a 12% decrease and a 4% increase at day 7 compared with day 0. Consistent with the RT-qPCR data, the RL sample showed the biggest decrease, with 47% and 0% increases at day 7 and day 0, respectively. The SL sample showed a 6% decrease and a 23% increase. RPS showed the minimum change in mRNA, with a 3% decrease and <1% increase [data not shown but were in part published in (3)]. The decrease in mRNA signal is likely due to mRNA degradation, and the increase in the signal may be due to gene induction in desquamated oral epithelial cells in the saliva. Such a gene induction phenomenon has been observed in whole blood incubated at room temperature (4). Together these data suggest that RPS can best stabilize salivary mRNAs at room temperature for up to 7 days with minimum change in RNA level and complexity.

To further test the stability of salivary RNA in RPS-incubated samples, we performed an extensive time course analysis at room temperature for up to 12 weeks. We used saliva without any preservation, incubated for the same periods of time as a control. The results of RT-qPCR for β-actin mRNA are shown in Fig. 1B. The control CT values increased after 2 weeks and remained high until the end of the time course. In the presence of RPS, however, there was no increase in the CT values for up to 12 weeks. Throughout the time course, we detected significant differences in CT values between these 2 sets of samples (overall P <0.001) at all time points. We observed similar RT-qPCR results for interleukin-8 mRNA (Fig. 1C). Together these data suggest that RPS can stabilize salivary RNA at room temperature for up to 12 weeks without significant degradation.

The ability to promptly stabilize and preserve salivary RNA on collection at room temperature is of particular clinical and practical relevance. Room-temperature compatibility allows sample collection, stabilization, and transportation without specialized conditions (4°C, −20°C or −80°C), permitting saliva samples to be collected in RPS anywhere in the world at ambient temperatures and mailed into a central reference facility for analyses. This capability is an important first step toward the use of saliva as a clinical biofluid for screening diseases such as oral cancer.

In conclusion, our data demonstrate that RPS reagent can promptly stabilize the RNA in saliva at room temperature. This finding is important for the clinical application of salivary RNA as a diagnostic analyte.

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Measurement of 25-Hydroxyvitamin D Revisited

To the Editor:

A notable shortcoming of the HPLC method for measuring 25-hydroxyvitamin D described by Lensmeyer et al. (1) is its failure to adequately remove interference by the C3-epimer. Although the authors dismiss this because the interference was less than the lowest limit of quantification, this would not be true in some samples collected from children >1 year old, in which the concentration of this metabolite can be as high as 92 μg/L (2). The only methods demonstrated not to cross-react with the 3-epimer are a specifically modified liquid chromatography-tandem mass spectrometry (LC-MS/MS) method and the DiaSorin RIA assay (2).

The relatively poor correlation between HPLC and the DiaSorin RIA for 25-OH vitamin D reported by Lensmeyer et al. (1) contradicts observations by several investigators (2–7) of good agreement between both the DiaSorin RIA and LIAISON® assays and either HPLC or LC-MS/MS methods. Indeed, several groups have used the DiaSorin RIA as the reference method to validate HPLC or LC-MS/MS methods with good agreement (3–5). The study of assay variability by Binkley et al. (7) with a small number of samples demonstrated good agreement and no significant bias between the DiaSorin RIA and an HPLC method. Furthermore, results from the International Vitamin D External Quality Assessment Scheme confirm the accuracy of the DiaSorin RIA in measuring both D3 and D2 forms of 25 OH vitamin D (8). The DiaSorin RIA gave results for 3 samples containing primarily

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D3 that were very close to the all-laboratory trimmed mean, and although the Diasorin RIA method means for the 2 samples containing endogenous D2 were lower than that for HPLC, the difference was not statistically significant based on the high CV of the latter method.

Diasorin has recently incorporated a panel of serum samples with 25-hydroxyvitamin D values measured by LC-MS/MS into the routine QC testing for both the RIA and LIASON 25-OH vitamin D assays. The use of these samples assures that both methods will continue to have the good correlation to a reference method that was demonstrated by Fenske et al. (6).

The authors of the article cited above respond:

To the Editor:

Dr. Schmidt’s comments give the false impression that our recently published HPLC method (1) is unable to identify the presence of the C-3 epimer apart from 25(OH)D3 or 25(OH)D2. We routinely achieve levels of chromatographic resolution illustrated in Fig. 1. Chromatogram A displays elution of the C-3 epimer alone. Chromatogram B shows separation of 25(OH)D3 and its C-3 epimer, a scenario that would be observed for samples from infants <1 year old. Chromatogram C is from a patient receiving vitamin D2 as a supplement. Here the compounds are not baseline resolved, but each has a different retention time, and clearly they do not coelute. Most importantly, the C-3 epimer is not mistaken for 25(OH)D2. Our method has detected the C-3 epimer in nearly 25% of the average 650 samples we test each month. Concentrations of the C-3 epimer are usually <10 μg/L. Thus, we can analyze samples from infants <1 year of age (as well as older patients) without modifying the method. In contrast, Singh et al. (2) require 2 liquid chromatography tandem mass spectrometry (LC-MS/MS) procedures, one for samples from patients <1 year of age and another for patients >1 year. Singh et al. (3) have also reported that the C-3 epimer is “indistinguishable from 25(OH)D3 by most LC-MS/MS assays”. In contrast, our HPLC method is one of a small number that can effectively resolve the C-3 epimer from 25(OH)D3 and 25(OH)D2.

The integrity of our comparison data (HPLC vs Diasorin RIA) has also been brought into question by Dr. Schmidt. Interestingly, Diasorin, with whom Dr Schmidt is associated, generated the RIA data for our study. Binkley et al. (4) report that the reproducibility of the Diasorin RIA measurements is highly dependent on the laboratory in which the assay was performed. Furthermore, other investigators have compared Diasorin RIA vs HPLC (5) or LC-MS/MS (6–8). On close examination of these reports, inconsistencies appear in the interpretation of the statistical data, which often lack a regression plot of the data (5, 7, 8) or a statistic Sy/x, often called standard error of estimate, to describe the scatter.

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Fig. 1. Chromatograms. (A), elution of 25(OH)-epi-D3 (not extracted); (B), a serum extract containing 25(OH)D3 (40 μg/L) and 25(OH)-epi-D3 (30 μg/L); and (C), a serum extract containing 25(OH)D2 (69 μg/L), 25(OH)-epi-D2 (15 μg/L), and 25(OH)D2 (12 μg/L).