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Factors affecting salivary α-amylase levels measured with a handheld biosensor and a standard laboratory assay

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Abstract

Objectives: A handheld biosensor for measuring salivary α-amylase (sAA) was developed for convenient on-site measurement. Previous studies reported some discrepancies in sAA levels measured with a biosensor and a standard assay. This study aimed to compare sAA levels measured with three different methods and the factors affecting its levels.

Methods: Thirty-eight participants collected saliva two times for three measurements. First, the collector strip was placed under the tongue for 2 minutes, then the strip was used to measure sAA level on-site immediately (intraoral biosensor; method 1). Then, a participant pooled the saliva for 4 minutes and collected the saliva into the tube which was aliquoted to measure in a laboratory with a handheld biosensor (extraoral biosensor; method 2) and with a standard enzyme kinetic assay (EKA; method 3). Additional experiments were carried out to compare the levels of sAA measured with differences in pooling time and positioning of the collector strip.

Results: A high correlation of sAA levels between an extraoral and an EKA measurement ($r = 0.989$) was observed, while sAA levels measured with an intraoral method showed a significant but weaker correlation with either an EKA ($r = 0.475$) or an extraoral method ($r = 0.436$). Saliva pooling time and positioning of the collector strip significantly affected sAA levels.

Conclusions: A handheld biosensor is valid to measure sAA levels extraorally. For an intraoral measurement, pooling time and positioning of the collector strip need to be taken into account.

1 | INTRODUCTION

Salivary α-amylase (sAA) has been regarded as a stress biomarker as it has been consistently shown to increase after psychological or physiological stress (Nater & Rohleder, 2009). The fact that stress can be examined from saliva is an important aspect of research in the field of psychology because it implies many advantages. First, the collection of saliva is noninvasive. Second, sAA is stable for a long period of time (up to 3 weeks) at room temperature (DeCaro, 2008) making it convenient for performing an experiment outside the laboratory setting. These reasons make sAA an excellent biomarker.

There are many methods to measure sAA levels (Rohleder & Nater, 2009). A standard automated analyzer commonly used in a hospital setting to measure serum amylase from the pancreas can be applied to measure sAA in an automated manner without having to prepare the sample. However, the machine is not portable. For a laboratory setting, there is another standard method of enzyme kinetic assay (EKA) that requires general laboratory equipment usually already available in a laboratory, that is, a spectrophotometer. EKA can

measure many sAA samples simultaneously but requires preparation of samples and skill to make the measurement reliable. The third method was more recently developed due to the need to be able to measure sAA in a real-time setting (Yamaguchi et al., 2004). This handheld machine employs a similar principle to the EKA and the automated analyzer, that is, enzyme kinetics to measure the activity of α-amylase to digest the substrate (not the amount of amylase itself). The advantage of a handheld biosensor is real-time measurement without having to process the saliva sample.

Previous studies have confirmed the validity of this handheld biosensor. sAA levels measured from a handheld biosensor and an automated analyzer or an EKA kit yielded highly correlated results (Robles et al., 2013; Shetty, Zigler, Robles, Elashoff, & Yamaguchi, 2011). However, one study found that saliva collection method could greatly affect the readings of a handheld biosensor (Robles et al., 2013). We measured sAA levels using three methods. One method was to measure intraorally, using a collector strip to soak the saliva, and measure on-site with a handheld biosensor. The second method was to aliquot the saliva from passive drooling to measure extraorally with a handheld biosensor. Third was to aliquot the saliva to measure with a standard EKA. Then we examined how different pooling time and positioning of the collector strip affected sAA levels.

2 | METHODS

2.1 | Participants

Thirty-eight healthy volunteers aged between 19 and 65 years (mean 35.08 ± 12.89; 60.5% female) took part in the saliva collection. All participants had no underlying diseases, were not taking any medications that could affect sAA levels (ie, drugs affecting autonomic nervous systems such as beta-blockers), did not smoke, and were not pregnant. Participants were asked to refrain from drinking alcoholic beverages for 12 hours and from eating or drinking an hour prior to saliva collection. Plain drinking water was allowed all the time. Informed consent was obtained from each participant after the full description of the study. This study was a part of a larger project aimed to assess dental anxiety. The study was approved by the ethics research committee of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University.

