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Title	Interlaboratory comparison with a reference measurement procedure (RMP) for determining 24,25-dihydroxyvitamin D3 in human serum using liquid chromatography-tandem mass spectrometry (LC-MS/MS)
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Publication date	2026-04-28
Original Citation	Wise, S A, Mineva, E, Pfeiffer, C M, Vesper, H, Cavalier, É, Peeters, S, Galvin, K, Cashman, K D, Hoofnagle, A N, Williams, E L, Briggs, L E, Souness, M E, Kuszak, A J, Hahm, G & Camara, J E 2026, 'Interlaboratory comparison with a reference measurement procedure (RMP) for determining 24,25-dihydroxyvitamin D3 in human serum using liquid chromatography-tandem mass spectrometry (LC-MS/MS)', Analytical and Bioanalytical Chemistry, pp. 1-16. https://doi.org/10.1007/s00216-026-06480-8
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://www.scopus.com/pages/publications/105037525020 - 10.1007/s00216-026-06480-8https://www.scopus.com/pages/publications/105037525020?origin=resultslist
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Download date	2026-11-06 13:49:46
Item downloaded from	https://hdl.handle.net/10468/18901



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Analytical and Bioanalytical Chemistry
https://doi.org/10.1007/s00216-026-06480-8

RESEARCH PAPER

Interlaboratory comparison with a reference measurement procedure (RMP) for determining 24,25-dihydroxyvitamin D_3 in human serum using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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Received: 4 February 2026 / Revised: 9 March 2026 / Accepted: 12 March 2026
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Abstract

An interlaboratory comparison study was conducted among five laboratories for determining 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) in human serum using liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods. The Centers for Disease Control and Prevention (CDC), Imperial College Healthcare NHS Trust, University College Cork, University of Liège, and University of Washington analyzed 50 single-donor samples and two new Standard Reference Materials (SRMs®). The results from each laboratory were compared with target values assigned by the National Institute of Standards and Technology (NIST) using a reference measurement procedure (RMP) and evaluated using Ordinary Deming linear regression and Bland-Altman analysis. Three of the five laboratory methods provided results that were in good agreement with the NIST RMP results showing linear regression slopes ranging from 0.972 to 1.003 and Bland-Altman mean bias of −0.092 nmol/L, 0.025 nmol/L, and 0.035 nmol/L. Two laboratories demonstrated a significant positive bias with linear regression slopes of 1.158 and 1.214 and Bland-Altman mean bias of 0.162 nmol/L and 0.708 nmol/L. The CDC method is currently used to assign "information only" values for $24,25(OH)_2D_3$ in the quarterly distributions of the Vitamin D External Quality Assessment Scheme (DEQAS). Given the agreement (linear regression slope = 0.984, $R^2 = 0.996$ and mean bias of −0.092 nmol/L) between the CDC method and the NIST RMP observed in this study, the CDC-assigned $24,25(OH)_2D_3$ values in DEQAS may provide a more accurate reference than the current participant consensus mean values.

Keywords Certified reference materials (CRMs) · Standard reference materials (SRMs) · 24,25-Dihydroxyvitamin D_3 · 25-Hydroxyvitamin D_3 · 25-Hydroxyvitamin D_2 · Vitamin D External Quality Assessment Scheme (DEQAS)

Introduction

The measurement of vitamin D metabolites, particularly 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$), to assess vitamin D status has evolved over the past five decades to primarily two current analytical methodologies: (1) a wide diversity of fully automated commercial ligand binding assays (primarily immunoassays) and (2) in-house (and several commercial) liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods [1–4]. While the primary focus has been on analytical methods for determining serum total 25(OH)D (defined as the sum of circulating $25(OH)D_2$ and $25(OH)D_3$) and the current marker for vitamin D status assessment, recent interest has emerged for the measurement of additional vitamin D metabolites [1–6]. Reviews by Alexandridou et al. [1], Alonso et al. [2], Altieri et al. [7], and Herrmann [4] provide comprehensive overviews of vitamin D metabolism that highlight the importance of various metabolites (sometimes referred to as the vitamin D metabolome) in understanding vitamin D deficiency, as well as the analytical challenges in measuring these metabolites. Most LC-MS/MS methods for determining vitamin D metabolites focus primarily on $25(OH)D_2$, $25(OH)D_3$, and the 3-*epimer* of $25(OH)D_3$. However, Jones and Kaufmann [8] make a case that the emergence of highly sensitive and specific LC-MS/MS technology allows for simultaneous monitoring of multiple components of the vitamin D metabolome, enhancing the understanding of

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vitamin D metabolism and providing additional diagnostic information without significant increase in cost.

The determination of two dihydroxyvitamin D metabolites, 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has received considerable attention. The production of the main bioactive form of vitamin D, $1,25(OH)_2D_3$, is tightly regulated with a corresponding short half-life (~ 4 h to 6 h), and its serum levels are three orders of magnitude lower than those of $25(OH)D_3$. These factors present significant analytical challenges in measuring this biomarker [9]. Levels of $24,25(OH)_2D_3$, the major product of $25(OH)D_3$ catabolism, are approximately 10 to 15% of those of $25(OH)D_3$, making it easier to measure [10–13] than $1,25(OH)_2D_3$. LC-MS/MS methods for determining $24,25(OH)_2D_3$ as one of several vitamin D metabolites have evolved during the past 15 years, with most methods using isotopically labeled $24,25(OH)_2D_3$ as one of several internal standards to provide an isotope dilution (ID) approach to quantify $24,25(OH)_2D_3$ [10, 14–21]. Tai et al. [21] at the National Institute of Standards and Technology (NIST) developed a Reference Measurement Procedure (RMP) for determining $24,25(OH)_2D_3$ and used it to assign $24,25(OH)_2D_3$ concentrations in Standard Reference Material® (SRM®) 972a and SRM 2973 [22]. The NIST ID LC-MS/MS method for $24,25(OH)_2D_3$ is currently the only RMP recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) (JCTLM ID C13RMP2) [23], and SRM 972a [24] and SRM 2973 [22] are currently the only SRMs available with values assigned for $24,25(OH)_2D_3$ [25]. Several recently reported LC-MS/MS methods for determining $24,25(OH)_2D_3$ were validated using SRM 972a [14, 16, 17, 26, 27].

There has been increasing interest in measuring $24,25(OH)_2D_3$ [6, 10, 20, 28, 29] due in part to its potential to provide additional information regarding vitamin D metabolism. The ratio between the amount of $25(OH)D_3$ and $24,25(OH)_2D_3$, known as the vitamin metabolite ratio (VMR), was proposed by Wagner et al. [19] as a predictor of serum 25(OH)D response to vitamin D_3 supplementation. The VMR has since found diagnostic utility to assess vitamin D status differences among different populations [30], to identify vitamin D deficiency [31–33] and genetic enzyme defects and mutations [34], and to investigate vitamin D_3 catabolism [34]. A review by Dirks et al. [35] highlighted the need for precise $24,25(OH)_2D_3$ measurements to use the VMR effectively. Wise et al. [36] demonstrated the importance of accurate measurements of $24,25(OH)_2D_3$ using an RMP versus a consensus mean from the Vitamin D External Quality Assessment Scheme (DEQAS).

While activities to improve the accuracy and comparability of total 25(OH)D measurements have made significant progress in the past two decades, e.g., DEQAS [37], the CDC Vitamin D Standardization Certification Program (VDSCP) [38], and SRMs [25], similar efforts for $24,25(OH)_2D_3$ have been limited. Since 2015, DEQAS has provided a consensus mean value for the concentration of $24,25(OH)_2D_3$, based on a limited number of participants (currently 11 laboratories) reporting LC-MS/MS results for $24,25(OH)_2D_3$, in the regular quarterly sample distribution for measurements of total 25(OH)D. Carter et al. [39] compared the performance of 10 LC-MS/MS assays for determining $24,25(OH)_2D_3$ in a 2018 DEQAS exercise with five samples ranging in concentration of $24,25(OH)_2D_3$ from 1.0 to 9.1 nmol/L and observed CVs of between 21 to 29.6%. This relatively poor agreement among laboratories in this exercise prompted the authors to call for the use of SRMs to improve performance [39]. Since 2018, the CDC has provided results for $24,25(OH)_2D_3$ in DEQAS samples using their routine LC-MS/MS method. Several recent papers [20, 40, 41] have reported the development of LC-MS/MS methods for determining $24,25(OH)_2D_3$ and have used DEQAS samples for method validation.

In 2017, the National Institutes of Health, Office of Dietary Supplements (NIH ODS), and NIST reported the results of an intercomparison study among six laboratories [13] for determining $24,25(OH)_2D_3$ using LC-MS/MS. The samples analyzed in the 2017 interlaboratory study were SRM 972a (four levels), SRM 2973, and 30 archived samples from the DEQAS program, which are pooled samples from several donors [37]. As a follow-up and extension to this intercomparison study for determining $24,25(OH)_2D_3$, a second intercomparison study was initiated by NIH ODS to compare results using the NIST RMP to results from selected laboratories who currently provide measurement services for vitamin D studies and/or who participate in DEQAS measuring $24,25(OH)_2D_3$. In this current study, participating laboratories analyzed a set of 50 single-donor serum samples and two new SRMs to determine $24,25(OH)_2D_3$ using their custom in-house LC-MS/MS methods.

