



# Simultaneous voltammetric analysis of tryptophan and kynurenine in culture medium from human cancer cells

Ilona Sadok<sup>a</sup>, Katarzyna Tyszczyk-Rotko<sup>b</sup>, Robert Mroczka<sup>c</sup>, Magdalena Staniszewska<sup>a,\*</sup>

<sup>a</sup> Laboratory of Separation and Spectroscopic Method Applications, Centre for Interdisciplinary Research, The John Paul II Catholic University of Lublin, Konstantynów 1J, 20-708, Lublin, Poland

<sup>b</sup> Faculty of Chemistry, Maria Curie-Skłodowska University, Maria Curie-Skłodowska Square 3, 20-031, Lublin, Poland

<sup>c</sup> Laboratory of X-ray Optics, Centre for Interdisciplinary Research, The John Paul II Catholic University of Lublin, Konstantynów 1J, 20-708, Lublin, Poland

## ARTICLE INFO

### Keywords:

Tryptophan  
Kynurenine  
Differential pulse voltammetry  
Bismuth film  
Boron-doped diamond electrode  
Cancer cells

## ABSTRACT

The paper outlines the first report of application of a differential pulse voltammetry for simultaneous quantification of clinically important molecular markers – tryptophan and its metabolite – kynurenine. The analytes were determined in less than 60 s at the boron-doped diamond electrode modified *in situ* with bismuth film (BiF/BDDE). Proper adjustment of a supporting electrolyte composition allowed to obtain good separation of tryptophan and kynurenine oxidation peaks that appeared at potential of 0.88 and 1.05 V (vs. Ag/AgCl), respectively. Studies using an optical profilometer have confirmed an increase in electrode surface area after deposition of Bi film. At the optimized conditions, the obtained detection limits of tryptophan and kynurenine were at 30 nM concentrations. The method was validated for linearity, precision, accuracy, selectivity and recovery. We have investigated an impact of numerous relevant interfering organic compounds (including amino acids and different tryptophan metabolites of kynurenine pathway) on voltammetric signals of the measured analytes. Finally, for proof-of-technology, the sensor was used for tryptophan and kynurenine quantification in culture medium collected from human cancer cell lines (breast MDA-MB-231 and ovary SK-OV-3). The target molecules were analyzed directly, without any sample preparation step. The sensor showed good accuracy in presence of the sample matrix components that was confirmed by high performance liquid chromatography measurements. Our work emphasizes the advantages of application of the herein proposed, easy to fabricate voltammetric sensor, instead of popular chromatographic assays or previously proposed potentiometric immunosensor. The method might serve for rapid assessment of kynurenine pathway activity in cancer cells.

## 1. Introduction

Tryptophan (Trp) is an essential amino acid playing a fundamental role in human nutrition and health. It is utilized by cells in protein synthesis and serves as an *in vivo* precursor for several bioactive compounds including neurotransmitters serotonin and tryptamine, the pellagra preventive vitamins niacin (vitamin B<sub>3</sub>) and nicotinamide (vitamin B<sub>6</sub>), the sleep wake cycle regulating hormone melatonin and other important molecules [1]. Stimulation by inflammatory molecules, such as interferon  $\gamma$ , is the trigger for Trp depletion via so called kynurenine pathway, which generates immunomodulatory, neuroprotective and neurotoxic intermediates, collectively called kynurenines. This metabolic route is initiated by enzyme indoleamine 2,3-dioxygenase 1 (IDO1) mediating Trp degradation into formylkynurenine (NFK) transformed subsequently into kynurenine (Kyn, the first stable

metabolite of the kynurenine pathway). Dysregulation of the kynurenine pathway is associated with several pathologies of nervous system, such as Parkinson's disease, schizophrenia, Huntington's disease, and brain cancers [2]. Changes in Trp metabolism effecting T-cell suppression play a role in pregnancy abnormalities [3]. Furthermore, overexpression of IDO1 can facilitate survival and growth of tumor cells by fostering immune suppression [4]. IDO1 inhibition represents one of the most potent therapeutic approaches to inhibit tumor growth [5].

Trp catabolism is linked to many disorders and development of sensitive, accurate, cheap, and rapid methods for simultaneous determination of Trp and the kynurenine pathway metabolites is an objective of the widespread interest. Liquid chromatography with respect to its selectivity and sensitivity became the method of choice for determination of Trp and kynurenine pathway metabolites. HPLC-UV systems have found a broad application in analysis of Trp and Kyn in

\* Corresponding author.

E-mail addresses: [ilonasadok@kul.lublin.pl](mailto:ilonasadok@kul.lublin.pl) (I. Sadok), [ktyszczyk@poczta.umcs.lublin.pl](mailto:ktyszczyk@poczta.umcs.lublin.pl) (K. Tyszczyk-Rotko), [rmroczka@kul.lublin.pl](mailto:rmroczka@kul.lublin.pl) (R. Mroczka), [staniszkul@kul.lublin.pl](mailto:staniszkul@kul.lublin.pl) (M. Staniszewska).

<https://doi.org/10.1016/j.talanta.2019.120574>

Received 4 September 2019; Received in revised form 13 November 2019; Accepted 15 November 2019

Available online 18 November 2019

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variety of biological milieus [6]. However, they suffer from laborious sample preparation such as extraction, protein precipitation, filtration, concentration, and extract purification. Despite that a small amount of sample is introduced into the chromatography system (the injection volume is frequently less than 50  $\mu$ L), the method requires a large volume of organic solvents to achieve proper separation of analytes, column rinsing and conditioning. This also makes such analysis time consuming. Chromatographic systems coupled with mass spectrometric detectors allow to shorten analysis time improving both sensitivity and selectivity, however, they require expensive and sophisticated equipment, laborious sample preparation, and use of isotopically labeled internal standards.

The main goal of this work was to develop a simple method for quantification of Trp and Kyn without a need for sophisticated equipment (like liquid chromatography system) and sample preparation. Electroanalytical methods, because of their sensitivity and simplicity and relatively low-cost instrumentation present the good alternative to chromatographic approaches. So far, many electrochemical sensors have been proposed for Trp determination in variety of samples. There are protocols available for simultaneous quantification of Trp and other biologically important compounds such as uric acid [7] or amino acids like tyrosine [8,9], cysteine [9]. Electrochemical sensors have been also applied for determination of Trp metabolites of methoxyindole pathway such as dopamine [7,10], serotonin [11], 5-hydroxyindoleacetic acid [10]. They use some modified electrodes based on the multiwalled carbon nanotubes or disposable screen-printed graphite. Regarding electrochemical determination of kynurenine pathway metabolites, including Kyn, the literature have been rather scarce. For example, liquid chromatography coupled to electrochemical detector was applied for measurements of Kyn at the multiwalled carbon nanotube-modified glassy carbon electrode [12] and 3-hydroxykynurenine at the carbon glassy electrode [13], in human plasma and brain samples. There is also one work proposing the use of potentiometric immunosensor for monitoring of Trp consumption and Kyn production by cancer cells [14].

For this study, we have chosen differential pulse voltammetry (DPV) – the electrochemical method based on voltage-current-time relationship arising in the electrochemical cell that gained numerous applications in analysis of a variety of biologically active compounds [15]. In general, voltammetric measurements might be carried out in the cell of three electrodes (working electrode, auxiliary electrode and reference electrode) dipped in an ionic electrolyte containing dissolved sample. After application of the appropriate potential, the compounds of interest are reduced or oxidized at the surface of the working electrode that results in the generation of a current. The plot of this current versus applied potential is called voltammogram and provides quantitative and qualitative information. We have shown that boron-doped diamond electrode (BDDE) might be used as a working electrode for voltammetric analysis of Trp and Kyn. The sensitivity of this method might be improved by the simple modification of an electrode surface with the Bi film. To the best of our knowledge, this is a first report describing the use of BDDE modified with Bi film (BiF/BDDE) for the voltammetric simultaneous determination of Trp and Kyn. Furthermore, for our knowledge, no studies have been reported for simultaneous voltammetric determination of Trp and Kyn. Our preliminary measurements have indicated that these two clinically important molecules show similar voltammetric behavior and give signals at similar potentials resulting in significant overlapping of peaks. Next, Trp amounts in a variety of biological samples are generally much higher than Kyn that provides another challenge during development of analytical protocol. We managed to overcome these problems by simple modification of the surface of the working electrode and careful selection of the supporting electrolyte composition. We believe, this work could inspire other researchers for further improvements of simultaneous voltammetric determinations of Trp and Kyn in regard to both selectivity and sensitivity.

## 2. Materials and methods

### 2.1. Apparatus

Voltammetric determinations were performed using the Autolab potentiostat/galvanostat (PGSTAT101, Metrohm, the Netherlands) operated with a 1.11 NOVA software. The BDDE (inner diameter of 3 mm, boron doping level of 1000 ppm, electrical resistivity of 0.075  $\Omega$ m) was purchased from Windsor Scientific Ltd (Berkshire, UK).

Chromatographic determinations were carried out using the 1200 Series high performance liquid chromatograph equipped with diode array detector (HPLC-DAD), autosampler, quaternary pump with vacuum degasser and column thermostat (Agilent Technologies, USA). Separation was achieved on the Zorbax Eclipse Plus C18 rapid resolution HT 4.6  $\times$  150 mm, 3.5  $\mu$ m column coupled with the Zorbax Eclipse Plus-C18 2.1  $\times$  12.5 mm, 5  $\mu$ m Narrow Bore Guard Column (Agilent Technologies, USA). Instrument control and data analysis were carried out using the Agilent ChemStation software v.B.04.02.

