Original Research Article

Saliva Sampling Method Affects Performance of a Salivary α-Amylase Biosensor

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ABSTRACT: Objectives: Prompted by the discordance between a standardized salivary alpha-amylase (sAA) biosensor applied in clinical settings and a reference laboratory analyzer, we examined the impact of the saliva sampling method on the analytic performance of the biosensor.

Methods: Direct mouth readings using the biosensor from 31 normal, healthy volunteers were compared to biosensor and conventional assay readings obtained from saliva samples collected concurrently by passive drool and processed in three different ways (unprocessed, thawed, and thawed and centrifuged).

Results: The direct readings from the mouth showed consistently lower sAA values (Ps < 0.01) compared to all other combinations of sample processing and quantification platforms (biosensor vs. conventional assay). Readings obtained from passive drool saliva were strongly correlated with one another, and Bland–Altman plots of agreement indicated a smaller discrepancy between conventional and biosensor readings obtained with passive drool when compared to the direct mouth readings.

Conclusions: Our study indicates that the analytical performance of the sAA biosensor is influenced significantly by the saliva sampling method. In contrast to the relatively imprecise direct mouth measurements, biosensor sAA levels established indirectly from passive drool saliva samples provide more accurate estimation of sAA levels, even after intermediary processing steps (e.g., freezing, thawing, centrifugation). Am. J. Hum. Biol. 25:719–724, 2013.

Advances in biosensing technology have begun to realize the much-heralded potential of saliva for point-of-care assessment of the stress response (Shetty and Yamaguchi, 2010; Yamaguchi and Shetty, 2012). The capacity for extracting biometric information from readily accessible saliva presents exciting opportunities to study the psychobiology of the stress response with a precision, temporal and spatial resolution and convenience not afforded by conventional, laboratory-based approaches. Similar to personal blood glucose meters, the rapid reporting of stress-response indicators by point-of-care salivary biosensors could permit timely clinical decisions in the management of stress-related disorders and allow self-monitoring. The capacity for bringing the analysis closer to the patient or participant is particularly appealing for field researchers who frequently work under nonideal conditions and have to contend with multiple quality failure points and the expenses associated with the collection, storage and processing of saliva samples in distant, centralized laboratories.

In a previous article (Shetty et al., 2011), we described the developmental validation of a point-of-care, user-friendly, biosensor system for rapid measurement of salivary α-amylase (sAA)—a putative indicator of adrenergic activity (Nater and Rohleder, 2009). The sAA biosensor prototype comprises of disposable saliva collector strips and a hand-held reader with integrated analytic and reporting capabilities. Bioanalytical validation of the biosensor, using saliva samples from a group of healthy male volunteers, demonstrated a close correspondence with a conventional laboratory-based clinical chemistry analyzer (Olympus AU 400, Olympus America, Center Valley, PA). The excellent performance characteristics (accuracy, precision, repeatability) of the biosensor and the ability for a truncated sampling-reporting cycle (<1 min) suggested potential use in field studies to establish evidentiary linkages between sAA and clinical endpoints of stress reactions.

Building on the promising results of the validation study conducted in a laboratory setting (Shetty et al., 2011), we initiated a field study to examine the ecological validity of the sAA biosensor in real-world settings. Specifically, we used the biosensor to study the temporal relationships between sAA levels and clinical symptoms of traumatic stress in a cohort of 80 subjects recovering from severe physical injury (unpublished data). Surprisingly,

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Conflict of Interest: Author Yamaguchi is inventor of the technology for the direct measurement of salivary alpha-amylase. As employee of the University of Toyama (Japan) where the technology was developed, he has assigned to the university the corresponding patents (United States Patent 7,183,069 & 7186696) and will be entitled to share any royalties earned from licensing the patents. Beyond this, he does not have any financial relationships, stock, board membership or part ownership with any entity involved with the commercialization of the technology nor has he received any honoraria or consulting fees related to the product. All other authors declare that they have no conflict of interest.

