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Response to reviewers

Influence of boron content on electrochemical properties of boron-doped diamond electrodes and their utilization for leucovorin determination

Renáta Šelešovská, Barbora Kränková, Michaela Štěpánková, Pavlína Martinková, Lenka Janíková, Jaromíra Chýlková, Marian Vojs

Reviewer 2

It is only recommeneded to add a minor revision related to commnent 8) by Reviewer 2 (to state clearly that the speculation about higher sp2 content in the 20 000 ppm BDD is based on previous literature data).

Specifically, sentence "The slight increase of <DELTA>Ep for electrode with B/C 20 000 ppm is the most likely caused by the worse BDD film quality - higher content of sp2 hybridized carbon [17, 28]."

can be rephrased e.g., "The slight increase of <DELTA>Ep for electrode with B/C 20 000 ppm may be explained by a higher content of sp2 hybridized carbon in these BDD films, as it was observed in previous studies [17, 28]".

This sentence was rephrased according to the reviewer's requirements.

Influence of boron content on electrochemical properties of boron-doped diamond electrodes and their utilization for leucovorin determination

Renáta Šelešovská,[a*] Barbora Kränková,[a] Michaela Štěpánková,[a] Pavlína Martinková,[a] Lenka Janíková,[a] Jaromíra Chýlková,[a] Marian Vojs[b]

[a] *University of Pardubice, Faculty of Chemical Technology, Institute of Environmental and Chemical Engineering, Studentská 573, 532 10 Pardubice, Czech Republic*

[b] *Slovak University of Technology in Bratislava, Faculty of Electrical Engineering and Information Technology, Institute of Electronics and Photonics, Ilkovičova 3, 812 19 Bratislava, Slovak Republic*

* e-mail: renata.selesovska@upce.cz

Abstract

Set of the lab-made boron-doped diamond electrodes (BDDEs) prepared with various B/C ratio (1000, 2000, 4000, 8000, 10 000, and 20 000 ppm) in the gas phase was subjected to the detailed characterization in the present paper. It was proved, that the B/C ratio influenced the electrochemical features of the working electrodes like a width of the potential window, which decreased with increasing B/C. Variations of reversibility of two redox systems ($Fe(CN)_6^{3-/4-}$ and $Ru(NH_3)_6^{2+/3+}$) depending on B/C were observed as well. The best electrochemical properties were found for BDDE with B/C 10 000 ppm. Moreover, the working electrodes were tested for voltammetric analysis of leucovorin based on its oxidation. The lab-made BDDEs were applied for voltammetric analysis of the pharmaceutical preparation containing leucovorin with excellent results (recovery 97.7-103.3 %, $RSD_5 \leq$ 2.1 %).

1 Introduction

Boron-doped diamond (BDD) represents remarkable material with various technical applications [1]. Due to the development of the still most used technique for BDD preparation − chemical vapor deposition (CVD) in the eighties of the twentieth century, the possibilities of its application have grown very fast [1-3]. The first mention about the use of a diamond doped with argon and nitrogen as a working electrode appeared in 1983 [4] but the later works focused mainly on boron-doped diamond electrodes (BDDEs) and their possible electroanalytical applications, *e.g.* [5-15]. Their very specific or unique features, like wide potential window, low background current, high mechanical and chemical stability, high resistance to passivation or non-toxicity, are the main reasons, why the use of these electrodes have been so expanded in the last twenty years, *e.g.* [5-15]. It was proved, that properties of the BDDEs depends on many factors, *e.g.* (*i*) concentration and type of the dopant, (*ii*) morphological properties, (*iii*) main crystallographic orientation, (*iv*) surface termination (H, O, F, *etc.*), (*v*) grain boundaries, and (*vi*) presence of the non-diamond carbon phases [16]. The effect of B concentration on the electrochemical features of BDDE have been widely studied, *e.g.* in [17-35]. It was ascertained, that concentration of B strongly influences the conductivity of the BDD films − heavily doped films have metallic type of conductivity and the lower doped BDD could be found as semiconductive, which intensively affects electrochemical properties of BDDEs [10, 19, 22, 28, 31, 33-35]. Besides conductivity, B content and a quality of the film in general also affects width of the potential window or the background current [18, 26, 28, 32, 35]. Moreover, it was confirmed, that amount of B and

B/C, respectively, in the gas phase influences the structure of the prepared films, specifically higher level of B caused smaller formed crystals [19, 25, 27, 28, 35].
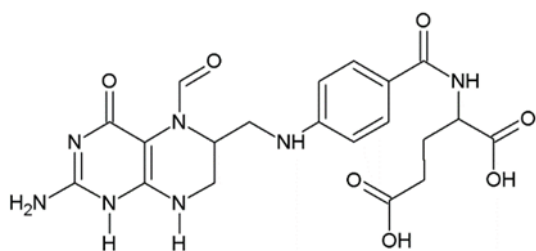
Voltammetric behavior of the particular redox systems could reveal important information about the surface of used working electrodes, *e.g.* in [7, 36-47]. Heterogeneous transfer of electron (transfer between the redox system and the electrode) can be categorized into two groups: (*i*) *outer sphere reaction*, where the electrode reaction proceeds only by mass transport and the working electrode serves as a donor or an acceptor of electrons. The electrode kinetics is not sensitive to the structure or surface of the electrode; and (*ii*) *inner sphere reaction*, which is strongly dependent on the electrode surface because it proceeds via some specific interaction with the surface [36, 37]. The most often employed redox system, typical representative of the inner sphere reactions, whose behavior has been widely studied on BDDEs is $Fe(CN)_6^{3-/4-}$ [7, 30, 31, 35, 37-39, 41-43]. Besides $Fe(CN)_6^{3-/4-}$, voltammetric behavior of aquated ions ($Fe^{3+/2+}$, $Ce^{4+/3+}$ or $Eu^{2+/3+}$), which proceed also through inner sphere reaction mechanism pathway, has been investigated on BDDEs as well [37, 42-45]. The redox probes, which typically provide outer sphere reaction and have been investigated on BDDEs are particularly $Ru(NH_3)_6^{2+/3+}$ and $IrCl_6^{2-/3-}$ [35, 37-41, 47].

Much less publications have been so far focused on the electroanalytical features of BDDEs with different boron content and their influence of analysis of specific electroactive compounds like nitrates [18, 23], benzophenone-3 [31], linuron [32], 4-chloro-3-methylphenol [33], 5-nitroquinoline [34], and 2-aminobiphenyl [35].

Leucovorin (LV, scheme 1) is a reduced derivative of folic acid [48]. It is administrated with methotrexate to reduce its negative side effects [49-51] or in combination with 5-fluorouracil to enhance its effectiveness during treatment of various serious diseases [52, 53]. It was chosen as a representative electroactive compound, because its voltammetric behavior has been already described only on mercury [54-56] and silver solid amalgam electrodes [56].

Considering the used working electrodes, the papers were focused on recording of LV reduction and thus interested in cathodic signals [54-56]. Our proposed voltammetric methods based on application of silver solid amalgam electrodes provided low detection limits specifically 2.2×10^{-8} and 5.0×10^{-8} mol L^{-1} for mercury meniscus modified (m-AgSAE) and polished silver solid amalgam electrode (p-AgSAE), respectively [56]. The obtained results were compared also with outputs from measuring with hanging mercury drop electrode (HMDE), which provided significantly higher limit of detection (9.8×10^{-7} mol L^{-1}) [56].

The present paper deals with characterization of the electrochemical features of BDDEs prepared under different conditions particularly with different B/C in the gas phase. Moreover, their applicability as an analytical tool for determination of the chemoprotectant drug LV based on its electrochemical oxidation is examined as well.

Scheme 1. Chemical structure of leucovorin.

2 Experimental

2.1 Chemicals

All of the used chemicals were of analytical grade purity and were obtained from Sigma Aldrich, if not stated otherwise. Standard solution of 0.001 mol L^{-1} leucovorin was prepared by simple dissolution of its calcium salt powder in the distilled water. It was stored in a refrigerator. Solutions of lower concentration were prepared by dilution with distilled water or the supporting electrolyte directly before analysis.

Britton-Robinson buffer (BRB) consisting of the mixture of acidic (H_3PO_4, H_3BO_3, and CH_3COOH – all three of the same concentration 0.4 mol L^{-1}) and alkaline component (0.2 mol L^{-1} NaOH from Lachema, Czech Republic) was used as a supporting electrolyte. 0.5 and 0.05 mol L^{-1} H_2SO_4 was prepared from 96 % H_2SO_4 (Penta-Švec, Czech Republic). 0.1 mol L^{-1} KCl was prepared by dissolution of its powder (Penta-Švec, Czech Republic) in the distilled water. Standard solutions of 2.5×10^{-3} mol L^{-1} $K_3[Fe(CN_6)]$ and $[Ru(NH3)_6]Cl_3$ (both of purity ≤ 99 %) were prepared in the solution of 0.1 mol L^{-1} KCl.

"Leucovorin Ca LACHEMA 10" for preparation of the injection solution originated from Pliva-Lachema, Czech Republic.

2.2 Instrumentation

Voltammetric measurements were carried out by computer controlled analyzer Eco-Tribo Polarograph (Polaro-Sensors, Czech Republic) and employing software POLAR.PRO version 5.1. The measuring system consisted of three electrodes – BDDE as a working electrode, Ag│AgCl│KCl (sat.) as a reference (Monokrystaly, Czech Republic), and platinum wire (Monokrystaly, Czech Republic) as an auxiliary electrode. As a working BDD electrode, we have used the commercially available one with working surface of 7.07 mm^2 and B/C ratio in the gas phase of 1000 ppm as declared by the producer Windsor Scientific (UK) or one of the laboratory prepared electrodes (Slovak University of Technology in Bratislava, Faculty of Electrical Engineering and Information Technology, Institute of Electronics and Photonics) with working surface of 0.43 mm^2 and B/C ratio in gas phase of 1000, 2000, 4000, 8000, 10 000, and 20 000 ppm. All measurements were carried out at laboratory temperature of 23±2 °C. Values of pH were measured by pH-meter Accumet AB150 (Fisher Scientific, Czech Republic).

All graphical dependences were constructed using Excel software (Microsoft, USA). Parameters of calibration curves and confidence intervals were calculated using OriginPro 9

software (OriginLab Corporation, USA) on the level of significance 0.05. Statistical parameters like limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration dependences as three times and ten times, respectively, of standard deviation of intercept divided by the slope.

2.3 Procedures

2.3.1 Electrochemical characterization of BDDEs

Preparation and surface characterization of the lab-made BDD electrodes is described in our previous paper [57]. All of the used working BDD electrodes (the lab-made and the commercial one as well) were activated and regenerated employing the same procedure. The chosen electrode underwent activation in 0.5 mol L^{-1} H_2SO_4 by applying negative (cathodic) potential ($E_c = -2000$ mV) for 60 s followed by insertion of positive (anodic) potential ($E_a = +2000$ mV) of the same duration. Then, 20 cyclic voltammograms between -1000 mV and $+2000$ mV were recorded to ensure stabilization of the activated electrode. After this step, the working electrode was ready for analysis. Besides the activation, the regeneration step, which consisted of the regeneration potential ($E_{reg} = +2000$ mV) applied for the regeneration time ($t_{reg} = 5$ s), was inserted between every scan. This process ensured the O-terminated surface and oxidation of eventually present impurities on the BDD electrode working surface.

For electrochemical characterization of BDD films cyclic voltammetry (CV) of redox systems of $[Fe(CN_6)]^{3-/4-}$ and $[Ru(NH_3)_6]^{3+/2+}$ was measured under the following parameters: scan rate (v) of 100 mV s^{-1}, potential range from -1600 mV to $+1800$ mV. CV of the above mentioned redox systems was used also for the measurements of dependences on v (10, 20, 50, 100, 200, 300, 400 and 500 mV s^{-1}).

2.3.2 Voltammetric determination of leucovorin

CV was applied for the investigation of the voltammetric behavior of LV depending on supporting electrolyte pH and scan rate (v). Measurements were carried out from initial potential (E_{in} = −1000 mV) to the switching potential (E_{switch} = +2000 mV) and back to −1000 mV employing the scan rate of 100 mV s^{-1}. In case of the scan rate study, the value of v varied from 25 to 500 mV s^{-1}. Based on our previous experiences, differential pulse voltammetry (DPV) and BRB of pH 3 was chosen for determination of LV. The optimized DPV parameters, which were used for all subsequent analysis, are as follows: E_{in} = −700 mV, final potential − E_{fin} = +1800 mV, E_{reg} = +2000 mV, t_{reg} = 5 s, v = 40 mV s^{-1}, pulse width 40 ms, sampling time 20 ms, pulse height 50 mV. The method was previously proposed and published for the commercial BDD electrode [58] and subsequently utilized in the present study for the lab-made working electrodes. Baseline for current signals evaluation was added as a tangent to the curve at minimum before and after the peak.