The optical profilometer (WYKO NT9800, Veeco, USA) was used to determine the surface microroughness in three dimensions. The objective lens magnification was set to 100  $\times$  corresponding to 0.19  $\mu$ m sampling (pixel) size. The whole post processing analysis was carried out using the Probe Image Processor (SPIP) v. 5.1.4 software (Image Metrology A/S, Denmark).

The cancer cells were cultured at 37  $^{\circ}$ C in humidified atmosphere of 5% CO<sub>2</sub> employing the HERAccl 150i Cu incubator (Thermo Fisher Scientific).

Crystalline chemicals were weighed using a XP6 microbalance supplied by Mettler Toledo (Switzerland). The pH measurements were made on the CI-316 pH meter (Elmetron) and on the SevenMulti dual meter pH/conductivity completed with InLab<sup>®</sup> Expert Pro (Mettler Toledo, Switzerland). Ultra-purified water (> 18 M  $\Omega$  cm) was produced by the Milli-Q system (Millipore, UK). Samples were shaken using the Stuart SSL4 see-saw rocker, centrifuged using the 5415 R Centrifuge (Eppendorf, Germany), and concentrated using the Genevac EZ-2 Elite Personal Evaporator (Genevac Ltd, UK).

### 2.2. Reagents

Crystalline L-tryptophan ( $\geq$ 98%), L-kynurenine ( $\geq$ 98%), 3-hydroxy-D,L-kynurenine, kynurenic acid ( $\geq$ 98%), nicotinic acid ( $\geq$ 99.5%), nicotinamide ( $\geq$ 99.5%), anthranilic acid ( $\geq$ 99.5%), 2-picolinic acid (99%), xanthurenic acid (96%), 3-hydroxyanthranilic acid (97%), quinolinic acid (99%), L-lysine ( $\geq$ 98%), L-glutamine ( $\geq$ 99%), L-aspartic acid ( $\geq$ 98%), L-alanine ( $\geq$ 98%), L-asparagine ( $\geq$ 98%), L-serine ( $\geq$ 99%), L-cysteine ( $\geq$ 98%), L-leucine ( $\geq$ 98%), L-proline ( $\geq$ 99%), L-histidine ( $\geq$ 99%), L-threonine ( $\geq$ 98%), L-valine ( $\geq$ 98%), L-tyrosine ( $\geq$ 98%), L-methionine ( $\geq$ 98%), L-phenylalanine ( $\geq$ 98%), L-arginine ( $\geq$ 98%), glycine ( $\geq$ 98.5%), glucose, L-ascorbic acid ( $\geq$ 99%), uric acid ( $\geq$ 99%), 3-nitro-L-tyrosine (3NT), trichloroacetic acid (TCA), activated charcoal, trypsin were purchased from Sigma-Aldrich (USA). Stock solutions of Kyn and Trp were prepared in dimethyl sulfoxide (DMSO, Uvasol<sup>®</sup>, Merck, Germany). Fetal bovine serum (FBS), L-glutamine and 10 000 U/mL penicillin/streptomycin were purchased from PAN Biotech (Aidenbach, Germany). Methanol (HPLC grade), glacial acetic acid (Suprapur<sup>®</sup>) and ammonium acetate (fractopure) were purchased from Merck (Germany). Dubelcco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g/L of D-glucose were from Institute of Immunology and Experimental Therapy PAS in Wrocław. Phosphate buffered saline (PBS) solution was prepared by dissolving 8 g of sodium chloride (Merck, Poland), 0.2 g of potassium chloride (Sigma-Aldrich, USA), 1.44 g of sodium phosphate dibasic (Sigma-Aldrich, Poznań, Poland), potassium dihydrogen phosphate (Suprapur<sup>®</sup>, Merck, Poland) in 1 L of water. pH was adjusted to 7.4 with hydrochloric acid (37%) from Merck (Poland).

### 2.3. Voltammetric measurements

The conventional three electrode quartz cell (volume of 10 mL) consisting of a platinum wire (counter electrode), Ag/AgCl (reference electrode) and bismuth film modified boron-doped diamond electrode (BiF/BDDE, working electrode), was used. Prior to use, the commercially available BDD electrode with diameter of 3 mm was polished on a Buehler polishing pad using series of alumina slurries (1.0, 0.3 and 0.05  $\mu\text{m}$ ), following washing with water and cleaning in an ultrasonic water bath for 2 min with distilled water to remove adsorbed impurities.

Voltammetric measurements were carried out from a supporting electrolyte containing: 0.25 M mixture of  $\text{CH}_3\text{COONH}_4$  and  $\text{CH}_3\text{COOH}$  (pH 6.5), 0.04 M potassium sodium tartrate, 0.5  $\mu\text{M}$  Bi(III) and variable concentrations of target compounds (Trp and Kyn). Bismuth film was deposited onto the BDDE surface *in situ* applying the potential of  $-1.35$  V for 30 s with stirring. The differential pulse voltammograms (DPVs) were registered in a range from 0.5 to 1.2 V (modulation amplitude: 25 mV, modulation time: 0.05 s, step potential: 0.01 V).

### 2.4. Liquid chromatography

The binary gradient consisting of solvent A (10 mM  $\text{CH}_3\text{COONH}_4$  in water acidified to pH 4.0 with  $\text{CH}_3\text{COOH}$ ) and solvent B (methanol) was applied for Trp and Kyn separation. Elution was carried out in the following manner: 0–17 min 0% solvent B; 17–20 min 0–5% solvent B; 20–30 min 5–20% solvent B; 30–35 min 20–30% solvent B; 35–40 min 30–60% solvent B; 40–45 min 60–0% solvent B; 45–50 min 0% solvent B. The flow rate was set at 0.5 mL/min with column temperature at 20 °C. Kyn elution was monitored at 360 nm (retention time 14.6 min), and Trp and 3NT as the internal standard (retention time 29.2 and 26.6 min, respectively) at 286 nm. The injection volume was 5  $\mu\text{L}$ .

### 2.5. Cancer cells culture

Studies were conducted using two different cancer cell lines: the human breast carcinoma cell line MDA-MB-231 and the human ovarian cancer cell line SK-OV-3 both purchased from American Type Culture Collection (ATCC). The cells were grown on Petri dishes in DMEM containing 4.5 g/L of D-glucose, 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin and passaged at 80% of confluency. For the experiment cells were detached from plates using trypsin, seeded onto 12-well ( $0.3 \times 10^6$  cells per well) and allowed to attach overnight. Next day the culture medium was gently aspirated and 500  $\mu\text{L}$  of the fresh medium was added. After 48 h the cell conditioned medium was collected and centrifuged (5 min,  $14\,000 \times g$ , 4 °C). The supernatant was immediately frozen at  $-80$  °C until analysis.

### 2.6. Sample preparation procedure for voltammetric measurements

The frozen samples of culture medium were thawed at room temperature and vortexed well. The aliquot (200–500  $\mu\text{L}$ ) was directly added to the supporting electrolyte in the voltammetric cell (final volume 5 mL). Trp and Kyn concentration was determined using a standard addition method.

### 2.7. Procedure for sample preparation for chromatographic analysis (HPLC-DAD)

The sample of medium (125  $\mu\text{L}$ ) fortified with 44.2  $\mu\text{M}$  3NT (internal standard) was deproteinized with 15  $\mu\text{L}$  of 30% (w/v) TCA, vortexed and centrifuged (15 min,  $14\,000 \times g$ , 4 °C). The supernatant (125  $\mu\text{L}$ ) was gently transferred into a glass vial and evaporated to dryness. The residual was reconstituted in 25  $\mu\text{L}$  of an aqueous solution of 10 mM  $\text{CH}_3\text{COONH}_4$  (pH 4.0), transferred to chromatographic insert vial and immediately analyzed in triplicate. If needed, samples were

diluted 10-fold with 10 mM  $\text{CH}_3\text{COONH}_4$  (pH 4.0) and reanalyzed. Quantitative analysis was conducted based on a matrix-matched calibration curve.

### 2.8. Statistical analysis

A paired Student's *t*-test was used to evaluate if Trp and Kyn concentrations determined in culture medium by voltammetric method differ from those found by a reference method (HPLC-DAD). Data were processed using the XLSTAT 2018.5.52140. The differences were considered significant at  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Simultaneous voltammetric determination of Trp and Kyn at the BDDE

Due to its unique properties (such as wide electrochemical potential window, low and stable background current), BDDE has been widely used as electrochemical sensor [16]. Unmodified BDDE has been so far applied for Trp determination in presence of tyrosine [8], however, there are no reports on its application for Kyn electrochemical analysis. Thus, we tested whether it is possible to conduct simultaneous voltammetric measurements of tryptophan and kynurenine using the commercially available BDDE.

