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Vanillylmandelic and Homovanillic acid: Electroanalysis at non-modified and polymer-modified carbon-based electrodes

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ABSTRACT

The electrochemical behaviour of two main catecholamine metabolites serving as diagnostic tumour biomarkers, homovanillic acid (HVA) and vanillylmandelic acid (VMA), on anodically oxidized boron doped diamond (BDD) electrodes, non-modified glassy carbon electrodes (GCE), and GCE modified by polymers, namely by Nafion (Nafion/GCE) and by poly(neutral red) (PNR/GCE), was investigated. The voltammetric responses of both biomarkers are strongly dependent on pH of the aqueous buffer solution and electrode material used. BDD electrodes exhibit more positive oxidation potentials and lower current densities for oxidation of both analytes, depending also on the surface termination. Concentration dependences were measured in 0.1 mol L^{-1} phosphate buffer pH 3.0, as optimal supporting electrolyte for differential pulse voltammetric determination. Achieved detection limits were 0.6, 0.9, 0.8, and 1.2 μmol L^{-1} for HVA and 0.4, 1.5, 2.4, and 1.1 μmol L^{-1} for VMA at BDD electrodes, non-modified GCE, Nafion/GCE, and PNR/GCE, respectively. The limits of quantification for both organic compounds on all tested electrode materials are sufficiently low for their determination in urine. Verification of the possibility of simultaneous voltammetric determination of the studied biomarkers in the mixture was also carried out.

1. Introduction

Vanillylmandelic acid (VMA) and homovanillic acid (HVA) are the major end products of the catecholamine metabolism [1]. The main endogenous catecholamines produced and released by the adrenal medulla, the sympathetic nerves, and the brain are epinephrine and norepinephrine, both primarily metabolized into VMA, and dopamine whose major metabolite is HVA. Both metabolites are structurally closely related (Fig. 1), differing only in the presence of a hydroxyl group in the alfa position in the structure of VMA.

The quantitation of catecholamines and their metabolites in physiological fluids has great importance, mainly in clinical chemistry, since these compounds serve as diagnostic markers for diverse metabolic [2,3] and neurological disorders [4,5]. Both organic acids occur in the various biological matrices and are determined frequently in urine [6–21], blood plasma [22,23], blood serum [24], cerebrospinal fluid [3,25], and brain tissue [26]. VMA and HVA represent two crucially essential biomarkers of neuroblastoma [27,28], the third most common cancer type affecting children under the age of five. Pheochromocytoma is the most serious neuroendocrinal tumour involving over-production of VMA and HVA among adults with the highest incidence between 40 and 50 years of life [5]. The HVA/VMA ratio [28,29] can also be used as a clinical marker, for instance as a screening tool for Menkes disease [2], a copper transport disorder that results in early death. Previously performed experimental studies have revealed that an increased concentration of HVA in the cerebrospinal fluid is related to suicide attempts [30], post-traumatic stress disease [31], and Parkinson's disease [3]. A high level of HVA in blood plasma arises in patients suffering from chronic schizophrenia [32] and it is also connected with the eating disorder bulimia [33]. Moreover, other researches have shown that HVA is a neuroendocrine marker of behavioural diseases and there is a tendency of developing drug addiction [34].

The determination of VMA and HVA faces several analytical problems including similar structures and low concentrations in biological matrices. Many analytical procedures have been developed for their separation and determination, including liquid chromatography in combination with electrochemical detection (ED) [11,12,22,23,25,26,35–37], fluorescence

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detection [10,38,39], chemiluminescence detection [40], mass spectrometry (MS) [24,41], hydrophilic interaction liquid chromatography connected to tandem MS [17], gas chromatography coupled with MS [16], capillary electrophoresis combined with ED [5,9,13], MS [42], and UV [8] detection, micellar electrokinetic chromatography with UV detection [6,7], and isotachophoresis with conductivity detection [15]. Thin-layer chromatography [14,39] and immunomethods [18,19,21] have also been used.

Electroanalytical methods rely on the oxidizability of VMA and HVA at the hydroxyl group attached to the aromatic ring [29,43–45]. This is a typical electrooxidation pathway for most phenolic compounds at carbonaceous electrode materials including, for instance, napth-2-ol [46], *p*-nitrophenol [47], chlorophenols [48], estrone [49], bisphenol A [50], clioquinol [51], *p*-coumaric acid [52], vanillin [53], capsaicin [54], ochratoxin A [55]. An overview of the electroanalytical techniques established for HVA and VMA determination up to now is given in Table 1. Carbon-based working electrodes such as edge-plane pyrolytic graphite [29], composite film [56], screen-printed carbon electrodes [57], and carbon paste electrodes [58] have been utilized. The electrochemical deposition of copper metal films [59], electropolymerized Alizarin Red S dye [43], and heterocyclic compound 3-amino-5-mercapto-1,2,4-triazol [44] on the surface of glassy carbon electrodes, and the adsorption of phosphatidylethanolamine lipid on carbon paste electrode [45], are previously employed electrode modifications that enhance the selectivity and sensitivity of HVA determination. Furthermore, voltammetric sensors based on glassy carbon electrodes covered by molecularly imprinted polymers have been prepared for VMA [60] and HVA [61]. A new diagnostic tool based on polymer films produced from Tröger base for potentiometric detection of VMA [62] has been recently developed.

The objective of the present work is mainly focused on the study of the electrochemical behaviour of HVA and VMA at various carbon-based electrodes, namely boron-doped diamond (BDD) electrode and glassy carbon electrode (GCE), both non-modified and polymer-modified by Nafion (Nafion/GCE) and by poly(neutral red) (PNR/GCE). Their utilization for the voltammetric determination of both of the studied metabolites and their determination in their mixture was evaluated.

2. Experimental

2.1. Reagents and solutions

All chemicals were of analytical reagent grade and used as received without any further purification. Deionized water (Millipore Mili plus Q system, USA) with resistance not less than 18.2 MΩ was used for the preparation of all aqueous solutions.

The 1.0 mmol L^{-1} stock solutions of VMA and HVA (both Sigma-Aldrich, USA) were prepared by dissolving the pure substance in deionized water and stored in the dark at 6 ± 2 °C. More dilute solutions of HVA and VMA were prepared by an appropriate dilution of the stock solutions with deionized water or buffer.

The following solutions were also prepared: 1.0 mol L^{-1} and 0.1 mol L^{-1} potassium chloride (Lachema, Czech Republic (CZ)), 0.1 mol L^{-1} sodium hydroxide, 0.1 mol L^{-1} sulfuric acid (both Penta, CZ), 0.1 mol L^{-1} potassium nitrate (Riedel-de Haën, Germany) in deionized water, 1.0 mmol L^{-1} potassium hexacyanoferrate (Lachema, CZ) in 0.1 mol L^{-1} KCl and in 1.0 mol L^{-1} KCl. Britton – Robinson (BR) buffers were prepared by mixing the acidic component consisting of three acids, 0.04 mol L^{-1} boric acid (Lach-Ner, CZ), 0.04 mol L^{-1} ortho-phosphoric acid (Penta, CZ), and 0.04 mol L^{-1} acetic acid (Lachema, CZ), with 0.2 mol L^{-1} sodium hydroxide to achieve desired pH value. A solution of 0.1 mol L^{-1} phosphate buffer pH 3.0 was prepared in an analogous way by diluting 85% ortho-phosphoric acid in deionized water and adjusting to the desired pH with the concentrated sodium hydroxide solution; 0.025 mol L^{-1} phosphate buffer pH 5.5 was prepared by weighing the required amount of potassium dihydrogen phosphate (Riedel-de Haën, Germany) and dipotassium hydrogen phosphate trihydrate (Panreac, Spain) and dissolving them in 0.1 mol L^{-1} potassium nitrate solution.

