The concept of the "universal slope": towards substantially shorter decentralized insulin immunoassays

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Abstract

Sensing of analytes in decentralized and remote locations is today a reality. However, the number of measurable analytes is still limited, mainly due to the requirement for time-consuming calibration prior to the analysis, resulting in delayed results, and usually requiring specialized instruments and reagents and highly-skilled operators. Most particularly, immunoassays require typically from 60 to 90 minutes of processing that hinders their implementation for routine diagnostic applications that often require rapid and frequent parameter measurements, such as decentralized point-of-care (POC) testing of insulin currently being developed as an adjunct to improve diabetes control. In this work we propose and demonstrate the theoretical framework behind the establishment of a universal slope for calibration-free direct insulin quantification in serum samples with an amperometric insulin immunosensor. This new quantitative analysis approach relies on the unique measurement of the sample without applying standards and can thus dramatically simplify and shorten the assay while significantly reducing the reagent costs. Such substantial improvements are expected to facilitate successful decentralized POC insulin testing. We demonstrate this concept for serum insulin quantification in samples from individuals with type 1 diabetes using meticulous statistical analysis to support the prospective applicability of the universal slope approach.

1. Introduction

Over the past two decades, research in the field of chemical sensors has focused on the development of miniaturized devices capable of providing measurements at the point of need, away from controlled centralized laboratory settings [Sempionatto, 2021]. In fact, decentralized sensing in decentralized and remote locations is presently a reality. Clinical measurements of many key blood parameters are now routinely performed at the point of primary care [Bakker, 2016]. Home self-testing glucometers [Klatman, 2019] and recent Cov-19 diagnostic strips [Pinheiro, 2021] are representative examples of successful implementation of decentralized measurements. An important part of efforts to enable decentralized monitoring has been directed towards the development of sensor devices built using scalable low-cost, yet robust, fabrication technology and ideally defined by portability, user-friendliness, and reliable quantitative analytical assays [Giannoulas, 2019]. Despite the tremendous promise offered by mobile sensor devices, the number of measurable analytes is still limited.

One of the main drawbacks limiting the widespread application of biosensors in decentralized and field locations is the need for individual and frequent recalibration, which leads to sample-to-answer delays and usually requires specialized instruments and high-skilled operators [Rousseau, 2021]. In the case of the "auto-calibrated" glucose meter this has been addressed by a factory pre-calibration of strips [Heller, 2008] or using a 'built-in' calibration, in which each blood glucose measurement is calibrated using an internal standard present in the capillary sample chamber [Noble, 2014]. The calibration of the measuring device is an essential part of performing an analytical methodology with quantitation capability [Hyk, 2013]. Comparison against calibration standards of known analyte content allows determination of the unknown analyte content in a sample. Among the different calibration methods, calibration by standard additions (SA) is the most common approach to account for matrix effects or the lack of sensitivity of a given analytical method limiting sample dilution. Indeed, it is the common operation in measuring physiological levels of biomolecules in real biological samples. In this case, the calibration standards are added directly to the sample prior to analysis. The unknown endogenous analyte concentration in the sample problem is determined by extrapolation to zero value of the instrument response [Brown, 2012]. However, the SA method is time-consuming, greatly increasing the total assay time, its complexity and cost of reagents, often requiring a full calibration curve constructed with several increasing standard additions for each sample to be analyzed [Hyk, 2013]. In practice,

when a SA-based analytical method is used routinely and high efficiency is required in terms of time, workload and resources, it is usually reduced to a simplified approach of one- or two-point calibration performing a reduced number of replicas at each level as long as the calibration curve has been previously well-characterized by proper statistical treatment and method validation to ensure the reliability and quality of the analytical results [Peters, 2007] [Cuadros-Rodriguez, 2007] [Hyk, 2013]. Recent efforts have been realized for reduction of the extent of the calibration step. These include a calibrant-loaded paper-based sensor device performing one-point for external and standard addition calibration [Giannoulas, 2019], and a simplified calibration-based immunosensor relying on an integrated calibration and analysis on board of the same biosensor through calculation of a constant [Hervas, 2010]. Furthermore, implementation of calibration-free sensors has been demonstrated as feasible, not without requiring of highly robust development processes involving the optimization of sensing materials, fabrication and assay protocols, and the use of the most adequate readout methodologies. [Bakker, 2016]. Several calibration-free sensors have been reported recently including SPR-based sensors relying on calibration-free concentration analysis (CFCA) [Hu, 2020] [Shah, 2015], or potentiometric aptamer-based methodologies [Li, 2017] [Li, 2019] [Idili, 2019], interdigitated electrodes [Kang, 2021], and ion selective electrodes [Rousseau, 2021].

The use of SA is particularly time consuming in the case of immunoassays, because each measurement typically requires 20-30 min, leading to complete assay times of over 60 or 90 min. Such immunoassays are widely used for routine decentralized diagnostic applications of diverse biomarkers that often require rapid timely information. One important example is decentralized point-of-care (POC) testing of insulin where effective management of diabetes is expected to rely on frequent insulin measurements with short sample-to-answer times [Wolkowicz, 2020]. Insulin has a critical role in the context of type 1 diabetes (T1D): quantifying this analyte could permit the understanding of insulin pharmacokinetics for individuals, especially under different physiological conditions. While insulin levels are currently measured using laboratory based methods, the development of point of care measurement of plasma levels of insulin would expand the ability to determine safe levels of insulin to guide further administration. In addition to the significantly long POC assays and corresponding delay in obtaining important timely clinical results, such standard-addition operations are labor intensive and costly (due to the use of additional reagents and steps).

