Supplementary Information (SI) for Analyst.
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Supporting information

S1. The impact of the Ag nanoparticle substrate on the variability of blood serum SERS signal.

Surface-enhanced Raman spectroscopy measurements may exhibit greater variability in the recorded data compared to Raman and fluorescence spectroscopy. This variability arises from two primary factors: (1) the inherent irregularity of the SERS substrates employed, and (2) the stochastic arrangement of the sample components on the substrate surface. The random geometry of the substrate, coupled with the unpredictable deposition of components, contributes to significant fluctuations in the collected signals. A major challenge in this context is the identification of effective "hot spots," where the experimental geometry is conducive to generating a strong plasmon resonance effect, thereby facilitating the detection of an enhanced Raman signal. Consequently, SERS measurements have predominantly been oriented towards qualitative analysis, as achieving quantitative results under these conditions has proven to be particularly challenging[1].

Despite the depicted problems, it is possible to minimize the registered signal variation (however, it is not possible to fully eliminate the problem). This requires (1) unification of the utilized SERS-substrate surface and (2) maximum leveling of the applied layer of tested sample. For this purposes it is possible to utilize colloids of noble metals to achieve required unification in the analysis of biofluids, specifically blood and blood serum[2].

Bonifacio et al demonstrated that Au and Ag colloids may be efficiently utilized for the analysis of blood and blood fractions; and that is noteworthy, they concluded that Ag nanoparticles may be utilized for repeatable SERS signal acquisition in case of near-infrared signal excitation. Further A. Bonifacio, J. Popp and co-authors provided a detailed explanation of suitable SERS strategies for the analysis of biofluids[3]. This review acknowledges the described approaches for the stable SERS signal acquisition.

Moreover, with the progress of SERS techniques, different research groups nowadays report about the possibility to apply SERS in quantitative analysis of biofluids. As example, Xu et al[4], provided a description of stable SERS approaches for quantitative analysis of hundreds of different analytes. In general, from the presented literature one may conclude that it is possible

to utilize Ag-based substrate to achieve stable SERS measurements (suitable even for quantitative measurements).

In our study we utilize an approach of SERS analysis based on Ag nanoparticles implementation. The full characterization of utilized SERS substrate is provided in[5]. Similarly to A. Bonifacio we prepare an Ag nanoparticles that further were dried on a metal surface to create a SERS substrate. Application of exact preparation conditions provides unification of Ag nanoparticles. Size of the nanoparticles was controlled by UV-Vis absorbance spectrum, while size of Ag nanoparticles agglomerates in the SERS substrate were controlled by electron-microscopic images. Altogether such approach resulted in a unification of utilized substrate surface. Centrifugation of serum sample ensures homogenization of the sample. Another important feature is utilization of trisodium citrate while the production of the substrate. This allows one to achieve the best contact angle between the drop of blood serum and surface of the substrate. As a result, the analyzed serum sample is distributed in a thin layer over the substrate. Such geometry enables acquisition of signal only from the area of direct analyte absorbance onto the SERS-active surface. The described methodology helps to minimize the coffee-ring effect that commonly affect SERS measurements[6]. Thus, the proposed approach provides both (1) the minimizing of the utilized SERS-substrate irregularity and (2) regular/known arrangement of tested samples components on the utilized substrate surface.

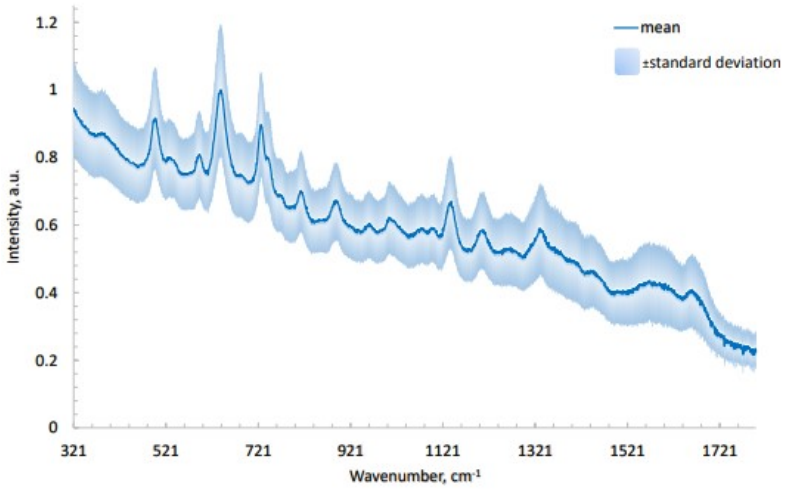

Figure S1 - Mean and standard deviation for blood serum sample examined for 5 different SERS substrates at 15 different spots.