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the biosensor-facilitated sAA measurements obtained directly from the mouth could not be corroborated by the analysis of whole saliva samples obtained concurrently by passive drool and analyzed subsequently by the laboratory-based clinical chemistry analyzer. Across saliva samples obtained at three different time points (~1, 3, and 5 weeks after the injury), the agreement between the two different measurement methods (R^2 range 0.06–0.23) was considerably less than established previously ($R^2 > 0.98$; Shetty et al., 2011).

Because the assay methods, Olympus analyzer as well as the sAA biosensor, are standardized, we suspected that the discordant findings derived from the saliva sampling methods and not the measurement techniques. Our precursor biosensor validation studies were performed under controlled conditions. Passive drool saliva samples were stored frozen at −20°C in the collection tubes and subsequently thawed and centrifuged and batch assayed in duplicate, using the collector strips and sAA biosensor as well as the Olympus analyzer. In contrast, the biosensor sAA levels in the trauma patients were established in naturalistic settings through direct reading from saliva samples obtained by placing the collector strips directly on the floor of the mouth. We therefore sought to examine the impact of the saliva collection method on the quantitative estimates of sAA concentration provided by the salivary biosensor. A secondary aim was to determine the extent to which the post-collection processing of saliva samples may contribute to measurement discrepancy.

METHODS

Experimental design

Unstimulated saliva samples and biosensor sAA readings were obtained from 31 healthy, adult student volunteers participating in a health psychology course or dental school at a large public university. Biosensor sAA reading was obtained directly from the mouth and a corresponding whole saliva sample was collected by passive drool into a 15 ml polypropylene tube. Prior consumption of food or beverages was not controlled. The order of biosensor reading and passive drool saliva was randomly counterbalanced between participants. The biosensor reading was collected using the conventional approach of allowing saliva to pool on the floor of the mouth and then placing the collector strip under the tongue (Direct—biosensor) for ~25 s in order to allow the tip to saturate with a fixed amount of saliva (≈25 μl).

Following passive drool saliva collection, 2 ml of the whole saliva was aliquoted into a 15-ml tube. A biosensor collector strip was dipped into the tube for 10 s and placed in the reader to obtain a reading (Unprocessed—biosensor) and the residual saliva and tube was discarded. An additional 1 ml was transferred to a microcentrifuge tube and delivered to the laboratory for assay (Unprocessed—conventional). The remaining whole saliva sample was frozen at −20°C. One week later, frozen samples were allowed to thaw on ice, a 2-ml aliquot of the whole saliva was placed into a 15-ml tube, tested with another biosensor collector strip (Thawed—biosensor) and an additional 1 ml aliquot was delivered to the laboratory for assay (Thawed—conventional assay). Finally, the remaining saliva sample was centrifuged (−4°C, 2,600 rpm, for 15 min), 2 ml transferred to a 15-ml tube, and retested with a biosensor collector strip (Thawed/centrifuged—biosensor), and an additional 1-ml aliquot was delivered to the laboratory for assay (Thawed/centrifuged—conventional assay). The thawed/centrifuged—conventional assay is considered the gold standard reading because it corresponds to standard saliva processing procedures. In our previous validation study, the thawed/centrifuged biosensor readings had a high correlation ($R^2 > 0.98$) with the gold standard readings from the clinical chemistry analyzer (Shetty et al., 2011). Because of lack of available sample remaining, complete biosensor and conventional assay data were available for 30 participants. The study protocol was approved by the UCLA Institutional Review Board.

Salivary alpha-amylase assessment

Biosensor measurements. As described in more detail elsewhere (Shetty et al., 2011), the biosensor employed in this study consisted of disposable colorimetric test strips and a hand held reader that uses a miniaturized optical platform to detect and quantify salivary alpha-amylase. The test strips collect 25 μl of saliva when saturated, minimizing the effect of salivary flow rate. Inputted algorithms normalize variations in ambient temperature and salivary pH. For direct readings, the small test strip is placed under the tongue for 15 s, and is then removed and inserted into the sensor unit. For indirect readings from passive drool samples, the test strip is placed into a 2-ml aliquot of saliva in a 15-ml test tube for 15 s, and is then removed and inserted into the sensor unit. The biosensor provides a date/time-stamped reading within 15 s.

