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HUMAN NUTRIENT METHODS

Assessment of Regulatory Compliance Testing for Vitamin D in Infant Formula—Impact of Delegated Regulation (EU) 2019/828

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Abstract

Background: Since the publication of *Standard Method Performance Requirements* (SMPR®) for vitamin D in infant formula (SMPR 2011.004) by AOAC INTERNATIONAL, revised vitamin D limits have been recommended by the European Food Safety Authority (EFSA) for infant formula and adopted in Commission Delegated Regulation (EU) 2019/828. The vitamin D range introduced, 2–2.5 μg/100 kcal, is significantly narrower than previous limits specified by Codex Standard 72–1981 and requires lower method reproducibility metrics to adequately assess regulatory compliance. The narrower limits for vitamin D present a significant challenge for current-generation reference analytical methods that comply with SMPR 2011.004.
Objective: We evaluate the impact of Delegated Regulation (EU) 2019/828 on the demonstrated performance of AOAC Method **2016.05**/ISO 20636:2018 to assess the likelihood that vitamin D results produced by the method would be found outside the EU limits when testing infant formula that is compliant as manufactured.
Methods: AOAC Method **2016.05**/ISO 20636:2018, specifically data generated during multi-laboratory study, was used as a basis for statistical evaluation of the impact of the narrower EU vitamin D limits.
Results: The review of AOAC Method **2016.05**/ISO 20636:2018 method performance against the vitamin D regulatory range introduced in (EU) 2019/828 indicates methods capable of performing in alignment with SMPR 2011.004 are likely to produce results that fail to meet EU requirements.
Conclusions: Our assessment illustrates the high probability that a well-manufactured product with vitamin D levels within the EU regulatory range would fail to meet the regulatory requirements due to analytical method variability when tested using fit-for-purpose methods. Further, required method performance cannot be expected with the future development of new methods. To avoid this, consideration should be given to aligning proposed regulatory limits with method

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performance metrics of current-generation compendial methods.

Highlights: Current, state-of-the-art methods cannot consistently verify infant formula product compliance for vitamin D in accordance with (EU) 2019/828.

Regulatory requirements for infant formula are designed to ensure product safety and include general food quality and fundamental nutritional composition; they may also address specific functional properties. In turn, manufacturers of infant formula must follow good manufacturing practices using well-defined procedures for ingredient qualification through to final product manufacture so that each infant formula product is safe for intended use, manufactured in accordance with formulation design, and compliant with all applicable regulatory requirements.

The determination of micronutrients in infant formula, including vitamin D, is inherently challenging. Regulatory limits for vitamin D levels in infant formula can differ from country to country and are restrictive to ensure adequate consumption at the lower regulatory limit and, for food safety reasons, to avoid overconsumption at the upper regulatory limit.

Vitamin D in infant formula, as either cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2), is found at relatively low levels (<0.02 mg/kg) in a complex fat/protein matrix of potentially interfering compounds. Complicated sample preparation procedures are required to effectively and quantitatively isolate vitamin D from the sample matrix prior to analysis. Choices of suitable analytical methodologies are limited since vitamin D has no useful chromophore, natural fluorescence, or significant electrochemical properties. Vitamin D also undergoes thermal isomerization to form a bioactive structural isomer, pre-vitamin D, which also must be measured to account for total vitamin D content (1). This makes compliance testing especially difficult relative to other vitamins and micronutrients present in these matrixes.

It is recognized that the validation of fit-for-purpose quantitative analytical methods should include several fundamental performance parameters including specificity, detection limits, precision, range, accuracy, recovery, and uncertainty (2). When measuring compliance against either product specifications or international regulatory limits, accuracy and reproducibility precision are arguably the more critical of method characteristics. There continues to be a plethora of analytical methods reported, including those adopting a combination of liquid chromatography and tandem mass spectrometry, LC-MS/MS, for the quantitative estimation of vitamin D in foods, including infant formula and adult nutritionals. These methods typically provide estimates for most of these parameters via single-laboratory validation but inherently exclude reproducibility precision (3–7).

