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LABEL-FREE VOLTAMMETRIC SCREENING OF HUMAN BLOOD SERUM[1]

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The current study presents a comprehensive voltammetric investigation into the direct analysis of untreated human blood serum in a phosphate buffer at an unmodified, graphite electrode by means of voltammetry. By employing advanced square-wave voltammetry at an edge plane pyrolytic graphite electrode (EPPGE), the basic principles were established for developing a sensitive, fast, simple, and label-free method for the simultaneous screening of uric acid, bilirubin, and albumin analytes that are present in human blood serum and are quite essential for rapid medical diagnostics. The electrochemical protocol utilizes the specific structural patterns of the EPPGE, the inherent redox and adsorption properties of the analyzed analytes, and the sensitivity and rapidity of the employed advanced voltammetric technique.

The methodology has been successfully applied for quantification of the considered analytes in a series of samples of human blood serum and was compared with the standard methods used in a clinical biochemical laboratory. This novel method represents a significant advancement towards the development of point-of-care devices aimed at swiftly and simultaneously quantifying uric acid, bilirubin, and albumin levels in human serum.

Keywords: human blood serum; label-free voltammetric biosensor; uric acid; bilirubin; albumin

ВОЛТАМЕТРИСКА АНАЛИЗА НА ЧОВЕЧКИОТ КРВЕН СЕРУМ БЕЗ ДОПОЛНИТЕЛНА УПОТРЕБА НА РЕАГЕНСИ

Во оваа студија е претставено комплетно волтаметриско испитување насочено кон директна анализа на човечкиот крвен серум во фосфатен пуфер со употреба на немодифицирана графитна електрода. Со примена на квадратнобранова волтаметрија на електрода од странично ориентиран пиролитичен графит се поставени основните принципи за развој на чувствителен, брз, едноставен и ефикасен метод за симултан скрининг на мочна киселина, билирубин и албумин, аналити кои се присутни во човековиот крвен серум и се многу важни за брза медицинска дијагностика. Електрохемискиот протокол се базира на специфичните структурни карактеристики на електродата од странично ориентиран пиролитичен графит, интегралните редокс и атсорпциски својства на анализираните аналити, како и чувствителноста и брзината на употребената напредна волтаметриска техника.

Методологијата е успешно применета за квантификација на разгледаните аналити во серија примероци на човечки крвен серум и споредена е со стандардните методи користени во клиничко-биохемиските лаборатории. Овој нов метод претставува значителен чекор кон развојот на уредите

[1] Dedicated on the occasion of the Golden Jubilee of the *Macedonian Journal of Chemistry and Chemical Engineering*

за point-of-care, кои се насочени кон брзо и симултано квантифицирање на концентрациите на мочна киселина, билирубин и албумин во човечкиот крвен серум.

Клучни зборови: човечки крвен серум; волтаметриски биосензор; мочна киселина; билирубин; албумин

1. INTRODUCTION

The ongoing demand for the precise determination of biochemical markers requires the design of cost-effective and reliable devices capable of swiftly providing analytical responses to specific analytes. This necessity is particularly pronounced in clinical analyses, where advanced devices are being developed to allow for label-free, in situ sensing of important physiological systems across diverse matrices.[1] The effectiveness of such instrumental devices is crucial for the rapid diagnosis of numerous pathologies. Among the instrumental devices being explored in clinical analyses, there is a noticeable increase in the design of simple amperometric biosensors capable of converting specific biochemical signals into measurable electric currents that correlate with defined analyte concentrations. Key considerations in the design process of electrochemical amperometric biosensors include their specificity, reliability, and prompt response to analyte levels within a given matrix. In a broader context, amperometric biosensors consist of specific electronic conductors (working electrodes) that serve as platforms for facilitating electron exchange with defined physiological systems in a selective manner. Literature indicates that the development of amperometric sensors is tailored for quantifying significant analytes such as haemoglobin, uric acid, cholesterol, glucose, hydrogen peroxide, and creatinine, among others.[2–8]

While the rapid response, cost-effectiveness, and selectivity are favourable attributes of amperometric sensors,[6–8] challenges persist due to the potential contamination of the working electrode and interferences arising from various analytes present in complex matrices like urine, blood, or serum. In our recent study, we elucidated a series of experimental findings concerning the direct square-wave voltammetry (SWV) of human blood serum.[9] Utilizing an edge plane pyrolytic graphite electrode (EPPGE) as the working electrode, we demonstrated the distinct and simultaneous identification of three voltammetric signals corresponding to uric acid (UA), bilirubin (BLR), and albumin (ALB), while referring to some of their specific redox characteristics.

The results presented in the current work build upon our previous results[9] by providing a comprehensive examination of the electrochemical characteristics inherent to these systems. This investigation is vital for the development of a voltammetric biosensor capable of simultaneously quantifying these three compounds directly in human blood serum. The findings outlined herein can be viewed as a crucial foundation for the development of an economically feasible and rapid amperometric biosensor tailored for the direct quantification of the three analytes in human blood serum.