2.3.3 Analysis of pharmaceutical sample

The sample "Leucovorin Ca LACHEMA 10" was prepared for analysis by the producer declaration: a content of the phial was dissolved in 1 mL of distilled water. The prepared solution of the calculated concentration of 1.955×10^{-2} mol L^{-1} was ten times diluted. 10 µL of the diluted solution was added to the polarographic cell with 10 mL of the supporting electrolyte (BRB pH 3) and the determination was carried out by the standard addition method and two standard additions (20 µL of 1×10^{-3} mol L^{-1} LV) were applied at least. The determination was five times repeated and relative standard deviation of repeated determination (RSD) was calculated.
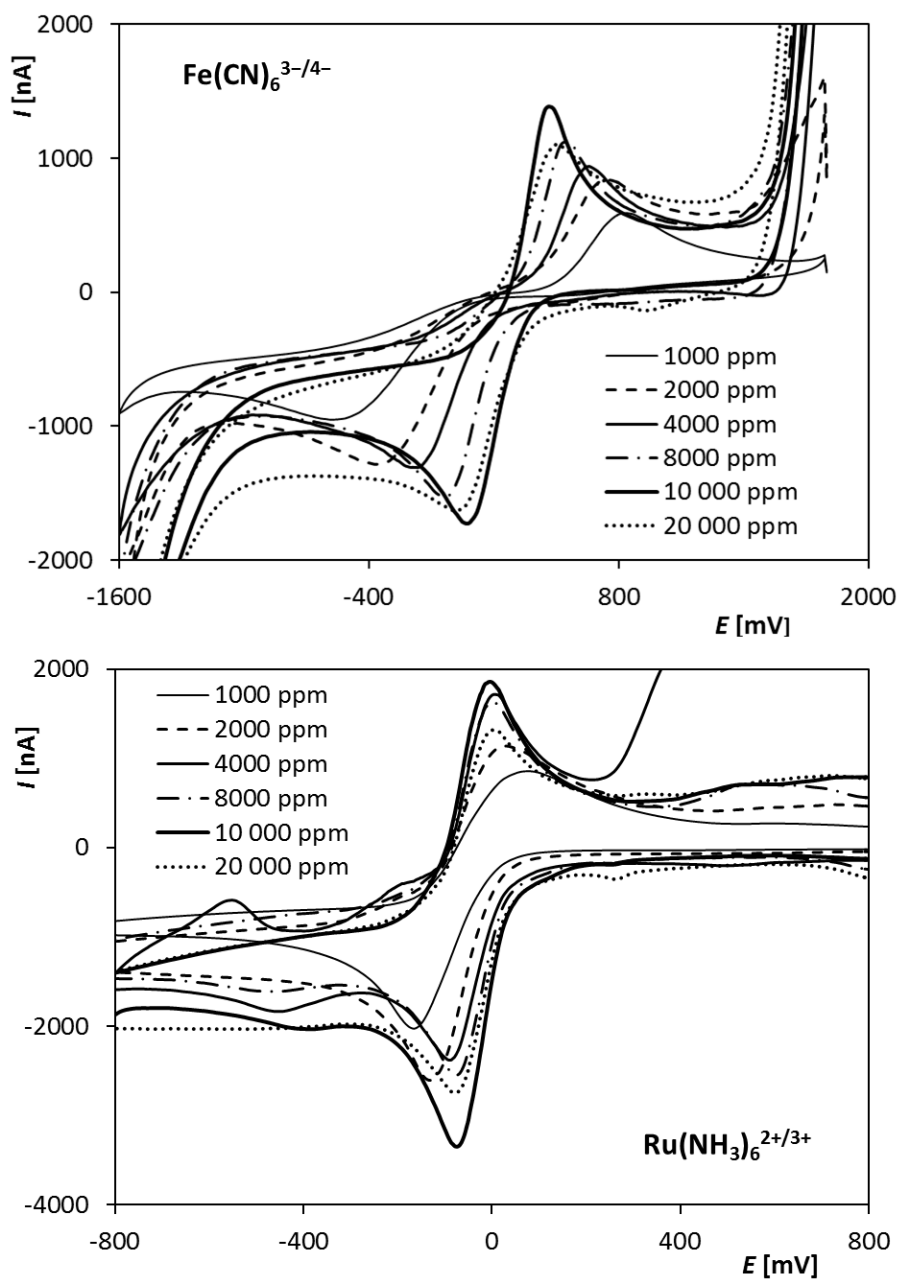
3 Results and discussion

3.1. Electrochemical characterization of BDDEs using cyclic voltammetry

Two redox systems, which are commonly applied for characterization of the working electrodes, namely $[Fe(CN)_6]^{3-/4-}$ and $[Ru(NH_3)_6]^{3+/4+}$, were employed for our experiments. $[Ru(NH_3)_6]^{3+/4+}$ is typical representative of redox systems provided outer sphere reactions [35, 37-41, 47]. On the other hand, it was proved, that $[Fe(CN)_6]^{3-/4-}$ ensured inner sphere reaction on BDD electrodes, discussed more in detail *e.g.* in [7, 30, 31, 35, 37-39, 41-43]. CV was utilized as a measuring method and various parameters like width of the potential window, reversibility of the electrode reaction, the electrochemically active surface, and the apparent electron transfer rate constant (k^0_{app}), respectively, were calculated.

The first necessary step of the characterization of the lab-made BDDEs was recording of the width of the potential window. $0.1\,mol\,L^{-1}$ KCl was employed as a supporting electrolyte. The border potential on the anodic and cathodic side as well was the value, when the current reached ±1500 nA. The increasing B/C ratio in the gas phase caused narrowing of the potential window. Moreover, the narrowing was more significant in the cathodic region, which is consistent with literature [18, 25, 28, 35, 57]. Particularly, the width decreased of about 900 mV comparing BDDE 1000 ppm and 20 000 ppm and the widest potential window, namely 3660 mV, was observed for BDDE with B/C 1000 ppm. These results are summarized in TAB S1 in Supporting materials. The potential window recorded on the commercial BDDE in the same medium was about 2800 mV [58].

Reversibility of the used redox markers was examined next. The obtained cyclic voltammograms are depicted in Figure 1. Considering reversibility, it is apparent that this parameter grown with increasing boron content, which is obvious from the increasing of the particular signals and decreasing of their potential differences. Particular values of the anodic and cathodic peak heights (I_{pa} and I_{pc}), their ratio (I_{pa}/I_{pc}), and values of the anodic and cathodic peak potential (E_{pa} and E_{pc}) and their difference (ΔE_p) are summarized in Table 1 and displayed in the inset of Figure 1.

Figure 1 Cyclic voltammograms of the studied redox systems recorded on the lab-made BDDEs. Method – CV, $E_{in} = E_{fin} = -1600$ mV, $E_{switch} = +1800$ mV, $v = 100$ mV s^{-1}; supporting electrolyte – 0.1 mol L^{-1} KCl; $c([Fe(CN)_6]^{3-/4-}) = c([Ru(NH3)_6]^{3+/4+}) = 2.5 \times 10^{-3}$ mol L^{-1}.

Table 1 Basic parameters of cyclic voltammograms measured on all tested BDDEs for redox systems $Fe(CN)_6^{3-/4-}$ and $Ru(NH_3)_6^{2+/3+}$ in 0.1 mol L^{-1} KCl.

B/C [ppm]	I_{pa} [nA]	I_{pc} [nA]	I_{pa}/I_{pc}	E_{pa} [mV]	E_{pc} [mV]	ΔE_p [mV]
			$Fe(CN)_6^{3-/4-}$			
1000	+500	−507	0.98	+829	−577	1406
2000	+510	−576	0.88	+720	−355	1075
4000	+672	−767	0.88	+659	−163	822
8000	+849	−947	0.89	+550	−24	574
10 000	+1039	−1053	0.98	+545	+72	473
20 000	+694	−710	0.98	+503	+12	491
			$Ru(NH_3)_6^{2+/3+}$			
1000	+1161	−1268	0.92	+71	−167	238
2000	+1226	−1389	0.88	+26	−132	158
4000	+1283	−1407	0.91	+26	−91	117
8000	+1400	−1530	0.92	+4	−71	75
10 000	+1560	−1612	0.97	−10	−71	61
20 000	+972	−1128	0.86	+8	−77	85

The parameter I_{pa}/I_{pc} ranged from 0.86 to 0.98 for both redox markers. The highest values limiting to the theoretical value 1 confirming reversibility of the electrode reaction were found for BDDE with the highest B/C (10 000 ppm for both redox systems and 20 000 ppm for $Fe(CN)_6^{3-/4-}$) and surprisingly for BDDE with B/C 1000 ppm. On the other hand the potential difference, which is the most important criteria for assessment of the reversibility, was especially for the system $Fe(CN)_6^{3-/4-}$ enormous (ranged from 1406 to
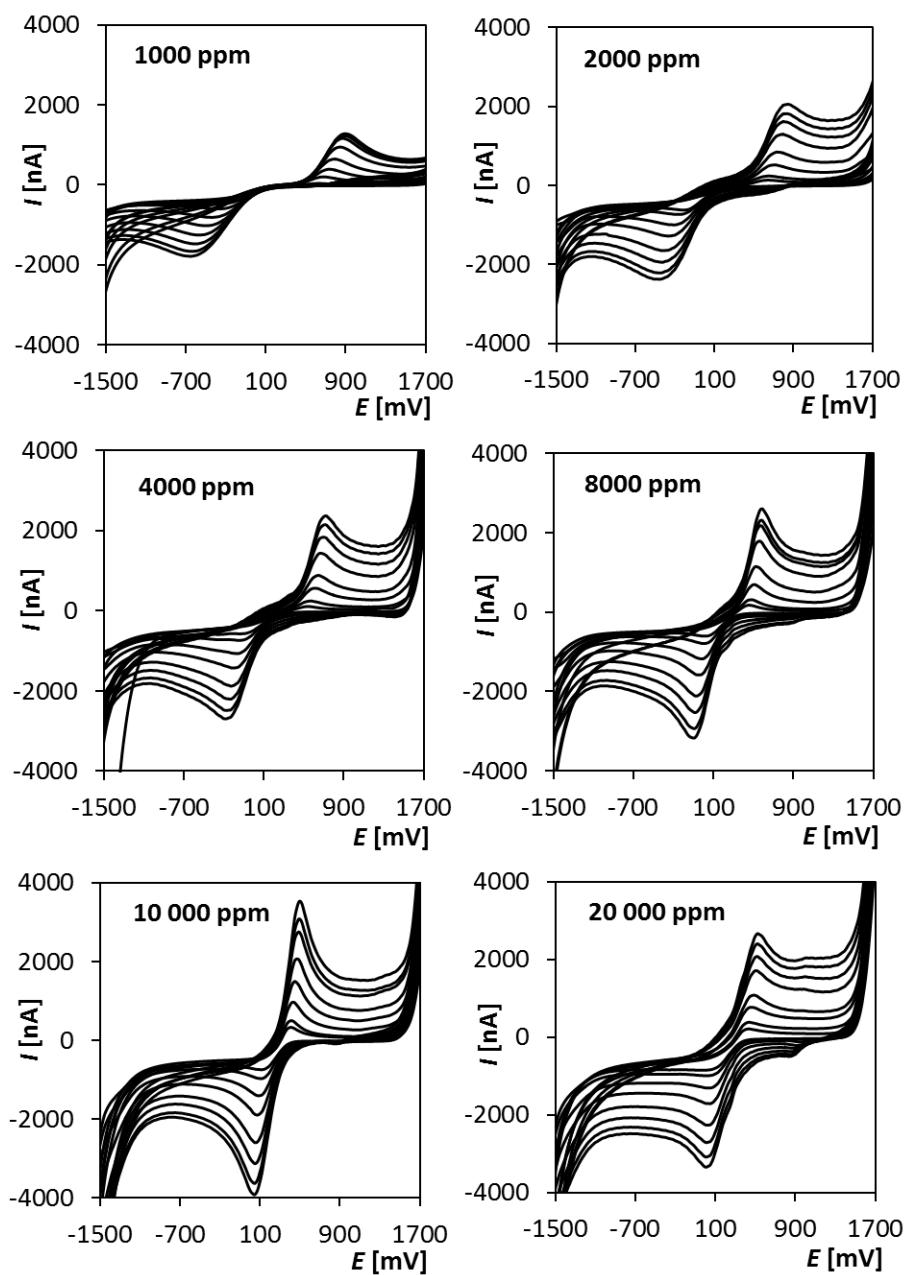
491 mV) and did not limit to the theoretical value of 59 mV. Considering a decreasing trend of the potential difference, it was again confirmed, that the reversibility increased with the boron content and the best result, namely the lowest value of ΔE_p, was obtained for BDDE with B/C 10 000 ppm. Moreover, ΔE_p defined for this electrode and $Ru(NH_3)_6^{2+/3+}$ limiting to the theoretical value 59 mV, which is typical for the one-electron reversible electrode reaction. The slight increase of ΔE_p for electrode with B/C 20 000 ppm may be explained by a higher content of sp^2 hybridized carbon in these BDD films, as it was observed in previous studies [17, 28]. The results of ΔE_p analysis obtained for the lab-made working electrodes were consistent with those calculated for the commercial BDDE, particularly 552 mV for $Fe(CN)_6^{3-/4-}$ and 67 mV for $Ru(NH_3)_6^{2+/3+}$ [58]. The lower values of ΔE_p for the redox probe $Ru(NH_3)_6^{2+/3+}$ could be explained by the nature of the electrode reaction. The outer sphere reaction pathway and the electron transfer as well is in general not influenced by the physicochemical properties of working surface of the electrode [59].

