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RESEARCH ARTICLE

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Feasibility of precise and reliable glucose quantification in human whole blood samples by 1 tesla benchtop NMR

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The standard procedure for blood glucose measurements is enzymatic testing. This method is cheap, but requires small samples of open blood with direct contact to the test medium. In principle, NMR provides non-contact analysis of body fluids, but high-field spectrometers are expensive and cannot be easily utilized under clinical conditions. Low-field NMR systems with permanent magnets are becoming increasingly smaller and more affordable. The studies presented here aim at exploring the capabilities of low-field NMR for measuring glucose concentrations in whole blood. For this purpose, a modern 1 T benchtop NMR spectrometer was used. Challenges arise from broad spectral lines, the glucose peak locations close to the water signal, low SNR and the interference with signals from other blood components. Whole blood as a sample comprises even more boundary conditions: crucial for reliable results are avoiding the separation of plasma and cells by gravitation and reliable reference values. First, the accuracy of glucose levels measured by NMR was tested using aqueous glucose solutions and commercially available bovine plasma. Then, 117 blood samples from oral glucose tolerance testing were measured with minimal preparation by simple pulse-acquire NMR experiments. The analysis itself is the key to achieve high precision, so several approaches were investigated: peak integration, orthogonal projection to latent structure analysis and support vector machine regression. Correlations between results from the NMR spectra and the routine laboratory automated analyzer revealed an RMSE of 7.90 mg/dL for the best model. 91.5% of the model output lies within the limits of the German Medical Association guidelines, which require the glucose measurement to be within 11% of the reference method. It is concluded that spectral quantification of glucose in whole blood samples by high-quality NMR spectrometers operating at 1 T is feasible with sufficient accuracy.

Abbreviations: CLab, Central Laboratory; FH, blood collection tube containing sodium fluoride and sodium heparin; FWHM, full-width-half-maximum; OGTT, oral glucose tolerance testing; (O)PLS, (orthogonal) projection to latent structures; PCA, principal component analysis; PoC, point of care; RMSE, root mean square error; SVM, support vector machine; ZKT, Zentrum für klinische Transfusionsmedizin Tübingen.

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KEYWORDS
applications, body, diabetes, methods and engineering, MRS and MRSI methods, post-acquisition processing, quantitation, spectroscopic quantitation

1 | INTRODUCTION

Assessment of blood glucose levels several times a day is crucial for diabetic patients in order to prevent serious consequences of hypo- or hyperglycemia and to optimize insulin therapy. Monitoring of glucose levels is also often necessary in patients with extra-corporal circulation during hemodialysis or in the course of some types of surgery. It is essential to keep the blood glucose concentration within the range of 70-180 mg/dL.[1] In addition, measuring glucose levels in units of stored blood is helpful for quality control.[2] Devices for measuring glucose in blood samples in laboratories are often stationary, leading to distinct time delays between drawing blood and providing quantitative results. Therefore, mobile test devices are often used for monitoring blood glucose levels in diabetic patients and for surveillance of medical interventions. Most common types of point-of-care (PoC) device, eg the HemoCue glucose analyzer (Ängelholm, Sweden), use enzymatic reactions and light transmission detection for glucose monitoring, but inherently depend on mixing the whole blood with additional substances prior to measurement. Continuous glucose monitoring systems allow tracking of glucose levels throughout the day, eg by a small sensor that is positioned in the subcutaneous interstitial fluid. Its glucose levels, which are slightly delayed compared with blood glucose levels, are read out by the sensor and wirelessly transferred to a receiver that stores the data and visualizes it to the patient.[3] However, all techniques require invasive procedures, potentially leading to an increased risk of contamination or infection for the patient and/or the personnel.

MR techniques are well known to provide fully non-contact assessment of the chemical composition of samples. Relaxometric NMR measurements were reported to be one option,[4,5] NMR spectroscopy revealing specific chemical shift components of interesting molecules another. The peak area of a substance directly correlates to its concentration, making NMR spectroscopy a non-destructive, robust and highly reproducible technique for quantitative analysis.[6] However, the assessment of blood glucose in an extracorporeal system or even inside the body is challenging.

Glucose molecules ($C_6H_{12}O_6$) exhibit a relatively complex spin system with NMR signal pattern details dependent on the magnetic field strength. High-field spectrometers provide high sensitivity and small linewidths and therefore good separation of the glucose signals from other signal components in blood, but conditions worsen for decreasing magnetic field strengths and lower field homogeneity. Newly developed very small permanent magnets provide homogeneous fields of limited field strengths up to approx. 1.5 T.[7] In order to explore the feasibility of such approaches for direct whole blood analysis, this work focuses on testing the applicability of low-field NMR spectrometers. For our study we chose a 1 T benchtop spectrometer and moderate acquisition times of less than 15 min, but confined ourselves to simple methods: ^{1}H FID acquisition after 90° RF pulse excitation (pulse-acquire spectroscopy) without any dedicated water suppression or sophisticated sample preparation.