In this work we propose and demonstrate the theoretical framework behind the establishment of a universal slope (US) for the case of an amperometric insulin immunosensor for calibration-free direct quantification in serum samples. Our new US-based quantitative analysis approach aims at dramatically shortening the assay time and simplifying the assay protocol while dramatically reducing the reagent costs. The US is presented as a constant estimated as the average of the slopes obtained in the calibrations constructed by SA in serum samples collected from numerous individuals (**Figure 1**). This constant will allow direct determination of serum insulin by recording solely the signal corresponding to the sample without the need for time-consuming insulin standards steps, similarly to what has been implemented for glucose strips with direct calculation of glucose through a constant. The concept has been demonstrated using our recently reported sandwich format enzyme (HRP)-based insulin immunosensor, whose analytical protocol commonly relied on SA calibrations with insulin standard-spiked serum samples [Vargas, 2022]. Such a sensor was applied for decentralized clinical testing, monitoring serum insulin variation profiles of individuals with type 1 diabetes and the results were compared with a centralized lab-based ELISA method [Aiello, 2022]. In this manuscript we explore the feasibility of using the US approach to quantify serum insulin in the samples that were run in those clinical tests, using robust statistical analysis to support the significance of the results obtained. Such US should incorporate the intra- and inter-personal variations given by the complexity of the serum sample matrix affecting the analytical performance of the immunosensor, besides alleviate the need of calibration by the end-user due to the sensor manufacturing process [Li, 2019]. The US provides an attractive approach to bring closer the implementation of disposable insulin immunostrips, coupled with hand-held readers, toward substantially faster, simpler and cheaper POC insulin testing, allowing timely interventions in the management of diabetes in particular [Wolkowicz, 2020], [Vargas, 2022] [Aiello, 2022]. While the new US concept is demonstrated here in connection to insulin diabetes testing, it can be readily adapted to the rapid decentralized immunoassays of a wide range

of clinically-relevant biomarkers (e.g., cardiac ones), where timely interventions are critical.

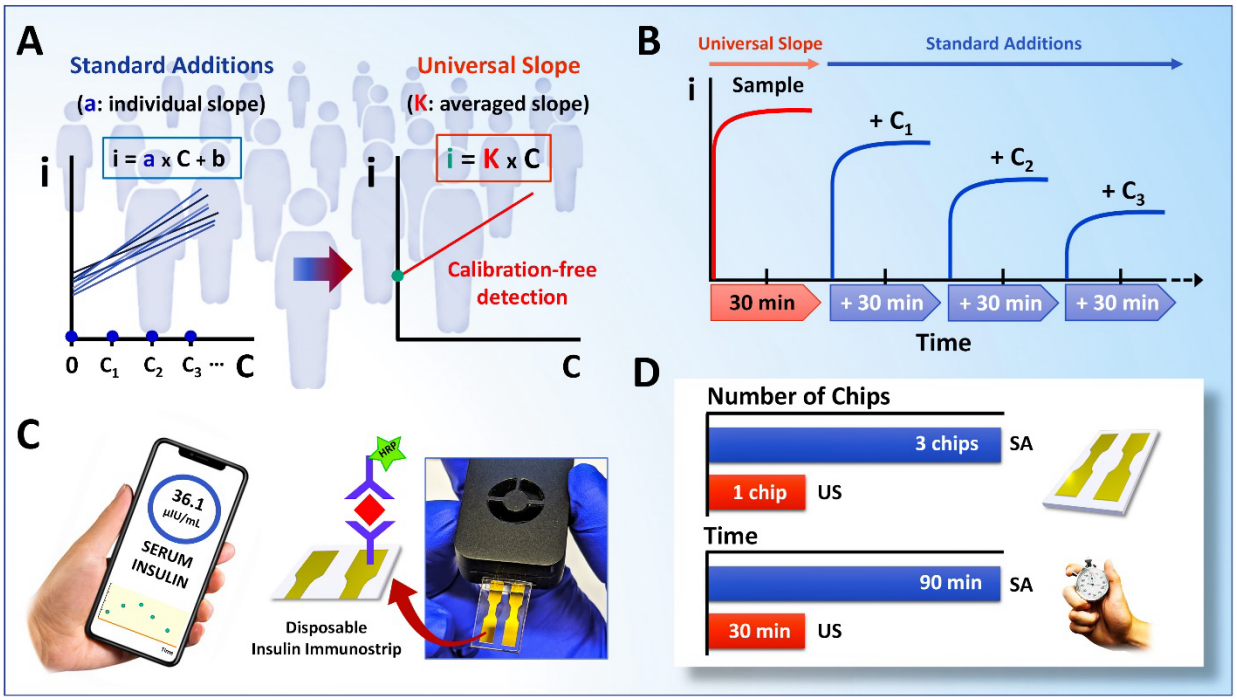

Figure 1. The concept of the universal slope. A) Comparison of the SA- and the US-based analytical approaches: the quantitation equation obtained by multi-point calibration realized in each sample with the measurement of several standards, and the single measurement-based expression for quantitation through the averaged US. B) The timeline representing the duration of each analytical protocol involving 30-min incubations for each concentration test run: the 30-min analysis of the US-based protocol vs. the 120 min of the three-point standard additions calibration. C) The conceptual idea of a disposable insulin strip interfaced with a hand-held reader for frequent on-site insulin measurements. D) Visual representation of the advantages of applying the US approach enabling to reduce the assay analysis and resources use compared to the traditional SA method. Abbreviations: HRP – horseradish peroxidase; SA – standard additions; US – universal slope.