A prerequisite for candidate analytical methods to be approved as Official Final Action by AOAC INTERNATIONAL is to meet requirements as documented in the *Standard Method Performance Requirements* (SMPR®). The SMPR for determination of vitamin D in infant and adult nutritionals was established by the Vitamin D Working Group, approved by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) and published in 2011 as SMPR 2011.004 (Table 1) (8). Nutrient definition and required method performance metrics were based on applicable global standards for required or allowable vitamin D content available at the time of publication (9–14). These standards defined vitamin D limits of 1–2.5 μg/100 kcal for infant formula and formulas for special medical purposes intended for infants defined for ages 0–12 months (Table 2).

Table 1. Standard method performance requirements AOAC **2011.004**[a,b]

Criteria	Value	
Analytical range	0.12–5.1	
Limit of detection (LOD)	≤0.02	
Limit of quantitation (LOQ)	≤0.12	
Repeatability (RSD_r)	0.12–1.5	≤15%
	>1.5	≤11%
Recovery	0.12–1.5	80–120%
	>1.5	90–110%
Reproducibility (RSD_R)	≤15%	

[a] Concentrations apply to (1) "ready-to-feed" liquids "as is"; (2) reconstituted powders (25 g into 200 mL water); and (3) liquid concentrates diluted 1:1 by weight.

[b] μg/100 g expressed separately as vitamin D_2 and vitamin D_3 in reconstituted final product.

Table 2. Vitamin D regulatory limits in infant formula

Standard	Regulatory limits	
	μg/100 kcal	μg/100 mL[a]
Codex, Stan 72-1981 (3)	1–2.5	0.65–1.63
EU 2006/141/EC (5)	1–2.5	0.65–1.63
1980 IFA (6)	1–2.5	0.65–1.63
FSANZ, Stan 2.9.1 (8)	1.05–2.63	0.65–1.71

[a] Conversion factor used: 65 kcal/100 mL, Codex range is 60–70 kcal/100 mL per Codex Stan 72–1981 §3.1.2.

To demonstrate eligibility as an AOAC *Official Method* SM compared to SMPR metrics, it is required to characterize method performance by single-laboratory validation and subsequently demonstrate method reproducibility through an international multi-laboratory collaborative study (15). These protocols were implemented for the method described in this study (AOAC Method **2016.05**/ISO 20636:2018) including the characterization of method reproducibility precision. This rigor differentiates this method from other, single laboratory validated methods reported in the literature and is why it is viewed by the global infant formula industry as the most suitable method available to ensure vitamin D compliance with current legislation.

In 2015, Commission Delegated Regulation (EU) 2016/127 amended the requirements for vitamin D content for infant and follow-on formulas from 1–2.5 μg/100 kcal to 2–3 μg/100 kcal (16), simultaneously increasing both the minimum and maximum vitamin D limits while narrowing the range of allowable vitamin D content in infant formulas.

In 2018, the European Food Safety Authority (EFSA) raised concerns that consumption of formula containing elevated levels of vitamin D could pose safety risks. In its Scientific Opinion (17), EFSA concluded that the use of infant formula containing 3 μg/100 kcal vitamin D may lead younger infants, aged up to 4 months, to consume amounts of vitamin D above the tolerable recommended daily upper intake level. The opinion further

Table 3. Vitamin D EU regulatory limits for infant formula per (EU) 2016/127

Standard	Original Issue Delegated Regulation (EU) 2016/127 (9) μg/100 kcal	Amended per Delegated Regulation (EU) 2019/828 (11) μg/100 kcal
Regulatory minimum	2	2
Regulatory maximum	3	2.5
Range (max−min)	1.0	0.5
Mid-point (min+max)/2	2.5	2.25

concluded that a maximum vitamin D content of 2.5 μg/100 kcal in infant formula does not result in intakes of vitamin D above the tolerable upper intake level from the formula alone and that the maximum vitamin D content permitted under Delegated Regulation (EU) 2016/127 for infant formula should be lowered to 2.5 μg/100 kcal in accordance with Article 6 and Article 9 of Regulation (EU) No. 609/2013. The new limits recommended by EFSA for infant formulas, especially those designed for consumption by infants aged up to 4 months, were adopted by the EU in 2019 as shown in Table 3 (18).

The revised limits, 2–2.5 μg/100 kcal, present a significant challenge to current-generation state-of-the-art analytical methods that perform in accordance with AOAC SMPR 2011.004, such as AOAC Method **2016.05**/ISO 20636:2018 (19–22). An evaluation of the impact of regulatory requirements for various micronutrients in fortified foods for infants and young children was previously discussed, concluding that a number of available reference methods are not fit to assess compliance with the narrow regulatory limits (23).