Electroactive area of the working electrodes (A) was calculated utilizing Randles-Ševčík equation [60-62] for the scan rate of 100 mV s^{-1} and the representative of the inner sphere reaction pathway − $Fe(CN)_6^{3-/4-}$. The outcomes are summarized in Table 2. It is evident that the active surface increased with the boron content and the largest area was calculated for BDDE with B/C 10 000 ppm. It could be assumed, that the higher active surface is related with the smaller formed crystals, in agreement with [19, 25, 27, 28, 35]. The significant reduction was observed for films with higher B/C ratio, which could be again caused by the worsen quality of the film. These results were also consistent with literature [19, 25, 27, 28, 35].
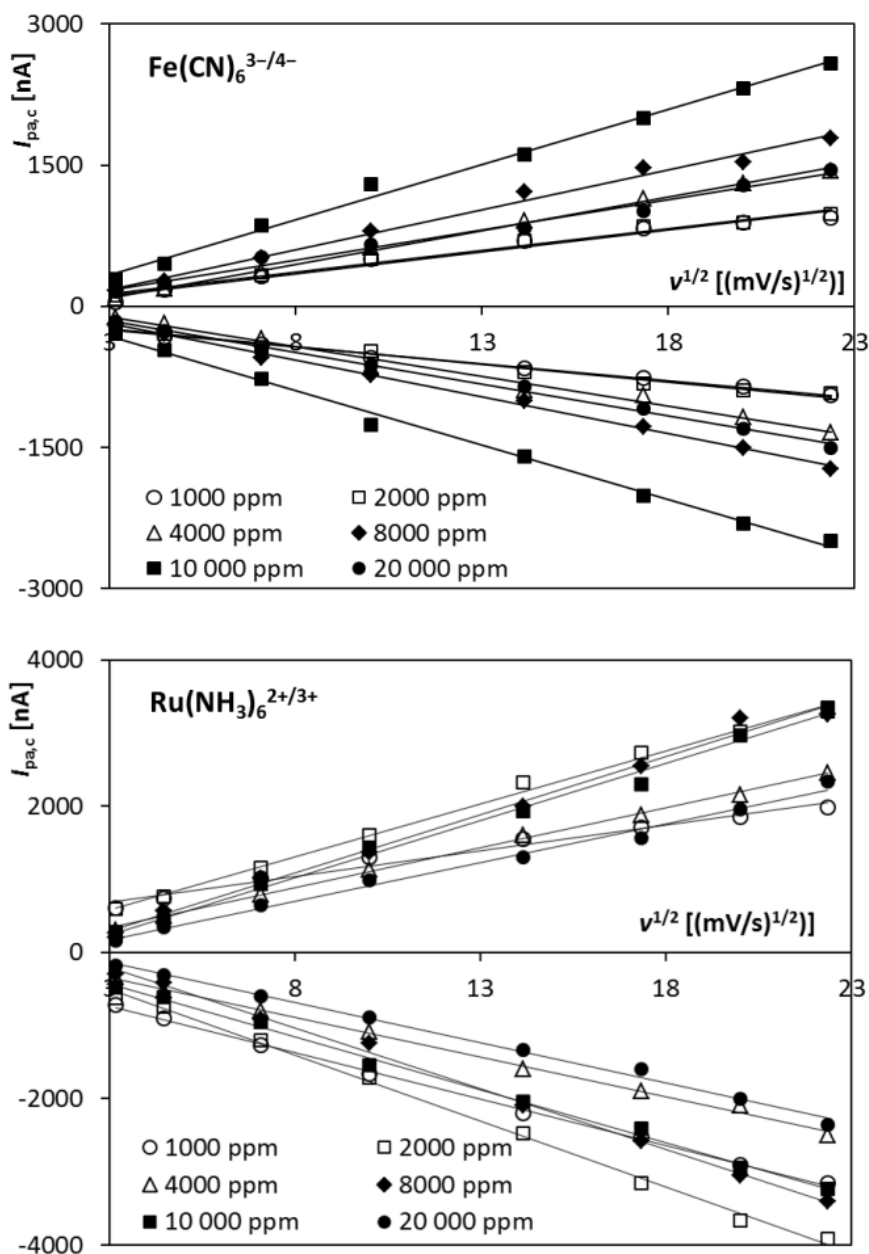
Table 2 Calculated values of the electrochemical active surfaces and the apparent heterogeneous electron-transfer rate constants for all tested BDDEs.

B/C	A(Fe(CN)$_6^{3-/4-}$)	k^0_{app}(Fe(CN)$_6^{3-/4-}$)	k^0_{app}(Ru(NH$_3$)$_6^{2+/3+}$)
[ppm]	[mm^2]	[cm s^{-1}]	[cm s^{-1}]
1000	0.25	1.8×10^{-9}	5.2×10^{-4}
2000	0.26	1.2×10^{-8}	2.7×10^{-3}
4000	0.30	7.0×10^{-7}	5.5×10^{-3}
8000	0.36	9.0×10^{-6}	1.3×10^{-2}
10 000	0.60	1.1×10^{-4}	9.7×10^{-2}
20 000	0.30	5.0×10^{-5}	7.7×10^{-3}

The influence of the scan rate was examined next. This parameter varied from 10 to 500 mV s^{-1} and obtained cyclic voltammograms for Fe(CN)$_6^{3-/4-}$ and all of the tested lab-made electrodes are depicted in Figure 2. Very similar outcomes were obtained for the second redox system as well. It is evident from Figure 2, that anodic and cathodic signal increased with the scan rate but the dependence was non-linear. On the other hand, the linearity was observed for the dependence between peak height and the square root of the scan rate ($v^{1/2}$), which is illustrated by Figure 3 and TAB S2 and S3 in supplementary materials containing parameters of the particular equations (slopes, intercepts, and correlation coefficients). This result is typical for the diffusion controlled electrode reaction, which is common for BDDEs due to their low ability to adsorb analytes on the working surface. The slopes (TAB S2 and S3) of the dependencies increased with the boron content and the maximum value was obtained for BDDE with B/C 10 000 ppm (Fe(CN)$_6^{3-/4-}$) and 8000 ppm (Ru(NH$_3$)$_6^{2+/3+}$), respectively. The diffusion-controlled pathway of the electrode reaction was further confirmed by the log(I_p)-log(v) analysis, particularly by the slopes (TAB S4 and S5) of these linear dependencies. They equalled or limited to the theoretical value 0.5, which is typical for the diffusion controlled reaction.

Figure 2 Cyclic voltammograms of $Fe(CN)_6^{3-/4-}$ recorded on the lab-made BDD electrodes at various scan rates. Method – CV, $E_{in} = E_{fin} = -1600$ mV, $E_{switch} = +1800$ mV, $v = 10, 20, 50, 100, 200, 300, 400,$ and 500 mV s^{-1}; supporting electrolyte – 0.1 mol L^{-1} KCl; $c([Fe(CN)_6]^{3-/4-}) = 2.5 \times 10^{-3}$ mol L^{-1}.

Figure 3 Dependence of the peak heights provided by $Fe(CN)_6^{3-/4-}$ and $Ru(NH_3)_6^{2+/3+}$, respectively, on the square root of scan rate recorded on the lab-made BDD electrodes at various scan rates. Method – CV, $E_{in} = E_{fin} = -1600$ mV, $E_{switch} = +1800$ mV, $v = 10, 20, 50, 100, 200, 300, 400,$ and 500 mV s^{-1}; supporting electrolyte – 0.1 mol L^{-1} KCl; $c([Fe(CN)_6]^{3-/4-}) = c([Ru(NH_3)_6]^{3+/4+}) = 2.5 \times 10^{-3}$ mol L^{-1}.

The last experiments in the electrochemical characterization was examination of the influence of scan rate and square root of the scan rate, respectively, on the anodic and
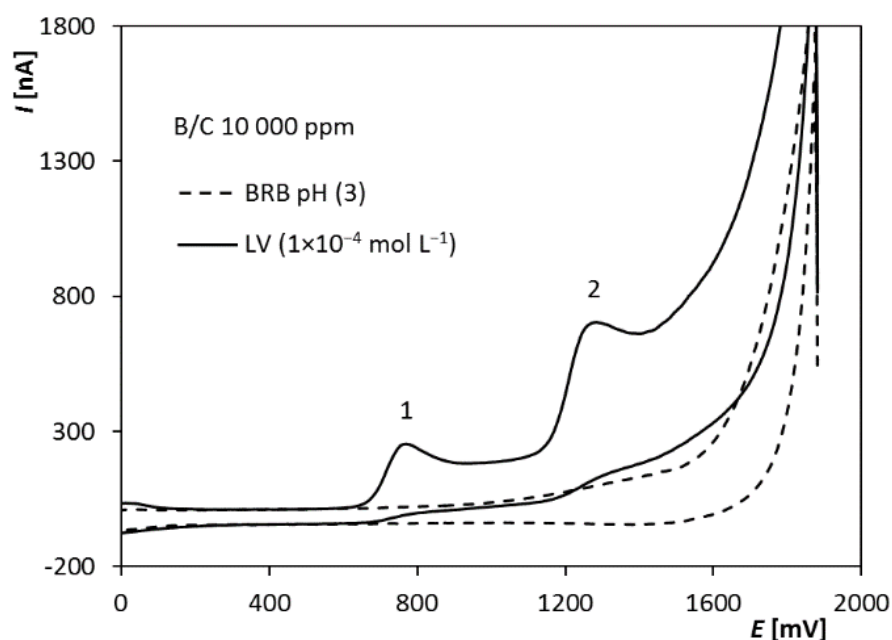
cathodic peak potential difference and calculation of the apparent heterogeneous electron transfer rate constants (k^0_{app}) employing the Nicholson approach [63]. It was found out, that the most differentiated signals were recorded on BDDE with B/C 1000 ppm and the difference decreased with increasing B/C. The lowest values were determined for BDDE with the highest B/C. Values of k^0_{app} were calculated for $v = 100$ mV s^{-1}. Values of Ψ (TAB S6) were directly subtracted from the graphical dependence in the article [63] for the redox system $[Ru(NH3)_6]^{3+/4+}$ and the calculated values of the apparent scan rate constant ranged from 5.2×10^{-4} (1000 ppm) to 1.3×10^{-2} cm s^{-1} (8000 ppm). It is obvious (Table 2), that k^0_{app} increased with growing B/C in the gas phase, which corresponded with the literature [35]. High values of ΔE_p recorded for the second redox system ($[Fe(CN)_6]^{3-/4-}$) corresponded with low Ψ the particular, which were not included by Nicholson in the reference [63]. Thus, we created linear extrapolation of the dependence (based on the outcomes in [63]), which could be described by equation (1). Values of Ψ for $[Fe(CN)_6]^{3-/4-}$ are summarized in TAB S7. Values of k^0_{app} (Table 2) ranged from 1.8×10^{-9} (1000 ppm) to 1.1×10^{-4} cm s^{-1} (10 000 ppm) and the similar behavior of k^0_{app} depending B/C in the gas phase as in the case of $[Ru(NH_3)_6]^{3+/4+}$ was observed – the value of the apparent rate constant increased with growing B/C. It could be concluded, that higher k^0_{app} indicated faster heterogeneous electron transfer and confirmed favorable electrochemical properties of the working electrodes. Thus, the electrodes of the best electrochemical features could be assumed for BDDEs with higher B/C in the gas phase namely 10 000 ppm.

$$\Psi = 10^{-0.0056048(\Delta E \times n) + 0.1882152}$$
(1)

3.2. Voltammetric determination of leucovorin using BDDEs with different boron content

The target of this work, besides the electrochemical characterization of the lab-made BDDEs, was examination and assessment of their analytical utilization for LV determination. LV was chosen as a model analyte because its electrochemical behavior is well-described only at mercury or amalgam working electrodes [54-56] and only our previous work focused on development of voltammetric method for LV oxidation on the commercial BDDE [58].

Cyclic voltammograms in absence and presence of 1×10^{-4} mol L^{-1} LV between potentials -1000 mV and $+1800$ mV were recorded in BRB of pH 3 on BDDE with B/C 10 000 ppm (Figure 4), which was chosen based on the results discussed above. Two well-developed oxidation signals, as in case of commercial BDDE [58] were recorded. The first signal was chosen for all subsequent analysis due to its well-developed shape, easy evaluation, and favorable position.

