

# Interlaboratory Trial for Measurement of Vitamin D and 25-Hydroxyvitamin D [25(OH)D] in Foods and a Dietary Supplement Using Liquid Chromatography–Mass Spectrometry

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## Supporting Information

**ABSTRACT:** Assessment of total vitamin D intake from foods and dietary supplements (DSs) may be incomplete if 25-hydroxyvitamin D [25(OH)D] intake is not included. However, 25(OH)D data for such intake assessments are lacking, no food or DS reference materials (RMs) are available, and comparison of laboratory performance has been needed. The primary goal of this study was to evaluate whether vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> concentrations in food and DS materials could be measured with acceptable reproducibility. Five experienced laboratories from the United States and other countries participated, all using liquid chromatography tandem–mass spectrometry but no common analytical protocol; however, various methods were used for determining vitamin D<sub>3</sub> in the DS. Five animal-based materials (including three commercially available RMs) and one DS were analyzed. Reproducibility results for the materials were acceptable. Thus, it is possible to obtain consistent results among experienced laboratories for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> in foods and a DS.

**KEYWORDS:** reference material, food, dietary supplement, vitamin D<sub>3</sub> (cholecalciferol), 25-hydroxyvitamin D<sub>3</sub> (25-hydroxycholecalciferol)

## ■ INTRODUCTION

Vitamin D deficiency is a worldwide concern because of its major health consequences, including rickets and the postulated increased risk of other diseases.<sup>1–3</sup> Numerous epidemiological evaluations of vitamin D intake and vitamin D status have been reported, as have studies on the relationship of these factors with occurrence of various diseases.<sup>4–8</sup> Humans and animals obtain vitamin D<sub>3</sub> (cholecalciferol) from dietary sources and from synthesis when 7-dehydrocholesterol in the skin is exposed to UV light.<sup>9</sup> Vitamin D<sub>3</sub> is the primary form in the diet, although vitamin D<sub>2</sub> (ergocalciferol) can be present in some foods and supplements.<sup>5–7,10–13</sup> These “parent” compounds of vitamin D are metabolized by humans and animals to

25(OH)D and other metabolites before they are used by the body. Therefore, animal tissue—and, in turn, animal-derived human foods—contain 25-hydroxyvitamin D [25(OH)D] as well as the parent vitamin D, and the 25(OH)D can contribute to the overall vitamin D value of the food. In fact, evidence suggests that the content of 25(OH)D may be 2–5 times more potent than the content of parent vitamin D.<sup>10–12</sup> Therefore,

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accurate estimates of dietary vitamin D intake require the inclusion of the 25(OH)D content of foods.<sup>13</sup>

Current estimates of vitamin D intake suggest that many people in the United States consume considerably less than the established dietary requirement.<sup>14</sup> The serum 25(OH)D concentrations of the U.S. population, an indicator of vitamin D status, are higher than expected on the basis of estimated vitamin D intakes alone.<sup>15,16</sup> Although sun exposure is often cited as a major factor in this discrepancy, another possibility is the failure to account for intakes of 25(OH)D in foods. This potentially important omission could result in a significant underestimation of vitamin D intake because the reliability of calculated vitamin D intake rests heavily on the trueness and availability of food composition data,<sup>13</sup> which do not typically include 25(OH)D assessments. For example, information on vitamin D in the major source of food composition data in the United States, the U.S. Department of Agriculture's (USDA's) National Nutrient Database for Standard Reference,<sup>17</sup> is currently being updated,<sup>18</sup> but it does not provide 25(OH)D values. Some limited data on the 25(OH)D content of foods are available in other databases outside the United States,<sup>19,20</sup> but the lack of data in the United States on 25(OH)D<sub>3</sub> in foods and the extent to which dietary 25(OH)D<sub>3</sub> contributes to vitamin D status merit further exploration.

In addition to limited 25(OH)D measurements in food composition data, the lack of reliable food composition data on vitamin D is also due to uncertainty about the accuracy (trueness and precision) of the methods used to generate such data. Furthermore, obtaining reliable estimates of average concentrations in foods and sources of variability in the food supply, as well as in dietary supplements (DSs), requires representative sampling, validated analytical methods, expert analysts, and accredited, documented quality-control measures.

In the 1990s and early 2000s, the methodology for quantification of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> used high-performance liquid chromatography (HPLC) with UV detection, which necessitated time-consuming cleanup steps to obtain accurate results.<sup>21,22</sup> Recent progress has been made in the development of methodology for the quantitation of vitamin D in foods using liquid chromatography–tandem mass spectrometry (LC-MS/MS) for measurements of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub>.<sup>23–27</sup>

When published values from different studies are compared, variability due to real differences in food composition versus those due to differences in analytical methodology and skill in assay performance cannot be discerned in the absence of common control or reference samples. In a 2008 study<sup>28</sup> that quantified vitamin D<sub>3</sub> in the same five food matrices at six experienced laboratories, each using their standard in-house methodology, the results showed various degrees of difference among the foods and laboratories. Whether this variability was due to differences in the methods used and/or individual skills in laboratory procedures is an open question. Differences in matrix and analyte levels can affect assay performance even when the same basic methodology is used. Therefore, control and reference materials representing different food matrices are needed to ensure the trueness and precision of quantitative results across a wide spectrum of foods.

The first specific aim of this study was to determine whether vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> concentrations in a range of animal-based food matrix materials and a DS could be measured with acceptable reproducibility by laboratories that are experienced in vitamin D analysis, without a common analytical protocol.

The secondary aim was to assess the potential for the use of the materials as control or reference materials in future research.

## MATERIALS AND METHODS

**Overview.** Through an interagency agreement between the Nutrient Data Laboratory (NDL; Beltsville, MD, USA) of the USDA Agricultural Research Service and the Office of Dietary Supplements of the National Institutes of Health (Bethesda, MD, USA), NDL designed and conducted a pilot study that measured vitamin D and 25(OH)D levels in foods and a DS. A “roundtable” group of five experts identified and critically evaluated scientific issues related to sample selection, preparation, distribution, and tracking as well as laboratory participation and evaluation of results. The roundtable members were representatives from Health Canada (Longueuil, Quebec, Canada), U.S. Food and Drug Administration (College Park, MD, USA), National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA), and USDA's Food Composition and Method Development Laboratory (Beltsville, MD, USA). In addition, scientists from the Office of Dietary Supplements participated in the roundtable discussions. Five animal-based foods and one DS were selected by NDL in consultation with the roundtable group. Samples were prepared at a central location and distributed to each laboratory on two occasions for analysis of vitamin D and 25(OH)D contents.