2.2 | Experimental design comparing three measurement methods (experiment 1)

Each participant provided two saliva samples. The first sample was from placing the collector strip under the tongue for 2 minutes and the measurement was performed immediately with a handheld biosensor (intraoral biosensor). After that, each participant pooled the saliva for 4 minutes and passively drooled the saliva into a 2-mL tube which was kept frozen for later measurement by two methods (extraoral biosensor and EKA) performed on the same day for each sample. A flow chart of the protocol is shown in Figure 1.

Some participants could not pool saliva within 4 minutes (saliva less than 0.5 mL). In this case, participants were allowed more time until they could collect enough saliva.

2.3 | Measurements of salivary alpha-amylase (sAA)

Three types of measurements were employed.

1. *Intraoral biosensor*: After the saliva was allowed to soak onto the collector strip for 2 minutes, the strip was directly placed into a handheld biosensor for a reading of the α-amylase level on-site. This handheld biosensor (developed by Dr. Yamaguchi et al. (2006) and produced by Nipro Co, Osaka, Japan) comprises a collection strip and a miniaturized optical reading machine. The

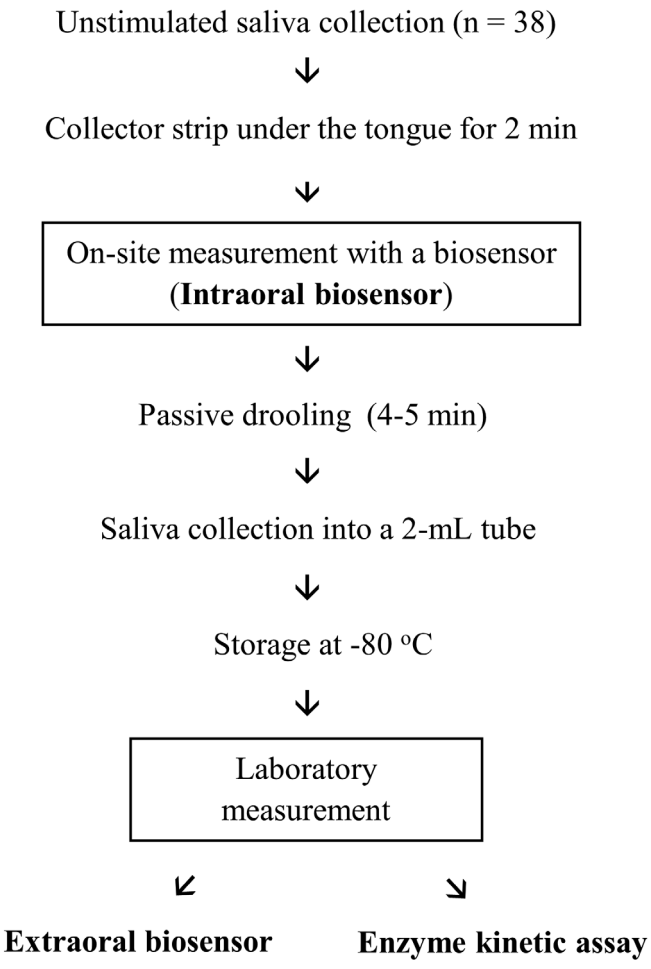

FIGURE 1 The experimental design to compare three measurement methods

collection strip consists of a pad for saliva collection at the tip and a reagent paper containing a chromogenic substrate, Gal-G2-CNP, for sAA. When the collector strip was saturated with saliva, it was inserted into the reader. The process of reading involves a simple manipulation of the lever on the machine and a 10-second waiting period for the enzymatic reaction to take place. The machine measures the reaction product at 430 nm. The whole process takes less than 1 minute to reveal sAA level per sample. The biosensor can measure sAA levels correctly in the linear range between 0 and 200 U/mL with 10.2% coefficient of variation (Yamaguchi, Hanawa, & Yoshida, 2007).

2. *Extraoral biosensor*: The frozen saliva from passive drooling was thawed and centrifuged at 1500 rpm for 15 minutes. A 25 μL aliquot of the saliva was pipetted onto the collector strip. Then, the strip was put into a handheld biosensor for a reading of the α-amylase level.
3. *Enzyme kinetic assay (EKA)*: This is a standard method for measuring sAA in a laboratory. The frozen saliva was thawed and centrifuged at 1500 rpm for 15 minutes on the same day of the extraoral biosensor measurement for each saliva sample. The measurement was performed according to the manufacturer's instruction (Salimetrics, State College, Pennsylvania). Briefly, all the reagents had to be brought to room temperature before the measurement. The aliquots of saliva were prepared at 1:200 dilution. Then 8 μL of the diluted sample was added into the 96-well plate. Amylase substrate (2-chloro-p-nitrophenol linked with maltotriose) was then added to start the enzymatic reaction and the plate was read at exactly 1 and 3 minutes with a spectrophotometer at 403 nm (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, Rockford, Illinois) at 37°C. The whole process takes about 1 to 2 hours to complete, but it can measure many samples at the same time. This EKA kit can measure sAA levels correctly in the range between 3.1 and 423.1 U/mL with 5.47% coefficient of variation (according to the manufacturer's instruction)