The aims of the current interlaboratory comparison study were to (1) directly compare measurements obtained from participating laboratories' custom in-house LC-MS/MS methods to the NIST $24,25(OH)_2D_3$ RMP using 50 single-donor samples and two new SRMs, (2) assess the comparability of results among selected laboratories providing measurement services and/or participating in DEQAS for determining $24,25(OH)_2D_3$, (3) assess differences in measurements between single-donor and pooled serum samples for $24,25(OH)_2D_3$, and (4) assess whether comparability among laboratories has improved over the past decade since the introduction of SRMs with assigned $24,25(OH)_2D_3$ values.

Experimental

Participating laboratories

Five laboratories with expertise in determining vitamin D metabolites including $24,25(OH)_2D_3$ were invited to participate in this intercomparison study: (1) Centers for Disease Control and Prevention (CDC), Division of Laboratory Sciences, Atlanta, GA, USA; (2) Imperial College Healthcare NHS Trust (ICHT), London, UK; (3) University College Cork (UCC), Cork Centre for Vitamin D and Nutrition Research, School of Food and Nutritional Sciences; Cork, IE; (4) University of Liège (U of Liège), Department of Clinical Chemistry, CIRM, CHU de Liège, Liège, BE; and (5) University of Washington (U of WA), Department of Laboratory Medicine and Pathology, Seattle, WA, USA. The results from these laboratories were compared with results obtained at the NIST Chemical Sciences Division using the RMP for determining $24,25(OH)_2D_3$ [21].

Samples analyzed

The participating laboratories in this intercomparison study analyzed a set of 50 single-donor samples and two recently issued SRMs as unknown samples and two SRMs with certified values for $24,25(OH)_2D_3$ as control materials. The set of 50 single-donor serum samples was used previously in two studies to assess the commutability of selected SRMs and External Quality Assessment (EQA) samples for the determination of total 25(OH)D [42, 43]. These single-donor samples were procured from Solomon Park Research Laboratories (Seattle, WA) and were prepared according to the Clinical Laboratory Standards Institute (CLSI) C37-A guidelines [44, 45]. The samples were from 50 human donors without known underlying health conditions (i.e., no known disease states, pregnancy, or renal failure) and contained only endogenous vitamin D metabolites with a distribution of total 25(OH)D concentrations across a clinically relevant range of 15 to 150 nmol/L and $24,25(OH)_2D_3$ concentrations ranging from 0.48 to 15.2 nmol/L as determined at NIST using their RMP. The set of 50 single-donor samples contained 8 samples with elevated levels of $25(OH)D_2$, i.e., > 20 nmol/L from donors supplementing their diets with ergocalciferol (vitamin D_2). Each single-donor sample vial contained 0.5 mL of serum. The details for the preparation and distribution of 25(OH)D concentrations for these 50 single-donor samples were described previously [12].

Two new SRMs for determining vitamin D metabolites were also analyzed in this study as unknown samples: (1) SRM 2969 Vitamin D Metabolites in Frozen Human Serum (total 25-hydroxyvitamin D low level) and (2) SRM 2970 Vitamin D Metabolites in Frozen Human Serum (25-hydroxyvitamin D_2 high level) [46]. SRM 2969 and SRM 2970 were developed to complement the levels of $25(OH)D_2$, $25(OH)D_3$, and total 25(OH)D in SRM 972a [24] and SRM 2973 [22]. SRM 2969 has a lower level of total 25(OH)D, i.e., 34.7 nmol/L (13.9 ng/mL), than prior existing SRMs, i.e., 47.1 nmol/L (18.9 ng/mL) in SRM 972a and at the lower range of "insufficient" vitamin D status [47]. SRM 2970 has a high endogenous $25(OH)D_2$ concentration of 56.9 nmol/L (23.5 ng/mL), which is higher than prior existing endogenous SRMs. These two SRMs have certified values assigned for only $25(OH)D_2$, $25(OH)D_3$, and total 25(OH)D. SRM 2969 and SRM 2970 [46] have low levels of $24,25(OH)_2D_3$ of 1.34 nmol/L and 1.70 nmol/L, respectively, based on screening analyses at NIST using the RMP, which are lower than any of the certified values for $24,25(OH)_2D_3$ in the four levels of SRM 972a (3.39 to 6.38 nmol/L). However, there are no assigned values for $24,25(OH)_2D_3$ on the NIST Certificate of Analysis for SRM 2969 or SRM 2970.

SRM 972a Level 2 and SRM 2973, which have certified values for $24,25(OH)_2D_3$ of 3.39 ± 0.12 nmol/L and 7.51 ± 0.26 nmol/L, respectively, were provided to the participants for use as control materials during the analysis of the unknown samples. All samples were shipped on dry ice from NIST to the participants during the weeks of October 10, 2022, and October 17, 2022. Results of the analyses were received from four laboratories in March 2023 and the remaining laboratory provided results in May 2025.

Methods

Analytical protocol and information reporting for participating laboratories

Each laboratory was requested to provide the following method information, which is summarized in Table 1: (1) LC-MS/MS instrumentation description, (2) LC column (manufacturer, dimensions, particle size), (3) LC conditions, (4) *m/z* transitions monitored, (5) source of calibration standards used for $24,25(OH)_2D_3$, and (6) limit of detection (LoD) and/or limit of quantification (LoQ) and reportable range. The participating laboratories were requested to follow an analysis protocol consisting of one run with duplicate measurements of the single-donor samples and of SRM 2969 (3 subsamples) and SRM 2970 (3 subsamples). For the SRM control samples (SRM 972a L2 and SRM 2973), four subsamples of each SRM (denoted as A, B, C, and D) were distributed in four different positions among the 50 single-donor, SRM 2969, and SRM 2970 samples and measured in duplicate. Following this protocol, the laboratories each provided 128 sample results. The laboratories were provided with an Excel data reporting template. Laboratories were

Table 1 Participants and LC-MS/MS assays used in intercomparison study for determination of $24,25(OH)_2D_3$