A detailed statistical evaluation of the impact of the new EU regulatory limits for vitamin D in infant formula on product compliance testing to EU 2019/828 using published single- and multi-laboratory validation results for AOAC Method **2016.05**/ISO 20636:2018 is presented. Amended SMPR metrics required to align method performance with these revised regulatory limits are also presented.

Delegated Regulation (EU) 2019/828 Impact Assessment

For the purpose of predicting the impact of the narrower EU vitamin D regulatory limits for infant formulas to current EN and AOAC methods, the following assumptions were made:

(1) the true value for vitamin D content in the product is at the regulatory mid-point, 2.25 μg/100 kcal;
(2) the product caloric density is assumed to be at the mid-point of the allowable range, 60–70 kcal/dL, or 65 kcal/dL;
(3) manufacturing and ingredient variability were excluded;
(4) measurement inaccuracy and/or bias is negligible;
(5) measured results are normally distributed around the true value;
(6) the estimated reproducibility values derived from multi-laboratory trial (MLT) studies are considered true values of the analytical method, and the uncertainty of reproducibility values arising from their estimation is negligible and can be ignored;
(7) the product assessment is carried out using a single result from a single sample test.

These assumptions assign the true vitamin D content at the mathematical mid-point of the EU regulatory range and allow the range to fully incorporate method imprecision. In this regard, these assumptions represent a best-case scenario, and any significant deviation of the true vitamin D content from the regulatory range mid-point will result in increased probability of noncompliance.

In practice, method reproducibility can also be considered a measure of the maximum difference, within a defined probability, between two determinations made randomly under typical reproducibility conditions. A plot of measured vitamin D results against method reproducibility for a product with a true vitamin D value at the regulatory range mid-point demonstrates that measured results are more variable with increased measurement imprecision (Figure 1).

The acceptable method reproducibility (RSD_R) of a fit-for-purpose method expressed in SMPR 2011.004 is ≤15% RSD. This value is less than the precision predicted by the Horwitz ratio of 22% RSD_R for concentration levels <0.1 mg/kg and significantly less than the allowable upper limit based on the Horwitz ratio of 2, which is 44% RSD_R as described in the Codex Procedural Manual (24). With a regulatory range of 1–2.5 μg/100 kcal as specified by Codex, the predicted noncompliance rate is less than 1% due to measurement imprecision using fit-for-purpose methods.

The revised vitamin D limits specified in EU 2019/828, 2–2.5 μg/100 kcal, are significantly narrower than both Codex and EU published guidance for acceptable tolerances around the label declaration in foods excluding infant formula (25). These tolerances allow for measurement uncertainty and set the tolerable levels for vitamins in foods to −35% and +50% of the required vitamin content. If considered acceptable and applied to infant formula with vitamin D content at the regulation mid-point, the acceptable range would be 1.5–3.4 μg/100 kcal.

Method Performance—AOAC 2016.05/ISO 20636:2018

AOAC *Official Method* **2016.05**/ISO 201636:2018 is the current, state-of-the-art method for determining vitamin D in infant formula. Samples are prepared using alkaline saponification at elevated temperature with the lipid-soluble fraction, including vitamin D, extracted into an organic solvent. Vitamin D is derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) producing a higher-molecular weight adduct, and the resulting analytical sample is analyzed by reversed-phase liquid chromatography (RPLC) and tandem mass spectrometry (MS/MS). Stable-isotope internal standards are used for quantification and to correct for sample preparation and instrument artifacts. This method was evaluated against SMPR 2011.004 via a nine-laboratory MLT study using 15 product types representing infant formulas, follow-on formulas, and child/adult nutritional products (26). A summary of method repeatability and reproducibility for infant formula products used in the MLT is provided in Table 4. The average reproducibility for these products is 8.2% RSD_R, with the expected distribution of results illustrated in Figure 2.