Since spectral complexity is reduced in blood plasma compared with whole blood as additional signals and line broadening effects resulting from blood cells are avoided, most former studies on assessment of glucose concentrations have been carried out in blood plasma or serum.[6,8] Luaibi et al investigated glucose level accessibility in whole blood at 400 MHz.[9] Urine glucose concentrations were successfully determined and plasma samples were analyzed by Percival et al using a benchtop NMR instrument operating at 60 MHz, motivating further investigations of bio fluids by low-field spectroscopy.[10] Our study is the first one aiming at NMR assessment of blood glucose in whole blood using a low-field spectrometer. NMR spectra of water-based solutions such as plasma or whole blood show a dominating water resonance—its intensity is approximately 10 000 times higher than that of the metabolite signals. Signals close to the water peak—here glucose—are superimposed by the flank of the water signal and contributions from other blood metabolites. This prevents direct integration and quantification of the glucose signals and requires more dedicated multivariate analysis approaches. Further challenges include low SNR, broad resonances and the sample preparation itself to handle temporal glucose degeneration, oxygenation state and minimizing blood sedimentation.

The feasibility test of the low-field NMR for assessing the glucose concentration in whole blood was carried out in a stepwise approach: in the first step, aqueous solutions with known glucose concentrations were analyzed, followed by studies in bovine plasma in a second step. Finally, human whole blood samples taken during oral glucose tolerance testing (OGTT) were assessed by NMR spectroscopy and the results were compared with those of established methods.

2 | EXPERIMENTAL

All glucose level measurements were carried out on a 1 T benchtop NMR spectrometer (Spinsolve Carbon, Magritek, Aachen, Germany), operating at a sample temperature of 301 K. The only exception (due to limited availability) is the comparison of whole blood and plasma spectra, which

was conducted on a Spinsolve Carbon Ultra operating at 1.5 T and thus exhibiting higher SNR and better spectral resolution. Shimming was performed according to the manufacturer guidelines with a 10% H_2O and 90% D_2O sample each day before the OGTT. The shim currents were maintained during the typically 5-10 NMR tube switches during the OGTT.

2.1 | Device sensitivity and accuracy by measurements of aqueous glucose solutions

A general feasibility study of glucose level measurement by benchtop NMR was carried out by preparing four glucose solutions of different concentrations. Glucose powder (1.011 g; D(+)-glucose anhydrous, cell culture grade, AppliChem, Darmstadt, Germany) dissolved in water for injection (25 mL; Ampuwa, Fresenius Kabi, Bad Homburg, Germany) was used as a stock solution (4,040 mg/dL). 150 μL of it was diluted with four different volumes of water (11.85, 3.85, 2.25 and 1.56 mL). The resulting glucose concentrations, 50, 150, 250 and 350 mg/dL, were measured five times using a HemoCue Glucose 201 RT, a commonly used PoC device for in vitro blood glucose determination. The HemoCue automatically multiplies the results by 1.11 to convert whole blood inputs to plasma equivalent values.[11] We undo this transformation to avoid bias as we left the default sample type of the HemoCue with aqueous solutions. Each sample was also measured five times in a row with a ^{1}H-pulse-acquire sequence with repetition time 9 s, pulse angle 90°, dwell time 500 μs, 16 384 points, 128 averages and four dummy scans. One measurement took 20 min. The sample tubes were inverted once in between the runs to ensure that the same measurement conditions are met. Free induction decay data was imported to MATLAB R2018b (MathWorks, Natick, MA), zero-filled to twice the total number of points and Fourier transformed. The ppm axis for this series was referenced to the glucose peak at 3.42 ppm. In order to reduce user-dependent evaluation steps an automatic phase correction using the area minimization method,[12] and baseline correction,[13] were applied by using available algorithms. Direct integration of the glucose peaks is difficult as the background signal of the water resonance in this region may shift from sample to sample. Thus, a baseline correction with the Whittaker smoother was used to remove the water background in the spectral region of interest. First, the data was smoothed with a Savitzky-Golay filter comprising a first degree polynomial within a five-point range. Then, the Whittaker baseline parameters λ and p needed to be determined, which also required visual checks by the user to ensure a proper correction as already mentioned by Eilers.[13] The Whittaker smoother calculated a smoothed version for each spectrum as a baseline. This baseline was then subtracted from the spectrum and the remaining signal in the range from 3.3 to 3.9 ppm was integrated. The area was compared with the reference values in an *xy* plot and the relationship was assumed to be linear over the whole range and therefore fitted with a linear function by Passing-Bablok regression[14] (code used [15]). The resulting fit allowed calculation of the glucose levels via the NMR spectra. Finally, the root mean square error (RMSE) value for the NMR glucose compared with the reference method was determined and minimized by the MATLAB in-build function "fminsearch" for the visually confirmed start points λ = 100 and p = 0.001.

2.2 | Reproducibility of glucose determination in bovine plasma

The same measurement setup and evaluation principle were applied to a more complex solution of control glucose bovine plasma (GlucoTrol NG levels 1-4, Eurotrol, Ede, The Netherlands), as plasma also contains most blood proteins.[6] A 2.0% methanoic acid solution was prepared as internal standard for these measurements and the following whole blood measurements. Further rationale for the choice of this internal standard originates from the preparation of whole blood samples and will follow in the next section. Methanoic acid with 99% purity from the university pharmacy was used (unknown origin); the other component was water for injection, Ampuwa (see above). The plasma samples (600 μL) were mixed with 70 μL methanoic acid solution and the NMR tube was filled with 600 μL of the solution. The spectra were referenced to the methanoic acid peak.