**Identification of Participating Laboratories.** NDL identified analytical laboratories with expertise in measuring vitamin D and 25(OH)D in various matrices. These laboratories had either published their vitamin D methodology or had previously provided NDL with vitamin D data that met data quality evaluation standards.<sup>29</sup> The participating laboratories were Covance Laboratories (Madison, WI, USA), Health Canada (Longueuil, Quebec, Canada), Heartland Laboratories (Ames, IA, USA), NIST (Gaithersburg, MD, USA), and the Technical University of Denmark (Søborg, Denmark). All of the laboratories were using LC-MS/MS for separation, detection, and quantitation but varied analytical protocols to analyze vitamin D and 25(OH)D contents. Two of the laboratories also had experience analyzing DSs for vitamin D content. The five laboratories are identified in this report using randomly assigned letters A–E.

**Selection of Food Samples.** Standard animal-based food reference materials from NIST that could contain 25(OH)D<sub>3</sub> were initially considered for the study, including NIST Standard Reference Material (SRM) 1577c Bovine Liver, NIST SRM 1546a Meat Homogenate, and NIST SRM 1845a Whole Egg Powder.

Foods included in the initial screening were cooked chicken liver, cooked beef liver, rotisserie chicken with skin, a cooked pork and egg yolk composite, and cooked ground beef. The materials chosen for screening represented a variety of food matrices with potential for measurable levels of 25(OH)D.

NDL collaborated with scientific partners at Virginia Tech (Blacksburg, VA, USA) to screen these materials and samples of other foods for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> contents. A homogeneous composite of locally procured (Blacksburg, VA, USA) retail samples of chicken liver, beef liver, and rotisserie chicken was prepared for each of these three foods using validated protocols.<sup>28,30</sup> The ground beef and the pork and egg yolk samples had been previously prepared using the same protocols for use as control materials in the USDA National Food and Nutrient Analysis Program.<sup>30</sup> Subsamples of all composites were maintained in glass jars, sealed under nitrogen at –60 °C and protected from light. The frozen samples were shipped on dry ice via express overnight delivery to Covance Laboratories, where they were analyzed for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> contents.

The screening results were evaluated by the roundtable experts who selected the following materials to represent a range of expected vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> concentrations and diverse matrices in this study: NIST SRM 1577c Bovine Liver; NIST SRM 1546a Meat Homogenate; NIST SRM 1845a Whole Egg Powder; cooked chicken liver; and cooked ground beef. The roundtable experts selected these materials because they were sufficiently diverse to represent variability in method performance based on specific matrix characteristics that

**Table 1a. Methodology Used by Participating Laboratories To Analyze Vitamin D Content in Foods and 25(OH)D Content in Foods and a Dietary Supplement<sup>b</sup>**