Figure 4 Cyclic voltammograms in absence and presence of LV recorded on the lab-made BDDE with B/C 10 000 ppm. Method – CV, $E_{in} = E_{fin} = -1000$ mV, $E_{switch} = +1800$ mV, $v = 100$ mV s^{-1}; supporting electrolyte – BRB (pH 3); $c(LV) = 1\times10^{-4}$ mol L^{-1}.
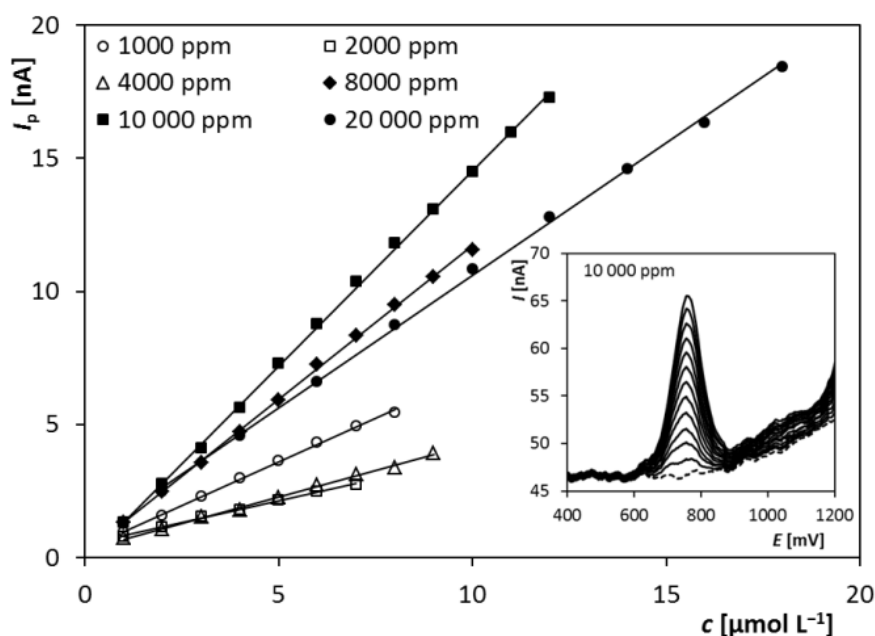
3.2.1. Analysis of model solutions

At the beginning, DP voltammograms of 1×10^{-6} mol L^{-1} LV were recorded on all tested lab-made BDDEs and the peak height were compared (FIG S1). The highest current responses were obtained on electrodes with higher B/C, namely 10 000 and 20 000 ppm, which corresponded to the previously described results and with literature dealing with analysis of organic compounds on BDDE prepared under different B/C in the gas phase [31, 33-35]. It could be concluded, that electrode with B/C 10 000 ppm provided comparable or even better electrochemical properties than BDDE with B/C 20 000 ppm and increasing of the B/C did not lead to improving of the electrode`s features.

Similar concentration dependencies on 10^{-6} mol L^{-1} level of LV were recorded on all of the tested electrodes. Example of the obtained curves of LV ranged from 1×10^{-6} to 1.2×10^{-5} mol L^{-1} recorded on BDDE with B/C 10 000 ppm is illustrated by the inset of Figure 5. It is evident from Figure 5 that the peak height increased linearly with LV concentration in the analyzed solution particularly. Parameters of the straight lines (slopes, intercepts, correlation coefficients (R)) are summarized in Table 3. The highest sensitivity could be supposed, based on the highest slope, for BDDE with B/C 10 000 ppm, which again confirmed our previous results. Moreover, peak of LV increased linearly with its concentration in the widest range employing this working electrode. On the other hand, sensitivity of the electrodes with lower B/C (1000 – 4000 ppm) is comparable and relatively low.

Table 3 Statistical parameters of concentration dependences of LV obtained with all particular BDDEs.

B/C [ppm]	Slope [nA L μmol^{-1}]	Intercept [nA]	r	LDR [mol L^{-1}]	LOD [mol L^{-1}]	LOQ [mol L^{-1}]	RSD$_{11}$ [%]
1000	(0.653±0.010)	(0.339±0.051)	0.9992	2.5×10^{-7}-2.5×10^{-5}	6.7×10^{-8}	2.2×10^{-7}	2.0
2000	(0.3267±0.0088)	(0.499±0.039)	0.9982	1.0×10^{-6}-3.0×10^{-5}	3.6×10^{-7}	1.2×10^{-6}	1.7
4000	(0.3951±0.0065)	(0.312±0.036)	0.9984	5.0×10^{-7}-3.0×10^{-5}	1.2×10^{-7}	3.7×10^{-7}	1.9
8000	(1.1545±0.0098)	(0.176±0.061)	0.9997	5.0×10^{-7}-3.5×10^{-5}	9.0×10^{-8}	3.0×10^{-7}	3.4
10 000	(1.466±0.012)	(0.114±0.085)	0.9997	5.0×10^{-7}-5.0×10^{-5}	1.0×10^{-7}	3.4×10^{-7}	1.1
20 000	(0.983±0.011)	(0.74±0.14)	0.9995	2.0×10^{-6}-4.0×10^{-5}	4.2×10^{-7}	1.4×10^{-6}	0.8

Figure 5 Linear concentration dependencies obtained on the lab-made BDDE with B/C 1000 – 20 000 ppm. Inset: Voltammetric curves of LV recorded on BDDE with B/C 10 000 ppm. Method – DPV, E_{in} = −700 mV, E_{fin} = +1800 mV, v = 40 mV s^{-1}; supporting electrolyte – BRB (pH 3); c(LV) = 1×10^{-6} – 8×10^{-6} mol L^{-1} (1000 ppm), 1×10^{-6} – 7×10^{-6} mol L^{-1} (2000 ppm), 1×10^{-6} – 9×10^{-6} mol L^{-1} (4000 ppm), 1×10^{-6} – 1×10^{-5} mol L^{-1} (8000 ppm), 1×10^{-6} – 1.2×10^{-5} mol L^{-1} (10 000 ppm), and 2×10^{-6} – 1.8×10^{-5} mol L^{-1} (20 000 ppm).

Statistical parameters like linear dynamic range (LDR), limit of detection (LOD), limit of quantification (LOQ), and relative standard deviation of 11 repeated measurements (RSD$_{11}$) were calculated next and could be found in Table 3. LDR is a concentration range in which the peak height linearly increased with concentration of the analyte. The widest LDR was again recorded on BDDE with B/C 10 000 ppm and very similar extent were measured on BDDE with B/C 4000 and 8000 ppm as well. The remaining electrodes provided narrower LDR. Moreover due to the poorly evaluable signal, which was mainly caused by the high background and the low ratio of the signal to noise, the lowest concentration, which could be reliably detected on BDDE with B/C 20 000 ppm, was 4 times higher than on BDDE with B/C 10 000 ppm. The lowest detection and quantification limits, respectively, were surprisingly calculated for BDDE with B/C 1000 ppm and expectably for BDDE with B/C 8000 and 10 000 ppm as well. The outcomes calculated for BDDE with B/C 1000 ppm could be explained by low background, high ratio signal to noise, and high linearity ($r = 1.000$) of the dependence employed for calculation of the limits, respectively.

Repeatability of the measurements was studied next. 11 repeated voltammograms of 1×10^{-5} mol L^{-1} were recorded using all of the tested working electrodes and RSD$_{11}$ was calculated from the obtained peak heights. All of the values are ≤3.4 %, which confirmed stable and repeatable response recorded on all of the electrodes.

Obtained LODs were lower than that obtained for analysis of LV on HMDE (9.8×10^{-7} mol L^{-1}) and comparable or about maximum one order of magnitude higher than those (2.2×10^{-8} and 5.0×10^{-8} mol L^{-1}) received for analysis with AgSAE [56]. In contrast with amalgam and mercury working electrode, where LV was accumulated on the electrode surface for 60 s (m-AgSAE), 100 s (p-AgSAE), and 40 s (HMDE), respectively, any accumulation time was not applied for analysis with BDDE. Therefore, the analysis was faster but the sensitivity was sufficient.

3.2.2. Analysis of leucovorin model solutions

Repeatability of the LV determination was studied using two model solutions for all of the examined electrodes. The standard addition method was employed for this experiment and 2 standard additions were added at least. Every determination was 5 times repeated and relative standard deviation of 5 repeated determinations (RSD_5), average value with particular confidence intervals ($\alpha = 0.05$), and recovery was calculated for each electrode (Table 4). It is obvious, that all of the obtained results were correct and repeatable ($RSD_5 \leq 3.8\%$). Therefore, all tested electrodes can be found suitable for the determination of LV based on its electrochemical oxidation when the analyte concentration ranges within the LDR obtained for particular electrodes. However, based on the above mentioned results (Table 3) BDDEs with higher boron content provide better statistical parameters including lower LOQ and wider LDR and thus allow lower LV concentrations to be determined.

Table 4 Results of the repeated determination of LV in model solutions using the lab-made BDDEs

B/C [ppm]	Added [mol L^{-1}]	Found[*] [mol L^{-1}]	Recovery [%]	RSD$_5$ [%]
1000	3.0×10^{-6}	$(3.020\pm0.030)\times10^{-6}$	99.0-101.3	1.5
	1.0×10^{-6}	$(1.010\pm0.012)\times10^{-6}$	99.5-104.0	1.8
2000	3.0×10^{-6}	$(3.040\pm0.043)\times10^{-6}$	98.3-103.6	2.1
	1.0×10^{-6}	$(1.020\pm0.024)\times10^{-6}$	97.0-105.0	3.5
4000	3.0×10^{-6}	$(3.020\pm0.043)\times10^{-6}$	98.3-104.0	2.2
	1.0×10^{-6}	$(1.020\pm0.025)\times10^{-6}$	96.5-107.0	3.7
8000	3.0×10^{-6}	$(3.030\pm0.031)\times10^{-6}$	99.3-103.3	1.5
	1.0×10^{-6}	$(1.010\pm0.025)\times10^{-6}$	95.5-106.0	3.8

10 000	3.0×10^{-6}	$(3.020\pm0.022)\times10^{-6}$	98.6-101.3	1.1
	1.0×10^{-6}	$(1.010\pm0.011)\times10^{-6}$	98.0-102.0	1.7
20 000	3.0×10^{-6}	$(3.010\pm0.039)\times10^{-6}$	97.3-102.3	1.9
	2.0×10^{-6}	$(2.000\pm0.018)\times10^{-6}$	98.5-101.0	1.4

*The number of valid digits is used accordance to the literature [64].

3.2.3. Analysis of pharmaceutical preparation

Applicability of the tested BDDEs with different boron content was verified by analysis of the pharmaceutical preparation "Leucovorin Ca LACHEMA 10". Standard addition method was employed again. The determinations were 5 times repeated and values of RSD_5 were calculated (Table 5). Example of the obtained voltammograms recorded using BDDE with 10 000 ppm and the graphical evaluation of the LV amount, respectively, are depicted in Figure 6.
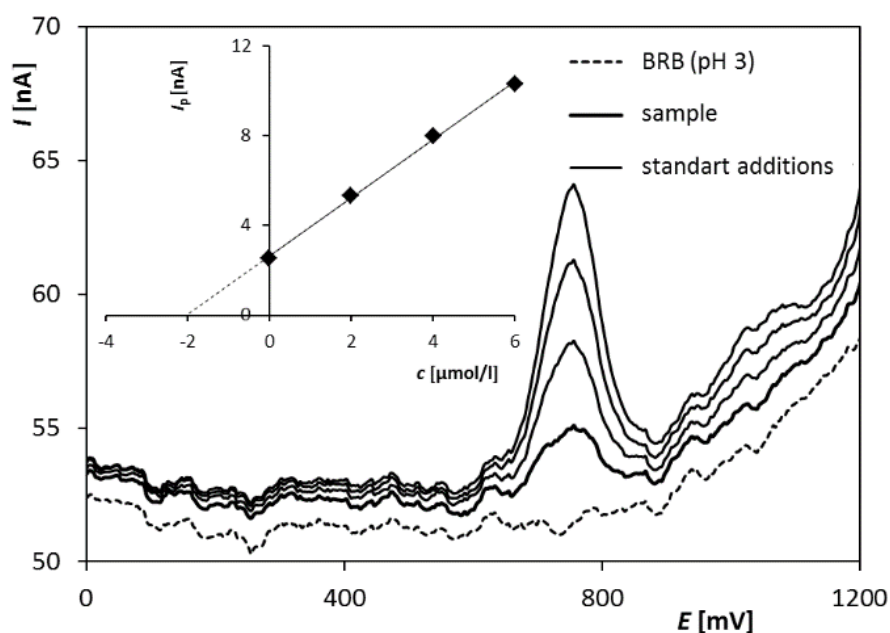
All of the tested electrodes provided accurate, correct, and repeatable results of the determination. The same variations of the peak height, background, and ratio of signal to noise, respectively, were observed as in case of model solutions analysis mentioned above. Considering the matrix of the sample, which was relatively simple (presence of no other electroactive compounds or interfering compounds), any negative effect caused by the low doping level was not observed and determination itself was not negatively influenced at al. Therefore, it could be concluded, that our proposed method in combination with all of the tested working BDDES is suitable for determination of LV in pharmaceutical samples.

Table 5 Results of the repeated determination of LV in pharmaceutical preparation "Leucovorin Ca LACHEMA 10".

B/C	Declared	Found*	Recovery	RSD_5

[ppm]	[mg]	[mg]	[%]	[%]
1000	10	(10.030±0.080)	98.2-100.8	1.2
2000	10	(10.09±0.12)	98.7-102.8	1.8
4000	10	(10.08±0.13)	98.2-102.8	2.0
8000	10	(10.03±0.10)	98.7-102.9	1.6
10 000	10	(10.03±0.14)	97.7-103.3	2.1
20 000	10	(10.041±0.099)	99.2-102.3	1.4

[*] The number of valid digits is used accordance to the literature [64].

Figure 6 Determination of LV in the pharmaceutical preparation "Leucovorin Ca LACHEMA 10" using BDDE with B/C in the gas phase 10 000 ppm. Inset: Graphical evaluation of the LV concentration. Method – DPV, E_{in} = −700 mV, E_{fin} = +1800 mV, v = 40 mV s^{-1}; supporting electrolyte – BRB (pH 3); standard additions - V = 20 µL, c = 0.001 mol L^{-1}.

4 Conclusion

The aim of the present paper was to characterize electrochemical properties of the lab-made BDDEs prepared with various B/C ratio in the gas phase. This parameter strongly influenced the features of the working electrodes, namely the width of the potential window, reversibility of the electrode reaction, height of the background, and the ration of signal to noise. The best results were obtained for electrodes with higher (not the highest) B/C in the gas phase, specifically 10 000 ppm.