Participant [citation]	Method details: MS/MS and LC instrumentation and conditions
NIST [21]	Applied Biosystems API 5000 or 4000 LC-MS/MS with Agilent 12601290 Series LC system or SCIEX QTRAP 6500+ LC-MS/MS system with Agilent 1290 Infinity II series LC system; Ascentis Express C18, 4.6 mm × 150 mm, 2.7 µm; isocratic elution with 0.75 mL/min of 30:70 water:methanol (MeOH) (v/v) for 40 min, followed by a 15 min column rinse with 100% methanol, and 12 min re-equilibration to initial conditions; column temperature 35 °C. Internal standard (IS): $24,25(OH)_2D_3$-d_6 (IsoSciences, King of Prussia, PA, USA) no purity assessment. *m/z* transitions monitored: $24,25(OH)_2D_3$: 417 → 381; IS $24,25(OH)_2D_3$-d_6: 423 → 387. Calibrant: $24,25(OH)_2D_3$, two lots (IsoSciences, King of Prussia, PA, USA), purity assessed in-house as 94.1% and 93.2%. Sample preparation: 2 g of serum spiked with IS solution, pH adjusted to 9.8 with carbonate buffer solution; LLE performed twice with 50% hexane/ethyl acetate and shaking; organic layer extract dried under N_2 and reconstituted with 100 µL MeOH. Serum volume required: 2 mL. Limit of quantitation: 0.5 nmol/L; reportable range: 0.5 to 25 nmol/L. Control materials: SRM 972a L1, L2, and L3
CDC (this paper)	Agilent Infinity II LC-6490 MS; Ascentis Express F5, 4.6 mm × 150 mm, 2.5 µm; gradient elution with 1.1 mL/min of 70% MeOH/DIW increased to 80% MeOH in 11 min followed by 100% MeOH at 11.1 min for 0.9 min and back to initial conditions at 13 min for run time of 13.1 min; column temperature 30 °C; IS: $24,25(OH)_2D_3$-d_6 (Entegris, Inc. Billerica, MA). *m/z* transitions monitored: $24,25(OH)_2D_3$: 417.3 → 399.3 (381.3); IS $24,25(OH)_2D_3$-d_6: 423.3 → 387.3. Calibrant: Prepared from SRM 2971. Sample preparation: 0.5 mL serum mixed with IS solution and 0.2 mL of 0.1 g/mL Na_2CO_3; mixed and equilibrated prior to LLE with 50% hexane/ethyl acetate; organic layer decanted and dried in vacuum centrifuge; reconstituted in 100 µL of 75% MeOH/water and transferred to 96-well plate. Serum volume required: 500 µL. Limit of detection: 0.3 nmol/L; reportable range 0.3 to 25 nmol/L. Control materials (in addition to SRM 2973 and SRM 972a L2); internal serum QC materials
Imperial College Healthcare NHS Trust (this paper)	Waters ACQUITY UPLC system and Xevo TQ Absolute Triple Quadrupole mass Spectrometer; Waters ACQUITY UPLC HSS PFP column (100 mm × 2.1 mm, 1.8 µm); Mobile Phase A (HPLC-grade water) and B (HPLC-grade MeOH) both containing 2 mmol/L ammonium acetate and 0.1% formic acid at flow rate of 0.4 mL/min, run time of 10 min; column temperature 50 °C; injection volume: 15 µL. Calibrant: $24,25(OH)_2D_3$ (ASI Chemicals, Lot no. ASI024A157, stated purity 99.14%, not assessed). IS: $24,25R(OH)_2D_3$-d_6 (IsoSciences, King of Prussia, PA, USA). *m/z* transitions monitored: $24,25(OH)_2D_3$: 417.5 → 159.2 (quantifier), 417.5 → 381.45 (qualifier); $24,25(OH)_2D_3$-d_6: 423.5 → 159.2. Sample preparation: 150 µL of serum mixed with IS solution and added to 96-well sample plate; SLE with 50% hexane/ethyl acetate; extract evaporated to dryness and reconstituted in 50 µL 60% 95:5 methanol:isopropanol and 40% water. Serum volume required: 150 µL. Limit of detection: 0.5 nmol/L; reportable range: 1.2 to 96 nmol/L. Control materials (in addition to SRM 2973 and SRM 972a L2): DEQAS samples 490, 495, 499, 500, 501, 503, 505, 508, 642, 656, 660, 662–670 with consensus means and NIST RMP values or CDC information values
University College Cork [14]	Waters Aquity TQD; Supelco Ascentis Express F5 (10 cm × 2.1 mm, 2.7 µm); Ascentis Guard column (0.5 cm × 2.1 mm); Mobile Phase A (water) and B (methanol) both containing 2 mmol/L ammonium acetate, 0.1% formic acid; gradient elution with 35% A, 65% B to 4.5 min followed by nonlinear gradient to 28% A and 75% B after 8 min 0.45 mL/min; column temperature 35 °C; IS: $24,25(OH)_2D_3$-d_6 (IsoSciences, King of Prussia, PA, USA). *m/z* transitions monitored: $24,25(OH)_2D_3$: 417.3 → 121; $24,25(OH)_2D_3$-d_6: 423.3 → 121.1. Calibrant: $24,25(OH)_2D_3$ (IsoSciences, stated purity > 98%; purity assessed in-house at 92%). Sample preparation: 150 µL serum mixed with IS solution and allowed to equilibrate; proteins precipitated with 0.2 M aqueous $ZnSO_4$ solution and MeOH; LLE performed using 30/70 (v/v) hexane/ethyl acetate; extract evaporated to dryness and reconstituted in 100 µL 32/68 (v/v) water/MeOH. Serum volume required: 150 µL. Limit of detection: 0.65 nmol/L; reportable range: 1.34 nmol/L to 69.54 nmol/L. Control materials (in addition to SRM 2973 and SRM 972a L2): DEQAS samples 422 and 423 with NIST RMP values of 1.97 nmol/L and 6.84 nmol/L, respectively; analyzed before and after analysis run with measured results of 1.38 to 2.41 nmol/L and 6.36 to 7.20 nmol/L, respectively
University of Liège [15]	LC-MS/MS QTRAP 6500; Phenomenex Kinetex PFP, 100 Å, 2.1 mm × 100 mm, 2.6 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH at 0.40 mL/min; column temperature 30 °C. IS: $24,25(OH)_2D_3$-d_3 (IsoSciences, King of Prussia, PA, USA); *m/z* transitions monitored: $24,25(OH)_2D_3$: 417.5 → 399.5 (qualifier); 417.5 → 381.5 (quantifier); IS $24,25(OH)_2D_3$-d_3: 423.5 → 405.6 (qualifier); 423.5 → 397.5 (quantifier). Calibrant: Prepared from SRM 2971. Sample preparation: 200 µL serum mixed with IS solution (acetonitrile) and 4.5% $ZnSO_4$ solution; 150 µL supernatant transferred to 96-well plate. Serum volume required: 200 µL; limit of detection: 0.624 nmol/L; reportable range: 0.624 to 61.4 nmol/L. Control materials (in addition to SRM 2973 and SRM 972a L2): DEQAS samples 616 and 618 with consensus means and CDC information values

Table 1 (continued)

Participant [citation]	Method details: MS/MS and LC instrumentation and conditions
University of Washington [18, 48]	Waters/Xevo TQ-S MS/MS with electrospray ionization source, Acquity UPLC, Waters Acquity UPLC HSS T3, 2.1 × 50 mm, 1.7 µm analytical column fitted with a Waters Acquity UPLC BEH C18 2.1 × 5 mm 1.7 µm guard column; Linear gradient starting at 31% mobile phase A (Optima grade water (Fisher, Pittsburgh, PA, USA)/0.1% formic acid (VWR, Radnor, PA, USA)) and 69% mobile phase B (Optima grade MeOH (Fisher)/0.1% formic acid), ending in 1 min at 18% mobile phase A and 82% mobile phase B at flow rates ranging from 0.2 to 1.0 mL/min (0.2 mL/min during analyte elution); IS: 24,25$(OH)_2D_3$-d_6; *m/z* transitions monitored: 24,25$(OH)_2D_3$, 623.50 → 298.20; 24,25$(OH)_2D_3$-d_6, 629.56 → 298.21. Sample preparation: 200 µL serum spiked with IS solution and immunoaffinity purified using anti-1α,25$(OH)_2D$ beads; after incubation, beads washed and analytes eluted with organic solvent; eluent dried and reconstituted with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivatizing agent in acetonitrile. Serum volume required: 200 µL. Calibrants: 24(R),25$(OH)_2D_3$ (Enzo Life Sciences, Farmingdale, NY, USA) and 24(S)25$(OH)_2D_3$ (MilliporeSigma, > 99.0% stated purity as determined by HPLC, not assessed in-house). Limit of detection: 1.15 nmol/L; reportable range: 1.2 to 240 nmol/L. Control materials (in addition to SRM 2973 and SRM 972a L2): in-house spiked charcoal-stripped serum

requested to provide results for 24,25$(OH)_2D_3$ in nmol/L with three significant figures. The U of WA reported values for all samples including nine samples that were below their LoD (1.15 nmol/L). UCC reported that 10 samples were below the LoD of 0.65 nmol/L, and the U of Liège reported that two samples were below the LoD of 0.62 nmol/L. ICHT reported results that were below the LoQ but above the LoD of 0.5 nmol/L. CDC reported results for all samples since they were higher than the LoD of 0.3 nmol/L. It should be noted that each laboratory followed their usual analytical method practice using different sample volumes for analysis, i.e., 150 µL (UCC and ICHT), 200 µL (U of WA and U of Liège), 500 µL (CDC), and 2000 µL for the NIST RMP. These differences in sample volume obviously impact the LoD/LoQ and may also have some impact on the accuracy of measurements for the low levels of 24,25$(OH)_2D_3$ present in several of the single-donor samples.

LC-MS/MS methods for 24,25$(OH)_2D_3$

Each laboratory used its custom in-house LC-MS/MS method for determining 24,25$(OH)_2D_3$ (Table 1). The laboratory methods used by UCC [14], University of Liège [15], and U of WA [18, 48] have been published previously. The methods used by CDC and ICHT have not been published previously and are described below in more detail.

For the CDC LC-MS/MS method, the serum sample (0.5 mL) was mixed with internal standard solution (0.1 mL, 50 nM 24,25$(OH)_2D_3$-d_6 in ethanol) and 0.1 g/mL Na_2CO_3 (0.2 mL). After mixing and equilibration for 1 h (room temperature), liquid-liquid extraction (LLE) was performed with 50% ethyl acetate/hexane (2 mL). After mixing and centrifugation, the organic layer was decanted by freezing the aqueous layer (dry ice ethanol bath, approximately −50 °C). The organic layer was dried in a vacuum centrifuge (40 min at 40 °C), the sample was reconstituted in 75% methanol/deionized water (0.1 mL), mixed, centrifuged, and transferred to a 96-well plate for injection on the LC-MS/MS instrument (20 µL). The LC-MS/MS analysis was performed with APCI+ ionization (gas flow, 11 L/min; nebulizer, 207 kPa (30 psi); gas temperature, 250 °C; vaporizer temperature, 300 °C; capillary voltage, 4500 V; corona current, 7.0 µA) and MRM detection with unit mass resolution and 150 ms dwell time (see Table 1 for additional method details). The method is linear from 0.3 to 25 nmol/L. Long-term (7 years) method performance features excellent accuracy: the mean bias from SRM 972a (4 levels) was −0.4% (n = 136), the mean recovery from spiking of three different levels of 24,25$(OH)_2D_3$ (5 to 15 nmol/L) into two single-donor serum materials was 98 ± 5% (n = 36). The long-term average inter-assay imprecision (CV) from three QC materials (endogenous concentrations between 2.7 and 9.5 nmol/L) ranged from 3.2 to 3.5% (n = 301). 24R,25$(OH)_2D_3$ was stable in two serum materials at room temperature for 24 h, for 3 freeze/thaw cycles, and for 7 years when stored deep frozen (−50 °C to −90 °C). Other endogenous serum vitamin D isobaric metabolites, i.e., 1,25$(OH)_2D_3$ and 23,25$(OH)_2D_3$, were chromatographically baseline separated from the analyte of interest.