|                         | Laboratory A   | Laboratory B   | Laboratory C  | Laboratory D   | Laboratory E   |
|-------------------------|--|--|---|--|--|
| Method reference        | Huang, M.; LaLuzerne, P.; Winters, D.; Sullivan, D., Measurement of vitamin D in foods and nutritional supplements by liquid chromatography/tandem mass spectrometry. <i>J AOAC Int</i> <b>2009</b> , 92, 1327–1335. | Not available  | Not available   | Burild, A.; Frandsen, H. L.; Poulsen, M.; Jakobsen, J., Quantification of physiological levels of vitamin D(3) and 25-hydroxyvitamin D(3) in porcine fat and liver in subgram sample sizes. <i>J. Sep. Sci.</i> <b>2014</b> , 37, 2659–2663. | Not available  |
| Sample size             | 3 g to 10 g depending on moisture content  | 0.8 g to 3 g depending on moisture content   | 0.6 g to 2 g  | 1 g  | 2 g to 3 g   |
| Internal standard       | ( <sup>2</sup> H <sub>3</sub> )vitamin D <sub>3</sub> and ( <sup>2</sup> H <sub>3</sub> )25(OH)D <sub>3</sub> (IsoSciences, King of Prussia, PA)   | ( <sup>2</sup> H <sub>6</sub> )vitamin D <sub>3</sub> and ( <sup>2</sup> H <sub>6</sub> )25(OH)D <sub>3</sub> (Chemaphor, Ottawa, Canada)      | ( <sup>3</sup> H)vitamin D <sub>3</sub> (in-house) and ( <sup>2</sup> H <sub>3</sub> )25(OH)D <sub>3</sub> (Sigma-Aldrich)                                      | ( <sup>2</sup> H <sub>6</sub> )vitamin D <sub>3</sub> and ( <sup>2</sup> H <sub>6</sub> )25(OH)D <sub>3</sub> (Chemaphor, Ottawa, Canada)  | ( <sup>13</sup> C <sub>5</sub> )vitamin D <sub>3</sub> and ( <sup>13</sup> C <sub>5</sub> )25(OH)D <sub>3</sub> (IsoSciences, King of Prussia, PA) |
| Initial treatment       | reagent-grade alcohol with 2 % pyrogallol acid, 50 % KOH added   | 10 mL pyrogallol 1% in EtOH and 8 mL KOH 60 %  | methanoic KOH   | sodium ascorbate, KOH, and ethanol   | lipase and ethanol   |
| Lipid treatment         | saponification under N <sub>2</sub> overnight  | saponification under N <sub>2</sub> at room temperature overnight  | saponification for 2 hours at 60°C  | saponification under N <sub>2</sub> at room temperature for 16–18 hours  | enzymatic digestion for 2 hours at 37° C   |
| Extraction step         | solution of 30 mL 20 % ether and 80 % hexane   | 3x petroleum ether/diethyl ether (80/20)   | 3x hexane/ethyl acetate (85/15)   | ethyl acetate/n-heptane (20/80)  | 2x hexane extracted, pooled, with addition of ~1g MgSO <sub>4</sub> to remove extra water; centrifuged; and dried                                  |
| Clean-up step           | dried and redissolved in 1 mL 70 % acetonitrile–H <sub>2</sub> O [not done for 25(OH)D]  | organic phase washed with water, taken to dryness, and dissolved in 800 µL 1-chlorobutane  | dried and redissolved in 1 mL hexane/methylene chloride   | dried and redissolved in 2-propanol in n-heptane   |  |
| Additional clean-up     |  | purified using HPLC normal phase (silica column) and fractions collection  | loaded into an SPE cartridge, eluted with methylene chloride/isopropanol, two cleanings through HPLC columns, final solution methylene chloride and isopropanol | loaded onto a silica SPE cartridge, rinsed with 0.5 % 2-propanol in n-heptane, eluted with 6% and 10 % 2-propanol in n-heptane   |  |
| Derivatization          | evaporated to dryness and 2 mg/mL PTAD in 100 % ACN added.   | fractions evaporated to dryness, PTAD reagent in ethyl acetate added, evaporated to dryness, reconstituted in 0.2 mL of methanol               | not derivatized   | dried under N <sub>2</sub> and derivatized with PTAD in anhydrous acetonitrile, solvent evaporated, sample reconstituted in 60% methanol   | derivatized with PTAD in acetonitrile, dried, reconstituted in 500 µL 70:30 methanol:water.  |
| HPLC column             | Thermo, UHPLC column, Hypersil GOLD aQ (1.9µm) 100 mm x 2.1 mm   | Phenomenex LUNA-C18 (3.0 µm) 150 mm x 2.1 mm   | Supelco-Sil LC-18 ODS column (5µm) 3.3 cm x 4.6 mm  | Ascentis Express C18 (2.1 mm x 10 cm, 2.7 µm particles) column and C18 (2.1 x 5 mm, 2.7 µm particles) guard column   | YMC-Pack Pro C18 RS (5µm, 8nm) 150 mm X 4.6 mm   |
| HPLC separation         | mobile phase: (1) 0.1% formic acid, 20% MeOH in ultrapure water; (2) 0.1 % formic acid in methanol   | mobile phase: (1) 45 % ACN/55 % H <sub>2</sub> O; (2) methanol, both with 2 mM ammonium acetate and 0.04 % formic acid                         | mobile phase: methanol/acetonitrile/water (48.5/48.5/3)   | mobile phase: (1) Milli-Q water, methylamine (5 mM), and formic acid (0.1 %); (2) methanol, methylamine (5 mM), and formic acid 0.1 %  | mobile phase: (1) water, (2) methanol  |
| Quantification          | 4000 LC-MS/MS System (Sciex): triple quadrupole tandem mass spectrometer   | LC-Agilent 1100 with binary pump coupled to a WATERS Quattro Premier XE MSMS system (ESI mode)   | Agilent 1290 UHPLC system coupled to an Agilent 6460 triple quadrupole LC/MS/MS ESI operated in positive MRM mode   | Agilent 1200 series HPLC coupled to an Agilent 6460 series triple quadrupole mass spectrometer operated in positive MRM mode   | Agilent 6410B MS/MS system, APCI, positive ionization mode   |
| Ion Masses <sup>a</sup> | D <sub>3</sub> - 560 (383, 298, 280)<br>25(OH)D <sub>3</sub> 558 (298, 280, 247)<br>D <sub>2</sub> 572 (298)<br>25(OH)D <sub>2</sub> N/A   | D <sub>3</sub> - 560 (298, 280)<br>25(OH)D <sub>3</sub> 593 (298, 280)<br>D <sub>2</sub> 572 (298, 280)<br>25(OH)D <sub>2</sub> 605 (298, 280) | D <sub>3</sub> - 385 (367, 259)<br>25(OH)D <sub>3</sub> 401 (383, 365)<br>D <sub>2</sub> 397 (379, 107)<br>25(OH)D <sub>2</sub> 413 (395, 355)                  | D <sub>3</sub> - 591 (298)<br>25(OH)D <sub>3</sub> 607 (298)<br>D <sub>2</sub> 603 (298)<br>25(OH)D <sub>2</sub> 619 (298)   | D <sub>3</sub> - 560 (298)<br>25(OH)D <sub>3</sub> 558 (298)<br>D <sub>2</sub> 572 (298)<br>25(OH)D <sub>2</sub> 570 (298)                         |
| Limit of quantification | Vitamin D <sub>3</sub> /D <sub>2</sub> : 0.05/0.20 µg/100g; 25(OH)D <sub>3</sub> : 0.012 µg/100g   | high moisture: 0.08 ng/g<br>low moisture: 0.2 ng/g   | 0.5 ng/g  | <0.1 ng/g  | vitamin D <sub>3</sub> /D <sub>2</sub> : 0.4/0.3 ng/g<br>25(OH)D <sub>3</sub> /D <sub>2</sub> : 0.2/0.7 ng/g                                       |

<sup>a</sup>Masses in parentheses are the product ions. <sup>b</sup>Abbreviations: HPLC, high-performance liquid chromatography; SPE, solid-phase extraction; PTAD, 4-phenyl-1,2,4-triazole-3,5-dione; ACN, acetonitrile; UHPLC, ultrahigh-performance liquid chromatography; MS/MS, tandem mass spectrometry; ESI, electrospray ionization; MRM, multiple reaction monitoring.