From this MLT study, the average method standard deviation, relative to the regulatory range mid-point of 2.25 μg/100 kcal, is 0.185 μg/100 kcal and equivalent to 1.35 standard deviations from the regulatory range midpoint as shown in Equation 1:

$$z = \frac{x - \mu}{\sigma} = \frac{2.5 - 2.25}{0.185} = 1.35 \quad (1)$$

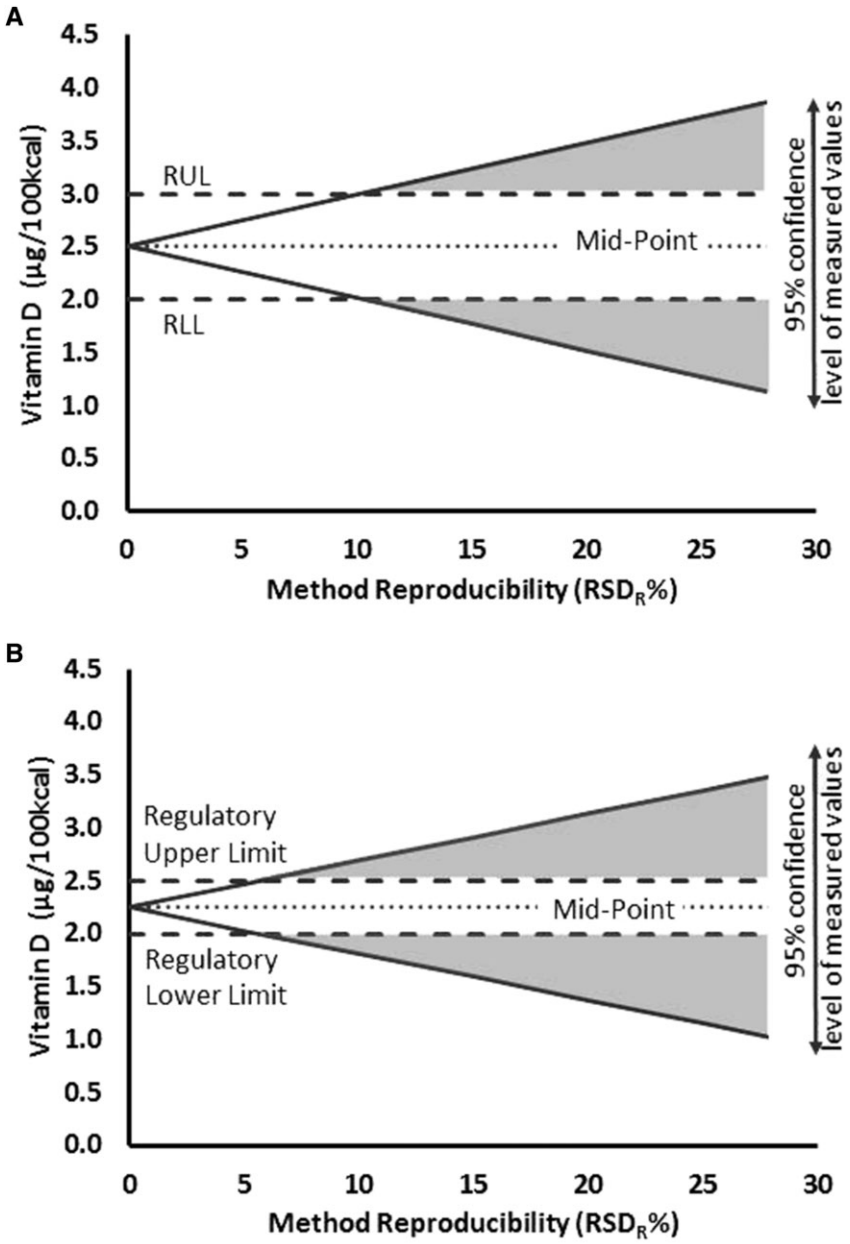

Figure 1. Expected results and method precision for product manufactured at mid-point of EU regulatory range. (A) Regulatory limits 2–3 µg/100 kcal; (B) regulatory limits 2–2.5 µg/100 kcal. RLL = regulatory lower limit; RUL = regulatory upper limit.

Table 4. Method precision from AOAC Method **2016.05** MLT for infant formula

Product description	Repeatability, RSD_r, %	Reproducibility, RSD_R, %
NIST SRM 1849a	3.2	7.2
Infant formula, RTF[a]	3.6	11.4
Infant formula, powder	1.9	6.5
PHP[b] infant formula, powder	3.2	6.4
Infant formula, powder	5.1	8.3
FOS/GOS[c] infant formula, powder	4.9	9.1
Average	3.7	8.2

[a] RTF = Ready-to-feed.
[b] PHP = Partially hydrolyzed protein.
[c] FOS/GOS = Fructo-oligosaccharides/galacto-oligosaccharides.