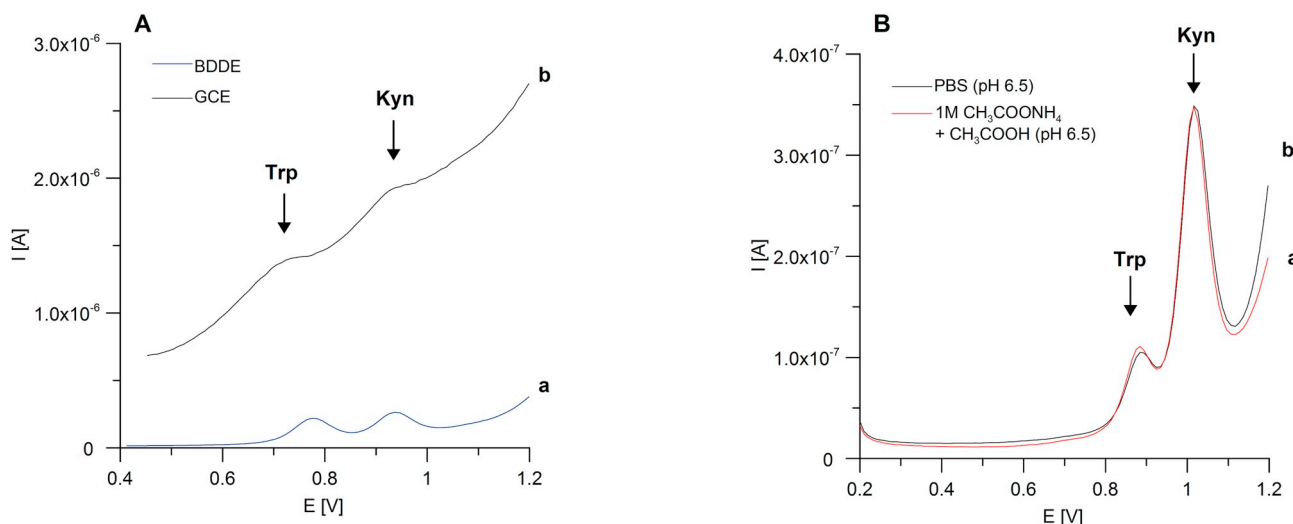
Preliminary studies were performed in PBS (pH 7.7), and as can be seen in Fig. 1A, two well separated peaks of Trp and Kyn were obtained on BDDE. Furthermore, BDDE offers lower background current and better shape of analyte peaks compared to a glassy carbon electrode (GCE) as emphasized in Fig. 1A. Next, we investigated the effect of PBS with different pH (3.0, 4.5, 6.0, 6.5, 7.0, 8.5) and 1 M  $\text{CH}_3\text{COONH}_4$  (pH 6.85) or its mixture with  $\text{CH}_3\text{COOH}$  of different pH (4.6, 5.9, 6.5) on 15  $\mu\text{M}$  Trp and Kyn signals registered on BDDE. DPVs obtained in different supporting electrolytes are presented in Fig. 1S. Generally, supporting electrolytes of pH lower than 4.6 and higher than 6.5 were found to be unsuitable for simultaneous Trp and Kyn determination because of significant peak overlapping or stretching, and lowering the analytes peak currents. We have also observed that the supporting electrolyte with pH from 4.6 to 6.5 provided similar Trp and Kyn peak resolution (about 130 mV under applied conditions of the analysis). After visual examination, supporting electrolyte composed of PBS (pH 6.5) and the mixture of 1 M  $\text{CH}_3\text{COONH}_4$  with  $\text{CH}_3\text{COOH}$  (pH 6.5) offered the most favorable conditions for simultaneous Trp and Kyn determinations. As can be seen in Fig. 1B, no significant differences in Trp and Kyn peak resolution or signal peak currents between these two supporting electrolytes were observed. The mixture of 1 M  $\text{CH}_3\text{COONH}_4$  with  $\text{CH}_3\text{COOH}$  (pH 6.5) has been selected for further measurements.

These results confirm that Trp and Kyn could be determined simultaneously by differential pulse voltammetry (DPV) without a need for sophisticated electrode surface modification (such as by specific monoclonal antibodies).

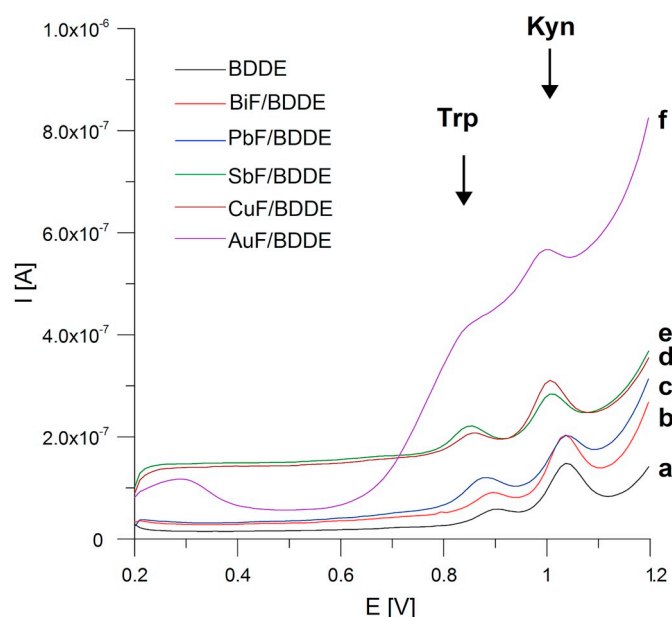
### 3.2. Modification of BDDE surface with metal film

The working electrode surface modification by electrodeposition of metal particles is a documented way for improvement of sensitivity and selectivity of voltammetric analysis. We have already emphasized the applicability of this strategy in case of biologically active compounds analysis in our previous works [17–19]. Thus, we asked whether such surface modification of BDDE could provide some benefits in case of simultaneous Trp and Kyn determination.

For this purpose, Pb(II), Bi(III), Cu(II), Sb(III) or Au(III) solution was introduced into the supporting electrolyte to reach the final concentration of 0.2  $\mu\text{M}$ , and metal ions were reduced under stirring by applying the potential of  $-1.55$  V for 60 s. To stabilize Pb(II) ions in electrolyte at pH 6.5, 0.02  $\mu\text{M}$  potassium sodium tartrate was added to



**Fig. 1.** (A) Comparison of DPVs obtained during 10 μM Trp and Kyn determination in PBS (pH 7.7) at (a) BDDE (d = 3 mm) and (b) GCE (d = 3 mm) expressed as the relationship between potential (E) measured in [V] and current (I) measured in [A]. (B) DPVs obtained in different supporting electrolytes (pH 6.5) in (a) mixture of 1 M CH<sub>3</sub>COONH<sub>4</sub> with CH<sub>3</sub>COOH (pH 6.5) and (b) PBS (pH 6.5) on 15 μM Trp and Kyn peak currents at bare BDDE.



**Fig. 2.** Comparison of Trp and Kyn signals (5 μM) expressed as potential (V) versus current (I) after modification of BDDE with metal film: (a) bare BDDE; (b) BDDE covered with Pb film (PbF/BDDE); (c) BDDE covered with Bi film (BiF/BDDE); (d) BDDE covered with Cu film (CuF/BDDE); (e) BDDE covered with Sb film (SbF/BDDE); (f) BDDE covered with Au film (AuF/BDDE). Metal film was deposited at -1.55 V for 60 s. The supporting electrolyte containing 1 M CH<sub>3</sub>COONH<sub>4</sub> and CH<sub>3</sub>COOH (pH 6.5) with 0.2 μM of proper metal ion was used for film deposition.

the supporting electrolyte [20]. The concentration of analytes was 5 μM. Modification with Au film resulted in background current increase and reduction of voltammetric response of Trp and Kyn (Fig. 2). For other modifiers, the current increase of both Trp and Kyn peaks, compared to bare BDDE (peak resolution about 160 mV) was observed. Electrode surface modification with Pb film caused 2-fold and 1.4-fold increase of Trp and Kyn peak currents, respectively (peak resolution about 170 mV). Bismuth film deposition allowed to obtain the same peak resolution and increase of both target compounds signals (2.4-fold and 1.14-fold, respectively). Modification with Sb film particles provided peak resolution of 160 mV and caused about 2.7-fold and

1.4-fold increase of Trp and Kyn peak, respectively. Deposition of Cu particles provided peak resolution about 140 mV and caused 1.7-fold and 1.11-fold increase of Trp and Kyn signals, respectively. Furthermore, a significant rise in a background current after Cu and Sb particles deposition onto the BDDE was observed. The obtained results are compared in Fig. 2. Across the surface modifications tested, only Pb and Bi films slightly improved Trp to Kyn peak resolution compared to unmodified BDDE. Considering all advantages and disadvantages of applied surface modifications (peak resolution, improvement of peak current, metal toxicity) we decided to select the BDDE modified with Bi film (BiF/BDDE) as the working electrode for simultaneous voltammetric determinations of Trp and Kyn.

