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VOLTAMMETRIC AND AMPEROMETRIC DETERMINATION OF HOMOVANILLIC, VANILLYLMANDELIC, AND 5-HYDROXYINDOLE-3-ACETIC ACID

VOLTAMETRICKÉ A AMPEROMETRICKÉ STANOVENÍ HOMOVANILOVÉ, VANILMANDLOVÉ A 5-HYDROXY-3-INDOLOCTOVÉ KYSELINY

Ph.D. Thesis

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I also declare that neither this PhD Thesis nor its significant part has been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Prague, 10th June 2020

RNDr. Anna Němečková

This dissertation is based on experiments carried out in the period from 2015 to 2020 at the Charles University, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry. During this period, the research visit in the laboratory of Prof. Dr. Frank-Michael Matysik at the University of Regensburg, Faculty of Chemistry and Pharmacy, Institute of Analytical Chemistry, Chemo- and Biosensors, Regensburg, Germany, was completed. Further experiments were done at the J. Heyrovský Institute of Physical Chemistry of the Czech Academy of Sciences.

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Abstract

Presented dissertation thesis is focused on the development of electrochemical methods for the determination of three important tumour biomarkers, namely homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindole-3-acetic acid (5-HIAA).

First part of the study is focused on electrochemical behaviour of these analytes in batch arrangement using differential pulse voltammetry (DPV) at screen-printed carbon electrodes (SPCEs). It has been proved that presented method is sufficiently sensitive for monitoring above mentioned analytes. Moreover, it can be used for determination of HVA and VMA in mixture. Obtained limits of detection (*LOD*s) were 0.24 $\mu mol \cdot L^{-1}$ for HVA, 0.06 $\mu mol \cdot L^{-1}$ for VMA, and 0.12 $\mu mol \cdot L^{-1}$ for 5-HIAA.

The requirements to speed up the analysis and at the same time to reduce its price initialized our study of the determination of tested biomarkers in flow systems. Firstly, flow injection analysis with amperometric detection was investigated for the determination of all three biomarkers at the same SPCE, and then an analogous determination of structural more similar pair, HVA and VMA, was performed at a boron doped diamond electrode (BDDE). Obtained *LOD*s of optimized methods were as follows: at SPCE 0.07 $\mu mol \cdot L^{-1}$ for HVA, 0.05 $\mu mol \cdot L^{-1}$ for VMA, and 0.03 $\mu mol \cdot L^{-1}$ for 5-HIAA, respectively; at BDDE 0.44 $\mu mol \cdot L^{-1}$ for HVA and 0.34 $\mu mol \cdot L^{-1}$ for VMA, respectively.

Furthermore, the determination of monitored biomarkers in human urine by HPLC with amperometric detection at a glassy carbon electrode was studied. After its optimization and the development of a simple urine samples pre-treatment procedure, a rapid determination of all three analytes in one chromatographic run of the urine sample was successfully performed with *LOD*s 11.0 $\mu mol \cdot L^{-1}$ for HVA, 5.0 $\mu mol \cdot L^{-1}$ for VMA, and 8.3 $\mu mol \cdot L^{-1}$ for 5-HIAA, respectively.

Abstrakt

Předložená disertační práce je zaměřena na vývoj elektrochemických metod stanovení biomarkerů nádorových onemocnění: homovanilové (HVA), vanilmandlové (VMA) a 5-hydroxy-3-indoloctové (5-HIAA) kyseliny.

V první části práce bylo zkoumáno elektrochemické chování těchto analytů ve vsádkovém uspořádání pomocí diferenční pulsní voltametrie (DPV) na sítotiskových uhlíkových elektrodách (SPCEs). Bylo prokázáno, že toto stanovení je dostatečně citlivé pro sledování těchto biomarkerů a že může být použito i pro stanovení HVA a VMA ve směsi. Získané meze detekce (*LOD*) byly 0,24 $\mu mol \cdot L^{-1}$ pro HVA, 0,06 $\mu mol \cdot L^{-1}$ pro VMA a 0,12 $\mu mol \cdot L^{-1}$ pro 5-HIAA.

Požadavky na zrychlení analýzy a zároveň snížení její ceny byly impulsem pro stanovení vybraných analytů v průtoku. Nejprve byla studována průtoková injekční analýza s amperometrickou detekcí pro stanovení všech tří biomarkerů na stejných SPCE, poté bylo podobné stanovení pro strukturně podobnější látky, HVA a VMA, v průtoku provedeno i na borem dopované diamantové elektrodě (BDDE). Po optimalizaci metod byly dosaženy následující *LOD*: s použitím SPCE 0,07 $\mu mol \cdot L^{-1}$ pro HVA, 0,05 $\mu mol \cdot L^{-1}$ pro VMA a 0,03 $\mu mol \cdot L^{-1}$ pro 5-HIAA; s použitím BDDE 0,44 $\mu mol \cdot L^{-1}$ pro HVA a 0,34 $\mu mol \cdot L^{-1}$ pro VMA.

Dále bylo studováno stanovení sledovaných biomarkerů v lidské moči metodou HPLC s amperometrickou detekcí na elektrodě ze skelného uhlíku. Po její optimalizaci a vyvinutí jednoduché předúpravy vzorků moči bylo úspěšně provedeno rychlé stanovení všech tří analytů v jedné analýze vzorku moči s *LOD* 11,0 $\mu mol \cdot L^{-1}$ pro HVA, 5,0 $\mu mol \cdot L^{-1}$ pro VMA a 8,3 $\mu mol \cdot L^{-1}$ pro 5-HIAA.

