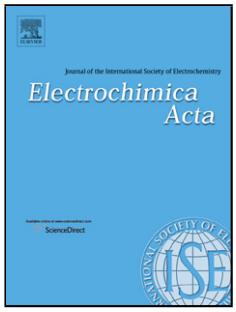

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Advanced electrochemical platform for determination of cytostatic drug flutamide in various matrices using a boron-doped diamond electrode

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Highlights

- First report dealing with electrochemical oxidation of flutamide is presented
- BDD electrode was used for the first time in sensing of cytostatic drug
- Advanced innovative platform for determination of flutamide was developed
- Submicromolar LOD values were achieved using bare BDD electrode
- Simple and effective alternative to mercury-based and modified electrodes

Abstract

An innovative, rapid and simple electrochemical approach for the reliable quantification of cytostatic drug flutamide (FLU) in various matrices is herein proposed. This platform involves coupling of differential pulse (DPV) and square-wave voltammetry (SWV) with a boron-doped diamond (BDD) electrode as the working electrode and 0.1 M sulphuric acid as the supporting electrolyte. For the first time, the ~~cyclic~~ voltammetric profile of FLU was manifested by three irreversible and diffusion-controlled oxidation peaks at +1.1 (P1), +1.4 (P2) and +1.9 V (P3). The analytical performance evaluation was assessed for all three peaks, using both pulse voltammetric techniques with the optimized operating parameters and the highest sensitivity of 1.76 nA/µM was accomplished for P2 using DPV and 3.54 nA/µM for P3 using SWV. The corresponding linear concentration ranges were found to be 0.99 – 42.9 and 4.8 – 35.5 µM with the detection limits of 0.42 and 0.18 µM, respectively. The repeatability varied, depending on the oxidation peaks of FLU, with the relative standard deviations in the range of 3.3 – 8.8% and 2.9 – 9.3% for DPV and SWV, respectively. The proposed electrochemical platform was successfully applied in the analysis of pharmaceutical formulations, spiked human urine and water samples with the significant mean recoveries. Using BDD electrode, the current work establishes an advanced, simple and rapid alternative platform to so far used toxic mercury-based electrodes and time demanding chemically modified electrodes in cytostatic sensing. Besides, BDD electrode represents a comfortable electrochemical sensor for routine analysis in pharmaceutical, clinical and environmental chemistry.

Abbreviations: AB – acetate buffer, AgN-GCE – silver nanoparticles modified glassy carbon electrode, BDDE – boron-doped diamond electrode, BR – Britton-Robinson buffer, CPE – carbon paste electrode, CV – cyclic voltammetry, DCP – direct current polarography, DME – dropping mercury electrode, DPP – differential pulse polarography, DPV – differential pulse voltammetry, E_p – peak potential, GO-GCE – graphene oxide modified glassy carbon electrode, LCR – linear concentration range, LOD – detection limit, LSAdSV – linear sweep adsorptive stripping voltammetry, LSV – linear sweep voltammetry, PGA-CPE – polyglutamic acid film modified carbon paste electrode, PGE – pencil graphite electrode, SDS-PGE - sodium dodecyl sulphate modified pencil graphite electrode, SPCE – screen-printed carbon electrode, SWV – square-wave voltammetry, SWCAdSV – square-wave cathodic adsorptive stripping voltammetry

Keywords: cytostatic drug flutamide; boron-doped diamond electrode; electrochemical sensor; differential pulse voltammetry; square-wave voltammetry

1. Introduction

Cytostatic agents are a broad group of carcinogenic, mutagenic and teratogenic pharmaceuticals used in the treatment of cancer, infections and skin diseases. Among them, flutamide (4-nitro-3-trifluoromethylisobutyranilide, here abbreviated to FLU, Scheme 1) represents a non-steroidal androgen receptor antagonist primarily used in treatment of advanced prostate cancer. During this treatment, FLU is excreted principally in urine and to lesser extent in feces (4.2%) as unchanged drug and hydroxylated metabolites (mainly 2-hydroxyflutamide) [1]. This drug also decreases the metabolism of C-19 steroids by the cytochrome P-450 system at the target cells in the secondary sex organs [2]. Similarly, it may also be used to treat excess androgen levels in women especially those with polycystic ovarian syndrome. In this viewpoint, declining fertility rates has emerged as a major social concern and therefore the excessive consumption of FLU and related anticancer drugs has recently attracted growing public and scientific interest. Based on this significance, advanced and perspective analytical methods and procedures of high sensitivity and selectivity for the continual control of cytostatic drugs in pharmaceuticals, biological and environmental samples are still desired.

Here Scheme 1

To date, different analytical methods, assays and protocols have been evolved and applied to detect and quantify FLU in miscellaneous kinds of samples. These particularly include high performance liquid chromatography coupled with ultraviolet [3] and mass detector [4], gas chromatography [5], spectrophotometry [6], fluorescence [7], chemiluminescence [8], immunohistochemistry [9] and radioimmunoassay [10]. Most of these methods offer good sensitivity and selectivity as well as the possibility for determining plenty of drugs simultaneously, but on the other hand, high price of the equipment, complicated sample

derivatization and clean-up steps make them oftentimes expensive, tedious and time-consuming.

Nowadays, electrochemical methods have attracted more attention for routine drug analysis, due to their advantages of cheap instrumentation, fast response, simple operation, time saving and high sensitivity accompanied by satisfactory selectivity, precision and accuracy [11]. Up to now, the literature survey has reflected a few electrochemical methods for detection and quantification of FLU, mostly employing mercury-based electrodes and solely based on the electrochemical reduction of the nitrobenzene moiety in this molecule. In 80s of the previous century, Snycerski developed first electrochemical method for determination of FLU on dropping mercury electrode (DME) in combination with direct current polarography (DCP) [12]. Álvarez-Lueje et al. focused on a detailed understanding of the different steps of electrochemical reduction process of FLU on DME in aqueous and mixed media with special attention to the feasibility of the high reactive nitro anion radical formation [13]. A cathodic stripping voltammetric procedure for determination of FLU in tablets and human serum samples was reported by Hammam et al. [14]. Temerk and Ibrahim studied the interaction of FLU with single and double stranded DNA at different temperatures using cyclic voltammetry (CV) and square-wave voltammetry (SWV) [15]. The electroanalytical procedures for rapid and reliable determination of FLU in pharmaceutical formulations, urine and serum samples were established by Subba Reddy et al. [16] and El-Shanawany et al. [17]. With regards to mercury-free and bare working electrode material for FLU sensing, screen-printed carbon electrodes (SPCEs) in combination with differential pulse voltammetry (DPV) were tested as electrochemical sensors for voltammetric determination of FLU [18]. Besides, Temerk et al. applied pencil graphite electrode (PGE) for individual and simultaneous determination of FLU and irinotecan in biological fluids using square-wave cathodic adsorptive stripping voltammetry (SWCASV) [19]. Brahman et al. interested in preparation and application of bare

[20] and polymer film modified carbon paste electrode (CPE) [21] for quantification of FLU, with the obtained detection limit (LOD) of 3.6 and 0.18 µM, respectively. A novel electroanalytical approach based on extraction of FLU from real samples and enrichment on the electrode surface, capable of detecting picomolar concentration levels of FLU (LOD = 34 pM), was introduced by Ensafi et al. [22]. Ag nanoparticles modified glassy carbon electrode (AgN-GCE) was used as a sensitive electrochemical sensor for assessment of FLU content in tablets and human urine samples [23]. Karthik et al. developed a selective and sensitive method for the detection of FLU in human and rat blood serum samples (LOD = 6 nM) based on electrocatalytic activity graphene oxide (GO) modified GCE [24]. Despite the useful utilization of conventional electrodes (DME, SPCE, PGE, CPE) and chemically modified electrodes, electrochemists are constantly pushed to explore the novel and advanced material platforms as reliable electrochemical sensors for detection and determination of FLU and related drugs.

In this respect, boron-doped diamond (BDD) has been found as a novel and perspective electrode material providing significant benefits unlike conventional carbon materials. This electrode material yields distinguished chemical stability (due to sp^3 hybridized carbon atoms in diamond structure), low background current, excellent biocompatibility and the widest potential range in both aqueous and non-aqueous media [25, 26]. In the past years, interesting scientific reports, based on BDD electrochemical sensors, have been published focusing on determination of various drugs such as vitamins [27], antibiotics [28], antihypertensives [29], opiates [30] and neurotransmitters [31]. Similarly, the possibilities of chemical modification of BDD electrode for construction of electrochemical biosensors have been recently reviewed by our group [32].

As mentioned above, electrochemical methods for FLU sensing published so far are explicitly established on reduction process on working electrode. Herein, for the first time, a novel concept is proposed based on electrochemical oxidation of FLU on BDD electrode. In

addition, to the best of our knowledge, the literature survey has indicated a few published papers dealing with the use of mercury-free electrochemical sensors for the determination of FLU. However, all these sensors are based on conventional and unmodified carbon-based electrode materials (SPCE, PGE, CPE) [18-20]. In view of this fact, the advanced electrochemical platform is introduced based on using bare BDD electrode coupled with differential pulse and square-wave voltammetric procedures for reliable determination of FLU in pharmaceutical, human urine and water samples.

2. Experimental

2.1. Reagents and solutions

Flutamide (CAS No. 13311-84-7, purity ≥ 98%) was purchased from Sigma Aldrich (Slovakia). 1 mM stock solution of FLU was prepared by dissolution of appropriate amount of its powder in 50 mL methanol (p.a., Lachema, Czech Republic) and without any further purification. When not used, this solution was stored in a glass flask in refrigerator, without any changes in consistency during a few weeks. H_2SO_4 of different concentrations and Britton-Robinson (BR) buffers were used as supporting electrolytes for purposes of this work. BR buffers were prepared by mixing of 40 mM H_3BO_3, H_3PO_4, CH_3COOH adjusted with 0.2 M NaOH (p.a., Lachema, Czech Republic). The working and calibration solutions of different concentrations of FLU were freshly prepared by dilution of the appropriate volume of stock solution in supporting electrolyte. All analyzed solutions were prepared in deionized water with resistivity above 18 MΩ cm.

2.2. Instrumentation

Electrochemical measurements were performed with an Autolab PGSTAT 101 (Metrohm Autolab B.V., The Netherlands) using a conventional three-electrode cell with a bare BDD electrode (film diameter of 3 mm, electrical resistivity of 0.075 Ω cm and B/C ratio in gaseous phase during the deposition step of 1000 ppm, Windsor Scientific Ltd., UK), an Ag/AgCl/3 M

KCl electrode as reference and a platinum wire as auxiliary electrode. All pH values were assessed using pHenomenal® pH 1100L meter (VWR, Slovakia) with a combined glass-reference electrode.