2. EXPERIMENTAL

The use of human blood serum samples was approved by the Ethical Committee of the Faculty of Medical Sciences, Goce Delčev University, Štip, North Macedonia, under Decision No. 0801–2/13, dated February 16, 2022. Voltammetric measurements were performed at room temperature, utilising a conventional three-electrode voltammetric cell, with square-wave voltammetry explored as a working technique. A PalmSens2 potentiostat/galvanostat (PalmSens BV, Netherlands) was the instrumental system used, under the control of PSTrace 5.9 software (PalmSens BV). An edge plane pyrolytic graphite electrode (EPPGE) with a geometrical surface area of 7.069 mm² (ALS Co., Ltd.) was used as the working electrode, a graphite rod as a counter electrode, and an Ag/AgCl electrode (3 M KCl) as the reference electrode. The EPPGE was meticulously cleaned through sequential treatment with abrasive paper, Al_2O_3 slurry, followed by rinsing with deionized water and subsequent air-drying. Unless specified otherwise, the instrumental parameters of SWV signal chosen for the purpose of analytical investigations included a frequency of 50 Hz, an amplitude of 50 mV, and a step potential of 4 mV. A phosphate buffer solution (0.1 M) with a pH of 7.34 was utilized as the supporting electrolyte in all experiments. In instances not explicitly specified, 500 µl of human blood serum sample were dissolved in 20 ml of phosphate buffer (2.4% (V/V)) for consecutive analyses in voltammetric experiments.

Stock solutions of uric acid (Reanal Budapest, p.a. > 99 %) and bovine serum albumin (Sigma Aldrich, ≥ 98 %) were prepared by dissolution in phosphate buffer (0.1 M) with pH of 7.34, while

the bilirubin stock solution (Alfa Aesar, 97 %) was prepared by dissolving it in dimethyl sulfoxide (Thermo Scientific, 99.7 %). All other solutions were prepared using deionized water from the Arium® mini Plus purification system provided by Sartorius. The interferences of various analytes (glucose, cholesterol, and triglycerides) were examined using aqueous primary standards obtained from Biosystems.

Serum preparation followed established protocols, involving the collection of whole blood and allowing coagulation with a clot-activator for 15 – 25 minutes at room temperature. Subsequent centrifugation at 1,000 – 2,000 rotations per minute for 10 minutes facilitated the separation of the serum as a supernatant fraction. The obtained serum samples were stored at −18 °C until required, without further treatment. The standard spectrophotometric protocols for the quantification of uric acid, bilirubin, and albumin in human blood serum were applied, following the procedures described in more details in our previous work.[9]

All concentrations reported in the experiments pertain to their respective values within the electrochemical cell.

To estimate the molar concentration of a given analyte in the electrochemical cell, we applied the following equation:

$$c_2 = c_1 \cdot \varphi, \text{ where } \varphi = V_{\text{serum}} / (V_{\text{buffer}} + V_{\text{serum}}) \quad (1)$$

and φ represents the volume fraction. Here, V_{serum} is the volume of serum used for analysis in the electrochemical cell, and V_{buffer} is the volume of the buffer solution present in the electrochemical cell. In this context, c_2 signifies the molar concentration of the specified analyte in the electrochemical cell, while c_1 is the molar concentration of that analyte in human blood serum. To determine the molar concentration of albumin, we utilized the following expression:

$$c(\text{ALB}) = \gamma(\text{ALB}) \,/\, M(\text{ALB}), \quad (2)$$

where γ(ALB) denotes the mass concentration of albumin, and M(ALB) stands for the molar mass of albumin (66,500 g/mol).

3. RESULTS AND DISCUSSION

3.1. *Basic voltammetric characterization of uric acid, bilirubin, and albumin in human blood serum*

Conducting voltammetry within complex matrices such as human blood serum poses considerable challenges. Large biomolecules like proteins, and other analytes such as cholesterol, triglycerides, etc., in human blood serum often exhibit strong adsorption onto the surfaces of many working electrodes, thereby impeding electron transfer between the working electrode and the numerous redox-active analytes of interest. While certain electrodes, primarily graphite-based ones, have demonstrated some efficacy in designing biosensors for various analytes in human serum,[10–13] recent exceptional studies have underscored EPPGE as a highly promising working electrode for conducting direct voltammetry within complex matrices.[9,14,15] Thus, by utilizing EPPGE, coupled with square-wave voltammetry[16,17] as the operative technique, our recent research has demonstrated the feasibility of conducting direct analysis in human blood serum.[9]

The methodology outlined in our previous work[9] facilitated rapid and label-free sensing of diagnostically important molecules, including uric acid (UA), bilirubin (BLR), and albumin (ALB), within human blood serum samples. This was achieved by dissolving only 2.4% (V/V) of human blood serum in a phosphate buffer solution (at physiological pH of 7.34 to 7.45). A representative example of the SW voltammetric response of human blood serum is depicted in Figure 1.