2. Experimental section

2.1.Insulin immunosensor fabrication and assay protocols

Full details on the chemicals, instrumentation, and procedures used in the fabrication, bioassays, and amperometric measurements of the insulin immunosensor mentioned in this work can be found in our previous articles [Vargas, 2022] [Aiello, 2022]. Briefly, sensors were batch-fabricated in UC San Diego by metal sputtering deposition on plastic substrates, with each sensor consisting of two rectangular gold electrodes acting as working and joint reference/counter electrodes, respectively. The working electrode of each chip was functionalized with an

alkanethiol-based self-assembled monolayer for the covalent attachment of the anti-insulin antibody and a deactivation reaction on the capture antibody-modified surface to prevent non-specific binding was performed. The immunostrips were brought to Sansum Diabetes Research Institute (SDRI) in Santa Barbara for on-the-spot insulin determination in untreated venous blood serum samples collected from individuals with type 1 diabetes. The quantitation of insulin was realized by SA-based calibration method using 1-step 30-min HRP (horseradish peroxidase)-labeled sandwich immunoassays in which the sample and the enzymatic tracer are incubated simultaneously. Thus, a 10-µL sample droplet of undiluted serum non-spiked or spiked with insulin standard and supplemented with the HRP-tagged anti-insulin detector antibody was incubated for 30 minutes onto the working electrode of the insulin immunostrip. Once the immunoreaction was finalized, the chip was washed and dried for subsequent amperometric readout in the presence of the H_2O_2 enzymatic substrate and the 3,3',5,5'-tetramethylbenzidine (TMB) redox mediator.

2.2.Insulin immunosensor quantification methods

Each insulin determination using the SA was carried out by a 3-point standard addition calibration, achieving the measurement of serum samples with and without spiked insulin standard at concentrations of 200 pM and 400 pM. For each calibration point, three replicate measurements using three different chips were performed. T Serum insulin concentrations were calculated by extrapolation using the calibration equations obtained from the sensor measurements, estimated by least squares linear regression method using Microsoft Excel. The error bars shown in the insulin concentration values quantified by SA method (shown in **Figure 3**) correspond to the error in the analyte concentration quantification estimated through the typical error of the calibration as indicated previously [Aiello, 2022]. In the case of the US-based approach we estimated the insulin concentrations by dividing the amperometric signal recorded for the serum samples without spiked insulin standard by the aaverage slope, described in detail in the results section below.. The error bars shown in the insulin concentration values calculated through the averaged slope were estimated as standard deviation of three replicates.

2.3.Statistical analysis

An ANalysis Of VAriance (ANOVA)test was performed for comparison of the slope data obtained by the SA method with the US method.. A Lilliefors test and a Q-Q plot were performed to assess the log normal distribution of the slope data set and accurate average slope value estimation. A Spearman rank-order correlation analysis was performed (MATLAB) to evaluate the correlation between the insulin concentration values estimated with the SA and the US methods of the decentralized insulin immunosensor and the ELISA method measured in a centralized lab (

The level of agreement between the different insulin quantitation methods was evaluated by representing Bland-Altman plots, and the differences in the accuracies in the insulin quantifications were compared through the estimation of the absolute error (AE) and by applying a Wilcoxon signed rank test for paired comparisons of the accuracies. These analyses were realized and represented using OriginPro 2016.

3. Results and discussion

3.1. Assessment of the universality of an averaged slope and its estimation

The demonstration of the feasibility of the US approach and the determination of its value represents the starting point of the analysis (**Figure 2**). The prospective universality of an averaged slope was assessed with statistical methods like ANOVA. For this analysis, all the subjects were included except for patient HS1-07 as this individual was tested using different experimental assay conditions [Aiello, 2022]. An ANOVA test is usually applied used to determine whether there are any statistically significant differences between the means of three or more independent groups using the F-distribution [Freedman,2007]. Specifically, we performed the ANOVA test to compare the means of the slope data between participants and determines whether any of those means are statistically significantly different from the mean within participants. The null hypothesis (H_0) for the test is that there is no difference in means between participants. The alternative hypothesis (H_a) is that at least one pair of means is different. If there are n participants:

- H_0: $\mu_1 = \mu_2 = \mu_3 = \ldots = \mu$
- H_a: At least two of the group means $\mu_1, \mu_2, \mu_3, \ldots, \mu_n$ are not equal

where μ represents the mean within participants, and μ_i, i=1,2…, n are the individual means.

