Supporting Information

Direct Low Field J-Edited DIffusional Proton NMR Spectroscopic Measurement of COVID-19 Inflammatory Biomarkers in Human Serum

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Materials and Methods

Chemicals and consumables

Phosphate buffer (75 mM Na_2HPO_4, 2 mM NaN_3, 4.6 mM sodium trimethylsilyl propionate-[2,2,3,3-$^2H_4$] (TSP) in H_2O/D_2O 4:1, pH 7.4 ±0.1), all NMR tubes (5 mm outer diameter SampleJet™ NMR tubes and regular 7 inch, 5 mm NMR tubes) with the corresponding sealing caps, and the Bruker Fourier 80 (F80) shimming sample (Doped Water 5% H_2O 0.6 mM $CuSO_4$ in D_2O) were purchased from Bruker A.G. Rheinstetten).

Autonomous Community of the Basque Country (Spain): The cohort consisted of i) patients who tested positive for SARS-CoV-2 infection from upper and/or lower respiratory tract swabs by RT-PCR (n=29) and ii) healthy control participants (n=28). All serum samples were collected by the Basque Biobank for research (BIOEF). Healthy serum samples were collected before the COVID-19 pandemic from the active population while the COVID-19 samples were collected at the Cruces University Hospital (Barakaldo, Spain) from patients who presented compatible symptoms, confirmed by a RT-PCR assay on nasal swab samples. All participants provided informed consent to clinical investigations, according to the Declaration of Helsinki, and all data were anonymized to protect their confidentiality. The sample handling protocol was evaluated and approved by the *Comité de Ética de Investigación con medicamentos de Euskadi* (CEIm-E, PI+CES-BIOEF 2020-04 and PI219130). Shipment of human samples to ANPC had the approval of the Ministry of Health of the Spanish Government. Samples were stored at -80 °C.

Sample preparation and data acquisition

^{1}H NMR sample preparation IVDr 600 MHz: Plasma samples were thawed at 4 °C for 2 h then centrifuged for 10 minutes at 13000 g at 4 °C. All plasma samples were prepared in 5 mm outer diameter SampleJet™ NMR tubes, following the recommended procedures for *in vitro* analytical and diagnostics procedures[1] using 250 µL of plasma mixed with 250 µL phosphate buffer . Additionally, the samples were sonicated for 5 minutes at ambient temperature prior to analysis.

The total volume of 500 uL is below the recommended volume threshold for the IVDr methods. Therefore, comparison spectra from 600-400 uL were recorded to ensure that the shimming (spectral) quality is unaffected for 500 uL (data not shown).

^{1}H NMR spectroscopy data acquisition and processing parameters IVDr 600 MHz: NMR spectroscopic analyses were performed on a 600 MHz Bruker Avance III HD spectrometer, equipped with a 5 mm BBI probe and fitted with the Bruker SampleJet™ robot cooling system set to 5 °C. A full quantitative calibration was completed prior to the analysis using a protocol described elsewhere[1]. All IVDr methods and JEDI experiments were acquired at 310 K. After all samples were measured, the temperature was switched to 298 K and all experiments were re-measured with the same experiments for better comparison with the F80, which operates at 298 K.
The standard one-dimensional (1D) experiments with solvent suppression (pp: noesygppr1d) were acquired with 32 scans (+4 dummy scans), 98k data points, relaxation delay of 4.0 s, and a spectral width of 30 ppm resulting in a total experiment time of 4 min 3s (according to the Bruker In Vitro Diagnostics research IVDR methods).

The JEDI sequences (JEDI-PGPE and JEDI-PGSE) were acquired with 64 scans, 98k data points, relaxation delay of 1.0 s, and a spectral width of 30 ppm resulting in a total experiment time of 4 min 23 s for JEDI-PGPE and 4 min 20 s for JEDI-PGSE. A more detailed explanation of the sequences can be found elsewhere[2].

Time domain data were Fourier transformed and processed in automation using Bruker Topspin™ 3.6.2 or Bruker Topspin™ 4.0.9 and ICON™ NMR to obtain phase and baseline corrected spectra. An exponential line broadening of 0.3 Hz was applied to the 1D water suppressed experiment, whereas a broadening of 1.0 Hz was applied to the JEDI free induction decays (FID). For the JEDI sequences, only the first 32K real data points were Fourier transformed to reduce unwanted noise contributions from the FID.

All DIRE and JEDI Spectra were calibrated by setting the spectral reference value to 0 (SR = 0), i.e. "no calibration". The *N*-acetyl residues of Glyc and the -NMe_3^+ moieties of SPC show constant chemical shift behaviour and are not perturbed by inter-sample variation found in serum or plasma[2]. ERETIC correction was applied to all spectra to ensure the observed intensities are quantitative[3,4].

^{1}H NMR sample preparation 80 MHz: After experiment acquisition of the IVDr methods at 600 MHz the plasma/buffer solutions were transferred with a syringe into standard 7 inch, 5mm NMR tubes. The tubes were sealed with standard NMR caps. The tubes were shaken and additionally, the samples were sonicated for 1 minute at ambient temperature prior to analysis.
The plasma only samples without buffer were thawed at 4 °C for 2 h then centrifuged for 10 minutes at 13000 g at 4 °C. 500 uL were directly transferred into a standard 7 inch, 5 mm NMR tube. The tubes were sealed with standard NMR caps and sonicated for 5 minutes at ambient temperature prior to analysis.

^{1}H NMR spectroscopy data acquisition and processing parameters 80 MHz: NMR spectroscopic analyses were performed on a 80 MHz Bruker Fourier 80 spectrometer, equipped with a F80 Two Channel (^{1}H +^{13}C) probe including a nominal 25 G/cm Z gradient system. The system operates at a constant temperature of 298.15 K. To shim the system a shimming sample (Doped Water 5% H_2O 0.6 mM $CuSO_4$ in D_2O) was inserted overnight and the Bruker shimming algorithm operated till measurements ensued or until the minimum line shape criteria (1.0 Hz at 50% and 20 Hz at 0.55% of the resonance line) for the water signal were reached. After the daily acquisition of samples was finished, the shimming sample was inserted again to shim overnight and maintain magnetic homogeneity. Shimming quality for a measurement day was found to be stable within the minimum line shape criteria.

Before every sample a regular ^{1}H 1D (pp: zg) was acquired to allow for temperature equilibration and act as a quality check for the shim and determine possible spectral offsets. The spectrum was acquired with 1 scan (0 dummy scans), 16384 data points, a relaxation delay of 300.0 s to ensure temperature equilibration, and a spectral width of 40 ppm resulting in a total experiment time of 5 min 2 s.

The standard one-dimensional (1D) experiments with solvent suppression (pp: noesygppr1d) were acquired with 32 scans (+4 dummy scans), 17646 data points, relaxation delay of 4.0 s, and a spectral width of 40 ppm resulting in a total experiment time of 4 min 4 s.