For the ICHT method, 150 µL of serum sample was added to a 96-well square sample plate followed by addition of 100 µL working internal solution [10 nM 24,25$(OH)_2D_3$-d_6]. Following plate mixing at 100 RPM for 5 min and room temperature equilibration for 10 min, supported liquid extraction (SLE) was performed with 50:50 n-hexane:ethyl acetate (v/v) (1 mL total). Eluate was collected in a 96-well round-bottomed collection plate and evaporated to dryness (1 h at 40 °C) before reconstitution in 50 µL 60% 95:5 methanol:isopropanol (v/v) and 40% LC-MS-grade water. Plates were sealed and mixed at 900 RPM for 5 min before loading onto the LC-MS/MS instrument. LC-MS/MS analysis was performed with 15 µL injection volume

with ESI+ ionization (capillary voltage, 0.75 kV; source temperature, 150 °C; desolvation temperature, 450 °C; cone gas flow, 150 L/h) and multiple reaction monitoring (MRM) detection with unit mass resolution (see Table 1 for additional method details). The method was linear from 1.2 to 96.0 nmol/L with a LoD of 0.5 nmol/L. The mean recovery from spiking of three different levels of $24,25(OH)_2D_3$ (9.6–39.4 nmol/L) into serum pools was 95.5 ± 8% ($n = 36$). Intra-assay imprecision (CV) from four EQA or specimen pool materials (9 to 38 nmol/L) ranged from 1.4 to 4.7% ($n = 48$) and inter-assay imprecision (CV) from three EQA or specimen pool materials (5 to 27 nmol/L) ranged from 5.1 to 9.1% ($n = 60$). Isobaric metabolites $1,25(OH)_2D_3$ and fludrocortisone were both shown to be chromatographically baseline resolved from $24,25(OH)_2D_3$ and $24,25(OH)_2D_3$-d_6.

NIST analyses

The NIST analyses of the 50 single-donor samples for determining $24,25(OH)_2D_3$ using the RMP [21] have been described previously [12], and the mean results are reported in Table 2 for comparison with the results from the participating laboratories. The mean concentrations of $24,25(OH)_2D_3$ in the 50 single-donor samples and the four SRMs, ordered from lowest to highest concentration, are shown in a bar graph plot in Figure S1 (Electronic Supplemental Material, ESM). For analysis of SRM 2969 and 2970, NIST reported the results for six replicates as part of the development of these SRMs [46].

Statistical analysis of results

The results from each of the laboratories were evaluated using Ordinary Deming regression as recommended by the CLSI guide [49] for the comparison of results from two analytical methods. The Ordinary Deming regression is recommended over the ordinary linear regression (OLR) for comparison of methods since the Deming regression assumes that both x and y have significant, non-negligible errors whereas the OLR assumes that x is measured without error [49]. All calculations for the Ordinary Deming regression and the Bland-Altman plots were performed using Analyse-it (Analyse-it Software, Leeds, UK). The Analyse-it Methods comparison Tab, which is based on CLSI guidelines [49–51], was used to generate Ordinary Deming Regression lines with 95% prediction intervals.

Results and discussion

A summary of the results from the five laboratories for determination of $24,25(OH)_2D_3$ in the 50 single-donor samples is provided in Table 2 and compared with the results from the NIST RMP. The overall absolute % bias of each of the laboratory methods for the 50 single-donor samples compared to the NIST RMP was 4.5%, 6.3%, 7.6%, 14.6%, and 21.5% (Table 2). A plot of % bias to the NIST RMP versus the single-donor samples and SRMs ordered from lowest to highest concentration of $24,25(OH)_2D_3$ is shown in Figure S2 (ESM). Each laboratory's results were compared to the NIST RMP through Ordinary Deming regression and Bland-Altman analysis as illustrated in Figs. 1, 2, and 3 and summarized in Table 3. The width of the prediction interval (PI) for the Ordinary Deming regression and the width of the limits of agreement (LoA) for the Bland-Altman analysis provide a measure of the variability of each laboratory's method ranging from 0.976 to 2.222 nmol/L and 0.860 to 3.794 nmol/L, respectively (Table 3). Overall, three of the five laboratory methods compare very favorably with the NIST RMP. As illustrated in Fig. 2, both the UCC and ICHT results were positively biased compared to the NIST results with regression line slopes of 1.214 and 1.158, respectively. UCC and ICHT both used the same source for their $24,25(OH)_2D_3$ calibrant and initially reported results based on the manufacturer's stated purity of 98%. However, UCC determined in-house that the standard had a purity of 92%, necessitating a recalculation of the results. ICHT also recalculated their results based on the same purity assessment. The Bland-Altman analysis of these two laboratories' results (Fig. 2B and D) indicates that a proportional constant error exists in the measurements as demonstrated by the increasing difference in the laboratory results and the NIST RMP, which indicates that a calibration error may still remain [52]. Two of the three laboratories with good agreement with the NIST RMP used a certified reference material (CRM) as the calibrant (see Table 1). The results of this intercomparison study emphasize the necessity of using either CRMs as calibrants or assessing the purity of non-CRM calibrants in-house to verify and/or establish an accurate concentration.

SRM 972a Level 2 and SRM 2973 were analyzed as controls by each laboratory concurrent with the single-donor samples, SRM 2969, and SRM 2970, and the results are summarized in Table 4 and plotted in Fig. 4. The results for the two SRMs used as controls from the two laboratories with the significant positive bias were also outside the uncertainties of the certified values based on NIST RMP measurements only ($n = 12$). The current COAs for SRM 2969 and SRM 2970 do not have assigned concentration values for $24,25(OH)_2D_3$. However, the certification report [46] provided values of 1.34 ± 0.02 nmol/L (± SD) (0.57 ± 0.01 ng/mL) and 1.70 ± 0.02 nmol/L (± SD) (0.73 ± 0.01 ng/mL), respectively, obtained from NIST RMP screening analyses ($n = 6$). The results from the four laboratories that provided results for SRM 2969 and SRM 2970 are summarized in Table 4 and plotted in Figure S5 (ESM). Consensus values for $24,25(OH)_2D_3$ in SRM 2969

Table 2 Mean concentration of $24,25(OH)_2D_3$ in 50 single-donor samples as determined by five laboratories and compared with results using the NIST RMP