2.4 | Pooling time experiment (experiment 2)

Originally, the handheld biosensor was designed for an intraoral measurement with the collector strip placed under the tongue for 30 seconds (Yamaguchi et al., 2007). This experiment aimed to examine if holding the strip under the tongue for a longer period would affect the sAA levels. To compare the effect of saliva pooling time, the participants (n = 16) held the collector strip under the tongue for 30 seconds twice and for 2 minutes twice with a randomized sequence. The collector strip was measured on-site right away. In total, each participant collected saliva four times for an intraoral measurement.

2.5 | Positioning of a collector strip experiment (experiment 3)

The handheld biosensor was designed for an intraoral measurement with the collector strip placed in an upward position, where the pad collecting the saliva faces the base of the tongue (Figure 2) (Yamaguchi et al., 2007). However, the collector strip can also be placed downward where the collector pad faces the floor of the mouth. In both directions, the collector strip is placed under the tongue. To examine

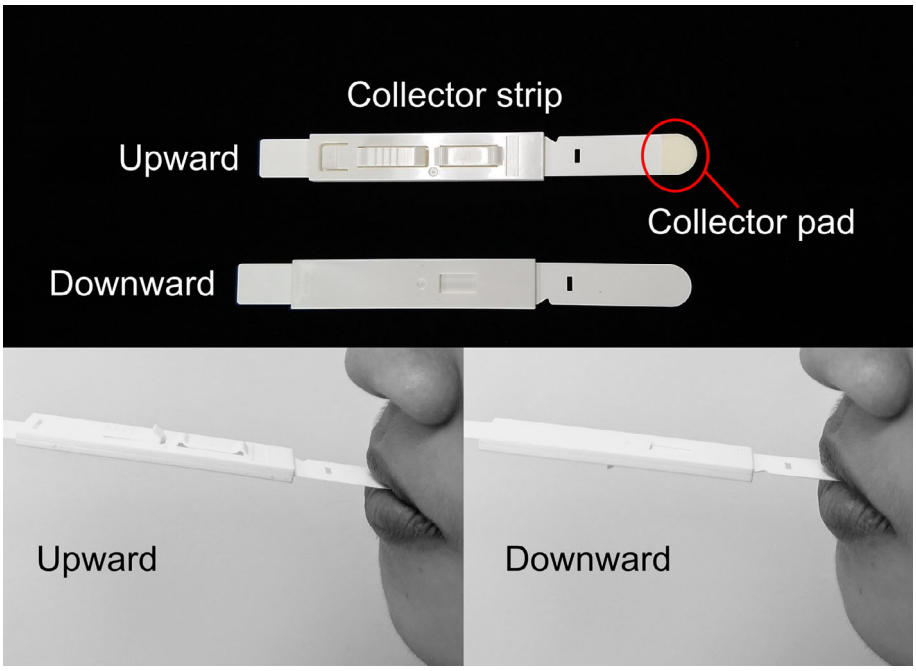

FIGURE 2 The picture of a collector strip. The placement of a collector strip in upward and downward position is demonstrated

whether placing the collector strip upward or downward would affect sAA levels, the participants (n = 11) were randomized to place the strip under the tongue in the upward and downward positions for 30 seconds each time for six consecutive times. Accordingly, each participant held the collector strip in the upward position three times and downward position three times with random sequences. The strips were used to measure sAA levels with an intraoral biosensor. This is the only experiment in the current study where the collector strips were also placed downward. In all other experiments, the collector strips were always placed upward.

2.6 | Statistical analysis

Data were presented as mean ± SEM. Kolmogorov-Smirnov test was used to determine normality of the samples. Correlations of sAA measured with different methods were assessed using Spearman nonparametric analysis. Agreements between different measuring methods were analyzed using Bland-Altman plots with log-transformation performed as the data were abnormally distributed. Wilcoxon Signed Ranks test was used to compare the means in the pooling time and the positioning of the collector strip experiments. Statistical analysis was performed using SPSS statistics program version 21 (IBM, New York).