For GCE surface modification, 1% (*v*/*v*) Nafion solution (5% *v*/*v*, Sigma Aldrich, UK) prepared in pure ethanol (Merck, Germany) and 1 mmol L^{-1} neutral red monomer (65% dye content, Aldrich, Germany) solution in 0.025 mol L^{-1} phosphate buffer pH 5.5 + 0.1 mol L^{-1} KNO_3 were used.

2.2. Instrumentation

Voltammetric measurements were carried out using a computer-driven Eco-Tribo Polarograph with PolarPro 5.1 software (Eco-Trend Plus, Prague, CZ) and an IviumStat electrochemical analyzer controlled by software IviumSoft version 2.024 (Ivium Technologies, The Netherlands).

A conventional three-electrode arrangement was used with a silver chloride reference electrode (Ag | AgCl | 3 mol L^{-1} KCl), a platinum wire serving as an auxiliary electrode (both Elektrochemické detektory, Turnov, CZ), and a commercially available BDD electrode with 3 mm diameter, area of 7.1 mm^2 and boron doping level ~0.1% (Windsor Scientific, UK) and a GCE with 1 mm diameter, area of 0.79 mm^2 (Bio-Logic Science Instruments, France), both non-modified and modified, were employed as the working electrodes.

The pH measurements were performed using digital pH-meters with a combined glass electrode (Jenway, UK, and Crison, Spain). All experiments were carried out at laboratory temperature (25 ± 1 °C).

2.3. Procedures

The BDD electrode was pre-treated at E_{ACT} = +2.4 V for t = 20 min in 0.1 mol L^{-1} H_2SO_4 to obtain an oxidized surface. Between individual scans, the BDD electrode was activated directly in the stirred analysed solution applying E_{ACT} = +2.4 V for t = 30 s if not mentioned otherwise. Differential pulse voltammetry (DPV) at BDD electrodes was performed using pulse height + 50 mV, pulse width 80 ms followed by sampling time 20 ms, and scan rate v = 20 mV s^{-1}.

DPV at GCE was carried out at the scan rate v = 4 mV s^{-1}, pulse height + 50 mV, pulse width 50 ms, and sampling time 2 ms. Before each experiment, the surface of non-modified GCE was polished manually using sequentially smaller particles of diamond spray (3 μm and 1 μm, Kemet International, UK) and an aqueous slurry of alumina powder (0.05 μm, BDH Chemicals, UK) for a total duration of approximately 5 min, then rinsed thoroughly with deionized water. When necessary, potential cycling in 0.1 mol L^{-1} sodium hydroxide solution within the potential range from −1.0 V to +1.0 V at scan rate v = 200 mV s^{-1} was used. All modified GCEs were employed directly; the polishing step was prior to surface modification. Modified GCEs were prepared freshly before each measurement.

Nafion/GCE was prepared applying 10 μL of 1% (*v*/*v*) Nafion solution, resulting from dilution of 60 μL 5% (*v*/*v*) Nafion monomer in 240 μL of pure ethanol, at polished GCE surface and leaving the single layer coating to dry at room temperature for two hours.

To prepare PNR/GCE, the polished GCE was first subjected to cycling in the potential range from −1.0 V to +1.0 V at the scan rate of v = 100 mV s^{-1} in supporting electrolyte of the same composition as used for electropolymerization, *i.e.* 0.025 mol L^{-1} phosphate buffer pH 5.5 + 0.1 mol L^{-1} KNO_3, until stable voltammograms were obtained (usually 15 cycles). Electrochemical polymerization of neutral red [63] was then carried out during 20 cycles in the potential range from −1.0 V to +1.0 V at the scan rate of v = 50 mV s^{-1} in 1 mmol L^{-1} neutral red monomer solution in 0.025 mol L^{-1} phosphate buffer pH 5.5 + 0.1 mol L^{-1} KNO_3. PNR film was thus formed directly on the surface of the GCE.

The concentration dependences were constructed from the average

of four replicate measurements for each calibration solution of HVA and VMA and evaluated by the least squares linear regression method. The limits of detection (LOD) were calculated as a threefold and the limits of quantification (LOQ) as a tenfold of the standard deviation *s* of the peak currents (ten runs) of the lowest measurable concentration, divided by the slope of corresponding calibration curve *k*.

3. Results and discussion

3.1. Cyclic voltammetry at GC and BDD electrode and oxidation mechanism of HVA and VMA

Cyclic voltammograms of HVA and VMA ($c = 0.1\ \mathrm{mmol\,L^{-1}}$ of each) at non-modified GCE were recorded at a scan rate of $v = 500\ \mathrm{mV\,s^{-1}}$ from 0 V to +1.9 V, *i.e.* to the end of the potential window (Fig. 1C+D). The HVA oxidation results in one anodic peak +0.670 V in the first scan in the positive direction, an indistinctive cathodic peak at +0.350 V in the reverse scan, and second anodic peak at +0.415 V in consecutive scans. A similar electrochemical behaviour of HVA was observed at GCE modified by the polymer of Alizarin Red S [43]. Presumably, oxidation of HVA leads to formation of 4-aceto-*o*-quinone, providing a base for the 3,4-dihydroxyphenylacetic acid/4-aceto-*o*-quinone redox couple being oxidized/reduced at lower potentials than the parent HVA. The formation of the quinone species is in agreement with a mechanism involving oxidation of substituted *o*-methoxyphenols [64]. During the two-electron two-proton oxidation in acidic media, first a phenoxy radical is formed and then a carbocation at the aromatic ring. The carbocation is attacked by a water molecule acting as a nucleophile resulting in methoxy group loss, and corresponding *o*-quinones are formed [65]. The other possibility is stabilization of the emerging phenoxy radical by reaction with the parent compound resulting in the formation of dimers and/or polymers possibly causing passivation of the electrode surface [66–68].

In contrast to HVA, the oxidation of VMA at non-modified GCE results in two anodic peaks at potential values of +0.665 V and +0.885 V in all successive cycles, but no cathodic peak is observed. Electrooxidation of VMA was closely examined at an edge-plane pyrolytic graphite electrode [29] and carbon composite film electrode [56] where VMA has two oxidation peaks in contrast to only one peak of HVA. This difference in HVA and VMA voltammetric responses is caused by the presence of the hydroxyl group in the alpha position in the VMA structure allowing molecule re-aromatization through tautomerization after the first $2e^-$, H^+ oxidation step, leading to a carbocation. It is unstable and readily undergoes decarboxylation under formation of vanillin. Vanillin is subsequently oxidized ($2e^-$, $2H^+$ oxidation, leading to substituted *o*-quinone species), thus creating the second peak at the voltammograms [29]. For HVA, which lacks the hydroxyl group in the alpha position, the rate of the re-aromatization step is much slower and requires an additional water molecule to produce vanillic alcohol [29]. Therefore, no further peaks are observed in the voltammograms of HVA recorded at the above-mentioned carbon-based electrode materials containing predominantly the sp^2-hybridized carbon atoms.