The JEDI sequences (JEDI-PGPE and JEDI-PGSE) were acquired with 256 scans (+4 dummy scans), 3224 data points, relaxation delay of 2.5 s, and a spectral width of 20 ppm resulting in a total experiment

time of 15 min 46 s for JEDI-PGPE and 15 min 34 s for JEDI-PGSE. A more detailed explanation of the sequences can be found elsewhere[2] and in the supplementary information.

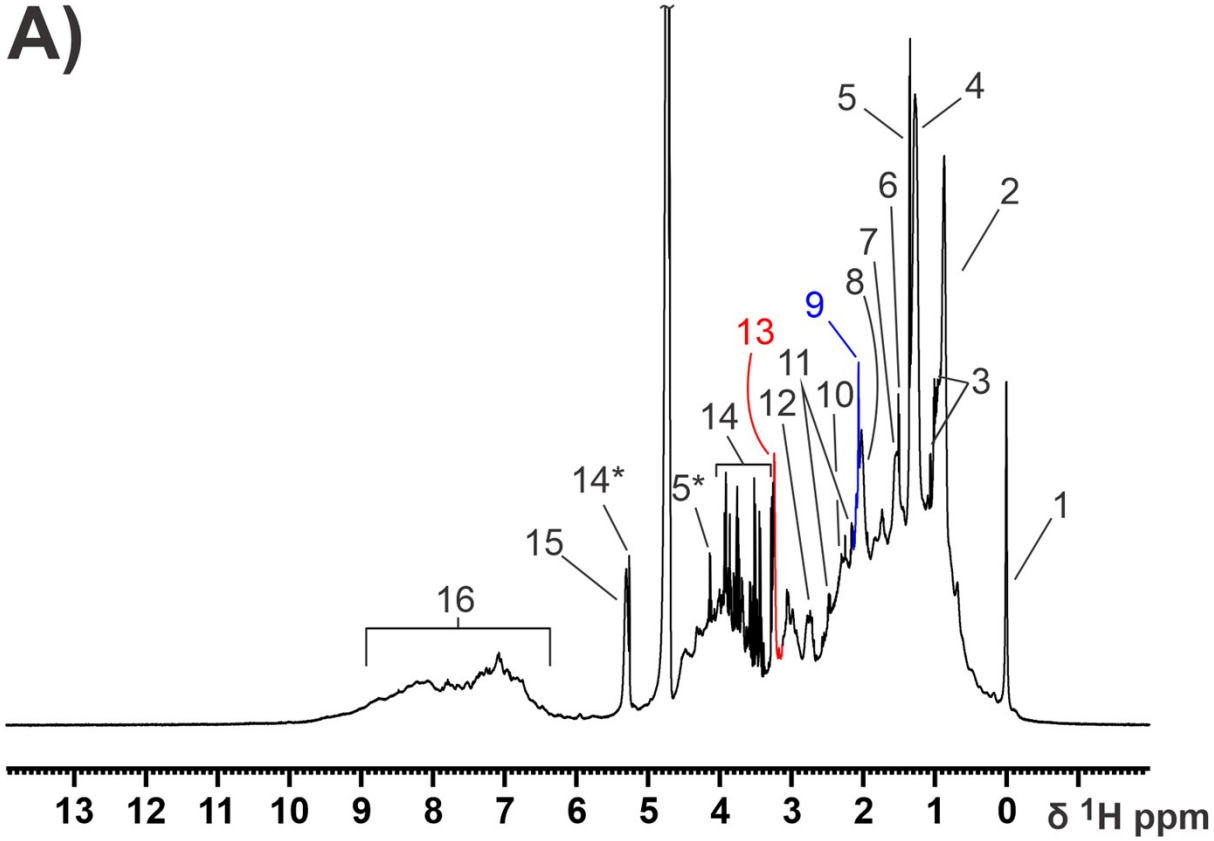
For the samples that contained plasma only and no buffer the number of scans for was reduced to 64. All other parameters were kept the same, resulting in a total experiment time of 4 min 9 s for DIRE, 4 min 7 s for JEDI-PGPE and 4 min 4 s for JEDI-PGSE. Some plasma only samples were additionally acquired with a varying number of scans (1 - 2048; with and without dummy scans).

Time domain data was Fourier transformed and processed in automation using Bruker Topspin™ 4.0.9 and ICON™ NMR to obtain phase and baseline corrected spectra. An exponential line broadening of 0.3 Hz was applied to the 1D water suppressed experiment, whereas a broadening of 1.0 Hz was applied to the JEDI free induction decays (FID). For the JEDI-PGSE sequence, only the first 1300 and for JEDI-PGPE the only first 2000 real data points were Fourier transformed to reduce unwanted noise contributions from the FID.

All spectra were calibrated to the residual water peak $\delta = 4.70$ ppm.