Moreover, voltammetric method for determination of the chemoprotectant drug LV based on its electrochemical oxidation was tested employing all of the studied electrodes. Obtained excellent results confirmed suitability of the proposed analytical tool for determination of LV in pharmaceutical samples. On the other hand, it was proved that the boron content in BDD films affects significantly the obtained analytical parameters for particular BDDEs.

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Influence of boron content on electrochemical properties of boron-doped diamond electrodes and their utilization for leucovorin determination

Renáta Šelešovská,[a*] Barbora Kränková,[a] Michaela Štěpánková,[a] Pavlína Martinková,[a] Lenka Janíková,[a] Jaromíra Chýlková,[a] Marian Vojs[b]

[a] *University of Pardubice, Faculty of Chemical Technology, Institute of Environmental and Chemical Engineering, Studentská 573, 532 10 Pardubice, Czech Republic*

[b] *Slovak University of Technology in Bratislava, Faculty of Electrical Engineering and Information Technology, Institute of Electronics and Photonics, Ilkovičova 3, 812 19 Bratislava, Slovak Republic*

* e-mail: renata.selesovska@upce.cz

Abstract

Set of the lab-made boron-doped diamond electrodes (BDDEs) prepared with various B/C ratio (1000, 2000, 4000, 8000, 10 000, and 20 000 ppm) in the gas phase was subjected to the detailed characterization in the present paper. It was proved, that the B/C ratio influenced the electrochemical features of the working electrodes like a width of the potential window, which decreased with increasing B/C. Variations of reversibility of two redox systems ($Fe(CN)_6^{3-/4-}$ and $Ru(NH_3)_6^{2+/3+}$) depending on B/C were observed as well. The best electrochemical properties were found for BDDE with B/C 10 000 ppm. Moreover, the working electrodes were tested for voltammetric analysis of leucovorin based on its oxidation. The lab-made BDDEs were applied for voltammetric analysis of the pharmaceutical preparation containing leucovorin with excellent results (recovery 97.7-103.3 %, $RSD_5 \leq$ 2.1 %).

Key words

Voltammetry, Boron-doped diamond electrode, Boron content, Leucovorin, Pharmaceutical samples

1 Introduction

Boron-doped diamond (BDD) represents remarkable material with various technical applications [1]. Due to the development of the still most used technique for BDD preparation − chemical vapor deposition (CVD) in the eighties of the twentieth century, the possibilities of its application have grown very fast [1-3]. The first mention about the use of a diamond doped with argon and nitrogen as a working electrode appeared in 1983 [4] but the later works focused mainly on boron-doped diamond electrodes (BDDEs) and their possible electroanalytical applications, *e.g.* [5-15]. Their very specific or unique features, like wide potential window, low background current, high mechanical and chemical stability, high resistance to passivation or non-toxicity, are the main reasons, why the use of these electrodes have been so expanded in the last twenty years, *e.g.* [5-15]. It was proved, that properties of the BDDEs depends on many factors, *e.g.* (*i*) concentration and type of the dopant, (*ii*) morphological properties, (*iii*) main crystallographic orientation, (*iv*) surface termination (H, O, F, *etc.*), (*v*) grain boundaries, and (*vi*) presence of the non-diamond carbon phases [16]. The effect of B concentration on the electrochemical features of BDDE have been widely studied, *e.g.* in [17-35]. It was ascertained, that concentration of B strongly influences the conductivity of the BDD films − heavily doped films have metallic type of conductivity and the lower doped BDD could be found as semiconductive, which intensively affects electrochemical properties of BDDEs [10, 19, 22, 28, 31, 33-35]. Besides conductivity, B content and a quality of the film in general also affects width of the potential window or the background current [18, 26, 28, 32, 35]. Moreover, it was confirmed, that amount of B and

B/C, respectively, in the gas phase influences the structure of the prepared films, specifically higher level of B caused smaller formed crystals [19, 25, 27, 28, 35].
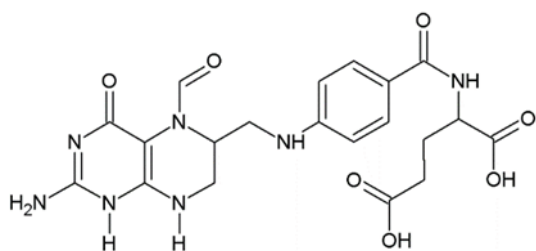
Voltammetric behavior of the particular redox systems could reveal important information about the surface of used working electrodes, *e.g.* in [7, 36-47]. Heterogeneous transfer of electron (transfer between the redox system and the electrode) can be categorized into two groups: (*i*) *outer sphere reaction*, where the electrode reaction proceeds only by mass transport and the working electrode serves as a donor or an acceptor of electrons. The electrode kinetics is not sensitive to the structure or surface of the electrode; and (*ii*) *inner sphere reaction*, which is strongly dependent on the electrode surface because it proceeds via some specific interaction with the surface [36, 37]. The most often employed redox system, typical representative of the inner sphere reactions, whose behavior has been widely studied on BDDEs is $Fe(CN)_6^{3-/4-}$ [7, 30, 31, 35, 37-39, 41-43]. Besides $Fe(CN)_6^{3-/4-}$, voltammetric behavior of aquated ions ($Fe^{3+/2+}$, $Ce^{4+/3+}$ or $Eu^{2+/3+}$), which proceed also through inner sphere reaction mechanism pathway, has been investigated on BDDEs as well [37, 42-45]. The redox probes, which typically provide outer sphere reaction and have been investigated on BDDEs are particularly $Ru(NH_3)_6^{2+/3+}$ and $IrCl_6^{2-/3-}$ [35, 37-41, 47].

Much less publications have been so far focused on the electroanalytical features of BDDEs with different boron content and their influence of analysis of specific electroactive compounds like nitrates [18, 23], benzophenone-3 [31], linuron [32], 4-chloro-3-methylphenol [33], 5-nitroquinoline [34], and 2-aminobiphenyl [35].

Leucovorin (LV, scheme 1) is a reduced derivative of folic acid [48]. It is administrated with methotrexate to reduce its negative side effects [49-51] or in combination with 5-fluorouracil to enhance its effectiveness during treatment of various serious diseases [52, 53]. It was chosen as a representative electroactive compound, because its voltammetric behavior has been already described only on mercury [54-56] and silver solid amalgam electrodes [56].

Considering the used working electrodes, the papers were focused on recording of LV reduction and thus interested in cathodic signals [54-56]. Our proposed voltammetric methods based on application of silver solid amalgam electrodes provided low detection limits specifically 2.2×10^{-8} and 5.0×10^{-8} mol L^{-1} for mercury meniscus modified (m-AgSAE) and polished silver solid amalgam electrode (p-AgSAE), respectively [56]. The obtained results were compared also with outputs from measuring with hanging mercury drop electrode (HMDE), which provided significantly higher limit of detection (9.8×10^{-7} mol L^{-1}) [56].

The present paper deals with characterization of the electrochemical features of BDDEs prepared under different conditions particularly with different B/C in the gas phase. Moreover, their applicability as an analytical tool for determination of the chemoprotectant drug LV based on its electrochemical oxidation is examined as well.

Scheme 1. Chemical structure of leucovorin.

2 Experimental

2.1 Chemicals

All of the used chemicals were of analytical grade purity and were obtained from Sigma Aldrich, if not stated otherwise. Standard solution of 0.001 mol L^{-1} leucovorin was prepared by simple dissolution of its calcium salt powder in the distilled water. It was stored in a refrigerator. Solutions of lower concentration were prepared by dilution with distilled water or the supporting electrolyte directly before analysis.

Britton-Robinson buffer (BRB) consisting of the mixture of acidic (H_3PO_4, H_3BO_3, and CH_3COOH – all three of the same concentration 0.4 mol L^{-1}) and alkaline component (0.2 mol L^{-1} NaOH from Lachema, Czech Republic) was used as a supporting electrolyte. 0.5 and 0.05 mol L^{-1} H_2SO_4 was prepared from 96 % H_2SO_4 (Penta-Švec, Czech Republic). 0.1 mol L^{-1} KCl was prepared by dissolution of its powder (Penta-Švec, Czech Republic) in the distilled water. Standard solutions of $2.5 \times 10^{-3} \text{ mol L}^{-1}$ $K_3[Fe(CN_6)]$ and $[Ru(NH3)_6]Cl_3$ (both of purity ≤ 99 %) were prepared in the solution of 0.1 mol L^{-1} KCl.

"Leucovorin Ca LACHEMA 10" for preparation of the injection solution originated from Pliva-Lachema, Czech Republic.

2.2 Instrumentation

Voltammetric measurements were carried out by computer controlled analyzer Eco-Tribo Polarograph (Polaro-Sensors, Czech Republic) and employing software POLAR.PRO version 5.1. The measuring system consisted of three electrodes – BDDE as a working electrode, Ag│AgCl│KCl (sat.) as a reference (Monokrystaly, Czech Republic), and platinum wire (Monokrystaly, Czech Republic) as an auxiliary electrode. As a working BDD electrode, we have used the commercially available one with working surface of 7.07 mm^2 and B/C ratio in the gas phase of 1000 ppm as declared by the producer Windsor Scientific (UK) or one of the laboratory prepared electrodes (Slovak University of Technology in Bratislava, Faculty of Electrical Engineering and Information Technology, Institute of Electronics and Photonics) with working surface of 0.43 mm^2 and B/C ratio in gas phase of 1000, 2000, 4000, 8000, 10 000, and 20 000 ppm. All measurements were carried out at laboratory temperature of 23±2 °C. Values of pH were measured by pH-meter Accumet AB150 (Fisher Scientific, Czech Republic).

All graphical dependences were constructed using Excel software (Microsoft, USA). Parameters of calibration curves and confidence intervals were calculated using OriginPro 9

software (OriginLab Corporation, USA) on the level of significance 0.05. Statistical parameters like limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration dependences as three times and ten times, respectively, of standard deviation of intercept divided by the slope.

2.3 Procedures

2.3.1 Electrochemical characterization of BDDEs

Preparation and surface characterization of the lab-made BDD electrodes is described in our previous paper [57]. All of the used working BDD electrodes (the lab-made and the commercial one as well) were activated and regenerated employing the same procedure. The chosen electrode underwent activation in 0.5 mol L^{-1} H_2SO_4 by applying negative (cathodic) potential ($E_c = -2000$ mV) for 60 s followed by insertion of positive (anodic) potential ($E_a = +2000$ mV) of the same duration. Then, 20 cyclic voltammograms between -1000 mV and $+2000$ mV were recorded to ensure stabilization of the activated electrode. After this step, the working electrode was ready for analysis. Besides the activation, the regeneration step, which consisted of the regeneration potential ($E_{reg} = +2000$ mV) applied for the regeneration time ($t_{reg} = 5$ s), was inserted between every scan. This process ensured the O-terminated surface and oxidation of eventually present impurities on the BDD electrode working surface.

For electrochemical characterization of BDD films cyclic voltammetry (CV) of redox systems of $[Fe(CN_6)]^{3-/4-}$ and $[Ru(NH_3)_6]^{3+/2+}$ was measured under the following parameters: scan rate (v) of 100 mV s^{-1}, potential range from -1600 mV to $+1800$ mV. CV of the above mentioned redox systems was used also for the measurements of dependences on v (10, 20, 50, 100, 200, 300, 400 and 500 mV s^{-1}).

2.3.2 Voltammetric determination of leucovorin

CV was applied for the investigation of the voltammetric behavior of LV depending on supporting electrolyte pH and scan rate (v). Measurements were carried out from initial potential ($E_{in} = -1000$ mV) to the switching potential ($E_{switch} = +2000$ mV) and back to -1000 mV employing the scan rate of 100 mV s^{-1}. In case of the scan rate study, the value of v varied from 25 to 500 mV s^{-1}. Based on our previous experiences, differential pulse voltammetry (DPV) and BRB of pH 3 was chosen for determination of LV. The optimized DPV parameters, which were used for all subsequent analysis, are as follows: $E_{in} = -700$ mV, final potential $- E_{fin} = +1800$ mV, $E_{reg} = +2000$ mV, $t_{reg} = 5$ s, $v = 40$ mV s^{-1}, pulse width 40 ms, sampling time 20 ms, pulse height 50 mV. The method was previously proposed and published for the commercial BDD electrode [58] and subsequently utilized in the present study for the lab-made working electrodes. Baseline for current signals evaluation was added as a tangent to the curve at minimum before and after the peak.