Cyclic voltammograms of HVA and VMA ($c = 0.1\ \mathrm{mmol\,L^{-1}}$ of each) at an anodically oxidized BDD electrode (Fig. 1A+B) show only one anodic peak at around +0.980 V and +1.160 V, respectively. On further cycling, no other peaks were observed that could be due to tested analytes, but the height of the peak decreases continuously, confirming electrode fouling. The oxidation potentials at BDD electrodes are approximately 0.3 V (HVA) and 0.5 V (VMA) more positive than at GCE, and also at other sp^2 carbon-based electrodes investigated previously (see Table 1). Regarding data in literature, peak potential values are +0.700 V, +0.720 V, and +0.380 V for HVA, and +0.680 V, +0.720 V, and +0.370 V for VMA (in acidic media, E_p *vs.*

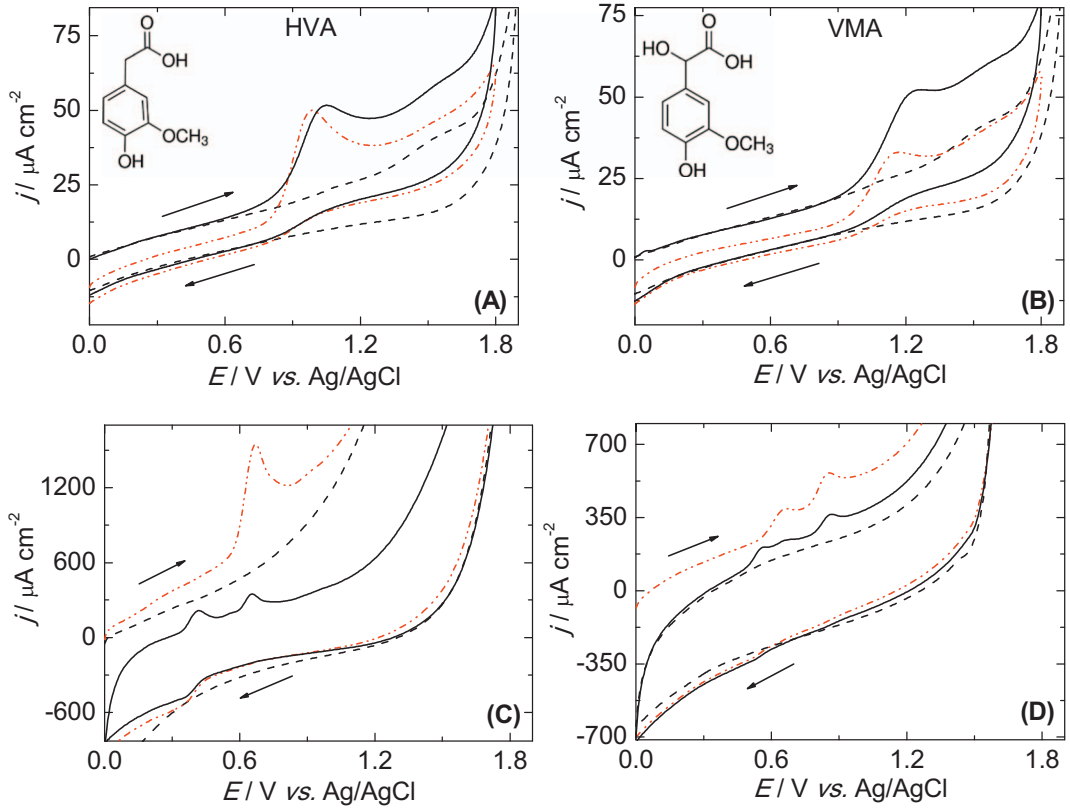

Fig. 1. Cyclic voltammogram of (A, C) HVA and (B, D) VMA ($c = 0.1\ \mathrm{mmol\,L^{-1}}$ of each) in $0.1\ \mathrm{mol\,L^{-1}}$ phosphate buffer pH 3.0 recorded at (A, B) anodically oxidized BDD electrode at the scan rate $v = 100\ \mathrm{mV\,s^{-1}}$; (C, D) non-modified GCE at the scan rate $v = 500\ \mathrm{mV\,s^{-1}}$. Displayed are the first (red, dot-dashed line) and the second (black, solid line) cycle. Dashed line corresponds to supporting electrolyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Overview of electroanalytical methods established for HVA and VMA determination using non-modified and modified carbon-based working electrodes.

Analyte	Modification/working electrode	Supporting electrolyte	Method	E_p *vs.* Ag/AgCl (V)	LOD (μmol L^{-1})	Ref.
HVA	poly(3-amino-5-mercapto-1,2,4-triazol)/GCE	0.2 mol L^{-1} PB pH 7.2	ChA	+0.420	$9.4{\cdot}10^{-5}$	[44]
	poly(Alizarin Red S)/GCE	0.1 mol L^{-1} PB pH 3.0	DPV	+0.662	$1.7{\cdot}10^{-2}$	[43]
	Cu/GCE	0.1 mol L^{-1} PB pH 7.2	SWV	+0.590[b]	$1.0{\cdot}10^{-2}$	[59]
	molecularly imprinted polymer/GCE	0.1 mol L^{-1} citric acid/HCl pH 1.1 with 40% acetonitrile	DPV	+0.810	$7.0{\cdot}10^{-3}$	[61]
	composite carbon film electrode	BR buffer pH 2.0	DPV	+0.700	0.1	[56]
	carbon paste electrode (CPE)	BR buffer pH 2.0	DPV	+0.720	0.4	[58]
	phosphatidylethanolamine/CPE	PB pH 7.4	CV	+0.550[b]	3.0	[45]
	screen-printed electrode	BR buffer pH 3.0	DPV	+0.380[c]	0.2	[57]
	BDD electrode	0.1 mol L^{-1} PB pH 3.0	DPV	+0.835	0.6	[a]
	GCE	0.1 mol L^{-1} PB pH 3.0	DPV	+0.542	0.9	[a]
	Nafion/GCE	0.1 mol L^{-1} PB pH 3.0	DPV	+0.540	0.8	[a]
	PNR/GCE	0.1 mol L^{-1} PB pH 3.0	DPV	+0.540	1.2	[a]
VMA	molecularly imprinted polymer/GCE	0.025 mol L^{-1} citric buffer pH 3.0 with 10% acetonitrile	DPV	+0.845	–	[60]
	composite carbon film electrode	BR buffer pH 2.0	DPV	+0.680	0.2	[56]
	carbon paste electrode	BR buffer pH 2.0	DPV	+0.720	0.6	[58]
	screen-printed electrode	BR buffer pH 3.0	DPV	+0.370[c]	0.1	[57]
	MWCNTs/screen-printed electrode	1 mmol L^{-1} PB pH 6.85	DPV	+0.380[b]	1.0	[29]
	edge-plane pyrolytic graphite electrode	1 mmol L^{-1} PB pH 6.85	DPV	+0.430[b]	1.7	[29]
	BDD electrode	0.1 mol L^{-1} PB pH 3.0	DPV	+1.005	0.4	[a]
	GCE	0.1 mol L^{-1} PB pH 3.0	DPV	+0.504	1.5	[a]
	Nafion/GCE	0.1 mol L^{-1} PB pH 3.0	DPV	+0.494	2.4	[a]
	PNR/GCE	0.1 mol L^{-1} PB pH 3.0	DPV	+0.494	1.1	[a]

PB (phosphate buffer); ChA (chronoamperometry); CPE (carbon paste electrode); MWCNTs (multi-walled carbon nanotubes).
[a] This work.
[b] E_p (V) *vs.* saturated calomel electrode.
[c] E_p (V) *vs.* silver electrode.