2.3. Measurement procedure

Cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square-wave voltammetry (SWV) were applied for the investigation of electrochemical behaviour of FLU and evaluation of analytical performance. At the beginning of every work day, the BDD electrode surface was firstly washed down with deionized water and then very softly rubbed with a piece of damp silk cloth until a mirror-like appearance of surface was obtained. Subsequently, it was rinsed with deionized water once again and used for the voltammetric measurements with repeatable signals (no cathodic and/or anodic pretreatment of BDD electrode was necessary to perform as no significant changes of current response of FLU were noticed after application of -2.5 V (cathodic) and/or +2.5 V in 1 M H_2SO_4 for 60 s). All voltammograms were recorded after addition of particular volumes of FLU stock and/or working solutions into the electrochemical cell already containing 20 mL of supporting electrolyte. The calibration curves were evaluated from the baseline-corrected (by application of Savitzky-Golay method with level of 2 and polynomial order of 2) differential pulse (DP) and square-wave (SW) voltammograms with the optimized operating parameters (modulation amplitude, modulation time and frequency). The calibration curves were statistically analyzed by OriginPro 8.0 (OriginLab, USA) and the relevant results (slope and intercept) were evaluated in a 95% confidence interval. LODs were calculated as standard deviation of intercept divided by slope of the particular calibration curve [27, 28]. All electrochemical measurements (except for repeatability assessment) were performed in triplicate at ambient temperature.

2.4. Preparation of real and model samples and procedures for their analysis

2.4.1. Pharmaceutical dosages

The pharmaceutical dosages of *Flutasin*® (declared amount of FLU according to the leaflet was 250 mg per tablet) were obtained in cooperation with the Faculty of Pharmacy at the Comenius University in Bratislava. The procedure for preparing the particular pharmaceutical sample for analysis was as follows: ten tablets of *Flutasin*® were weighed and powdered using mortar and pestle. 205 mg of this powder was dissolved in 25 mL methanol and filtered through the filter paper (the pore size of 20 µm). The clear filtrate was filled up with methanol to 100 mL volumetric flask to get stock sample solution. 100 µL of stock sample solution was added to the electrochemical cell to 20 mL of supporting electrolyte. The analysis was undertaken using standard addition method with three consecutive additions of 200 µL of 1 mM FLU working solution into the electrochemical cell.

2.4.2. Human urine samples

Drug-free human urine samples were taken from three non-smoking and healthy volunteers (V1: male, 33 years; V2: female, 23 years; V3: female, 22 years) on an empty stomach on the day of the experiment. In this sense, these experiments were undertaken in compliance with respective law (Parliamentary Act no. 40/1964 Coll. Civil Code as amended) with the informed consent obtained from the volunteers prior to the experiments. The urine samples were prepared by following procedure: ~~100 µL~~ 1 mL of particular fresh urine was transferred to electrochemical cell already containing 20 mL of supporting electrolyte. Afterwards, the solution in cell was enriched with aliquot of FLU working solution to form model (spiked) human urine sample with total FLU concentration of ~~4.97~~ 4.74 µM. The quantification of FLU in this kind of sample was carried out using standard addition method with respective volumes of ~~100, 200 and 300~~ 70, 140 and 210 µL of stock solution.

2.4.3. River, well and tap water samples

In the next step, the different water samples (river, well and tap) were used for analysis as an example of the feasibility of the proposed method in environmental sensing. The river water

sample (W1) was collected from the Slovak district Piešťany from the river Váh flowing through this town. The well water sample (W2) was obtained from the community Ostrov near Piešťany. The tap water sample (W3) was taken from the electroanalytical laboratory in which the experiments were conducted (Slovak University of Technology in Bratislava). Concerning the sample preparation, all samples were firstly filtered through a filter paper to get a clear filtrate and the particular volume (~~100 µL~~ 1 mL) was quantitatively transferred into 20 mL of supporting electrolyte in electrochemical cell. The quantification of FLU was performed as follows: an aliquot of FLU working solution was added to the electrochemical cell to get ~~4.95~~ 4.74 µM FLU. Subsequently, DPV and SWV procedures were launched. After this procedure, the aliquots of stock solution (100, 200 and 300 µL) were added and the analyses were undertaken.

3. Results and discussion

3.1. Study of electrochemical behaviour of FLU on BDD electrode

3.1.1. Effect of supporting electrolyte and reversibility study

To acquire the most favorable experimental conditions for sensitive and selective determination of FLU, CV measurements were executed employing various supporting electrolytes with different pH values such as BR buffers (pH 2.25 – 7.02) and strong acids (sulphuric acid, nitric acid and perchloric acid in the concentration range from 0.1 to 2 M). The experiments showed that the best results were obtained in acidic media. The well-defined voltammetric profile of FLU with the highest magnitude and the lowest background current were noticed in the presence of sulphuric acid when compared to BR buffers, nitric and perchloric acid (the results are not shown). Hence, sulphuric acid was chosen as an optimal medium for further studies in this work. Fig. 1A demonstrates the CV records of 1 mM FLU on BDD electrode with the most distinctive voltammetric profile achieved in the presence of 0.1 M sulphuric acid. For comparison, the typical CV records, registered in the absence and

presence of 1 mM FLU in 0.1 M H_2SO_4 and BR buffer at pH 2.25, are displayed in Fig. 1B. It is apparent that three oxidation peaks P1, P2 and P3 with the peak potentials of +1.1, +1.4 and +1.9 V, respectively, were recorded in forward scan ~~in 0.1 M H_2SO_4~~. One reduction peak was observed at +0.3 V in reverse scan revealing information about total irreversibility of electrode reaction of FLU on BDD electrode. With regards to BR buffer at pH 2.25, only two oxidation peaks of FLU were occurred, the first oval-shaped peak with the maximum at around +1.25 V and the second well-shaped peak at +2.0 V. Obviously, due to the undesirable voltammetric profile of FLU in BR buffer pH 2.25, this electrolyte was not applied in further experiments. It is obvious from Fig. 1B that the background current of BDD electrode was found to be low at very positive potentials (higher than +1.5 V) using CV. Hence, it corroborates the favourable properties of BDD electrode material for detection and quantification of highly oxidizing analytes.

Taking so far published data into account, the electrochemical behaviour of FLU presented in this work is not consistent with those reported for mercury [12-17], conventional carbon-based [18-20] and chemically modified working electrodes [21-24] because in these cases the electrode reaction was explicitly based on reduction of FLU. In the contrast, a novel concept presented in this work introduces the oxidation reaction of FLU on BDD electrode material.

Here Fig. 1

3.1.2. Effect of effective scan rate (frequency)

The effect of effective scan rate (frequency *f)* on the peak currents of 1 mM FLU (I_p) was explored by SWV in the potential window from 0 to +2 V in 0.1 M H_2SO_4 using the BDD electrode. We preferred a sensitive SWV to CV technique as oxidation peaks P1 and P2 were partially overlapping when using CV (Fig. 1B) which could affect the reliable evaluation of their current responses. Fig. 2 reflects the SWV records for the frequencies ranging from 5 to 150 Hz (at fixed step potential of 1 mV and amplitude of 50 mV). As evidenced by

voltammograms, the current responses of FLU got higher with increasing frequency and distinct voltammetric profiles of all oxidation peaks (P1, P2 and P3) were observed. Besides, no significant mutual influence of these signals was present. The current responses of P1, P2 and P3 (I_{p1}, I_{p2}, I_{p3}) with distinct peak potentials (E_{p1}, E_{p2}, E_{p3}) were considered for evaluation. Fig. S1 (in Supporting Information) displays the particular linear relationships such as: log I_p against log f and E_p against log f. The former is expressed by following equations (Eqs. 1-3), as evidenced below:

$$\log I_{p1} = (0.590 \pm 0.034)\times\log f - (1.357 \pm 0.055) \qquad R^2 = 0.985 \qquad (1)$$

$$\log I_{p2} = (0.476 \pm 0.026)\times\log f - (0.681 \pm 0.041) \qquad R^2 = 0.983 \qquad (2)$$

$$\log I_{p3} = (0.516 \pm 0.030)\times\log f - (0.928 \pm 0.048) \qquad R^2 = 0.981 \qquad (3)$$

The results clearly indicate a diffusion-driven mechanism of the electrode reaction of FLU on BDD electrode as the slope values (0.590, 0.476 and 0.516) in Eqs. 1-3 are close to the theoretical one (0.5) [26]. In the light of this fact, the influence of adsorption phenomenon as a rate-determining step during the redox reaction of FLU seemed to be minor. Additionally, as can be seen in Fig. 2, the slight shift of all peak potentials towards positive direction was recorded as the frequency gradually increased. This behaviour confirms the irreversible nature of the electrode reaction of FLU on BDD electrode. The irreversible electrode reaction of analyte was also characterized by the linear relationships of E_p against log f (Eqs. 4-6):

$$E_{p1}\ (\mathrm{V}) = (0.029 \pm 0.001)\times\log f + (0.738 \pm 0.002) \qquad R^2 = 0.993 \qquad (4)$$

$$E_{p2}\ (\mathrm{V}) = (0.031 \pm 0.002)\times\log f + (1.033 \pm 0.003) \qquad R^2 = 0.984 \qquad (5)$$

$$E_{p3}\ (\mathrm{V}) = (0.033 \pm 0.001)\times\log f + (1.561 \pm 0.002) \qquad R^2 = 0.999 \qquad (6)$$

From the slopes of these dependences ($2.3RT/\alpha nF$), αn values for P1, P2 and P3 were found to be 2.04, 1.91 and 1.79. Considering irreversible system with α value equal to 0.5, the number of electrons involved in the redox reaction of FLU on BDD electrode was calculated to be roughly 4 for all three peaks (4.08, 3.82 and 3.58). However, the proposed work does not focus

on the direct clarification of the detailed mechanism of the oxidation of FLU. For elucidation, galvanostatic or potentiostatic coulometry for total conversion of analyte should be carried out with consecutive spectral analysis (NMR, MS) for identification of oxidation products of FLU.

Here Fig. 2

3.2. Analytical performance evaluation

3.2.1. Establishment of differential pulse and square-wave voltammetric procedures

To ensure the significant analytical sensitivity and selectivity for the determination of FLU on BDD electrode, the DPV and SWV procedures have been developed. The optimization was carried out using 0.1 mM FLU in 0.1 M H_2SO_4 and the operating parameters such as modulation amplitude and modulation time for DPV and amplitude and frequency for SWV were explored. The optimization was undertaken in such a manner that one parameter was always altered within the procedure while the second one was remained unchanged. As depicted in DP voltammograms in Fig. 3A, an alteration of modulation amplitude in the range of 10 – 200 mV (with the modulation time and the scan rate fixed at 50 ms and 10 mV/s, respectively) indicated the increase of current responses for P1, P2 and P3 with a slight shift of their peak maximums to the less positive values. Besides this, the voltammetric signals became considerably wider as the modulation amplitude increased, with the higher background current to be accompanied. In the view of adequate sensitivity and selectivity, the optimal modulation amplitude for all oxidation peaks of FLU was set at 100 mV. With regards to modulation time, the studied values ranged from 10 to 100 ms. The inset of Fig. 3A images the decrease of the background current as the modulation time increased. The optimal modulation time of 25 ms was considered as suitable owing to the highest current responses for all oxidation peaks. Concerning the SWV procedure, the corresponding operating parameters were optimized with the following values selected for further voltammetric measurements: frequency of 25 Hz (studied range of 10 – 150 Hz, Fig. 3B) and amplitude of 50 mV (10 – 200 mV, inset of Fig. 3B).