NMR data analysis and statistical evaluation

Integral values were directly extracted from Topspin using the "intser" command application. Integration of the peaks is achieved readily by summation of a fixed spectra region (600 MHz: Glyc $\delta = 2.04 - 2.18$ ppm; SPC: $\delta = 3.18\text{-}3.30$ ppm; 80 MHz: Glyc $\delta = 1.80 - 2.08$ ppm; SPC $\delta = 2.95 - 3.30$ ppm). The statistical evaluation of the integrals for the box plots and linear fits ($y = a+b*x$) were done using OriginPro. The nonparametric Wilcoxon ranked test was performed with OriginPro. Signal to noise determination was done in Topspin using the ".sino" command application. On the F80, the signal regions for Glyc $\delta = 1.80 - 2.08$ ppm and SPC $\delta = 2.95 - 3.30$ ppm were used. The used noise region was $\delta = 10.00 - 12.00$ ppm.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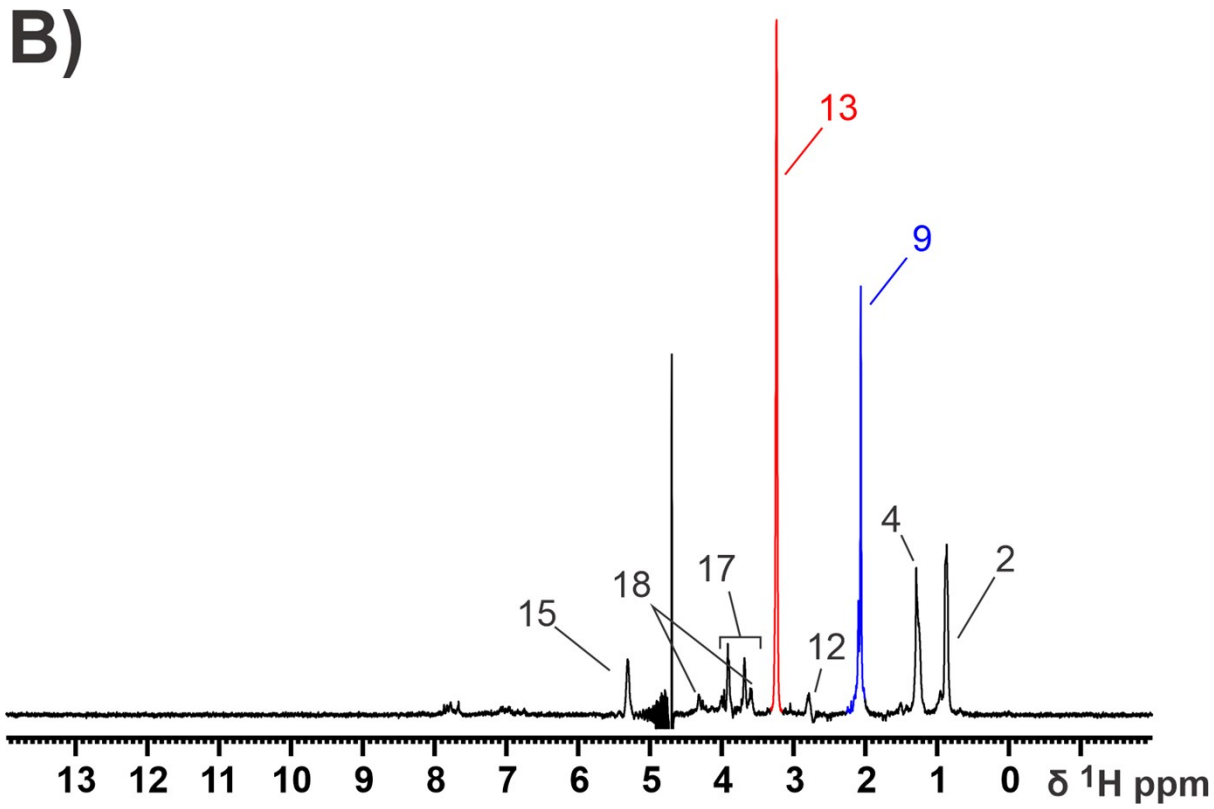
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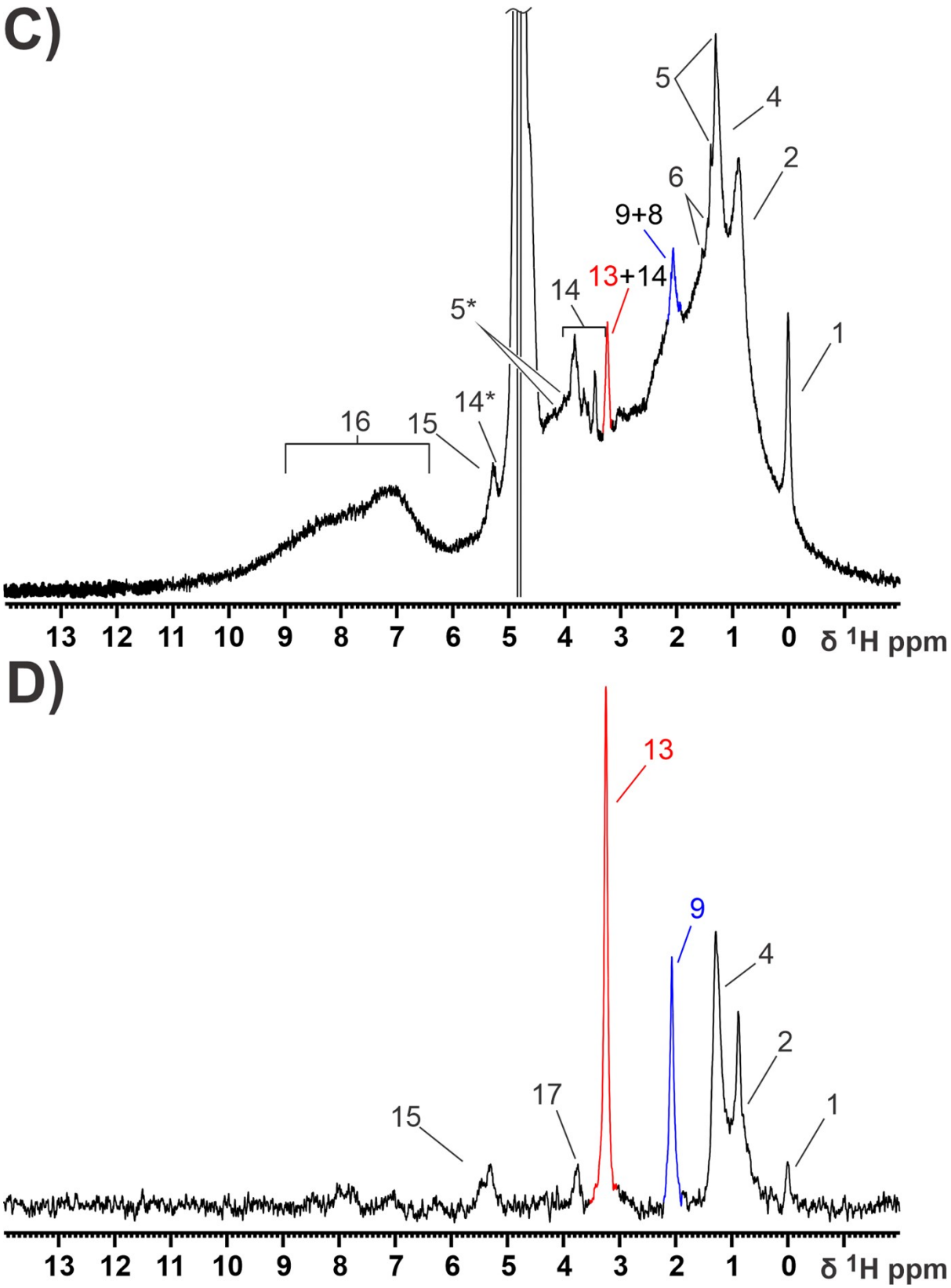

Figure S1: Comparison of solvent suppressed proton spectra and JEDI for a healthy control at 600 and 80 MHz. The biomarkers SPC (red) and Glyc (blue) are highlighted.