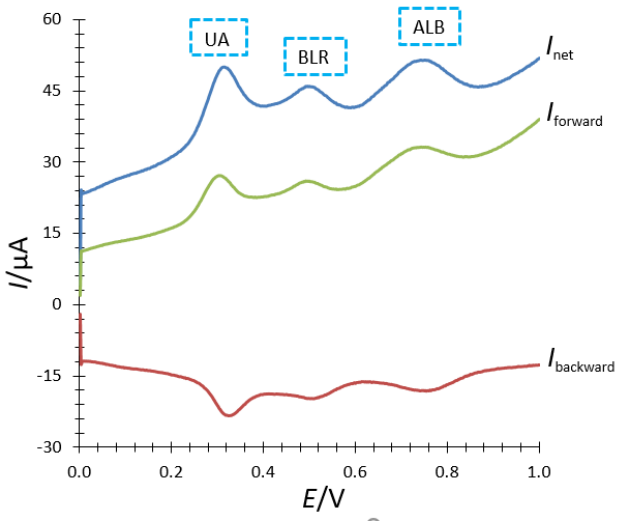

Fig. 1. Square-wave voltammogram of 2.4% (V/V) human blood serum in a phosphate buffer solution with pH = 7.34. The graph displays forward, backward, and net component of the recorded square-wave voltammogram. The concentrations of uric acid (UA), bilirubin (BLR), and albumin (ALB) in the electrochemical cell were 10.7 μM, 5.5 μM, and 7.6 μM, respectively. Experimental conditions included a square-wave amplitude (E_{sw}) of 50 mV, step potential (dE) of 4 mV, square-wave frequency (f) of 50 Hz, deposition potential (E_{dep}) of 0.00 V, and deposition time (t_{dep}) of 30 s.

As comprehensively elaborated in our recent work,[9] the initial process occurring at a potential of about +0.26 V corresponds to the redox activity associated with uric acid, while a second process taking place around +0.46 V is attributed to the redox activity of bilirubin. The third process, occurring at approximately +0.73 V, has been identified as originating from the redox activity of albumin. The three separate electrode processes are associated with a significant degree of electrochemical reversibility, as evidenced by distinct forward and backward current components. Additionally, redox active species exhibit notable affinity for adsorption onto the surface of the working electrode.[9] The notable electrochemical reversibility of bilirubin, uric acid, and albumin present in the serum correlates with their intrinsic attributes to undergo rapid electron exchange with the surface of the working EPPGE.

Figure 2 illustrates a series of SW voltammograms of human blood serum recorded following extended deposition periods at the initial potential of 0.0 V vs. Ag/AgCl reference electrode. The rather sharp definition of net SW voltammetric peaks, associated with the variations in their magnitudes relative to deposition time, frequently serve as compelling indicators of electrode processes of surface immobilized redox species (refer to Fig. 2).

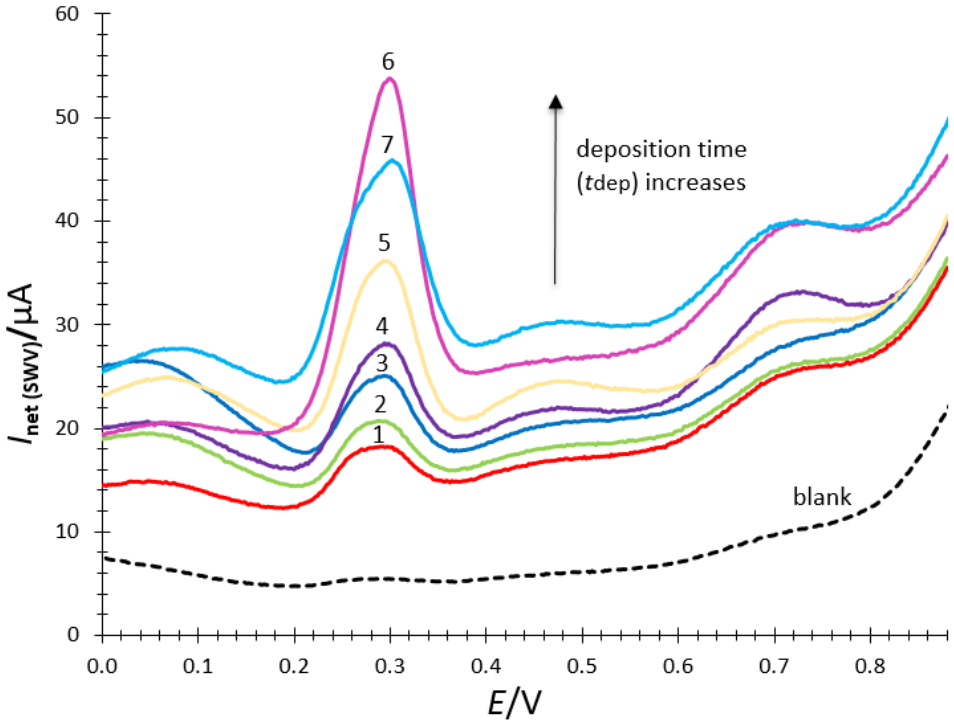

Fig. 2. Net square-wave voltammograms illustrating the changes observed as a function of deposition time for 2.4% (V/V) of human blood serum in a phosphate buffer solution with pH = 7.34. The concentrations of uric acid, bilirubin, and albumin in the electrochemical cell were 14.2 μM, 2.3 μM, and 20.4 μM, respectively. Applied deposition time was 0 s (1), 15 s (2), 20 s (3), 30 s (4), 45 s (5), 60 s (6), and 120 s (7). The curve at the bottom represents the blank of the phosphate buffer recorded in the absence of human blood serum. Refer to Figure 1 for additional experimental conditions.