Key words

5-Hydroxyindole-3-acetic acid

Amperometry

Boron doped diamond electrode

Differential pulse voltammetry

Flow injection analysis

Glassy carbon electrode

Homovanillic acid

HPLC

Screen-printed carbon electrode

Tumour biomarkers

Vanillylmandelic acid

Klíčová slova

5-Hydroxy-3-indoloctová kyselina

Amperometrie

Biomarkery nádorových onemocnění

Borem dopovaná diamantová elektroda

Diferenční pulsní voltametrie

Homovanilová kyselina

HPLC

Průtoková injekční analýza

Sítotisková uhlíková elektroda

Uhlíková elektroda

Vanilmandlová kyselina

List of symbols and abbreviations

5-HIAA	5-hydroxyindole-3-acetic acid
AD	amperometric detection
BDDE	boron doped diamond electrode
BRB	Britton-Robinson buffer
CV	cyclic voltammetry
DPV	differential pulse voltammetry
ED	electrochemical detection
FIA	flow injection analysis
GCE	glassy carbon electrode
HPLC	high performance liquid chromatography
H-terminated	hydrogen-terminated
HVA	homovanillic acid
LOD	limit of detection
MPA	multiple pulse amperometry
O-terminated	oxygen-terminated
pH	negative of the decadic logarithm of the activity of hydronium ions
RSD	relative standard deviation
SPCE	screen-printed carbon electrode
SPE	solid phase extraction
UV	ultraviolet spectrophotometry
VMA	vanillylmandelic acid

Contents

1 INTRODUCTION

This dissertation thesis has been worked out at the Department of Analytical chemistry, Faculty of Science at the Charles University within the framework of a long-term research at the UNESCO Laboratory of Environmental Electrochemistry in Prague to develop sensitive and selective analytical methods applicable for the determination of biologically active organic compounds important from the medicinal, pharmaceutical, toxicological, and environmental point of view.

The dissertation thesis presents results obtained in the last five years and it is based on following five scientific publications [1-5], which are attached as Appendix parts I – V (Chapters 7 – 11). To distinguish the references to these publications in entire text of this dissertation thesis, corresponding numbers in square brackets are in bold.

[1] **A. Makrlíková**, J. Barek, V. Vyskočil, T. Navrátil, Electrochemical methods for the determination of homovanillic, vanillylmandelic, and 5-hydroxy-3-indoleacetic acid as cancer biomarkers (in Czech), Chem. Listy 112 (2018) 605–615.

[2] **A. Makrlíková**, E. Ktena, A. Economou, J. Fischer, T. Navrátil, J. Barek, V. Vyskočil, Voltammetric determination of tumor biomarkers for neuroblastoma (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) at screen-printed carbon electrodes, Electroanalysis 29 (2017) 146–153. doi:10.1002/elan.201600534.

[3] **A. Němečková-Makrlíková**, F.-M. Matysik, T. Navrátil, J. Barek, V. Vyskočil, Determination of three tumor biomarkers (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) using flow injection analysis with amperometric detection, Electroanalysis 31 (2019) 303–308. doi:10.1002/elan.201800540.

[4] **A. Němečková-Makrlíková**, T. Navrátil, J. Barek, P. Štenclová, A. Kromka, V. Vyskočil, Determination of tumour biomarkers homovanillic and vanillylmandelic acid using flow injection analysis with amperometric detection at a boron doped diamond electrode, Anal. Chim. Acta 1087 (2019) 44–50. doi:10.1016/j.aca.2019.08.062.

[5] **A. Němečková-Makrlíková**, J. Barek, T. Navrátil, J. Fischer, V. Vyskočil, H. Dejmková, Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection – *submitted to Journal of Electroanalytical Chemistry* (2020).

The dissertation thesis has been submitted as a contribution to the increasing demand for inexpensive methods for monitoring of tumour biomarkers in biological fluids which can be miniaturized and simplified even up to the point-of-care testing (bedside testing) [6].

The use of electroanalytical methods is beneficial in the case of monitoring large numbers of samples on routine basis. The main advantage is low-cost instrumentation and running costs in comparison with separation and spectrometric techniques. Electroanalytical methods provide short time of analyses with sufficient sensitivity and selectivity and the possibility of miniaturization and automation [7].

Tumour biomarkers are biological molecules found in biological fluids mostly used for screening population for the presence of tumours, especially in patients with increased risk of tumours (or other diseases). Screening of biomarkers can help to diagnose occurrence of a tumour and predict its future behaviour; monitor malignancy at the time of remission or response to a current therapeutic intervention [8, 9].

Tumour biomarkers could be determined in a variety of biological fluids including saliva, serum, plasma, blood, sperm, breath, and urine. Unlike other body fluids, urine analysis has several advantages. Sampling is non-invasive and repeated sampling is not a problem, moreover, it does not require medical auxiliary staff. The analytical advantage is lower urinary protein content and hence a sample with less interferents. However, urine volume is variable in certain time unit and may vary with water consumption, physiological, and external factors. Consequently, the concentration

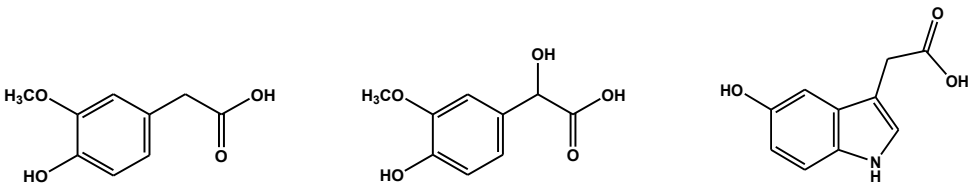
of biomarkers in urine varies and normalization is required in most cases, typically normalization related to urine volume, osmolality, or creatinine concentration [10, 11].

With respect to the above mentioned facts, the main aim of presented dissertation thesis was the development of sensitive electrochemical methods for the determination of three important tumour biomarkers, namely homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindole-3-acetic (5-HIAA) at different electrode materials. Second aim was to test simultaneous determination of three analytes in human urine. Biomarkers were determined at disposable screen-printed electrodes (SPCEs) in batch **[2]** and in flow **[3]** arrangement, at boron doped diamond electrode (BDDE) in flow arrangement **[4]**, and at glassy carbon electrode (GCE) in flow arrangement (combination with HPLC) **[5]**.

2 OVERVIEW OF TESTED TUMOUR BIOMARKERS

2.1 Analytes

Homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid), vanillylmandelic acid (VMA, D,L-4-hydroxy-3-methoxymandelic acid), and 5-hydroxyindole-3-acetic acid (5-HIAA, 2-(5-hydroxy-1*H*-indol-3-yl)acetic acid) are well-known biomarkers of various diseases, including tumours. Biomarkers are biological molecules present in biological fluids or tissues, and represent pathological changes in human body, so monitoring of all changes ongoing in organism during the treatment is not only possible, but very easy. HVA and VMA are structurally related final products of catecholamine metabolism, HVA is a dopamine metabolite, VMA is an epinephrine (adrenaline) and norepinephrine metabolite. 5-HIAA is a breakdown product of serotonin (5-hydroxytryptamine) (Fig. 1) [12, 13].



