can not be calculated by simply combining the two uncertainty components. Some of uncertainty sources included in $u_{\text{s.p}}$ contribute to the variation of measurement results among subsamples. Therefore, uncertainty sources of a single IDMS measurement and their effects to $u_{\text{s.p.}}$ and s have to be carefully evaluated.

The uncertainty of the IDMS measurement ($u_{\text{s.p}}$) consists of two groups of uncertainty components. Group I: uncertainty components common to all subsamples. With the measurement protocol adopted in the determination of folic acid in infant formula in this study, the uncertainties of the calibration standard solution and the isotope ratio standard and the uncertainty in measuring the ratio of folic acid/$^{13}C_5$-folic acid of the isotope ratio standard solution by LC/MS belong to this group. Those components give same systematic effects to all subsamples and do not contribute to the variation among measurement results of those subsamples as the same values are applied to them. We denote the uncertainty due to these components as $u_{\text{s.p.,systematic}}$, which can be calculated by combining uncertainty components of Group I according to Eq. (1). Group II: uncertainty components that are unique to each subsample such as uncertainties in weighing sample taken for analysis and aliquot of the internal standard solution spiked to it, and the uncertainty in measuring the ratio of folic acid/$^{13}C_5$-folic acid of each subsample by LC/MS. The components in Group II give random effects to each single IDMS measurement and make contribution to the

variation of the measurement value among subsamples. The measurement uncertainty due to the components in this group is denoted to $u_{\text{s.p.,random}}$. The uncertainty of a single ID-LC/MS measurement ($u_{\text{s.p.}}$) can be calculated by following equation as discussed in the previous article.[8]

$$u_{s.p.} = \sqrt{u^2_{\text{s.p.,systematic}} + u^2_{\text{s.p.,random}}} \quad (2)$$

In the case of a "well-performed measurement and proper evaluation of uncertainty arising from random effects", $u_{\text{s.p.,random}}$ is equivalent to the standard deviation (s) of n multiple measurements. In this case, the difference among the n multiple measurements is not statistically significant ($s \cong u_{\text{s.p.,random}}$), and the uncertainty of the mean of n multiple measurements, $u(C_{\text{mean}})$ can be expressed as following.[8,9]

$$u(C_{\text{mean}}) = \sqrt{u^2_{\text{s.p.,systematic}} + \frac{u^2_{\text{s.p.,random}}}{n}} \quad (3)$$

By substituting $u_{\text{s.p.,random}}$ by s, Eq. (3) can be written as following.

$$u(C_{\text{mean}}) = \sqrt{u^2_{\text{s.p.,systematic}} + \frac{s^2}{n}} \quad (4)$$

Though the standard deviation (s) of the individual results should reflect the uncertainty resulting from random effects, $u_{\text{s.p.,random}}$, the difference among the individual n measurement results is sometimes statistically significant ($s > u_{\text{s.p.,random}}$). The difference between s and $u_{\text{s.p.,random}}$ is due to uncertainties arising from *unrecognized* random effects,[9] and can be considered as an additional uncertainty ($u_{\text{u,random}}$) with $s = \sqrt{u^2_{\text{s.p.,random}} + u^2_{\text{u,random}}}$. We note that sample inhomogeneity is one possible source of $u_{\text{u,random}}$ in real world of chemical analysis. The statistically estimated measurement repeatability (s) includes all uncertainties of random effects either recognized or not. In this respect, Eq. (4) can be used whether the difference among the individual n measurement results is statistically significant or not, and the logical explanation of this conclusion could be found at the previous article.[9] In retrospect, it is rather safe choice for analytical chemist to carry out multiple measurements in order to report an average value and to statistically evaluate its uncertainty due to random effects. In the statistical approach, the uncertainty of the mean of multiple measurement results can be calculated by combining the uncertainty resulting from sources of the systematic effects ($u_{\text{s.p.,systematic}}$) and the standard deviation of the individual results (s) according to Eq. (4).

The above strategy for evaluating the uncertainty of multiple measurement results were adopted in following section to the ID-LC/MS measurement results of folic acid in infant formula.