Table 1b. Methodology for Analysis of Vitamin D<sub>3</sub> Content in the Dietary Supplement<sup>a</sup>

|                         | Laboratory A   | Laboratory B   | Laboratory C   |
|-------------------------|--|--|--|
| Subsampling             | fill from 30 softgels  | 30 softgels dissolved in 20 mL of water at 50 °C   | 10 softgels dissolved  |
| Internal standard       | none   | ( <sup>2</sup> H <sub>6</sub> )vitamin D <sub>3</sub> (Chemaphor, Ottawa, Canada)  | ( <sup>3</sup> H)vitamin D <sub>3</sub> (in-house)   |
| Initial treatment       | none   | 20 mL water at 50 °C; 70 mL 1 % pyrogallol in ethanol added, followed by 50 mL KOH 60 % (w/v)  | methanolic KOH   |
| Saponification          | none   | at room temperature overnight under N <sub>2</sub>   | 2 hours at 60 °C   |
| Extraction step         | weighed and dissolved in hexane  | further extraction of an aliquot of the solution using 3x petroleum ether/diethyl ether (80/20)  | 3x hexane/ethyl acetate (85/15)  |
| Clean-up step           |  | organic phase is washed with water before evaporation to dryness, dissolution of residue in 2.0 mL 1-chlorobutane                        | dried and redissolved in 1 mL hexane/methylene chloride  |
| Additional clean-up     |  | purification using HPLC normal phase (silica column) and fractions collection, evaporation of fraction evaporated reconstitution in MeOH | loaded into an SPE cartridge, eluted with methylene chloride/isopropanol, two clean-ups through HPLC columns |
| HPLC column             | Partisil (250 mm x 4.6 mm), 5 micron                                   | ACE-C18 3.0um, 150 x 3.0 mm  | Supelco-Sil LC-18 ODS column   |
| HPLC separation         | mobile phase: (1) 1.5 % isopropanol/hexane; (2) 5 % isopropanol/hexane | mobile phase: (1) 50 % methanol/H <sub>2</sub> O + 0.1 % acetic acid; (2) methanol 95 % + isopropanol 5 % + 0.1 % acetic acid            | mobile phase: methanol/acetonitrile/water (48.5/48.5/3)  |
| Quantification          | HPLC-UV detection at 265 nm  | LC-MS/MS APCi Agilent 1100 LC coupled to a Thermo Quantum Ultra triple quad MS/MS  | HPLC-UV with liquid scintillation counting for internal standard   |
| Limit of quantification | 0.05 µg/100g   | 700 µg/100 g vitamin D <sub>3</sub>  | 5 ng by ratio of weight to calculated concentration  |

<sup>a</sup>Abbreviations: HPLC, high-performance liquid chromatography; SPE, solid-phase extraction; UV, ultraviolet; LC-MS/MS, liquid chromatography-tandem mass spectrometry; APCi, atmospheric pressure chemical ionization.

might affect extraction and quantitation [e.g., fat content, concentration of vitamin D<sub>3</sub>, and 25(OH)D<sub>3</sub>, and animal tissue type (e.g., muscle, liver, or egg)].

**Selection of the DS.** The DS product for this study was selected with the aim of developing a DS in-house control material. Scientists on the Dietary Supplement Ingredient Database team at NDL studied current vitamin D<sub>3</sub> supplements on the market, evaluating claimed composition and manufacturing information for both animal- and non-animal-based vitamin D<sub>3</sub> supplements (Supplemental Table 1). Their goal was to purchase supplements that represented a diversity of vitamin D source materials, production methods, matrices, and supplement strength (vitamin D<sub>3</sub> labeled content) and test them for 25(OH)D<sub>3</sub> content. On the basis of these criteria, six products sold as capsules, tablets, softgels, and powder labeled as having vitamin D<sub>3</sub> content and one product labeled as having 25(OH)D<sub>3</sub> content were chosen for preliminary analyses. These products had a wide range of labeled vitamin D content per serving (from 0 to 12,500 IU). Subsamples of 90 units of each product were placed in amber plastic bottles with desiccant packs, sealed under ambient conditions, and shipped via next-day delivery to Covance Laboratories for screening analysis of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> content.

The screening results (presented in Supplemental Table 2) also indicated that all six DS products sold with vitamin D<sub>3</sub> content claims contained 25(OH)D<sub>3</sub> at levels similar to those in the foods tested for inclusion in the study. However, their 25(OH)D<sub>3</sub> levels were minute compared to the amount of vitamin D<sub>3</sub> in the products. The label of the product ultimately chosen for the study claimed that it contained 2000 IU vitamin D<sub>3</sub>/serving, a level commonly taken by the U.S. population. This product also contained a measurable amount of 25(OH)D<sub>3</sub>. It was a private-brand softgel capsule sold in warehouse stores and was produced using a common manufacturing process of lanolin recrystallization.<sup>31</sup> Thus, purchasing this product by the case provided a large amount of serving units of the same lot, enabling its use as an in-house control material in future studies.

**Sample Preparation and Shipment.** Separate sample sets of the selected materials were shipped to each laboratory on two occasions (hereafter referred to as “trial 1” and “trial 2”) separated by

approximately 2 months. The NIST materials were shipped in their original packaging. The DS for the study was stored at −30 °C in its original containers, and individual bottles were opened only as needed. Each shipment included a total of 40–100 g of material per food and 90 DS softgel capsules. Sample bottle labels identified the material type. The samples were shipped on dry ice in an insulated cooler via express overnight delivery to each laboratory. General estimates of the expected concentrations of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> in each sample were given to the laboratories to assist in determining the assay parameters.

**Sample Analysis.** The laboratories were asked to use their usual methodology to analyze vitamin D in duplicate or triplicate in each sample. Although results for vitamin D<sub>2</sub> were not requested, some laboratories also included vitamin D<sub>2</sub> in their assays. All laboratories spectroscopically characterized their standards before use; the only materials supplied to the laboratories were the samples to be analyzed. Laboratories were asked to describe their methodology in detail and to answer a set of questions on the analyses that they performed. For the DS, the laboratories were advised to combine 30 units (i.e., capsules, tablets, softgels) for each assay. (U.S. Pharmacopeia provides guidelines on the amounts of capsules or tablets to be combined for analysis that range from at least 10 to at least 30 for products with vitamin D only or with vitamin D in multivitamin matrices.<sup>32–34</sup>) Guidelines for subsampling the DS were provided. The guidelines indicated that each laboratory had the option to analyze for each replicate assay the contents (fill only) of the combined 30 softgels or to dissolve 30 whole softgels before subsampling and analysis. Laboratories that conducted their analyses after dissolving the whole softgels calculated results for the amount of fill based on the weights of the whole capsules and their fill. Thus, for this paper, DS concentrations from all laboratories are reported as micrograms per 100 g of fill contents.