3 | RESULTS

3.1 | Levels of sAA measured with three different methods

A different set of saliva was used to measure sAA levels with an intraoral biosensor (saliva collected under the tongue) and with an extraoral biosensor or an EKA (whole saliva from passive drooling). The intraoral biosensor method gave the lowest levels of sAA compared to extraoral and EKA methods (Table 1), while the extraoral biosensor method yielded the highest values.

3.2 | Correlations of sAA levels between different measurement methods

As shown in Table 2, sAA levels measured with all three methods showed significant positive correlations ($P < .01$). SAA measured with the intraoral biosensor showed moderate correlation with levels measured using an extraoral biosensor ($r = 0.436$) or EKA ($r = 0.475$). The levels of sAA from the same saliva aliquots measured with extraoral biosensor and EKA showed the strongest correlation ($r = 0.989$). At very high levels of sAA, the correlation of sAA measured with an extraoral biosensor vs an EKA was less than at lower levels (Figure 3B). This could be because these levels were out of the linear range of the biosensor's capacity (Shetty et al., 2011).

The Bland-Altman plots of agreement between methods were performed and showed good agreement between an extraoral biosensor and an EKA (Figure 4). The levels of sAA from an intraoral biosensor showed larger mean differences when compared with either an extraoral biosensor or an EKA.

3.3 | The effect of pooling time on sAA levels

Most of the participants could pool their saliva for about 0.5 to 1.5 mL after 4 minutes; however, some participants needed a longer time to pool the saliva. We postulated that the stress involved in pooling the saliva might be a factor underlying the difference in sAA levels. The range of time participants needed to pool saliva was from 4 to 30 minutes. Nine volunteers (24%) took longer than 5 minutes to pool saliva and most of them (8 out of 9) spent no more than 10 minutes. Only one participant spent 30 minutes to pool the saliva. Participants who took a longer time to pool saliva, that is, more than 5 minutes, tended to have higher sAA levels (Table 3). As shown in the first experiment, extraoral measurement showed higher sAA levels compared with intraoral measurement; however, the increase in sAA levels from an intraoral measurement was significantly higher in participants who needed more than 5 minutes to pool saliva ($P = 0.041$). While, for intraoral measurement, all participants held the strip under the tongue for the same duration (2 minutes) and participants who took a longer time to pool saliva did not have higher sAA levels as shown from the intraoral measurement.

We then had a question whether this would be the same if the participant held the strip under the tongue for a different period. As shown in Figure 5, the sAA levels from the

TABLE 1 Levels of sAA measured with different methods (U/mL)

Group (n = 38)	Mean	SEM	Range
Intraoral biosensor	33.13	4.21	5-150
Extraoral biosensor	116.53	17.01	10-409
EKA	77.27	13.41	1.80-361.50

Abbreviations: EKA, enzyme kinetic assay; sAA, salivary α-amylase.

TABLE 2 Spearman correlations between sAA levels measured with different methods

	Correlation coefficient	*P*-value
Intraoral vs extraoral biosensor	0.436	.006
Extraoral biosensor vs EKA	0.989	<.001
EKA vs intraoral biosensor	0.475	.003

Abbreviations: EKA, enzyme kinetic assay; sAA, salivary α-amylase.