Here Fig. 3

3.2.2. Calibration curve and analytical parameters

The feasibility of the developed DPV and SWV procedures was explored by construction of calibration curves. In this respect, the analytical performance was assessed (sensitivity, linearity, LOD and repeatability) by examining the oxidation peak current values (I_{p1}, I_{p2} and I_{p3}) as a function of concentration of FLU (c_{FLU}) under the optimized experimental conditions (number of measurements n = 3, except for repeatability). Fig. 4 (A-B) shows the respective DP and SW voltammograms (after baseline correction using Savitzky-Golay method) demonstrating distinct oxidation signals P1, P2 and P3 (+0.9, +1.3 and +1.9 V for DPV; +0.9, +1.2 and +1.8 V for SWV) with good peak-to-peak potential separation and without any substantial mutual influence of their current responses. The corresponding calibration curves are shown in the insets of Fig. 4. The analytical performance evaluation of the proposed methodology is comprehensively summarized in Table 1. Regarding the DPV procedure, the highest sensitivity (1.76 nA/μM), the lowest LOD (0.42 μM) and the widest linear concentration range (0.99 – 42.9 μM) were achieved for the oxidation peak P2 of FLU. The sensitivity for P1 and P3 seemed to be lower which resulted in the higher LOD values. Concerning the SWV procedure, the peaks P2 and P3 exhibited the highest sensitivity (3.15 and 3.54 nA/μM, respectively) with the lowest LOD values (0.21 and 0.18 μM, respectively). Moreover, it is evident that LOD values for the particular peaks accomplished by the SWV procedure are approximately twice lower than those determined by the DPV.

Here Fig. 4

Here Table 1

Taking the repeatability into account, the highest relative standard deviation (RSD) values were achieved for P2 (8.8 and 9.3%) by performing ten consecutive measurement in both DPV

and SWV modes for 5 μM FLU under the optimum experimental conditions. On the other hand, RSDs for ~~the~~ P1 and P3 were lower than 5% in both cases confirming the significant repeatability of the developed method. To conclude, the proposed advanced electrochemical platform offers the significant sensitivity and low LODs as well as satisfactory repeatability for the quantification of FLU.

3.2.3. Examination of selectivity

The effects of potentially interfering agents, mostly present in biological samples or pharmaceuticals, were investigated by SWV for 20 μM FLU under the optimum experimental conditions. The maximum concentration of the potential interfering agent, which may cause an absolute error less than 10% for the peak currents of FLU (for all oxidation peaks) was considered as the tolerance limit. The results revealed that the effect of 200-fold excess of inorganic ions Na^+, K^+, Ca^{2+} and Mg^{2+} on the current response of FLU was found to be negligible. With regards to organic species such as glucose and sucrose, no significant impact on FLU signals was observed in their 100-fold excess (the results are not shown). Nevertheless, the effect of uric acid (UA), dopamine (DOP), ascorbic acid (AA, vitamin C) and folic acid (FA, vitamin B9) was appeared to be substantial even at their equimolar and 10-times higher concentration levels. The respective SW voltammograms are depicted in Fig. 5 (A-D). Clearly, the oxidation peak currents of individual solutions of UA, DOP, AA and FA (in equimolar concentration to FLU) ranged from +1.0 to +1.8 V, thus significantly affecting all FLU signals by strong overlapping. Furthermore, when the 10-fold excess of these substances was present, the oxidation signals P1 and P2 of FLU were completely overlaid by the distinct voltammetric profile of these interfering compounds. This observation made impossible the reliable evaluation of analyte current response. Concerning the potentials higher than +1.7 V it was found that signal P3 could also not be completely evaluated in the presence of UA and FA. Even when their peak potentials were far enough from P3, this signal was substantially affected

at equimolar and 10-times higher concentration levels of the interfering agents. In the case of AA and DOP, the results of the interference study signified that P3 could be reliably recognized. Herein, 10-fold excess of AA and DOP gave rise to slight increase and decrease of the peak currents of P3, respectively. To sum up, the direct application of the proposed method for the determination of FLU could be slightly restricted in the analysis of real biological samples in the presence of these interfering substances. However, this issue could be resolved prior to voltammetric determination of FLU on BDD electrode by applying suitable separation techniques and/or methods.

Here Fig. 5

3.2.4. Analysis of pharmaceutical dosages

To explore the accuracy of the method and to validate its practical applicability, the analysis of pharmaceutical dosages *Flutasin*® was carried out by both pulse techniques using the standard addition method [33-35]. The preparation of the sample is described in detail in section 2.4.1. As an illustrative example of analysis, the particular SW voltammograms are given in Fig. 6 with the graphical evaluation of standard addition method for the oxidation peak P2 of FLU. The recovery values for all oxidation peak currents determined by the DPV a SWV procedures are summarized in Table 2. It is evident that the accomplished values ranged from 97.2 to 102.8% and from 95.6 to 106.4% for DPV and SWV, respectively. Apparently, the results of the analysis of pharmaceutical dosages testified the fact that the proposed protocol did not suffer from any considerable matrix effect. Hence, the amount of cytostatic drug FLU can be accurately quantified in pharmaceutical dosages by the developed method. This fact opens the promising possibilities of utilization of BDD electrode for reliable determination of cytostatic drugs in routine pharmaceutical analysis.

Here Fig. 6

Here Table 2

3.2.5. Analysis of human urine samples

The applicability of the method was also tested on the set of various samples with more complicated matrix in comparison with pharmaceutical dosages. The developed methodology was applied to quantify FLU in spiked human urine samples of volunteers (V1-V3) by both pulse techniques exploiting the standard addition method. The sample preparation is mentioned in detail in section 2.4.2. According to Table 3, satisfactory recovery values (~~96.6 – 104.0%~~ 95.8 – 106.3% and ~~91.5 – 98.6%~~ 95.4 – 101.7% for DPV and SWV, respectively) were obtained when the oxidation peak currents of P1 and P3 were evaluated manifesting the fact that no notable interference was observed during the analysis. Fig. 7 typifies the analysis of model human urine sample of V2 by the SWV procedure with the standard addition procedure applied to the evaluation of P1 at +1.4 V. As far as the evaluation of the peak current for P2, it rendered the slight interference with the unknown oxidation peak at +1.2~~5~~ V arising from the urine sample. Our effort was also to characterize this peak. On the basis of several standard additions of common urinary compounds it was likely to be attributed to UA. Obviously, this interference was noticed in all analyzed human urine samples resulted in considerable decrease of recovery values (~~46.3 – 60.2%~~ 39.9 – 49.2% and ~~79.1 – 87.7%~~ 72.6 – 83.3% for DPV and SWV, respectively). For this reason, P2 was not suitable for quantification and P1 and P3 were preferentially more appropriate for the reliable quantitative assessment of FLU. Regarding this fact, the benefit of the proposed methodology could consist in the possibility to select the most suitable oxidation peak of FLU when the effect of the particular interfering agent present in analyzed solution is considered as minor or negligible. Overall, the developed method exhibited the good accuracy for the quantification of FLU in model human urine samples. However, for closer view and clarification of analysis of human urine samples, e.g. after following a single 250 mg oral dose of FLU to adult volunteers and/or patients, the quantification of the

biologically active hydroxylated metabolites would be required to be undertaken. These metabolites reach maximum in about 2 h indicating that they are rapidly formed from FLU [36].

Here Table 3

Here Fig. 7

3.2.6. Analysis of water samples

In order to evaluate the accuracy of the proposed methodology also in environmental sensing, the standard addition method was utilized for the analysis of river (W1), well (W2) and tap (W3) water samples spiked with FLU (~~4.95~~ 4.74 μM). The preparation of this kind of sample is consistently described in section 2.4.3. The average results of three replicate DPV and SWV measurements for the peaks P1, P2 and P3 are summarized in Table 4. Evidently, the satisfactory recovery values ranged from ~~92.5 to 109.5%~~ 93.9 to 105.9% and from ~~97.0 to 110.9%~~ 95.4 to 104.6% for DPV and SWV, respectively, were obtained for all water samples. It is evident that no relevant matrix interferences of the analyzed water samples were noticed. Fig. 8 shows the analysis of the river water sample (W1) by the SWV procedure under the optimum experimental conditions. Herein, the peak P3 was illustratively evaluated (recovery of ~~102.0~~ 97.3%). To conclude, the proposed methodology is satisfactory accurate and suitable for the reliable quantification of FLU in various water samples.

Here Table 4

Here Fig. 8

3.2.7. Comparison with mercury, carbon and chemically modified working electrodes

Table 5 compares the selected experimental and analytical parameters of electrochemical methods for the determination of FLU published so far with those presented by advanced platform. Clearly, the mercury electrodes have been considered as conventional sensitive electrochemical platforms for quantifying FLU in tablets, urine and serum, with LOD typically below 1 μM [14-17]. In these cases, the outstanding analytical performance of FLU sensing lies

in high overpotential of hydrogen evolution and self-renewing atomically smooth electrode surface, which can facilitate the reduction process of FLU on this electrode material. However, mercury itself has been recently considered as poisonous and toxic substance and there are some indispensable issues for an adequate handling, especially with regards to its mechanical stability in electrochemical experiments in flow systems and on-site measurements. Nevertheless, modern electroanalytical chemistry within the state-of-the-art trends mostly classifies mercury as obsolete electrode material.

Hence, several analysts aimed at emergence of the perspective and environmental-friendly electrode materials which could be used as platforms for construction of reliable electrochemical sensors in trace determination of FLU. In this viewpoint, the application of solid materials, mainly bare carbon-based electrodes (SPCE, PGE, CPE) [18-20], has been efficient. Unlike mercury electrodes, they are mechanically more stable and compatible for the usage in flow systems. Recently, the use of PGE as variant electrode material for very sensitive quantification of FLU (with LOD of 16 nM) by square-wave cathodic adsorptive stripping voltammetry (SWCAdSV) has been demonstrated [19]. Concerning the supporting electrolyte, in the case of all carbon-based working electrodes, neutral and/or weakly alkaline environment was the best medium for FLU reduction with the more negative peak potential values when compared to mercury electrodes (Table 5). Furthermore, the chemically modified carbonaceous electrodes have also been found as efficient electrochemical tools for the determination of FLU [21-24], mostly due to the enhancement of the electron transport leading to substantial improvement of sensitivity. To date, the most sensitive electrochemical method with LOD for FLU of 34 pM has been developed in mildly acidic medium (BR at pH 2.5) using a sodium dodecyl sulphate modified PGE (SDS-PGE) [22]. In this case, FLU was extracted from real samples and enriched electrochemically (reduction) on the modified surface of the working electrode and analyzed based on its oxidation peak current.