Analysis of the proposed SERS substrate provided two main findings. Firstly, the SERS signal acquisition in the single point of the substrate is stable (differences between spectra are less than 4%). This indicates that no blinking phenomenon of SERS spectra from Ag nanoparticles was

detected[7]. The term “blinking” is used to describe temporal fluctuations in the Raman spectral signal. It is typically accepted that the observation of blinking effect in the SERS spectrum is indicative of a single molecule undergoing adsorption/desorption cycles at a SERS “active” site. Secondly, the maximum observed standard deviation (SD) of the SERS signal registered in different SERS substrates and different areas of substrates was not exceeding 19%. The data was measured for 5 different SERS substrates in 15 different areas (Fig. S.1). Note here that in the proposed methodology we utilize detector operating at the ambient temperature. Thus, partly deviation of the registered SERS signal is due to the utilized detector characteristics. The proposed methodology provides an opportunity for quantitative analysis of human blood serum components. For example, determination of urea and creatinine content is possible with R^2 more than 0.8 , as demonstrated by Bratchenko, et al. [8].

In general, the obtained results indicate accuracy and stability of the proposed SERS technique and SERS substrates. However, due to the nature of SERS measurements some deviation in the registered SERS signal is still possible.

S2. UV-fluorescence and optical density of fresh and FTC blood serum samples.