Conventional assay. Aliquots of passive drool saliva were analyzed at the UCLA Clinical and Translational Research Laboratory using an automated clinical chemistry analyzer (Olympus AU 400, Olympus America, Center Valley, PA), which is considered an established method for measuring alpha-amylase (Rohleder and Nater, 2009) in addition to the enzyme kinetic assays used in smaller scale laboratories.

Data analysis

Data analysis was performed using SPSS (IBM SPSS 20, Armonk, NY). We used repeated measures ANOVA to test for differences in mean biosensor values between the assay methods (Table 1), followed by pairwise comparisons with a Bonferroni correction to adjust for multiple comparisons. To examine the correspondence among assay methods, we computed Pearson correlations as well as Spearman rank correlations. We also computed

TABLE 1. Variation in sAA activity levels by sample processing/assay methods

Reading	Mean	SD	Range
Biosensor			
Direct[a]	40.3	24.2	8–132
Unprocessed[b]	75.4	32.9	23–187
Thawed[c]	100.5	48.8	31–232
Thawed/centrifuged[b]	87.1	38.8	23–158
Conventional			
Unprocessed[b]	83.8	44.4	17–211.6
Thawed[b]	82.6	40.36	17.6–182.6
Thawed/centrifuged[b]	79.5	36.04	17.6–181.6

Note: Units are in U ml^{-1}. Readings with the same subscripts were not significantly different from one another at $P < 0.05$, Bonferroni adjusted for multiple comparisons.

correlations using $\log_{10}(1+x)$ transformed data based on the heterogeneity of the variability between methods across the range of the values. Because correlations between methods of measurement do not fully capture agreement (Bland and Altman, 1986), we computed the bias, estimated from the mean difference between methods ($\bar{d}$), and the standard deviation (s) of those differences. Next, for each pair of measurement methods, we constructed Bland-Altman plots graphing the averages versus the differences along with the 95% limits of agreement ($\bar{d} \pm 1.96\ s$) which show the range within which most of the differences between the measurements are expected to lie.

RESULTS

Table 1 shows the descriptive statistics for sAA values across the 31 participants. Obtained values were within the dynamic range of the biosensor (5–450 U ml^{-1}), and the passive drool values were within the linear range of the biosensor (10–230 U ml^{-1}) with the exception of one outlier (one thawed-biosensor reading at 232 U ml^{-1}). The processing/assay methods significantly differed from one another, $F(6,174) = 23.93$, $P < 0.001$, and follow-up pairwise comparisons showed that the direct-biosensor reading was significantly lower compared to all other processing/assay methods (Ps < 0.001). The thawed-biosensor reading was significantly higher compared to all other processing/assay methods (Ps < 0.05). The unprocessed-biosensor, thawed-centrifuged-biosensor, and the conventional samples did not significantly differ from one another.

Table 2 shows the correlations between the processing/assay methods. The pattern of correlations showed that direct readings showed small to modest, but not statistically significant correlations with biosensor readings derived from passive drool samples and conventional assay readings from passive drool samples. By contrast, biosensor readings derived from passive drool samples showed strong and significant correlations with each other and with conventional assay readings from passive drool samples. The results were similar when using $\log_{10}(1+x)$ transformed data (Table 3).

The relationship between the thawed/centrifuged—biosensor readings, which showed high correlations with conventional assay in our prior study (Shetty et al., 2011), and other three biosensor readings are shown graphically in Figure 1A–C. In addition, Figure 2A–C depict Bland–Altman plots of agreement. Both Table 2 and Figures 1A–C and 2A–C show the stronger correspondence between passive drool readings and the thawed/centrifuged—biosensor reading when compared to the direct reading. Moreover, the slopes of the best-fit regression lines differed substantially for the direct reading (unstandardized $\beta = 0.15$, $P = 0.21$) compared to the unprocessed (unstandardized $\beta = 0.62$, $P < 0.001$) and thawed readings (unstandardized $\beta = 1.13$, $P < 0.05$). Furthermore, the Bland–Altman plots indicated a smaller discrepancy between the thawed/centrifuge—biosensor reading and the passive drool readings when compared to the direct reading, and a narrower 95% limits of agreement band around those differences. Moreover, in contrast to the restricted range of sAA values obtained from the direct reading, the sAA values from passive drool samples were consistently higher.