With regards to the herein presented protocol it is worth noting from data in Table 5 that LODs accomplished by the proposed methodology are lower than those obtained by DME [12], SPCE [18], CPE [20] and AgN-GCE [23] and comparable with PGA-CPE [21]. Furthermore, sulphuric acid was used for the first time as supporting electrolyte for the electrochemical sensing of FLU. Accordingly, the presented electrochemical platform based on the application of BDD electrode in connection with the DPV and SWV procedures satisfies all required characteristics for the modern electrochemical sensors (low-cost, no chemical modification, rapidity, simplicity, sufficient sensitivity and good repeatability). Besides, it can be successfully used as an alternative platform to mercury and chemically modified electrodes for determination of FLU.

Here Table 5

4. Conclusion

So far published scientific papers dealing with sensing of cytostatic drug flutamide are predominantly focused on the application of mercury-based and chemically modified electrodes whilst these determinations are explicitly based on electrochemical reduction of nitro moiety of this compound. Herein, the electrochemical oxidation of FLU on BDD electrode is introduced for the first time and elaboration of a novel and advanced electrochemical platform for reliable determination of this analyte in various kinds of samples is presented. CV measurements revealed three irreversible and diffusion-controlled oxidation signals at around +1.1, +1.4 and +1.9 V in 0.1 M H_2SO_4. Using the DPV and SWV procedures with optimized operating parameters, the LOD values for FLU were determined at submicromolar concentration levels for all oxidation peaks (0.18 – 0.83 μM). The RSD values characterizing the repeatability varied from 3.3 to 8.8% and from 2.9 to 9.3% for DPV and SWV, respectively, depending on the particular oxidation peak of FLU. The effect of possible interfering agents such as uric acid, dopamine, ascorbic acid and folic acid appeared to be substantial even in equimolar

concentration level to FLU, thus slightly restricting the feasibility of this method in analysis of biological samples with complicated matrix. The developed methodology was applied to the analysis of the pharmaceutical dosages without a demanding sample pretreatment with the achieved recoveries in the range of 97.2 – 102.8% and 95.6 – 106.4% by DPV and SWV procedure, respectively. The satisfactory recovery values were also reached in analysis of the spiked human urine samples and water samples. The contemporary aspect of the herein presented work lies in the utilization of the bare BDD electrode as advanced, simple and effective alternative platform in cytostatic sensing to so far used toxic mercury-based electrodes and time and cost demanding chemically modified electrodes. Obviously, the coupling of BDD electrode with pulse techniques may represent a simple, low-cost and comfortable electrochemical tool for routine analysis in pharmaceutical, clinical and environmental chemistry.

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Table 1

The analytical performance evaluation of the developed method using the DPV and SWV procedures with the optimized parameters on the BDD electrode (n = 3, except for repeatability).

Analytical performance evaluation	**Pulse technique**					
	DPV			**SWV**		
	P1	**P2**	**P3**	**P1**	**P2**	**P3**
Peak potential (V vs. Ag/AgCl/3 M KCl)	+0.9	+1.3	+1.9	+0.9	+1.2	+1.8
Linear concentration range (µM)	4.8 – 42.9	0.99 – 42.9	4.8 – 42.9	4.8 – 35.5	0.99 – 35.5	4.8 – 35.5
Sensitivity (nA/µM)	0.94 ± 0.02	1.76 ± 0.01	1.18 ± 0.02	1.16 ± 0.03	3.15 ± 0.04	3.54 ± 0.01
Intercept (nA)	-2.26 ± 0.26	-0.72 ± 0.25	-2.56 ± 0.27	-1.21 ± 0.20	-2.09 ± 0.22	-1.76 ± 0.21
Coefficient of determination	0.997	0.997	0.997	0.988	0.998	0.990
Detection limit (µM)	0.83	0.42	0.69	0.52	0.21	0.18
Repeatability (%, for 5 µM, n = 10)	3.3	8.8	7.5	3.8	9.3	2.9

Table 2

The analysis of the pharmaceutical dosages *Flutasin*® using the proposed method (n = 3).

Pharmaceutical dosage	Declared amount (mg)	Evaluated oxidation peak	Determined amount* (mg)		Recovery (%)	
			DPV	SWV	DPV	SWV
Flutasin®	250	**P1**	257 ± 18	239 ± 12	102.8	95.6
		P2	243 ± 7	244 ± 7	97.2	97.6
		P3	242 ± 15	266 ± 16	96.8	106.4

*Confidence interval for 95% probability calculated as [$\bar{x} \pm t_{n-1,\alpha}\, SD/n^{1/2}$]; $t_{2;\,0.05} = 2.92$

Table 3

The analysis of the spiked human urine samples of volunteers (V1-V3) using the proposed method (n = 3).

Human urine sample	Expected concentration (µM)	Evaluated oxidation peak	Determined concentration* (µM)		Recovery (%)	
			DPV	SWV	DPV	SWV
V1	4.74	**P1**	4.66 ± 0.23	4.82 ± 0.33	98.3	101.7
		P2	1.89 ± 0.64	3.55 ± 1.54	39.9	74.9
		P3	4.78 ± 0.45	4.80 ± 0.41	100.8	101.3
V2	4.74	**P1**	4.54 ± 0.28	4.59 ± 0.18	95.8	96.8
		P2	2.33 ± 0.94	3.95 ± 1.31	49.2	83.3
		P3	4.71 ± 0.31	4.63 ± 0.47	99.4	97.7
V3	4.74	**P1**	5.04 ± 0.42	4.58 ± 0.27	106.3	96.6
		P2	2.02 ± 0.97	3.44 ± 1.03	42.6	72.6
		P3	4.68 ± 0.33	4.52 ± 0.41	98.7	95.4

*Confidence interval for 95% probability calculated as [$\bar{X} \pm t_{n-1,\alpha}\, SD/n^{1/2}$]; $t_{2;\,0.05} = 2.92$

Table 4

The analysis of the different kinds of the spiked water samples (W1-W3) using the proposed method (n = 3).

Water sample	Expected concentration (µM)	Evaluated oxidation peak	Determined concentration* (µM)		Recovery (%)	
			DPV	SWV	DPV	SWV
W1	4.74	**P1**	4.65 ± 0.41	4.84 ± 0.54	98.1	102.1
		P2	4.79 ± 0.24	4.89 ± 0.15	101.1	103.2
		P3	4.95 ± 0.28	4.71 ± 0.29	104.4	99.4
W2	4.74	**P1**	4.61 ± 0.31	4.75 ± 0.15	97.3	100.2
		P2	4.78 ± 0.18	4.73 ± 0.22	100.8	99.8
		P3	4.45 ± 0.29	4.52 ± 0.32	93.9	95.4
W3	4.74	**P1**	4.82 ± 0.26	4.96 ± 0.35	101.7	104.6
		P2	5.02 ± 0.37	4.72 ± 0.19	105.9	99.6
		P3	4.65 ± 0.15	4.61 ± 0.28	98.1	97.3

*Confidence interval for 95% probability calculated as [$\bar{x} \pm t_{n-1,\alpha}\, SD/n^{1/2}$]; $t_{2;\,0.05} = 2.92$

Table 5

The comprehensive survey of electrochemical methods for the determination of FLU using mercury, carbonaceous and chemically modified electrodes.

Working electrode	Supporting electrolyte	Electrode process	E_p (V)	Technique	LCR (μM)	LOD (LOQ*) (μM)	Analyzed samples	Ref.
DME	PB pH 7.05	reductio	–0.48	DCP	0.216 – 3.65	1.8	tablets	[12]
DME	BR pH 2-12	reductio	–	DPP	–	–	tablets	[13]
DME	AB pH 5.0	reductio	–	LSAdSV	–	0.19	tablets, serum	[14]
DME	BR pH 4.0	reductio	–0.20	DPP	0.002 – 15	0.00125	tablets, urine,	[16]
DME	PB pH 8.0	reductio	–0.55	CV	0.1 – 1.3	0.178	tablets, urine,	[17]
SPCE	BR pH 7.0	reductio	–0.80	DPV	2 – 10	0.8*	tablets	[18]
PGE	BR pH 8.0	reductio	–0.65	SWCAdSV	0.398 – 6.36	0.016	tablets, urine,	[19]
CPE	PB pH 7.0	reductio	–0.75	DPV	72 – 580	3.6	tablets	[20]
PGA-CPE	PB pH 7.0	reductio	–0.75	DPV	72 – 580	0.18	tablets	[21]
SDS-PGE	BR pH 3.5	oxidatio	+0.20	DPV	0.0001 – 0.1	0.00003 4	plasma, urine	[22]
AgN-GCE	PB pH 6.0	reductio	–0.65	DPV	10 – 1000	9.33	tablets, urine	[23]
GO-GCE	PB pH 7.0	reductio	–0.54	LSV	0.009 – 1.9	0.006	serum	[24]
BDDE	0.1 M H_2SO_4	oxidation	+0.9, +1.3, +1.9 +0.9	DPV SWV	0.99 – 42.9** 0.99 – 35.5**	0.42** 0.21**	tablets, urine, water	This work