2.3 | From plasma to whole blood

To gain insight into the differences between benchtop whole blood and plasma spectra, whole blood and plasma of the same sample were measured (at 1.5 T): whole blood was collected before blood donation at the Zentrum für klinische Transfusionsmedizin Tübingen (ZKT) into sodium fluoride (NaF) sodium heparin (Na heparin) blood tubes, FH (2 mL, Greiner Bio-One, Kremsmünster, Austria). The ethical committee of the University Hospital Tübingen granted ethical approval for blood collection for this study at the ZKT and the German Center for Diabetes Research (see below). Tubes containing heparin were used instead of the commonly used EDTA tubes for glucose analysis because of additional EDTA signals between 3 and 4 ppm.[6] The enzyme toxin NaF alone may not be sufficient to completely inhibit glycolysis in blood collection tubes, but glycolysis is greatly reduced by keeping blood pH between 5.3 and 5.9.[16] Therefore, it was beneficial to use methanoic acid as internal standard: we titrated the amount of methanoic acid (2%) to reach approximately pH 5.6. About 70 μL of methanoic acid had to be added to 600 μL of whole blood. Within the blood pH range of healthy humans (7.35-7.45) and even at the pH reduced by the addition of

the internal standard the pH shifts of glucose are small.[17,18] Furthermore, whole blood itself is a biological buffer, which caused us to refrain from pH buffer solutions. Further positive characteristics of methanoic acids are a single well separated spectral line and low natural abundance in blood samples.[19] The glucose concentration within the FH tubes did not decay significantly over 2 h (for a detailed analysis see Reference [20]), which is much longer than the spectrum acquisition time of 14 min. Methanoic acid combines the internal standard with acidification of the blood and therefore we consider it a good solution for blood spectra acquisition, especially if focused on glucose quantification. Plasma was generated from whole blood by centrifugation at 10 000 *g* and 22 °C for 10 min. In the same manner as for bovine plasma, 600 μL whole blood or plasma was mixed with 70 μL methanoic acid solution and then the NMR tube was filled with 600 μL of the solution. All NMR tubes were vortexed for 60 s before insertion and measurements started after 4 min temperature equilibrium. ^{1}H NMR one-pulse spectra were acquired with the following parameters: repetition time 4.5 s, pulse angle 90°, dwell time 500 μ, 8 192 points, 128 averages and four dummy scans (total duration roughly 10 min).

2.4 | Analysis of human whole blood samples

In the last step, 122 whole blood samples were measured. The common glucose concentration in healthy blood donors is roughly settled between 60 and 100 mg/dL. In collaboration with the diabetes department of the University Hospital Tübingen, German Center for Diabetes Research, we also gained access to samples with higher concentrations from adults during the course of OGTT. Blood was collected into FH blood tubes, via venipuncture by experienced staff every 30 min for 2 h. Reference glucose values were determined by the hospital's Central Laboratory (CLab) via an automated analyzer (ADVIA XPT clinical chemistry analyzer, Siemens Healthineers, Eschborn, Germany) in NaF-Na_2EDTA tubes (S-Monovette FE, 2.7 mL, Sarstedt, Nümbrecht, Germany) by the hexokinase method. Additionally, the glucose concentration in the FH tubes was measured with HemoCue Glucose 201 RT using a modified glucose dehydrogenase method. Two different reference data sets were utilized for validation and analysis. A Bland-Altman plot[21,22] (version[23]) with the CLab and PoC values was used to detect outliers: all points that are outside the 1.96 standard deviation range (Figure 1) are indicated. Five samples were thus excluded from further analysis, resulting in 117 whole blood specimens.

Sample preparation steps and sequence parameters from the whole blood and plasma comparison were identical with the ones described above. All samples were prepared and measured within 90 min of collection. In addition, post-processing steps and analysis by integration were performed in the same manner as before for glucose solution and bovine plasma, in order to ensure comparability.

2.5 | Comparison of different data processing strategies for glucose determination in whole blood

An internal intensity calibration of the spectra automatically applied by Spinsolve allows a quantitative comparison of different measurements without further need for normalization of the data. Nonetheless, it is possible to normalize the spectral intensities to the integral of the internal standard (methanoic acid with known concentration), allowing insight into experimental pipetting errors, but also requiring a stable baseline around the reference line to allow proper integration. This is related to a common method to analyze NMR spectra by deconvolution[24]: the