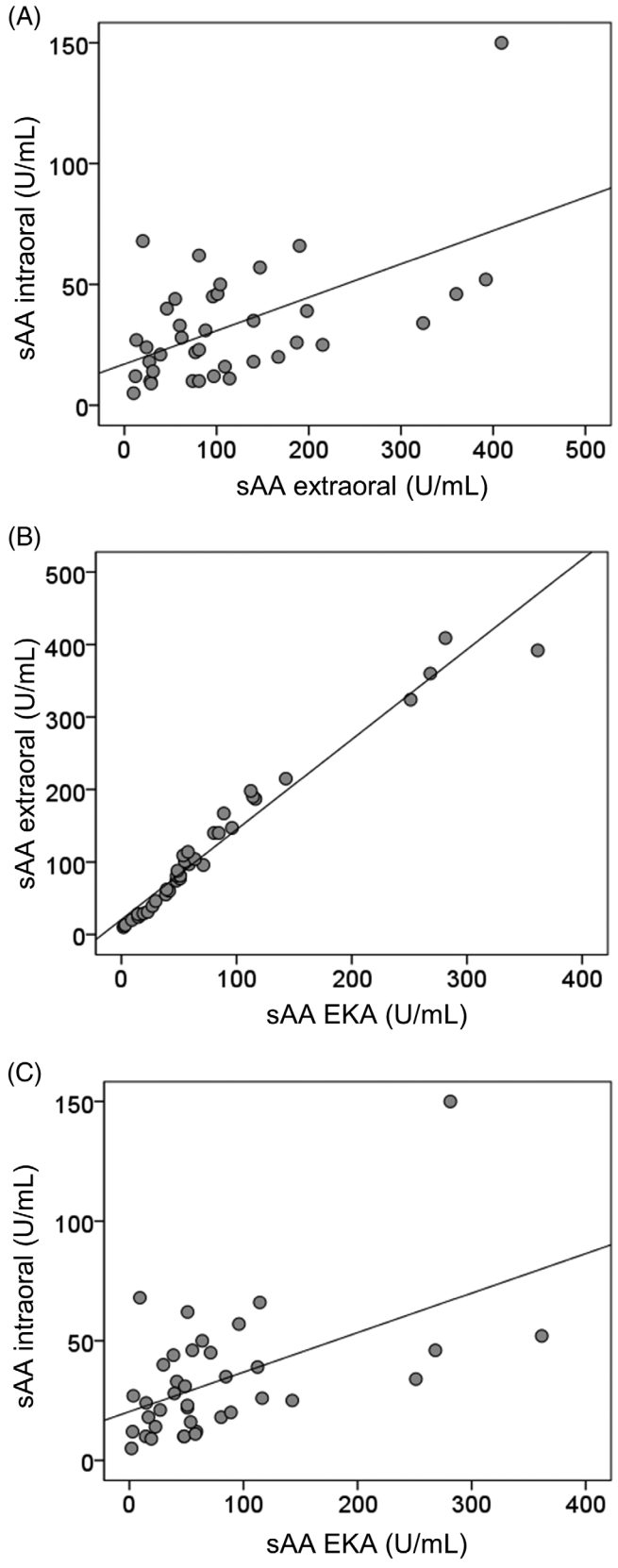

FIGURE 3 Correlations of sAA levels measured with (A) an intraoral vs an extraoral biosensor, (B) an extraoral biosensor vs an EKA, and (C) an intraoral biosensor vs an EKA. EKA, enzyme kinetic assay; sAA, salivary α-amylase