HVA VMA 5-HIAA

Fig. 1. Structural formulae of HVA, VMA, and HIAA

HVA, VMA, and 5-HIAA are present in various biological matrices, most often they are determined in urine [14], blood plasma or serum [15-17], cerebrospinal fluid [18], and brain tissues [19, 20]. Urine is the most common matrix for the determination of HVA, VMA, and 5-HIAA, their reference urinary concentrations vary from 8.2 to 41.0 μmol·L^{-1} for HVA, from 11.6 to 28.7 μmol·L^{-1} for VMA, and 17.8 to 58.3 μmol·L^{-1} for 5-HIAA [21]. Blood plasma or serum could be alternative to determination of these analytes in urine [22, 23], although plasma concentration values are

approximately 400 times lower and urine collection is easier to handle and it is easier to obtain in large quantities.

Non-physiologically high values of concentration levels of HVA, VMA, and 5-HIAA in biological fluids can indicate several diseases including tumours.

Concentrations of HVA and VMA are increased at patients suffering neuroendocrine catecholamine-producing tumours; neuroblastoma [24, 25] and pheochromocytoma [26, 27]. Neuroblastoma is the most common malignant tumour in babies; it is typically seen in patients less than 5 years old. Neuroblastoma arises from sympathetic ganglion cells and it has wide range of symptoms. Metastases could occur in bones, liver or lymph nodes [28, 29]. Pheochromocytoma is in the most cases benign tumour of adults between 40 and 50 years of life. It arises from the chromaffin cells of the adrenal gland and can metastasize into lungs, bones, liver, and lymph nodes [30, 31].

Elevating urinary level of 5-HIAA is associated with carcinoid tumours. Many carcinoid tumours are asymptomatic, 30-40% of patients with tumours have carcinoid syndrome, which is manifestation of carcinoid tumours with syndromes including skin flushing, asthma like wheezing attacks, and diarrhoea. Tumours are rare in adult population, sometimes they can metastasize [32, 33].

Measuring HVA, VMA, and 5-HIAA in biological fluids can be useful for the diagnosis not only of tumours. They are (individually or together) connected with schizophrenia [34], Parkinson's disease [35], autism [36], Tourette syndrome [37], Menkes disease [38], suicide attempts [39], depression [40], and posttraumatic stress disorder [41].

2.2 Methods of determination

The determination of HVA, VMA, and 5-HIAA requires selective and sensitive analytical methods because of their similar structures and low concentration levels in biological fluids. The most frequently used methods for the determination of all three mentioned biomarkers are HPLCs with different types of detection. The most common is electrochemical detection (ED) [42-46], e.g., amperometric [47] or coulometric [48], and extremely sensitive fluorescence detection [49-51], mostly after their derivatization [52, 53]. More sensitive and selective is chemiluminescence detection [54],

and the most sensitive and the most selective is mass spectrometric detection [16, 55-58].

Other methods for the determination of HVA, VMA, and 5-HIAA are gas chromatography [59, 60], thin-layer chromatography [61, 62], immunoanalytical methods [63-65], spectrophotometry [66, 67], capillary electrophoresis [21, 68], often connected with amperometric detection [68, 69], micellar electrokinetic chromatography [70, 71], and isotachophoresis [72].

An overview of the most recent trends in the quantification of biogenic amines as biomarkers in biofluids with special focus on liquid chromatography, gas chromatography, and capillary electrophoresis with various applications can be found in the review [73].

The above mentioned methods are mostly time consuming and require complicated instrumentation, therefore electrochemical arrangements and methods present a suitable alternative [74]. Beneficial features of the electroanalytical methods are simplicity and user-friendliness with sufficient sensitivity and selectivity. They are less expensive also because of negligible solvent consumption. Applicability of electrochemical methods for pharmaceutical and drug analysis is summarized in [75]. Special attention was devoted to voltammetric and potentiometric techniques, but also to the application of electrochemical detectors coupled with flow system. Potentiometric determination of biomarkers was also published in [76] as well as flow injection analysis with amperometric detection [3]. Electrochemical methods for the determination of HVA, VMA, and 5-HIAA focusing on the most common voltammetric and amperometric techniques are summarized in review [1].

2.3 Electrochemical oxidation of studied biomarkers

Due to the presence of hydroxyl group on aromatic system, HVA, VMA, and 5-HIAA are electrochemically active and can be easily oxidized on various electrode materials [77].

Even though HVA and VMA are structurally similar, their electrochemical behaviour is different. It was found that oxidation of VMA, e.g., at an edge-plane pyrolytic graphite electrode, basal-plane pyrolytic graphite electrode, or GCE using DPV, leads to the formation of vanillin, which is formed through decarboxylation of the molecule, and then can be oxidized at a higher potential connected with the

production of the *o*-quinone species and their oxidation to form a mixture of other products (Fig. 3). These two steps result in two distinct voltammetric peaks. HVA oxidation pathway is similar (Fig. 2), but due to slower speed of decarboxylation and rearomatization leading to the formation of vanillic alcohol, only one oxidation peak is observed [77, 78].