A) 600 MHz proton spectrum with solvent suppression at 310K. B) 600 MHz JEDI spectrum at 310K C) 80 MHz proton spectrum with solvent suppression. D) 80 MHz JEDI spectrum. Common metabolites are assigned in A-D and represent the following: 1 = TSP -CH_3; 2 =

Lipoprotein -CH_3; 3 = Valinev -CH_3; 4 = Lipoprotein -CH_2-; 5 = Lactate -CH_3; 5* = Lactate -CH-; 6 = Alanine -CH_3; 7 = Lipoprotein -CH_2CH_2CO-; 8 = Lipoprotein -CH_2CH=CH-; 9 = Glyc -CH_3; 10 = Lipoprotein -CH_2CO-; 11 = Glutamine -CH_2-; 12 = Lipoprotein -CH=CH-CH_2-CH=CH-; 13 = SPC -CH_3; 14 = Glucose -CH-; 14* = Glucose -CH- (anomeric); 15 = Lipoprotein -CH=CH-; 16 = aromatic protein background (mostly albumin) -CH=CH- (also contains protein amide residues); 17 = Sugar protons of Glyc -CH-; 18 = Choline moiety of SPC -CH_2-;

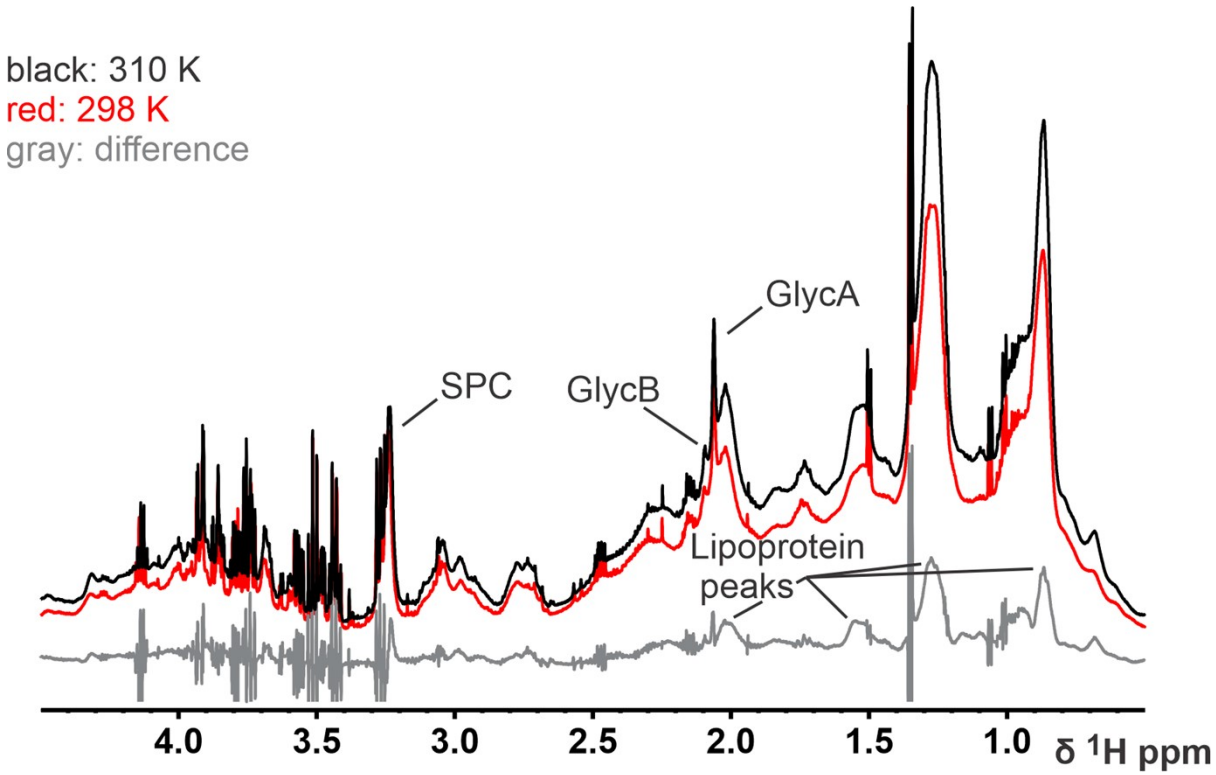

Figure S2: Aliphatic 600 MHz ^{1}H NMR region of serum at 310 and 298 K.
The solvent-suppressed ^{1}H NMR spectra were acquired at 310 (black) and 298 (red) K. The spectra were both aligned to Glucose δ = 5.23 for better comparability. A difference spectrum between 310 and 298 K is shown in gray and highlights the main changes between the spectra. The broad protein background and all lipoprotein peaks are significantly reduced at the lower temperature, mainly due to decreased mobility. The biomarkers SPC and Glyc stemming from lipoproteins (SPC) and glycoproteins (Glyc) are significantly reduced as well.

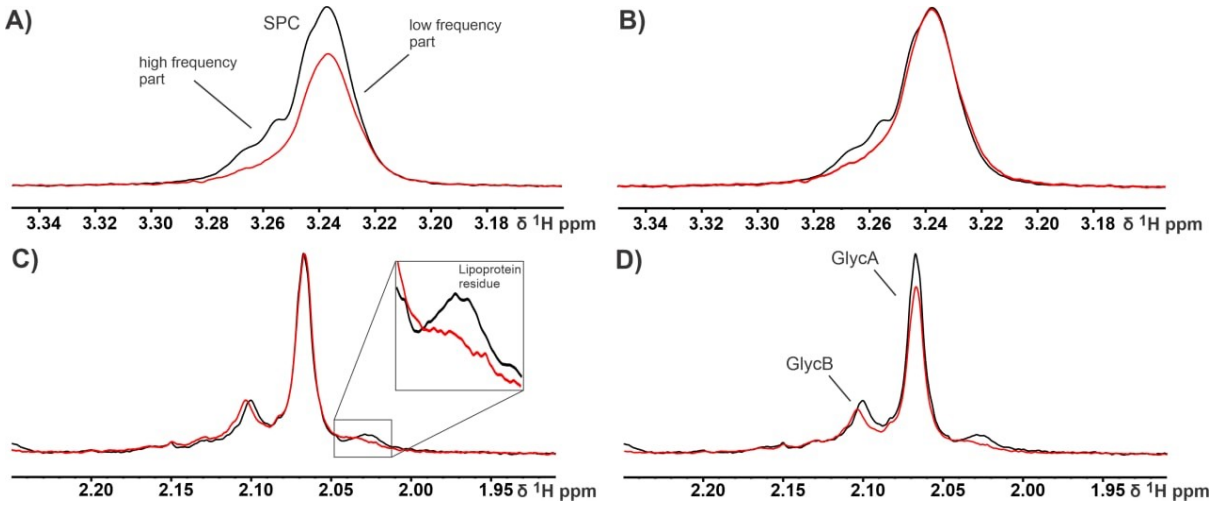

Figure S3: 600 MHz JEDI spectra of SPC and Glyc at 310 and 298 K.

A) SPC at 310 K (black) and 298 K (red). SPC ~30% reduced. B) Same as SPC spectrum as in A but scaled to maximum. It can be seen that especially the high-frequency region is reduced. C) Glyc at 310 K (black) and 298 K (red). Glyc is overall 20% reduced and the respective chemical shift difference between GlycA and GlycB is increased by 2.4 Hz at 298 K. D) Same as the Glyc spectrum in C but scaled to maximum. It can be noticed that residual lipoprotein peak is reduced.

Basic pulse sequences

Slight changes were made for the JEDI-PGSE and JEDI-PGPE pulse sequences and their respective parameters for the two investigated temperatures 310 K and 298 K at 600 MHz and the 80 MHz experiments.