Additionally, the adsorption curves delineated in Figure 3 pertaining to uric acid, bilirubin, and albumin reveal distinct evidence of surface saturation on the working electrode beyond deposition time of about 85 s for uric acid (1), 50 s for bilirubin (2) and 90 s for albumin (3) under the specified experimental conditions referred to in Figure 3.

The observations regarding the adsorption characteristics of all three analytes on carbon-based electrodes are consistent with literature data,[18–20] as well as with our previous findings.[9] As can be inferred from Figure 3, the adsorption of uric acid dominates. Moreover, the intensity of the response of uric acid is a consequence of rapid electron exchange and, thus, its significant degree of electrochemical reversibility, which is particularly important for electrode processes under conditions of SWV.[16] The saturation of the electrode with bilirubin takes place at a significantly shorter deposition time compared to uric acid (curve 2 in Fig. 3), yet the response of bilirubin is much weaker due to the significantly more complex electrode

mechanism, accompanied by follow-up chemical reactions and the fouling effect of the reaction products.[21,22] Saturation of the electrode with albumin occurs at a comparable time to uric acid (compare curves 1 and 3 in Fig. 3). It should be noted that even though the concentration of albumin dominates in the medium in comparison to uric acid and bilirubin, the diffusion coefficient of the large albumin molecules is expected to be significantly lower than for other small analytes,[23,24] which effects the morphology of the adsorption isotherm (curve 3 in Fig. 3).

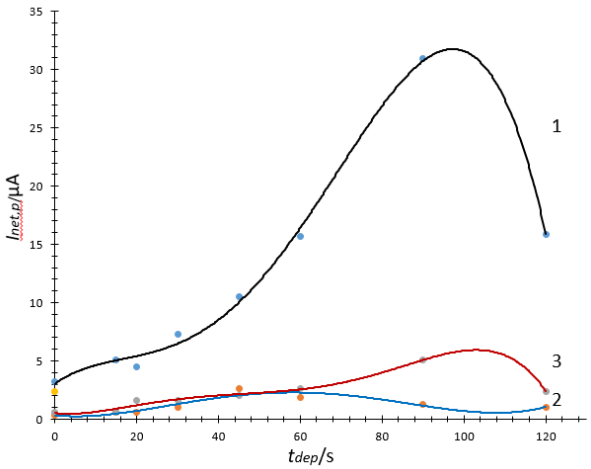

Fig. 3. Adsorption curves demonstrating the relationship between net SWV peak currents and deposition time (t_{dep}) for uric acid (1), bilirubin (2), and albumin (3). For additional experimental conditions, refer to Figure 1.

Given the complexity of the studied medium, it should be noted that the decreasing intensity of any voltammetric peak at prolonged deposition times could be a consequence of competitive adsorption with other analytes,[25,26] as well as the competitive adsorption of the studied analytes. Thus, the decrease in response beyond a certain deposition time does not necessarily imply saturation of the electrode with a particular analyte only. Instead, it reflects the multifaceted surface phenomena occurring in such a complex medium.

3.2. *Effect of the instrumental parameters on the voltammetric signals of uric acid, bilirubin, and albumin in human blood serum*

The optimization of instrumental parameters is regarded as a pivotal stage in the comprehensive process of designing amperometric biosensors, particularly in samples where multivariate analysis is conducted. In the current scenario, wherein the redox transformation of the three studied biomolecules in human blood serum originates predominantly from an adsorbed state, factors such as frequency, square-wave amplitude, step potential, deposition potential, and deposition time are critical parameters essential to delineating voltammetric peaks exhibiting desirable measurable characteristics. With respect to the deposition potential, it should be noted that the influence was tested in regions between –0.30 V and +0.40 V. Because the maximum current magnitudes were obtained at a deposition potential of 0.00 V, this value was selected as optimal for analytical purposes. It has been extensively elucidated that frequency stands as a critical instrumental parameter across all pulse voltammetric methodologies,[16,27] with particular significance in square-wave voltammetry.[16,28] Its influence extends beyond merely modulating the rate of electron exchange between the working electrode and the redox analyte molecules, but it also exerts an impact on the adsorption phenomena occurring throughout the temporal duration of each applied potential pulse at surface-confined redox systems.[16] Figure 4 depicts the relationships between the currents associated with the net square-wave voltammetric peaks of uric acid, presented as a function of the logarithm of the applied SW frequency.

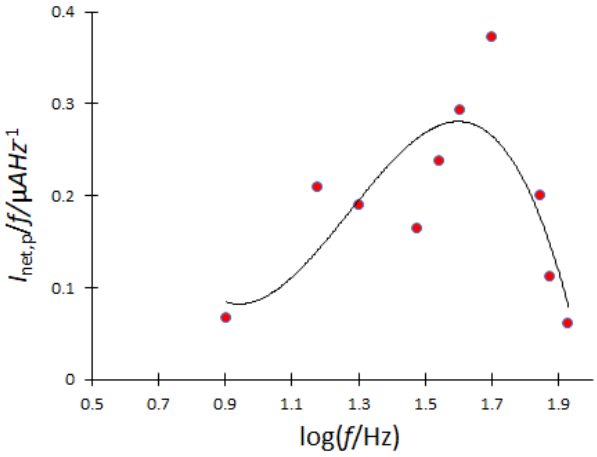

Fig. 4. The relationships between the ratio of $I_{net,p}/f$ of the net SW voltammograms for uric acid analysed in terms of the logarithm of the applied SW frequency. The concentration of uric acid in the electrochemical cell was 14.6 μM, while the concentrations of bilirubin and albumin were 2.21 μM and 21.2 μM, respectively. No deposition was applied for this set of experiments. For additional experimental conditions refer to Figure 1.