Single-donor sample no.[a]	NIST RMP		CDC			Imperial College			Univ. College Cork			Univ. of Liège			Univ. of Washington		
	Concentration (nmol/L)																
	Mean[b]	SD[c]	Mean[d]	SD	% bias	Mean[d]	SD	% bias	Mean[d]	SD	% bias	Mean[d]	SD	% bias	Mean[d]	SD	% bias
9	0.48	0.01	0.455	0.020	−5.0	0.65	0.18	36.0	< 0.65	-	0.0	< 0.62	-	-	0.640[f]	0.012	33.6
23	0.53	0.03	0.492	0.009	−6.9	0.80	0.05	50.8	< 0.65	-	-	< 0.62	-	-	0.647[f]	0.070	22.7
34	0.58	0.04	0.606	0.006	5.3	0.63	0.03	8.6	< 0.65	-	-	0.65	0.14	12.6	0.608[f]	0.146	5.7
3	0.65	0.03	0.605	0.076	−6.6	0.90	0.05	39.3	< 0.65	-	-	0.767	0.059	18.6	0.761[f]	0.070	17.7
39	0.72	0.03	0.730	0.006	0.8	0.90	0.09	24.3	< 0.65	-	-	0.738	0.086	2.0	0.763[f]	0.088	5.4
2	0.76	0.02	0.751	0.016	−1.1	0.94	0.04	24.1	< 0.65	-	-	0.933	0.057	23.0	0.846[f]	0.019	11.5
22	0.78	0.03	0.703	0.023	−9.4	0.96	0.01	23.4	< 0.65	-	-	0.776	0.024	−0.1	0.874[f]	0.188	12.7
32	0.78	0.02	0.801	0.008	2.9	0.96	0.02	24.0	< 0.65	-	-	0.95	0.03	22.4	0.989[f]	0.010	27.2
1	0.89	0.03	0.900	0.033	0.8	1.34	0.30	50.1	< 0.65	-	-	1.03	0.10	15.2	1.04[f]	0.06	16.0
31	1.29	0.03	1.03	0.06	−20.2	1.40	0.10	8.5	1.23	0.01	−4.4	0.97	0.02	−24.7	1.06[f]	0.02	−18.1
18	1.38	0.02	1.42	0.02	3.6	1.72	0.02	24.7	1.35	0.17	−2.2	1.48	0.01	8.0	1.38	0.07	0.3
24	1.53	0.03	1.32	0.08	−13.8	1.69	0.09	10.1	< 0.65	-	-	1.51	0.09	−1.4	1.52	0.23	−0.6
17	1.59	0.03	1.47	0.14	−7.4	1.98	0.04	24.4	1.59	0.02	−0.1	1.59	0.11	−0.1	1.51	0.01	−5.0
26	1.79	0.01	1.72	0.01	−3.7	1.77	0.09	−1.2	1.94	0.19	8.8	1.90	0.05	6.3	1.94	0.11	8.8
48	1.84	0.02	1.92	0.05	4.2	2.51	0.10	36.6	1.52	0.34	−17.4	1.91	0.02	4.0	1.88	0.13	2.2
42	1.87	0.03	1.72	0.08	−8.2	2.22	0.02	18.5	1.45	0.24	−22.5	1.83	0.02	−2.0	1.89	0.19	1.2
43	2.50	0.01	2.62	0.16	4.5	3.23	0.10	29.0	2.08	0.07	−16.7	2.60	0.02	4.0	3.05	0.08	22.0
40	2.79	0.04	2.82	0.01	1.1	3.51	0.00	25.9	2.43	0.10	−12.9	2.87	0.09	3.0	3.04	0.42	8.9
25	2.93	0.05	2.88	0.01	−1.7	3.79	0.08	29.0	2.63	0.24	−10.5	2.99	0.04	2.0	2.94	0.26	0.2
14	2.94	0.01	3.00	0.01	2.3	3.89	0.05	32.2	3.39	0.24	15.4	3.14	0.07	7.0	3.05	0.48	3.8
28	3.00	0.04	2.67	0.13	−10.9	3.48	0.07	16.1	2.61	0.11	−13.0	2.46	0.20	−17.8	2.75	0.17	−8.2
29	3.11	0.02	3.12	0.04	−0.2	3.74	0.21	20.2	3.29	1.29	5.6	3.23	0.04	3.8	3.06	0.22	−1.5
33	3.43	0.05	3.30	0.04	−4.0	4.24	0.05	23.4	4.60	2.20	34.1	3.63	0.13	5.9	3.58	0.14	4.4
45	3.64	0.01	3.44	0.01	−5.7	4.30	0.01	18.0	4.15	0.70	13.8	3.88	0.07	6.5	3.78	0.10	3.6
35	3.70	0.05	3.95	0.07	6.9	4.91	0.01	32.7	4.17	0.98	12.9	4.24	0.07	14.7	4.01	0.03	8.4
20	4.18	0.03	4.14	0.10	−0.8	5.15	0.03	23.4	4.63	0.49	10.8	4.12	0.22	−1.3	4.29	0.36	2.6
21	4.50	0.04	4.45	0.01	−1.2	5.45	0.19	20.9	4.52	1.36	0.5	4.41	0.12	−2.1	4.48	0.08	−0.6
16	4.54	0.08	3.96	0.06	−12.8	4.91	0.04	8.1	5.19	0.18	14.3	3.98	0.17	−12.2	4.02	0.13	−11.4
46	5.12	0.03	4.94	0.08	−3.5	5.93	0.09	15.6	6.33	0.80	23.5	4.72	0.21	−7.9	5.14	0.08	0.3
41	6.01	0.05	5.44	0.09	−9.4	6.44	0.06	7.1	6.82	0.60	13.4	5.54	0.13	−7.9	6.12	1.79	1.8
36	6.03	0.14	6.04	0.06	0.2	7.09	0.07	17.5	8.12	0.75	34.5	6.18	0.13	2.5	6.32	0.58	4.7
15	6.06	0.07	6.41	0.08	5.8	7.51	0.11	23.8	7.38	0.05	21.7	5.90	0.31	−2.6	6.12	0.04	1.0
7	6.32	0.07	6.16	0.08	−2.6	7.71	0.04	22.0	5.99	0.28	−5.2	6.39	0.12	1.1	6.83	0.44	8.1
8	6.47	0.08	6.72	0.15	3.9	7.77	0.33	20.0	8.06	1.34	24.6	6.83	0.10	5.6	8.42	1.75	30.1

Table 2 (continued)

Single-donor sample no.[a]	NIST RMP		CDC			Imperial College			Univ. College Cork			Univ. of Liège			Univ. of Washington		
	Concentration (nmol/L)																
	Mean[b]	SD[c]	Mean[d]	SD	% bias	Mean[d]	SD	% bias	Mean[d]	SD	% bias	Mean[d]	SD	% bias	Mean[d]	SD	% bias
30	6.90	0.08	6.81	0.30	−1.3	8.25	0.06	19.5	6.38	0.44	−7.6	6.88	0.12	−0.4	6.39	0.64	−7.5
13	6.95	0.12	6.97	0.01	0.3	8.09	0.04	16.5	8.63	0.80	24.2	7.39	0.18	6.4	6.21	0.44	−10.7
50	6.95	0.05	6.30	0.30	−9.4	7.84	0.09	12.8	8.18	0.70	17.8	6.84	0.08	−1.5	6.92	0.18	−0.4
5	7.03	0.07	6.96	0.02	−0.9	8.43	0.01	19.8	7.75	0.86	10.2	6.94	0.53	−1.2	7.16	0.44	1.8
11	7.24	0.15	7.36	0.02	1.6	8.58	0.05	18.4	8.79	1.21	21.4	6.84	0.04	−5.6	7.46	0.44	3.1
12	7.30	0.16	6.91	0.01	−5.4	8.46	0.38	15.8	7.70	1.04	5.4	7.05	0.22	−3.5	6.62	0.25	−9.4
27	7.52	0.06	7.60	0.34	1.1	9.02	0.17	20.0	8.89	-	18.3	7.46	0.07	−0.7	7.13	0.23	−5.1
19	8.69	0.07	8.47	0.10	−2.6	10.35	0.07	19.1	9.22	1.06	6.1	8.65	0.19	−0.5	8.76	0.01	0.7
49	9.20	0.18	9.01	0.18	−2.0	11.05	0.07	20.2	10.34	2.25	12.5	9.17	0.35	−0.3	9.54	0.38	−3.7
10	9.26	0.07	9.44	0.08	2.0	11.20	0.14	21.0	11.27	0.65	21.7	9.46	0.09	2.1	9.59	0.10	3.6
44	9.41	0.13	9.28	0.11	−1.4	11.25	0.07	19.6	9.80	0.78	4.1	9.66	0.02	2.7	9.34	0.56	−0.7
6	10.9	0.1	11.25	0.1	3.0	13.05	0.21	19.5	13.42	2.83	22.8	11.6	0.4	6.5	11.6	0.3	5.9
38	12.4	0.2	12.0	0.2	−3.1	14.40	0.14	15.8	14.89	0.74	19.8	12.3	0.9	−0.7	11.8	0.1	−5.4
37	13.8	0.1	14.2	0.6	2.8	16.15	0.07	16.9	16.88	2.60	22.2	14.6	0.3	5.6	13.6	0.2	−1.5
4	14.4	0.1	13.25	0.2	−7.9	16.05	0.07	11.6	16.41	1.40	14.1	12.8	0.7	−10.8	13.0	0.8	−9.3
47	15.2	0.2	14.8	0.6	−2.4	17.85	0.21	17.7	17.84	0.12	17.6	16.1	0.6	5.9	15.0	0.3	−1.0
% bias[g]					**−2.4**			**21.4**			**9.0**			**1.9**			**3.8**
SD[h]					**5.6**			**9.8**			**14.3**			**8.9**			**10.5**
|% bias|[i]					**4.5**			**21.5**			**14.6**			**6.3**			**7.6**
|SD|[j]					**4.0**			**9.6**			**8.3**			**6.5**			**8.1**

[a]Samples ordered from lowest to highest concentration of $24,25(OH)_2D_3$; sample numbers are listed in the analysis order

[b]Mean of duplicate sample preparations ($n = 2$), each with duplicate injections

[c]Standard deviation of the measurements

[d]Mean of duplicate sample preparations ($n = 2$), each with one injection

[e]Percent bias compared to NIST RMP results, i.e., % bias = 100 × (Lab results − NIST result)/NIST result

[f]U of WA reported this result even though it was less than the LoD of 1.15 nmol/L

[g]Mean percent bias for all 50 single-donor samples; when results were listed as below the LoD (a dash in the table cell), they were not included in the bias assessment

[h]SD of mean bias

[i]Absolute percent bias for all 50 single-donor samples

[j]SD of absolute mean percent bias

Cells containing a dash indicate no calculated SD or bias because the results were reported as below the LoD