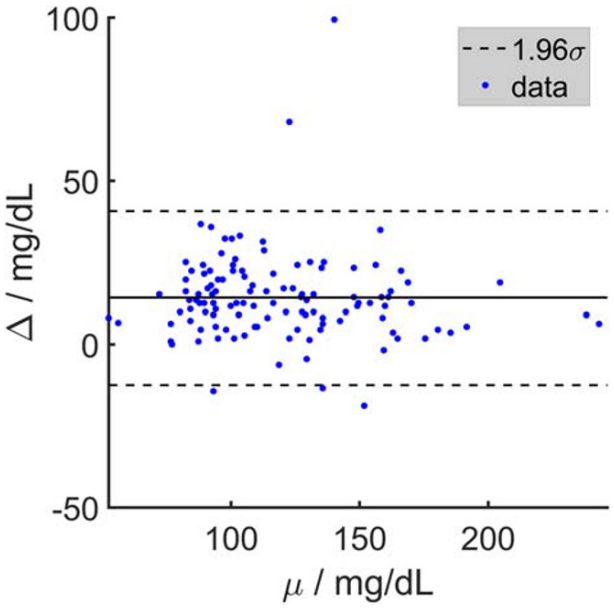

FIGURE 1 Bland-Altman plot of the glucose concentration values of the PoC device (HemoCue glucose 201 RT) and the CLab automated analyzer (Advia XPT)

spectral lines are fitted with Lorentzian or Gaussian functions to model the acquired signal and then the fit is integrated for quantification. We used the function "peakfit" to demonstrate this kind of analysis (code available online [25]). Lorentzian lines were fitted for methanoic acid and Gaussian lines for the glucose peaks at 3.79 and 3.42 ppm. The strong signal overlap with the water peak makes it difficult to fit the complete spectrum as a whole. The complexity was reduced by only submitting an interval of 0.25 ppm around the peak centers to the line fitting algorithm, which allowed a first-order polynomial approximation of the background. Usually spectra consist of several thousand data points, but only a few are required for quantification. These points may not be identified easily by the human eye in the presence of huge background signals and thus line fitting cannot be used properly as it requires prior knowledge about the peak positions and lineshapes. Projection to latent structures (PLS) analysis is related to the well known principal component analysis (PCA) and achieves a reduction in dimensionality of the data set by identification of main components.[26] First, the data is split into training and test sets, both of which cover the whole range of observations in the best case. Second, a model for the observations is created with the training set and challenged by the test set. Then the RMSEs of the resulting regressions, scores and loadings are evaluated for outliers and quality. An expansion of the method is the orthogonal projection to latent structures (OPLS), which decomposes the data into two different kinds of component (each of them characterized by their "loadings"): first, those making a major contribution to the quantification, and second, those not contributing ("orthogonal"). For spectral data this facilitates the interpretation of the components by showing which signals contribute to the model and which do not. As typical glucose spectra can be easily obtained, we preferred OPLS over PLS or PCA for our analysis.[27] The processed spectra and the reference values were handed over to MATLAB for OPLS (Cluster Toolbox[28]). The OPLS algorithm works on training and test sets. In order to obtain suitable training and test data with coverage of the entire range of glucose concentrations we used the "duplex algorithm" for splitting, which alternately adds the pair of the minimum and maximum values in the reference data to the test and the training sets and then removes it from the following iterations. Prior knowledge about the glucose spectra allowed reduction of the relevant spectral range to the section between 3.0 and 4.0 ppm. One PLS component and two orthogonal ones were selected after checking the explained variance and RMSE trend for up to 10 components. Finally, machine learning was applied on the data set to check for improvements in terms of speed and accuracy. For supervised learning the data was transferred to MATLAB's "Regression Learner" application. Cross-validation was set to 50-fold and the linear support vector machine (SVM) regression model, which searches for the best linear correlation in the data while allowing an error range in the observations, was trained.

3 | RESULTS

The most common and straightforward method for NMR quantification is integration of spectral lines, but this procedure requires proper baseline correction. OPLS can handle spectra without major baseline problems, similarly to SVM regression. We compare the results of the three mentioned methods for the OGTT samples in Table 1. The guidelines of the German Medical Association for laboratory assays[29] were checked on the quantification models: the guidelines request no more than 11% difference from the reference device in the glucose concentration range between 40 and 400 mg/dL.

3.1 | Signal patterns in aqueous glucose solution, bovine plasma and human whole blood

Figure 2 shows typical spectra of whole blood and plasma at 1.5 T at a common blood glucose concentration around 100 mg/dL (5.6 mmol/L). The water peak dominates the spectrum at 4.75 ppm; other visible signals in plasma and whole blood were identified as follows (disregarding potential contributions from lower-concentration components): lipid signals occur at 1.26 ppm (CH_2), 0.9 ppm (CH_2CH_3) and 2.0 ppm ($CH_2C{=}C$ group).[30] Protein signals (mainly albumin) are covering the whole range from 0.8 to 4.0 ppm with a maximum around 3.2 ppm. Finally, broad glucose signals resulting from the overlap of several ^1H atomic positions are seen at 3.42, 3.61 and 3.79 ppm. Note that the glucose peak at 5.23 ppm is overlaid by the water signal. Protein contributions, eg from albumin, histidine and arginine, overlay the glucose signals at approximately 3.20 ppm. The metabolites taurine at 3.41 ppm and myo-inositol at 3.63 ppm may be influencing the peak area of the glucose signals. Taurine concentration in

TABLE 1 OGTT analysis overview: RMSE values for all discussed evaluation methods and comparison with the RiliBÄK guidelines: the percentages of our samples that are within 11% of the reference values

	CLab (2.7 mL NaF-EDTA)		PoC (4.25 µL NaF-Na heparin)	
	RMSE (mg/dL)	Fulfillment of RiliBÄK (%)	RMSE (mg/dL)	Fulfillment of RiliBÄK (%)
Line fitting	9.22	86.3	9.42	84.6
Baseline correction	9.72	81.2	10.0	82.9
OPLS: train|test	8.05|11.6	84.6	8.77|10.2	83.8
Linear SVM	7.90	91.5	9.07	90.6