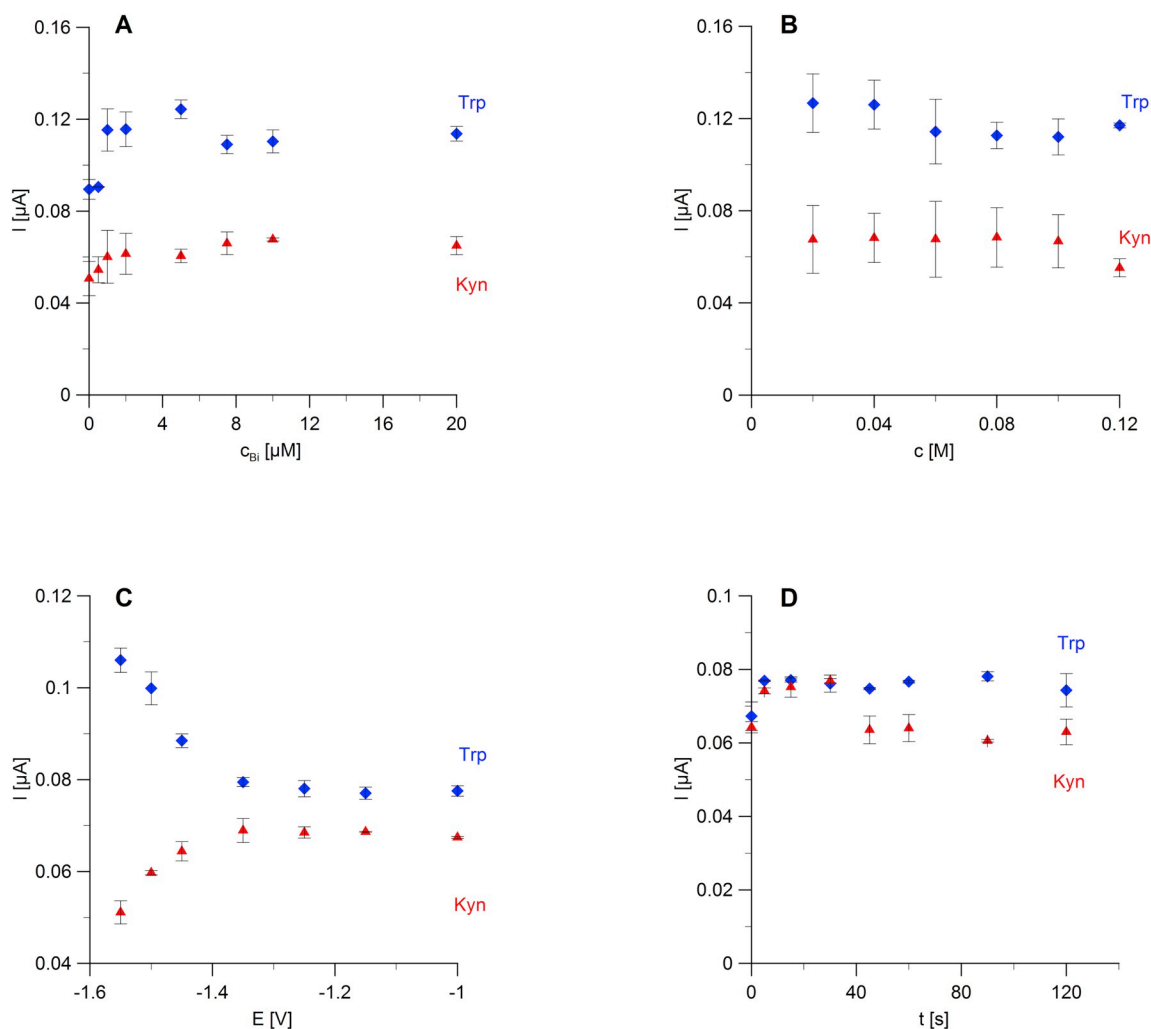
### 3.3. Optimization of voltammetric conditions

In this section, we collected the results regarding an impact of various measurement conditions (including composition of the supporting electrolyte, parameters of Bi film deposition) on the target compound signal.

All studies were carried out in presence of 5 μM Trp and Kyn in the supporting electrolyte initially composed of the mixture of 1 M CH<sub>3</sub>COONH<sub>4</sub> with CH<sub>3</sub>COOH (pH 6.5) and 0.1 M potassium sodium tartrate. Many papers emphasized advantages of using tartrate for stabilization of Sb(III), Pb(II) and Bi(III) before being deposited on the electrode surface at neutral or mild alkaline media [21–23]. Under these conditions, tartrate prevents hydrolysis of these metal ions by formation water-soluble complexes and allows for their reduction on the electrode surface. Beneficial effect of potassium sodium tartrate in case of Bi or Sb films formation in acidic conditions has also been reported. In this case, tartrate provided a wide cathodic operational potential window necessary for detecting low concentrations of Zn(II) [24,25]. Our preliminary studies confirmed that addition of potassium sodium tartrate to the supporting electrolyte allows for more reproducible Bi deposition onto the BDDE surface. Estimated repeatability of analyte signal (assessed as relative standard deviation (RSD) of 10 successive measurements of peak currents of 5 μM Trp and Kyn at BiF/BDDE) were 15.7% for Trp and 11.5% Kyn determined without presence of potassium sodium tartrate, and 6.7% for Trp and 5.8% for Kyn analyzed after introduction of 0.1 M potassium sodium tartrate into the supporting electrolyte.

At first, the influence of CH<sub>3</sub>COONH<sub>4</sub> concentration in the mixture with CH<sub>3</sub>COOH (pH 6.5) within the range from 0.1 to 1.0 M on the Trp





**Fig. 3.** Influence of: (A) Bi(III) concentration, (B) potassium sodium tartrate concentration (C); (C) potential of Bi film deposition (E); (D) Bi film deposition time (t) on 5  $\mu\text{M}$  Trp and Kyn peak currents (I). The supporting electrolyte contained 0.25 M  $\text{CH}_3\text{COONH}_4$  in the mixture with  $\text{CH}_3\text{COOH}$  (pH 6.5).

and Kyn peak currents (I) was studied. No effect on Kyn voltammetric response was observed, while Trp signal attained maximum and nearly constant value for the concentration of  $\text{CH}_3\text{COONH}_4$  in the range from 0.25 M to 1.0 M. Furthermore, no peak shift within studied concentration range of  $\text{CH}_3\text{COONH}_4$  was observed. Following measurements were carried out in the supporting electrolyte composed of 0.25 M  $\text{CH}_3\text{COONH}_4$  in the mixture with  $\text{CH}_3\text{COOH}$  (pH 6.5).

Next, the effect of Bi(III) concentration on the Trp and Kyn voltammetric signals was studied. Different concentrations of Bi(III) from 0 to 20  $\mu\text{M}$  were introduced into the electrolyte after the electrode surface was electrochemically cleaned by applying the potential of 0.5 V for 30 s. Bi film was deposited under stirring at potential  $-1.55$  V for 60 s. Presence of Bi film onto the electrode surface improved both Trp and Kyn peak currents as confirmed in Fig. 3A. Started from Bi(III) concentration of 0.5  $\mu\text{M}$  in the supporting electrolyte, Trp and Kyn signals appeared to reach maximal and nearly stable values, and was selected for further studies.

The effect of potassium sodium tartrate concentration on Trp and Kyn peak currents (I) was also investigated in the concentration range from 0.02 to 0.12 M. The results presented in Fig. 3B indicates that the potassium sodium tartrate concentration had no significant effect on Kyn peak current. However, a slight decrease of Trp signal was observed for potassium sodium tartrate concentration of 0.05 M and higher. For further experiments the reagent concentration of 0.04 M was chosen.

The influence of the Bi film deposition onto the electrode surface on analytes peak current was investigated in the potential range from  $-1.55$  to  $-1.0$  V. As can be seen in Fig. 3C, the oxidation peak of Trp attained maximum value at the Bi deposition potential of  $-1.55$  V, then started to decrease with increased potential to finally reach a stable value in the potential range from  $-1.35$  to  $-1.0$  V. Simultaneously, reduction of Trp signal was accompanied by increase in Kyn peak current, which attained maximum and stable value at metal deposition potentials from  $-1.35$  to  $-1.0$  V. Bi deposition potential of  $-1.35$  V provided the optimal conditions for simultaneous measurements of Trp and Kyn signals, and was selected for further experiments.

Finally, the influence of the Bi film deposition time (t) on the Trp and Kyn peak currents in the range of 0–120 s was investigated. It was observed that Trp signal remained nearly stable for Bi deposition time from 5 to 120 s. On the other hand, Kyn signal reached maximum value during deposition from 5 to 30 s, and then tended to decrease (Fig. 3D). Based on the obtained results, Bi film deposition time of 30 s was chosen for further studies.

The need for introducing additional potential for cleaning the electrode surface after previous measurement was also verified. For this purpose, we compared repeatability of 10 successive measurements of voltammetric signals of 5  $\mu\text{M}$  Trp and Kyn obtained with or without the purification potential of 0.5 V for 30 s. The relative standard deviations (RSD) were 1.4% and 1.0% for Trp, and 1.5% and 1.6% for Kyn with and without the use of purification, respectively. The results have

confirmed that electrode surface cleaning step might be omitted, so the purification potential was removed from the final protocol.

### 3.4. Optimization of DPV parameters

Differential pulse and square wave voltammograms were recorded in order to select the best registration technique for Trp and Kyn determinations. DPV showed lower background current and better shape of Kyn oxidation signal, thus was used in further studies (Fig. 2S). Next, the influence of the instrumental parameters of differential pulse scan mode on peak currents of 5  $\mu\text{M}$  Trp and Kyn was also tested. The step potential was changed in the range from 10 to 50 mV, modulation amplitude from 10 to 125 mV and modulation time from 0.01 to 0.08 s. Increase in the step potential value resulted in the decrease of number of points in recorded signals and their poor shape. Despite improvement in Trp and Kyn peak currents, a change in modulation time to higher values caused gradual increase of background current and poor peak shapes. In case of modulation time, Trp and Kyn peak currents attained the maximum values for the lowest time tested. All data are collected in the Supporting Information file (Fig. 2S). The optimum values of DPV parameters were as followed: modulation amplitude of 25 mV, modulation time of 0.05 s, step potential of 0.01 V.