Ag/AgCl) at composite carbon film [56], carbon paste [58], and screen-printed electrodes [57], respectively. This positive potential shift in comparison with other carbonaceous electrode materials has also been observed for other organic molecules, *e.g.*, dopamine [69,70], ascorbic acid [70,71] or phenolic compounds as dimethoxyphenol [72], dichlorophenol, or dimethylphenol [73], presumably due to the heterogeneous surface of BDD possessing reduced number of charge carriers and lower density of electronic states (DOS). It is at least two orders of magnitude lower for BDD electrodes than for metals such as Pt, Au, typically having DOS of $\sim 10^{23}\,cm^{-3}\,eV^{-1}$ [74], and disordered graphitic sp^2 structures as glassy carbon [75] and may lead to decreased electron transfer rates and higher overpotentials for redox reactions at BDD electrodes in comparison to other electrode materials nearing metallic conductivity. As consequence, the second oxidation signal of VMA present at other carbon-based materials is not developed at BDD within its potential window.

3.2. Influence of pH on the electrochemical behaviour of HVA and VMA

DP voltammetric responses of HVA and VMA (c = 0.1 mmol L^{-1} of each) were recorded at non-modified GCE and BDD electrode in BR buffer of pH 2.0–12.0. Marked differences between the investigated electrode materials were revealed in the number of voltammetric signals, their potentials, and current densities as is clear from Fig. 2.

HVA shows only one oxidation peak at non-modified GCE over the whole studied pH range, within the potential range from +0.210 V to +0.630 V (Fig. 2C). The peak is best developed in acidic media of pH 2.0 and 3.0. With increase of pH, the peak potential of HVA shifts to lower values and the peak current dramatically decreases; in neutral and basic solutions the magnitudes of the obtained signals are just several tens of nA (Fig. A1 in Supplementary material).

Dependences of peak potential, E_p, of HVA on pH (Fig. 2E) are linear in the ranges pH 2.0–6.0 and pH 7.0–12.0 and can be described by the following equations (Eq. 1, Eq. 2):

$$E_p(mV) = (-60 \pm 4){\cdot}pH + (741 \pm 16)(R = 0.992) \quad (1)$$

$$E_p(mV) = (-55 \pm 4){\cdot}pH + (864 \pm 35)(R = 0.989) \quad (2)$$

Both slope values are close to the theoretical value of 59 mV/pH which indicates oxidation mechanism comprising an equal number of electrons and protons, *i.e.* two-electron two-proton overall oxidation yielding the 4-aceto-*o*-quinone and releasing methanol as described in [43,45,64] and in chapter 3.1. This mechanism is supported by the fact that the peak widths of DP voltammograms at half height are 45–50 mV for the whole investigated pH range.

In contrast, at the BDD electrode, one to three oxidation peaks of HVA are observable and their potential is either independent of pH or shifts slowly to more positive values with increase of pH. The first oxidation peak is best developed in acidic solutions of pH 2.0–4.0 in the potential range from +0.805 V to +0.825 V. The position of this main peak gradually shifts to higher potential values up to +1.010 V with increasing pH. However, from pH 5.0 the shape of the peaks deteriorates and a smaller peak in the region of more positive potentials appears from +1.420 V to +1.440 V. In basic media of pH 10.0–12.0, a third signal appears in the region from +1.240 V to +1.280 V. Importantly, these changes in electrochemical behaviour visualized by change in number of oxidation signals occur in media of pH values corresponding to pK_A values of HVA (pK_{A1} = 4.35 for dissociation of the carboxylic group, pK_{A2} = 10.34 for the phenolic group). Two factors should be considered to give an insight into differences in oxidation mechanism of HVA on GCE and BDD: (i) different chemical functionalization of the electrode surface; (ii) structure of the compound in media of varying pH in dependence on pK_A values of acidic groups in the molecule.

For BDD, anodic pre-treatment resulting in O-terminated surface containing C–OH, C–O–C, C=O and COOH [76] groups was applied. Such surface has low conductivity, it is relatively hydrophilic and bears partially negative charge [77]. Naturally, these properties influence strongly interactions between the surface and the analyte or its reaction intermediates, as well as electron transfer kinetics. This is obvious from Fig. 4, where is depicted the comparison of HVA signals at anodically pre-treated and polished BDD surface. On the latter surface, lacking the COOH functionalities and having the other carbon-oxygen bonds reduced in number according to [76], more positive oxidation potential was obtained for HVA. Interestingly, for *m*-cresol opposite effect, *i.e.* negative shift of peak potential on polished surface in comparison with