Specifically, a parabola-like dependency exists between the frequency normalized net peak currents and the SW frequency, exhibiting a maximum at around 50 Hz. Beyond this critical fre-

quency, the response decreases. This behaviour is characteristic of not only of uric acid but also of bilirubin and albumin. It implies that at higher frequencies (commonly larger than 70 Hz), artifacts arising from uncompensated resistance due to the blocking of the electrode with products of the electrode reactions for all three analytes become significant. This observation is further supported by the morphological analysis of the net SW peaks at frequencies higher than 70 Hz for all three analytes, correlating well with theoretical data.[29]

It is important to note that the parabolic curve presented in Figure 4, where the ratio of the peak current and the frequency is presented as a function of the logarithm of the frequency can be also analyzed in the context of the notable feature of the surface electrode processes under conditions of SWV known as the 'quasi-reversible maximum'.[16] The position of the maximum could be related to the standard rate constant of the electrode reaction, as discussed in the previous study.[9] More importantly for the present study, from an analytical perspective, the SW frequency corresponding to the maximum of the curve in Figure 4 is considered as the optimal parameter for quantifying the studied analytes. It provides the highest response while remaining relatively unaffected by artifacts arising from the complexity of the electrode mechanisms of the studied analytes.

Besides frequency, the square-wave amplitude stands out as one of the most adjustable parameters in square-wave voltammetry, influencing two primary characteristics of the resulting voltammograms: the background signal and the signal-to-noise ratio of the resultant peaks.[16,28] More importantly, the amplitude of applied pulses (E_{sw}) serves as a parameter predominantly influencing the rate of heterogeneous electron transfer between the working electrode and the analyte molecules.[16] For square-wave amplitudes up to approximately 70 mV, the intensity of all three voltammetric peaks increases in proportion to E_{sw}. However, as the square-wave amplitude exceeds 70 mV, all voltammetric peaks begin to broaden significantly, leading to some degree of mutual overlapping when the amplitude surpasses 80 mV. A square-wave amplitude of 50 mV yielded well-resolved peaks with readily measurable features; thus, this value was adopted for analytical purposes.

Moreover, the resolution and some of the major characteristics of voltammetric peaks were found to be influenced by the step potential. Besides resolution of voltammetric peaks, step potential affects the overall degree of electrochemical reversibility of electrode processes.[30] An optimisation study of this instrumental parameter revealed that a potential step of 4 mV proved to be quite suitable for analytical evaluations related to all voltammetric peaks. At potential steps larger than 8 mV, all voltammetric peaks became distorted to some extent.

3.3. *Effect of interferences*

Conducting a voltammetric analysis within a complex system such as human blood serum presents significant challenges, particularly when no pre-treatment or additional complexing reagents are introduced to the sample. It is anticipated that numerous molecules within human blood serum will exhibit an inclination to adsorb onto the surface of the working electrode, alongside uric acid, bilirubin, and albumin. In this context, many of these systems may exert considerable effects regarding adsorption forces, and potentially influencing the electron transfer step as well. Furthermore, concurrent interactions at the working electrode surface among the adsorbed molecules of uric acid, bilirubin, and albumin may also occur.

Considering various significant parameters found in human blood serum, we examined how glucose, cholesterol, and triglycerides affect the voltammetric behaviour of the analyzed substances. Through a series of experiments conducted in human blood serum, we discovered that glucose has no discernible impact on voltammetric peaks when its concentration is below 300 μM. Conversely, when cholesterol concentrations surpass 200 μM in the electrochemical cell, there is a notable reduction in the intensity of all voltammetric peaks (not shown). This observation generally applies to patients with elevated cholesterol levels in their blood. It is well documented that cholesterol, being highly lipophilic, tends to strongly adsorb onto the surface of carbon-based electrodes.[12]

This strong adsorption of cholesterol leads to significant insulation of the working electrode's surface, thereby impeding the adsorption of other analyte molecules and hindering the electron exchange between the working electrode and the molecules under study.

Regarding the impact of triglycerides on the voltammetric profiles of the investigated molecules, an intriguing trend is observed in the context of the uric acid (Fig. 5). An elevation in triglyceride concentration within the range from 44 μM to 52 μM results in a notable increase in the voltammetric peak of uric acid. However, a subsequent increase in triglycerides leads to a significant reduction in the intensity of the voltammetric peak

(refer to Fig. 5). Evidently, a moderate concentration of triglycerides facilitates adsorption and electron communication between the working electrode and the uric acid, whereas triglycerides present at concentrations exceeding 52 μM exhibit substantial adsorption at the working electrode surface, thereby significantly diminishing the surface concentration of uric acid. It is important to note that the concentrations of triglycerides depicted in Figure 5 are beyond the upper range of reference values of a healthy person. It should be noted that triglycerides in this concentration range have a minor impact on the peak of albumin (see the peak of albumin in the inset in Fig. 5).