### 3.5. Cyclic voltammetric studies

To study the nature of the reaction occurring at the surface of BDDE modified with Bi film, the scan rate measurements were carried out from 0.05 to 0.35 V/s in solution containing 5  $\mu\text{M}$  Trp or Kyn. In the forward scan, one anodic peak of Trp at about 0.85 V, and no peak in the reverse scan was observed. This corresponds to the irreversible process. Furthermore, the anodic peak currents of Trp linearly increased with the increasing of scan rate (Fig. 4A). It suggested that within the applied conditions the diffusion-controlled reduction of Trp occurs at the BiF/BDDE. This observation was confirmed by studying the relationship between Trp peak current and square root of scan rate. The obtained plots were linear ( $R^2$  was 0.999) suggesting that the process is diffusion-controlled (Fig. 4B). Regression of the logarithm of the peak current ( $\log I$ ) and the logarithm of the scan rate ( $\log v$ ) resulted in slopes equal to 0.40 ( $R^2 = 0.999$ ). This confirms a diffusion-controlled nature of the processes in case of Trp. Furthermore, a linear relationship between the Trp peak position ( $E_p$ ) and the logarithm of the scan rate ( $\ln v$ ) was obtained with a regression equation of  $E_p(\text{V}) = 0.0603 \ln v (\text{mV s}^{-1}) + 0.8942$  ( $R^2 = 0.994$ ). In order to evaluate the number of transferred electrons, the Laviron's equation was used:

$$E_p = E^{0'} - \frac{2.303RT}{\alpha nF} \ln \frac{RTk^0}{\alpha nF} + \frac{2.303RT}{\alpha nF} \ln v \quad (1)$$

where:  $R$  – the universal gas constant ( $R = 8.314 \text{ J mol}^{-1} \text{ K}^{-1}$ ),  $T$  – the absolute temperature ( $T = 298 \text{ K}$ ),  $F$  – Faraday constant ( $F = 96485 \text{ C mol}^{-1}$ ),  $\alpha$  – transfer coefficient (for irreversible processes  $\alpha = 0.5$ ),  $n$  – number of transferred electrons,  $k^0$  – standard heterogeneous rate constant,  $v$  – scan rate. According to Laviron's theory, the slope of  $E_p$  versus  $\ln v$  plot is equal to  $RT/\alpha nF$ . Thus,  $\alpha n$  value calculated was 0.43 resulting in the number of transferred electrons on BiF/BDDE equal to 0.86 ( $\sim 1$ ).

In analogy, the behavior of Kyn onto the BiF/BDDE was studied utilizing cyclic voltammetry. One peak in anodic run (at potential about 1.05 V) and no peak in reduction scan was observed indicating that oxidation of Kyn is an irreversible process. Anodic peak of Kyn increased linearly with a scan rate suggesting also a diffusion-controlled process (Fig. 4C). Furthermore, Kyn peak currents were found to increase linearly with a square root of scan rate ( $R^2$  was equal to 0.994) (Fig. 4D). Regression of the logarithm of the Kyn peak current ( $\log I$ ) and the logarithm of the scan rate ( $\log v$ ) resulted in slopes equal to 0.48 ( $R^2 = 0.995$ ). The data suggested that in case of Kyn analysis a

diffusion-controlled processes occurs on the BiF/BDDE. The relationship between the Kyn peak position ( $E_p$ ) and the logarithm of the scan rate ( $\ln v$ ) was linear with a regression equation of  $E_p(\text{V}) = 0.0438 \ln v (\text{mV s}^{-1}) + 1.026$  ( $R^2 = 0.988$ ). The number of electron transfer ( $n$ ) involved in the Kyn oxidation process on BiF/BDDE based on the Laviron's theory was 1.17 ( $\sim 1$ ).

### 3.6. Electrode surface characterization using an optical profilometer

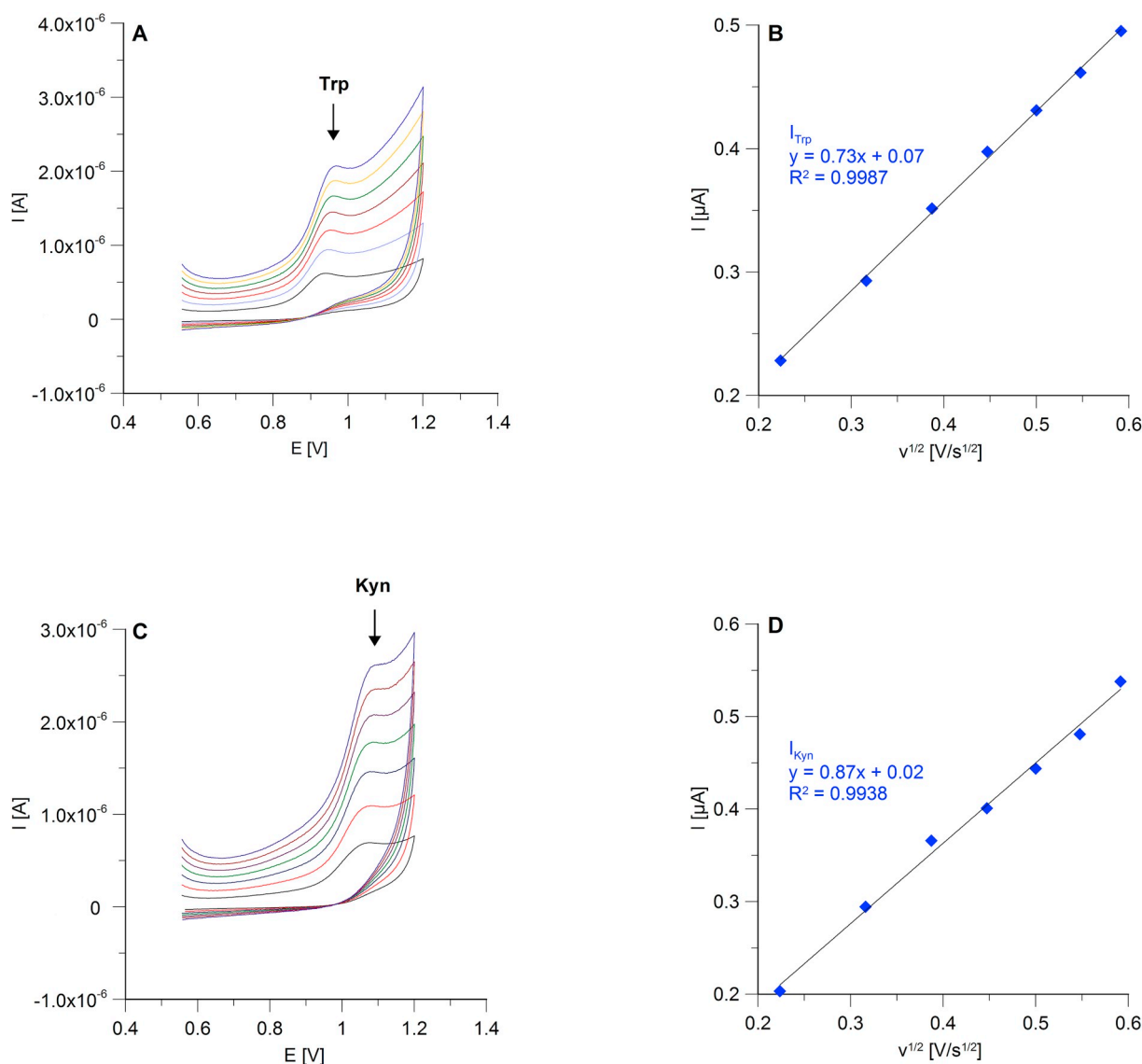
Surface of the bare BDDE after mechanical polishing (cleaning) demonstrated homogenous, smooth surface (Fig. 5A). However, few scratches with irregular shape (indicated on Fig. 5A by the red arrows) were visible. It presumably is an effect of surface cleaning. Surface roughness ( $S_q$ ) of unmodified BDDE was equal to 2.92 nm. Closer inspection within the box area visible in Fig. 5A demonstrated homogenous, amorphous character without defects (Fig. 5B) and low surface roughness ( $S_q = 1.8 \text{ nm}$ ). Profile deviation along white line (shown in Fig. 5B) exhibits regular profile with deviation in the range from  $-2.0 \text{ nm}$  to  $1.5 \text{ nm}$  (Fig. 5C). Overall, electrode surface after mechanical polishing was sufficiently smooth for Bi deposition and for further characterization of the metal particles in nanoscale dimensions.

Electrode surface after electrochemical deposition of Bi (Fig. 5D) demonstrated higher surface roughness ( $S_q = 4.41 \text{ nm}$ ) in comparison to the surface of the bare BDDE ( $S_q = 2.92 \text{ nm}$ ). Similarly, as it was for the unmodified BDDE, a few scratches are still visible on the surface (see red arrows in Fig. 5D). The higher surface roughness after metal deposition are determined by the presence of Bi visible within the blue box marked in Fig. 5D. Closer inspection of Bi particles present within blue box is shown in Fig. 5E. Surface profile along white line that shows cross section of the example particle (marked by white line in Fig. 5E) is presented in Fig. 5F. Bismuth particle is characterized by asymmetrical shape with dimensions as follows: diameter about  $2 \mu\text{m}$ , height from left side about  $6 \text{ nm}$ , height from right side about  $15 \text{ nm}$ . Overall, deposited Bi particles demonstrated very likely a tendency to aggregation in lateral direction and diameter about  $2 \mu\text{m}$ . On the other hand, aggregation in vertical direction does not take place, as vertical size of particles is maintained in the range of several nm.