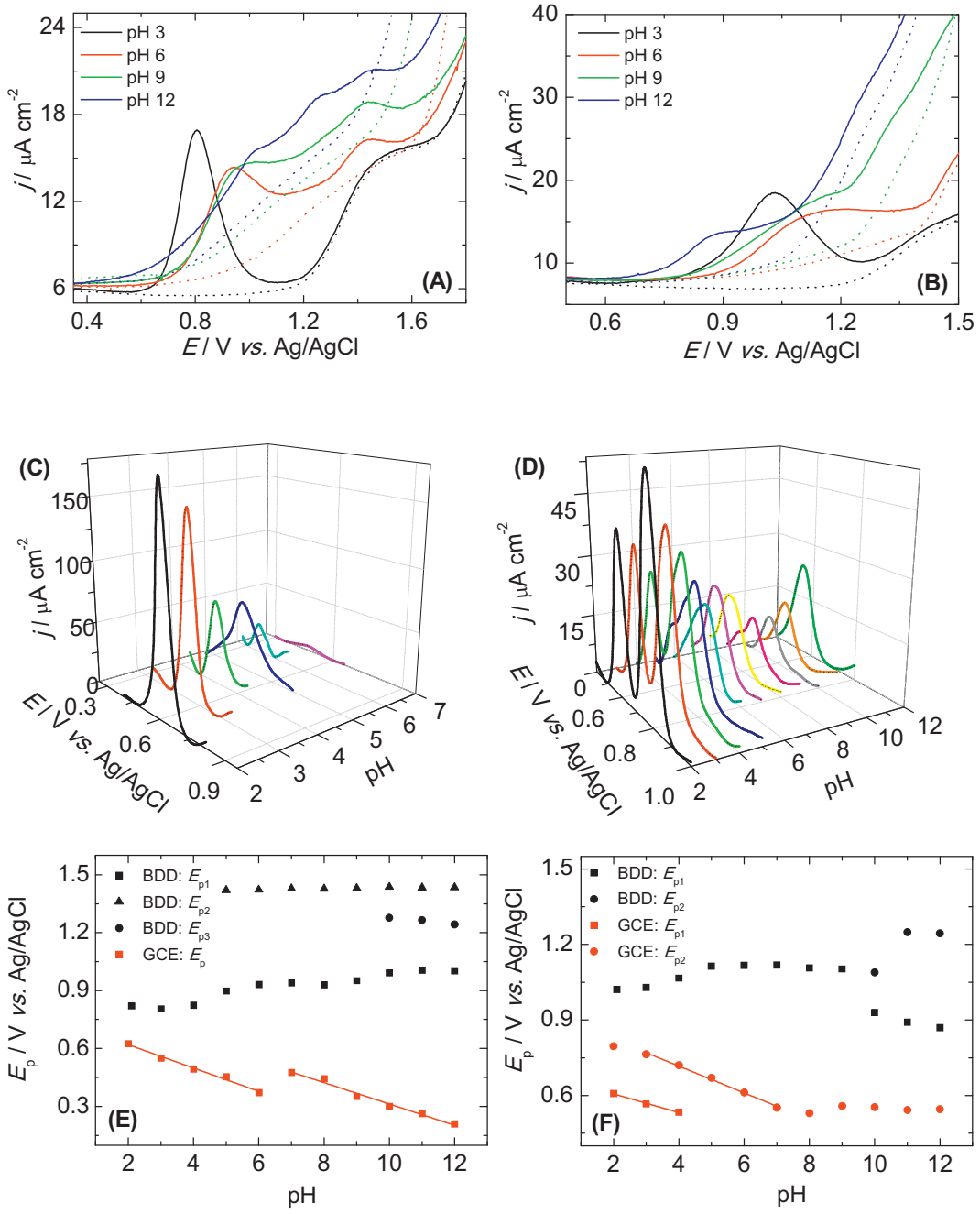

Fig. 2. DP voltammograms of (A, C) HVA, (B, D) VMA (c = 0.1 mmol L^{-1} of each; depicted by solid lines) recorded at (A, B) anodically oxidized BDD electrode, and (C, D) non-modified GCE in BR buffer solutions of pH values in the range of 2.0–12.0. Dotted lines in (A) and (B) present supporting electrolytes. The dependences of peak potentials E_p on pH for (E) HVA and (F) VMA (c = 0.1 mmol L^{-1} of each) recorded at (black) anodically oxidized BDD electrode and (red) non-modified GCE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

O-terminated surface was reported [78], emphasizing the importance of carboxylic group in the molecule for the analyte-BDD surface interaction. Naturally, these interactions can influence also the stability of the intermediates, and thus have an impact on the oxidation mechanism. It seems that at anodically pre-treated BDD, in contrast to GCE, corresponding *o*-quinones are not the final oxidation products. This can be deduced from the absence of their voltammetric signals in CVs in Fig. 1A + B and from Fig. 3 depicting consecutive DPV scans of HVA. On the other hand, DP voltammograms of HVA recorded at BDD exhibit one oxidation peak only in acidic media and more oxidation signals in anodic scan at higher pH values, changing in number around pK_A values of HVA as described above. These peaks are indistinctive and correspond probably to further oxidation processes of the products formed by reaction of phenoxy radical, the first intermediate of HVA oxidation, with the parent compound or other intermediates. Nevertheless, the negative charge due to dissociation of carboxylic and phenolic moiety of HVA is presumably the reason for decrease of the signals as electrostatic repulsion between the anodized HVA and the partially negative charge at BDD surface is expected, as reported for other carboxylic acids [79].

Apparently, explanation of oxidation mechanism of HVA at BDD electrode requires more extended study involving the examination of the influence of surface pre-treatment. This could explain not only the changes in number of oxidation peaks in the pH range close to pK_A values of HVA but also the slight positive shift of the first oxidation peak, corresponding to initial oxidation of phenolic moiety and

typically exhibiting negative potential shift as observed for GCE in this study and for other carbonaceous materials [56,58].

VMA, similarly to HVA, shows the best voltammetric responses in acidic medium at both non-modified GCE and anodically oxidized BDD electrode. At GCE, dependences of peak potentials E_{p1} of VMA on pH (Fig. 2F) in the range of pH 2.0–4.0 and for its second peak E_{p2} on pH within the range 3.0–7.0 are linear (see Eq. 3 and Eq. 4):

$$E_{p1}(mV) = (-37 \pm 3)\cdot pH + (680 \pm 9)(R = 0.994) \quad (3)$$

$$E_{p2}(mV) = (-53 \pm 2)\cdot pH + (930 \pm 10)(R = 0.997) \quad (4)$$

Two clearly developed peaks as a result of VMA oxidation at GCE are observed only in acidic media of pH 2.0–4.0 (Fig. 2D and F). According to literature [29], the first peak can be assigned to the $2e^-$, H^+ oxidation process through unstable carbocation and re-aromatization of the molecule to vanillin, the second peak presumably corresponds to the further oxidation of formed vanillin (see Section 3.1). With increasing pH, *i.e.* from pH 5.0 and higher, overlaying of the recorded peaks and their deformation arises, hence VMA oxidation results in only one peak, with almost constant potential value of +0.545 ± 0.015 V within the pH range 7.0–12.0.

Unlike at GCE, at anodically oxidized BDD only one peak appears as the result of VMA oxidation, at relatively high positive potentials from +1.020 V to +1.120 V. The peaks are well-developed only in acidic media pH 2.0–4.0 for the non-ionized form of the acid (pK_{A1} = 3.44 for carboxylic group). At higher pH values, when VMA occurs in anionic form and can be repelled from anodically activated BDD surface containing negatively charged oxygen functionalities, the elongated shape of the DPV signals is observed, indicating slow electron transfer kinetics. Further, subtle second peaks appear in the range of pH 10.0 to 12.0 for dianion of the acid (pK_{A2} = 9.93 for the phenolic group). As in the case of HVA, obviously the form of the acid influences its electrochemical behaviour at O-terminated BDD and further studies are required to fully understand this phenomenon.

3.3. Optimization of electrode activation conditions in the presence of HVA

Six consecutive DP voltammograms of HVA (c = 0.1 mmol L^{-1}) in acidic media at GCE and BDD electrode were recorded (Fig. 3). The predictable fouling of surfaces of both carbon electrodes was confirmed; decreases in voltammetric responses were 52% at GCE and 55% at BDD electrode. Simultaneously, two additional peaks at +0.444 V and +0.576 V of increasing height can be discerned from the second scan in DP voltammograms of HVA at GCE. The fouling of both electrodes is caused by the adsorption of reaction intermediates, dimers or polymers formed as intermediates and which are products of reactions of the highly reactive phenoxy radical, the first oxidation product of HVA and VMA (see Section 3.1).

Based on the aforementioned findings, various activation techniques to prevent unfavourable electrode fouling were investigated. Mechanical polishing of the surface of GCE using successively smaller particles of diamond spray (3 μm and 1 μm) and alumina (0.05 μm) was proven to be the only suitable way for its cleaning. The HVA peak parameters from six DP voltammetric scans measured at GCE while applying the mechanical polishing procedure between the individual scans were as follows: E_p (V) = +0.542 ± 0.004 (s_r = 0.73%) and j_p (μA cm^{-2}) = 126.9 ± 14.2 (s_r = 10.99%). For the BDD electrode, four activation approaches were tested (compare DP voltammograms in Fig. 4 and peak potentials and current densities in Table 2). These were stirring of the analysed solution, polishing on alumina slurry (0.3 μm), and electrochemical activation *in-situ* both in cathodic regime (at a potential corresponding to hydrogen evolution [80,81]) and anodic regime (at the potential of water decomposition thus leading to production of highly reactive hydroxyl radicals [76,82,83]).

