In their article in the current issue of Clinical Chemistry, Farrell et al. (1) describe the latest in a number of studies (2, 3) highlighting the method-related variability in serum 25-hydroxyvitamin D (25-OHD) results. There is common agreement that 25-OHD is a "difficult" analyte. That quality is generally ascribed to its hydrophobic nature, its existence in several different molecular forms, its tight binding to vitamin D–binding protein (VDBP) and, until recently, the absence of reference materials or a reference measurement procedure (RMP) against which assays could be standardized. This last problem was mitigated to some extent with the introduction in 2009 of the NIST Standard Reference Materials (SRM 972 and SRM 2972) and the acceptance of the NIST and University of Ghent liquid chromatography–tandem mass spectrometry (LC-MS/MS) assays as RMPs (4). Unfortunately, 3 of the 4 SRM 972 reference materials are either spiked with exogenous metabolites or diluted with equine serum, characteristics that render these reference materials unsuitable for many immunoassays (5, 6).

The history of 25-OHD methodology could serve as a case study of the consequences of transferring a rigorous but labor-intensive method from the unhurried atmosphere of the research laboratory to the bustle of a routine clinical laboratory having to meet tight deadlines. The pioneering competitive protein-binding (CPB) method of Haddad and Chyu (7) involved solvent extraction and chromatography, with the results of every sample corrected for procedural losses. Subsequent attempts at simplifying a CPB method by omitting the chromatography stage proved unsuccessful (8), and an automated version (Nichols Advantage) introduced in 2004 was withdrawn in 2006. The successive abandonment of sample extraction, chromatography, and correction for procedural losses in immunoassays has undoubtedly contributed to the inconsistencies reported by Farrell et al., as have differences in assay standardization. Nevertheless, results submitted to the international Vitamin D External Quality Assessment Scheme (DEQAS) have shown a gradual reduction in interlaboratory imprecision (CV) in recent years—from >30% in 1995 to 15% in 2011. This reduction may be due to the increasing dominance of automated immunoassays, in particular the DiaSorin LIAISON Total method and, more recently, greater use of the NIST SRMs in standardization. Whatever the explanation, it appears that there is currently reasonable agreement, with 1 or 2 notable exceptions, between most 25-OHD methods (9). Nevertheless, the fact that a change of method can affect the continuity of results has implications for the monitoring of patients on vitamin D supplements and for long-term epidemiologic studies. Even within the same method, the consistency of results can be compromised by subtle lot-to-lot changes in reagent kits or more overtly by a change of antibody or in a reformulation of reagents.

With the introduction of the RMPs and the expected release in 2012 of a new generation of human serum–based SRMs, assay standardization should become less of an issue, and attention is likely to focus on the reliability of individual results, a far more intractable problem. The use of summary statistics in method comparisons, particularly the correlation coefficient, and linear regression equations can disguise a marked variance in the results given by 2 methods for the same sample. Method differences are best revealed by the use of Bland–Altman plots, which allow discrepancies between results for individual samples to be seen at a glance. These apparently random errors undermine the confidence in the veracity of all 25-OHD results submitted to the international Vitamin D External Quality Assessment Scheme; 24,25-(OH)D, 24,25-dihydroxyvitamin D; 3-epi-25-OHD, 3-epi-25-hydroxyvitamin D. 

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2 Nonstandard abbreviations: 25-OHD, 25-hydroxyvitamin D; VDBP, vitamin D–binding protein; RMP, reference measurement procedure; SRM, Standard Reference Material; LC-MS/MS, liquid chromatography–tandem mass spectrometry; CPB, competitive protein-binding (method); DEQAS, Vitamin D External Quality Assessment Scheme; 24,25-(OH)_{2}D, 24,25-dihydroxyvitamin D; 3-epi-25-OH_{2}D, 3-epi-25-hydroxyvitamin D.
susceptible to variations in VDBP concentration. Manufacturers have been coy about disclosing details of the separation step used in these assays, but most appear to involve the denaturation of VDBP via a pH change. Whatever the detail, there is an assumption—with little or no published evidence—that 100% of 25-OHD is displaced from VDBP or, if not, that the percentage displaced in different samples is consistent. The data presented in the report of Heijboer et al. suggest this is not the case. Four of the 5 nonextraction immunoassays compared with the reference LC-MS/MS method had a highly significant correlation of bias to the VDBP concentration. Moreover, the scatter of results indicates that the bias was far from consistent; this finding offers one explanation for the arbitrary nature of method differences with individual samples.