The ANOVA uses the F-statistic, that computes the ratio of the variability between participants and the variability within participants. If F is small, the hypothesis of equal means is accepted, so

the means are significantly different because the variability between participants is larger than the variability within participants. If F is large, the null hypothesis is rejected, and it means that all means are the same and the differences are due to random variation. As in other test statistics the determination of the width of F is given by the p-value, whose significance level was set equal to 0.05. If the p-value is less than 0.05, the null hypothesis is rejected, although it will not tell you which participants were different. The ANOVA test on the slope data set accepted the null hypothesis with p-value=0.30, providing the statistical evidence that there is not a significant statistical difference in the slope data between different participants. **Figure 2A** shows a pairwise comparison of the slope data between participants. The blue circles and blue dashed bars represent the means (μ_i, i=1,2…, n) and corresponding confidence intervals for the participants. The red solid vertical line represents the global mean value estimated (μ) and the red shaded area limited by the two red dashed vertical lines represents the confidence interval for the slope data set. It is worthwhile to note that the confidence intervals for the eight participants intersect with the intervals for the mean of the slope data set. This intersection indicates that the variance of the overall slope data set is approximately the same as the variance of each of the participants.

To estimate the value of an averaged slope fairly representing the sensitivity of the insulin sensor in human serum samples, we identified the density function of the slope samples. To achieve this task, we considered a wide set of 68 slope values obtained with our insulin immunosensor in SA calibrations realized to quantify the serum insulin in samples acquired from San Diego Blood Bank and SDRI during sensor development and applicability tests. From the histogram of the data (**Figure 2B**), it can be assumed that the data are lognormally distributed. To verify this hypothesis, a Lilliefors test of normality is applied to the data samples with significance level 0.05. A Lilliefors test computes the maximum discrepancy between the empirical distribution function and the cumulative distribution function of the Normal distribution and assess whether the maximum discrepancy is large enough to be statistically significant [Lilliefors, 1967]. We log transformed the data and tested the null hypothesis that the log-transformed data follows a normal distribution. The null hypothesis was accepted with p-value=0.0029. Additionally, the acceptance of the null hypothesis can be visually verified by using a Normal Q-Q plot, or quantile-quantile plot. A Q-Q plot is created by plotting two sets of quantiles against one another and if both sets of quantiles came from the same distribution, the values lie along a straight. **Figure 2C** shows that the log-transformed points seem to fall about a straight line, verifying that the assumption on the

nature of the density function of the slope samples is correct. Maximum likelihood estimation method was applied to estimate the parameters of the probability distribution. Specifically, the estimated mean of the log-normal distribution was $e^{\mu+\frac{\sigma^2}{2}}$=0.11 (nA/pM), with a confidence interval = [0.1015, 0.1350] (nA/pM), resulting in the parameter for the averaged slope.