All approaches have led to highly repeatable signals characterized by $s_r \leq 4\%$ for the current densities; however, the highest signal/background ratio was achieved when anodic activation was applied. At the hydrogen-terminated BDD electrode, the increment of the supporting electrolyte background current reduced the peak current of HVA by almost 28%, and the peak potential was shifted by approximately 0.035 V to more positive values in comparison with the oxidized BDD surface. A bigger shift of the peak potential of HVA to more positive values by more than 0.260 V and a decrease of the voltammetric response by roughly 50% occurs on a polished BDD surface, where the number of carbon-oxygen bonds is either significantly reduced (C—OH, C—O—C, C═O) or they are practically non-existent (COOH) in comparison with an anodically oxidized BDD surface [76]. Such a positive potential shift is surprising since for other phenolic compounds, for instance *m*-cresol, the opposite trend has been observed [78]. Thus, the side chain attached to the aromatic core, rather than phenolic moiety, influences interaction with the polished surface leading to unfavourable oxidation. In general, phenolic compounds seem to be very sensitive to BDD surface termination as differences in oxidation potentials or even their adsorption was observed as strongly depending on surface pretreatment [53,84,85].

3.4. Voltammetric study of HVA and VMA at polymer-modified GC electrodes

Three different modifications of the GCE surface were tested to investigate enhancement of the voltammetric signal in the determination of HVA and VMA. Preliminary measurements on the GCE modified by multi-walled carbon nanotubes revealed such modification not to be appropriate for the electrochemical determination of HVA and VMA because peak currents were almost 50-times lower than at non-modified GCE. This is in contrast to hundreds of research articles claiming about

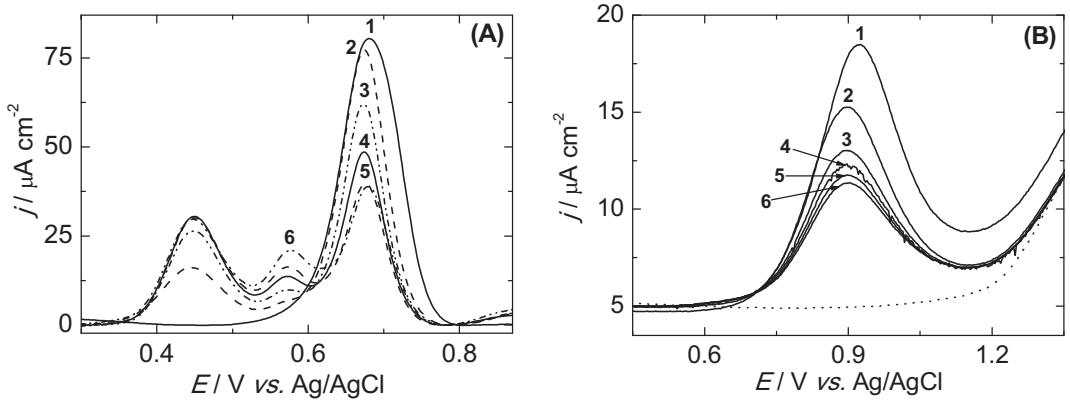

Fig. 3. Six consecutive DP voltammograms of HVA (c = 0.1 mmol L^{-1}) recorded (A) in BR buffer pH 2.0 at GCE, and (B) in BR buffer pH 3.0 at BDD electrode. The numbers at curves denote the scan order. No activation used between the individual scans.

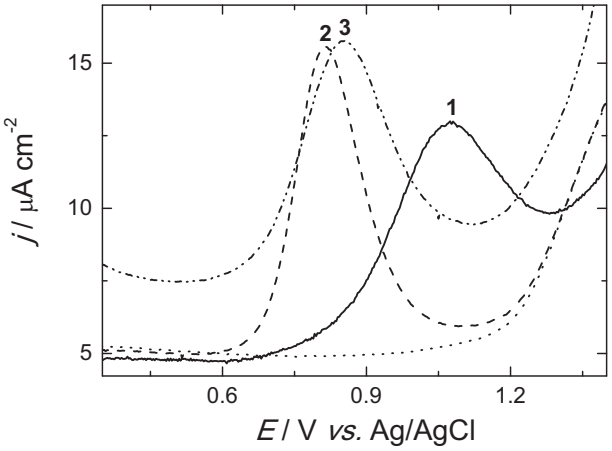

Fig. 4. DP voltammograms of HVA (c = 0.1 mmol L^{-1}) at BDD electrode in BR buffer pH 3.0 using various activation techniques between consecutive scans: (1) polishing on alumina slurry, t = 30 s, (2) stirring, anodic activation, E_{ACT} = +2.4 V, t = 60 s, (3) stirring, cathodic activation, E_{ACT} = −2.4 V, t = 120 s. Dotted line represents supporting electrolyte.

Table 2
Influence of the activation technique applied between the scans recorded at BDD electrode by DP voltammetry on peak potentials E_p and peak current densities j_p of HVA (c = 0.1 mmol L^{-1}) in BR buffer pH 3.0. Medians, confidence intervals (α = 0.05), and relative standard deviations expressed for six measurements (n = 6).

Activation conditions	E_p (V)	s_r (E_p) (%)	j_p (μA cm^{-2})	s_r (j_p) (%)
Polishing on alumina slurry, t = 30 s	1.077 ± 0.017	1.54	4.99 ± 0.19	3.81
Anodic activation + stirring (+2.4 V, t = 60 s)	0.816 ± 0.010	1.17	10.15 ± 0.34	3.34
Cathodic activation + stirring (−2.4 V, t = 120 s)	0.851 ± 0.009	1.07	7.36 ± 0.22	2.89

the advantages of CNTs, such as increased voltammetric currents, electrocatalytic effects and reduced fouling. Nevertheless, it has been pointed out [86,87] that uncertainties still remain regarding the origin of the electrochemical signal obtained at CNTs, hence care should be taken when describing their electrochemistry. It is often stated that the effects are due to the "inherently unique" properties of CNTs, however, fundamental reasons for these "special" characteristics and the apparent improvements in electrode performance have not yet been fully elucidated. Thus, findings regarding electrochemical behaviour of HVA and VMA at functionalised multi-walled CNTs in our study could contribute to the clarification and understanding of the CNT electrochemistry.

Cyclic voltammograms of HVA and VMA recorded in the 0.1 mol L^{-1} phosphate buffer pH 3.0 at Nafion-coated GCE and PNR-modified GCE are shown in Fig. 5. Again, substantial electrode fouling is obvious. Importantly, the electrochemical behaviour at modified GCEs resembles that observed at bare GCE concerning the number of peaks in the first and consecutive cycles (compare with GCE at Fig. 1), and further modifications of GCE have little impact on the peak positions of the examined organic acids. Peak positions of HVA and VMA at Nafion/GCE and PNR/GCE in 0.1 mol L^{-1} phosphate buffer pH 3.0 medium are not influenced by electrostatic interactions due to non-ionized forms of the biomarkers and polymers, so only a slight shift to lower potential values was observed.

In the case of HVA, during the first scan only one anodic peak is discerned at potentials +0.670 V, +0.630 V, and +0.625 V at non-modified GCE, Nafion/GCE and PNR/GCE, respectively. The number of peaks doubles in the second and consecutive cycles, an explanation for which was already given in Section 3.1, and the second peak ensued at following values of potentials +0.415 V, +0.410 V, +0.405 V at non-modified GCE, Nafion/GCE, and PNR/GCE, respectively. VMA shows two anodic oxidation peaks at all electrodes at potential values E_{p1} and E_{p2}: +0.665 V and +0.885 V at non-modified GCE, +0.575 V and +0.845 V at Nafion-coated GCE, +0.555 V, and +0.840 V at GCE modified by PNR, respectively.