Uncertainty sources of the IDMS measurement.

Each of the parameters, x_i, in equation 1 has an associated uncertainty, $u(x_i)$, which contributes to the final measurement results. Uncertainty sources of each parameter were evaluated as following and summarized at Table 1.

Calibration standard solution: Two components make major contributions to the uncertainty of the calibration standard solution, $u(C_{\text{s-sol}})$. Based on the certificated purity as stated in experimental section, the standard uncertainty of the purity of the

Table 1. Sources of uncertainty in IDMS measurements

components $u(x_i)$[a]	Source of Uncertainty	Quantification of Uncertainty	Remark
$u(C_{\text{s-sol}})$	Gravimetrically preparing calibration standard solution.	Self-consistency test of replicate standard solutions prepared independently: repeatability	Group I: Same systematic effect to the individual results of multiple measurements.
	Uncertainty of Purity	Certificate of the reference material or purity assay results (in this study)	
$u(MR_{\text{std. mix.}})$[b]	Gravimetrically preparing isotope standard solution	Self-consistency test of two isotope ratio standards from each single standard solution and pooled for four replicate standard solutions: repeatability	
$u(AR_{\text{std. mix.}})$	Area ratio of folic acid/$^{13}C_5$-folic acid for the calibration standard mixture, observed by LC/MS	Standard deviation of the mean of repeated LC/MS analysis	
$u(AR_{\text{sample}})$	Area ratio of folic acid/$^{13}C_5$-folic acid for sample extract, observed by LC/MS	Standard deviation of the mean of repeated LC/MS analysis	Group II: Random effect and contributing to the variation among multiple measurement results. Related with the repeatability (s).
$u(M_{sample})$	Weighing	Readability, repeatability, and linearity of the balance used (from its certificate)	
$u(M_{is\text{-sol, spiked}})$	Weighing	Readability, repeatability, and linearity of the balance used (from its certificate)	

[a]See text for details. [b] $MR_{\text{std,mix}} = \frac{M_{\text{s-sol, std. mix.}}}{M_{\text{is-sol, std. mix.}}}$, ratio of the weight of the folic acid standard solution ($M_{\text{s-sol,std. mix}}$) and the $^{13}C_5$-folic acid standard solution ($M_{\text{is-sol, std. mix}}$) added into the isotope ratio standard.

Table 2. Results of testing consistency of independently prepared standard solutions and isotope ratio standards

Standard solution No.	Isotope ratio standards	Isotope ratio (Weighed-in)[a]	Area ratio (observed)[b]	R.F.[c]	Average R.F.	RSD (between A & B)[e]
1	1A	0.9721	0.9816	1.0097	1.0079	0.26%
	1B	0.9794	0.9853	1.0060		
2	2A	0.9848	0.9868	1.0020	1.0006	0.20%
	2B	0.9877	0.9869	0.9993		
3	3A	0.9872	0.9849	0.9977	0.9978	0.02%
	3B	0.9910	0.9889	0.9979		
4	4A	0.9848	0.9879	1.0031	1.0005	0.38%
	4B	0.9931	0.9909	0.9978		
Overall Mean/Pooled					1.0017	0.25%[f]
RSD (among four standard solution)[d]					0.43%	

[a]Folic acid/$^{13}C_5$-folic acid ratio of the corresponding standard solution, calculated on the basis of gravimetrical preparation of the standard solution and the isotope ratio standard. [b]Area ratio of folic acid and $^{13}C_5$-folic acid peaks of the corresponding isotope ratio standards, measured by LC/MS. [c]R.F. = Area ratio (observed)/isotope ratio (weighed-in). [d]Relative standard deviation (RSD) of average R.F. among the four standard solutions. [e]RSD (A&B) = RSD between R.F.s of A and B isotope ratio standards for the corresponding standard solution. [f]Pooling of RSD(A&B) of the four standard solutions.