2.3.3 Analysis of pharmaceutical sample

The sample "Leucovorin Ca LACHEMA 10" was prepared for analysis by the producer declaration: a content of the phial was dissolved in 1 mL of distilled water. The prepared solution of the calculated concentration of 1.955×10^{-2} mol L^{-1} was ten times diluted. 10 µL of the diluted solution was added to the polarographic cell with 10 mL of the supporting electrolyte (BRB pH 3) and the determination was carried out by the standard addition method and two standard additions (20 µL of 1×10^{-3} mol L^{-1} LV) were applied at least. The determination was five times repeated and relative standard deviation of repeated determination (RSD) was calculated.
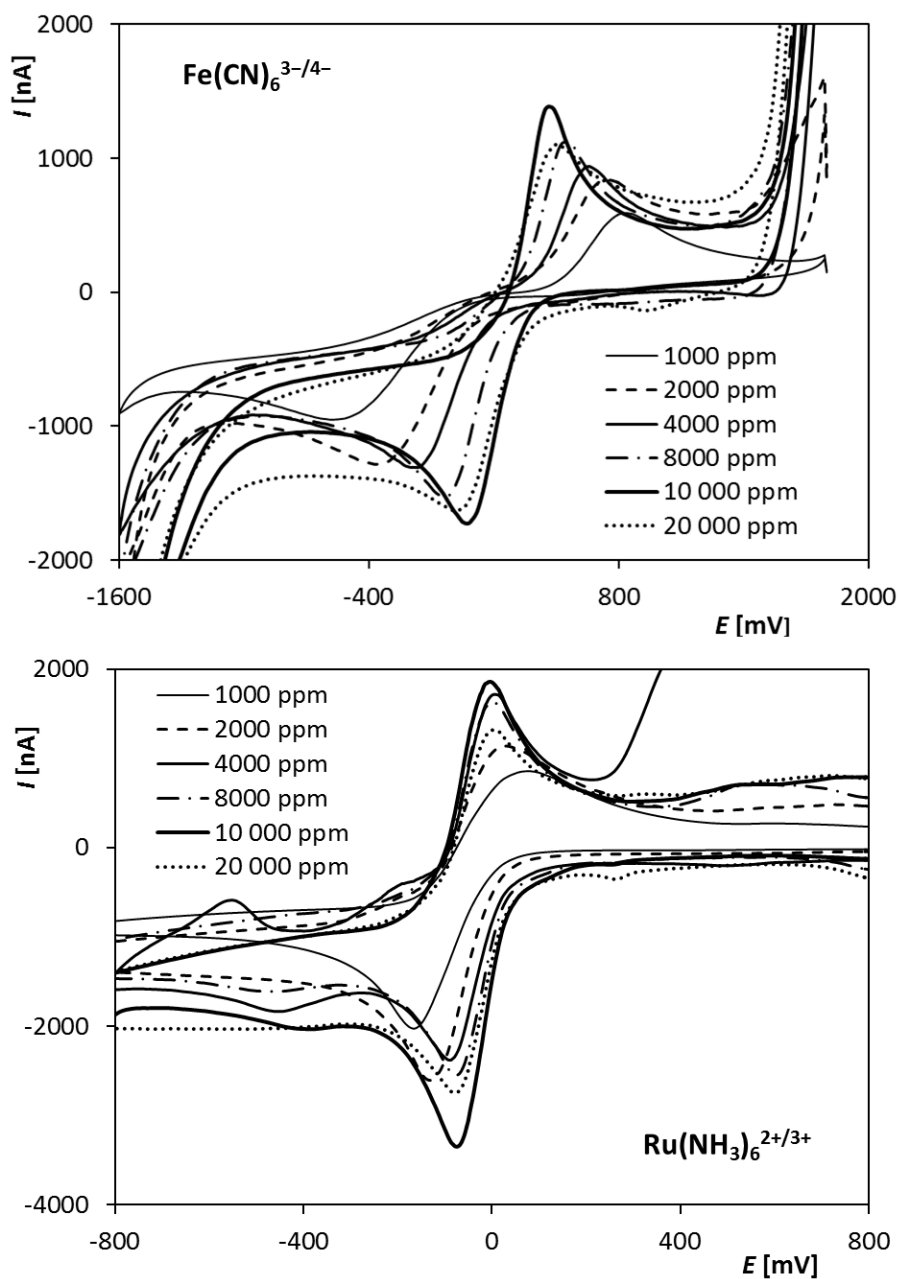
3 Results and discussion

3.1. Electrochemical characterization of BDDEs using cyclic voltammetry

Two redox systems, which are commonly applied for characterization of the working electrodes, namely $[Fe(CN)_6]^{3-/4-}$ and $[Ru(NH_3)_6]^{3+/4+}$, were employed for our experiments. $[Ru(NH_3)_6]^{3+/4+}$ is typical representative of redox systems provided outer sphere reactions [35, 37-41, 47]. On the other hand, it was proved, that $[Fe(CN)_6]^{3-/4-}$ ensured inner sphere reaction on BDD electrodes, discussed more in detail *e.g.* in [7, 30, 31, 35, 37-39, 41-43]. CV was utilized as a measuring method and various parameters like width of the potential window, reversibility of the electrode reaction, the electrochemically active surface, and the apparent electron transfer rate constant ($k^0{}_{app}$), respectively, were calculated.

The first necessary step of the characterization of the lab-made BDDEs was recording of the width of the potential window. $0.1 \, \text{mol} \, L^{-1}$ KCl was employed as a supporting electrolyte. The border potential on the anodic and cathodic side as well was the value, when the current reached ± 1500 nA. The increasing B/C ratio in the gas phase caused narrowing of the potential window. Moreover, the narrowing was more significant in the cathodic region, which is consistent with literature [18, 25, 28, 35, 57]. Particularly, the width decreased of about 900 mV comparing BDDE 1000 ppm and 20 000 ppm and the widest potential window, namely 3660 mV, was observed for BDDE with B/C 1000 ppm. These results are summarized in TAB S1 in Supporting materials. The potential window recorded on the commercial BDDE in the same medium was about 2800 mV [58].

Reversibility of the used redox markers was examined next. The obtained cyclic voltammograms are depicted in Figure 1. Considering reversibility, it is apparent that this parameter grown with increasing boron content, which is obvious from the increasing of the particular signals and decreasing of their potential differences. Particular values of the anodic and cathodic peak heights (I_{pa} and I_{pc}), their ratio (I_{pa}/I_{pc}), and values of the anodic and cathodic peak potential (E_{pa} and E_{pc}) and their difference (ΔE_p) are summarized in Table 1 and displayed in the inset of Figure 1.

Figure 1 Cyclic voltammograms of the studied redox systems recorded on the lab-made BDDEs. Method – CV, $E_{in} = E_{fin} = -1600$ mV, $E_{switch} = +1800$ mV, $v = 100$ mV s^{-1}; supporting electrolyte – 0.1 mol L^{-1} KCl; $c([Fe(CN)_6]^{3-/4-}) = c([Ru(NH3)_6]^{3+/4+}) = 2.5 \times 10^{-3}$ mol L^{-1}.

Table 1 Basic parameters of cyclic voltammograms measured on all tested BDDEs for redox systems $Fe(CN)_6^{3-/4-}$ and $Ru(NH_3)_6^{2+/3+}$ in 0.1 mol L^{-1} KCl.

B/C [ppm]	I_{pa} [nA]	I_{pc} [nA]	I_{pa}/I_{pc}	E_{pa} [mV]	E_{pc} [mV]	ΔE_p [mV]
			$Fe(CN)_6^{3-/4-}$			
1000	+500	−507	0.98	+829	−577	1406
2000	+510	−576	0.88	+720	−355	1075
4000	+672	−767	0.88	+659	−163	822
8000	+849	−947	0.89	+550	−24	574
10 000	+1039	−1053	0.98	+545	+72	473
20 000	+694	−710	0.98	+503	+12	491
			$Ru(NH_3)_6^{2+/3+}$			
1000	+1161	−1268	0.92	+71	−167	238
2000	+1226	−1389	0.88	+26	−132	158
4000	+1283	−1407	0.91	+26	−91	117
8000	+1400	−1530	0.92	+4	−71	75
10 000	+1560	−1612	0.97	−10	−71	61
20 000	+972	−1128	0.86	+8	−77	85

The parameter I_{pa}/I_{pc} ranged from 0.86 to 0.98 for both redox markers. The highest values limiting to the theoretical value 1 confirming reversibility of the electrode reaction were found for BDDE with the highest B/C (10 000 ppm for both redox systems and 20 000 ppm for $Fe(CN)_6^{3-/4-}$) and surprisingly for BDDE with B/C 1000 ppm. On the other hand the potential difference, which is the most important criteria for assessment of the reversibility, was especially for the system $Fe(CN)_6^{3-/4-}$ enormous (ranged from 1406 to
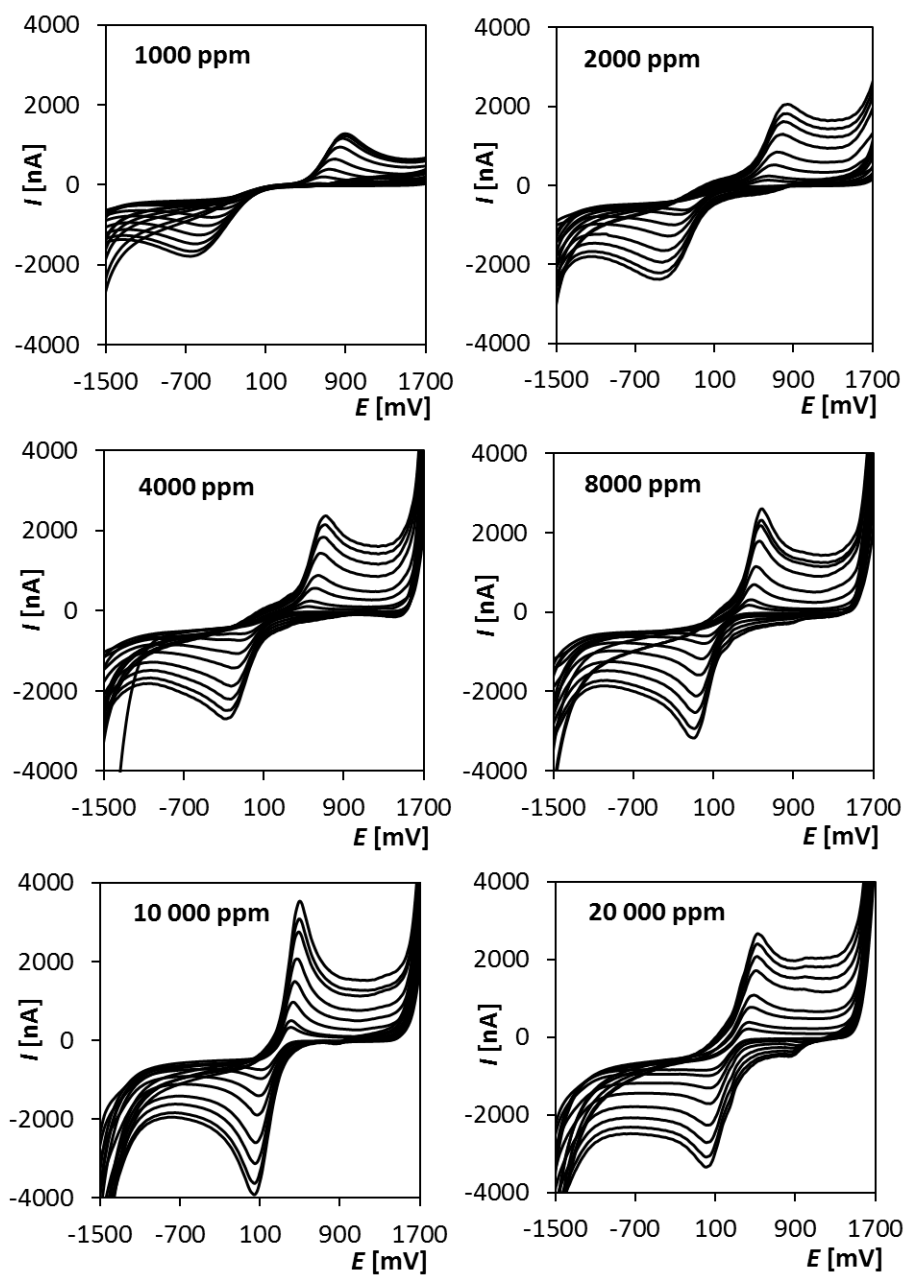
491 mV) and did not limit to the theoretical value of 59 mV. Considering a decreasing trend of the potential difference, it was again confirmed, that the reversibility increased with the boron content and the best result, namely the lowest value of ΔE_p, was obtained for BDDE with B/C 10 000 ppm. Moreover, ΔE_p defined for this electrode and $Ru(NH_3)_6^{2+/3+}$ limiting to the theoretical value 59 mV, which is typical for the one-electron reversible electrode reaction. The slight increase of ΔE_p for electrode with B/C 20 000 ppm may be explained by a higher content of sp^2 hybridized carbon in these BDD films, as it was observed in previous studies [17, 28]. The results of ΔE_p analysis obtained for the lab-made working electrodes were consistent with those calculated for the commercial BDDE, particularly 552 mV for $Fe(CN)_6^{3-/4-}$ and 67 mV for $Ru(NH_3)_6^{2+/3+}$ [58]. The lower values of ΔE_p for the redox probe $Ru(NH_3)_6^{2+/3+}$ could be explained by the nature of the electrode reaction. The outer sphere reaction pathway and the electron transfer as well is in general not influenced by the physicochemical properties of working surface of the electrode [59].