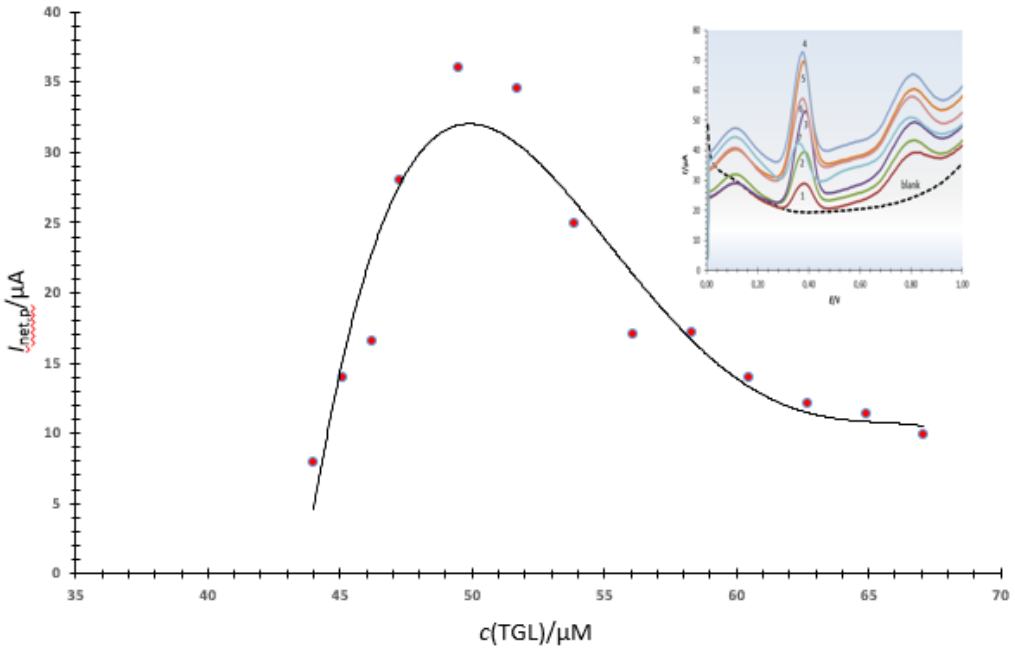

Fig. 5. A working curve demonstrating the impact of triglyceride concentration on the net SWV peak currents of uric acid. The experiments involved dissolving 2.4% (V/V) of human blood serum in a phosphate buffer, with conditions specified in Fig. 1. The concentration of uric acid in the electrochemical cell was maintained at 6.1 μM. The inset displays net square-wave voltammograms recorded at various concentrations of triglycerides: 44 μM (1), 45.1 μM (2), 46.2 μM (3), 49.5 μM (4), 51.7 μM (5), 53.9 μM (6), and 58.3 μM (7). The concentration of albumin in the electrochemical cell was 16 μM.

Given that the voltammetric activity exhibited by the examined systems (uric acid, bilirubin, and albumin) present in human blood serum occurs from an adsorbed state, it is reasonable to infer that competitive adsorption and interactions among the adsorbed analytes at the EPPGE surface are quite feasible. To investigate these effects, voltammetric analyses were conducted whereby the concentration of one analyte was systematically varied, while the concentrations of the remaining two analytes were held constant. It was observed that concentrations of uric acid exceeding 65 μM resulted in a concurrent reduction in the intensities of voltammetric peaks associated with both bilirubin and albumin. These findings agree with the general tendency of uric acid to adsorb more strongly than bilirubin, as can be inferred from data in Figure 3. Yet, concentrations of bilirubin exceeding 40 μM led to a decrease in the intensity of the uric acid peak as well. Obviously, competitive adsorption between bilirubin (c > 40 μM) and uric acid (c > 65 μM) is significant, which can have a negative impact on the magnitude of both peaks.

Conversely, concentrations of albumin exceeding 60 μM resulted in a modest augmentation of the intensity of the voltammetric peak associated with bilirubin. Given the established role of albumin as a protein mediating the membrane transfer of highly lipophilic bilirubin molecules,[31] it is reasonable to infer that elevated albumin concentrations within the voltammetric cell facilitate enhanced adsorption of bilirubin and promote its interaction with the working electrode. These interferences are significant in the context of voltammetry in human blood serum, as they contribute to a deeper comprehension of the voltammetric patterns of uric acid, bilirubin, and albumin. It is crucial to emphasise that more pronounced interactions between the analyte molecules are primarily expected in patients with elevated concentrations

of all three considered compounds in the serum. Yet, the problem with competitive interactions between the three analytes can be easily mitigated by decreasing the volume concentration of the studied serum in the voltammetric cell, i.e., to perform the analysis with less than 2.4% (V/V) serum content.

3.4. *Constructing plots showing the concentration dependences of the voltammetric peaks of UA, BLR, and ALB*

Figure 6 illustrates three curves demonstrating the concentration dependences of the voltammetric signals, constructed for all analyzed components.