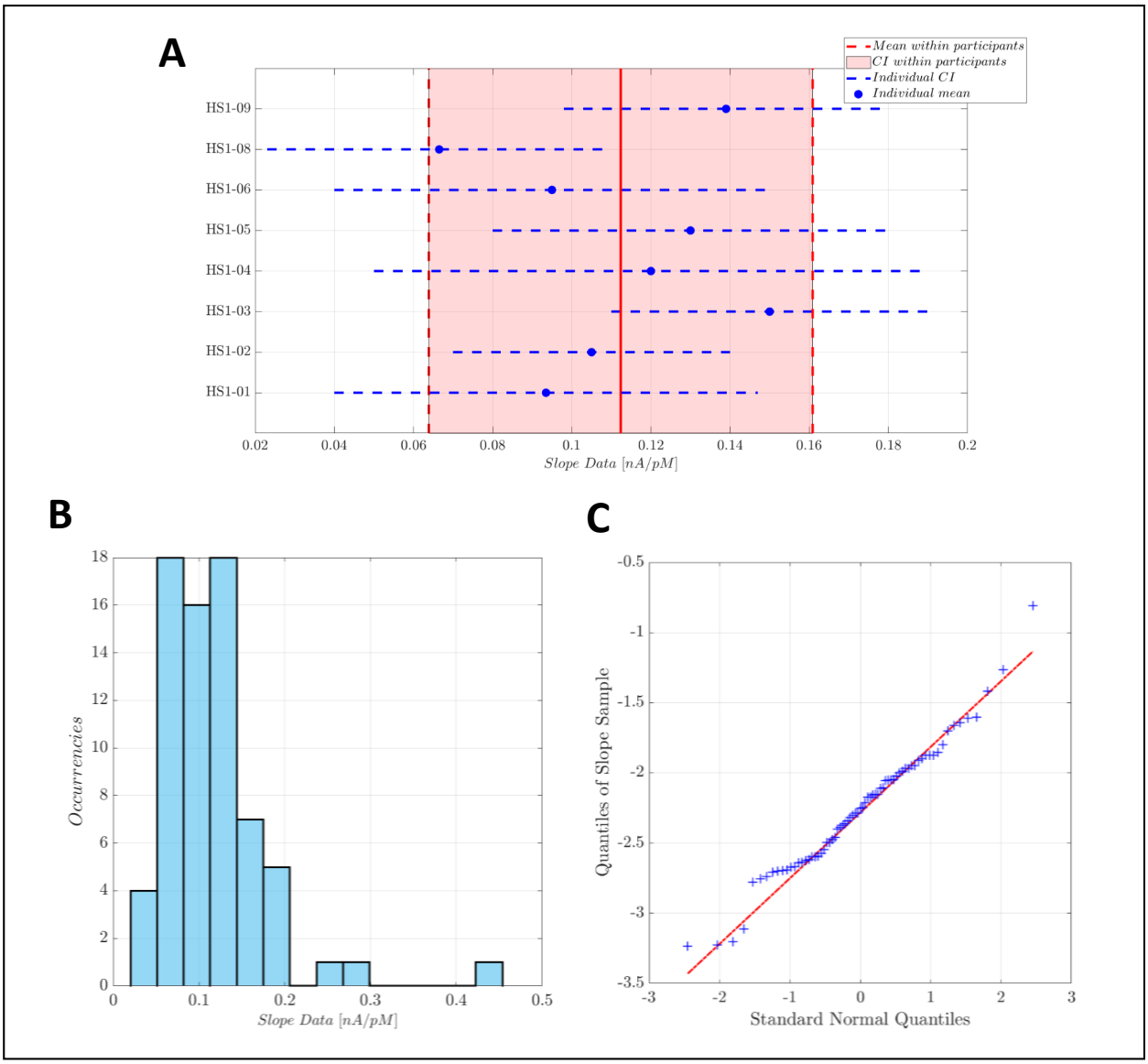

Figure 2. Demonstration of the viability of the Universal Slope and identification of its value. A) Pairwise comparison of the slope data between participants: blue circles and blue dashed bars are the means and confidence intervals (CI) for each one of the participants, respectively; the red solid vertical line is the mean within participants and the red shaded area limited by the two red dashed vertical lines is the confidence interval (CI) for the overall slope data set. Probability density function of the slope data set: B) histogram in which the x-axis of the data samples (the

slopes data, N = 68) has the bins, whose number is equal to the square root of the number of data samples, while the frequency of the data samples is reported on the y-axis. C) Q-Q plot displaying the sample data (blue) against the expected value for a Normal distribution (red) at each quantile in the sample data. The sample data closely follows the red straight line, suggesting that the sample has an approximately normal distribution.

3.2.Decentralized calibration-free serum insulin quantification through the universal slope

Once the value of the averaged slope was estimated, we applied the US analytical method approach to the data collected on-site at the clinic for insulin measurements in serum samples from the participants with type 1 diabetes using our immunostrips. To achieve this task, we used the estimated average slope value of 0.11 to calculate the insulin concentration contained in each serum sample, considering in this case only the sensor responses recorded for the samples in absence of insulin standard, in other words, the measurements registered during the first 30 minutes of analysis of each sample. The concentration values determined using the averaged slope approach have been compared to results obtained by a traditional SA protocol performed on-site at the clinic with our insulin immunosensor, and to ELISA results that were provided by a centralized lab from the shipped samples (**Figure 3**). These results illustrate that the insulin levels estimated through the averaged slope closely correlate with the values obtained by the SA method.Comparison with the insulin levels obtained by the reference ELISA method suggest potential of the US parameter for correcting the sensor insulin levels by bringing them closer to those provided by the ELISA assay. It is noteworthy that the US method potentially would allow reducing the analysis time from the ~ 90 min needed with the three-point SA calibration-based determination developed for our insulin immunosensor, involving measuring two standard-spiked serum samples, to the 30 min that would involve the single measurement of the signal corresponding to the sample.