Electroactive area of the working electrodes (A) was calculated utilizing Randles-Ševčík equation [60-62] for the scan rate of 100 mV s^{-1} and the representative of the inner sphere reaction pathway – $Fe(CN)_6^{3-/4-}$. The outcomes are summarized in Table 2. It is evident that the active surface increased with the boron content and the largest area was calculated for BDDE with B/C 10 000 ppm. It could be assumed, that the higher active surface is related with the smaller formed crystals, in agreement with [19, 25, 27, 28, 35]. The significant reduction was observed for films with higher B/C ratio, which could be again caused by the worsen quality of the film. These results were also consistent with literature [19, 25, 27, 28, 35].
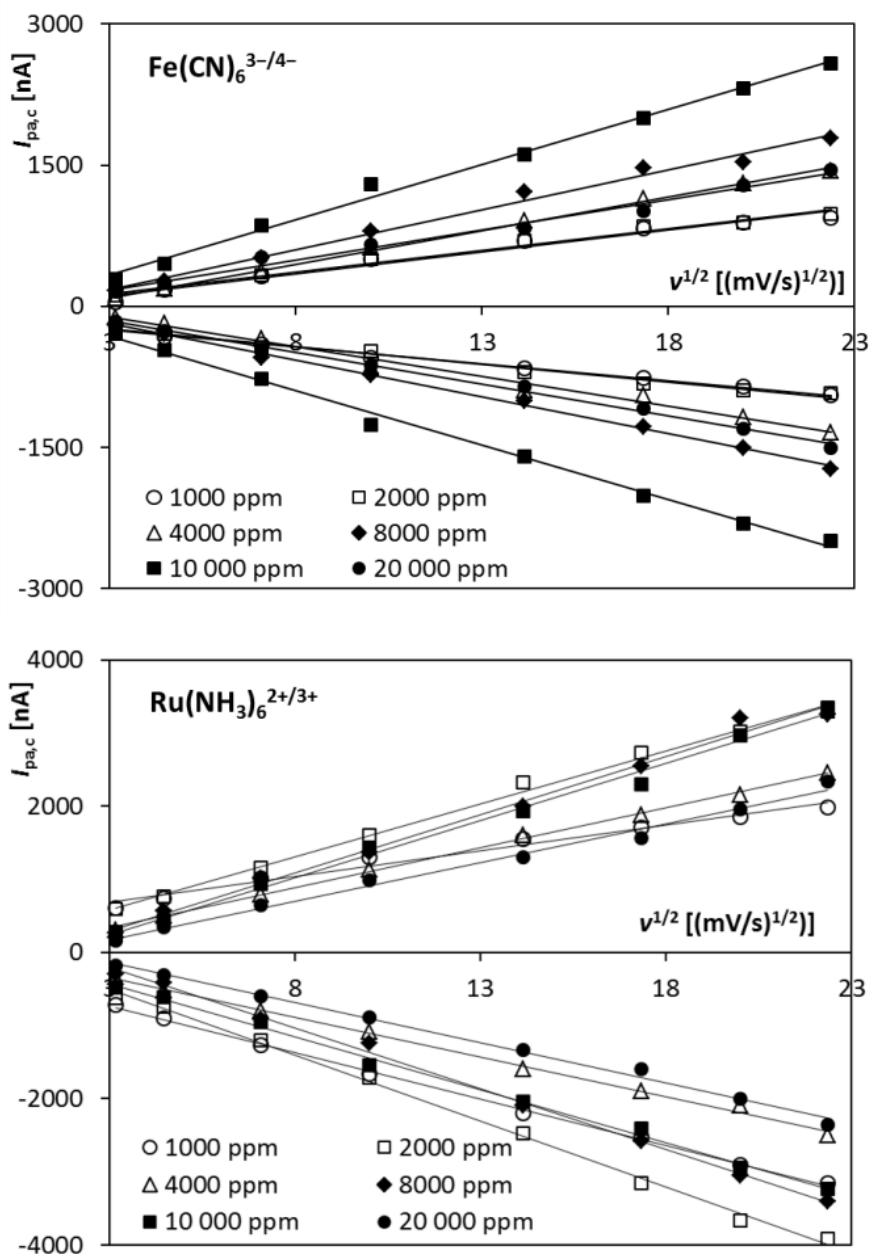
Table 2 Calculated values of the electrochemical active surfaces and the apparent heterogeneous electron-transfer rate constants for all tested BDDEs.

B/C [ppm]	$A(Fe(CN)_6^{3-/4-})$ [mm^2]	$k^0_{app}(Fe(CN)_6^{3-/4-})$ [cm s^{-1}]	$k^0_{app}(Ru(NH_3)_6^{2+/3+})$ [cm s^{-1}]
1000	0.25	1.8×10^{-9}	5.2×10^{-4}
2000	0.26	1.2×10^{-8}	2.7×10^{-3}
4000	0.30	7.0×10^{-7}	5.5×10^{-3}
8000	0.36	9.0×10^{-6}	1.3×10^{-2}
10 000	0.60	1.1×10^{-4}	9.7×10^{-2}
20 000	0.30	5.0×10^{-5}	7.7×10^{-3}

The influence of the scan rate was examined next. This parameter varied from 10 to 500 mV s^{-1} and obtained cyclic voltammograms for $Fe(CN)_6^{3-/4-}$ and all of the tested lab-made electrodes are depicted in Figure 2. Very similar outcomes were obtained for the second redox system as well. It is evident from Figure 2, that anodic and cathodic signal increased with the scan rate but the dependence was non-linear. On the other hand, the linearity was observed for the dependence between peak height and the square root of the scan rate ($v^{1/2}$), which is illustrated by Figure 3 and TAB S2 and S3 in supplementary materials containing parameters of the particular equations (slopes, intercepts, and correlation coefficients). This result is typical for the diffusion controlled electrode reaction, which is common for BDDEs due to their low ability to adsorb analytes on the working surface. The slopes (TAB S2 and S3) of the dependencies increased with the boron content and the maximum value was obtained for BDDE with B/C 10 000 ppm ($Fe(CN)_6^{3-/4-}$) and 8000 ppm ($Ru(NH_3)_6^{2+/3+}$), respectively. The diffusion-controlled pathway of the electrode reaction was further confirmed by the $\log(I_p)$-$\log(v)$ analysis, particularly by the slopes (TAB S4 and S5) of these linear dependencies. They equalled or limited to the theoretical value 0.5, which is typical for the diffusion controlled reaction.

Figure 2 Cyclic voltammograms of $Fe(CN)_6^{3-/4-}$ recorded on the lab-made BDD electrodes at various scan rates. Method – CV, $E_{in} = E_{fin} = -1600$ mV, $E_{switch} = +1800$ mV, $v = 10, 20, 50, 100, 200, 300, 400,$ and 500 mV s^{-1}; supporting electrolyte – 0.1 mol L^{-1} KCl; $c([Fe(CN)_6]^{3-/4-}) = 2.5 \times 10^{-3}$ mol L^{-1}.

Figure 3 Dependence of the peak heights provided by $Fe(CN)_6^{3-/4-}$ and $Ru(NH_3)_6^{2+/3+}$, respectively, on the square root of scan rate recorded on the lab-made BDD electrodes at various scan rates. Method – CV, $E_{in} = E_{fin} = -1600$ mV, $E_{switch} = +1800$ mV, $v = 10, 20, 50, 100, 200, 300, 400,$ and 500 mV s^{-1}; supporting electrolyte – 0.1 mol L^{-1} KCl; $c([Fe(CN)_6]^{3-/4-}) = c([Ru(NH_3)_6]^{3+/4+}) = 2.5 \times 10^{-3}$ mol L^{-1}.

The last experiments in the electrochemical characterization was examination of the influence of scan rate and square root of the scan rate, respectively, on the anodic and
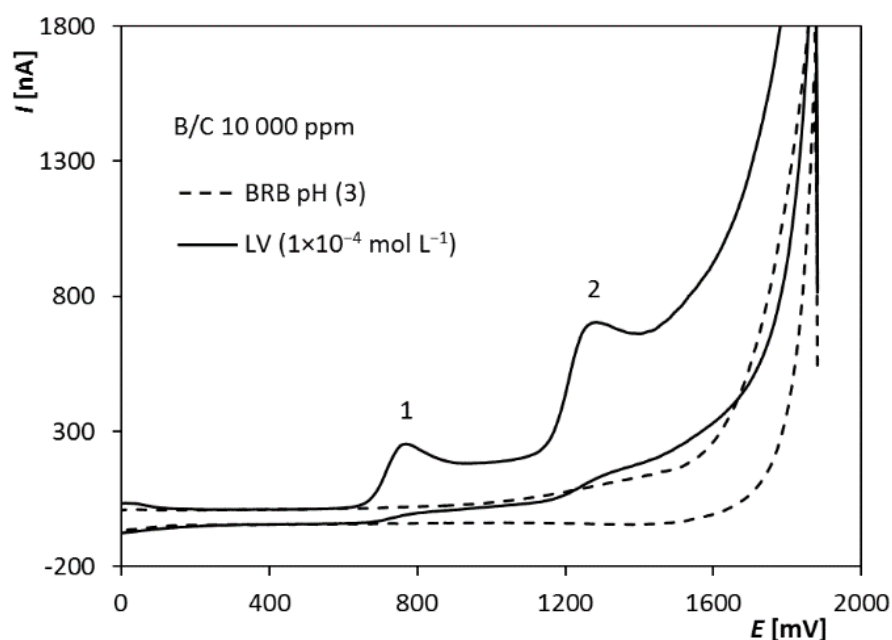
cathodic peak potential difference and calculation of the apparent heterogeneous electron transfer rate constants (k^0_{app}) employing the Nicholson approach [63]. It was found out, that the most differentiated signals were recorded on BDDE with B/C 1000 ppm and the difference decreased with increasing B/C. The lowest values were determined for BDDE with the highest B/C. Values of k^0_{app} were calculated for $v = 100$ mV s^{-1}. Values of Ψ (TAB S6) were directly subtracted from the graphical dependence in the article [63] for the redox system $[Ru(NH3)_6]^{3+/4+}$ and the calculated values of the apparent scan rate constant ranged from 5.2×10^{-4} (1000 ppm) to 1.3×10^{-2} cm s^{-1} (8000 ppm). It is obvious (Table 2), that k^0_{app} increased with growing B/C in the gas phase, which corresponded with the literature [35]. High values of ΔE_p recorded for the second redox system ($[Fe(CN)_6]^{3-/4-}$) corresponded with low Ψ the particular, which were not included by Nicholson in the reference [63]. Thus, we created linear extrapolation of the dependence (based on the outcomes in [63]), which could be described by equation (1). Values of Ψ for $[Fe(CN)_6]^{3-/4-}$ are summarized in TAB S7. Values of k^0_{app} (Table 2) ranged from 1.8×10^{-9} (1000 ppm) to 1.1×10^{-4} cm s^{-1} (10 000 ppm) and the similar behavior of k^0_{app} depending B/C in the gas phase as in the case of $[Ru(NH_3)_6]^{3+/4+}$ was observed – the value of the apparent rate constant increased with growing B/C. It could be concluded, that higher k^0_{app} indicated faster heterogeneous electron transfer and confirmed favorable electrochemical properties of the working electrodes. Thus, the electrodes of the best electrochemical features could be assumed for BDDEs with higher B/C in the gas phase namely 10 000 ppm.

$$\Psi = 10^{-0.0056048(\Delta E \times n) + 0.1882152} \tag{1}$$

3.2. Voltammetric determination of leucovorin using BDDEs with different boron content

The target of this work, besides the electrochemical characterization of the lab-made BDDEs, was examination and assessment of their analytical utilization for LV determination. LV was chosen as a model analyte because its electrochemical behavior is well-described only at mercury or amalgam working electrodes [54-56] and only our previous work focused on development of voltammetric method for LV oxidation on the commercial BDDE [58].

Cyclic voltammograms in absence and presence of 1×10^{-4} mol L^{-1} LV between potentials -1000 mV and $+1800$ mV were recorded in BRB of pH 3 on BDDE with B/C 10 000 ppm (Figure 4), which was chosen based on the results discussed above. Two well-developed oxidation signals, as in case of commercial BDDE [58] were recorded. The first signal was chosen for all subsequent analysis due to its well-developed shape, easy evaluation, and favorable position.

Figure 4 Cyclic voltammograms in absence and presence of LV recorded on the lab-made BDDE with B/C 10 000 ppm. Method – CV, $E_{in} = E_{fin} = -1000$ mV, $E_{switch} = +1800$ mV, $v = 100$ mV s^{-1}; supporting electrolyte – BRB (pH 3); $c(LV) = 1\times10^{-4}$ mol L^{-1}.
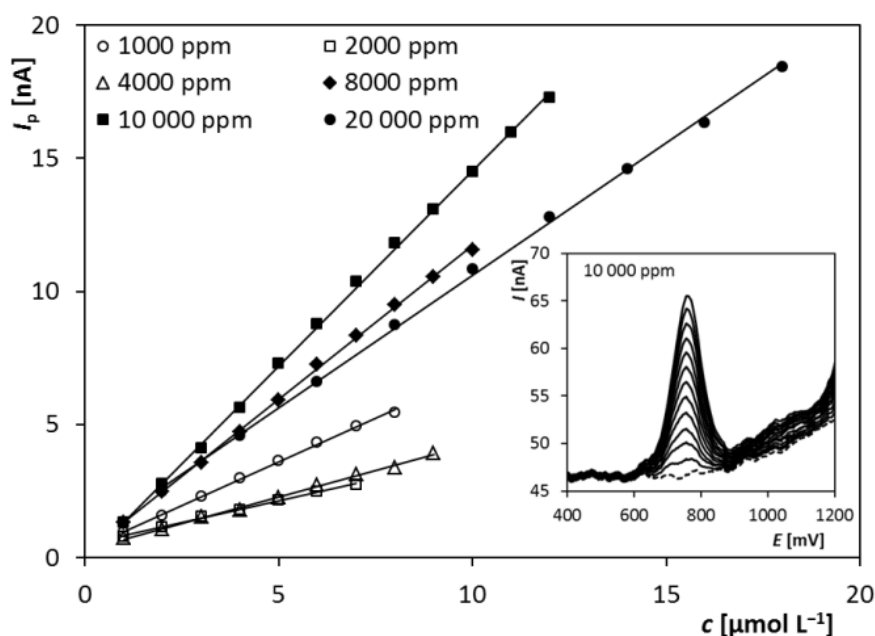
3.2.1. Analysis of model solutions

At the beginning, DP voltammograms of 1×10^{-6} mol L^{-1} LV were recorded on all tested lab-made BDDEs and the peak height were compared (FIG S1). The highest current responses were obtained on electrodes with higher B/C, namely 10 000 and 20 000 ppm, which corresponded to the previously described results and with literature dealing with analysis of organic compounds on BDDE prepared under different B/C in the gas phase [31, 33-35]. It could be concluded, that electrode with B/C 10 000 ppm provided comparable or even better electrochemical properties than BDDE with B/C 20 000 ppm and increasing of the B/C did not lead to improving of the electrode`s features.