The relationship between the gold standard reading (thawed/centrifuged—conventional assay) and the

TABLE 2. Correlations between collection/processing methods

		Biosensor				Conventional		
Instrumentation	Reading	Direct	Unprocessed	Thawed	Thawed/ centrifuged	Unprocessed	Thawed	Thawed/ centrifuged
Biosensor	Direct	–	0.40	0.13	0.24	0.24	0.21	0.20
	Unprocessed	0.45	–	**0.82**	**0.74**	**0.84**	**0.82**	**0.80**
	Thawed	0.26	**0.77**	–	**0.90**	**0.91**	**0.88**	**0.90**
	Thawed/centrifuged	0.27	**0.77**	**0.89**	–	**0.81**	**0.83**	**0.88**
Conventional	Unprocessed	0.30	**0.81**	**0.89**	**0.85**	–	**0.95**	**0.90**
	Thawed	0.26	**0.76**	**0.87**	**0.84**	**0.94**	–	**0.88**
	Thawed/centrifuged (gold standard)	0.21	**0.76**	**0.86**	**0.87**	**0.95**	**0.90**	–

Note. Values above the diagonal are Pearson correlations; values below the diagonal (shaded region) are Spearman correlations ($N = 31$). Statistically significant correlations at $p < .01$ are in **bold.**

TABLE 3. Correlations between collection/processing methods using $\log_{10}(1+x)$ transformed values

		Biosensor				Conventional		
Instrumentation	Reading	Direct	Unprocessed	Thawed	Thawed/ centrifuged	Unprocessed	Thawed	Thawed/ centrifuged
Biosensor	Direct	–	0.45	0.24	0.27	0.33	0.30	0.27
	Unprocessed	0.45	–	**0.85**	**0.84**	**0.84**	**0.83**	**0.80**
	Thawed	0.26	**0.77**	–	**0.93**	**0.92**	**0.91**	**0.91**
	Thawed/centrifuged	0.27	**0.77**	**0.89**	–	**0.86**	**0.87**	**0.88**
Conventional	Unprocessed	0.33	**0.84**	**0.92**	**0.86**	–	**0.97**	**0.96**
	Thawed	0.30	**0.83**	**0.92**	**0.87**	**0.97**	–	**0.95**
	Thawed/centrifuged (gold standard)	0.27	**0.80**	**0.91**	**0.88**	**0.96**	**0.95**	–

Note. Values above the diagonal are Pearson correlations; values below the diagonal (shaded region) are Spearman correlations ($N = 31$). Statistically significant correlations at $P < 0.01$ are in **bold.**