**Data Review.** Each laboratory's trial 1 data report was reviewed by an internal NDL group to ensure that all required information was received and to detect any potential problems with analyses, such as deviations from the study protocol or major discrepancies in results. NDL contacted laboratories that had report deviations or discrep-

ancies to alert them of any nonrepresentative analytical errors before these laboratories analyzed the second set of samples. Trial 2 data sets were also reviewed by an internal NDL group.

**Statistical Methods.** Results for each material included the replicate values for both trials from all laboratories. Data analysis was performed using PROLAB Plus software (QuoData Quality & Statistics, Dresden, Germany). Analyses included calculations of reproducibility relative standard deviations (RSDs) and repeatability RSDs for each material according to ISO 5725-2.<sup>35</sup> The reproducibility RSD expresses the precision of the average of the results from different laboratories and was calculated using the SD between the averages of replicate measurements by each laboratory in each trial. On the basis of the probability of detection (POD) model, predicted reproducibility RSD is calculated as  $2 \times C^{-0.15}$ , where  $C$  is a mass fraction of analyte, and estimated RSD is expressed as percent. Predicted repeatability RSD is considered to be half of this value. As the concentration decreases, the POD decreases and predicted RSD increases, as illustrated by the Horwitz curve.<sup>36,37</sup>

The repeatability RSD for each material expresses the variability between independent test results obtained within each laboratory. In this study, the repeatability RSD was calculated using the SD between the replicate measurements of each laboratory within each trial (the square root of the sum of the squares of the within-laboratory SD divided by 2, followed by multiplication by the number of laboratory/trials) as an estimate of overall within-laboratory variability for each analyte in each sample.

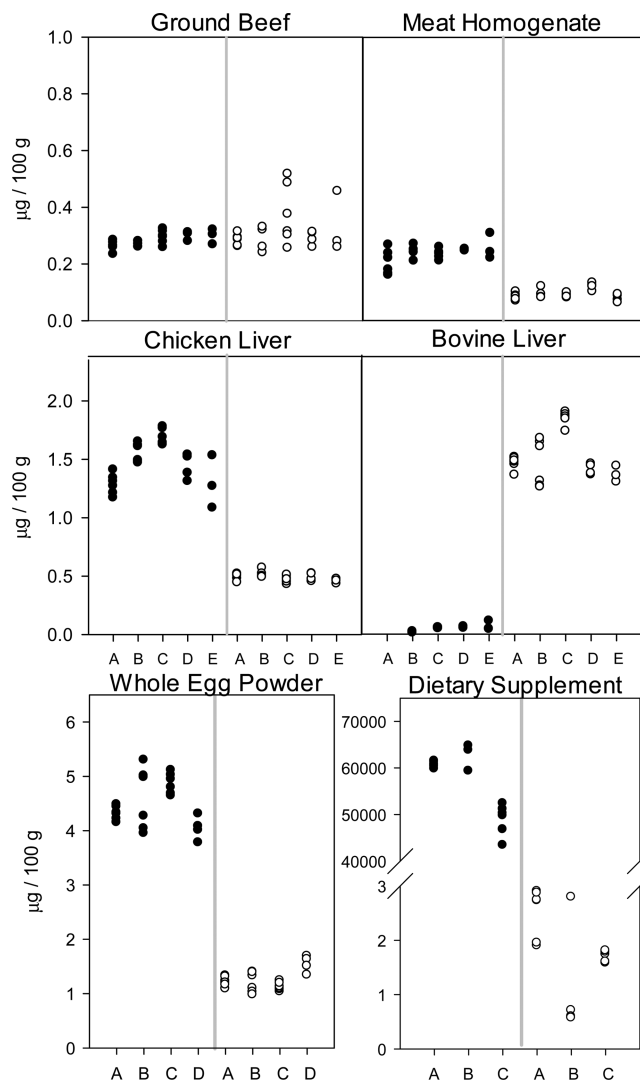
The Horwitz ratio (HorRat) was used to evaluate the reproducibility RSDs from data provided by the different laboratories for the materials.<sup>37,38</sup> The HorRat indicates the acceptability of the reproducibility of interlaboratory measurements, taking into consideration the analyte concentration. This ratio is the RSD of the mean divided by a predicted reproducibility RSD based on the concentration of the analyte. An acceptable HorRat is  $\leq 2.0$ .<sup>37</sup>

Outliers were identified using the Cochran test (analysis of variance) and Grubbs test (analysis of mean value deviations) according to ISO 5725-2.<sup>35</sup> Because the purpose of this study was to evaluate both inter- and intralaboratory variability for each material, the outliers identified from these tests were not excluded from calculation of overall mean and SD. One exception, however, was a value identified as an outlier by the Grubbs test that had already been manually excluded as a result of the analyst's data evaluation (laboratory D's vitamin D<sub>3</sub> measurement of the meat homogenate in trial 1). This outlier is explained under Results.

## RESULTS

**Analytical Methods Used for Foods.** After receipt of trial 1 data, it was evident that results from laboratory E differed markedly from those of the other laboratories. Laboratory E was not given details on the results from the other laboratories. However, because this laboratory had experience primarily in determining vitamin D and 25(OH)D levels in serum, the laboratory optimized its method to measure these analytes in food matrices before analyzing additional study samples. A new set of samples was then sent to laboratory E, which reported values for the meat homogenate, ground beef, and liver samples. Laboratories A, B, C, and E analyzed each food material in triplicate (three subsamples from the same mixture on the same day), and laboratory D submitted duplicate data.

Table 1a summarizes the methodology used by each laboratory. All laboratories used LC-MS/MS to quantify vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> levels without using a common sample preparation protocol. The laboratories labeled their internal standards in varied ways as well, using, for example, deuterium, tritium, or carbon-13. All laboratories calibrated their standards before use, which is important for obtaining accurate results. They also optimized their methodologies to measure the low concentrations of 25(OH)D in the foods and



**Figure 1.** Results for analyses of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> ( $\mu\text{g}/100\text{ g}$ ) levels in six matrices in two trials from five laboratories (A–E): (●) vitamin D<sub>3</sub>; (○) 25(OH)D<sub>3</sub>.