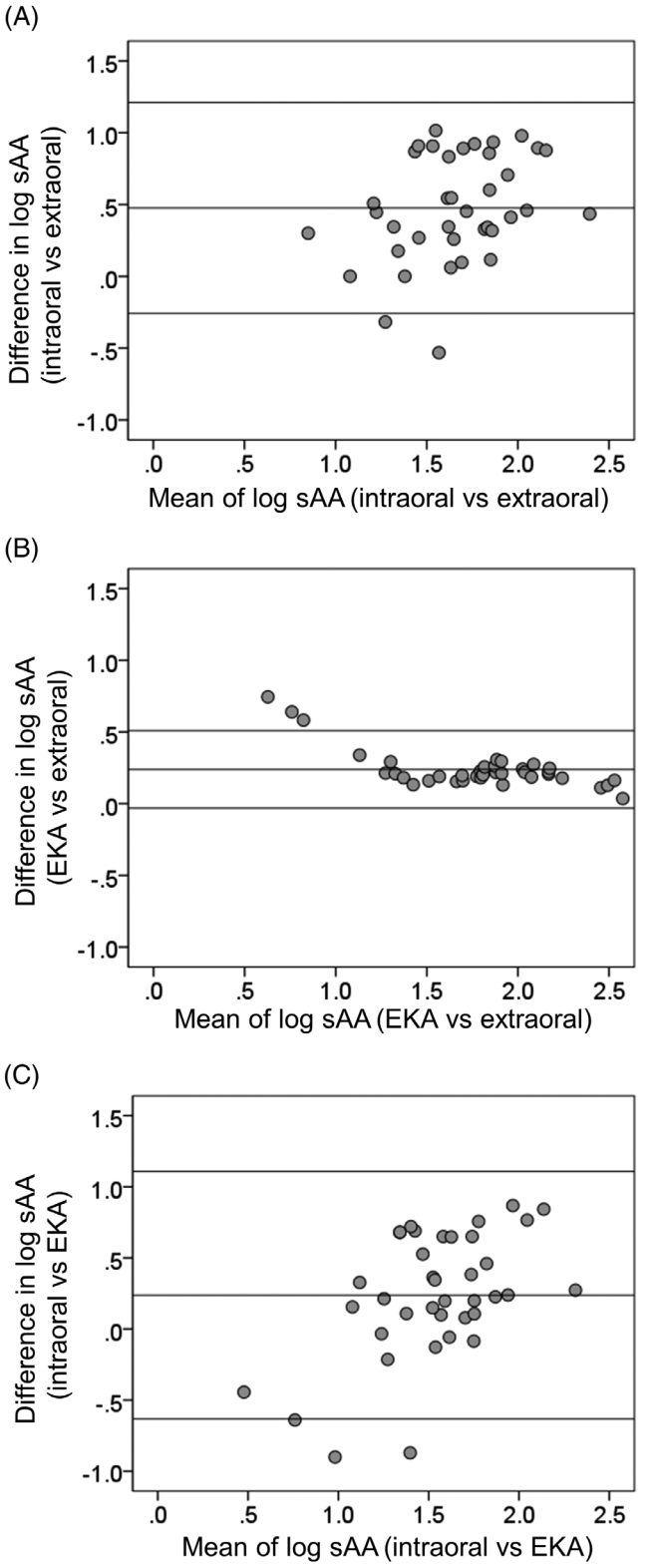

FIGURE 4 Bland-Altman plots (log transformed) to determine agreement between sAA levels measured with (A) an intraoral vs an extraoral biosensor, (B) an extraoral biosensor vs an EKA, and (C) an intraoral biosensor vs an EKA. The horizontal lines indicate mean ± 1.96 SD (95% confidence interval). EKA, enzyme kinetic assay; sAA, salivary α-amylase

participants holding the strip under the tongue for 30 seconds and 2 minutes were 18.91 ± 3.50 U/mL and 30.03 ± 3.28 U/mL, respectively ($P = 0.012$).

TABLE 3 Mean sAA levels between different pooling times (±SEM)

Group	4-5 minutes (n = 29)	>5 minutes (n = 9)
Intraoral biosensor[a]	33.28 ± 5.35	32.67 ± 4.85
Extraoral biosensor	99.66 ± 18.16	170.89 ± 38.08
Increase from intraoral	66.38 ± 15.17	138.22 ± 36.73*

Abbreviation: sAA, salivary α-amylase.
[a]No difference in pooling time, 2-minutes collection for all cases are shown here for comparison.
*$P < .05$.

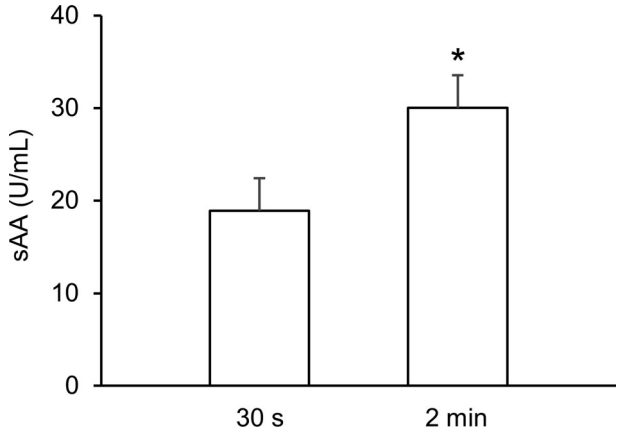

FIGURE 5 The effect of pooling time on sAA levels in the same participants after holding the strip under the tongue for 30 seconds and 2 minutes. The experiment was duplicated. n = 16, *$P < .05$. sAA, salivary α-amylase

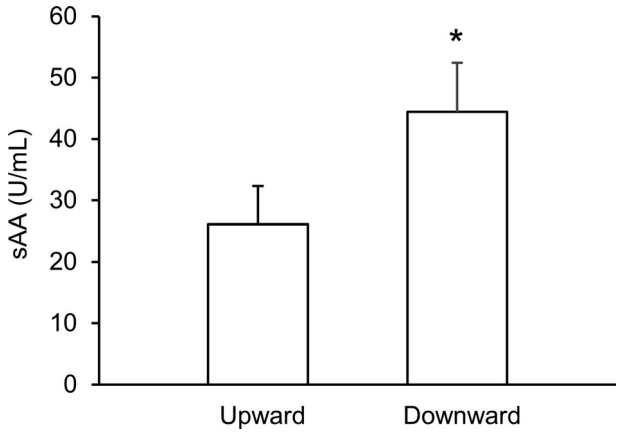

FIGURE 6 Levels of sAA with different positioning of a collector strip. The experiment was triplicated. n = 11, *$P < .001$. sAA, salivary α-amylase