### 3.7. Calibration graphs

Five independent experimental batches were conducted in order to investigate method linearity. For this, we investigated linear relationships when: (1) only one analyte was present in the supporting electrolyte, (2) the concentration of one species changed, whereas the other was kept constant, (3) the concentration of both analytes increased. Each calibration point was measured five times. The equations of the calibration plots were expressed as  $y = ax + b$ , where  $y$  corresponds to the peak current ( $\mu\text{A}$ ),  $a$  is a slope,  $x$  is the analyte concentration ( $\mu\text{M}$ ) and  $b$  refers to an intercept. The limits of detection (LOD) and quantification (LOQ) were calculated as a standard deviation of intercepts ( $n = 5$ ) divided by the slope of the calibration function and multiplied by 3.3 or 10, respectively. All calibration plots had coefficient of determination ( $R^2$ )  $> 0.990$ . The parameters of the analytical curves are collected in Table 1. Typical voltammograms obtained in the course of determination of different concentration of Trp and Kyn for each studied case and corresponding calibration plots are collected in Supporting Information (Fig. 3S–7S).

By simple modification of BDDE surface with Bi film and a proper choice of the supporting electrolyte composition, we were able to achieve satisfactory Trp and Kyn peak resolution and conduct simultaneous measurement of these two analytes at low concentration levels. Using the herein proposed approach, we obtained better LOD for Trp compared to many other sensors (Table 2). Majidi and coworkers, however, have received more impressive LOD for Trp determination using biosensor based on modified gold electrode [26]. Nevertheless, the protocol requires more sophisticated electrode surface modification



**Fig. 4.** Cyclic voltammetry (CV) studies of 5  $\mu\text{M}$  Trp or Kyn at the BiF/BDDE. The cyclic voltammograms of (A) Trp or (C) Kyn obtained for different scan rates (0.05 – 0.35 V/s). Relationships between (B) Trp or (D) Kyn anodic peak currents ( $I$ ) and the square root of the scan rates [ $\text{V/s}^{1/2}$ ] are shown. The supporting electrolyte composition included 0.25 M mixture of  $\text{CH}_3\text{COONH}_4$  with  $\text{CH}_3\text{COOH}$  (pH 6.5), 0.04 M potassium sodium tartrate, and 0.5  $\mu\text{M}$  Bi(III).

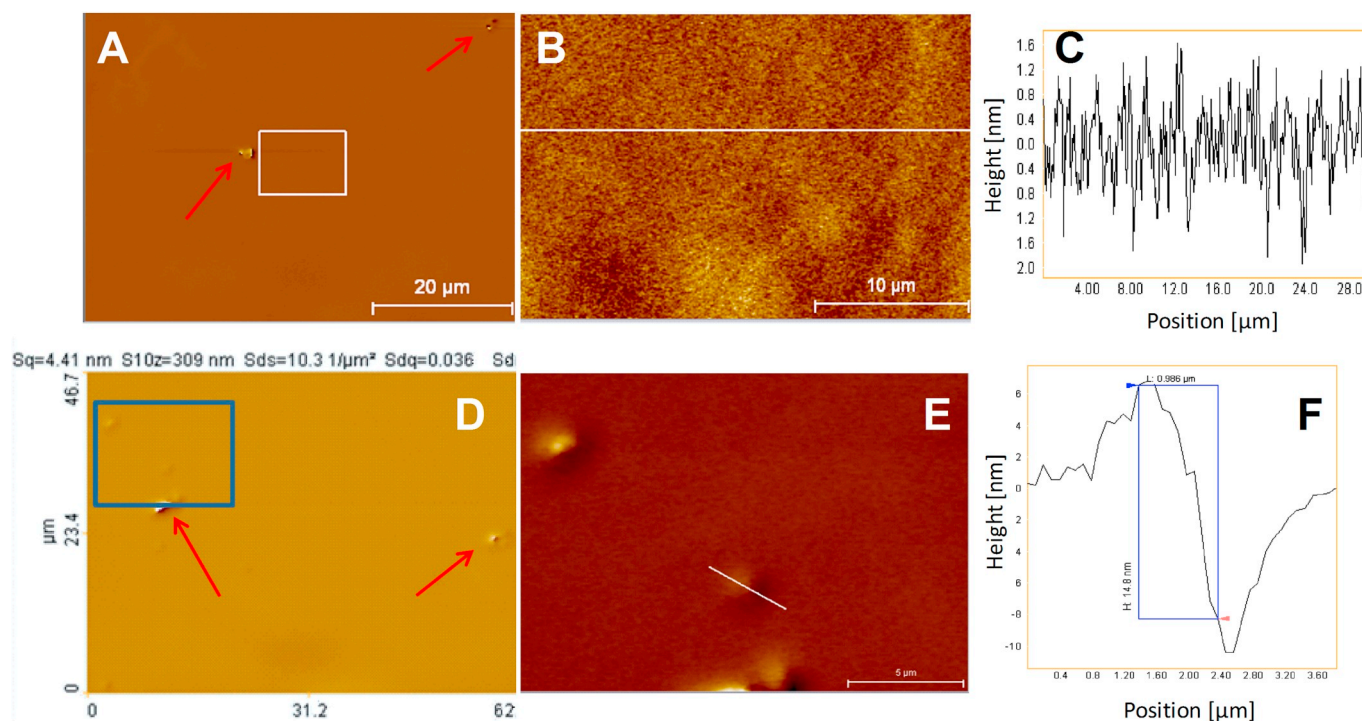
including attachment of carboxylated multiwall carbon nanotubes and deposition of Trp-specific RNA aptamer, and importantly, allows to only Trp determination [26]. This group has also developed a screen-printed potentiometric immunosensor for monitoring of Trp consumption and Kyn production by cancer cells reaching excellent LOD for Kyn (0.5 nM) and a broad linear range of the calibration curve [14]. However, the obtained LOD for Trp (0.12  $\mu\text{M}$ ) was higher than that reported by our group on BiF/BDDE. Furthermore, the protocol for the immunosensor preparation requires laborious modification of a gold screen-printed electrode surface by deposition of carboxylated multiwall carbon nanotubes and immobilization of the proper monoclonal antibody (mAb) specific to kynurenine. For comparison, the proposed here BiF/BDDE preparation requires only quick refreshing of the electrode surface by polishing before starting a set of experiments, and further modification with Bi film is carried out *in situ* during the voltammetric analysis taking only 30 s. The presented results indicate that voltammetric analysis using BiF/BDDE may be worth considering in terms of rapid estimation of differences in kynurenine pathway activation between cancer cell lines.

### 3.8. Method precision

The precision was investigated by measuring the current response of 2  $\mu\text{M}$  Trp and Kyn, determined as relative standard deviation of ten replicates. The test was carried out on the same day (intraday precision, repeatability) or on three different days (interday precision). The established values of intraday precision for Trp and Kyn were 1.3 and 1.5% ( $n = 10$ ), respectively. The inter-day precision was 3.7 and 5.4% ( $n = 30$ ) for Trp and Kyn, respectively. All obtained results did not exceeded thresholds of  $\pm 15\%$  confirming good precision of the method.

### 3.9. Recovery

Studies were carried out utilizing DMEM culture medium supplemented with 10% (v/v) FBS and known amount of Trp or Kyn. For this, 500  $\mu\text{L}$  of culture medium spiked or not with Trp and Kyn at two different concentration levels was directly added into the supporting electrolyte (10 mL) and analyzed by standard addition method. The results are compared in Table 3. Satisfactory recoveries for both



**Fig. 5.** Optical profilometer micrographs of BDDE surface unmodified (A–C) and modified with Bi film (BiF/BDDE, D–F): (A) BDDE the surface area  $47 \times 63 \mu\text{m}$ ; (B) BDDE surface area  $20 \times 30 \mu\text{m}$  within the box marked in (A); (C) BDDE surface profile along the line shown in (B); (D) BiF/BDDE surface area  $47 \times 63 \mu\text{m}$ ; (E) BiF/BDDE surface area  $15 \times 18 \mu\text{m}$  within the blue box shown in (D); (F) Bi/BDDE surface profile along the line shown in (E). Red arrows point out surface scratches, white line in panel (E) indicates the deposited particle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**  
Parameters of the calibration plots for Trp and Kyn.

Analyte	LOD [ $\mu\text{M}$ ]	LOQ [ $\mu\text{M}$ ]	Linear range [ $\mu\text{M}$ ]	Slope	Intercept	R <sup>2</sup>
Trp <sup>a</sup>	0.03	0.09	0.09 - 100.00	0.02	0.04	0.997
Kyn <sup>b</sup>	0.03	0.10	0.10 - 50.00	0.02	0.02	0.997
Trp <sup>c</sup>	0.03	0.09	0.09 - 1.00	0.06	0.01	0.992
	0.30	0.91	1.00 - 75.00	0.02	0.07	0.999
Kyn <sup>d</sup>	0.03	0.10	0.10 - 20.00	0.02	0.01	0.999
Trp <sup>e</sup>	0.03	0.10	0.10 - 1.00	0.10	0.01	0.992
	0.33	1.00	1.00 - 50.00	0.02	0.10	0.995
Kyn <sup>e</sup>	0.03	0.09	0.09 - 1.00	0.06	-0.01	0.996
	0.31	0.93	0.93 - 50.00	0.01	0.04	0.999

SD - standard deviation for  $n = 5$ .

<sup>a</sup> Increasing concentrations of Trp.

<sup>b</sup> Increasing concentrations of Kyn.

<sup>c</sup> Increasing concentrations of Trp in presence of constant Kyn amount (2  $\mu\text{M}$ ).