** assigned to the 2nd oxidation peak of FLU

Scheme 1

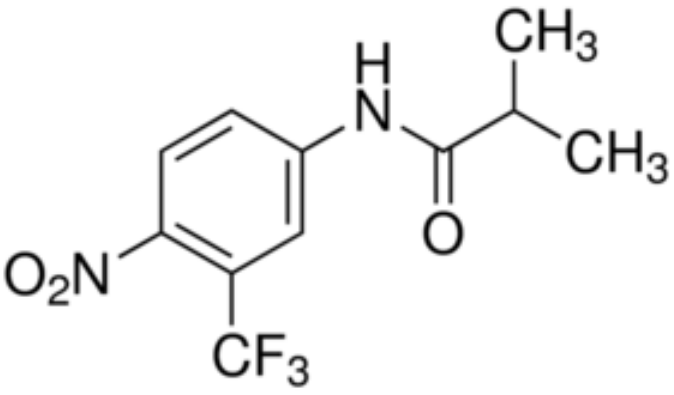
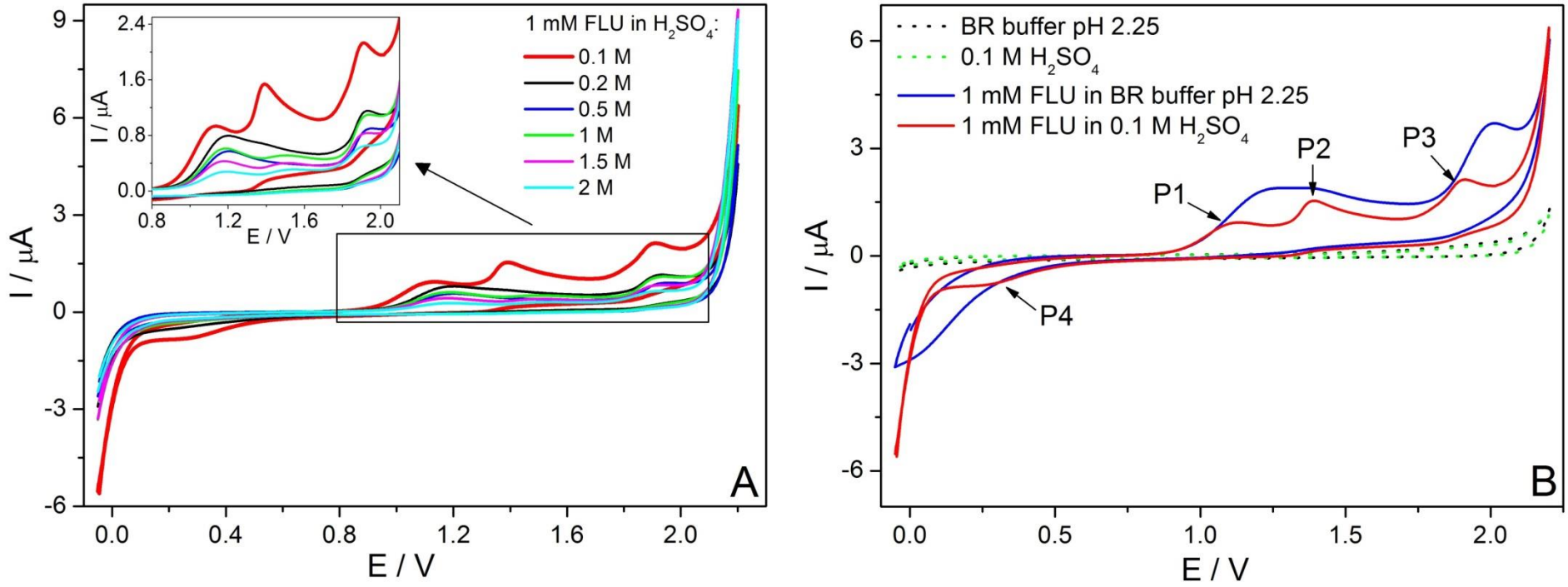