reference material (folic acid) is calculated to be 0.7%, whose degree of freedom (v) is regarded as infinity. The uncertainty in gravimetrically preparing a standard solution can be estimated by testing the consistency among replicate standard solutions. To test self consistency of the four calibration standard solutions prepared independently, the ID-LC/MS measurements were carried out. For each of the four standard solutions, two isotope ratio standards were prepared by gravimetrically mixing with proper aliquots of the $^{13}C_5$-folic acid standard solution. Total of eight isotope ratio standards were analyzed by the LC/MS method and the results were summarized at Table 2. Response factor of each isotope ratio standard solution was determined by dividing observed peak area ratio of folic acid/$^{13}C_5$-folic acid by the gravimetrically prepared isotope ratio. The relative standard deviation of the response factors (average of two isotope ratio standards from each) of the four standard solutions (0.43% with degree of freedom (v) equal to 3 in this case) represents the uncertainty due to the repeatability of gravimetrically preparing standard solution. Then, $u(C_{\text{s-sol}})$ was calculated by combining the uncertainties of the purity and gravimetric preparation method. $u(C_{\text{s-sol}})$ was 0.83%, and its effective degree of freedom was calculated to be 40 by using the Welch-Satterthwaite equation.[5]

Isotope ratio standard: Gravimetrical preparation of isotope ratio standards also has its own uncertainty. The repeatability of the preparation method is a major uncertainty source and which can be evaluated statistically. As shown in the previous section and at Table 2, two isotope ratio standard solutions for each of the four folic acid standard solutions were prepared and analyzed by the LC/MS method. The relative standard deviation of response factors between two isotope ratio standards, RSD (A&B), within a single folic acid standard solution ranges from 0.02% to 0.4%. Pooled RSD (A&B) among the four standard solution is 0.25% ($v = 4$), which represents the repeatability of the gravimetric preparation of isotope ratio standards and is assigned to the uncertainty associated with the ratio of $M_{\text{s-sol,std.mix.}}$ and $M_{\text{is-sol,std.mix.}}$, $MR_{\text{std,mix}} = \dfrac{M_{\text{s-sol, std. mix.}}}{M_{\text{is-sol, std. mix.}}}$.

Based on the self-consistency test results summarized at Table 2, standard solution No. 4 and isotope ratio standard 4A were selected as calibrants for the analysis of the infant formula sample.

Weighing sample and spiked internal standard: Amounts of sample taken for analysis and the $^{13}C_5$-folic acid standard solution spiked into it were estimated by measuring the mass difference of the vial before and after adding them into it. According to the certificate of the balance used in this study (Mettler Toledo, AT 201), its linearity and repeatability are 0.03 mg and 0.015 mg in the weighing range. These two contributions are assumed to show rectangular distributions and can be converted to the standard uncertainty (u_{w}) of the mass measured by weighing-by-difference;[15]

$$u_{\text{w}} = \sqrt{2\left(\frac{0.03\text{ mg}}{\sqrt{3}}\right)^2 + 2\left(\frac{0.015\text{ mg}}{\sqrt{3}}\right)^2} = 0.017\text{ mg} \quad (5)$$

Note that the linearity and the repeatability are counted twice. $u(M_{\text{sample}})$ and $u(M_{\text{is-sol,spiked}})$ are equal to u_{w}.

Isotope ratio measurements: The standard uncertainty in measuring the area ratio of folic acid/$^{13}C_5$-folic acid for the isotope ratio standards, $u(AR_{\text{std. mix.}})$ was estimated from the standard deviation from five repeated LC/MS runs. The standard uncertainty in measuring the area ratio for each subsample, $u(AR_{\text{sample}})$, was also evaluated in the same way.

Categorizing and combining uncertainty sources.

The measurement value (C_j) for the j-th subsample (j = 1, 2, ⋯, n) by the ID-LC/MS method can calculated by using Eq (1). Thus, the uncertainty of C_j was estimated as following.

$$\frac{u_{\text{s.p.}}(C_j)}{C_j} = \sqrt{\sum_i \left(\frac{u(x_{i,j})}{x_{i,j}}\right)^2} \quad (6)$$

Table 3. ID-LC/MS results of the measurement of folic acid in infant formula by ID-LC/MS