Similar concentration dependencies on 10^{-6} mol L^{-1} level of LV were recorded on all of the tested electrodes. Example of the obtained curves of LV ranged from 1×10^{-6} to 1.2×10^{-5} mol L^{-1} recorded on BDDE with B/C 10 000 ppm is illustrated by the inset of Figure 5. It is evident from Figure 5 that the peak height increased linearly with LV concentration in the analyzed solution particularly. Parameters of the straight lines (slopes, intercepts, correlation coefficients (R)) are summarized in Table 3. The highest sensitivity could be supposed, based on the highest slope, for BDDE with B/C 10 000 ppm, which again confirmed our previous results. Moreover, peak of LV increased linearly with its concentration in the widest range employing this working electrode. On the other hand, sensitivity of the electrodes with lower B/C (1000 – 4000 ppm) is comparable and relatively low.

Table 3 Statistical parameters of concentration dependences of LV obtained with all particular BDDEs.

B/C [ppm]	Slope [nA L µmol^{-1}]	Intercept [nA]	r	LDR [mol L^{-1}]	LOD [mol L^{-1}]	LOQ [mol L^{-1}]	RSD$_{11}$ [%]
1000	(0.653±0.010)	(0.339±0.051)	0.9992	2.5×10^{-7}-2.5×10^{-5}	6.7×10^{-8}	2.2×10^{-7}	2.0
2000	(0.3267±0.0088)	(0.499±0.039)	0.9982	1.0×10^{-6}-3.0×10^{-5}	3.6×10^{-7}	1.2×10^{-6}	1.7
4000	(0.3951±0.0065)	(0.312±0.036)	0.9984	5.0×10^{-7}-3.0×10^{-5}	1.2×10^{-7}	3.7×10^{-7}	1.9
8000	(1.1545±0.0098)	(0.176±0.061)	0.9997	5.0×10^{-7}-3.5×10^{-5}	9.0×10^{-8}	3.0×10^{-7}	3.4
10 000	(1.466±0.012)	(0.114±0.085)	0.9997	5.0×10^{-7}-5.0×10^{-5}	1.0×10^{-7}	3.4×10^{-7}	1.1
20 000	(0.983±0.011)	(0.74±0.14)	0.9995	2.0×10^{-6}-4.0×10^{-5}	4.2×10^{-7}	1.4×10^{-6}	0.8

Figure 5 Linear concentration dependencies obtained on the lab-made BDDE with B/C 1000 – 20 000 ppm. Inset: Voltammetric curves of LV recorded on BDDE with B/C 10 000 ppm. Method – DPV, E_{in} = −700 mV, E_{fin} = +1800 mV, v = 40 mV s^{-1}; supporting electrolyte – BRB (pH 3); c(LV) = 1×10^{-6} – 8×10^{-6} mol L^{-1} (1000 ppm), 1×10^{-6} – 7×10^{-6} mol L^{-1} (2000 ppm), 1×10^{-6} – 9×10^{-6} mol L^{-1} (4000 ppm), 1×10^{-6} – 1×10^{-5} mol L^{-1} (8000 ppm), 1×10^{-6} – 1.2×10^{-5} mol L^{-1} (10 000 ppm), and 2×10^{-6} – 1.8×10^{-5} mol L^{-1} (20 000 ppm).

Statistical parameters like linear dynamic range (LDR), limit of detection (LOD), limit of quantification (LOQ), and relative standard deviation of 11 repeated measurements (RSD$_{11}$) were calculated next and could be found in Table 3. LDR is a concentration range in which the peak height linearly increased with concentration of the analyte. The widest LDR was again recorded on BDDE with B/C 10 000 ppm and very similar extent were measured on BDDE with B/C 4000 and 8000 ppm as well. The remaining electrodes provided narrower LDR. Moreover due to the poorly evaluable signal, which was mainly caused by the high background and the low ratio of the signal to noise, the lowest concentration, which could be reliably detected on BDDE with B/C 20 000 ppm, was 4 times higher than on BDDE with B/C 10 000 ppm. The lowest detection and quantification limits, respectively, were surprisingly calculated for BDDE with B/C 1000 ppm and expectably for BDDE with B/C 8000 and 10 000 ppm as well. The outcomes calculated for BDDE with B/C 1000 ppm could be explained by low background, high ratio signal to noise, and high linearity ($r = 1.000$) of the dependence employed for calculation of the limits, respectively.

Repeatability of the measurements was studied next. 11 repeated voltammograms of 1×10^{-5} mol L^{-1} were recorded using all of the tested working electrodes and RSD$_{11}$ was calculated from the obtained peak heights. All of the values are ≤ 3.4 %, which confirmed stable and repeatable response recorded on all of the electrodes.

Obtained LODs were lower than that obtained for analysis of LV on HMDE (9.8×10^{-7} mol L^{-1}) and comparable or about maximum one order of magnitude higher than those (2.2×10^{-8} and 5.0×10^{-8} mol L^{-1}) received for analysis with AgSAE [56]. In contrast with amalgam and mercury working electrode, where LV was accumulated on the electrode surface for 60 s (m-AgSAE), 100 s (p-AgSAE), and 40 s (HMDE), respectively, any accumulation time was not applied for analysis with BDDE. Therefore, the analysis was faster but the sensitivity was sufficient.

3.2.2. Analysis of leucovorin model solutions

Repeatability of the LV determination was studied using two model solutions for all of the examined electrodes. The standard addition method was employed for this experiment and 2 standard additions were added at least. Every determination was 5 times repeated and relative standard deviation of 5 repeated determinations (RSD_5), average value with particular confidence intervals ($\alpha = 0.05$), and recovery was calculated for each electrode (Table 4). It is obvious, that all of the obtained results were correct and repeatable ($RSD_5 \leq 3.8\ \%$). Therefore, all tested electrodes can be found suitable for the determination of LV based on its electrochemical oxidation when the analyte concentration ranges within the LDR obtained for particular electrodes. However, based on the above mentioned results (Table 3) BDDEs with higher boron content provide better statistical parameters including lower LOQ and wider LDR and thus allow lower LV concentrations to be determined.

Table 4 Results of the repeated determination of LV in model solutions using the lab-made BDDEs

B/C [ppm]	Added [mol L^{-1}]	Found[*] [mol L^{-1}]	Recovery [%]	RSD$_5$ [%]
1000	3.0×10^{-6}	$(3.020\pm0.030)\times10^{-6}$	99.0-101.3	1.5
	1.0×10^{-6}	$(1.010\pm0.012)\times10^{-6}$	99.5-104.0	1.8
2000	3.0×10^{-6}	$(3.040\pm0.043)\times10^{-6}$	98.3-103.6	2.1
	1.0×10^{-6}	$(1.020\pm0.024)\times10^{-6}$	97.0-105.0	3.5
4000	3.0×10^{-6}	$(3.020\pm0.043)\times10^{-6}$	98.3-104.0	2.2
	1.0×10^{-6}	$(1.020\pm0.025)\times10^{-6}$	96.5-107.0	3.7
8000	3.0×10^{-6}	$(3.030\pm0.031)\times10^{-6}$	99.3-103.3	1.5
	1.0×10^{-6}	$(1.010\pm0.025)\times10^{-6}$	95.5-106.0	3.8

10 000	3.0×10^{-6}	$(3.020\pm0.022)\times10^{-6}$	98.6-101.3	1.1
	1.0×10^{-6}	$(1.010\pm0.011)\times10^{-6}$	98.0-102.0	1.7
20 000	3.0×10^{-6}	$(3.010\pm0.039)\times10^{-6}$	97.3-102.3	1.9
	2.0×10^{-6}	$(2.000\pm0.018)\times10^{-6}$	98.5-101.0	1.4

[*] The number of valid digits is used accordance to the literature [64].

3.2.3. Analysis of pharmaceutical preparation

Applicability of the tested BDDEs with different boron content was verified by analysis of the pharmaceutical preparation "Leucovorin Ca LACHEMA 10". Standard addition method was employed again. The determinations were 5 times repeated and values of RSD_5 were calculated (Table 5). Example of the obtained voltammograms recorded using BDDE with 10 000 ppm and the graphical evaluation of the LV amount, respectively, are depicted in Figure 6.
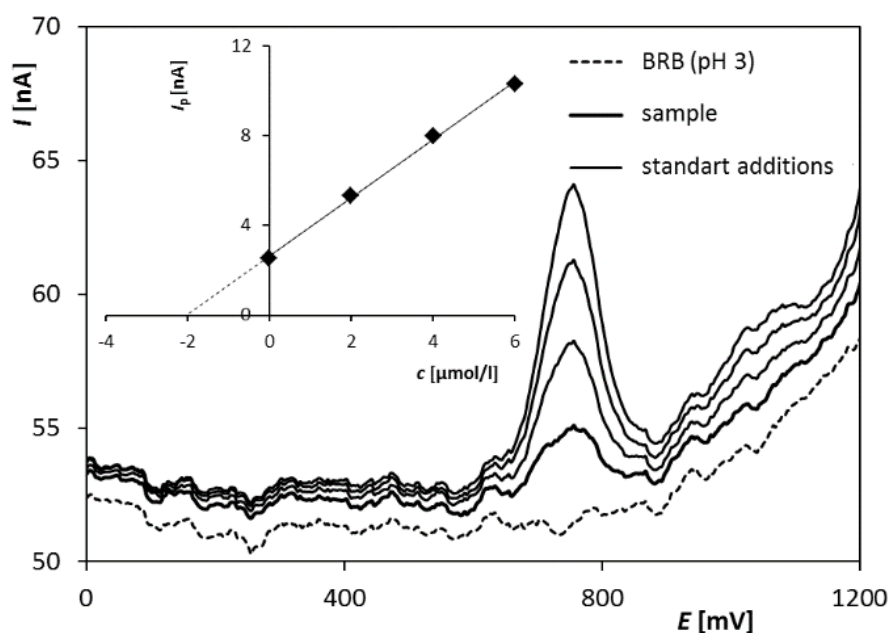
All of the tested electrodes provided accurate, correct, and repeatable results of the determination. The same variations of the peak height, background, and ratio of signal to noise, respectively, were observed as in case of model solutions analysis mentioned above. Considering the matrix of the sample, which was relatively simple (presence of no other electroactive compounds or interfering compounds), any negative effect caused by the low doping level was not observed and determination itself was not negatively influenced at al. Therefore, it could be concluded, that our proposed method in combination with all of the tested working BDDES is suitable for determination of LV in pharmaceutical samples.

Table 5 Results of the repeated determination of LV in pharmaceutical preparation "Leucovorin Ca LACHEMA 10".

B/C	Declared	Found*	Recovery	RSD_5

[ppm]	[mg]	[mg]	[%]	[%]
1000	10	(10.030±0.080)	98.2-100.8	1.2
2000	10	(10.09±0.12)	98.7-102.8	1.8
4000	10	(10.08±0.13)	98.2-102.8	2.0
8000	10	(10.03±0.10)	98.7-102.9	1.6
10 000	10	(10.03±0.14)	97.7-103.3	2.1
20 000	10	(10.041±0.099)	99.2-102.3	1.4

[*] The number of valid digits is used accordance to the literature [64].

Figure 6 Determination of LV in the pharmaceutical preparation "Leucovorin Ca LACHEMA 10" using BDDE with B/C in the gas phase 10 000 ppm. Inset: Graphical evaluation of the LV concentration. Method – DPV, $E_{in} = -700$ mV, $E_{fin} = +1800$ mV, $v = 40$ mV s^{-1}; supporting electrolyte – BRB (pH 3); standard additions - $V = 20$ μL, $c = 0.001$ mol L^{-1}.

4 Conclusion

The aim of the present paper was to characterize electrochemical properties of the lab-made BDDEs prepared with various B/C ratio in the gas phase. This parameter strongly influenced the features of the working electrodes, namely the width of the potential window, reversibility of the electrode reaction, height of the background, and the ration of signal to noise. The best results were obtained for electrodes with higher (not the highest) B/C in the gas phase, specifically 10 000 ppm.

Moreover, voltammetric method for determination of the chemoprotectant drug LV based on its electrochemical oxidation was tested employing all of the studied electrodes. Obtained excellent results confirmed suitability of the proposed analytical tool for determination of LV in pharmaceutical samples. On the other hand, it was proved that the boron content in BDD films affects significantly the obtained analytical parameters for particular BDDEs.

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