Fig. 1



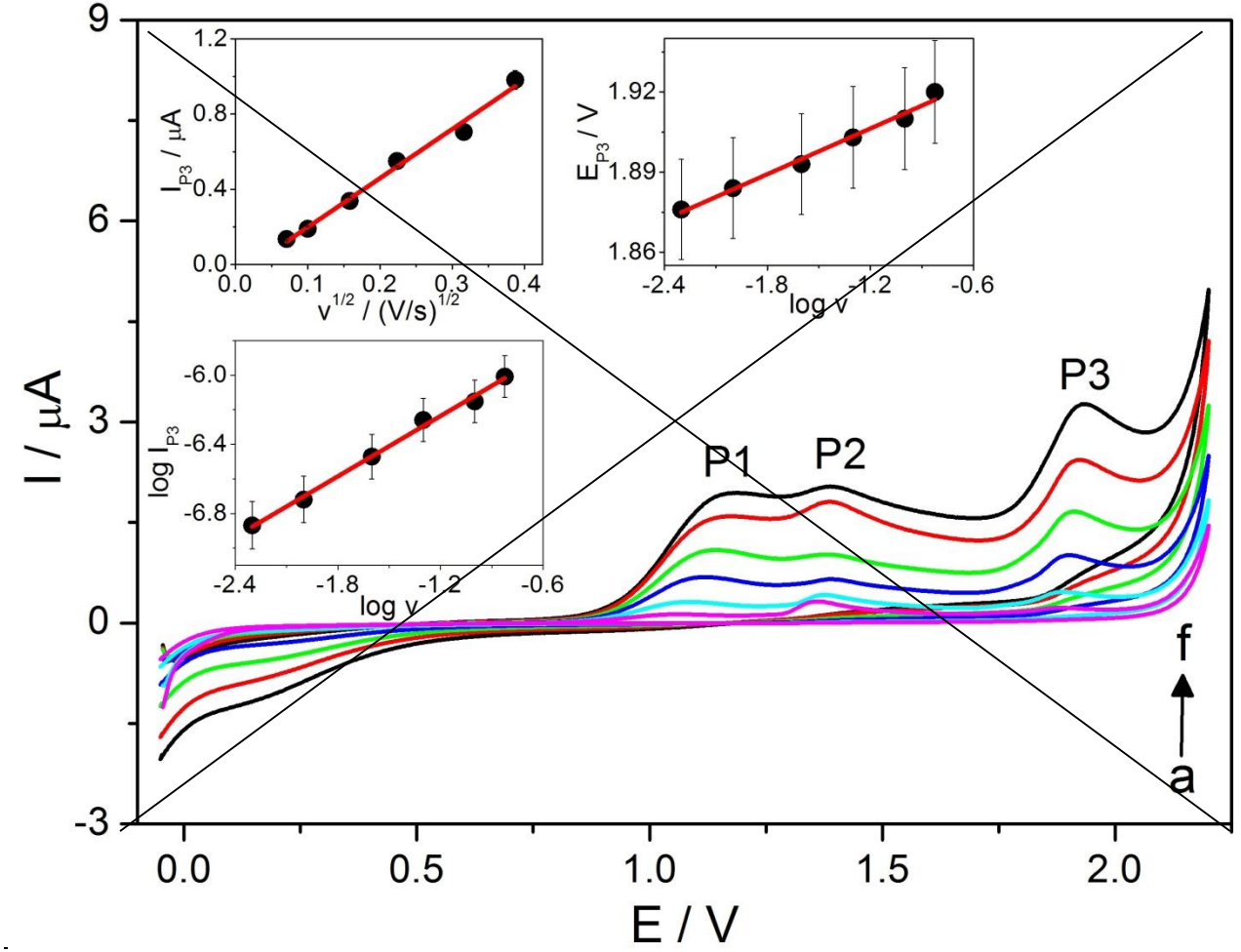


Fig. 2

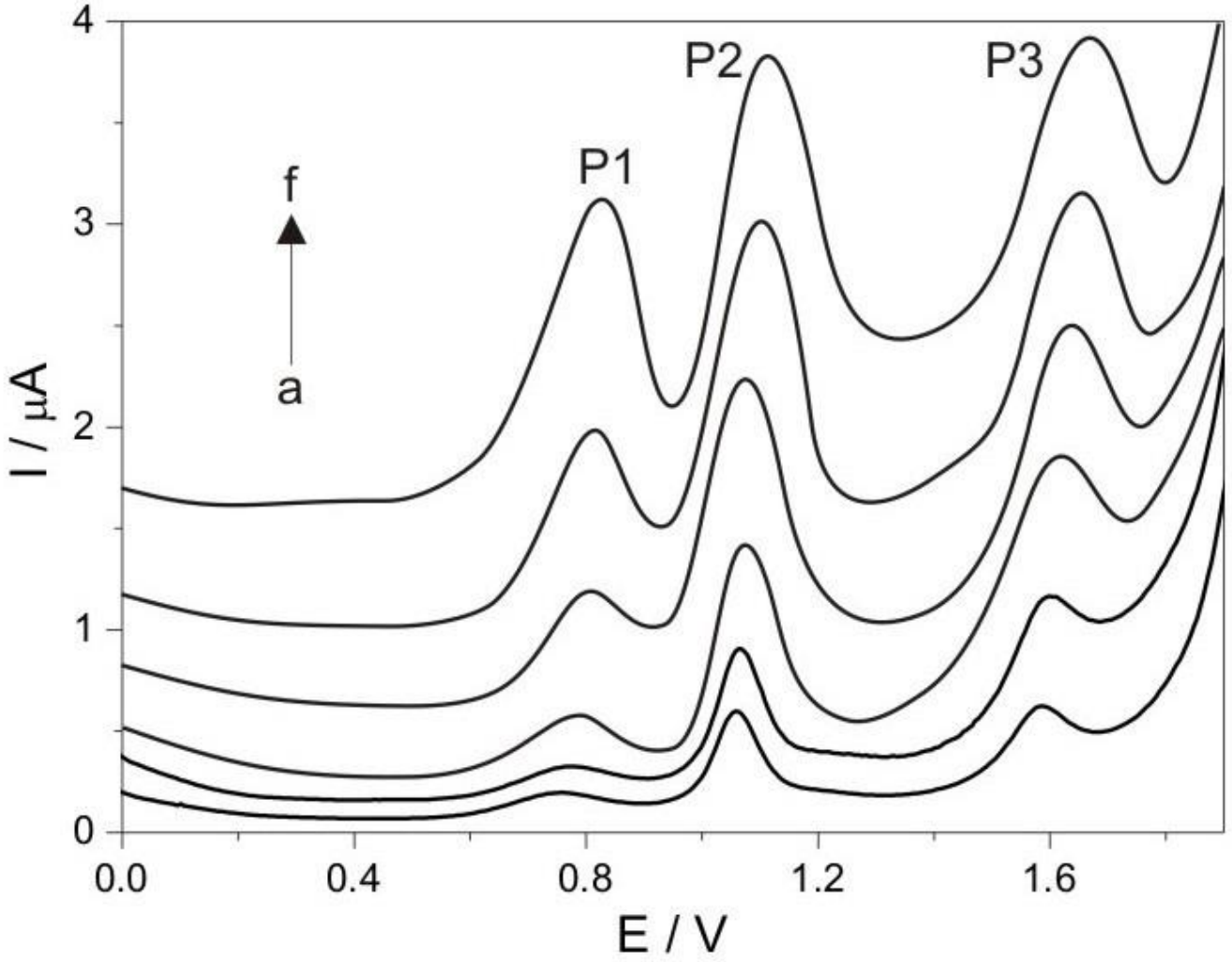


Fig. 3

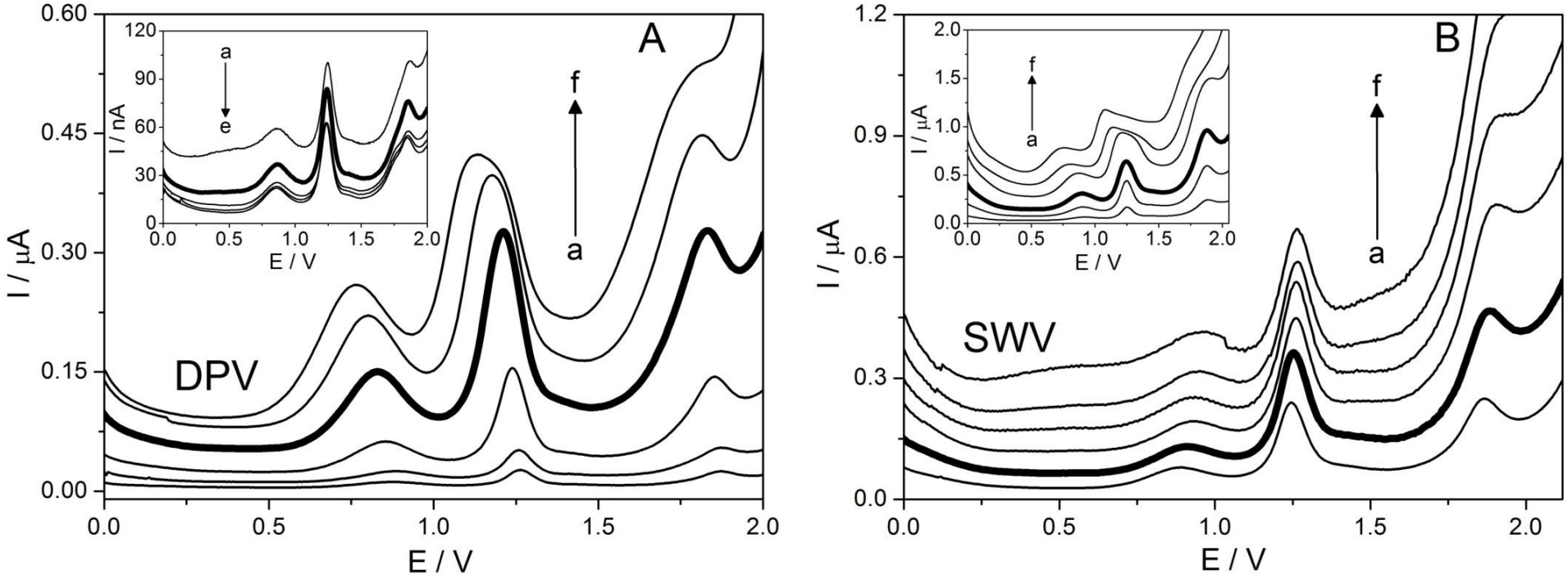


Fig. 4