3.4 | Positioning of a collector strip affected sAA levels

We noticed that the tongue could press down onto the collector strip making it difficult for the saliva to soak the collector pad. We postulated that placing the strip in the downward position where the collector pad faces the floor of mouth would allow the saliva to soak the collector pad better since it directly faces the pool of saliva on the floor of mouth and close to the opening of the sublingual and submandibular salivary glands. Participants were asked to place the strip in upward and downward positions for three times in each position. Then sAA levels were measured with an intraoral biosensor. As shown in Figure 6, putting the strip in the downward position resulted in significantly higher readings of sAA levels (44.45 ± 7.99 U/mL) than in the upward position (26.09 ± 6.25 U/mL).

4 | DISCUSSION

The present study found a strong correlation of sAA levels between an extraoral biosensor and an EKA ($r = 0.989$). Our findings were in agreement with a previous study that compared sAA levels measured between a biosensor and an automated analyzer with correlations in the range of 0.971 to 0.99 (Shetty et al., 2011). Another study that compared sAA levels measured between a biosensor and an EKA also found strong correlations in the range of 0.889 to 0.951 (Peng et al., 2016). These confirm the validity of a handheld biosensor to measure sAA compared with standard laboratory methods. On the other hand, sAA levels measured with an intraoral biosensor were less strongly but significantly correlated with the other two measurement methods (extraoral and EKA; range of correlation = 0.436-0.475). This is in contrast with the other two studies that found nonsignificant correlations (range of correlation = 0.113-0.30) between sAA levels measured intraorally with the collector strip placed under the tongue and measured extraorally from whole saliva (Peng et al., 2016; Robles et al., 2013). These results suggest that the saliva itself is responsible for the reading of a handheld biosensor since the readings were consistent with the same pool of saliva and less consistent with the different pool of saliva.

These discrepancies could possibly be accounted for by two factors we performed differently from the previous studies. One was that the duration of time the collector strip was allowed to soak with the saliva was quite short in the other two studies (25 and 30 seconds). In our pilot study, we found some strips to report error readings due to not enough saliva (dry strip) when the saliva collection time was 30 seconds. Indeed, Peng et al. (2016) reported error strips from no amylase activity. Yamaguchi, Kanemaru, Kanemori, and Mizuno

(2003) also found that the time it took to collect the same volume of saliva could vary greatly among individuals. For example, to collect 100 μL of saliva, the participants needed between 36 and 183 seconds. Xerostomia or oral dryness might explain how some collector strips reported error readings because some participants might not have enough saliva. The condition was observed in more than 20% of the general population (Sreebny, 2000).

Another important factor is the position in which the collector strip is placed under the tongue. We noticed in our pilot study that some participants placed the strip very close to the base of tongue. The tongue could press down onto the collector strip, making it difficult for the saliva to soak the collector pad. The collector pad should be placed at the anterior floor of mouth, close to the teeth, where there is more space and is also closer to the opening of the submandibular and sublingual salivary glands. In our experiment, a researcher was the one who placed the collector strip under the tongue of a participant to ensure the strip was positioned properly. When we increased the time to collect the saliva to 2 minutes and placed the collector strip in the anterior position, there was no error strip. These two factors we performed differently could be the reasons why we found a significant and higher correlation of sAA levels between an intraoral biosensor vs an extraoral biosensor or an EKA from the previous studies. Moreover, as we confirmed by comparing the position of the collector strips, we found that placing the strip downward increased the sAA levels. The results suggest that positioning of the strip could also play a role in reducing error readings from dry strips.

Nonetheless, the levels of correlation between an intraoral vs an extraoral or an EKA method were not strong. Robles et al. (2013) have discussed that sampling methods affected the readings of a handheld biosensor. When the collector strip is placed under the tongue, the saliva mainly comes from sublingual and submandibular salivary glands which secrete less sAA. However, with pooling and passive drooling, the whole saliva is from all salivary glands, including the parotid gland which is a major gland that secretes the most sAA (Harmon, Towe-Goodman, Fortunato, & Granger, 2008). This could account for lower readings of sAA levels with the method that collects saliva from under the tongue and not the whole saliva. An active collection method could also greatly affect the composition and the flow of the saliva. It was found that the flow rate of stimulated saliva could be 3 to 10 times higher than that of unstimulated saliva (Sreebny, 2000). In a study by Peng et al. (2016), the participants chewed on the synthetic swab. Stimulation by chewing can greatly increase sAA levels compared with passive collection by placing the strip under the tongue (Beltzer et al., 2010).