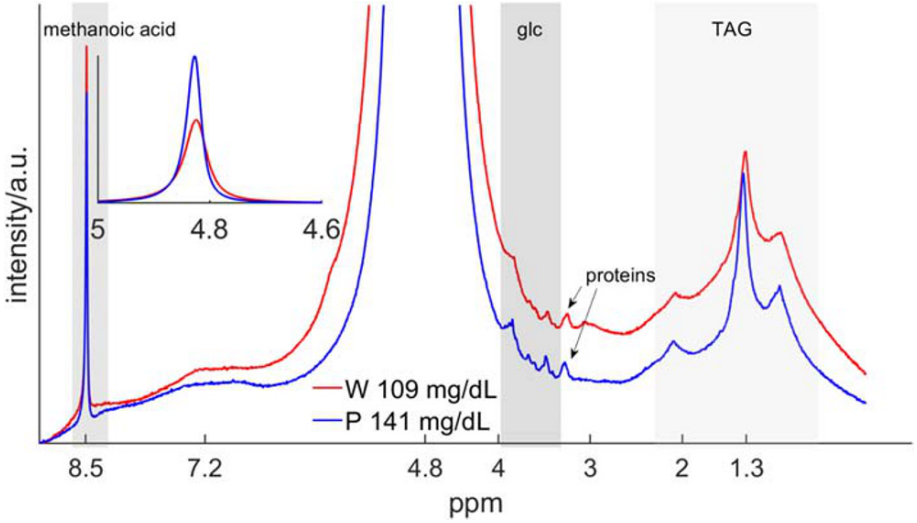

FIGURE 2 Comparison of a whole blood (blue) and plasma (red) spectrum from the same blood sample. Spectral signals: internal standard methanoic acid (8.49 ppm), water (4.75 ppm), proteins (overall background and 3.23), lipids (TAG, 0.9, 1.3 and 2.0 ppm) and glucose (3.45, 3.65 and 3.83 ppm). The HemoCue glucose values of the whole blood and plasma sample are given in the legend. The inset shows the water peak

blood was reported as 227 ± 35 μmol/L (measured in whole blood with lysed erythrocytes),[31] and myo-inositol shows 44 ± 9 μmol/L (measured in plasma).[32] According to these concentrations we expect their contributions to be at least 10 times lower than that of glucose. Since concentrations of these concomitant metabolites are relatively low and quite constant they were not considered during our quantification. On the left-hand side of the water signal are no quantitatively targetable peaks except the internal standard methanoic acid with its peak maximum at 8.45 ppm for our setup at slightly reduced physiological pH.[33] This value was determined in a separate measurement using the reference 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP).

3.2 | Reference measurements of aqueous glucose solution and bovine plasma by a PoC device

Aqueous glucose solutions used for general device sensitivity exhibited mean glucose levels (expected values in brackets) of 91 ± 2.9 (50), 199 ± 12 (150), 294 ± 4.5 (250) and 396 ± 5.8 (350) mg/dL and thus cover a broad detection range. Measured values were found to be higher than expected, with a constant offset of about 50 mg/dL, which is most likely caused by the underlying device errors of the pipette and the scale as well as the unconventional sample for the HemoCue. The bovine plasma samples contained 43 ± 2.8 (53 ± 14), 98 ± 2.6 (96-130), 149 ± 9.1 (168-212) and 269 ± 6.8 (292-376) mg/dL glucose and span the usual biological range in the OGTTs. The flasks were opened several weeks before this experiment, which could have led to changes in the glucose levels compared with the expected values given in the corresponding manuals. Note that the reference values were obtained before the dilution with the internal standard and the values for analysis were corrected. The standard deviation for five consecutive measurements is within the device tolerance and sets an error range to be achieved by the NMR quantification.

3.3 | Spectral analysis by integration

The NMR spectral integrals between 3.3 and 3.9 ppm after the optimized baseline correction via the Whittaker smoother and the reference glucose values for the glucose solution (Figure 3A), bovine plasma (Figure 3B) and OGTT series (Figure 3C) show a linear correlation. Noteworthy is the effectiveness of the applied baseline correction, which leads to partial spectra with an almost flat baseline. The overall accuracy of the NMR method can be estimated using Bland-Altman plots (Figure 4A). The 95% confidence intervals are roughly within 20 mg/dL over the whole range of concentrations. The Bland-Altman plot for the line fitting analysis is given in Figure 4B.