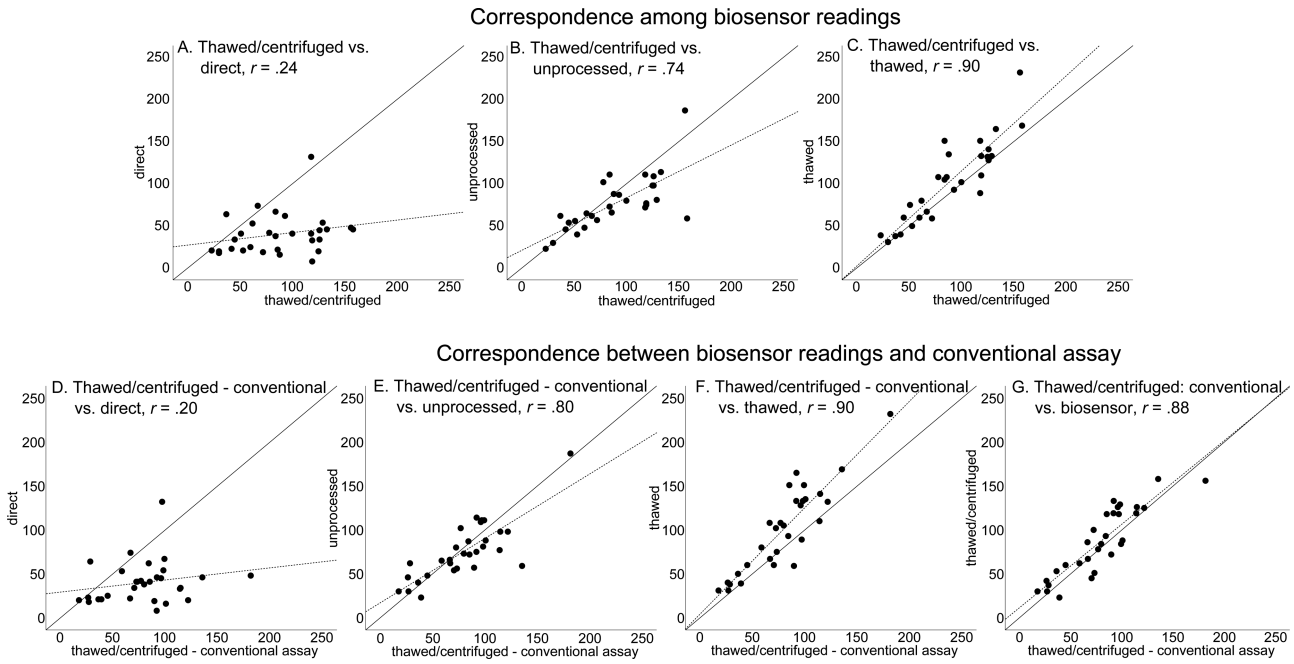

Fig. 1. Scatter plots to visualize the correlation between the sAA levels from different sampling and quantification approaches (biosensor and conventional assay). A–C: compare sampling approaches with the thawed/centrifuged—biosensor sample. D–G: compare sampling approaches with the thawed/centrifuged—conventional assay sample. The scatter plots show a clear positive correlation between the various biosensor methods but the data does not fall perfectly on the line of equivalence ($y = x$). Correspondence with gold standard was lowest for the direct reading (A).

biosensor readings are shown graphically in Figure 1D–G, with Bland–Altman plots in Figure 2D–G. The direct reading showed poorer correspondence (unstandardized $\beta = 0.14$, $P < 0.001$) compared to the other biosensor readings (unstandardized βs from 0.75 to 1.21, all Ps < 0.001). The closest correspondence was for the thawed/centrifuged—biosensor reading (unstandardized $\beta = 0.95$, $P < 0.001$). The Bland–Altman plots similarly indicated a