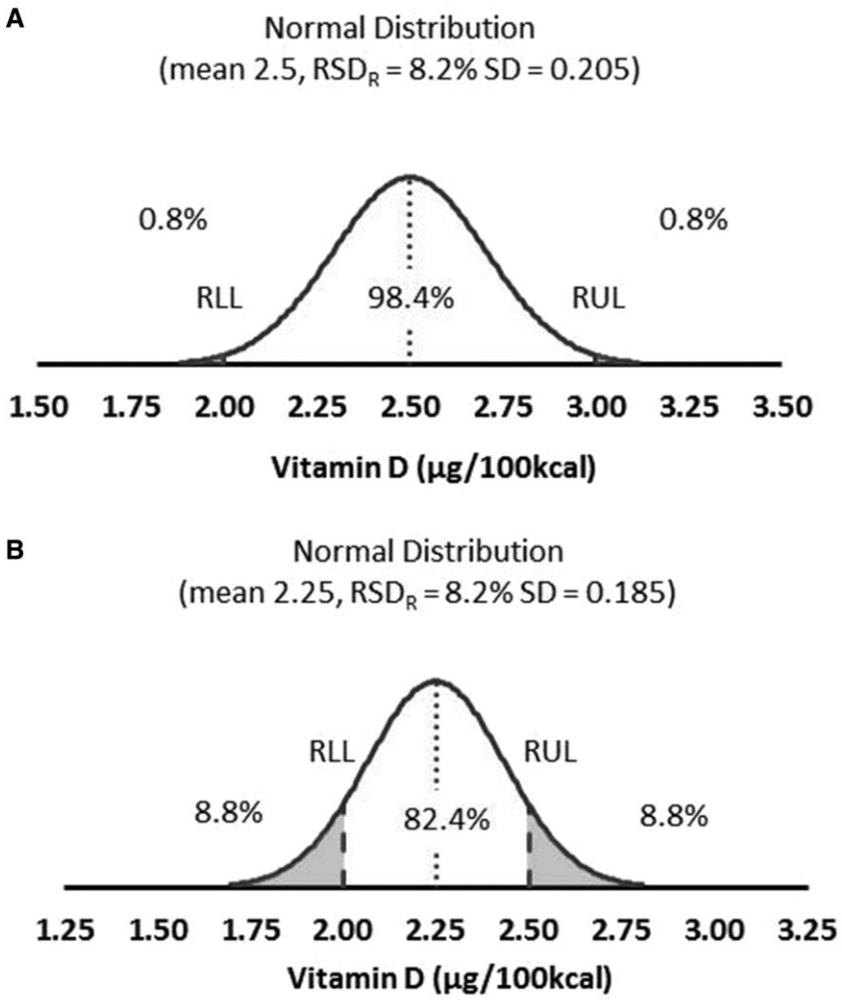

Figure 2. Normal distribution of test results and expected failure rate for AOAC 20.16.05/ISO20636:2018 assuming method reproducibility RSD_R =8.2%. (A) Regulatory limits 2–3 µg/100 kcal; (B) regulatory limits 2–2.5 µg/100 kcal. RLL = Regulatory lower limit; RUL = Regulatory upper limit.

Based on the performance of AOAC Method **2016.05** as demonstrated during MLT using infant formula matrixes, the expected percentage of test results that will fall within the EU regulatory limits is estimated to be 82.4% as shown in Equation 2:

$$P(-a < z < b) = P(z < b) - P(z < -a) = 0.912 - 0.088 = 0.824 = 82.4\% \quad (2)$$

The noncompliance, or failure, rate for a sample manufactured with a true vitamin D content at the center of the regulatory range is approximately 17.6% due exclusively to random testing error and is consistent with other assessments (23). When the same calculation is performed on the upper acceptable limit for reproducibility in AOAC SMPR 2011.004 of 15% RSD_R (SD_R = 0.338 at 2.25 µg/100 kcal), the product failure rate is considerably higher at 46% (Figure 3). Further, predicted rates of noncompliance are expected to be even higher when including typical manufacturing and ingredient variability as well as vitamin D degradation that can occur during product shelf-life. These factors typically result in true vitamin D content that is not centered within the regulatory range.