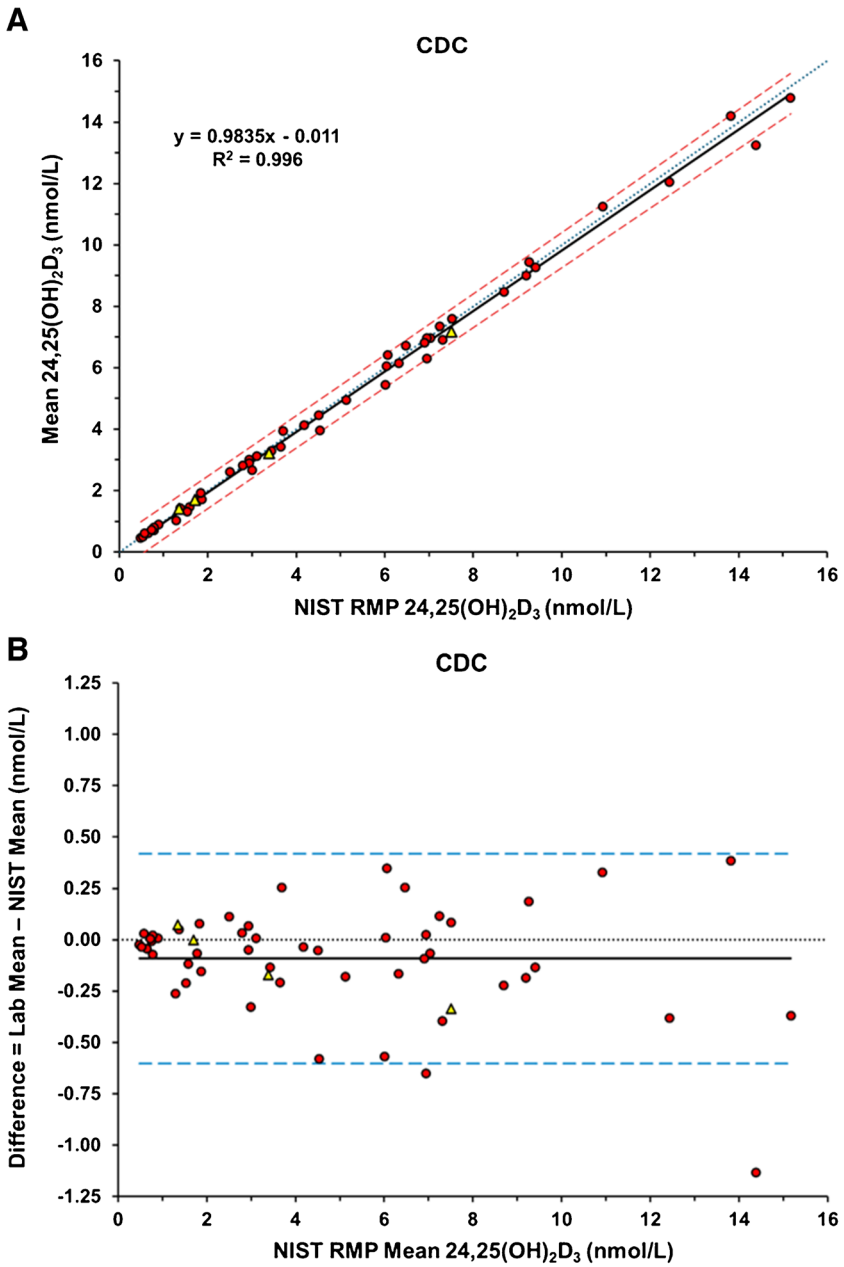


Fig. 1 **A** Ordinary Deming regression analysis for CDC versus NIST RMP results for the determination of $24,25(OH)_2D_3$ in 50 single-donor samples and four SRMs. Red circles are the single-donor samples and yellow triangles are the SRMs. Black solid line is the Ordinary Deming regression line; blue dotted line is the identity line ($x = y$), and red dashed lines are the 95% prediction interval. **B** Bland-Altman plot showing the difference in the laboratory mean and the NIST mean for the 50 single-donor samples and the four SRMs. Red circles are the single-donor samples and yellow triangles are the SRMs. Black solid line is the mean bias, blue dashed lines are the 95% confidence interval, and the black dotted line is the identity line ($x = y$)

and SRM 2970 based on the results of this study, including the NIST values, are 1.41 nmol/L ± 0.18 nmol/L (SD) and 1.79 nmol/L ± 0.24 nmol/L (SD), respectively, which could be considered as more robust target values for $24,25(OH)_2D_3$ in these two SRMs compared to the initial NIST RMP screening measurements [46].

Comparison with previous $24,25(OH)_2D_3$ intercomparison study

In the 2017 intercomparison study, 30 DEQAS samples (which were pooled serum samples rather than single-donor samples) and five SRM levels (SRM 972a and SRM 2973) were evaluated among five laboratories and NIST. Three of the laboratories in the current intercomparison study participated in this earlier comparison study, i.e., ICHT, UCC, and U of WA. The Ordinary Deming regression (Figure S3) and Bland-Altman (Figure S4) evaluation of the 2017 results for these three laboratories are summarized in Table S1 (ESM). Bland-Altman evaluation of the results from the previous study indicates that all three laboratories had a positive mean bias and an increasing trend in the difference plots in 2017 (Figure S4, ESM). In comparison, Bland-Altman evaluation of the current study results indicates an improvement for two of the three laboratories (ICHT and U of WA) based on a reduction in mean difference (0.445 to 0.162 nmol/L and

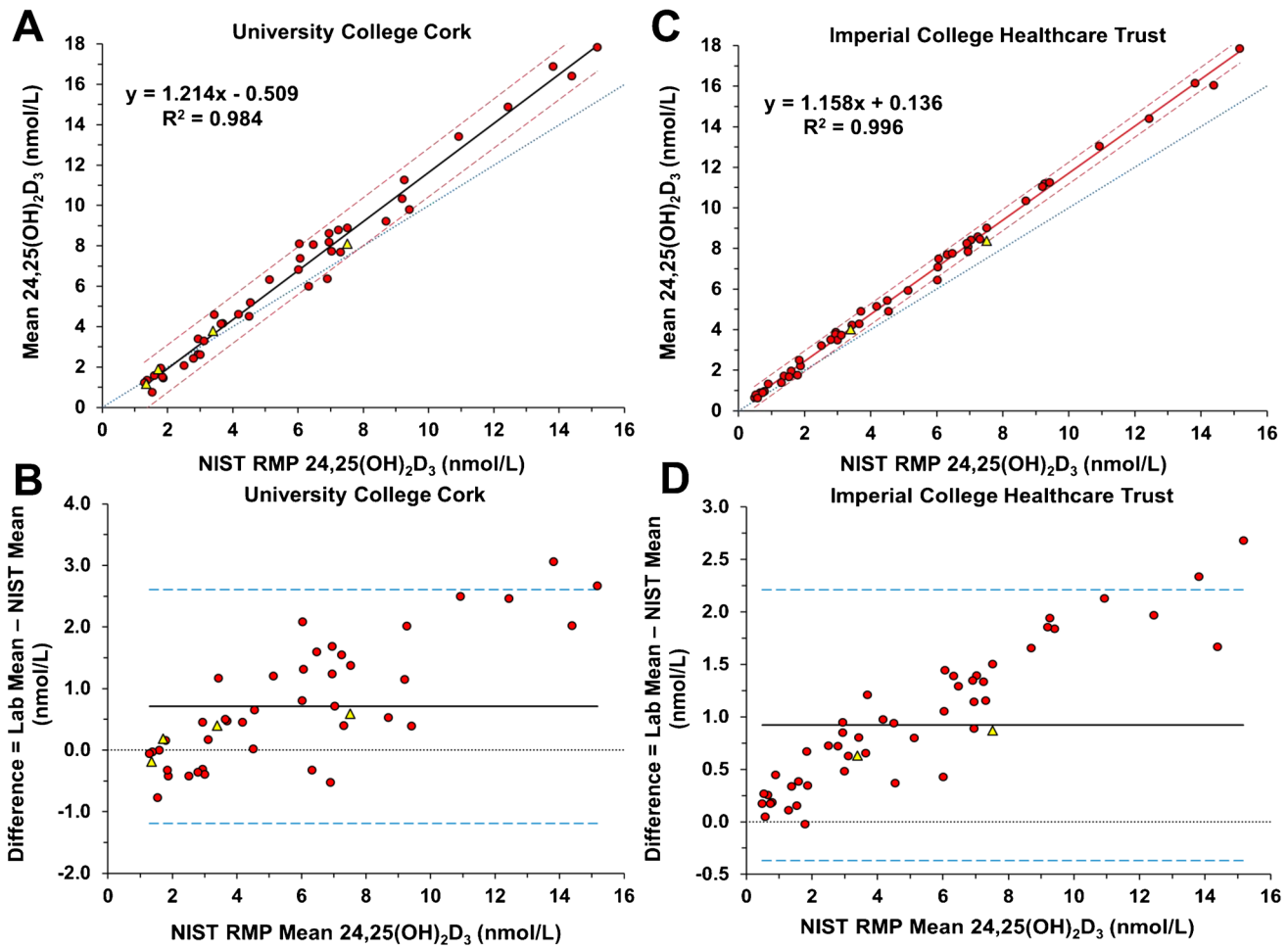


Fig. 2 Ordinary Deming regression analysis for University College Cork (**A**) and Imperial College Healthcare NHS Trust (**C**) versus NIST RMP results for the determination of $24,25(OH)_2D_3$ in 50 single-donor samples and four SRMs. Red circles are the single-donor samples and yellow triangles are the SRMs. Black solid line is the Ordinary Deming regression line; blue dotted line is the identity line ($x=y$), and red dashed lines are the 95% prediction interval. Bland-Altman plots showing the difference in the laboratory mean and the NIST mean for the 50 single-donor samples and the four SRMs for University College Cork (**B**) and Imperial College Healthcare NHS Trust (**D**). Red circles are the single-donor samples and yellow triangles are the SRMs. Black solid line is the mean bias; blue dashed lines are the 95% confidence interval, and the black dotted line is the identity line ($x=y$)

0.298 to 0.035 nmol/L, respectively) with UCC increasing only slightly (0.615 to 0.708 nmol/L). In the current study, the U of WA did not show an increasing trend in the Bland-Altman plot as observed in the previous study while ICHT and UCC retained the increasing trend in their difference plots. In addition, ICHT lowered the LoD for their method in the current study compared to the method used in the previous study.