For the JEDI-PGSE the diffusion delay δ was slightly increased from 3.0 to 3.5 ms at 298 K compared to the 310 K measurement in order to efficiently remove all small molecule contributions. As a result the total diffusion time Δ also changed slightly from 63 ms to 62.5 ms. For the 80 MHz measurement δ was increased to 5.0 ms and its relative power was increased from 80 % to 100 % compared to the 600 MHz experiment in order to ensure the removal of all small molecule contributions. This also led to a slight change in Δ. In addition, the JEDI-PGSE for the 80 MHz measurement contained no z-filter to avoid re-introduction of water signal interference. As a result the phase cycle was adjusted accordingly.

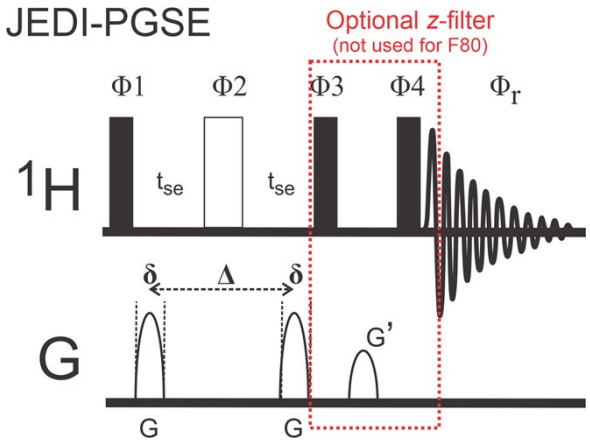

Figure S4: Sequence of Pulsed Gradient Spin Echo (PGSE).

Narrow, black rectangular bars are non-selective 90° pulses and broad, white rectangular bars present non-selective 180° pulses. G and G' are smoothed-square shape gradients.

600 MHz: For 310K, G as the diffusion delay δ is set to 3.0 ms with a strength of 80% and G' is set to 600 µs with a strength of 10% (nominal gradient strength at 100% = 53.5 g/cm). t_{se} is a spin-spin relaxation period set to 33 ms. The total diffusion time Δ accumulates to 63 ms. For 298K, G as the diffusion delay δ is set to 3.5 ms with a strength of 80% and G' is set to 600 µs with a strength of 10% (nominal gradient strength at 100% = 53.5 g/cm). t_{se} is a spin-spin relaxation period set to 33 ms. The total diffusion time Δ accumulates to 62.5 ms. During the *z*-filter, an optional Eddy current delay can be included with a duration of 5 ms. The phase cycling is Φ1 = x; Φ2 = y; Φ3 = x,-x, x, -x; Φ4 = (x)4; Φr = x, -x, x, -x.

80 MHz: G as the diffusion delay δ is set to 5.0 ms with a strength of 100% (nominal gradient strength at 100% = 25.0 g/cm). t_{se} is a spin-spin relaxation period set to 31.5 ms. The total diffusion time Δ accumulates to 57.4 ms. The phase cycling is Φ1 = x, (-x)2, x, y, (-y)2, y; Φ2 = y, -y, y, -y, x, -x, x, -x; Φr = x, (-x)2, x, y, (-y)2.

The JEDI-PGPE was kept the same for 310 K and 298 K as the diffusion strength was still sufficient to remove all small molecule contributions. For the 80 MHz measurement, the power was increased from 80 % to 100 % compared to the 600 MHz experiment in order to ensure the removal of all small molecule contributions. In addition, the JEDI-PGPE for the 80 MHz measurement contained no z-filter to avoid re-introduction of water signal interference. As a result the phase cycle was adjusted accordingly.

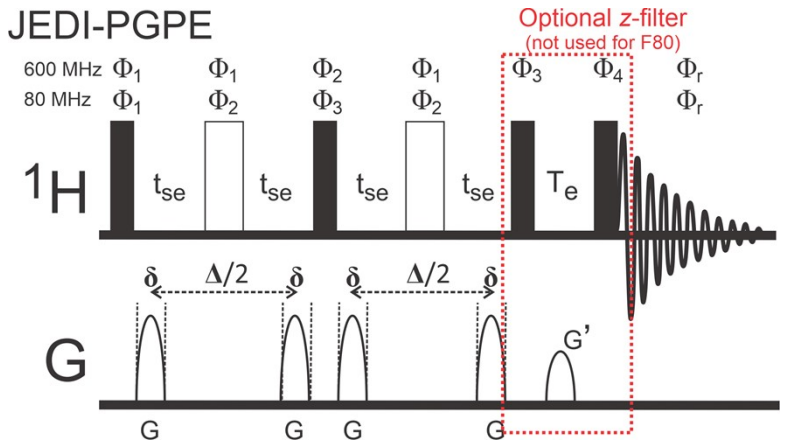

Figure S5: Sequence of Pulsed Gradient Perfect Echo (PGPE).

Narrow, black rectangular bars are non-selective 90° pulses and broad, white rectangular bars present non-selective 180° pulses.

600 MHz

G and G' are smoothed-square shape gradients, whereas G as the diffusion delay δ is set to 2.5 ms with a strength of 80% and G' is set to 600 µs with a strength of 70% (nominal gradient strength at 100% = 53.5 g/cm). t_{se} is a spin-spin relaxation period set to 27.5 ms. The total diffusion time Δ accumulates to 105 ms. T_e is an optional Eddy current delay of 5 ms. The phase cycling is Φ1 = x; Φ2 = y; Φ3 = x, -x; Φ4 = (x)2; Φr = x, -x.

80 MHz

G and G' are smoothed-square shape gradients, whereas G as the diffusion delay δ is set to 2.5 ms with a strength of 100% (nominal gradient strength at 100% = 25.0 g/cm). t_{se} is a spin-spin relaxation period set to 27.5 ms. The total diffusion time Δ accumulates to 105 ms. T_e is an optional Eddy current delay of 5 ms. The phase cycling is Φ1 = x, -x, x, -x; Φ2 = (y)4; Φ3 = (y)2, (-y)2, -x; Φr = x, -x, x, -x.