<sup>d</sup> Increasing concentrations of Kyn in presence of constant Trp amount (2  $\mu\text{M}$ ).

<sup>e</sup> Simultaneous increasing concentrations of Trp and Kyn.

analytes were obtained in full culture medium (from 91.3 to 101.3% and from 88.4 to 97.8% for Trp and Kyn, respectively). These results showed that culture medium samples might be analyzed with herein proposed voltammetric method without any sample preparation step.

### 3.10. Selectivity

The effect of different interfering compounds on Trp (2  $\mu\text{M}$ ) and Kyn (2  $\mu\text{M}$ ) signals was investigated in the concentration range from 0.2 to 100  $\mu\text{M}$ . The tolerance limit was  $\pm 10\%$  of the target compounds peak current in presence of interfering compounds.

At first, the effect of several different metabolites produced during Trp degradation along kynurenine pathway (kynurenines) on voltammetric behavior of Trp and Kyn was investigated. Quinolinic acid (QA), picolinic acid (PIC), nicotinic acid (NA), nicotinamide (NAM) did not

give voltammetric signals at BiF/BDDE in applied conditions and did not disturb Trp and Kyn analysis over the studied concentration range. No peak of kynurenic acid (Kyna) was observed, but this metabolite influences both Trp and Kyn signals in manner detailed in Table 4. Xanthurenic acid (XA), 3-hydroxyanthranilic acid (3HAA), 3-hydroxykynurenine (3HKyn) and anthranilic acid (AA) could be oxidized at 0.65, 0.38, 0.42 and 0.85 V, respectively at BiF/BDDE. The tolerance limits for these kynurenines were also collected in Table 4. It is worth mentioning that Kyna, 3HKyn, 3HAA, XA, AA amounts in biological samples are substantially lower than that of Trp and Kyn. While Trp and Kyn occur in human plasma at about 60 and 2  $\mu\text{M}$ , respectively, other kynurenines are at tens of nM [27]. Furthermore, metabolites of kynurenine pathway (except Kyn) are usually not detected or hardly detected in culture medium collected from untreated human glioma cells [28], monocytes, mature dendritic cells [29], and cancer cells as well [30]. Therefore, we assumed that interference of other kynurenines on Trp and Kyn voltammetric analysis should be negligible in case of culture medium matrix.

The objective of the study was quantification of Trp and Kyn in culture medium collected from cancer cell cultures. A typical cell culture medium contains of amino acids, glucose, salts, vitamins, and additional supplements. The requirements for these components vary among cell lines. Essential amino acids, required for the cell proliferation, are obligatory ingredients of all cell culture media as cells are unable to synthesize these by themselves. Thus, we investigated an effect of different amino acids on Trp and Kyn signals. Some noticeable influence was caused by the presence of 15 amino acids, including L-aspartic acid (Asp), L-lysine (Lys), L-threonine (Thr), L-valine (Val), L-glutamine (Gln), L-proline (Pro), L-phenylalanine (Phe), L-serine (Ser), L-histidine (His), L-asparagine (Asn), L-arginine (Arg), L-methionine (Met), L-leucine (Leu), L-alanine (Ala) and glycine (Gly) (up to 50-fold excess). Tolerable limits of L-cysteine (Cys) and L-tyrosine (Tyr) are set in Table 4. In applied conditions, L-tyrosine (Tyr) gave voltammetric signal at potential about 0.80 V and tends to overlap with



**Table 2**

Comparison of electrochemical sensors proposed for Trp and Kyn determination available in literature.

Electrode	Technique	Linear range [ $\mu\text{M}$ ]		LOD [ $\text{nM}$ ]		Application	Ref.
		Trp	Kyn	Trp	Kyn		
CPE-MWCNTs	DPV	0.6–100.0	–	70.0	–	serum	[7]
BDDE	DPV	20.0–1000.0	–	10000.0	–	amino acid injection	[8]
CNF-CPE	CPA	0.1–119.0	–	100.0	–	–	[9]
Ag@C-GCE	LSV	0.1–100.0	–	40.0	–	amino acid injection, serum	[31]
Apt-MWCNT-AuE	CC-PSA	0.1 <sup>a</sup> – 10.0	–	0.1	–	milk, blood, serum, saliva, urine, cancer cell culture medium	[26]
mAb-MWCNT-AuSPE	CC-PSA	10.0–300.0	–	–	–	cancer cell culture medium	[14]
		0.1–300.0	1.0 <sup>b</sup> – 1.0	120.0	0.5		
BiF/BDDE	DPV	0.1–1.0	0.1–20.0	30.0	30.0	cancer cell culture medium	This work
		1.0–75.0	1.0–100.0	–	–		

Ag@C-GCE – glassy carbon electrode modified with Ag nanoparticles-carbon shell composite; Apt-MWCNT-AuE – gold electrode modified with carboxylated multiwall carbon nanotubes and Trp aptamer molecules; BiF/BDDE – boron-doped diamond electrode modified with bismuth nanoparticles; CC-PSA – constant current-potentiometric stripping analysis; CNF-CPE – carbon paste electrode modified with carbon nanofiber; CPA – constant potential amperometry; CPE-MWCNTs – carbon paste electrode modified with multi-walled carbon nanotubes; DPV – differential pulse voltammetry; LSV – linear sweep voltammetry; mAb-MWCNT-AuSPE – gold screen-printed electrode modified with carboxylated multiwall carbon nanotubes and monoclonal antibody.

<sup>a</sup> - value in nM.

**Table 3**

Recovery of Trp and Kyn analyzed by DPV in cell culture medium.

Added [ $\mu\text{M}$ ]	Found $\pm$ SD [ $\mu\text{M}$ ]	Recovery [%]
Trp		
40	41 $\pm$ 9 <sup>a</sup>	101
80	73 $\pm$ 5 <sup>a</sup>	91
Kyn		
40	35 $\pm$ 1	88
80	78 $\pm$ 7	98

SD - standard deviation for n = 3.

<sup>a</sup> - value obtained after subtracting the Trp concentration found in the blank sample (without analyte spike).

**Table 4**

Tolerable excess of different organic compounds on Trp and Kyn voltammetric signals.

Interference compounds	Tolerable excess ( $\pm$ 10%)	
	Trp	Kyn
Metabolites of kynurenine pathway		
QA, PIC, NA, NAm	50.0	50.0
Kyna	10.0	5.0
XA	2.5	5.0
3HKyn	25.0	2.5
3HAA	2.5	Equal concentration
AA	Equal concentration	10.0
Amino acids		
Ala, Arg, Asp, Asn, Gln, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Val	50.0	50.0
Cys	5.0	10.0
Tyr	Equal concentration	5.0
Other		
Glucose, Vit C	50.0	50.0
UA	50.0	5.0

Trp signal. However, Trp and Kyn signals are measurable up to 25-fold excess of Tyr. Importantly, commercially available cell culture media usually contain about 5-fold higher amount of Tyr and Cys relative to Trp concentration. Furthermore, the sample is diluted after transferring

**Table 5**

Results of Trp and Kyn concentrations in culture medium of different human cancer cell lines determined by DPV and HPLC-DAD methods.

Trp $\pm$ SD [ $\mu$ M]			Kyn $\pm$ SD [ $\mu$ M]		p
Sample	DPV	HPLC-DAD	DPV	HPLC-DAD	
MDA-MB-231 cancer cell line					
#1	69 $\pm$ 2	71 $\pm$ 2	ND	D	0.184
#2	58 $\pm$ 0	58 $\pm$ 1	ND	D	0.905
#3	58 $\pm$ 2	55 $\pm$ 1	ND	D	0.054
#4	65 $\pm$ 2	68 $\pm$ 1	ND	D	0.136
SK-OV-3 cancer cell line					
#1	ND	D	35 $\pm$ 3	32 $\pm$ 0	0.104
#2	ND	D	58 $\pm$ 1	59 $\pm$ 1	0.072
#3	ND	D	39 $\pm$ 1	38 $\pm$ 0	0.222
#4	ND	D	41 $\pm$ 1	39 $\pm$ 2	0.223
#5	ND	D	39 $\pm$ 4	40 $\pm$ 1	0.639
#6	ND	D	31 $\pm$ 0	31 $\pm$ 1	0.637

SD – standard deviation (n = 3).

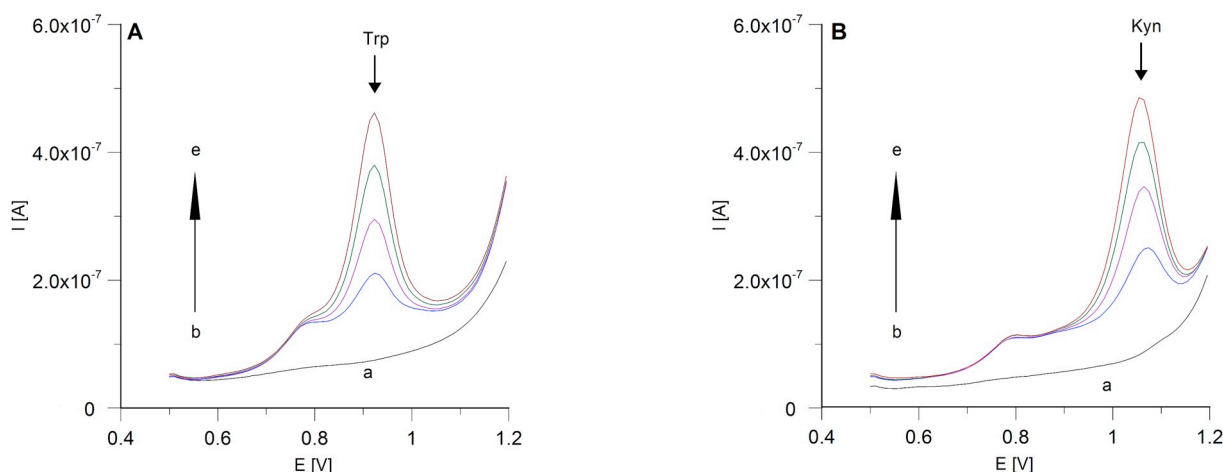
ND – not detected.