3.5. Influence of scan rate

Cyclic voltammograms of HVA and VMA (c = 0.1 mmol L^{-1} of each) in 0.1 mol L^{-1} phosphate buffer pH 3.0 were recorded at the scan rates 5–5120 mV s^{-1} at BDD electrode and 5–2000 mV s^{-1} at non-modified GCE, Nafion/GCE, and PNR/GCE. For both analytes at each electrode, a shift of the signal to more positive potentials with increasing scan rate was observed. Plots of the logarithm of peak current *versus* logarithm of scan rate were constructed since their slope values give information on the type of interfacial process. The theoretical value of 0.5 for diffusion control was approached for HVA at non-modified GCE (0.58), Nafion/GCE (0.59), and PNR/GCE (0.56). Conversely, for VMA the theoretical value of 1.0 for surface-confined processes was approached at non-modified GCE (0.99) and Nafion/GCE (0.88). At PNR/GCE, a mixed adsorption-diffusion process with a slope of 0.73 was observed for VMA. Interestingly, at the BDD electrode values lower than 0.5 were obtained for HVA (0.38) and VMA (0.40). The results indicate that the electrochemical process is markedly influenced by the presence of the hydroxyl group in the alpha position in the structure of VMA. It increases the proclivity to adsorption at non-modified and modified sp^2 carbon electrodes, probably due to the additional dipole-dipole interaction of the VMA molecule with the oxygen functionalities at the GCE surface or the polymeric films. On the other hand, the BDD electrode, considered as rather resistant towards adsorption of small organic compounds at both hydrogen- and oxygen-terminated surfaces exhibits sluggish kinetics of diffusion-controlled process for both studied compounds.

3.6. Concentration dependence

The concentration dependences of HVA and VMA were recorded in 0.1 mol L^{-1} phosphate buffer pH 3.0 within the concentration range of 1–100 μmol L^{-1} using DP voltammetry at all tested electrodes. DP voltammograms corresponding to concentration dependences of both clinical markers recorded at Nafion/GCE are depicted in Fig. A2. Pilot experiments using square-wave voltammetry were performed with both biomarkers at the BDD electrode but no significant improvement of the results was obtained in comparison with DP voltammetry, in agreement with the irreversible character of the redox signals. The analytical parameters and calculated values of LOD and LOQ are summarized in Table 3.

Concentration dependences are divided into two linear regions at non-modified GCE (both HVA and VMA), Nafion/GCE (only HVA), and at PNR/GCE (only VMA). Similar behaviour was also observed for other phenolic compounds at non-modified and modified carbon-based electrodes [88,89]. For VMA itself, two linear sections were obtained at an edge-plane pyrolytic graphite electrode [29]. The BDD electrode shows superiority over the other tested electrodes. Among the advantages clearly belongs ascertained linearity within the whole tested concentration range, which is related to the diffusion-controlled oxidation of HVA and VMA at this type of electrode. The lower sensitivity at the BDD electrode can be ascribed to the lower number of charge carriers at the heterogeneous BDD surface. Additionally, the lowest detection limits for both compounds were obtained at this electrode due to reasonable signal repeatability for the lowest detectable concentrations of HVA and VMA (c = 2 μmol L^{-1}, s_r (HVA) = 8.8% and s_r (VMA) = 11.8%, n = 10). A high signal-to-background current ratio is another recognizable property of BDD electrodes. However, all assessed

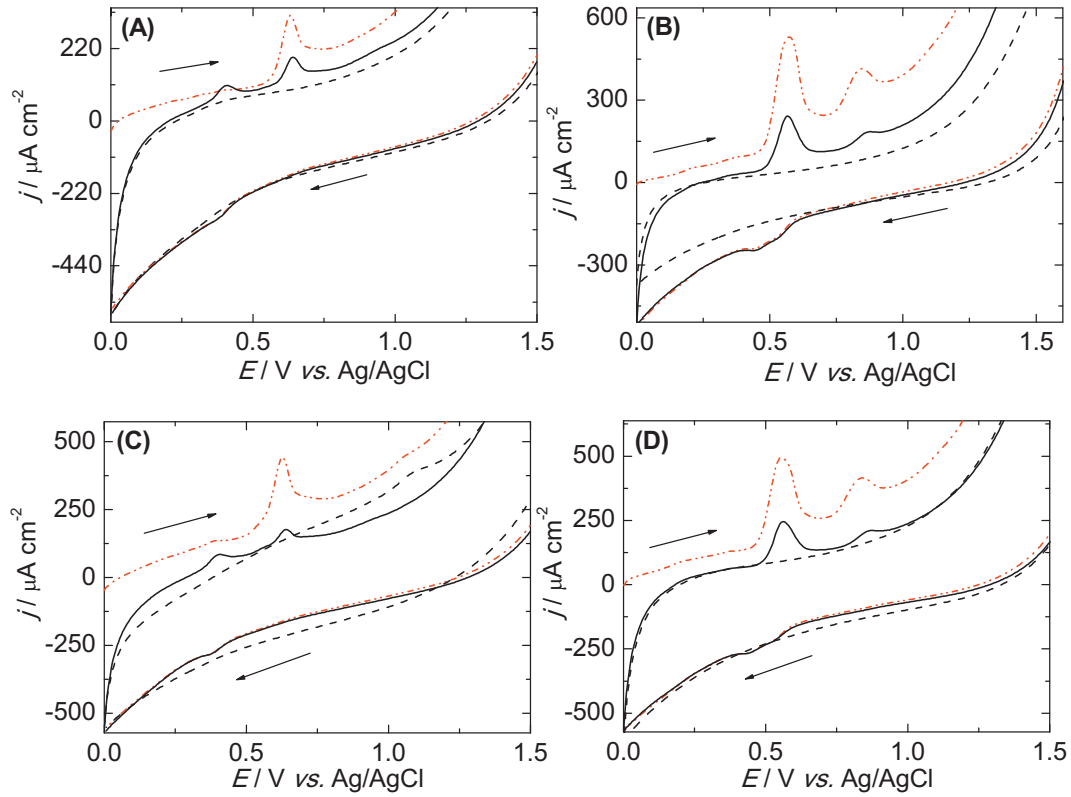

Fig. 5. Cyclic voltammograms of (A, C) HVA and (B, D) VMA (c = 0.1 mmol L^{-1} of each) in 0.1 mol L^{-1} phosphate buffer pH 3.0 recorded at (A, B) Nafion/GCE and (C, D) PNR/GCE at the scan rate v = 500 mV s^{-1}. Displayed are the first (red, dot-dashed line) and the second (black, solid line) cycle. Dashed line corresponds to supporting electrolyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LOQ values at tested carbon-based electrodes are sufficient for the determination of studied diagnostic markers in urine, where the physiological concentration of HVA and VMA is usually at the level of 8.2–41.0 µmol L^{-1} and 11.6–28.7 µmol L^{-1} [7], respectively.