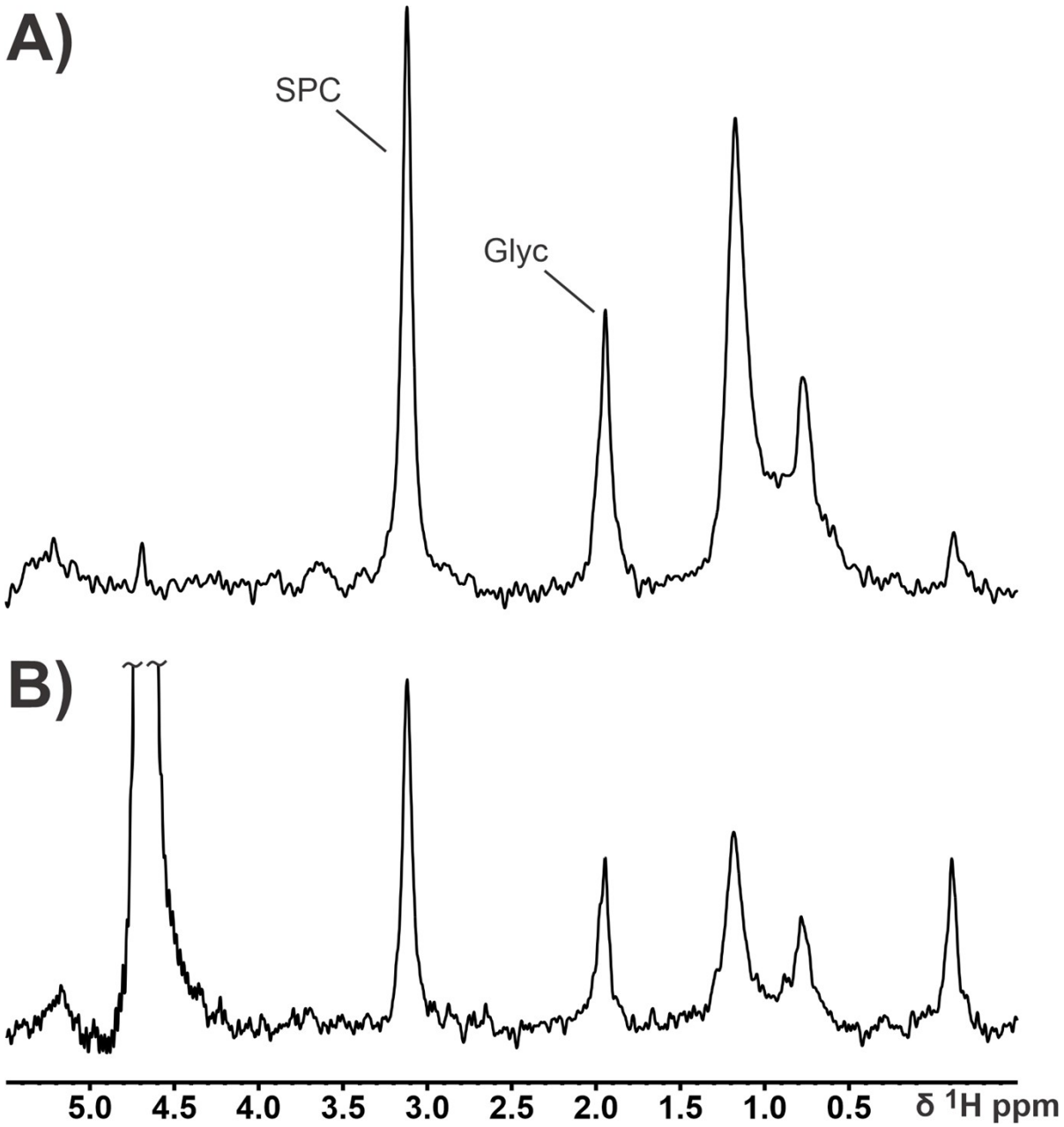

Figure S6: JEDI-PGSE and JEDI-PGPE of serum at 80 MHz.

A) JEDI-PGSE acquired with 256 scans (~15 min) at 80 MHz. B) JEDI-PGPE acquired with 256 scans (~15min) at 80 MHz. Both sequences yield a clean JEDI spectrum showing pronounced peaks for SPC and Glyc free of overlap. The JEDI-PGSE yields a higher S/N compared to the JEDI-PGPE (factor ~1.6), which in turn yields a more even baseline.

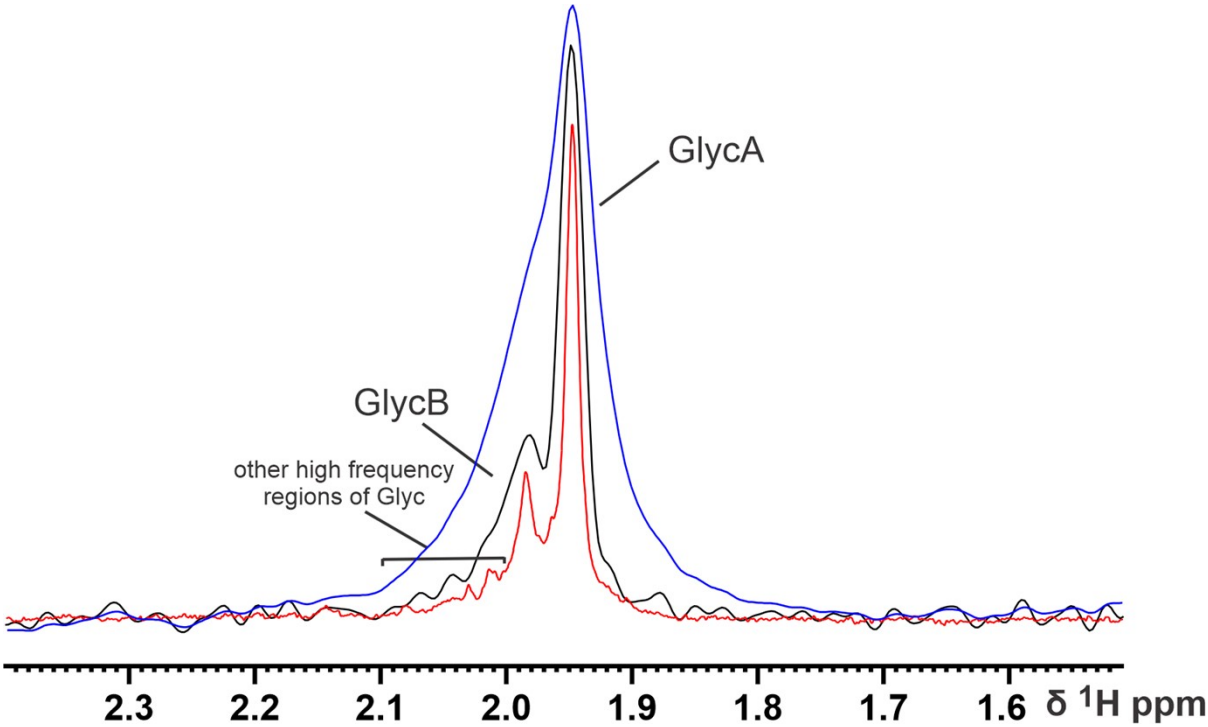

Figure S7: Comparison of the Glyc region in a serum sample at 80 MHz and 600 MHz by JEDI. The 80 MHz spectrum is shown with the two apodization functions exponential (blue, lb 1.0, standard processing used for JEDI in this work) and gaussian (black, gb 0.6, lb -2). The 600 MHz spectrum (red) shows a clear distinction between GlycA, GlycB and further high frequency regions of Glyc, whereas the 80 MHz under standard processing conditions (blue) does not resolve GlycA and GlycB and appears as broad singlet slightly mis-shaped indicating a second signal at high frequency. Using gaussian apodization it can be shown that the 80 MHz can still distinguish between GlycA and GlycB explaining the "distorted" peakshape using an exponential line broadening function. The 80 MHz spectrum was acquired with 2048 scans.