DS and of vitamin D in the foods. Laboratories A, B, D, and E derivatized the samples prior to LC-MS/MS analysis, but laboratory C did not. Another significant difference was that laboratory E did not saponify the samples but used an enzymatic digestion with lipase prior to LC-MS/MS analysis. Laboratory E indicated that its method had not been sufficiently validated for eggs and supplements, so it did not report results for those materials. In addition, laboratory D's analyst indicated concern about the validity of the vitamin D<sub>3</sub> value for the meat homogenate when reporting its trial 1 results. Therefore, laboratory D's data were excluded from these study results.

**Analytical Methods Used for the DS.** The DS was analyzed by laboratories A, B, and C (Tables 1a and 1b). Laboratory A analyzed the fill contents, whereas laboratories B and C analyzed whole softgels. Laboratories A and C analyzed the DS in triplicate. Laboratory B submitted one value for trial 1 and values from duplicate analysis for trial 2. Laboratory C conducted both of its trials for 25(OH)D<sub>3</sub> on the same day, rather than 2 months apart. Because laboratory C used a separate set of material and assay batches for each sample set, the data are reported here for two separate trials, even though

Table 2a. Vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> in Food Samples Analyzed by Five Participating Laboratories<sup>a,b</sup>

|                       | mean vitamin D <sub>3</sub> (μg/100 g) |                                 |                               |             |                              |                                | mean 25(OH)D <sub>3</sub> (μg/100 g) |                                 |                               |             |                              |                                |
|-----------------------|--|---------------------------------|-------------------------------|-------------|------------------------------|--------------------------------|--------------------------------------|---------------------------------|-------------------------------|-------------|------------------------------|--------------------------------|
|                       | chicken liver                          | dried bovine liver (NIST 1577c) | whole egg powder (NIST 1845a) | ground beef | meat homogenate (NIST 1546a) | dietary supplement (fill only) | chicken liver                        | dried bovine liver (NIST 1577c) | whole egg powder (NIST 1845a) | ground beef | meat homogenate (NIST 1546a) | dietary supplement (fill only) |
| <b>Trial 1</b>        |  |                                 |                               |             |                              |                                |                                      |                                 |                               |             |                              |                                |
| Laboratory A          | 1.35                                   | <0.05 <sup>c</sup>              | 4.42                          | 0.28        | 0.24                         | 61 200                         | 0.48                                 | 1.42                            | 1.29                          | 0.28        | 0.09                         | 2.79                           |
| Laboratory B          | 1.63                                   | 0.016                           | 5.10                          | 0.27        | 0.257                        | 59 357                         | 0.57                                 | 1.62                            | 1.37                          | 0.33        | 0.12                         | 1.75                           |
| Laboratory C          | 1.65                                   | 0.05                            | 5.03                          | 0.31        | 0.23                         | 51 256                         | 0.50                                 | 1.81                            | 1.12                          | 0.29        | 0.09                         | 1.78 <sup>d</sup>              |
| Laboratory D          | 1.46                                   | 0.06                            | 4.20                          | 0.31        | <sup>e</sup>                 |                                | 0.46                                 | 1.39                            | 1.66                          | 0.27        | 0.11                         |                                |
| <b>Trial 2</b>        |  |                                 |                               |             |                              |                                |                                      |                                 |                               |             |                              |                                |
| Laboratory A          | 1.22                                   | <0.05 <sup>c</sup>              | 4.23                          | 0.25        | 0.17                         | 60 200                         | 0.52                                 | 1.46                            | 1.15                          | 0.29        | 0.08                         | 2.24                           |
| Laboratory B          | 1.48                                   | 0.014                           | 4.09                          | 0.28        | 0.23                         | 64 280                         | 0.50                                 | 1.26                            | 1.04                          | 0.25        | 0.09                         | 0.58                           |
| Laboratory C          | 1.72                                   | 0.05                            | 4.71                          | 0.28        | 0.24                         | 46 653                         | 0.45                                 | 1.83                            | 1.14                          | 0.46        | 0.08                         | 1.66 <sup>e</sup>              |
| Laboratory D          | 1.42                                   | 0.05                            | 3.90                          | 0.29        | 0.25                         |                                | 0.52                                 | 1.39                            | 1.46                          | 0.31        | 0.13                         |                                |
| Laboratory E          | 1.29                                   | 0.07                            |                               | 0.30        | 0.26                         |                                | 0.46                                 | 1.34                            |                               | 0.33        | 0.08                         |                                |
| <b>combined</b>       |  |                                 |                               |             |                              |                                |                                      |                                 |                               |             |                              |                                |
| mean                  | 1.47                                   | 0.043                           | 4.49                          | 0.284       | 0.233                        | 56 390                         | 0.495                                | 1.51                            | 1.25                          | 0.315       | 0.093                        | 1.82                           |
| SD                    | 0.20                                   | 0.025                           | 0.45                          | 0.023       | 0.034                        | 7160                           | 0.041                                | 0.21                            | 0.20                          | 0.074       | 0.019                        | 0.82                           |
| reproducibility % RSD | 13.3                                   | 58.0                            | 10.0                          | 8.0         | 14.8                         | 12.7                           | 8.3                                  | 14.1                            | 16.0                          | 23.5        | 20.7                         | 45.1                           |
| Horwitz ratio         | 0.4                                    | 1.1                             | 0.4                           | 0.2         | 0.4                          | 2.1                            | 0.2                                  | 0.5                             | 0.5                           | 0.6         | 0.5                          | 1.6                            |
| repeatability % RSD   | 6.9                                    | 38.7                            | 2.8                           | 6.2         | 9.7                          | 2.9                            | 3.4                                  | 3.6                             | 5.4                           | 15.6        | 10.6                         | 14.8                           |