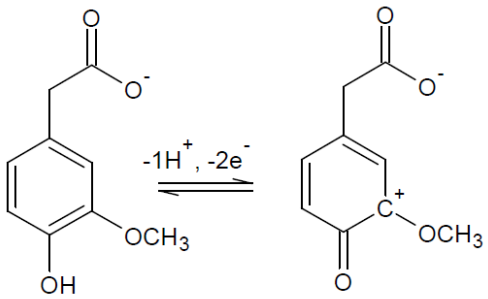


Fig. 2. Mechanism of the electrochemical oxidation of HVA [78]

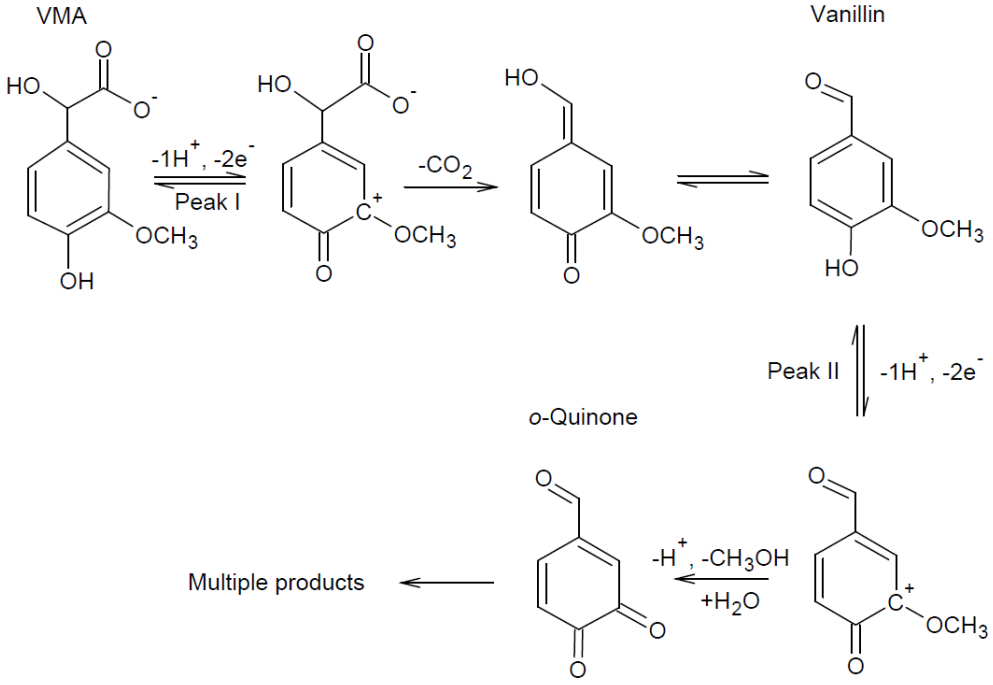


Fig. 3. Mechanism of the electrochemical oxidation of VMA [77]

In contrast, oxidation mechanism of 5-HIAA (Fig. 4) is slightly different and results in three voltammetric peaks. Electrochemical oxidation results in quinoneimine structure in which the C=O double bond (formed from the –OH group on the benzene ring) is conjugated with the C=N double bond (formed in the heterocyclic part of the molecule) [79].

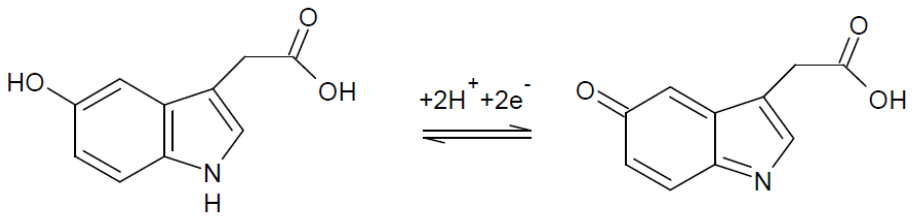


Fig. 4. Mechanism of the electrochemical oxidation of 5-HIAA [79]

According to [80], overall oxidation mechanism of 5-HIAA is complicated and voltammetric peaks correspond to irreversible reactions involving transfer of one electron and one proton.

DPV peak positions and peak heights of studied biomarkers depend on pH, composition of the supporting electrolyte, and electrode material. Corresponding peak potentials are in the range from +1.2 V (VMA) or +0.9 V (HVA) on anodically oxidized BDDE to +0.6 V (VMA) or +0.3 V (HVA) on GCE. 5-HIAA is oxidized at lower potentials. Therefore, it is possible to determine all these analytes simultaneously under certain conditions.

3 WORKING ELECTRODES AND TECHNIQUES

3.1 Working electrodes used

Three types of electrodes based on carbon (BDDE, GCE, and SPCE) were employed for determination of the discussed tumour biomarkers in this research.

The attractiveness of carbon electrodes for electroanalytical chemistry stems from advantages such as wide potential window (usually a wider potential range than in the case of solid metal electrodes), relatively chemical inertness, electrocatalytic activity for a variety of redox reactions, lower background oxidation currents, and low cost. Carbon has several allotropic forms, e.g., graphite, diamond, and fullerene. The enormous range of carbon materials (natural and synthetic) have been considered as variants of one or other of these allotropes [81, 82].

3.1.1 Boron doped diamond electrode

Unlike carbon with sp^2 bonds, diamond's structure is formed with sp^3-bonded carbon. Diamond is valued for its properties such as extreme hardness and high mechanical stability, corrosion resistance, chemical inertness, high thermal conductivity, and optical transparency. Normally, diamond is electrically insulating, but due to its large bang gap (5.47 eV) it is wide band gap semiconductor and exhibits semimetallic to metallic behaviour upon doping it with certain elements. Boron is dopant in the most cases (results in formation of p-semiconductor). Nevertheless, phosphorus or nitrogen can be also used (resulting in n-semiconductor) [83, 84].

BDDE possesses unique features as compared to other solid electrodes, such as chemical inertness and corrosion resistance, microstructural stability at extreme anodic and cathodic potentials, low and stable background current, wide working potential window, low sensitivity to dissolved oxygen, and electrochemical stability in acidic and alkaline media. BDDE provides good response for many oxidizable and reducible compounds in voltammetric or amperometric determination. BDDE in most

cases resists to passivation and electrode fouling, which is stated in many reviews, e.g., in [81, 85-89] (nevertheless, this is not true in all cases).

Electrochemical properties depend on the boron doped diamond structure involving boron doping concentration, content of sp^2 impurities, and the electrode's surface termination [90-92]. The BDDE surface could be hydrogen-terminated (H-terminated) or oxygen-terminated (O-terminated). The nonpolar H-terminated diamond surface shows hydrophobic tendency with negative electron affinity, high conductivity and low charge transfer resistance, whereas O-terminated diamond surface is hydrophilic with positive electron affinity, polar, and manifest lower electric conductivity. H-terminated surface can be easily changed to an O-terminated surface in several ways, e.g. by exposing the H-terminated surface to oxygen plasma, ozone treatment, wet chemical oxidation, exposure to high temperatures or electrochemical exposure to the high anodic potential [91-93].