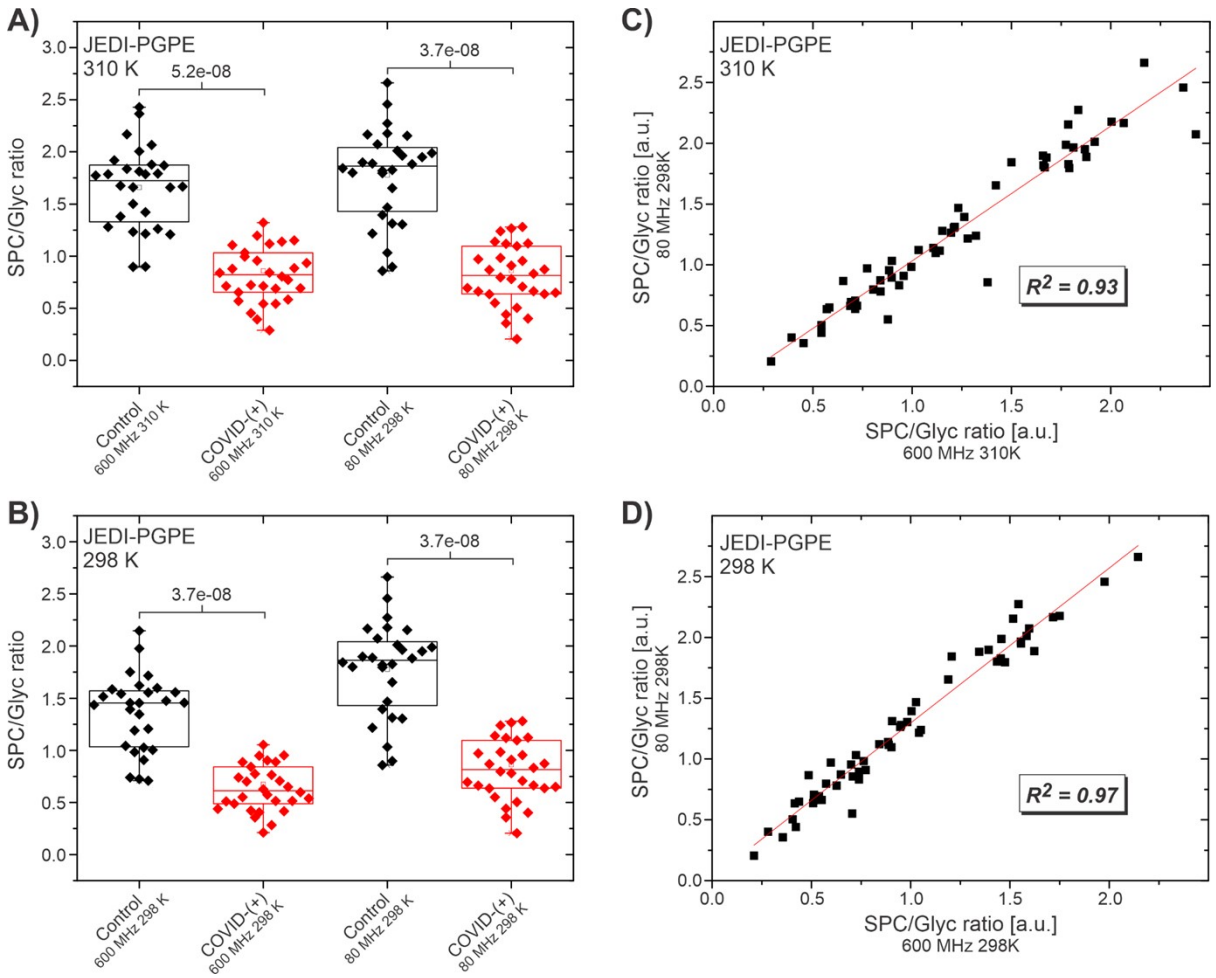

Figure S8: Figure 2: Statistical analyses comparing SPC/Glyc JEDI-PGPE measurements at 600 MHz 310 K (IVDr) and 600 MHz 298 K with SPC/Glyc measurements at 80 MHz 298 K.

A) Box plots showing the SPC/Glyc ratio for healthy controls (black) and COVID-(+) samples measured by JEDI-PGPE at 600 MHz 310 K and 80 MHz 298 K; both readily distinguishing between controls and COVID-(+) samples. K B) Box plots showing the SPC/Glyc ratio for healthy controls (black) and COVID-(+) samples at 600 MHz 298 K and 80 MHz 298 K; both readily distinguishing between controls and COVID-(+) samples. C) Linear fit for the relationship of SPC/Glyc at 600 MHz, 310 K and 80 MHz. D) Linear fit for the relationship of SPC/Glyc at 600 MHz 298 K and 80 MHz.