3.4 | OPLS and SVM results for whole blood samples

Figure 5 demonstrates the MATLAB OPLS outputs for glucose determination via NMR spectra: the processed spectral data fed to OPLS analysis is shown in A. B shows the resulting OPLS regression for glucose (for CLab reference values) with the RMSE values for training and testing: 8.05 mg/dL and 11.6 mg/dL, respectively. One PLS component explained nearly all variance in the data set when combined with two orthogonal ones, so in total three components were chosen. The two orthogonal components represent the water and protein background in the

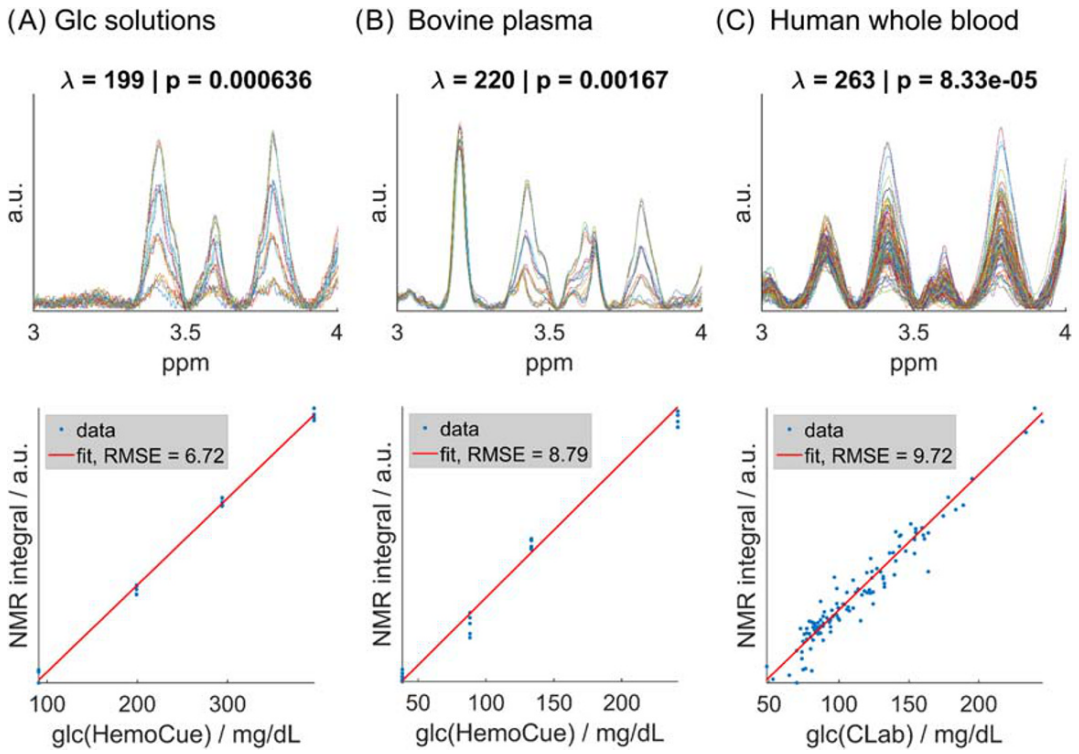

FIGURE 3 A, B, NMR analysis by integration of glucose-water solution (A) and bovine plasma-glucose solution (B). Five measurements were made by NMR and results were compared with HemoCue glucose testing. Spectra were processed with the Whittaker smoother for baseline correction before integration. The integrals of glucose signals between 3.3 and 3.9 ppm are plotted as dots. Linear fitting allows the calculation of the glucose concentrations and RMSE values. C, Effectiveness of the Whittaker-smoother-based baseline correction prior to integration for the OGTT samples: the glucose signals can be clearly identified. The corresponding Bland-Altman plot to displayed in Figure 4A

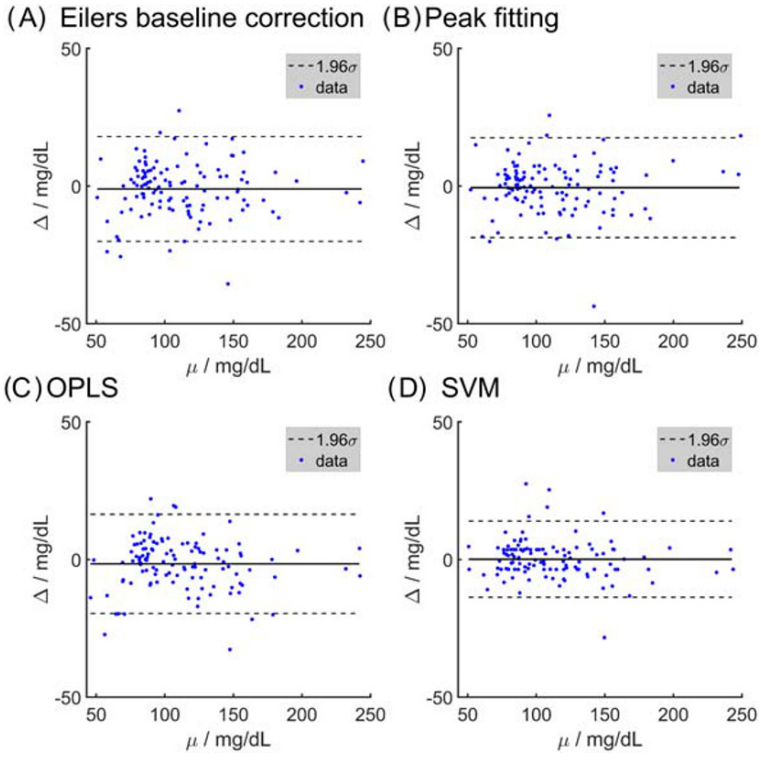

FIGURE 4 Overview of all analysis methods by Bland-Altman plots: integration after a Whittaker smoother baseline correction (A), peak fitting (B), OPLS (C) and SVM (D)