Required Method Precision for (EU) 2019/828 Amended Regulatory Limits

In the absence of manufacturing variability and with a known measurement standard deviation (SD) and negligible analytical error or bias, 95% of measured values will lie within ±2 method standard deviations (SD) of the distribution mean. As such, an estimate of the required method precision is approximately one-fourth of the regulatory range, or range midpoint ±2 SD with 95% confidence interval coverage (Equation 3).

$$\text{method standard deviation (est.)} = \text{regulatory range}/4 = 0.125\ \mu g/100\text{kcal} \quad (3)$$

An estimate of method reproducibility (RSD_R) required to meet the EU 2019/828 vitamin D limits can be calculated as follows (Equation 4):

$$\text{method relative reproducibility, } RSD_R(\text{est.}) = 0.125/2.25 \times 100 = 5.6\% \quad (4)$$

The RSD_R value obtained from an MLT is an estimate of the true value and, given that it is based on data generated by a

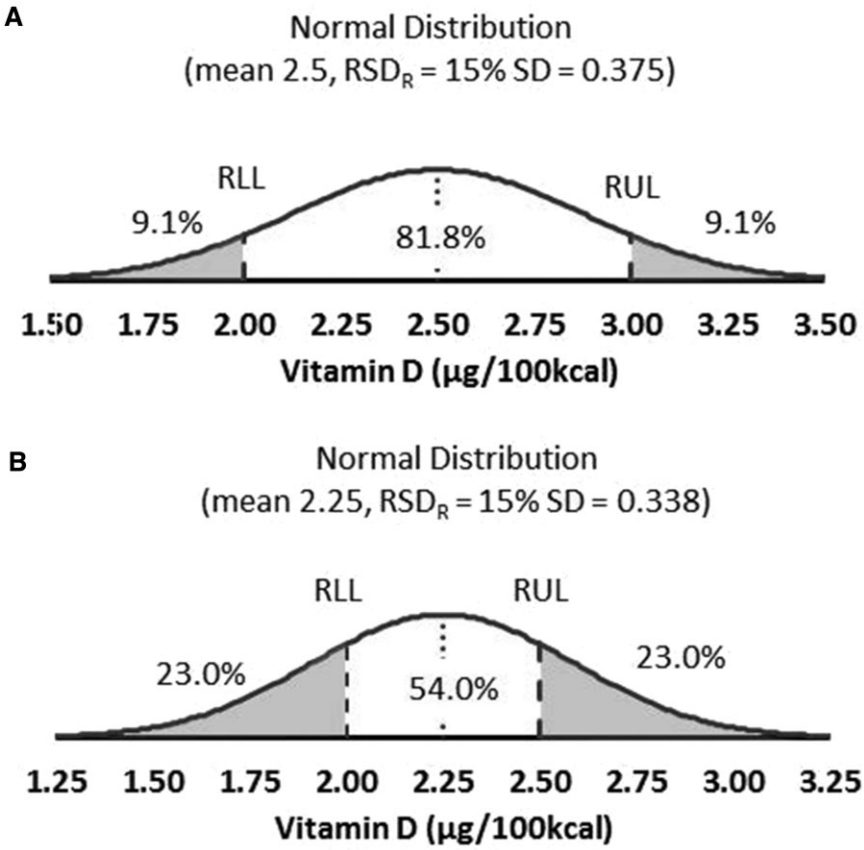

Figure 3. Normal distribution of test results and expected failure rate with method reproducibility $RSD_R = 15\%$. (A) Regulatory limits 2–3 µg/100 kcal; (B) regulatory limits 2–2.5 µg/100 kcal. RLL = Regulatory lower limit; RUL = Regulatory upper limit.

relatively few laboratories, may have considerable uncertainty. To minimize the uncertainty in the RSD_R value, an MLT study involving 200 or more laboratories would be required and is generally impractical to carry out. Therefore, one must allow for this uncertainty in the calculation of the target estimated standard deviation obtained from an MLT.