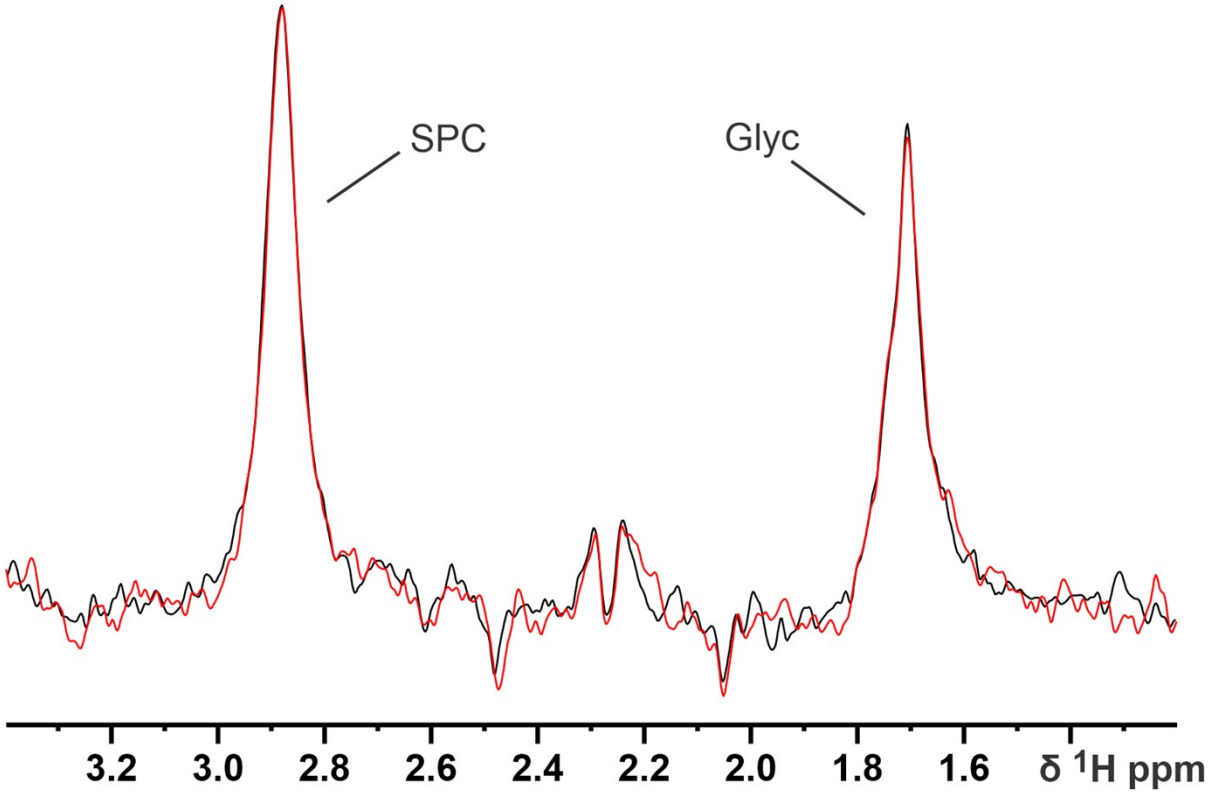

Figure S9: Chemical shift sensitivity of SPC and Glyc in a serum sample at 80 MHz.
Two JEDI spectra of the SPC and Glyc region were recorded directly after insertion (black) and after 30 min of temperature equilibration (red) showing identical chemical shifts for SPC and Glyc. The spectra were not referenced in any way explaining the low frequency shift of both signals.

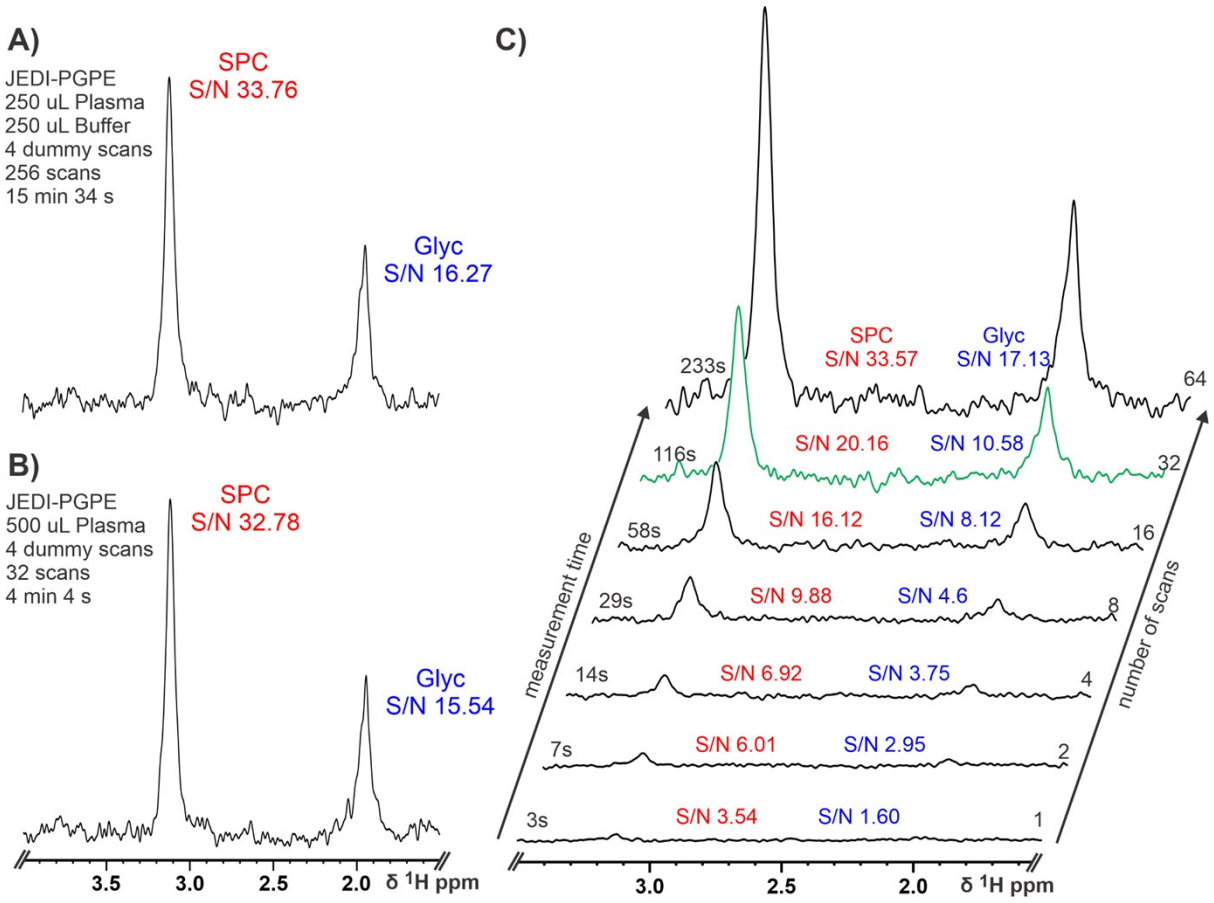

Figure S10: Effects of varying plasma concentration and number of scans/experimental time on JEDI-PGPE at 80 MHz.

A) Excerpt of a healthy control JEDI-PGPE spectrum at 80 MHz focusing on SPC and Glyc. The sample was prepared according to IVDr procedures, diluting the plasma 1:1 with a buffer (see Material and Methods) and the resulting required experimental parameters are shown. B) Excerpt of the same healthy control JEDI-PGPE spectrum as in A at 80 MHz focusing on SPC and Glyc. The sample was prepared without buffer dilution (see Materials and Methods) resulting in a four times reduction in measurement time, while retaining a similar S/N for SPC and Glyc. C) Excerpts of healthy control of JEDI-PGPE spectra at 80 MHz with increasing number of scans (no dummy scans)/ increasing experimental time. A limit of detection for SPC and Glyc ≥ 10 is reached after 32 scans with an experimental time of ~2 minutes.

References

1 A. C. Dona, B. Jimenez, H. Schafer, E. Humpfer, M. Spraul, M. R. Lewis, J. T. Pearce, E. Holmes, J. C. Lindon and J. K. Nicholson, *Anal. Chem.*, 2014, **86**, 9887–9894.

2 P. Nitschke, S. Lodge, T. Kimhofer, R. Masuda, S.-H. Bong, D. Hall, H. Schäfer, M. Spraul, N. Pompe, T. Diercks, G. Bernardo-Seisdedos, J. M. Mato, O. Millet, D. Susic, A. Henry, E. M. El-Omar, E. Holmes, J. C. Lindon, J. K. Nicholson and J. Wist, *Anal. Chem.*, 2022, **94**, 1333–1341.

3 S. Akoka, L. Barantin and M. Trierweiler, *Anal. Chem.*, 1999, **71**, 2554–2557.

4 G. Wider and L. Dreier, *J. Am. Chem. Soc.*, 2006, **128**, 2571–2576.