Measurement Number (j)	Concentration, C_j (mg/kg)	$u_{s.p.,random}(C_j)^a$ (mg/kg)	$u_{s.p.,systematic}(C_j)^b$ (mg/kg)	$u_{s.p.}(C_j)^c$ (mg/kg)
1	2.300	0.010 (0.43 %, v = 4)	0.022 (0.97 %, v = 40)	0.024 (1.06 %, v = 23)
2	2.342	0.017 (0.74 %, v = 4)	0.023 (0.97 %, v = 40)	0.029 (1.22 %, v = 22)
3	2.341	0.027 (1.15 %, v = 4)	0.023 (0.97 %, v = 40)	0.035 (1.50 %, v = 23)
4	2.346	0.028 (1.18 %, v = 4)	0.023 (0.97 %, v = 40)	0.036 (1.53 %, v = 22)
5	2.319	0.010 (0.43 %, v = 4)	0.022 (0.97 %, v = 40)	0.025 (1.06 %, v = 23)
Mean(C_{mean})/Pooled uncertainty	2.329	0.020 (0.84 %, v = 20)^d	0.023 (0.97 %, v = 40)	0.030 (1.29 %, v = 57)^e
Standard Deviation (s)	0.020 (0.84 %, v = 4)			
Combined Standard uncertainty, $u(C_{mean})^f$	0.024 (1.0 %, v = 13)			
Expanded Uncertainty	0.049 (2.1 %) (k = 2.02, in 95 % level of confidence)			

*v denotes the effective degree of freedom of the corresponding uncertainty. [a-c]See text for the notations.

[d] $u_{s.p.,random(pooled)} = \sqrt{\left(\sum_j v_j \cdot u^2_{s.p.,random}(C_j)\right) \Big/ \sum_j v_j}$. [e]Calculated by Eq. (2). [f]Calculated by Eq. (4).

Where, the parameters, $x_{i,j}$, are M_{sample}, $M_{is\text{-}sol,spiked}$, $MR_{std,mix} = \frac{M_{s-sol,std}}{M_{is-sol,std}}$, AR_{sample}, $AR_{std.\ mix.}$, and $C_{s\text{-}sol}$ for the corresponding subsample (j). The uncertainty of the measurement value of each subsample was estimated and listed in Table 3.

Among the uncertainty sources listed above, $u(MR_{std,mix})$, $u(AR_{std.\ mix.})$, and $u(C_{s\text{-}sol})$ belong to Group I, uncertainty components common to all subsamples, which give the same systematic effects to all subsamples in the case of the measurement protocol used in the model system, determination of folic acid in infant formula. $u(M_{sample})$, $u(M_{is\text{-}sol,\ spiked})$, and $u(AR_{sample})$ belong to Group II, are unique to each subsample. Those uncertainty components give random effects to the measurement results of each subsample and contribute to the variation of multiple measurement results in addition to the sample inhomogeneity. As in Eq (2), $u_{s.p.}(C_j)$ can be written as following.[16]

$$u_{s.p.}(C_j) = \sqrt{u^2_{s.p.,systematic}(C_j) + u^2_{s.p.,random}(C_j)} \tag{7}$$

Where, $u_{s.p.,systematic}(C_j)$ and $u_{s.p.,random}(C_j)$ can be calculated by combining the uncertainty components in groups I and II, respectively, using the following equations.

$$\frac{u_{s.p.,systematic}(C_j)}{C_j} = \sqrt{\left(\frac{u(MR_{std,mix})}{M_{std,mix}}\right)^2 + \left(\frac{u(AR_{std})}{AR_{std}}\right)^2 + \left(\frac{u(C_{s-sol})}{C_{s-sol}}\right)^2} \tag{8}$$

$$\frac{u_{s.p.,random}(C_j)}{C} = \frac{}{\sqrt{\left(\frac{u(M_{sample,j})}{M_{sample,j}}\right)^2 + \left(\frac{u(M_{is-sol,spiked,j})}{M_{is-sol,spiked,j}}\right)^2 + \left(\frac{u(AR_{sample,j})}{AR_{sample,j}}\right)^2}} \tag{9}$$