Anodically and cathodically pretreated surface can increase sensitivity of the measurements at BDDE [94].

Anodic pre-treatment (based on application of the high positive potential directly in the measured solution) is very effective way of pre-treatment and in the case of passivation of the BDDE surface. This strategy for determination of HVA and VMA was used in [95].

Cathodic pre-treatment and activation of the electrode surface (applying the high negative potential in the measured solution) ensure lower limits of detection (*LOD*s) and quantification (*LOQ*s), sensitivity, reliable, and reproducible signals [96-98]. Cathodically pretreated BDDE was used for example for determination of vanillin [99].

Another possible cleaning step is mechanical polishing, usually performed by polishing the surface of the BDDE by alumina slurry and silk cloth [100, 101].

BDDEs are used for determination of wide range of oxidizable and reducible compounds, for example of neurotransmitters and their metabolites and precursors – determination of HVA and VMA is described in [95], determination of VMA in [102] and of dopamine in [103], phenolic compounds (hydroquinone) [104], pharmaceuticals and therapeutics [105, 106], food and beverage additives (including vanillin) [99, 107], and pesticides [108].

In this research **[4]**, working BDDE (D-517-SA, Ø 3 mm, Windsor Scientific, UK) was used for flow injection analysis (FIA) with amperometric detection. Working electrode was in a three electrode "wall-jet" arrangement with reference Ag│AgCl electrode (3 mol·L^{-1} KCl, Elektrochemicke Detektory, Czech Republic) and with a platinum wire auxiliary electrode (Ø 0.5 mm, Monokrystaly, Czech Republic). BDDE was anodically activated and pretreated by applying potential of +2.4 V on the electrode (activation was carried out for 20 minutes before the whole series of measurements and the application of high positive potential for 30 second was done before each particular measurement to avoid the surface passivation).

3.1.2 Glassy carbon electrode

GCEs are the most commonly used carbon electrodes thanks to their excellent mechanical and chemical properties in particular wide potential range (approximately +1200 mV to −800 mV vs. saturated calomel electrode in acidic medium [109]), chemical resistance, gas-impermeability, low cost, and compatibility with most common solvents. They are commonly used in voltammetric, coulometric, and amperometric methods in aqueous and non-aqueous media with reproducible responses. These electrodes are characterized by high density and very small pore size [81, 82, 109, 110]. The surface of GCE suffers from contamination and loss of response due to electrode surface fouling, so the pre-treatment procedure should contain polishing. It could be performed by polishing on alumina particles on a smooth polishing cloth (widely used technique) or electrochemically (thermal) activation [82], or by their combination.

GCE surface can be easily modified. Due to the above mentioned features bare or modified GCEs have been widely spread in electrochemistry in general, even in determination of HVA, VMA, and 5-HIAA [95, 111-115].

A commercially available GCE (Ø 3 mm, Metrohm, Switzerland) was employed for HPLC with amperometric detection **[5]** and FIA with multiple pulse amperometric detection **[4]** in a "wall-jet" arrangement with a reference Ag│AgCl (3 mol·L^{-1} KCl, Monokrystaly **[5]**/ Elektrochemicke Detektory **[4]**, Czech Republic) electrode, and a platinum wire auxiliary electrode (Ø 0.5 mm, Monokrystaly, Czech Republic). The surface of the working electrode was mechanically cleaned before each measurement by polishing with aluminium oxide suspense and then rinsing with deionized water.

3.1.3 Screen-printed carbon electrode

Screen-printed electrodes are very attractive analytical devices for rapid, inexpensive, sensitive, and selective detecting a wide range of analytes in electroanalytical chemistry. Nowadays is a tendency to replace conventional electrodes by SPEs due to their many advantages. They play important role in miniaturization of measuring devices (with low sample consumption) and could be a part of portable apparatus or point-of-care system with user-friendly and equipment-free tests, where analyses are performed close to the patient. They are disposable, so they can eliminate contamination or biofouling [116, 117].

Apart from medical and pharmaceutical diagnosis [118-120], screen-printed electrodes are applied for example in environmental assays [121-123] and in food analysis [124-126].

Screen-printed technology uses a woven mesh defining the geometry of the sensor. Electrodes usually contain a three-electrode configuration (working, reference, and counter electrode) printed in two ink layers on a solid substrate (mostly plastic or ceramic). At the end, a protective ink is printed on the top of these layers to insulate the conductive track. Most commonly used inks are silver ink for conductive track and carbon ink (consisting of graphite particles, polymeric binder and other additives) for working electrode [116, 127].

The increasing interest in the use of green chemistry has given rise also to recycled and recyclable disposable electrodes where eco-friendly and sustainable materials, electrode modifiers, and alternative fabrication processes are suggested [128].

Apart from advantages of screen-printed technology as a design flexibility and process automation, the printed working electrodes enable a vast range of different combination in terms of electrode materials used (e.g. carbon, silver, platinum, gold) and its modification or altering the geometry of the electrode and used inks to improve selectivity and sensitivity towards determined analytes [129, 130]. Electrodes can be easily modified in several ways; in general, various substances can be deposited on the surface of the electrode (such as films, polymers, enzymes) or substances can be added into printing inks (e.g. metals, enzymes, polymers, complexing agents) [131-133]. Nanogold modified screen-printed carbon electrodes were used for simultaneous determination of dopamine and 5-HIAA [134].

In this research **[2, 3]** used commercially available screen-printed carbon electrodes (SPCEs, type DRP-110) were manufactured by DropSens, Spain. Working (Ø 4 mm) and counter electrodes were made of carbon; reference electrode was made of silver. All three electrodes were printed on a ceramic substrate (L33 × W10 × H0.5 mm).

3.2 Electrochemical techniques used

Electrochemical methods are for its advantageous properties routinely used for determination of wide range of biologically active compounds for variety of purposes, e.g., monitoring of harmful substances in environmental matrices or pharmaceuticals in human body and for studies of oxidation and reduction processes. Electrochemical detection in combination with spectrophotometric and chromatographic methods represents effective tool for the analysis of complex mixtures in various matrices [135-137].