Assignment of values for $24,25(OH)_2D_3$ for DEQAS

DEQAS currently provides one of the most widely recognized quality assessment schemes for serum vitamin D metabolites [37]. DEQAS has included measurements of $24,25(OH)_2D_3$ in its quarterly distributions for determining 25(OH)D since 2015 and provides a consensus mean value based on a limited number of participants (currently 11 laboratories) using LC-MS/MS. Since October 2018, DEQAS has also provided an "information only" value for $24,25(OH)_2D_3$ based on results from the CDC routine LC-MS/MS method used in this study. Each information value from the CDC is a mean of four independent measurements (duplicates analyzed in two runs). Typically, in each run at CDC, at least five quality assurance materials are prepared along with the DEQAS unknowns, i.e., one level of SRM 972a, three levels of internal quality control (QC) material, and one or two levels of internal blind QC material. The in-house QC materials are all characterized

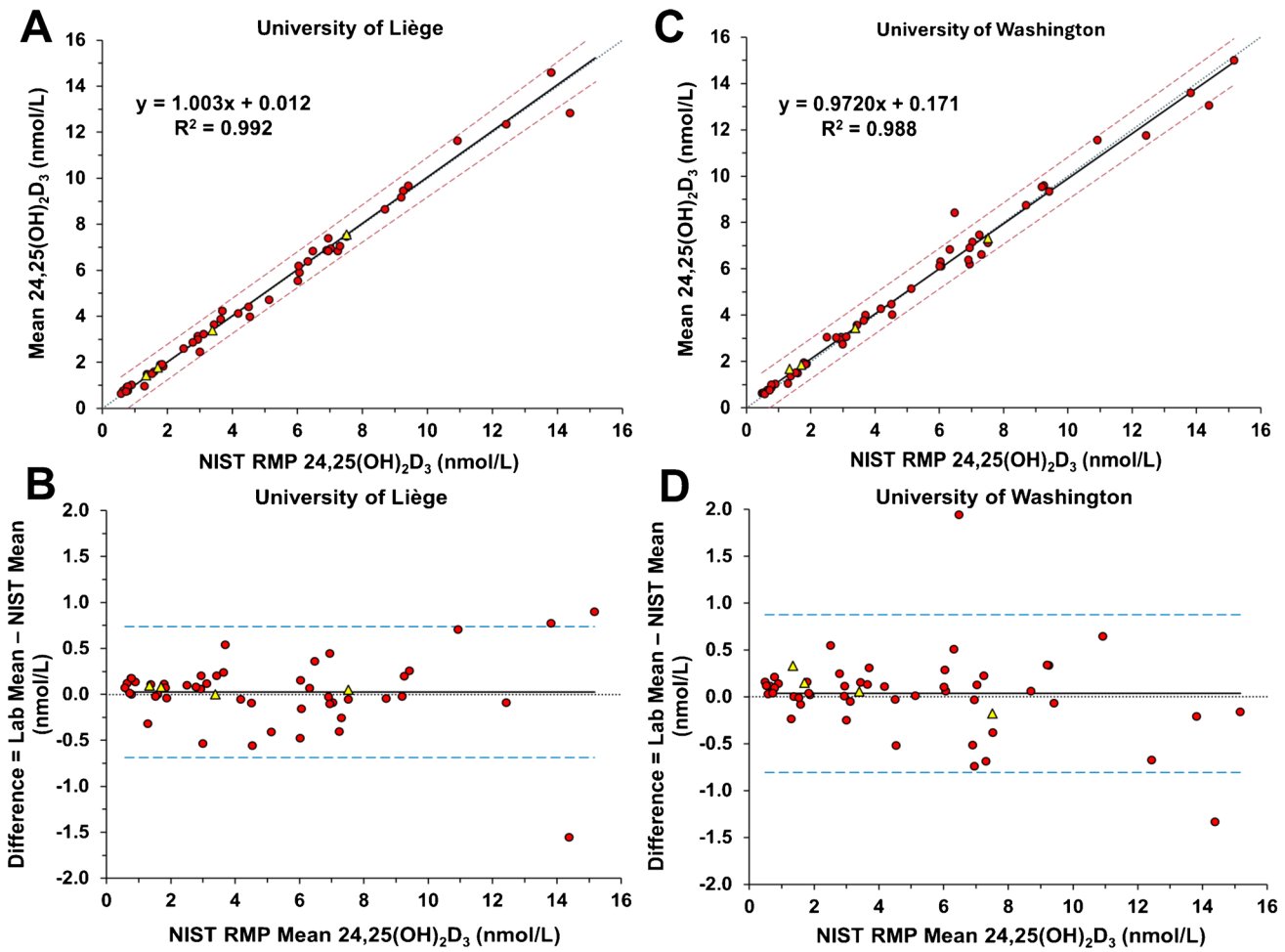


Fig. 3 Ordinary Deming regression analysis for University of Liège (**A**) and University of Washington (**C**) versus NIST RMP results for the determination of $24,25(OH)_2D_3$ in 50 single-donor samples and four SRMs. Red circles are the single-donor samples and yellow triangles are the SRMs. Black solid line is the Ordinary Deming regression line; blue dotted line is the identity line ($x=y$), and red dashed lines are the 95% prediction interval. Bland-Altman plots showing the difference in the laboratory mean and the NIST mean for the 50 single-donor samples and the four SRMs for University of Liège (**B**) and University of Washington (**D**). Red circles are the single-donor samples and yellow triangles are the SRMs. Black solid line is the mean bias, blue dashed lines are the 95% confidence interval, and the black dotted line is the identity line ($x=y$)

Table 3 Ordinary Deming regression and Bland-Altman mean difference of laboratory results compared to NIST RMP results for 50 single-donor samples and SRM samples

Laboratory	Ordinary Deming regression						Bland-Altman[b]			
	Regression line			Prediction interval (PI) $24,25(OH)_2D_3$ (nmol/L)[a]			$24,25(OH)_2D_3$ (nmol/L)			
	Slope	Int	R^2	Min	Max	Width	Mean difference	Lower 95% LoA	Upper 95% LoA	Width
CDC	0.984	−0.011	0.996	−0.508	0.485	0.993	−0.092	−0.603	0.419	1.022
Imperial College Healthcare	1.158	0.136	0.996	−0.352	0.624	0.976	0.162	−0.268	0.592	0.860
University College Cork	1.214	−0.509	0.984	−1.620	0.602	2.222	0.708	−1.189	2.605	3.794
University of Liège	1.003	0.013	0.992	−0.716	0.742	1.458	0.025	−0.687	0.738	1.425
University of Washington	0.972	0.171	0.988	−0.636	0.978	1.614	0.035	−0.806	0.877	1.683

[a]Min, minimum value at which $x=0$ on the y-scale; *Max*, maximum value at which $x=0$ on y-scale; *width*, distance from Min to Max

[b]*LoA*, 95% limits of agreement; *width*, distance from lower to upper LoA on y-axis

Table 4 Concentrations of $24,25(OH)_2D_3$ in SRMs as determined by five laboratories and compared with measurements using the NIST RMP

	SRM 2969		SRM 2970		SRM 972a L2		SRM 2973		Mean bias[e]
	nmol/L								
	Mean[b]	SD[b]	Mean[b]	SD[b]	Mean[c]	Unc[c]	Mean[c]	Unc[c]	
NIST target value[a]	1.34	0.02	1.70	0.03[b]	3.39	0.12	7.51	0.26	
Laboratory results	**Mean[d]**	**SD[d]**	**Mean[d]**	**SD[d]**	**Mean[d]**	**SD[d]**	**Mean[d]**	**SD[d]**	
CDC	1.42	0.02	1.70	0.03	3.22	0.01	7.18	0.20	
% bias[f]	5.4	-	0.0	-	−5.0	-	−4.4	-	1.0%
Imperial College	NA	-	NA	-	4.02	0.07	8.38	0.10	-
% bias[f]	NA	-	NA	-	18.5	-	11.5	-	15%
Univ. College Cork	1.16	0.25	1.89	0.61	3.79	0.18	8.10	0.64	-
% bias[f]	−13.6	-	10.9	-	11.7	-	7.9	-	4.2%
Univ. of Liege	1.44	0.10	1.78	0.08	3.39	0.09	7.56	0.09	-
% bias[f]	7.1	-	4.7	-	0.0	-	0.6	-	3.1%
Univ. of Washington	1.67	0.06	1.85	0.06	3.44	0.20	7.33	0.20	-
% bias[f]	24.6	-	8.8	-	1.6	-	−1.6	-	8.3%
Mean all lab results[g]	1.42[h]	-	1.81[h]	-	3.57	-	7.71	-	-
SD of lab means	0.21	-	0.08	-	0.33	-	0.51	-	-
% bias of mean[i]	5.5	-	5.7	-	5.3%	-	2.7%	-	4.0%
Mean incl. NIST[j]	**1.41**	-	**1.79**	-	**3.54**	-	**7.68**	-	-
SD[j]	**0.18**	-	**0.24**	-	**0.30**	-	**0.46**	-	-