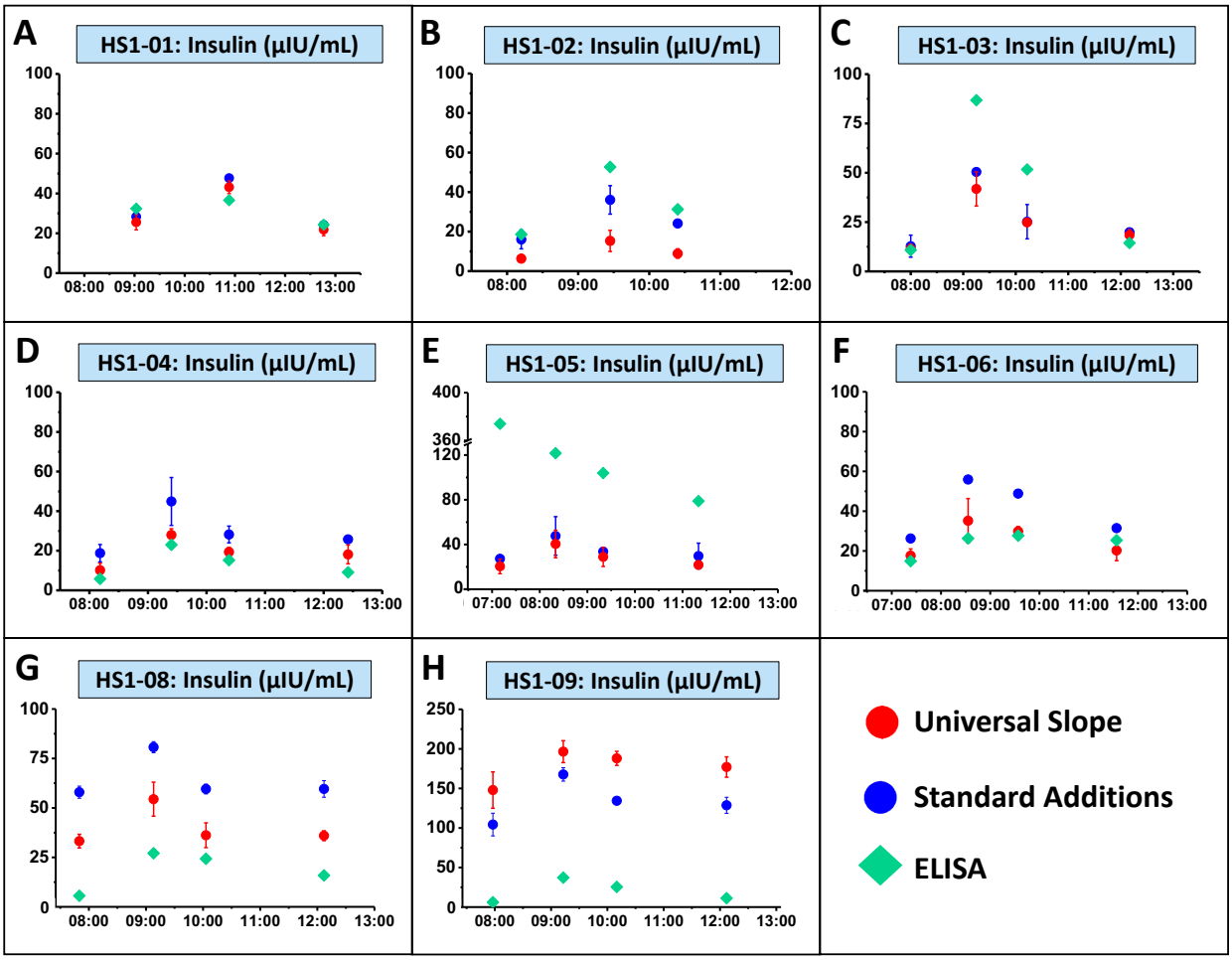

Figure 3. Serum insulin levels determination using the universal slope. Insulin concentrations quantified (expressed in µU/mL) in serum samples collected from eight different individuals with type 1 diabetes and personal profiles of insulin variation over time. Blue and red spots are the insulin concentrations determined using the SA- and US-based methods performed with the insulin immunosensor, respectively, and the green diamonds are the insulin levels determined by an ELISA kit method in a centralized lab. The error bars shown in the insulin concentration values quantified by SA method correspond to the error in the analyte concentration quantification estimated through the typical error of the calibration. The error bars shown in the insulin concentration values calculated through the US were estimated as standard deviation of three replicates measured with three different chips.

3.3. Validation of the averaged slope with correlation analysis

To assess the validity of the averaged slope method, we compared the insulin levels obtained by the new approach with the insulin concentrations provided by the ELISA, as well as the SA method. Correlation analysis was performed using the Spearman rank-order correlation method to assess the relationship among different methods considered for insulin determination.

The Spearman rank correlation coefficient (ρ_s) measures the probability of concordance between random variables based on the ranked values for each variable rather than the raw data. Therefore, ρ_s is invariant to strictly increasing transformations, such as a log-normal transformation. It can be shown that that when the definition of the Pearson correlation is applied to ranked data [Hotelling, 1936], it results in:

$$\rho_s = 1 - \frac{6\ \sum_{i-1}^{d} D^2}{k(k^2 - 1)}$$

where $D = r(x_i) - r(y_i)$ is the difference between the two ranks of each observation, $r(\cdot)$ is the rank corresponding to the scores from greatest to smallest value, and k is the number of samples. Spearman rank approach allows reduction of the effect of potential outliers on the correlation, because it measures the degree to which large or small values of each random variable is associated with large or small values of the other random variable. The ρ_s range from –1 to +1, where 0 indicates that there is no monotonic association, and the relationship gets stronger as the coefficient approaches an absolute value of 1. Hypothesis tests can be used to address the statistical significance of the results. We verified whether the novel method could replicate the strength of the association present between the insulin levels quantified by the SA methods and those from the laboratory insulin ELISA. The correlation coefficient between the averaged slope and the ELISA quantification of insulin levels ($\rho_s = 0.60$) was similar to the coefficient describing the relationship between the SA and ELISA approaches ($\rho_s = 0.72$). Specifically, both correlations were statistically significant ($p<0.01$) , showing that the novel US approach is reliable for insulin quantification compared with the ELISA method. An additional verification was conducted to assess the equivalence of the novel method with the well-known SA approach. With a correlation coefficient close to 1 ($\rho_s = 0.91$) and p-value less than 0.01, we tested that the averaged slope approach can reliably quantify insulin levels, which are in accordance with those obtained by the SA method. **Figure 4** shows the correlation plots for the ranked variables together with the correlation coefficients corresponding to each association (ELISA vs. SA (**Figure 4A**); ELISA vs. US (**Figure 4B**); and SA vs. US (**Figure 4C**)) where the shaded areas highlight the orientation and the strength of the association: stronger relationships mean relationships where the points are denser on the bisector.

On the other hand, the Bland-Altman method allow to quantify the level of agreement between two analytical methodologies by providing the mean bias for all measured data points