Other features and applications of electroanalytical methods with proper references are mentioned in Paragraph 2.2.

The following methods have been used to meet the objectives of presented dissertation thesis: cyclic voltammetry (CV) **[4]**, differential pulse voltammetry (DPV) **[2]**, flow injection analysis (FIA) with amperometric detection (AD) **[3, 4]** and multiple pulse amperometric (MPA) detection **[4]**, and high performance liquid chromatography with electrochemical (amperometric) and spectrophotometric detection (HPLC-ED/UV) **[5]**.

Cyclic voltammetry is usually used to investigate reduction and oxidation processes, reaction intermediates, and reaction products [138]. CV has been used in **[4]** for control of the BDDE surface after activation by high positive potential.

In contrast, differential pulse voltammetry is a sensitive voltammetric method for determining trace amounts of analytes. DPV provides low *LOD* values at submicromolar concentrations because of suppressed background currents [139].

Amperometric detection is used for the detection of electroactive compounds in flow-based analytical systems such as FIA, capillary electrophoresis, and liquid chromatography. A potential is applied between working and reference electrode. When solutes pass over the working electrode, an electrical current arises during oxidation or reduction of an electrochemically active substance. AD requires the control

of temperature, pH, and flow rate of the eluent [140, 141]. MPA detection uses multiple potential pulses on a single working electrode and monitors the current at several applied potentials, so it can detect different analytes in mixture simultaneously in one analysis or it enables prevent passivation (fouling) of the working electrode by the application of a cleaning potential pulses in the potential program [141].

In this dissertation thesis FIA in combination with AD, FIA in combination with MPA, and HPLC in combination with AD have been used. These combinations have been abundantly published for the determination of different compounds. Examples can be found in [142-146] for FIA with AD, in [147-151] for FIA with MPA, and in [152-156] for HPLC with AD.

4 RESULTS AND DISCUSSION

4.1 Differential pulse voltammetric determination of homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid at screen-printed carbon electrodes

This part of research was aimed to development of a fast, simple, and inexpensive DPV method for the determination of the three tumour biomarkers at commercially available SPCEs (Appendix II) **[2]**. SPCEs are not only selective, sensitive, disposable (and thus prevent cross-contamination and passivation), and due to its mass production relatively inexpensive sensors, moreover, they are also fully complying with point of care concept and miniaturization in general.

Firstly, differential pulse voltammograms of HVA in Britton-Robinson buffer (BRB) of pH 2.0 to 12.0 were measured. At pH 3.0 two well-developed peaks of HVA were formed, therefore, this pH value was selected as an optimum. Because of similar structure of VMA and HVA, optimum pH was found to be 3.0 as well. The peak of 5-HIAA at differential pulse voltammograms in alkaline media (pH >9.0) was not evaluable; on the contrary, in pH 3.0 two well-separated peaks of 5-HIAA were observed, therefore pH 3.0 was selected as optimum (Appendix II, Fig. 1 and 2) **[2]**.

Subsequently, the dependences of the peak current on the analyte concentration were measured (Appendix II, Fig. 3, 4, and 5) in BRB (0.04 $mol \cdot L^{-1}$, pH 3.0) in concentration range from 0.1 to 100 $\mu mol \cdot L^{-1}$. At differential pulse voltammograms of HVA and VMA two significant peaks applicable to analytical purposes were observed. Three peaks occur for 5-HIAA, it is caused probably by its different structure and mechanism of electrochemical oxidation described in Paragraph 2.3. However, only the first peak was suitable for evaluation of calibration dependences. Evaluation of the other two peaks was more difficult and leading to less precise (correct) results. Dependences were linear in the whole tested concentration range and obtained *LOD*s

were 0.24 μmol·L^{-1} for HVA (2nd peak), 0.06 μmol·L^{-1} for VMA (2nd peak), and 0.12 μmol·L^{-1} for 5-HIAA (1st peak), respectively.

Fig. 6 and 7 in Appendix II document an attempt to determine HVA and VMA in mixture. It is possible to find the peaks of HVA in the mixture where height is linear function of the concentration of HVA at constant concentration of the VMA and vice versa. Thus, DPV at SPCE can be used for the determination of the two biomarkers in their mixture by standard addition of these analytes in the concentration range between 10^{-5} and 10^{-6} mol·L^{-1}.

The findings of this study suggest that DPV at SPCE proved to be sensitive enough for monitoring of HVA, VMA, and 5-HIAA. With some proper preliminary separation and preconcentration, the newly developed method could be used even for monitoring and determination of tested biomarkers in various biological matrices **[2]**.

4.2 Flow injection determination of homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid at screen-printed carbon electrodes

Monitoring of biomarkers requires easy automated method with short time analysis. FIA meets these criteria. Moreover, in combination with amperometric detection (AD) it is inexpensive analytical method with sufficient sensitivity necessary for clinical, pharmaceutical, and environmental analysis. Compared to conventional batch methods, FIA has some significant advantages such as ability to analyse high number of samples in time with low sample and reagent consumption.

The purpose of this study was to investigate electrochemical behaviour of frequently monitored biomarkers, HVA, VMA, and 5-HIAA, and to develop methods for their determination using FIA-AD method at SPCE (Appendix III) **[3]**. To the best of my knowledge, FIA-AD for determination of HVA, VMA, and 5-HIAA had not been used yet. Combination of FIA and monolithic columns and chemiluminescence detection for determination of all three above mentioned biomarkers was published in [157] and [158], for FIA with chemiluminescence detection for VMA in [159].

For FIA measurements, commercially available wall jet flow cell with SPCEs was used. Although SPCE are disposable, it was verified that up to 20 measurements

can be repeated at a single SPCE at micromolar concentrations of the tested analytes without problems with passivation.

Parameters as flow rate of carrier solution, potential of detection, and pH of BRB used as a carrier solution had to be optimized in order to obtain the best results for the determination of HVA, VMA, and 5-HIAA.