[a]NIST target values for SRM 2969 and SRM 2979 are measurements using the RMP ($n=6$) and for SRM 972a L2 and SRM 2973 are the certified values from the Certificate of Analysis (COA), which are based solely on measurements from the NIST RMP ($n=12$)

[b]Mean and standard deviation (SD) of the measurements for SRM 2969 and SRM 2970 based on six measurements

[c]Mean is the certified value and uncertainty (Unc) for SRM 972a and SRM 2973 based on 12 measurements; uncertainty is the value associated with the certified values from the COA

[d]Mean and SD for SRM 2969 and 2970 are based on three measurements; mean and SD for SRM 972a L2 and SRM 2973 are based on four measurements

[e]Mean percent bias of measurements compared to the NIST target values for the four SRMs

[f]Percent bias compared to the NIST RMP, i.e., % bias = 100 × (Lab result − NIST result)/NIST result

[g]Mean of the means of results from all laboratories

[h]Mean of the results from four laboratories, i.e., excluding Imperial College Healthcare NHS Trust, which did not analyze SRM 2969 and SRM 2970

[i]Percent bias of the mean of results from all laboratories providing results compared to the NIST RMP

[j]Mean and SD of the results from all laboratories providing results plus the NIST RMP value

NA, samples not analyzed

Dash in cell indicates no data or calculation intended in the cell

in parallel with SRM 972a. Researchers have used DEQAS samples for $24,25(OH)_2D_3$ method validation, comparing their results with the CDC information values. Zelzer et al. [20, 41] used 20 DEQAS samples (nos. 551 to 570 and nos. 571 to 590) in two different studies, and Alshabrawy et al. [40] used five samples (nos. 546 to 550), comparing their results to the $24,25(OH)_2D_3$ information values.

This current interlaboratory comparison study provides a direct comparison of the NIST RMP with the routine CDC method used to assign the DEQAS $24,25(OH)_2D_3$ information values. The CDC method and the NIST RMP are in good agreement as demonstrated by linear regression analysis (slope = 0.984; $R^2 = 0.996$) and the Bland-Altman analysis (–0.09 nmol/L mean bias) (Fig. 1 and Table 3) and 4.5% absolute bias (Table 2) for the 50 single-donor samples. Based on the agreement of the two methods, the CDC information values for $24,25(OH)_2D_3$ for DEQAS can be considered reliable and accurate offering a better measure of accuracy and a guide to a likely target value compared to the consensus mean from the participating laboratories, which can be impacted by the observed variability among the laboratory results.

Use of SRMs to improve $24,25(OH)_2D_3$ measurements

Certified values for $24,25(OH)_2D_3$ were assigned to SRM 972a and SRM 2973 in 2017 based on measurements using

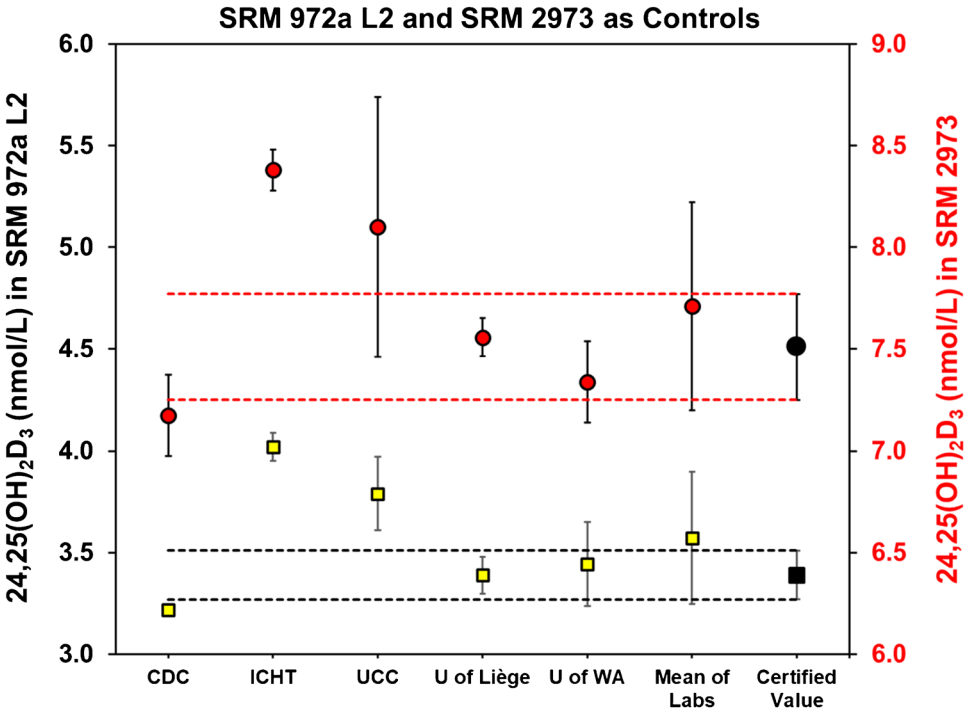


Fig. 4 Comparison of results from five laboratories for determination of $24,25(OH)_2D_3$ in SRM 972a L2 and SRM 2973 used as control materials. Yellow squares are the results for SRM 972a L2 and red circles are results for SRM 2973. The certified values as reported on the Certificate of Analysis for SRM 2973 and SRM 972a L2 are represented by the black circle and black square, respectively. Error bars on the laboratory results and laboratory means are ± SD; error bars on the NIST RMP value represent the uncertainty associated with the certified value reported on the Certificate of Analysis, which was determined based on measurements using the NIST RMP ($n = 12$). Mean of Labs ($n = 5$) is the mean of results for all five laboratories

the NIST RMP. Initially in 2015, the values assigned for $24,25(OH)_2D_3$ in SRM 972a were denoted as reference values prior to recognition of the NIST method as an RMP by the JCTLM in 2017. In the past decade, these commercially available SRMs with assigned $24,25(OH)_2D_3$ values have been used by various researchers for validating $24,25(OH)_2D_3$ methods [5, 14, 16, 17, 27, 53, 54] as summarized in Table S2 (ESM). CDC's ID LC-MS/MS method for $24,25(OH)_2D_3$, employed in this study, also used SRM 972a for accuracy assessment during method validation. They observed a mean bias (three independent measurements over multiple days) of −3.1%, −2.9%, −2.2%, and −1.9% for $24,25(OH)_2D_3$ in SRM 972a levels 1 through 4, respectively (overall mean bias of −2.5%). Recently, Kandler et al. [27] developed candidate RMPs for $24,25(OH)_2D_2$ and $24,25(OH)_2D_3$ based on an ID LC-MS/MS method. The candidate RMP for $24,25(OH)_2D_3$ was validated using SRM 972a (Levels 1 and 2) and a comparison with 31 samples supplied by the CDC VDSCP with values determined using the CDC ID LC-MS/MS method. Using SRM 972a (Levels 1 and 2), the candidate RMP for $24,25(OH)_2D_3$ had mean bias of −2.4% and −3.7%, respectively.

Conclusions

The LC-MS/MS methods reported in this study represent methods that focus only on $24,25(OH)_2D_3$ (RMP, CDC, and ICHT) and methods that measure multiple vitamin D metabolites including $24,25(OH)_2D_3$ (UCC, U of Liège, and U of WA). The results from three of the five participating laboratories in this study were in good agreement with the NIST RMP with absolute bias of 4.5 to 7.6% for 50 single-donor serum samples. Although two laboratories improved their results after in-house assessment and correction of the purity of their $24,25(OH)_2D_3$ calibrants, their results still had absolute bias of 14.6% and 21.5% in the analysis of the 50 serum samples. This study emphasizes the importance of using calibrants of known purity (e.g., CRMs or accurately assessed in-house materials). The agreement of the routine CDC method with the NIST RMP has positive implications for the CDC-assigned $24,25(OH)_2D_3$ information values for the DEQAS program.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-026-06480-8.

Author contribution S.A. Wise: conceptualization, data analysis and visualization, writing—original draft, review and editing; E. Mineva: conceptualization, methodology/investigation, writing, review and editing; C.M. Pfeiffer and H. Vesper: conceptualization, review and editing; E. Cavalier, K.D. Cashman, A.N. Hoofnagle, and E.L. Williams: supervision, writing, review and editing; J.E. Camara: conceptualization, provision of materials/samples, review and editing; S. Peeters, K. Galvin, L.E. Briggs, M.E. Souness, and G. Hahm: methodology/investigation, review and editing; A. Kuszak: conceptualization, review and editing. All authors read and approved the manuscript.

Data availability All data related to this paper are contained in either the paper or the Electronic Supplementary Material (ESM).

Declarations

Ethics approval The National Institute of Standards and Technology Research Protections Office reviewed the protocol for this project and determined it is "not human subjects research" as defined in 15 CFR 27, the Common Rule for the Protection of Human Subjects.

Conflict of interest The authors declare no competing interests.

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