between the two quantitative measurements for the same variable and by establishing limits of agreement. For proper evaluation, a graphical approach is employed. The constructed graph is a scatter plot XY where the x-axis is the average of two paired measurements, and the y-axis is the difference between such measurements. The statistical limits of agreement are calculated through the average and the standard deviation (SD) of the difference between the paired measurements, so that the 95% of the point of the data set should be found within ± 1.96 SD of the mean difference [Giavarina, 2015] [Bland, 1999]. In this case, we used the ELISA measurements as reference for comparison with the insulin concentration values determined with the SA and the US methods performed with the immunosensor, and the SA method for reference vs. the US approach. **Figure 4D** and **4E** displays the Bland-Altman comparison plots for ELISA insulin concentration values vs. those obtained with the immunosensor by applying the SA and US approaches, respectively. In both cases, the insulin data points would be similarly distributed within the limits of agreement, showing just one outlier. The mean bias values obtained are -5 µiU/mL and -1 µiU/mL for the SA and US comparisons, respectively; both values quite close to 0, meaning that the difference between the ELISA and both immunosensor methods are minimal. The negative signal in the bias describes the tendency of getting higher concentration values for the immunosensor compared to the reference ELISA, which can be ascribed to the fact that the ultralow insulin levels to be quantified would be close to the limit of detection of our insulin immunosensor [Vargas, 2022]. It is worth to point out that such bias value is lower in the case of the US-derived data, reflecting the previously mentioned capability of the averaged slope parameter to correct the insulin measurements. This is also observed in the Bland-Altman plot for comparison of the SA method with the new US approach (**Figure 4F**), where the mean bias value of 4 µiU/mL confirm the systematically higher insulin concentrations determined by the SA method. In this case, three insulin data points would lie beyond the lower limit as exceptionally higher insulin values provided by the US method, since most of the data remains in the upper half of the agreement area. Such area is remarkably narrower compared to the ELISA-immunosensor plots indicative of a high correlation between both immunosensor quantitation approaches. Finally, the sensor accuracy in the quantification of the insulin concentrations in the 30 serum samples analyzed using the SA and the US methods has been compared with the ELISA through the estimation of the absolute error (AE) as $AE=|ELISA-Sensor|$. Likewise, the AE in the determination of the insulin levels using the US new approach has been compared with the established SA as well. The distribution of the

resulted AE values for each comparison have been represented in **Figure 4 (G-I)**, whose mean difference values are (47.5 ± 25.2) µiU/mL, (49.6 ± 28.9) µiU/mL, and (15.6 ± 5.2) µiU/mL, corresponding to α=0.05, for the difference between ELISA – SA (**Figure 4G**), between ELISA – US (**Figure 4H**), and between SA – US (**Figure 4I**), respectively. In the case of the ELISA vs. sensor's methods comparisons the distributions are quite similar. In order to verify if there are statistically significant differences between the accuracy provided by the distinct analytical methods, firstly, a D'Agostino-Pearson test was realized to evaluate the normality of the AE data [Yap, 2011], obtaining p-values < 0.005 in all cases, meaning that the data set is not normally distributed. According to this, we run a Wilcoxon signed rank test as nonparametric tests for paired comparisons of the accuracies [Rosner, 2006]. The Wilcoxon test yielded p-values of 0.5133, 0.0155, and 0.2132 for the paired comparison of the AE between the ELISA and sensor (SA & US) approaches (Figure 4G vs. Figure 4H), between the ELISA – SA vs. SA – US (Figure 4G vs. Figure 4I), and between ELISA – US vs. SA – US (Figure 4H vs. Figure 4I), indicating that the difference in the absolute error committed would be statistically significant only when the ELISA and the US methods are compared with the SA, whereas this assumption is not applicable when comparing the accuracy provided by both the sensor's SA and US methods with the ELISA, or even both the ELISA and the SA methods with the new US approach. These results suggest that the quality of the analysis performance provided by the immunosensor when applying the US approach for quantitative determination of insulin is not impaired compared to that provided by the traditional SA method.