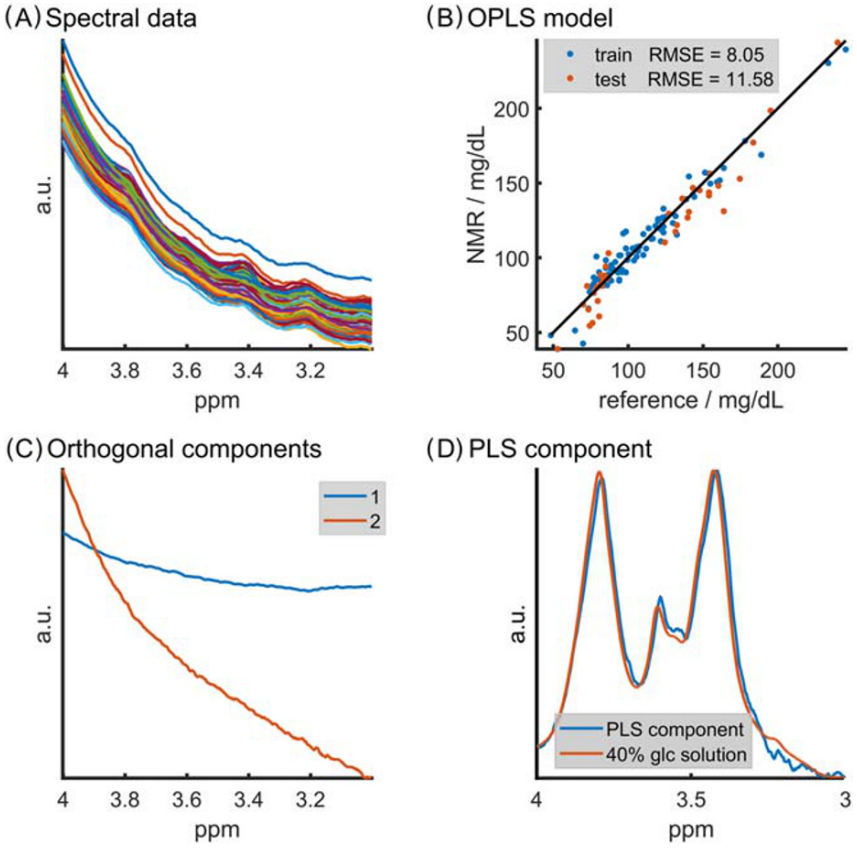

FIGURE 5 OPLS and SVM anaylsis of the OGTT series with CLab reference values: processed spectra in the range handed over to analysis (A), the loadings of the PLS (D) and orthogonal (C) components of the OPLS model, and the resulting regression for glucose concentration in whole blood (B). In the legend, the RMSE values for the train and the test set are displayed. The orthogonal components do not contribute to the glucose model. The loadings of the single PLS component show the characteristic glucose pattern. For direct comparison, the spectrum of a pure glucose solution acquired with the same sequence, but with longer inter-experimental delays due to the higher T_1 relaxation time, is superimposed

measurements (C). The glucose pattern is clearly shown in the loadings of the first PLS component (D), best seen when compared with the spectrum of a pure glucose solution sample (40% mass concentration). The linear SVM model resulted in an RMSE of 7.90 mg/dL, which is in line with previous results. Bland-Altman plots (Figure 4C and 4D) were also used here for visual representation of the model quality and are comparable to the integration analysis (Figure 4A and 4B), but with smaller confidence intervals. Bland-Altman plots for all methods using the reference values from the PoC device show very similar results in Figure S2.

4 | DISCUSSION

Low-field NMR spectroscopy generally suffers from relatively low sensitivity, broad spectral lines and signal overlap. These effects might considerably impair the quality of blood spectra recorded at low field and the subsequent assessment of glucose signals, when compared with high-field spectroscopy of blood plasma as reported by Nicholson et al.[34] The spectra from whole blood without further sample preparation mainly revealed signals from water, glucose and lipids. Since lipid signals are not always prominent peaks in the benchtop blood spectra and the ppm axis of the spectra may shift due to temperature and pH, we went for the internal standard methanoic acid to calibrate the chemical shifts.[6,35] It should be mentioned here that chemical shift referencing with an added substance was helpful for the measurements performed in our studies, but making use of a natural internal standard (eg the water signal position in non-water-suppressed spectra) seems feasible for future applications on NMR in extracorporal systems or in vivo.