Each data point on the concentration dependences lines in Figure 6 represents the average value derived from a minimum of nine measurements. Experimental procedures for generating the plots in Figure 6 involved the analysis of 2.4% (V/V) of human blood serum associated with a high bilirubin concentration, alongside normal concentrations of uric acid and albumin. The first point in each calibration line in Figure 6 refers to the concentration of a particular analyte in the serum determined by the UV-Vis method. The concentration was gradually increased in parallel for the three analytes, by introducing corresponding standard additions. Thus, a higher concentration of a particular analyte was measured in the presence of higher concentrations of the other two analytes in order to present the dependencies presented in Figure 6 in a more rigorous way, considering potential interactions between analytes. It is worth noting that subsequent to each voltammetric scan, the working electrode was cleaned in accordance with the procedure outlined in our earlier study.[9] The performance assessment of the SW voltammetric method is comprehensively presented through Table 1.

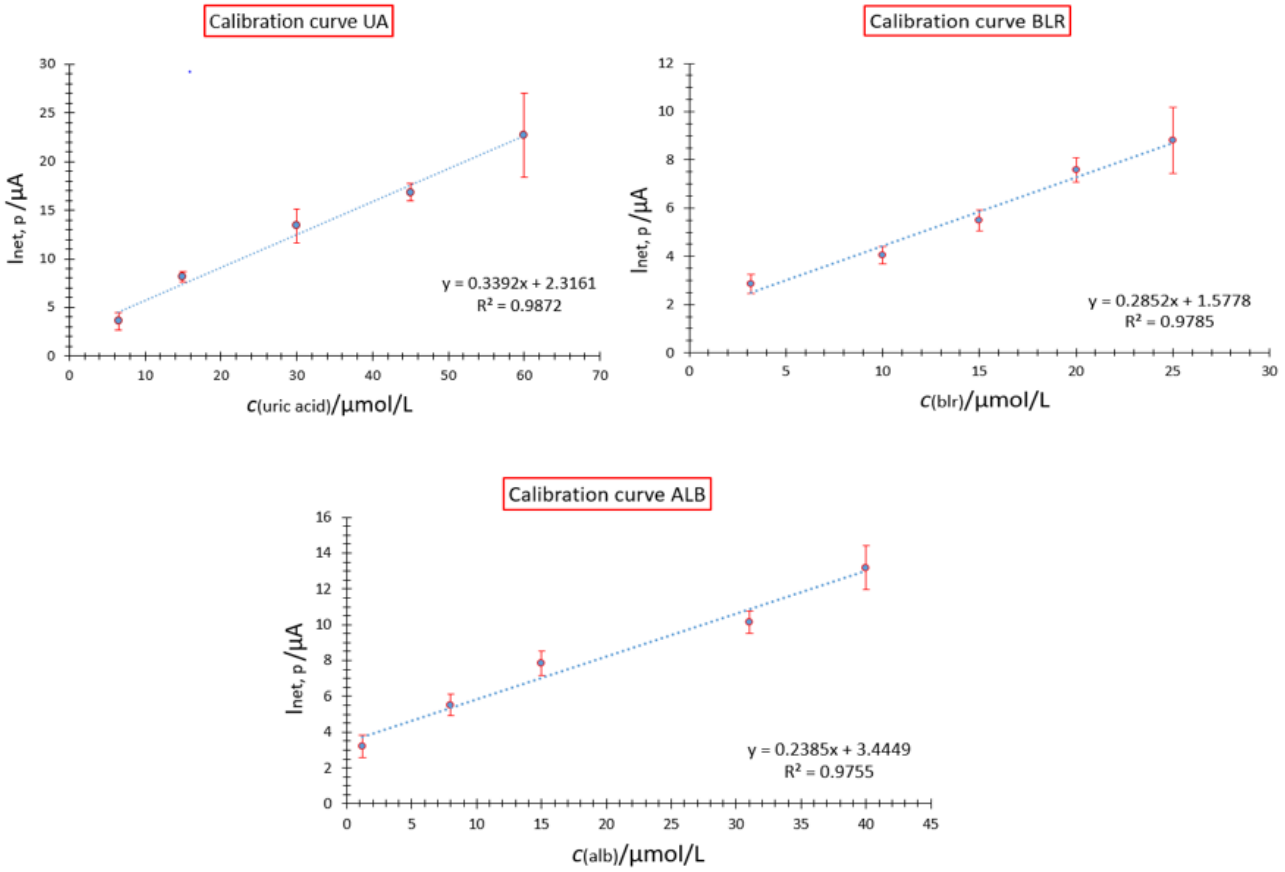

Fig. 6. Plots showing concentration dependences of voltammetric signals constructed for uric acid (UA), bilirubin (BLR), and albumin (ALB). Curves are constructed by the standard addition method performed with 2.4% (V/V) of human serum initially dissolved in phosphate buffer. The instrumental parameters used were SW frequency f = 50 Hz, SW amplitude E_{sw} = 50 mV, step potential dE = 4 mV, deposition potential E_{dep} = 0.0 V, and deposition time t_{dep} = 30 s. Each point presented on the graphs is an average of 9 measurements. The initial data points on each line shown represent their concentrations in human blood serum, as determined by the UV-Vis method.