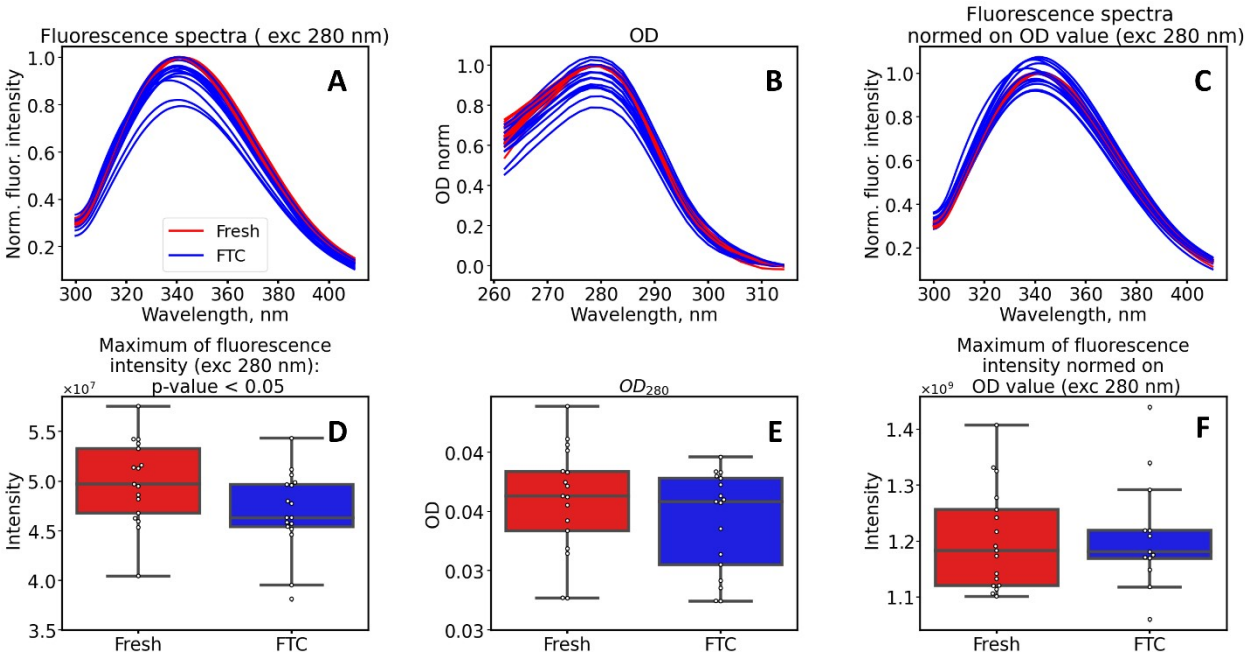

Figure S2 A. Fluorescence spectra of fresh and FTC blood serum samples at 280 nm excitation normalized on the maximum of the signal of fresh samples. B. Optical density spectra of fresh and FTC blood serum samples normalized on the OD maximum of the fresh samples.C. Fluorescence spectra of fresh and FTC blood serum samples at 280 nm excitation normalized on OD at 280 nm normalized on the maximum of the signal of fresh samples. D. Fluorescence spectra maximum values of fresh and FTC blood serum samples at 280 nm excitation. E. OD values of fresh and FTC blood serum samples in the UV spectral region. F. Fluorescence spectra maximum values of fresh and FTC blood serum samples at 280 nm excitation normalized on OD values at 280 nm.

S3. PARAFAC of fluorescence EEM in the excitation range of 320-480 nm range.

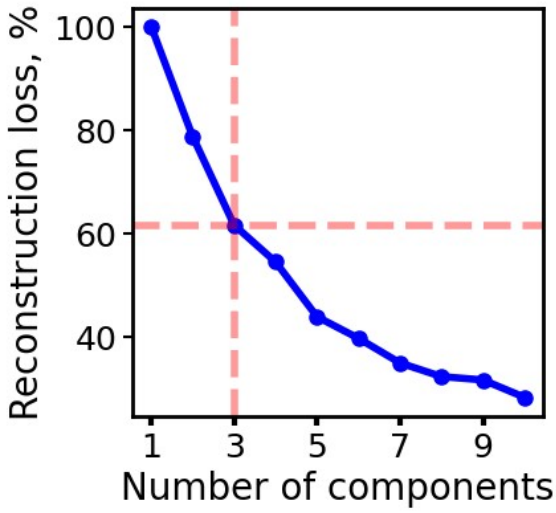

Figure S3. Reconstruction loss function of PARAFAC decomposition of blood serum EEM.

S4. The evaluation of Ag nanoparticle substrate-specific variability in blood serum SERS signal.

The substrate-specific variability was evaluated in a manner similar to our assessment of FTC-induced variability. We made use of the ANOVA F-test to analyze the grouping trends of the PCA amplitudes of the SERS spectra of blood serum based not only on the presence or absence of FTC and patient ID but also considering both parameters simultaneously. The purpose was to determine whether there was a significant grouping trend based on both these parameters, which would suggest that the SERS spectra of fresh samples from individual patients were similar to each other, as well as those FTC-treated samples, indicating minimal substrate-specific variability. Our results, presented in Figure 6D in the main article, demonstrate a strong grouping trend for all components. This suggests that substrate-specific variability is < 1%, which is significantly lower than the FTC-induced variability of 15% and the patient-specific variability, which is ~ 90%.

S5. The evaluation of FTC effect on blood serum SERS signal in the range starting from 425 cm^{-1}.

In our study, the SERS signal of blood serum samples was initially measured within the spectral range starting from 425 cm^{-1} (Fig. S4A). Here, we present a similar analysis applied to the SERS spectra in the reduced region, as outlined in the main text of the article. We first conducted

principal component analysis (PCA) on both fresh and FTC blood serum samples, resulting in the identification of 10 components, components 1-5 are illustrated in Figure S4B. Subsequently, we