4.1 | Comparison of reference data sets: PoC versus CLab

The measurement accuracy achieved with benchtop NMR for the pure glucose solution and the bovine plasma is limited not only by the NMR but also by the reference method. For example, the PoC device is not designed to measure pure glucose solutions, whch may cause offsets for the corresponding series. For the OGTT the reference glucose values were on the one hand determined in CLab by a gold standard measurement (hexokinase reaction in EDTA-plasma). On the other hand, the glucose concentration was directly measured in the FH tubes before NMR tube preparation. Both glucose reference tubes were drawn one after the other, first NaF-Na_2EDTA, second FH. The blood tubes used for NMR were FH, so they also contained NaF. Minor differences in the absolute values had to be expected (tested for five samples, Table 2). The effect of NaF

TABLE 2 Comparison of glucose levels in mg/dL in NaF-Na_2EDTA and NaF-Na heparin blood collection tubes. Blood was obtained from the same patient at the same time and then analyzed in CLab's usual workflow

NaF-Na_2EDTA	NaF-Na heparin
70	74
134	139
74	78
73	90
72	82

may take up to four hours before it inhibits all glycolysis,[35] but Fobker found that FH tubes are even slightly superior to the NaF-Na_2EDTA versions.[20] Our quick test shows higher glucose concentrations in FH than in the default tubes used for measurements in CLab. Either way, the time difference between the NMR measurement and the glucose determination in the laboratory might cause a difference between the remaining glucose concentrations, and is the main source of the observed deviations. Figure 1 shows a direct comparison of the two reference data sets in the two different tube types, PoC in FH and CLab in NaF-Na_2EDTA, demonstrating the timing issues when samples are submitted to CLab during the daily routine. In terms of accuracy for the NMR method, we see no significant difference between the two reference glucose datasets (Table 1).

4.2 | Plasma versus whole blood spectroscopy

Differences between plasma and whole blood spectra (Figure 2D) were observed regarding linewidth of water as well as lipids and in the protein background over the whole spectral range, which leads to an offset of the whole blood spectra. Linewidths were calculated by the full-width-half-maximum (FWHM) values for water. Plasma has smaller water lines at 1.5 T with an FWHM of 1.9 Hz than whole blood with 3.0 Hz, which is even further increased during the measurements using the more inhomogeneous 1 T spectrometer used for the OGTT series. These differences are attributed to the removal of hematocrit and larger proteins that results in fewer interactions with other components. Intermediate effects between whole blood and plasma can be observed during the slow sedimentation of cells in whole blood samples over several minutes due to gravitation, which degrades the sample and magnetic field homogeneity. To avoid separation within the measurement time of 14 min a preceding 60 s mixing proved to be effective. During the mixing the oxygenation of the whole blood increases, leading to increased T_2 values in the sample[36] and thus smaller linewidths.

4.3 | NMR setup

A very simple pulse-acquire sequence was used for this series. No water suppression was applied, which made the presented method easy to use. Further improvements in quantification could possibly be achieved by using adequate water suppression.[37] Note however, that the suppression of the broad water resonance at 1 T field strength also significantly reduces the glucose signals as the suppression bandwidth cannot be chosen small enough. The SNR provided by the 1 T spectrometer when combined with sophisticated analysis methods reaches acceptable accuracy. The smaller linewidth provided by the Spinsolve spectrometer at 1.5 T (in comparison to the standard Spinsolve spectrometer at 1.0 T) reduces the water peak background in the spectra significantly and therefore may increase the stability and accuracy of the measurements, as presaturation of the water signal becomes a feasible option. Overall, we see the limiting factor of our setup for assessment of glucose more in the linewidth and spectral resolution than in SNR issues.

4.4 | Analysis methods

Signal integration after optimized baseline correction led to reasonable results. Nevertheless, OPLS results are easier to interpret, as the loadings clearly show the glucose pattern, which confirms the underlying assumption of the correct identification of the three glucose peaks. Especially important to note is the basically non-existent contribution of the PLS component representing the spectral line at 3.2 ppm, which mainly originates from blood proteins, eg the amino acids arginine and histidine. Supervised machine learning was applied and resulted in the best correlation between spectral data and reference glucose values. The increment in accuracy determined by RMSE refines the overall tendency in the dataset seen by integration and OPLS analysis. Therefore, different evaluation methods can be applied, resulting in comparable outputs. Differences can be attributed to the stability and reproducibility of the NMR setup including the preparation steps. The RMSE values reached by the SVM model come close to the guidelines of the German Medical Association, with 91.5% of our samples within the required 11% difference range.

It should be mentioned that the quality of our reference values was limited due to the different blood tubes and unforeseen time delays in daily CLab routine, during which the glucose concentrations in either the NMR tube or the tube submitted for CLab analysis may change.

Nevertheless, the results come close to the German regulations with this simple setup. Furthermore, outlier values have a major influence on the resulting RMSE values, since the residuals are first squared and then averaged, in contrast to more outlier resistant measures such as the mean absolute error, which calculates the mean of the absolute values of the residuals. This mean absolute error tends to be lower than RSME by about 2 mg/dL for each model created by the presented data.

Issues to address during experimental setup for in vitro whole blood analysis include handling the separation of plasma and cells by gravitation for longer measurements, and the limitation of substances due to line broadening, overlapping and background signals. It seems very unlikely that NMR-based PoC devices will become competitive with enzymatic test strips in the near future, but NMR devices are being further miniaturized and becoming cheaper. Thus, NMR blood testing could become feasible for special applications, for example in devices with extracorporeal blood circulation or for quality checks of units of stored blood. There is still a long way to go for NMR-based blood tests in vivo, and it remains to be seen whether the conditions in the body are adequate in terms of glucose concentration and magnetic field distribution. For now, we conclude that reliable NMR quantification of glucose in whole blood samples is possible with minor sample preparation, a modern 1 T benchtop spectrometer[38] and well established evaluation methods.

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