T a b l e 1

Validation parameters for the determination of uric acid, bilirubin, and albumin obtained by square-wave voltammetry

Analyte	Concentration range/μM	Equation of linear regression line	Correlation coefficient R^2	Standard deviation SD/μA	Relative standard deviation RSD/%	LOD/μM	LOQ/μM
UA	6 – 60	$y = 0.3392x + 2.3161$	0.99	0.32	2.56	2.80	9.24
BLR	3 – 25	$y = 0.2852x + 1.5778$	0.98	0.25	2.60	2.20	7.26
ALB	2 – 40	$y = 0.2385x + 3.4449$	0.98	0.23	2.08	1.99	6.56

*The limit of detection (LOD) was estimated according to following equation: LOD = 3*S*/*a*; where *S* is the standard deviation of the net current of the blank, and *a* is the slope of the corresponding linear regression line. Limit of quantification (LOQ) is estimated as: LOQ = 3.3 × LOD.

Upon optimisation of experimental parameters, a linear correlation between the net SWV peak currents and concentration was observed for uric acid, bilirubin, and albumin across concentration ranges between 6 μM and 60 μM, 3 μM and 25 μM, and 2 μM and 40 μM, for uric acid, bilirubin, and albumin, respectively. The determined detection limits, as detailed in Table 1, are 2.80 μM, 2.20 μM, and 1.99 μM for uric acid, bilirubin, and albumin, respectively. Furthermore, the correlation coefficients (R^2) exceeded 0.99 and 0.98 for all examined analytes, affirming the method's robust linearity. Moreover, the relative standard deviation ranged between 2.00% and 2.60%, which indicates rather good precision of the method, keeping in mind the entire complexity of the studied matrix. The error bars depicted in the dependencies of Figure 6 reveal elevated standard deviations, observed particularly at higher analyte concentrations. This phenomenon can be attributed to the pronounced mutual interferences among all three components, which manifest more prominently at elevated analyte concentrations, as discussed earlier in this work.

3.5. *Analytical applications*

The voltammetric methodology developed in this study was employed to simultaneously determine the concentrations of studied analytes in seven samples of human serum. The results obtained from these voltammetric experiments are delineated in Table 2, alongside data acquired from the standard UV-Vis method used in the clinical laboratory. Evidently, notable agreement is observed between both datasets, with more pronounced disparities emerging necessary at elevated concentrations of uric acid (see serum No.4, for example). This outcome was anticipated, given the influence elucidated earlier in this investigation regarding the impact of heightened uric acid concentrations. It is pertinent to note that the data presented in Table 2 denote the concentrations of the analytes in the electrochemical cell.

T a b l e 2

A comparison between determined values for concentrations of UA, BLR, and ALB with UV/Vis protocol and with voltammetric methodology described in this work

Serum No.	UA/UV-Vis μmol/l	UA in this work μmol/l	BLR/UV-Vis μmol/l	BLR in this work μmol/l	ALB/UV-Vis μmol/l	ALB in this work μmol/l
1	5.2	6.9	/	/	15.5	16.2
2	/	/	/	/	20.8	21.6
3	4.3	8.8	/	/	18.5	15.7
4	64.5	54.2	3.43	4.3	23.6	24.1
5	/	/	10.1	9.2	/	/
6	5.6	5.8	7.2	6.6	/	/
7	35.1	42.8	/	/	/	/

*Values refer to the concentrations in electrochemical cell.

**For this set of measurements, 2.4% (V/V) of human serum were dissolved in phosphate buffer with pH of 7.34. Voltammetric measurements were conducted at deposition time t_{dep} = 30 s, frequency f = 50 Hz, amplitude E_{sw} = 50 mV, and step potential dE = 4 mV. For each serum, a minimum of 5 measurements were performed, and the average values of the peak currents were used for the evaluation of concentrations of UA, BLR, and ALB, by using the equations of linear lines from Table 1.

***For samples with numbers 4 and 7, 4.8% (V/V) human serum was used

4. CONCLUSIONS

This study presents a comprehensive voltammetric investigation into the direct analysis of untreated human blood serum. By employing advanced square-wave voltammetry at an unmodified, edge plane pyrolytic graphite electrode, a sensitive method for simultaneous quick screening of three crucial analytes (uric acid, bilirubin, and albumin), essential for rapid medical diagnostics, was established. The findings presented in this work demonstrate the feasibility of designing a cheap, fast, and reliable amperometric biosensor, even in highly complex media such as human blood serum. Without the need for any chemical pretreatment of the serum sample, the developed methodology enables simultaneous label-free quantification of the three analytes.

While the methodology developed relies to some extent on the overall composition of the matrix, it offers a streamlined approach, circumventing the need for expensive and time-intensive procedures traditionally employed in the electrochemical detection of targeted analytes.[6–8,31,32] The efficacy and reliability of the simple method were successfully validated by quantifying the three analytes in a series of samples of human blood serum from several patients, providing quantitative data that was in agreement with the standard methods used in a clinical biochemical laboratory.

The developed analytical protocol stands out due to its importance, particularly considering the predominant use of enzymatic-based methods and the widespread adoption of nanoparticle-modified electrodes in the majority of electrochemical biosensors designed for quantifying these systems.[11,13,14,19,33–36] The reported methodology is, indeed, envisioned as a pivotal step towards the establishment of point-of-care devices for the rapid and simultaneous quantification of uric acid, bilirubin, and albumin in human serum, which to the best of our knowledge, is a unique finding.

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