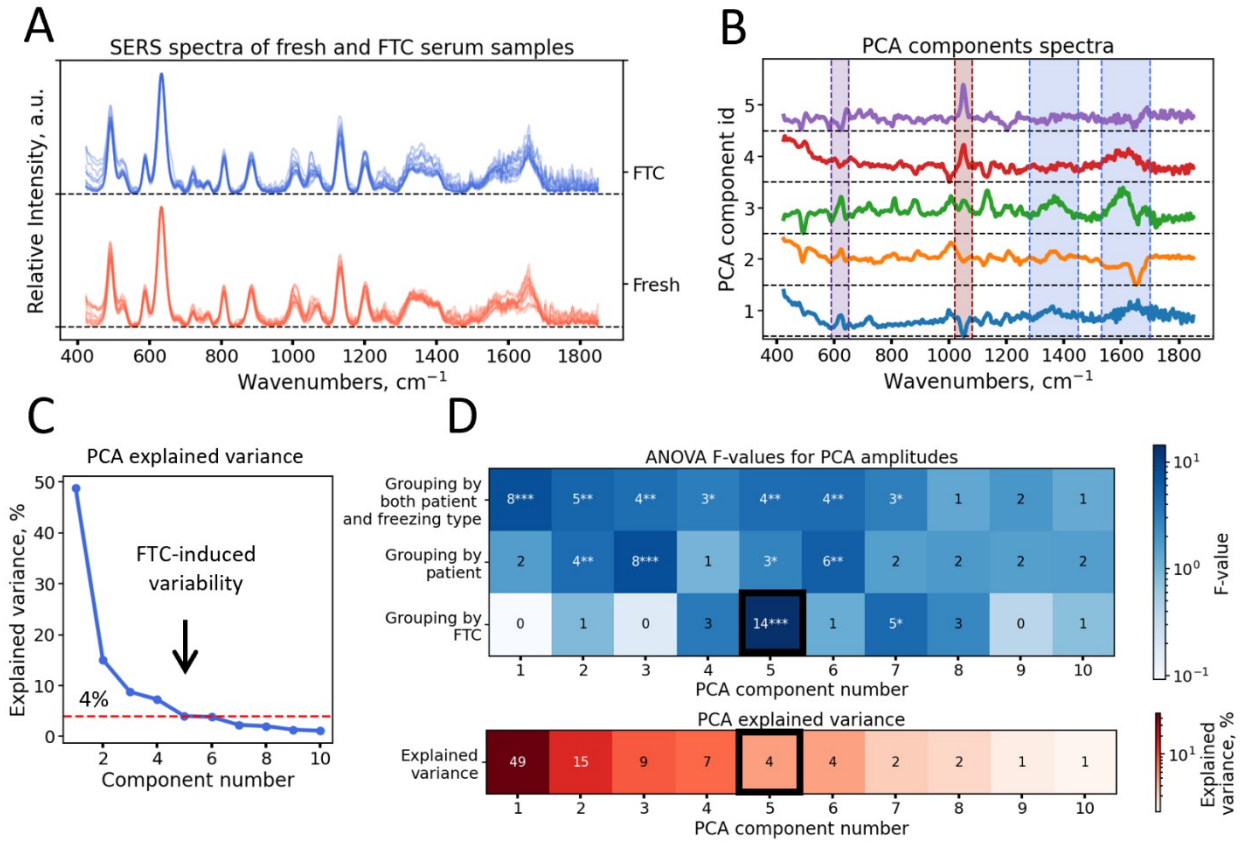

Figure S4.A. SERS spectra of fresh and FTC-treated blood serum samples measured in the spectral region of 425-1850 cm^{-1}. B. The PCA-derived components 1-5 of blood serum SERS spectra. The most pronounced bands are colour-marked. C. The PCA explained variance for blood serum SERS spectra. The dashed line indicates the explained variance of 4%. D. The results of the ANOVA test implementation for the assessment of variability of PCA amplitudes. The numbers of the most freezing-sensitive PCA components are highlighted by white rectangles. The F-values that surpass the critical F-value for a significance level of $p < 0.05$ are denoted with a single asterisk (*), those for a significance level of $p < 0.01$ are indicated by double asterisks (**), and values for a significance level of $p < 0.001$ are marked with triple asterisks (***).

performed the analysis of variance (ANOVA) F-test on the amplitudes of the obtained PCA components to determine whether there were significant grouping trends based on patient ID or the presence or absence of FTC. Our analysis revealed a significant grouping trend based on patient ID, with F-values exceeding the F-critical value for a significance level of $p < 0.01$ (indicated by double (**) and triple (***) asterisks) for PCA components 2, 3, and 6, which together account for 28% of the variance in the data (Fig. S4C-D). This indicates that patient-specific variability in the blood serum SERS signal may be estimated as 28%. Conversely, when examining grouping trends based on the presence or absence of FTC, only PCA component 5 exhibited an F-value larger than the F-critical value for a significance level of $p < 0.001$ (marked by triple (***) asterisks). This component accounts for merely 4% of the variance in the data (see

Fig. S4C-D), suggesting that FTC-induced variability in the SERS signal of blood serum is limited to 4% within this spectral region. Therefore, regarding the initial spectral region spanning from 425 cm^{-1}, our findings, presented in main text of the article, were validated: the variability in the blood serum SERS signal specific to individual patients was less than that induced by FTC. This supports the feasibility of implementing FTC prior to blood serum SERS measurements.

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