First of all, preliminary experiments with potassium ferrocyanide were made to optimize flow rate and to obtain general information about behaviour of an assembled FIA apparatus. Experiments leading to optimized parameters were tested in the following ranges: flow rate within 0.2 to 4 $mL \cdot min^{-1}$, working electrode potential within 0.1 V to 1.2 V, and pH of carrier solution (BRB, 0.04 $mol \cdot L^{-1}$) within 2.0 to 10.0. Injected volume was kept constant 20 µL. Calibration dependences were tested in the range from 10 to 1000 $\mu mol \cdot L^{-1}$. Optimum pH was found to be 3.0 and optimum detection potential was +0.5 V. Afterwards, these experiments were performed also with tumour biomarkers.

Optimized parameters for each biomarker were tested step by step in following ranges: flow rate within 0.5 to 2.5 $mL \cdot min^{-1}$, working electrode potential within 0.4 V to 1.2 V, and pH of carrier solution within 2.0 to 8.0. Dependences of the peak height on the potential of detection, on the flow rate, and on the pH for biomarkers are shown in Appendix III, in Fig. 1, Fig. 2, and Fig. 3. Optimum values are magnified; they were selected based on the highest possible peak height and the lowest relative standard deviation (*RSD*s).

Under optimum parameters for HVA, VMA, and 5-HIAA (flow rate 1 $mL \cdot min^{-1}$, detection potential +0.6 V for HVA and +0.8 for VMA and 5-HIAA, BRB (0.04 $mol \cdot L^{-1}$) at pH 2.0, current range 1-100 µA, injected volume 20 µL) calibration dependences were constructed. The measurements were repeated three times at each concentration in the concentration range 0.05-100 $\mu mol \cdot L^{-1}$. FIA recording of 5-HIAA (HVA and VMA are similar) are depicted in Fig. 4 in Appendix III **[3]**.

In the tested concentration range, all dependences were linear with *LOD*s 0.065 $\mu mol \cdot L^{-1}$ for HVA, 0.053 $\mu mol \cdot L^{-1}$ for VMA, and 0.033 $\mu mol \cdot L^{-1}$ for 5-HIAA, respectively, (calculated from peak heights), and 0.024 $\mu mol \cdot L^{-1}$ for HVA, 0.020 $\mu mol \cdot L^{-1}$ for VMA, and 0.012 $\mu mol \cdot L^{-1}$ for 5-HIAA, respectively (calculated from peak areas). In Table 3 in Appendix III, all figures of merit of the calibration straight lines are summarized.

This study has demonstrated for the first time that FIA-AD at SPCE presents a suitable technique for the determination of HVA, VMA, and 5-HIAA as frequently monitored biomarkers. Moreover, continuous flow in FIA method at SPCE was able to remove any potential interfering products by washing them away from the working electrode surface, thus minimize the risk of passivation, so no passivation or cross-contamination was observed **[3]**.

4.3 Flow injection determination of homovanillic acid and vanillylmandelic acid at boron doped diamond electrode

The purpose of this part of research was to verify the applicability of FIA-AD at BDDE for determination of HVA and VMA and also to investigate this approach for the analysis of the mixture of HVA and VMA using BDDE (Appendix IV) **[4]**. HVA and VMA levels are useful to diagnose tumours and different diseases. Determination of one biomarker must be possible in the presence of the other one. Moreover, in some cases, the absolute values of HVA and VMA are not significant and HVA/VMA ratio is needed. In addition, HVA and VMA are structurally more similar compounds than 5-HIAA and for an attempt of determining them in the mixture using FIA-MPA more convenient.

Possibilities of the BDDE use in flow systems are discussed in [160]. Advantages of FIA arrangement were discussed in Section 4.2. BDDE was chosen due to its unique properties and also due to better compatibility with principles of green analytical chemistry in comparison with SPCE used in previous research **[3]**. BDDE can be used repeatedly many times.

Prior to the measurements, BDDE surface was activated by applying high positive potential +2.4 V for 20 min in H_2SO_4 (0.5 $\mu mol \cdot L^{-1}$) and then by CV in BRB (0.04 $mol \cdot L^{-1}$, pH 7.0 from –0.8 V to +2.3 V at scan rate 100 $mV \cdot s^{-1}$. CV scans were repeated until CV curve was stable. Before each measurement, a potential +2.4 V for 30 sec was applied on the BDDE surface to eliminate passivation.

AD was carried out at a constant potential. Optimum applied potential and pH were optimized in terms of recording hydrodynamic voltammograms of HVA and VMA in the range between +0.5 V to +1.7 V in the pH range from 2.0 to 7.0 in BRB, depicted in Appendix IV, Fig. 2. FIA-MPA assumes that at the potential of the first pulse only

first analyte (HVA) is oxidized and at the potential of the second pulse both analytes (HVA and VMA) are oxidized. Then FIA-MPA response of VMA can be calculated by subtraction of HVA response from total response (HVA + VMA) using a correction factor (ratio of FIA-MPA peak responses of HVA obtained at the first pulse potential (lower) and at the second pulse potential (higher) [151]. According to Fig. 2 in Appendix IV, optimum pulse potentials were found to be +1.1 V for HVA and +1.5 V for VMA at pH 3.0. Similarly, the constant potentials +1.1 V for HVA and +1.5 V for VMA were found to be optimum for FIA-AD calibration dependences for individual analytes alone and for their mixture. A passivation of BDDE was observed, probably due to the adsorption of electrode reaction products on BDDE surface. Therefore, it was eliminated by applying cleaning potential prior each measurement (+2.4 V for 30 sec).

In the next part of research, optimum injection volume and flow rate of BRB were sought. Injection volume was tested from 20 to 100 μL, 20 μL was chosen as optimum due to better elimination of passivation, sufficiently high signal, and lower sample consumption. According to previous results of FIA-AD determination of HVA and VMA at SPCEs, flow rate was set to 1 $mL \cdot min^{-1}$ **[3]**. Under these optimum conditions, 25 consequent measurements were done. Injections gave amperometric responses with *RSD*s lower than 4% for both analytes at concentration 100 $\mu mol \cdot L^{-1}$.