D – detected, but not quantified (< LOQ).

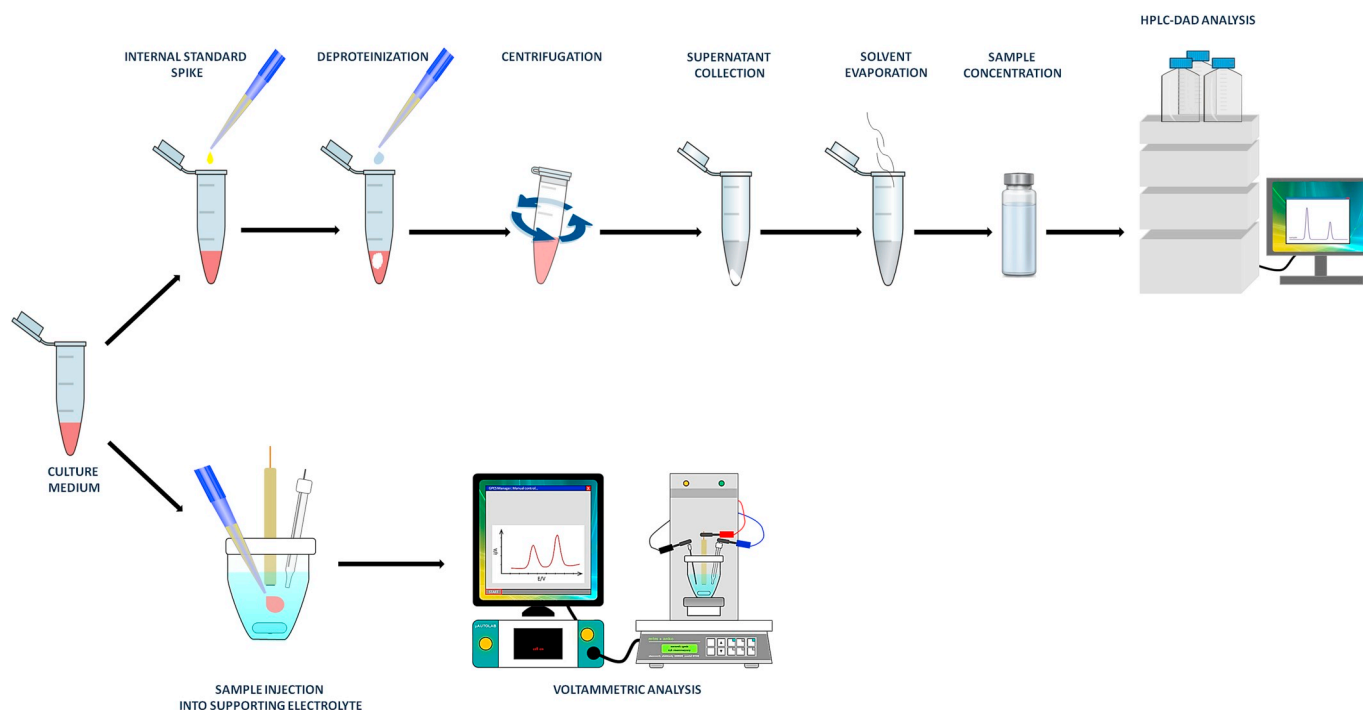
into the supporting electrolyte in the voltammetric cell, thus the negative effects of co-existing compounds are reduced.

Additionally, the influence of glucose (the main energy source for growing cells) was studied. It was found that this hexose did not significantly interfere with the measurement of Trp and Kyn over the studied concentration range (recoveries 100.5 – 104.2% and 101.5 – 104.6%, respectively). An effect of other electroactive compounds that are commonly present in biological fluids uric acid (UA) and ascorbic acid (vit C) have also been investigated. Vit C and UA signals appeared at 0.42 V and 0.44 V, respectively. Vit C did not cause any noticeable effect on the voltammetric behavior of Trp and Kyn towards the Bi/BDDE in the whole studied concentration range. UA did not disturb the voltammetric response of Trp and Kyn up to 50-fold and 5-fold excess, respectively. Kyn signal, however, was still measurable up to 50-fold excess of UA.

The results presented above indicated that the proposed DPV method shows satisfactory selectivity towards many coexisting compounds and might be applied for Trp and Kyn measurements in samples of cell culture medium.



**Fig. 6.** (A) DPVs obtained during Trp quantification in culture medium from MDA-MB-231 cancer cells: (a) background, (b) as (a) + sample (300  $\mu$ L), (c) as (b) + 4  $\mu$ M Trp, (d) as (b) + 8  $\mu$ M Trp, (e) as (b) + 12  $\mu$ M Trp. (B) DPV results obtained in course of Kyn determination in culture medium from SK-OV-3 cancer cells: (a) background, (b) as (a) + sample (240  $\mu$ L), (c) as (b) + 2  $\mu$ M Kyn, (d) as (b) + 4  $\mu$ M Kyn, (e) as (b) + 6  $\mu$ M Kyn. The supporting electrolyte composition was the same as in Fig. 5.



**Fig. 7.** Comparison of protocols for culture medium preparation before voltammetric and chromatographic analysis.

### 3.11. Analysis of culture medium from cancer cells

The utility of the herein proposed voltammetric method was demonstrated by determination of Trp and Kyn amounts in culture medium from different human cancer cell lines (MDA-MB-231 and SK-OV-3). For DPV measurements, a raw sample was directly injected into the supporting electrolyte and quantification was carried out by standard addition method. Additionally, Trp and Kyn concentration was assessed and compared by voltammetric (DPV) and chromatographic (HPLC-DAD) methods. Both sets of data are collected in Table 5.

The results have shown significant differences in Trp and Kyn levels in culture medium from breast and ovarian cancer cells. DPV analysis indicated that MDA-MB-231 culture medium contained from 58 to 69  $\mu$ M (mean:  $62 \pm 5 \mu$ M,  $n = 4$ ) of Trp, whereas Kyn amounts were below the LOD of DPV method. HPLC-DAD analysis confirmed the

results obtained for Trp (no statistically significant difference), and was able to detect small amounts of Kyn, however, at concentrations below the LOQ value. The results suggest that utilization of Trp by MDA-MB-231 cancer cells through kynurenine pathway is very low, as observed by Kyn generation at inconsiderable level. In contrast, a high Kyn generation and secretion into culture medium was observed in SK-OV-3 cell line. Ovarian cancer cells produced Kyn at amounts in the range from 31 to 58  $\mu$ M (mean:  $40 \pm 9 \mu$ M,  $n = 6$ ), while Trp was not detected in any studied samples (DPV results). Fig. 6 presents typical voltammograms obtained during Trp and Kyn determination in culture medium from breast and ovarian cancer cell lines. The Student t-test did not show statistically significant differences between Kyn concentrations quantified by DPV and HPLC-DAD methods (all  $p$  values were above 0.05). Additionally, Trp peaks were hardly visible at HPLC-DAD chromatograms (Fig. 8S) and we were not able to quantify this analyte.

## 4. Conclusion

Study of human metabolic pathways and the role of their disorders in pathogenesis in variety of human diseases is currently of great interest to scientists from around the world. Tryptophan catabolism via kynurenine pathway has been associated with promotion of the survival of the cancer cells and measurement of Kyn production is an indicator for assessment of this metabolic pathway activation. Considering an importance of analytical tools in the detection of clinically important molecular markers, we proposed simple voltammetric protocol for simultaneous Trp and Kyn monitoring in culture medium of human cancer cell lines, as an alternative to popular chromatographic tools.

The voltammetric method allows to omit a time-consuming sample preparation step by direct sample introduction into the supporting electrolyte of the voltammetric cell. Furthermore, single analysis on BiF/BDDE lasts about 1 min, while duration of chromatographic run was 45 min. The results obtained by both methods were in good agreement, confirming acceptable sensor accuracy in cell culture medium matrix. In addition, the proposed procedure displayed other advantages like significant reduction of usage of organic solvents – all experiments were carried out in aqueous solutions, and organic solvents were used only for dissolving crystalline analyte standards. Fig. 7 emphasizes the advantages of our voltammetric approach versus chromatographic one.

## Funding

This work was supported by Grant No. 2018/02/X/ST4/00187 from the National Science Centre, Poland for Ilona Sadok. The authors also gratefully acknowledge use of the facilities of the Centre of Interdisciplinary Research of the John Paul II Catholic University of Lublin founded by the European Union from European Regional Development Fund under the Operational Programme Development of Eastern Poland 2007–2013 (agreement POPW.01.03.00-06-003/09-00).

## Acknowledgements

Authors thank Agnieszka Ścibior, PhD, DSc from the Centre for Interdisciplinary Research of the John Paul II Catholic University of Lublin for sharing the culture equipment, Kamila Rachwał, PhD, from University of Life Sciences in Lublin for cell culture.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2019.120574>.

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