Guidance is available from ISO 5725-1 on the uncertainty of estimates of reproducibility and repeatability obtained from collaborative studies, expressed in terms of the number of laboratories and the number of replicates per laboratory (27). The relative uncertainty of the estimated method reproducibility standard deviation, relative to the true value (A_R), can be interpolated from table values for uncertainty estimates of repeatability and reproducibility standard deviations using values for:

p = the number of laboratories;
n = the number of replicate tests on each sample performed by each laboratory;
γ = the ratio of the reproducibility, σ_R, and repeatability, σ_r, standard deviations (Equation 5).

$$\gamma = \frac{\sigma_R}{\sigma_r} \tag{5}$$

A preliminary analysis of the MLT data for AOAC Method **2016.05** produces a value for $\gamma = 1.93$, close to the usual assumption $\gamma = 2$, that the method reproducibility is twice its repeatability. The calculation of A_R is based on the assumption that the standard deviation of the reproducibility standard deviation is normally distributed.

On this basis, the ratio s/σ is normally distributed with mean, $\mu = 1.00$ and standard deviation, $\sigma = A_R$ (Equation 6):

$$Probability\left(\frac{s_R}{\sigma_R} \geq 1 - 1.645A_R\right) = 0.95 \tag{6}$$

or equivalently (Equation 7):

$$Probability\left(\sigma_R \leq \frac{s_R}{1 - 1.645A_R}\right) = 0.95 \tag{7}$$

so that the term shown in Equation 8

$$\frac{s_R}{1 - 1.645A_R} \tag{8}$$

is the 95% upper confidence bound (UCB) for σ_R.

For the current MLT study, values of p, n, and γ are 10, 2, and 2, respectively. The calculated value of A_R is 0.41 so that the calculated UCB = 3.07. From this, the estimated reproducibility of this MLT must be no greater than one-third of the estimated target reproducibility true value in order that the true value (at 95% confidence) is not exceeded. That is, a method with the required reproducibility of 5.6% would need to demonstrate an RSD_R of 1.8% to account for RSD_R imprecision. This level of performance is not reasonable to expect based on experience with precision estimates as well as published performance metrics for methods targeting analytes at similar concentrations (28).

Conclusions

The vitamin D limits recommended per Delegated Regulation (EU) 2019/828 for infant formulas as defined in (EU) 2016/127 are significantly narrower than the published regulatory limits used for the development of AOAC SMPR 2011.004. Current state-of-the-art analytical methods that comply with SMPR 2011.004, including AOAC **2016.05**/ISO20636:2018 and other compendial methods such as AOAC **2002.05**/EN12821:2009, have not demonstrated method precision necessary to meet the revised vitamin D requirements. The average reproducibility obtained for a selected range of infant formula products tested as part of the MLT of AOAC Method **2016.05** was 8.2%. A product properly formulated and manufactured with a true vitamin D level at exactly the middle of the EU range would appear to fail to meet these regulatory requirements approximately 18% of the time. With additional allowances for typical manufacturing variability, instability over product shelf-life, or intra-laboratory method inaccuracy, the noncompliance rate is expected to exceed 18% and could be as high as 46% using current analytical methodology. Using the vitamin D limits put forward by EU 2019/828, the vitamin D AOAC SMPR 2011.004 would require modification specifying a decrease in method reproducibility from 15% to 5.6% and potentially as low as 1.8%, allowing for uncertainty in method reproducibility. Required method repeatability would be half these values.

The conclusions presented above describe method performance metrics necessary for fit-for-purpose methods to comply with EU 2019/828 rely on the most ideal situation: i.e., true vitamin D content in the middle of the regulatory range with no manufacturing or ingredient variability and a test method with very low measurement uncertainty. Reference methods including AOAC Method **2016.05**/ISO20636:2018 as well as other methods with similar performance are suitable for vitamin D compliance testing to regulations/standards in US, Codex, and Australia/New Zealand. However, it is unrealistic to expect that such methods can consistently demonstrate product compliance for vitamin D to (EU) 2019/828.

Based on our experience with analytical testing and determination of vitamin D content in nutritional products, including infant formulas, analytical methods using state-of-the-art technology with enough precision to consistently meet the limits set forth by Delegated Regulation (EU) 2019/828 are not available. In addition, the level of method performance required cannot be achieved with new method development. Consequently, there is an excessively high risk of infant formula products with compliant vitamin D levels being erroneously identified as noncompliant. Risk can be mitigated by giving consideration to aligning regulatory limits with method performance metrics of current-generation compendial methods.

Conflict of Interest

None declared.

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