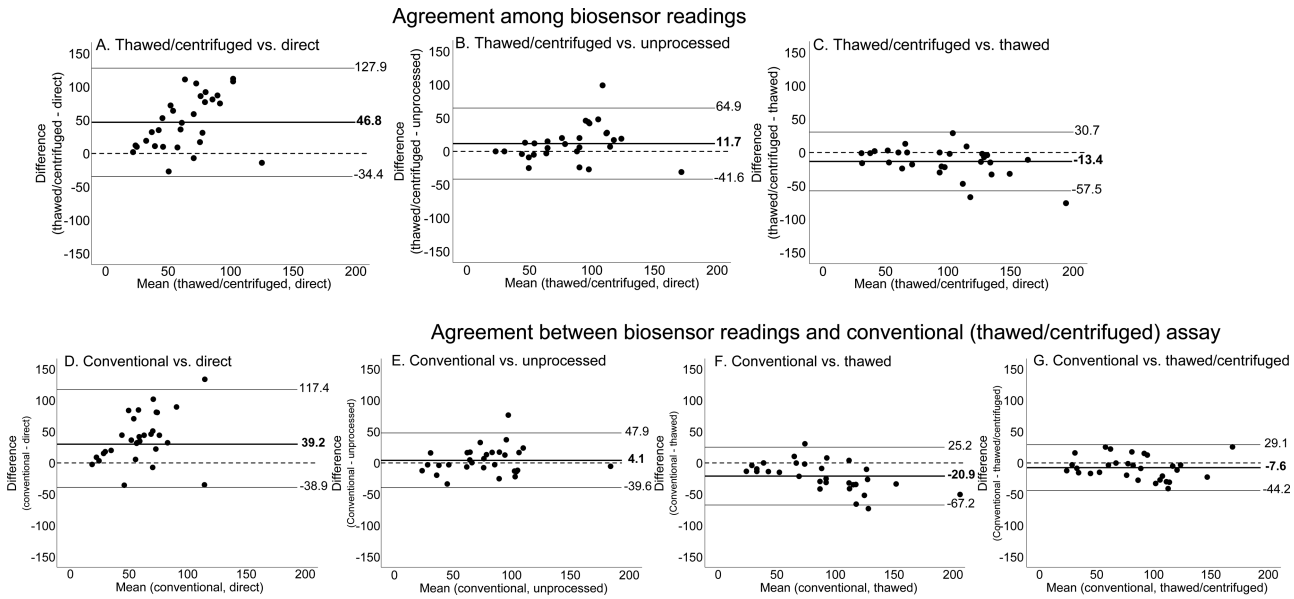

Fig. 2. Bland–Altman plots to visualize agreement between sAA levels from different saliva sampling and quantification approaches. A–C: compare sampling approaches with the thawed/centrifuged—biosensor sample. D–G: compare sampling approaches with the thawed/centrifuged—conventional assay sample. The measurement bias (thick, solid line indicating mean difference between methods) is greatest for the direct reading (A) and lowest for passive drool samples. The thin solid lines define the upper and lower limits of agreement (± 2 SD).

smaller discrepancy between biosensor readings involving passive drool saliva compared to the direct readings, and a narrower 95% limit of agreement around those differences. Similar to the biosensor readings, sAA values from passive drool samples were consistently higher than the direct reading.

DISCUSSION

Our results indicate that the performance of the point-of-care sAA biosensor is influenced significantly by the saliva sampling method. The quantitative estimates of sAA levels provided by the biosensor varied according to the source of the sample (e.g., measured directly from mouth vs. passive drool into a collection tube) and to a lesser degree, the processing of specimen matrix (centrifuged vs. whole saliva). Specifically, biosensor measurements of sAA obtained directly from the mouth showed low correlations and agreement with sAA levels from saliva collected through passive drool. In contrast, biosensor sAA levels established from passive drool saliva samples showed stronger agreement with each other and when compared to conventional assay. The low to modest correspondence between the direct and the passive drool measurements echo the findings of other investigators that implicate saliva sampling technique as a major source of measurement variability as it relates to sAA measurements (Harmon et al., 2008). Moreover, strong correlations were observed despite differences in intermediary processing steps (e.g., freezing, thawing, centrifugation), which suggests that employing the sAA biosensor to assess passive drool saliva in the field, where freezers or centrifuges may not be immediately available, may provide acceptable sAA values.

Point-of-care biosensors such as the sAA biosensor are very attractive to the clinician and the researcher because of their capacity to readily measure putative stress biomarkers and provide actionable data in near real-time. The latest version of the sAA biosensor incorporates numerous technical advances (e.g., fixed amount of saliva sample, correction for pH and temperature variations, small form-factor, user-friendly interface to minimize operator error) to suppress analytical variability between devices and to optimize measurement of biological variability. Indeed, precursor analytical validation of the sAA biosensor prototypes, conducted in controlled settings using whole saliva samples obtained by passive drool, indicated a high correspondence with sAA measurements conducted on the same samples in an automated clinical chemistry analyzer (Shetty et al., 2011). The measurement consistency and operating characteristics of the sAA biosensor led us to explore its utility for directly assessing sAA in naturalistic settings.

In hindsight, the lower sAA values obtained by the biosensor during direct in-mouth assessments were not unexpected. Recent work suggests that, unlike other salivary biomarkers that are serum constituents transported into saliva, the levels of the locally produced sAA are impacted significantly by the area from which the saliva is sampled as well as the collection technique (Beltzer et al., 2010; Harmon et al., 2008). Although saliva sampled directly from the mouth or by passive drool is often used interchangeably, sAA is unique in that its concentration can vary substantially depending on the differential contribution of the local salivary glands to the saliva volume. The parotid and submandibular glands secrete much higher levels of sAA relative to the sublingual glands. In one study, parotid saliva contained 4–10 fold higher concentrations of sAA compared to submandibular saliva (Veerman et al., 1996), while in another study, parotid and submandibular saliva had similar concentrations, and sublingual saliva had lower sAA concentrations (Harmon et al., 2008). Consistent with our findings of lower sAA levels for the direct reading, the saliva collector strip placed briefly (20 s) on the floor of the mouth tends to sample saliva primarily produced by the sublingual and submandibular glands (Humphrey and Williamson, 2001; Schenkels et al., 1995).