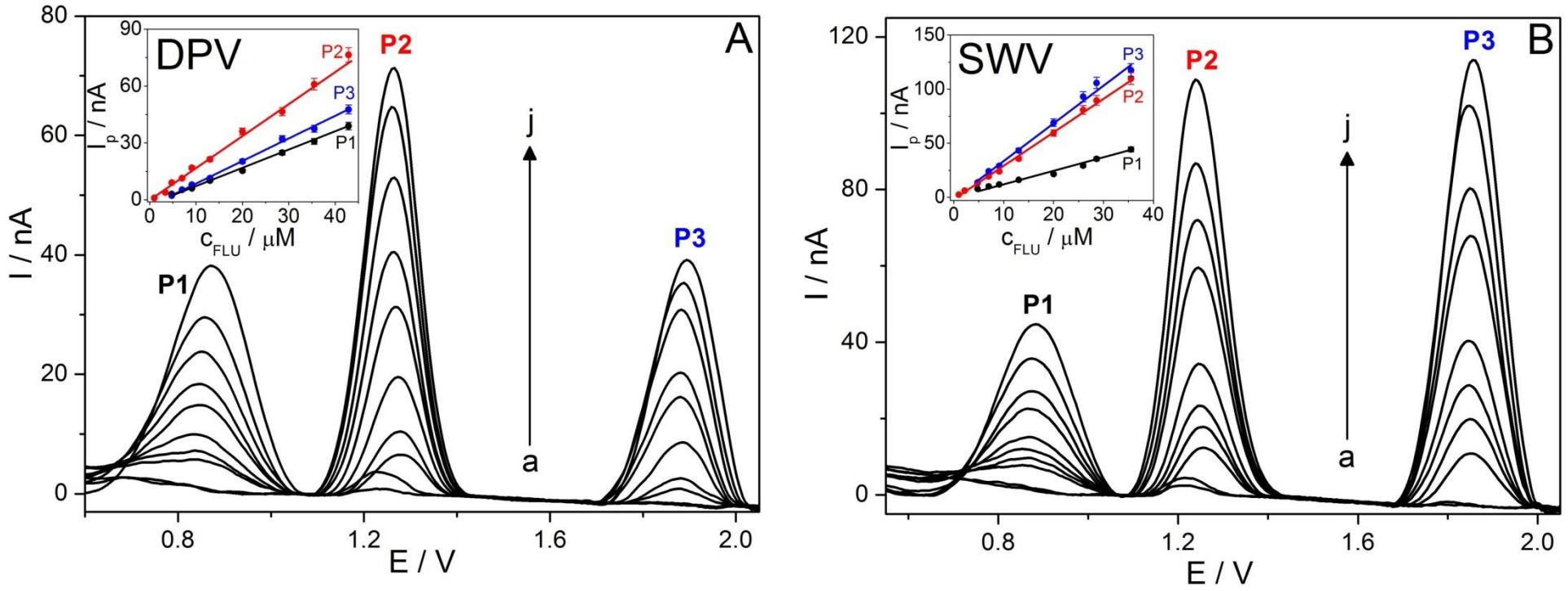


Fig. 5

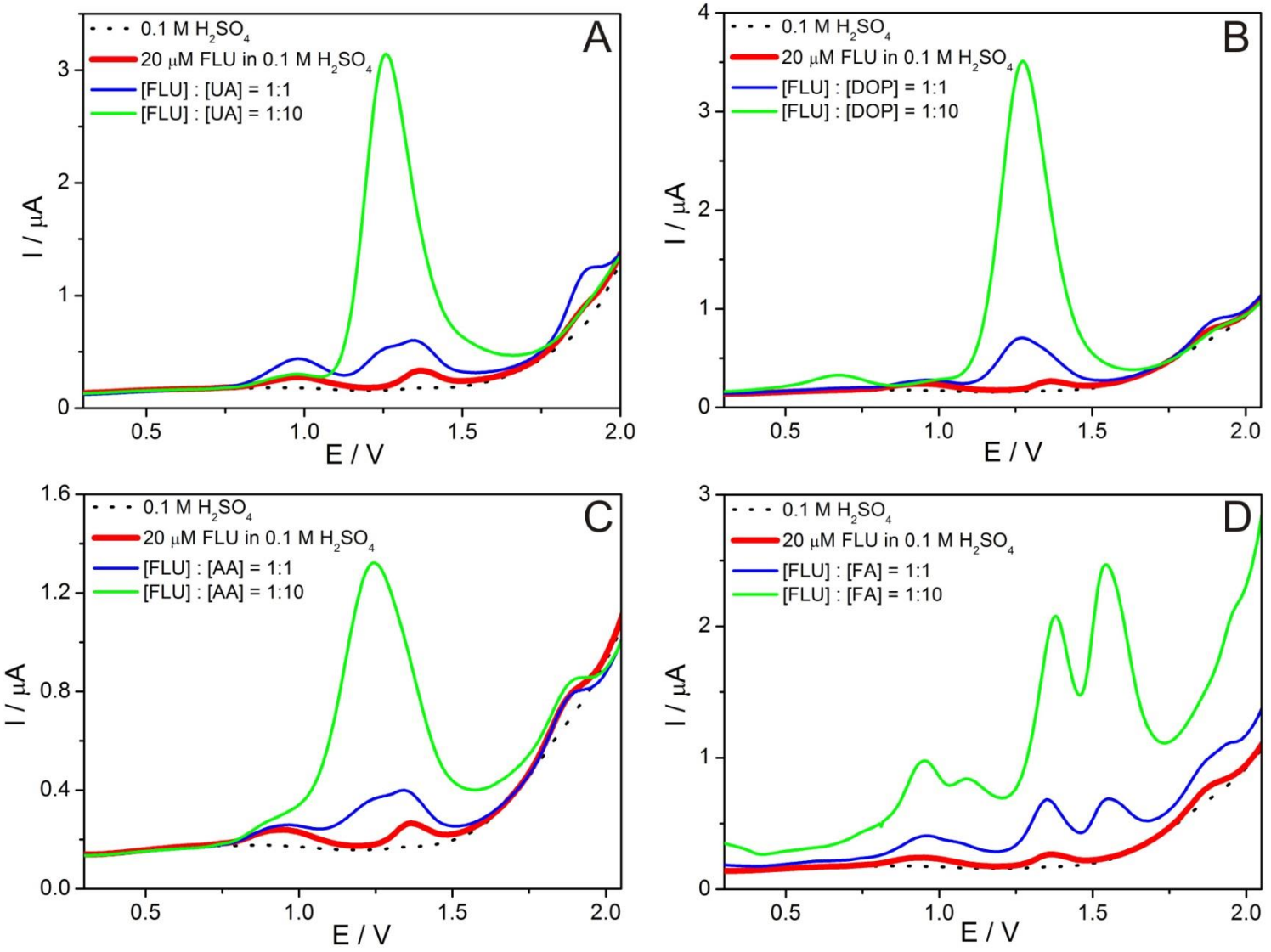
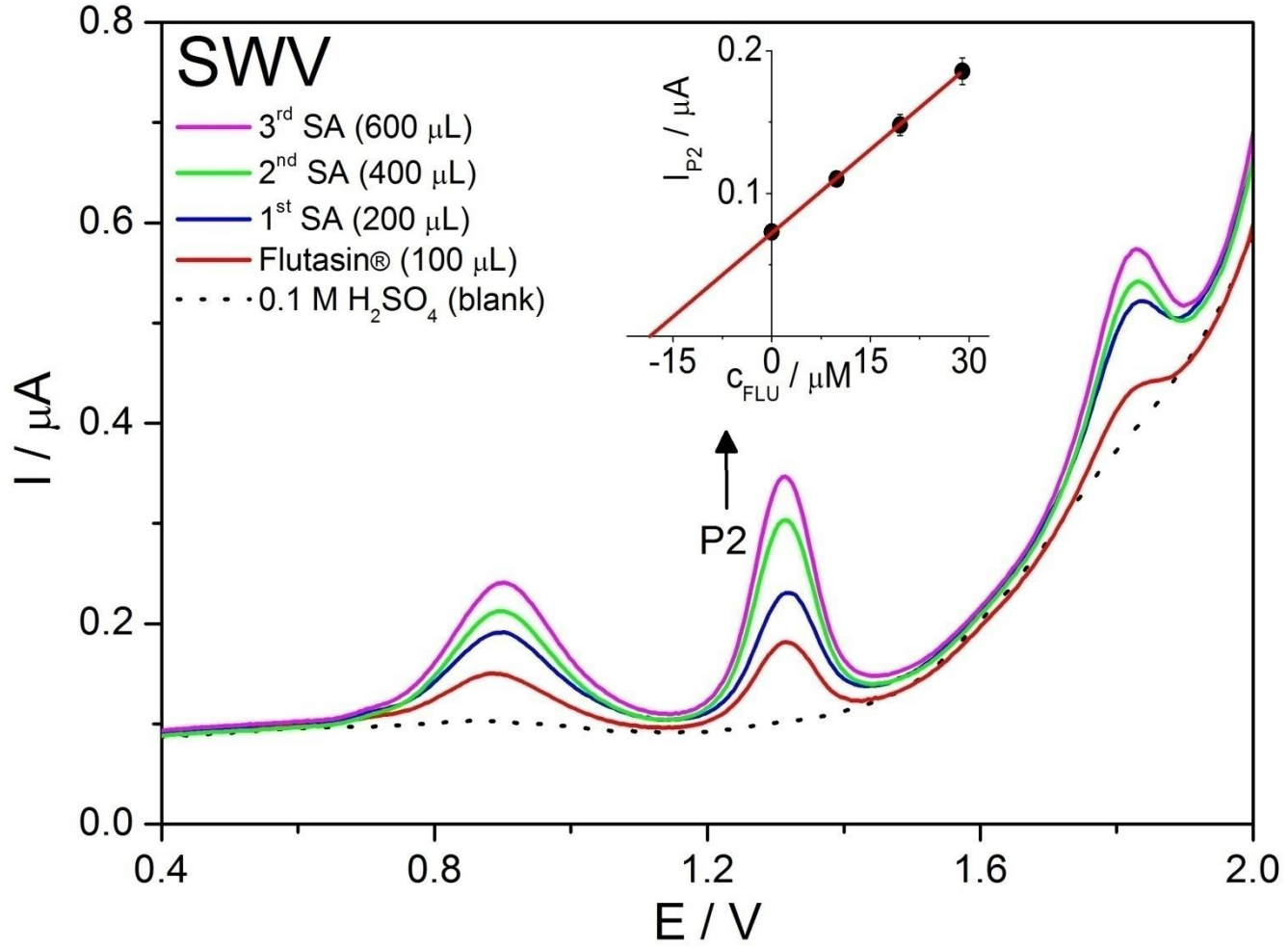
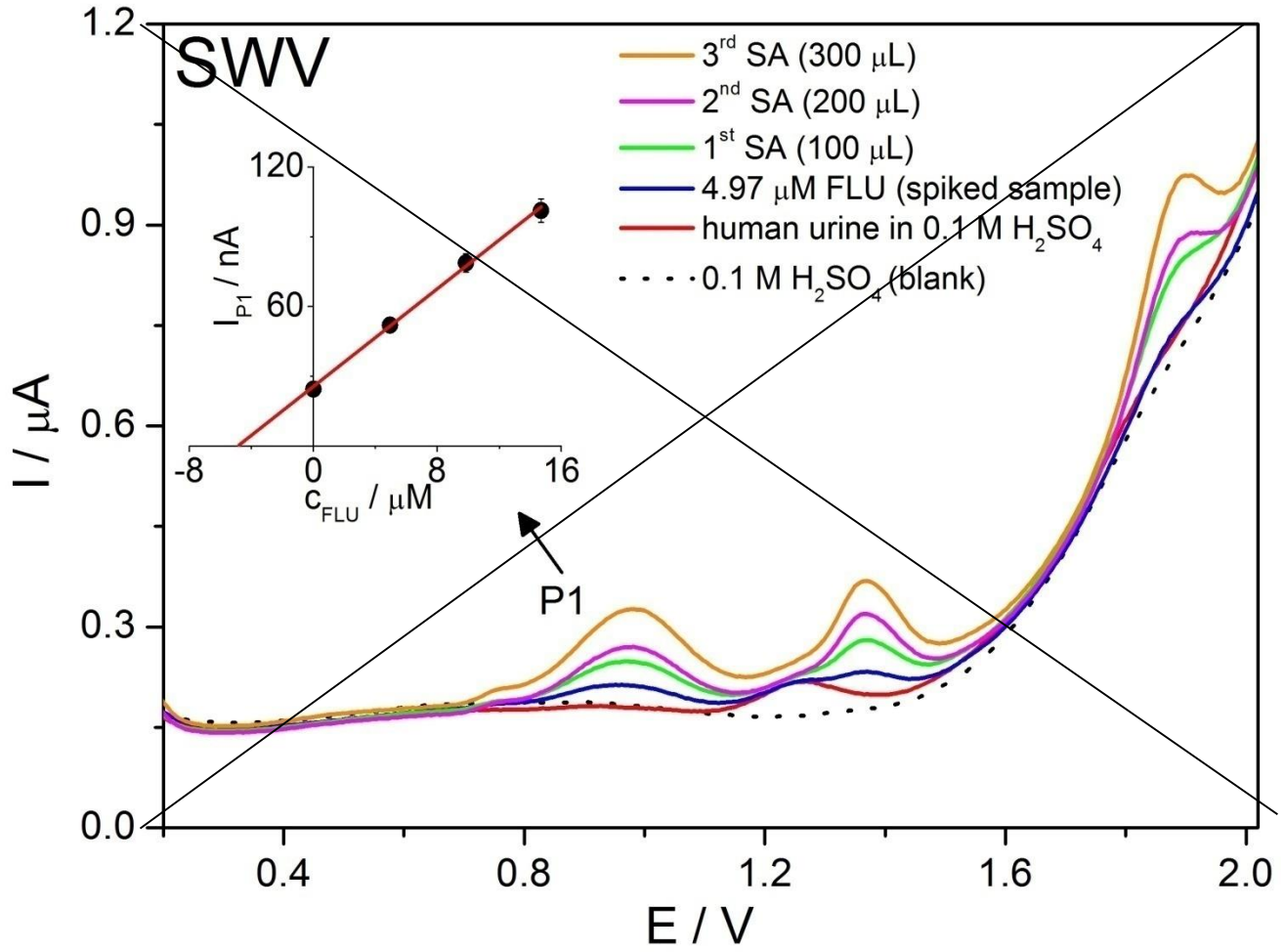