A recent and surprising twist in the debate on matrix effects was the report by Cavalier et al. (11) of interference in the DiaSorin LIAISON method by human antianimal antibodies. Interference from heterophilic antibodies is a problem more generally associated with noncompetitive immunoassays than with the competitive assays used to measure 25-OHD. When the affected samples were reassayed by the DiaSorin RIA method, the problem largely disappeared, presumably because of the initial protein-precipitation step used in this method. DiaSorin subsequently produced a modified version of the LIAISON method, which was apparently unaffected by heterophilic antibodies.

A limitation of the studies of Farrell et al. and Heijboer et al., which is explained by local prescribing conventions, is their failure to recruit more than a few individuals supplemented with vitamin D$_3$. Heijboer et al. acknowledge that the detection of 25-OHD$_3$ is method dependent [the Abbott ARCHITECT assay has only a 52% cross-reactivity with 25-OHD$_2$ (12)], and it is interesting to speculate whether VDBP, to which 25-OHD$_3$ binds less strongly than 25-OHD$_2$ (13), influences the detection of this metabolite in nonextraction assays.

Neither of these reports addresses the effects of interference from other hydroxylated vitamin D metabolites, such as 24,25-dihydroxyvitamin D [24,25-(OH)$_2$D] or 3-epi-25-hydroxyvitamin D$_3$ (3-epi-25-OHD$_3$). All immunoassays for 25-OHD show high cross-reactivity with 24,25-(OH)$_2$D, which can be present in serum at concentrations of up to about 12 nmol/L (14). A recent DEQAS investigation (9) has confirmed manufacturers’ assertions that 3-epi-25-OHD$_3$ is not detected by any of the widely used immunoassays, including the ones used in the Farrell et al. study. Interestingly, the Roche CPB method for total 25-OHD used in the investigations of Heijboer et al. did show significant cross-reactivity (approximately 57%) with the 3-epimer. Most LC-MS/MS and HPLC/UV assays did not resolve the 3-epimer. Although the amounts in adult serum are generally small, very high concentrations have been reported in some individuals (15). No mention is made of the 3-epimer in the report by Heijboer et al.

With the introduction of LC-MS/MS assays for 25-OHD came the opportunity for routine clinical laboratories to return to a more rigorous technique in which sample extraction, chromatography, and correction for procedural losses were restored and in which specificity was enhanced by the addition of mass spectrometry. LC-MS/MS is now widely used as the reference method in comparison studies such as the ones published in the current issue of this journal. Manufacturers are also beginning to use LC-MS/MS to calibrate their assay standards, and the perceived virtues of this technique are in danger of being exaggerated. In practice, the supposed specificity conferred by the MS/MS component is limited by its being an achiral technique, which by definition cannot distinguish between 25-OHD$_3$ and its epimer, 3-epi-25-OHD$_3$. Shah et al. (16) have demonstrated the need to chromatographically separate other isobaric substances, in particular 7-α-hydroxy-4-cholesten-3-one, an endogenous precursor of bile acids found in 4 of their 5 healthy volunteers. The authors claim that this substance would have inflated 25-OHD results by as much as 39% if not separated chromatographically, although there is no evidence of this degree of interference in routine LC-MS/MS assays. Failure to resolve the 3-epimer and other isobars, however, probably accounts for much of the persistent positive bias shown by “routine” LC-MS/MS methods in DEQAS and other method comparisons. Unfortunately, increasing the specificity of LC-MS/MS will necessitate longer chromatography run times, making the technique less suitable for the busy routine clinical laboratory. Nevertheless, LC-MS/MS is currently used by approximately 11% of DEQAS participants, and its popularity is likely to increase as the specificity problems are addressed.

The consequences of systemic differences between 25-OHD assays are not confined to clinical laboratories. Epidemiologic studies are an important source of normative 25-OHD data, but differences in methods used by national surveys have limited the extent to which results could be pooled. Recognizing the advantages to be gained from creating an international database, the US Office of Dietary Supplements (NIH) has organized a project (the Vitamin D Standardization Program), the goal of which is to standardize measurements done in national health surveys to those of the RMPs developed by NIST and the University of Ghent. It also allows for participation of clinical, public health, research, and commercial organizations in the standardization effort (17).

In summary, the studies of Farrell et al. and Heijboer et al. (1, 10) have identified general and specific problems
in measuring 25-OH D. The standardization of 25-OH D assays is slowly being addressed, but the elimination of matrix effects, of which VDBP is an example, will require the continual vigilance of analysts and clinicians and an ongoing dialogue with manufacturers.

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