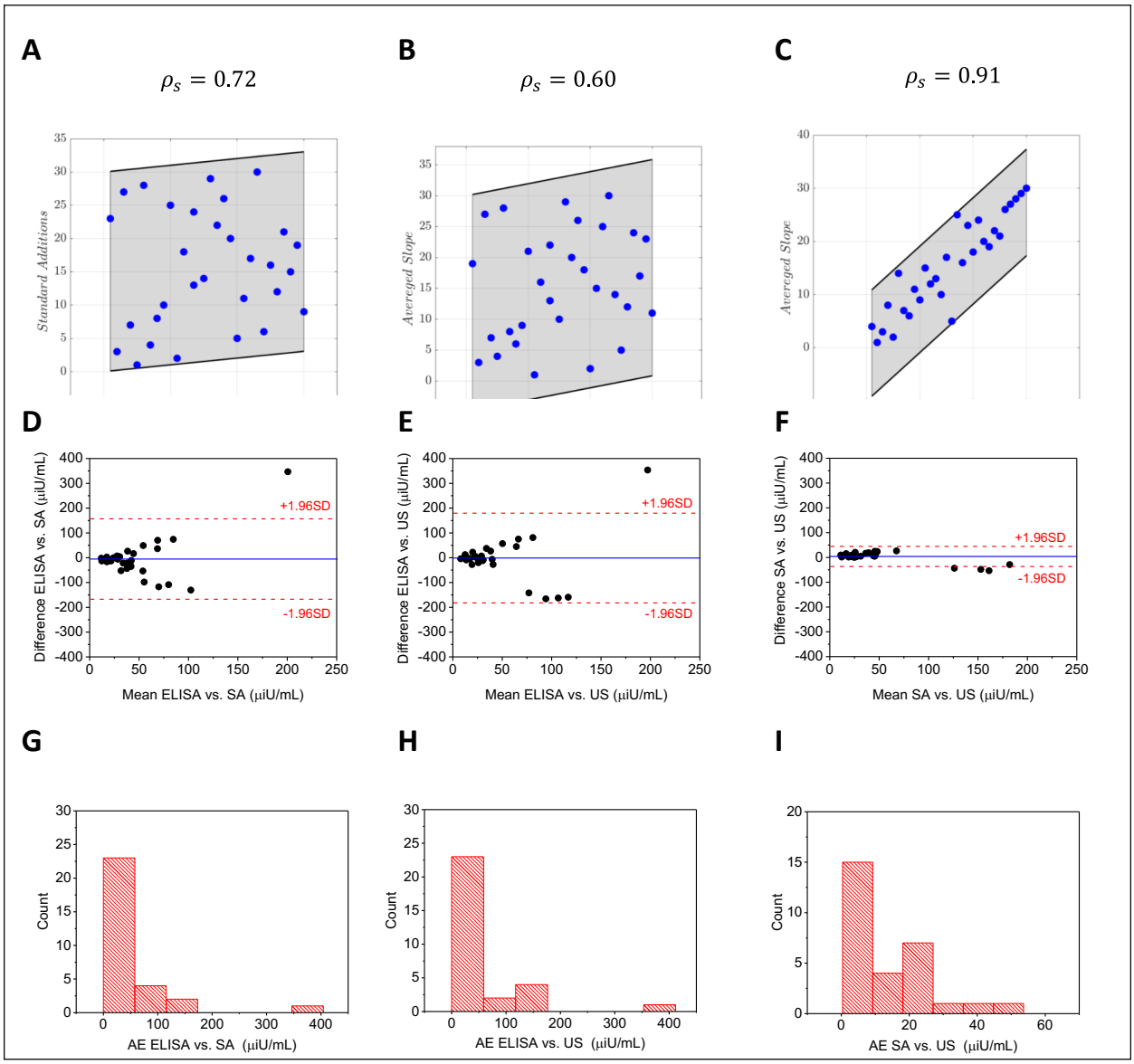

Figure 4. Correlation plots comparing the three quantification approaches. (A-C) Correlation plots for the three variables representing the ranked insulin levels obtained by a centralized ELISA method, and by the decentralized immunosensor operating through the SA and through the US methods in the serum samples collected from eight individuals with T1D. (D-F) Bland Altman comparison plots on the serum insulin concentrations data obtained by the ELISA, and by the insulin immunosensor using the SA and the US approaches. Blue solid and red dashed lines correspond to the mean bias values and the upper/lower limits of agreement, respectively. (G-I) Histograms displaying the distribution of the AE in the quantification of the serum insulin when comparing the reference ELISA method vs. the immunosensor's SA and US methods, and the US approach vs. the traditional SA based immunosensor method.

4. Conclusions

In this work, we have demonstrated by thorough statistical analysis the feasibility of establishing an averaged slope for calibration-free direct quantification of insulin in serum samples through the insulin measurements performed with an amperometric immunosensor chip, as an alternative to the SA calibration method. Our new analytical approach based on direct insulin quantification through a US constant would allow direct quantitation of the insulin concentration by performing just the measurement of the sample with the immunostrip without the need for subsequent time-consuming insulin standard additions. Such a greatly simplified immunoassay operation reduces dramatically the total assay time and costs and facilitate the implementation of POC insulin measurements. Factors such as personnel hand skills, environmental conditions, or workplace equipment would affect to a much lesser degree the insulin quantification outcomes. Furthermore, since the new US-based analysis method would minimize the serum sample volume requirements for the quantification, the potential applicability of our insulin immunosensor for capillary blood could be easily explored. The use of capillary blood samples would add great convenience to the analysis protocol by less invasive sampling procedures that can be performed at any remote location and allow reduced sample-to-answer times and costs. Moreover, the US-based method would reduce the error committed during the SA-based insulin quantification protocols ascribable to the cumulative sample dilution effects due to the various additions of insulin standards made to the different sample aliquots for the numerous incubations for the construction of the calibration. To advance this US-based insulin analytical approach toward truly auto-calibrated insulin strips development, further correlation analyses with a wider group of participants with diabetes should be performed, in order to assess whether any physiological condition would affect the reliability of the quantification with the insulin immunosensor. In this sense, clarification of the goals of the application will be important - whether the rapid delivery of frequent insulin measurements is more important than providing accurate absolute concentration values, which makes the accuracy of the data results provided by the US approach less rigorous. Furthermore, in the process of validation and correlation analysis of the US-based insulin sensor method, the choice of distinct existing analytical methodologies of reference (e.g., ELISA kits) that provide different accuracy and robustness towards the matrix of interest could greatly affect the outcome. Such substantial improvements in the speed, simplicity and costs will facilitate the realization of decentralized insulin testing towards improved management of diabetes. While the concept of US has been

presented in connection to inulin immunostrips, it can be readily expanded to decentralized testing of broad range of target biomarkers.

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Competing interests

J.E.P. is currently an employee and shareholder of Tandem Diabetes Care, Inc. The work presented in the manuscript was performed as part of his academic appointment at Sansum Diabetes Research Institute and is independent of his employment with Tandem Diabetes Care.

E.D. reports receiving grants from JDRF, NIH, and Helmsley Charitable Trust, personal fees from Roche and Eli Lilly, patents on artificial pancreas technology, and product support from Dexcom, Insulet, Tandem, and Roche. E.D. is currently an employee and shareholder of Eli Lilly and Company. The work presented in this manuscript was performed as part of his academic appointment and is independent of his employment with Eli Lilly and Company.

F.J.D. reports equity, licensed IP and is a member of the Scientific Advisory Board of Mode AGC.

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All other authors report no conflict of interest.

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