In contrast, the composition of unstimulated saliva collected by passive drool is more representative of whole saliva (Rohleder and Nater, 2009) and best captures sAA activity levels. Whole saliva obtained through passive drool is more typical of mixed saliva in that it contains a high proportion of saliva from the parotid gland. As shown in Figures 1 and 2, passive drool samples showed higher sAA concentrations compared to direct readings, suggesting a greater contribution from the parotid salivary gland which tends to produce large amounts of sAA. Indeed, in another study, sAA concentrations in parotid saliva were the strongest predictor of sAA concentrations in whole saliva, followed by sublingual saliva, with submandibular levels related to whole saliva in the opposite direction (Harmon et al., 2008).

Unfortunately, no studies to our knowledge have explicitly tested systematic relationships between psychological stress and sAA obtained from different salivary glands. Such work is particularly important because the composition of saliva varies by the relative influence of sympathetic (more viscous saliva) and parasympathetic (more "watery" saliva) activity. In addition, salivary glands are differentially innervated by sympathetic and parasympathetic nerves, with parotid glands mostly innervated by parasympathetic nerves (Proctor and Carpenter, 2007). Thus, future attempts to sample saliva using direct, in-mouth biosensor methods would need to systematically document how sAA varies when sampled from different locations (e.g., sublingual vs. parotid) under different conditions of sympathetic and parasympathetic activation (perhaps involving systematic pharmacological stimulation or blockade of the sympathetic and parasympathetic nervous systems). While the heterogeneity of sAA sources presents a methodological challenge for research on stress biomarkers, assessing such heterogeneity may in fact provide insights into the relative contributions of sympathetic and parasympathetic responses to stress.

These data represent ongoing efforts to develop and refine point-of-care biosensor systems for rapidly measuring salivary stress indicators such as sAA. Technical developments in the newer generation devices have greatly improved the speed, accuracy and reliability of sAA measurement, mainly through analytical improvements and enhancements in user interfaces that reduce operator error (Yamaguchi and Shetty, 2012). The measurement issues associated with the current sAA biosensor have little to do with the device itself. Our study showed that preanalytical factors such as sampling and processing of the saliva can cause a deterioration of the biosensor performance. For example, although thawed saliva readings showed larger correlations with conventionally assayed saliva, the Bland–Altman plot showed a

greater discrepancy between biosensor readings of thawed saliva and conventionally assayed saliva compared to biosensor readings of thawed/centrifuged saliva. A strong possibility is that mucins and small particles of various substances (cell debris, bacteria, food) that would ordinarily be cleared from the fluid through centrifuging, coupled with the small volume of saliva collected for the biosensor reading (25 μl) increased the possibility of interference with the quantification procedure.

Thus, in the future, investigators utilizing the sAA biosensor or other salivary biosensors need to be aware that variation in saliva sampling technique contributes to measurement errors in recorded sAA activity levels. Our early field experiences have significant implications for measurement strategy for biobehavioral studies involving the sAA biosensor. Ongoing refinements in the sAA biosensor technology and the sampling protocol will eventually redress the measurement variability. In the meantime, we suggest that early adopters of the technology consider measuring sAA indirectly from whole saliva collected by passive drool. We recommend freezing and centrifuging samples prior to analysis, although the feasibility of these processing steps depends on the population, sampling environment (the field vs. the laboratory), and equipment availability. If freezing and centrifuging is not possible, biosensor readings from passive drool samples may still yield reasonable results. As our findings suggest, sAA levels measured directly from the floor of the mouth are less accurate in comparison to readings from whole saliva.

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