The time required to collect the saliva under the tongue and whole saliva from passive drooling could be quite different. Usually, the under the tongue method requires only 25 to 30 seconds, while passive drooling may take more than 10 minutes or even up to 30 minutes depending on the amount of saliva collected considering the average flow rate of unstimulated saliva to be about 0.3 mL/min (Dawes, 1987; Humphrey & Williamson, 2001). In our study, we allowed participants to pool saliva for 4 minutes to collect less than 2 mL of saliva. This duration was not much longer than the intraoral method that collected the saliva for 2 minutes. We also postulated that the pooling time to collect the saliva could affect the levels of sAA due to increased stress. Indeed, some participants reported feeling stressed or anxious from not being able to pool enough saliva in the specified time. As shown in the results, participants who took longer than 5 minutes to pool the saliva had significantly larger differences in sAA levels between an extraoral and an intraoral biosensor method compared with participants who took 4 to 5 minutes to pool the saliva (Table 3). Our results are in contrast with the results observed in a previous study that found longer times to collect the whole saliva were associated with lower levels of sAA (Beltzer et al., 2010). However, that study compared the duration of time within only 1 to 5 minutes, which was shorter than our study and some other studies that needed to collect more amount of saliva (Robles et al., 2013). It is possible that 5-minutes period is not long enough to induce stress in the participants. Similarly, in our second experiment, sAA levels were higher when the participants held the strip under the tongue for a longer period of time (30 seconds vs 2 minutes; Figure 5). This could be from the contribution of the parotid salivary gland as more time passes. Saliva comes from all three glands. Also, as mentioned, some participants may have oral dryness and require more time to pool saliva. We found that the 2-minutes collection time gave more consistent results with fewer error readings.

These results suggest that a handheld biosensor is a valid instrument, but the collection method has to be taken into consideration. If the collection method is passive drooling (whole saliva collected in a tube), the sAA levels can be measured later with a handheld biosensor by pipetting the saliva onto the collector strip (an extraoral biosensor method). This is practical to perform in a field outside the laboratory setting as it does not require a special instrument and takes less than 1 minute to measure each sample. Moreover, a researcher can collect the saliva from many participants at the same time as each participant can pool the saliva individually. The EKA is also a practical method as it can measure many samples at the same time. However, there are many steps to prepare and process the saliva before measurement. The measurement has to be performed in a laboratory setting. It also requires skills and practice.

If the researcher wishes to collect saliva with the collector strip placed under the tongue (an intraoral biosensor method), there are a few issues of concern. The duration of time the collector strip is placed under the tongue should be long enough to ensure the strip is saturated with saliva. Also, the position of a collector strip is important as placing it downward will decrease the chance of error readings. This under-the-tongue collection method is practical, provided there are not many saliva samples to measure at the same time. This is because, even though it takes less than 1 minute to measure each sample, it can only measure one sample at one time. If there are many participants, they have to wait to take turns unless there are many biosensors and operators in the field. Another limitation is that if the sAA level is too high (out of linear range of the biosensor), the results might not be accurate and the saliva cannot be diluted as in other methods. However, this is unlikely to happen as the biosensor is designed for an intraoral measurement where it collects the saliva briefly under the tongue. So, the levels of sAA collected with this method are usually lower than by passive drooling with whole saliva. The advantage is that the researcher gets the results right away, and there is no need to prepare or store the saliva. The limitation of the current study is that the experiment to support the hypothesis that longer pooling times could result in increased stress was not performed.

In summary, all methods for measuring sAA levels (intraoral biosensor, extraoral biosensor, EKA, and automated analyzer) have their own advantages and limitations. Each researcher should take the nature of the experiment and the limitations of each method into account when considering which method to use. Different pools of saliva, that is, whole saliva vs under the tongue, should not be compared as they come from different methods of saliva collection. Enough time and the position of the collection strip should be taken into consideration when choosing to measure sAA levels with an intraoral biosensor. More studies should be carried out to examine the effect of stress from saliva pooling as a factor that might contribute as a confounding factor in stress research.

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AUTHOR CONTRIBUTIONS

K.T. performed the experiment and analyzed the data. N.J. performed the experiment, analyzed the data, and drafted the manuscript. P.P. designed the study, analyzed the data, and finalized the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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