$u_{s.p.,systematic}(C_j)$ and $u_{s.p.,random}(C_j)$ were calculated and also listed in Table 3. $u_{s.p.,random}(C_j)$ of all subsample were then pooled to estimate $u_{s.p.,random}$ in average. The relative value of $u_{s.p.,systematic}(C_j)$ to C_j is the same for all subsamples. Pooling of $u_{s.p.,systematic}(C_j)$ is $u_{s.p.,systematic}$ in average for the method. As the standard deviation among five subsamples and $u_{s.p.,systematic}$ were evaluated, u(C_{mean}) were calculated by Eq. (4) and the results were given in Table 3.

$$u(C_{mean}) = \sqrt{0.023^2 + \frac{0.020^2}{5}} = 0.024 \quad \text{in mg/kg (1.0\% of } C_{mean}) \tag{10}$$

By placing $u_{s.p.,random}$ and $u_{s.p.,systematic}$ into Eq. (2), we can calculate $u_{s.p.}$ which represents the standard uncertainty "expected" in a single ID-LC/MS measurement of the target analyte in the sample.

$$u(C_{mean}) = \sqrt{0.020^2 + 0.023^2} = 0.030 \quad \text{in mg/kg (1.29\% of } C_{mean}) \tag{11}$$

Findings from the uncertainty evaluation.

As shown in Table 3, s and $u_{s.p.,random}$ are 0.84% of the mean (C_{mean}) in the case of the ID-LC/MS measurement of folic acid in infant formula sample. As s and $u_{s.p.,random}$ are close to each other ($s \cong u_{s.p.,random}$), the ID-LC/MS measurements in this study were proven to be "well- performed" and uncertainty arising from random effects was properly evaluated. Also, it indicates that there was no unrecognized significant uncertainty source with random effects. In retrospect, this results along with many other IDMS studies, which were already published in different articles,[15-17] proves superiority of IDMS as a primary method which provides higher-order metrological quality because the operating procedure are well characterized and most significant uncertainty sources are identified.

$u_{s.p.}$ is 1.2% of the mean, and that is the relative standard uncertainty expected for a single measurement of folic acid in infant formula by the ID-LC/MS method. Equations (3) and (4) indicate that the contribution of $u_{s.p.,random}$ to $u(C_{mean})$ can be minimized by increasing the number of measurement (n) and near to zero if n is very large. However, the contribution of $u_{s.p.,systematic}$ to $u(C_{mean})$ does not decrease as increasing n. $u(C_{mean})$ gets close to $u_{s.p.,systematic}$ with very large n.

Conclusion

In general, multiple measurements are carried out for a single sample in many of chemical analyses. The basic concept underlying this common practice has been that the repeatability of the analytical method can be evaluated statistically. However, evaluation of the uncertainty for a final value of multiple measurements has been always complicated as some of uncertainty components in the measurement method are correlated with the statistically evaluated repeatability. In our previous articles,[8,9] this aspect was theoretically examined and an approach to determine the overall uncertainty by combining the uncertainties of the individual results from multiple measurements was proposed. In this article, the conceptual approach is extended to develop a practical model system to apply to IDMS measurements. The model system was applied to the analysis of folic acid in infant formula by the ID-LC/MS used in our laboratory. According to the model system, uncertainty sources in the ID-LC/MS measurement were categorized to two groups, one with systematic effects and the other with random effects to each of multiple measurements. The uncertainty of the mean of multiple measurements was calculated by combining uncertainties from sources of systematic effects with the repeatability of the measurement, which was statistically evaluated from the standard deviation of the mean of multiple measurements. It is shown that this type of approach can even include unrecognized uncertainties of random effects into the uncertainty of final value.

It should be stated here that whether an uncertainty component give systematic or random effect to each individual results of multiple measurements depends on the overall measurement protocol. When the protocol is changed, each uncertainty components should be re-categorized based on the new protocol.

Though this article applies the proposed approach to the IDMS method, which is known to be metrologically a higher-order method, this kind of approach can be applied to broad range of analytical methods. For this kind of approach, uncertainty sources should be identified and evaluated, and their relationship to multiple measurements should be analyzed based on the specific measurement protocol used.

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