<sup>a</sup>Shaded cells indicate no data reported. <sup>b</sup>Values for foods in this table are means from triplicate analyses within each trial reported by laboratories A, B, C, and E and duplicate analyses from laboratory D. Abbreviations: NIST, National Institute of Standards and Technology (Gaithersburg, MD, USA); SD, standard deviation; RSD, relative standard deviation. <sup>c</sup>Laboratory A reported a vitamin D<sub>3</sub> level of <0.05 for dried bovine liver (NIST 1577c) in trials 1 and 2, which is lower than the laboratory's limit of quantification. Therefore, these values were omitted from mean and SD calculations. <sup>d</sup>Laboratory C conducted both of its trials on the same day, not 2 months apart as the other laboratories did. <sup>e</sup>Laboratory D indicated uncertainty about the quality of its data on meat homogenate (NIST 1546a). Therefore, this value was considered an outlier and excluded from the results reported here.

the assays were not separated in time as was the case at the other laboratories. Because the 25(OH)D<sub>3</sub> content of the DS was similar to that of the foods, the laboratories measured it as described in Table 1a. The DS's vitamin D<sub>3</sub> content was orders of magnitude higher than its 25(OH)D<sub>3</sub> content and the vitamin D<sub>3</sub> in foods. As a result the laboratories optimized their methodologies to measure the high levels of vitamin D<sub>3</sub> in the DS (Table 1b). Laboratory B used LC-MS/MS to measure vitamin D<sub>3</sub> but did not derivatize the sample (because it is not necessary for high levels), whereas laboratories A and C used high-performance liquid chromatography–UV (a less sensitive detection method).

**Vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> Results for the Food Materials.** Figure 1 and Table 2a summarize the results for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> levels in the foods analyzed. The mean assayed vitamin D<sub>3</sub> concentrations ranged from 0.043 μg/100 g (dried bovine liver) to 4.49 μg/100 g (whole egg powder). The mean vitamin D<sub>3</sub> levels in the meat homogenate, whole egg powder, and chicken liver were higher than the corresponding 25(OH)D<sub>3</sub> levels in these foods, and the 25(OH)D<sub>3</sub> levels were higher than the vitamin D<sub>3</sub> levels in the dried bovine liver and ground beef.

The mean assayed 25(OH)D<sub>3</sub> concentrations ranged from 0.093 μg/100 g (meat homogenate) to 1.51 μg/100 g (dried bovine liver). Dried bovine liver had the lowest vitamin D<sub>3</sub> content of all materials analyzed (0.043 μg/100 g), near the limit of quantitation for all of the laboratories. However, dried bovine liver had the highest 25(OH)D<sub>3</sub> content (1.51 μg/100 g) of the food materials.

The reproducibility for vitamin D<sub>3</sub> ranged from 8% (ground beef) to 58% RSD (dried bovine liver; predicted reproducibility RSD for this very low analyte concentration is 51.28%). The corresponding HorRat values were all acceptable, ranging from 0.2 to 1.1. Repeatability for vitamin D<sub>3</sub> ranged from 2.8% RSD (whole egg powder) to 38.7% RSD (dried bovine liver; predicted repeatability RSD for this low analyte concentration is 25.64%, Table 2a).

The reproducibility for 25(OH)D<sub>3</sub> ranged from 8.3% RSD (chicken liver) to 23.5% RSD (ground beef). The HorRat values ranged from 0.2 (chicken liver) to 0.6 (ground beef). Repeatability for 25(OH)D<sub>3</sub> ranged from 3.4% RSD (chicken liver) to 15.6% RSD (ground beef; Table 2a).

**Vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> Results for the DS.** The mean assayed concentration of vitamin D<sub>3</sub> in the DS was 56,400 μg/100 g. The mean 25(OH)D<sub>3</sub> value was 1.82 μg/100 g, which was comparable to the levels found in the foods. The mean vitamin D<sub>3</sub> amount for the DS in this study was 14.8% above the labeled level [% difference for screened supplements ranged from 10.8 to 57.5% (see Supplemental Table 2)]. Reproducibility was 12.7% RSD for vitamin D<sub>3</sub> and 45.1% RSD for 25(OH)D<sub>3</sub>. Repeatability was 2.9% RSD for vitamin D<sub>3</sub> and 14.8% RSD for 25(OH)D<sub>3</sub>. For comparison, predicted reproducibility and repeatability RSDs for 25(OH)D<sub>3</sub> were 40.93 and 20.46%, respectively. However, the three laboratories handled the DS differently. Two laboratories dissolved the capsules and provided the data in micrograms per 100 g (whole capsule). The other laboratory cut the capsules open, emptied the contents, and provided the data in micrograms per 100 g of



**Table 2b. Vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub> in Dried Bovine Liver and Ground Beef Measured by Three Participating Laboratories<sup>a,b</sup>**

|                         | mean Vitamin D <sub>2</sub><br>(μg/100g)    |                | mean<br>25(OH)D <sub>2</sub><br>(μg/100g)   |                |
|-------------------------|---|----------------|---|----------------|
|                         | dried<br>bovine<br>liver<br>(NIST<br>1577c) | ground<br>beef | dried<br>bovine<br>liver<br>(NIST<br>1577c) | ground<br>beef |
| <b>Trial 1</b>          |   |                |   |                |
| Laboratory B            | <0.01                                       | 0.071          | 0.08  | 0.072          |
| Laboratory D            |   | 0.067          | 0.053                                       | 0.0435         |
| <b>Trial 2</b>          |   |                |   |                |
| Laboratory B            | <0.03                                       | 0.078          | 0.07  | 0.073          |
| Laboratory D            |   | 0.055          | 0.040                                       | 0.044          |
| Laboratory E            | 0.033                                       | 0.184          | 0.063                                       | 0.090          |
| <b>combined</b>         |   |                |   |                |
| mean                    |   | 0.099          | 0.064                                       | 0.067          |
| SD                      |   | 0.058          | 0.015                                       | 0.024          |
| reproducibility%<br>RSD |   | 59.0           | 24.2  | 35.2           |
| Horwitz ratio           |   | 1.3            | 0.5   | 0.7            |
| repeatability %<br>RSD  | 27.6  | 22.3           | 13.4  | 22.7           |

<sup>a</sup>Shaded cells indicate no data reported or not applicable. <sup>b</sup>Values for foods in this table represent means from triplicate analyses within trials reported by laboratories B and E and duplicate analyses from laboratory D. Abbreviations: NIST, National Institute of Standards and Technology (Gaithersburg, MD, USA); <, value below laboratory's limit of quantitation; SD, standard deviation; RSD, relative standard deviation.

fill as well as the total weights of the fill and capsule shell. The weight data were applied to the first laboratory's results to calculate the results in micrograms per 100 g of fill for direct comparison. For the DS results reported based on fill weight, the HorRat values were 2.1 for vitamin D<sub>3</sub> and 1.6 for 25(OH)D<sub>3</sub> (see Table 2a). The HorRat values for the DS results based on the weight of the whole capsule were 1.8 and 1.3, respectively (see Supplemental Table 3).

**Vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub> Results for the Foods and DS.** Only three laboratories reported results for vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub> levels, and only laboratory B reported values for vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub> in all materials. Therefore, results for vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub> are provided for descriptive purposes only, and only values above the limit of quantification are reported (Table 2b). The vitamin D<sub>2</sub> level reported by laboratory B was below the limit of quantitation in all materials except ground beef, and 25(OH)D<sub>2</sub> was measurable only in ground beef and dried bovine liver. The vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub> levels reported by laboratory B were considerably lower than those of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> (<0.1 μg/100 g).

## DISCUSSION

For the foods in this study, the HorRat values for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> (ranging from 0.2 to 1.1) demonstrated acceptable between-laboratory reproducibility at the analyte concentration present for all of the matrices (ground beef, chicken liver, whole egg powder, meat homogenate, and dried bovine liver). In our study, the low repeatability RSDs for vitamin D<sub>3</sub> and for 25(OH)D<sub>3</sub>, seen in all of the matrices except vitamin D<sub>3</sub> in bovine liver, suggest that the laboratories

had consistent within-laboratory results and, thus, acceptable within-laboratory precision for all study materials. Therefore, our study shows that it is possible to obtain results with acceptable overall reproducibility among experienced laboratories using LC-MS/MS to measure levels of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> for a variety of food matrices having naturally occurring vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub>.

For the DS, the three laboratories demonstrated that they were able to measure vitamin D<sub>3</sub> at high levels as well as very low levels of 25(OH)D<sub>3</sub>. The HorRat values of 2.1 and 1.8 for vitamin D<sub>3</sub> and 1.6 and 1.3 for 25(OH)D<sub>3</sub> indicate acceptable or close to acceptable reproducibility relative to the analyte concentration. The elevated RSDs obtained experimentally are close to theoretically predicted RSDs at such low analyte concentrations, and this is reflected in acceptable HorRat ratios. For example, the % RSD for the very low level 25(OH)D<sub>3</sub> in bovine liver seems very high. However, it is an acceptable RSD because as the concentration decreases, the predicted RSD increases, as illustrated by the Horwitz curve.<sup>37</sup>

The amount of 25(OH)D<sub>3</sub> in the vitamin D supplement was very low but was consistently present in all DSs analyzed in the screening and in the study. Vitamin D levels above label claims (overages) were seen in the DSs selected for this study and in the products screened for inclusion in this study. Manufacturers may add ingredient overages to provide the minimum content stated on the label at the end of the product shelf life.<sup>39</sup>

Vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub> were found in small quantities in ground beef and bovine liver, with acceptable HorRat ratios, although our study's focus was on measuring vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub>. Future studies need to identify whether different samples of beef in the food supply have variable vitamin D<sub>2</sub> content and identify sources of vitamin D<sub>2</sub> in food samples such as components of animals' diets.

The source of 25(OH)D<sub>3</sub> in meat, poultry, and eggs could be due to the animal's exposure to UV light, resulting in *in vivo* production of vitamin D<sub>3</sub>, or derived from the animal's feed containing vitamin D<sub>3</sub> or 25(OH)D<sub>3</sub>. Thus, variation in animal diet and environment can affect vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> levels in animal-based foods.<sup>21,40–48</sup> Unfortunately, data on the 25(OH)D<sub>3</sub> content of foods are limited and existing data can vary greatly.<sup>21,49,50</sup>

Comparison of studies of 25(OH)D<sub>3</sub> in foods is hindered by the lack of relevant certified reference materials,<sup>51</sup> which makes it difficult to ascertain whether the differences between studies are due to methodological or physiological factors. Matrix-matched reference or control samples make it possible to quantify the contribution of analytical differences that are separate from differences in food composition. Developing reference materials for use in analyses, for foods and for DSs, is relevant to ensuring the trueness of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> estimates. The data from this study will be used to help assign reference values for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> in several NIST SRMs—Bovine Liver (SRM 1577c), Meat Homogenate (SRM 1546a), and Whole Egg Powder (SRM 1845a).<sup>52</sup> As for reference values for DSs, this study provided data on a vitamin D product that could be used as in-house control material in future studies.

Our study had several limitations. Because it included only experienced analysts, validated methods, and appropriate equipment to obtain results, we cannot draw conclusions about the likely performance of other laboratories in analyzing the same materials. We also did not seek to quantify any bias between laboratories. Although the overall reproducibility was

acceptable for all materials, some systematic bias between laboratories could have affected the comparison of nutrient content results for foods analyzed at different laboratories.

Continued efforts to determine amounts of vitamin D and 25(OH)D in foods and supplements using validated methodologies and well-characterized control and reference materials will help answer questions about discrepancies between circulating amounts of 25(OH)D in the body and vitamin D intakes calculated from composition data for foods and DSs. These endeavors can also help ensure that public health policy decisions about vitamin D are based on accurate estimates of intake.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b05016.

Descriptions and analytical results of seven dietary supplements screened for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> and per-capsule analytical results from three laboratories for the dietary supplement (PDF)

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### Notes

Trade names are included for the reader's information only and are not meant to imply endorsement or preferential treatment by the U.S. government.

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

25(OH)D, 25-hydroxyvitamin D; DS, dietary supplement; USDA, U.S. Department of Agriculture; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NDL, Nutrient Data Laboratory; NIST, National Institute of Standards and Technology; SRM, standard reference material; RSD, relative standard deviation; HorRat, Horwitz ratio

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