Under pathological conditions, the increase of concentration of both biomarkers may occur and thus for correct diagnosis the determination of HVA/VMA ratio is essential. Hence, the possibility of the simultaneous determination of HVA and VMA (both c = 0.1 mmol L^{-1}) in their mixture prepared in 0.1 mol L^{-1} phosphate buffer pH 3.0 was verified at all tested electrodes by DP voltammetry (Fig. 6). In the pH 3.0 medium, the HVA oxidation peak and the first peak of VMA occur at very close potentials and could not be separated at non-modified GCE. At Nafion/GCE and PNR/GCE the individual signals of the two biomarkers are observable. At the BDD electrode, the difference of peak potentials reaches nearly 0.22 V and enables distinguishing the responses of HVA and VMA even though partial overlapping of the peaks is still present. Therefore, there is a chance for simultaneous voltammetric determination of both biomarkers at a properly selected working electrode. This topic is under further investigation.

4. Conclusion

This work comprises the electrochemical investigation of the products of the catecholamine metabolism that serve as important clinical tumour biomarkers, HVA and VMA, at BDD electrode, at non-modified GCE, and GCE modified by Nafion and by poly(neutral red).

The electrochemical behaviour of HVA and VMA is significantly influenced not only by the pH value of the aqueous medium, but also by the electrode material. Anodic oxidation of HVA and VMA at non-modified and modified GCE took place at considerably lower potentials and peak current densities are higher than at BDD electrodes, presumably due to the hindrance of electron transfer at the heterogeneous

Table 3
Parameters of concentration dependences of HVA and VMA recorded by DP voltammetry in 0.1 mol L^{-1} phosphate buffer pH 3.0, with evaluated LOD and LOQ values.

Compound	Electrode	Linear dynamic range (µmol L^{-1})	Intercept (µA cm^{-2})	Slope (µA L µmol^{-1} cm^{-2})	R	LOD (µmol L^{-1})	LOQ (µmol L^{-1})
HVA	BDD	2–100	−0.34 ± 0.06	0.093 ± 0.001	0.999	0.6	1.9
	GCE	2–10	−0.73 ± 0.62	2.80 ± 0.09	0.998	0.9	3.0
		10–100	21.15 ± 2.61	1.11 ± 0.04	0.996		
	Nafion/GCE	2–10	0.83 ± 0.43	1.76 ± 0.06	0.997	0.8	2.8
		10–100	9.16 ± 1.38	1.07 ± 0.03	0.999		
	PNR/GCE	4–100	0.99 ± 0.93	0.82 ± 0.01	0.998	1.2	4.1
VMA	BDD	2–100	−0.44 ± 0.09	0.101 ± 0.001	0.999	0.4	1.4
	GCE	6–10	−2.62 ± 0.46	1.13 ± 0.05	0.998	1.5	5.0
		10–100	3.26 ± 1.17	0.48 ± 0.03	0.996		
	Nafion/GCE	8–100	1.45 ± 0.68	0.50 ± 0.01	0.998	2.4	8.1
	PNR/GCE	4–10	−1.96 ± 0.14	1.36 ± 0.03	1.000	1.1	3.5
		20–100	2.48 ± 0.50	0.55 ± 0.01	1.000		

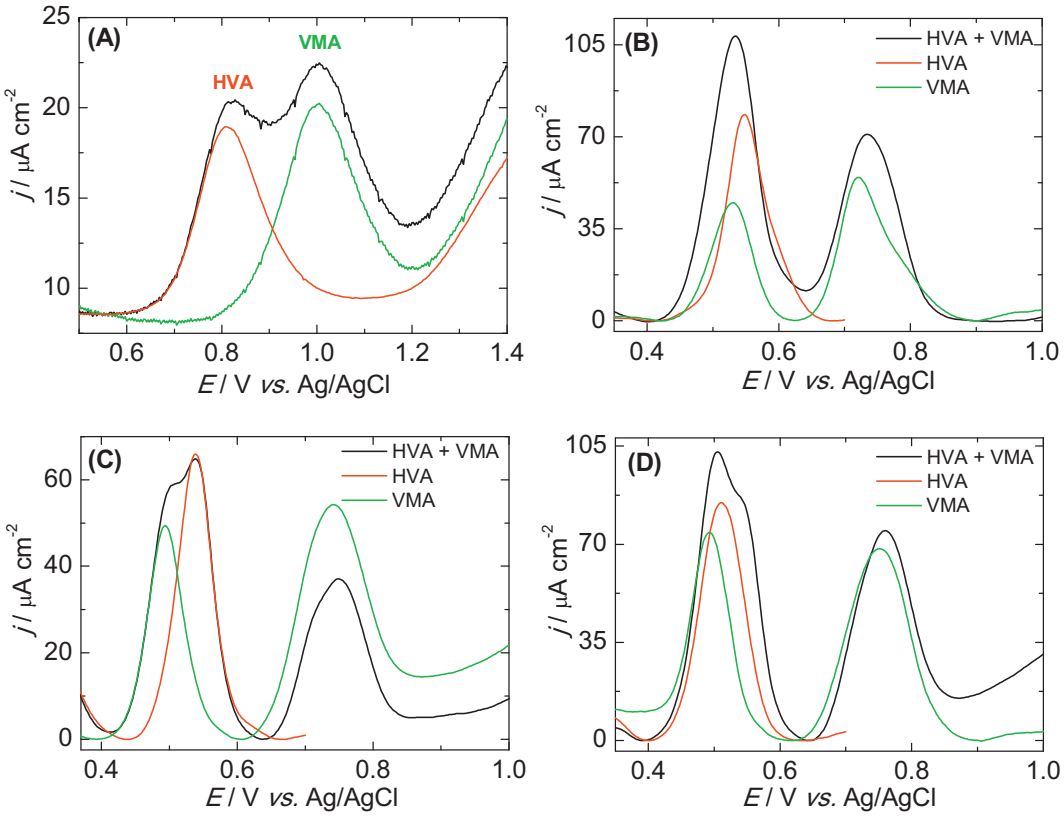

Fig. 6. DP voltammograms of HVA and VMA mixture (both c = 0.1 mmol L^{-1}) in 0.1 mol L^{-1} phosphate buffer pH 3.0 recorded at (A) anodically oxidized BDD electrode, (B) non-modified GCE, (C) Nafion/GCE, and (D) PNR/GCE.

BDD surface. The voltammetric response of HVA and VMA is also strongly dependent on the pre-treatment of the BDD surface, confirming the sensitivity of phenolic compounds to the content of oxygen-containing groups at the BDD surface. For all electrode materials, the highest and most well-defined voltammetric signals of HVA and VMA were obtained in acidic media where the analytes are in their non-ionized form, 0.1 mol L^{-1} phosphate buffer pH 3.0 being selected as optimal supporting electrolyte. In such a medium, the anodically oxidized BDD electrode is also able to partially distinguish the individual voltammetric responses of the biomarkers; nevertheless, another separation step is necessary for reliable resolution of both compounds.

Limits of quantification at all investigated electrodes are sufficient for the determination of both metabolites in urine. A linear dynamic range from 2 to 100 µmol L^{-1} was achieved and the lowest values of LOD of 0.6 µmol L^{-1} and 0.4 µmol L^{-1} for HVA and VMA, respectively, were obtained at anodically oxidized BDD electrode using DP voltammetry thus over performing the other tested electrodes from the analytical point of view. Exceptional advantages of BDD electrodes are undeniably no need to modify its surface, so time-consuming modification procedures are not required. The BDD electrode possesses sufficient sensitivity, and due to the possibility of electrochemical activation *in-situ* also shows resistance towards electrode fouling leading to a stable response and good repeatability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jelechem.2018.03.011.

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