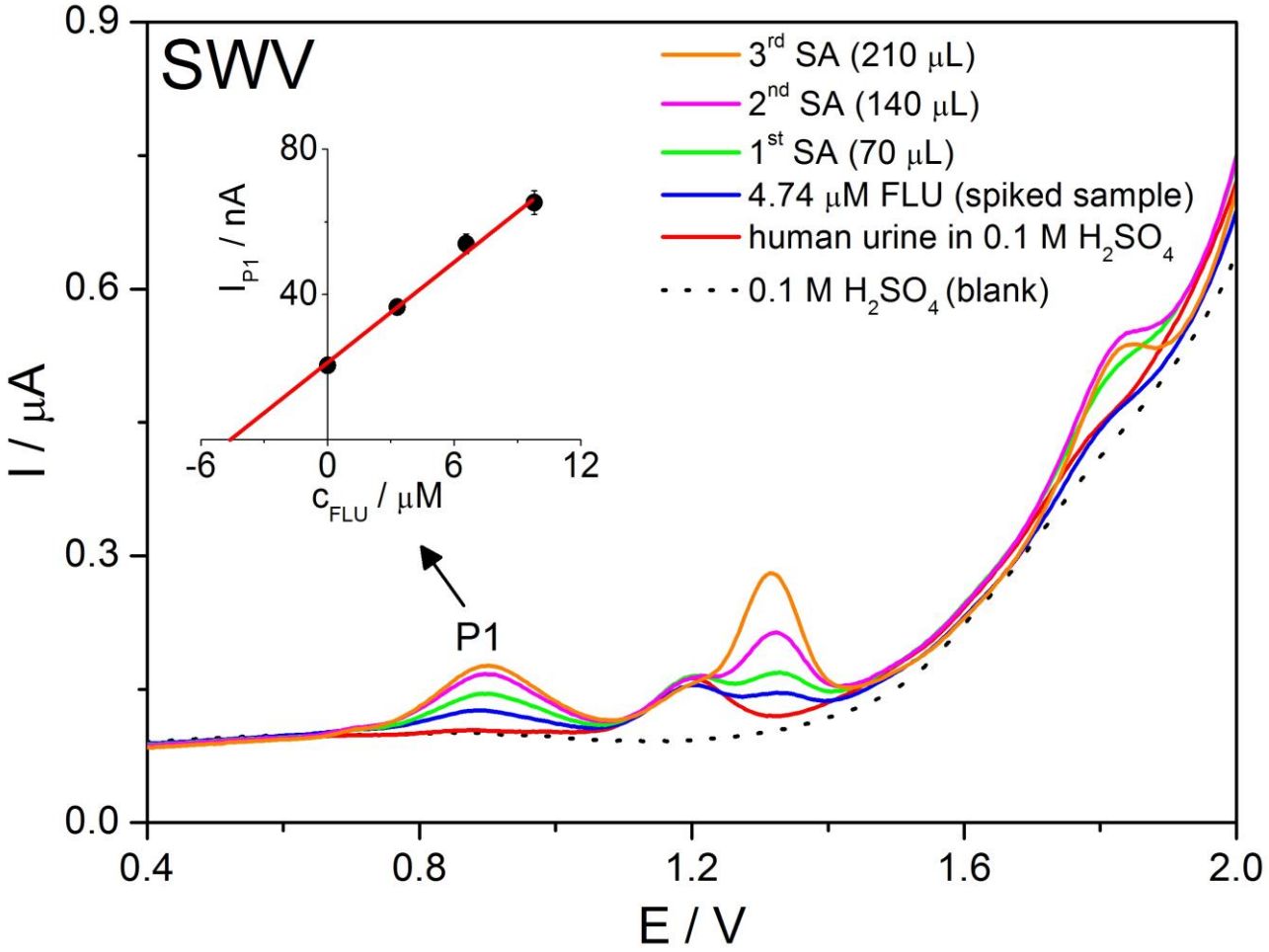
Fig. 6





*

Fig. 7



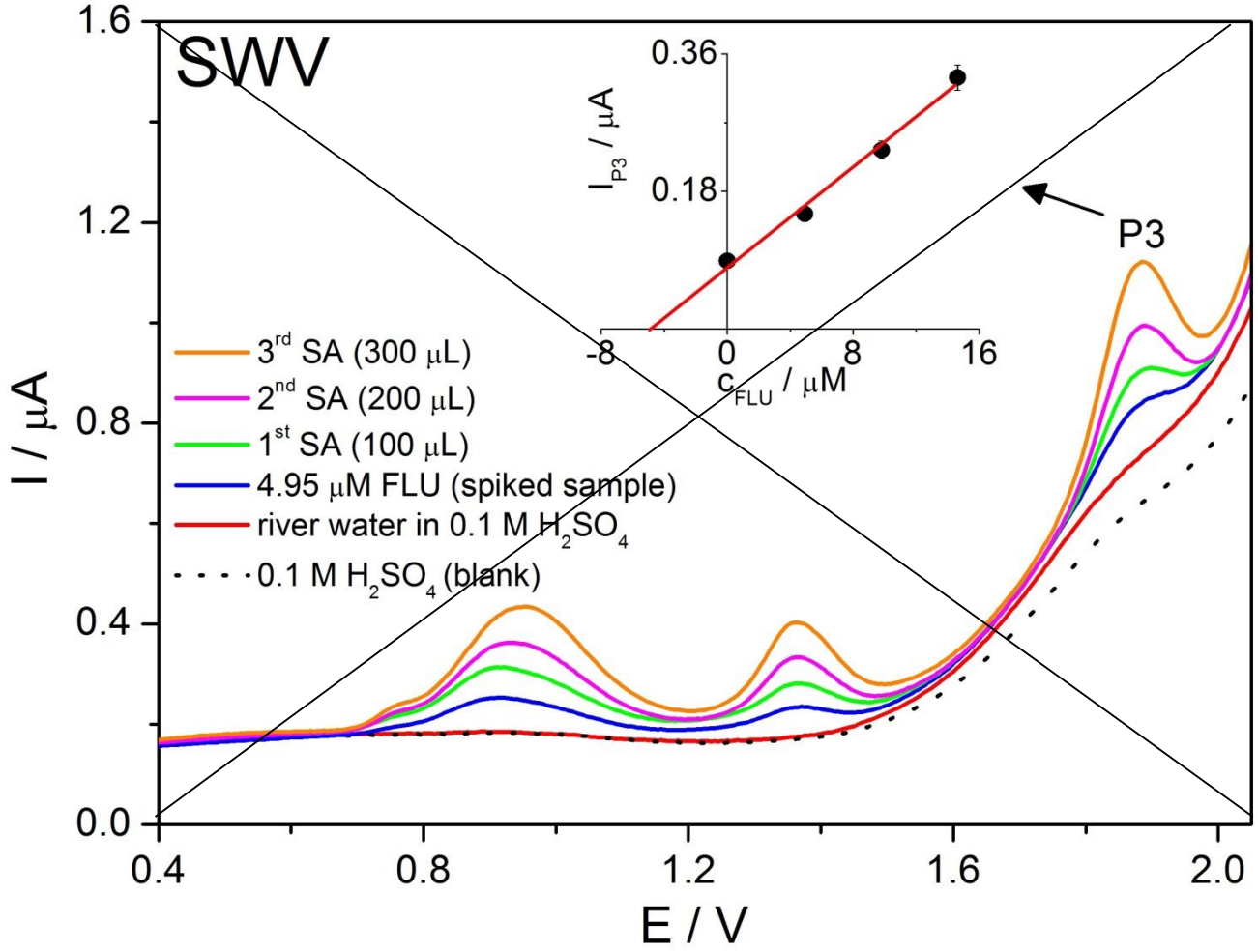
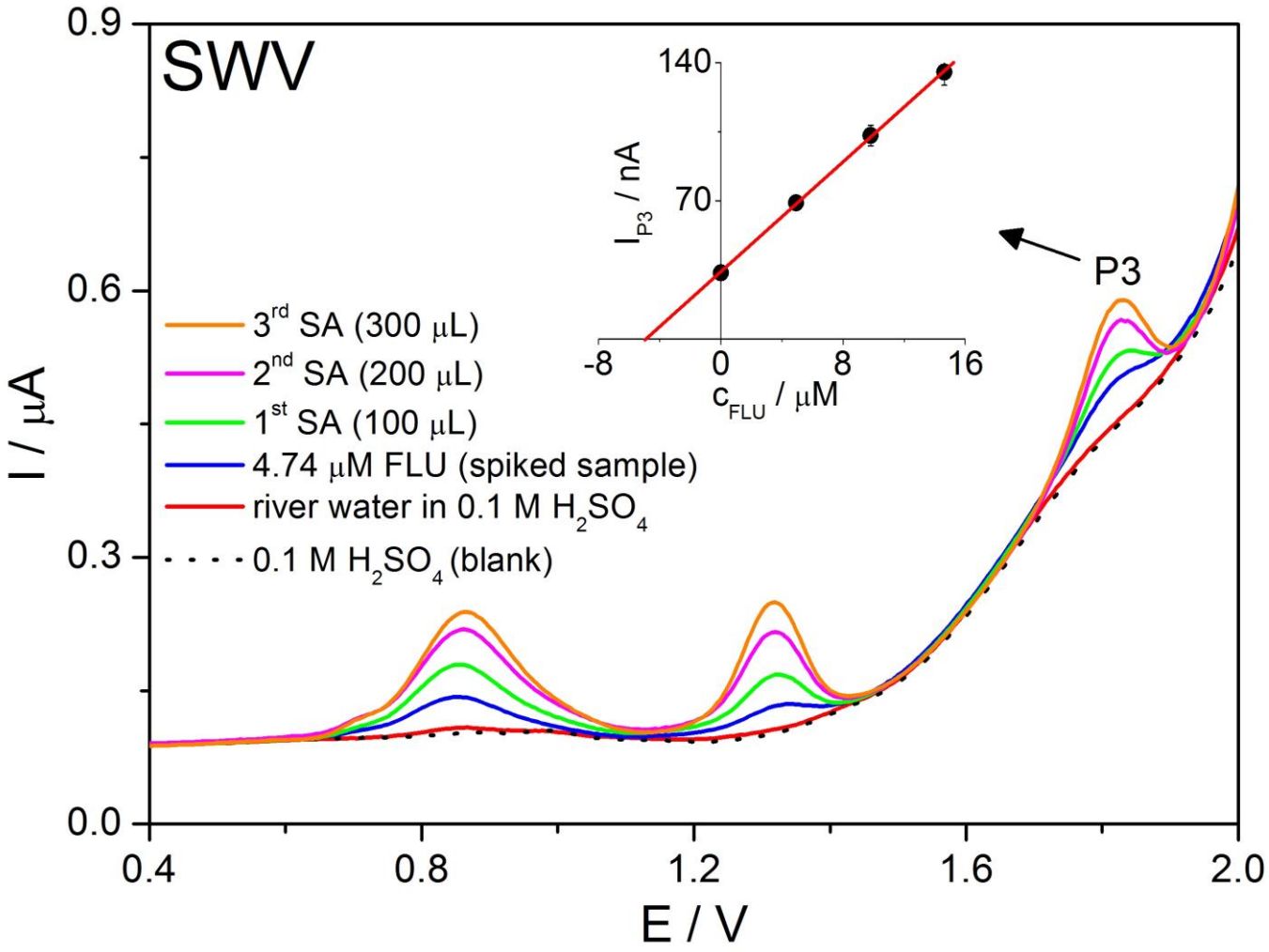


Fig. 8



Captions for figures

Fig. 1.

(A) CV records of 1 mM FLU in various concentrations of H_2SO_4 (0.1 – 2 M) on the BDD electrode in the potential range from –0.05 to +2.2 V with the scan rate of 100 mV/s.

(B) CV records of blank (BR buffer pH 2.25 and 0.1 M H_2SO_4) and 1 mM FLU in 0.1 M H_2SO_4 as well as 1 mM FLU in BR buffer pH 2.25 with oxidation peaks (P1, P2, P3 and P4) in the potential range from –0.05 to +2.2 V on the BDD electrode with the scan rate of 100 mV/s.

Fig. 2.

SWV records of 1 mM FLU for the set of the frequencies (*f*): (a) 5, (b) 10, (c) 25, (d) 50, (e) 100 and (f) 150 Hz in 0.1 M H_2SO_4 on the BDD electrode. The other SWV parameters: amplitude of 50 mV and step potential of 1 mV.

Fig. 3.

(A) DPV records of 0.1 mM FLU in 0.1 M H_2SO_4 on the BDD electrode for various modulation amplitudes: (a) 10, (b) 25, (c) 50, (d) 100, (e) 150 and (f) 200 mV. The optimization of modulation time: (a) 10, (b) 25, (c) 50, (d) 75 and (e) 100 ms is appended in the inset.

(B) SWV records of 0.1 mM FLU in 0.1 M H_2SO_4 on the BDD electrode for various frequencies: (a) 10, (b) 25, (c) 50, (d) 75, (e) 100 and (f) 150 mV. The optimization of amplitude: (a) 10, (b) 25, (c) 50, (d) 100, (e) 150 and (f) 200 ms is appended in the inset.

Fig. 4.

(A) DPV records (after background correction) for different concentrations of FLU: (a) 0.99, (b) 3.38, (c) 4.76, (d) 6.98, (e) 9.09, (f) 13.5, (g) 20.0, (h) 28.6, (i) 35.5 and (j) 42.9 µM in 0.1 M H_2SO_4 on the BDD electrode. The optimized DPV parameters: modulation amplitude of 100 mV, modulation time of 25 ms and scan rate of 10 mV/s. The corresponding calibration curves $I_p = f(c_{FLU})$ for P1, P2 and P3 are appended in the inset.

(B) SWV records (after background correction) for different concentrations of FLU: (a) 0.99, (b) 2.18, (c) 4.76, (d) 6.98, (e) 9.09, (f) 13.0, (g) 20.0, (h) 25.9, (i) 28.6 and (j) 35.5 µM in 0.1 M H_2SO_4 on the BDD electrode. The optimized SWV parameters: frequency of 25 Hz,

amplitude of 50 mV. The respective calibration curves I_p = f(c_{FLU}) for P1, P2 and P3 are appended in the inset.

Fig. 5.

SWV records demonstrating the effect of the presence of various interfering agents on 20 µM FLU in 0.1 M H_2SO_4 on the BDD electrode. The studied interfering agents were: **(A)** uric acid, **(B)** dopamine, **(C)** ascorbic acid and **(D)** folic acid. The optimized SWV parameters: frequency of 25 Hz, amplitude of 50 mV.

Fig. 6.

SWV records of analysis of the pharmaceuticals dosages *Flutasin*® with declared content of 250 mg FLU using standard addition method in 0.1 M H_2SO_4 on the BDD electrode. The corresponding standard additions: 200, 400 and 600 µL (c_{FLU} = 1 mM). The optimized SWV parameters: frequency of 25 Hz, amplitude of 50 mV. The quantification of FLU by standard addition method using the oxidation peak P2 is depicted in the inset.

Fig. 7.

SWV records of analysis of the model human urine sample of the volunteer 2 (V2) spiked with 4.97 µM FLU and evaluated by using standard addition method in 0.1 M H_2SO_4 on the BDD electrode. The corresponding standard additions: ~~100, 200 and 300~~ 70, 140 and 210 µL (c_{FLU} = 1 mM). The optimized SWV parameters: frequency of 25 Hz, amplitude of 50 mV. The quantification of FLU by standard addition method using the oxidation peak P1 is depicted in the inset.

Fig. 8.

SWV records of analysis of the model river water sample (W1) spiked with 4.95 µM FLU and evaluated by using standard addition method in 0.1 M H_2SO_4 on the BDD electrode. The corresponding standard additions: 100, 200 and 300 µL (c_{FLU} = 1 mM). The optimized SWV parameters: frequency of 25 Hz, amplitude of 50 mV. The quantification of FLU by standard addition method using the oxidation peak P3 is depicted in the inset.