Calibration dependences measured at optimum conditions were constructed when concentration of HVA was changed and concentration of VMA was constant and vice versa, in the concentration range from 1 to 10 $\mu mol \cdot L^{-1}$, concentration of VMA (HVA) was 5 and 10 $\mu mol \cdot L^{-1}$ (Appendix IV, Fig. 3 and Fig. 4), in concentration range from 10 to 100 $\mu mol \cdot L^{-1}$ concentration of VMA (HVA) was 50 and 100 $\mu mol \cdot L^{-1}$ (Appendix IV, Fig. 5 and Fig. 6). Calibration dependences were linear with *LOD*s 0.44 $\mu mol \cdot L^{-1}$ for HVA and 0.34 $\mu mol \cdot L^{-1}$ for VMA. Figs. 3-6 compare calibration dependences for HVA (VMA) alone and with additions of constant concentrations of VMA (HVA) and show a gradual linear increase in intercepts. All figures of merit of the calibration dependences are summarized in Appendix IV, Table 1 **[4]**.

FIA-MPA enables simultaneous determination of compounds with sufficiently different oxidation potentials. Pulses are altering optimum potentials selected for each analyte from hydrodynamic voltammograms. At pulse potential +1.1 V (optimum for HVA) the signal of HVA is high with only small contribution of VMA while

at pulse potential +1.5 V signal of VMA is at its maximum level. Various pulse widths (50 to 150 ms) were tested with no significant differences, so pulse width 100 ms was used as an optimum. At measurements alternating pulses of +1.1 V, +1.5 V were used with cleaning pulse +2.2 V (lower than in the case of amperometric detection, but sufficient enough). Other conditions were the same as in the case of amperometric detection. Calibration dependences consisted of varying concentration of HVA or VMA (10, 20, 40, 60, 80, and 100 μmol·L^{-1}) and 100 μmol·L^{-1} of the other biomarker. FIA-MPA calibration dependence of HVA in the presence of 100 μmol·L^{-1} of VMA in each solution can be seen in Fig. 8. Peak heights of HVA should be linearly increasing with increasing HVA concentration and peak heights of VMA should be constant due to constant concentration of VMA. However, calibration dependence of HVA was not linear and VMA at constant concentration had increasing tendency. Under the same conditions, FIA-MPA calibration dependence for HVA without addition of constant concentrations of VMA into solutions and also VMA at 100 μmol·L^{-1} separately in the absence of HVA was constructed. In that case, the calibration dependence was linear and amperometric signals of VMA at constant concentration were reproducible.

This shows that FIA-MPA cannot be used for the determination of HVA and VMA in a mixture simultaneously. Presumably, that is because of a mutual interaction of analytes and/or products of their electrochemical oxidation with each other or with the parent compounds. This conclusion was supported by the same experiments performed also on electrodes from different materials; SPCE (type DRP-110, DropSens, Spain) and a GCE (Ø 3 mm, Metrohm, Switzerland) with similar negative results for FIA-MPA, moreover, with stronger passivation which could not be eliminated by several tested methods usually used.

The results of this study indicate that the newly developed method for the determination of HVA and VMA using FIA with amperometric detection at BDDE has sufficient precision, sensitivity, and short time of analysis. FIA-AD at BDDE provides linear calibration dependences in wide concentration range. HVA can be determined in the presence of VMA and vice versa using FIA-AD, nevertheless, FIA-MPA was not applicable for the determination of the mixture of them, probably due to interaction of products of electrochemical reaction and initial analytes **[4]**.

4.4 HPLC of homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid using amperometric and spectrophotometric detection

Even though HPLC is common method for the determination of urinary HVA, VMA, and 5-HIAA used in clinical laboratories, this research is devoted to determination of these biomarkers by HPLC-ED (Appendix V) **[5]**. In articles published so far, for example in review **[1]**, only few of them allow simultaneous determination of all three biomarkers. In addition, they require expensive instrumentation and complicated sample pre-treatment leading to quite time-consuming analyses. Due to the mentioned facts, the aim of this work was to develop fast, simple, and inexpensive analytical method for simultaneous determination of the three tumour biomarkers in urine employing HPLC with AD without complicated and time-consuming sample pre-treatment.

Measurements were performed at a commercially available GCE in a simple lab-made wall-jet arrangement. The surface of the working electrode was mechanically polished prior to each measurement with aqueous slurry of alumina powder to eliminate passivation. For the purposes of comparison, apart from electrochemical detection the spectrophotometric one was carried out.

Before dosing biological samples into HPLC column appropriate sample pre-treatment is required to avoid its damage due to many organic compounds present in the sample. Partial sample clean up can also improve the HPLC part of the determination, especially in the case of relatively polar compounds, which is the case of studied biomarkers. Therefore, solid phase extraction (SPE) at commercially available poly(styrene-divinylbenzene) based SPE columns (LiChrolut EN 200 mg 3 mL standard PP-tubes; Merck Millipore, Germany) were chosen as a simple and straightforward technique, much faster and more „green“ than previously used and published liquid-liquid extraction.

Firstly, pilot experiments were carried out using partly adopted chromatographic conditions from [161] where HPLC-ED was successfully used for determination of HVA and VMA alone. The mobile phase consisted of a mixture of acetate-phosphate buffer at pH 2.5 using gradient with linearly increasing content of acetonitrile from 5 to 25% in 10 minutes. Optimum flow rate was 1 $mL \cdot min^{-1}$, detection potential +1.1 V, and injected volume 20 μL. Spectrophotometric detection was performed

at 279 nm. Under these optimum conditions, analytes were well separated in less than 10 minutes (Appendix V, Fig. 2). Calibration dependences were linear in the whole tested concentration range from 0.5 to 10 $\mu mol \cdot L^{-1}$ and obtained *LOD*s were 0.2 $\mu mol \cdot L^{-1}$ (HVA), 0.3 $\mu mol \cdot L^{-1}$ (VMA), and 0.3 $\mu mol \cdot L^{-1}$ (5-HIAA) for HPLC-ED (Appendix V, Table 1) and 0.2 $\mu mol \cdot L^{-1}$ (HVA), 0.6 $\mu mol \cdot L^{-1}$ (VMA), and 0.3 $\mu mol \cdot L^{-1}$ (5-HIAA) for HPLC-UV (Appendix V, Table 2). Molar absorptivity at 279 nm is 2900, 3100, and 7000 $mol^{-1} \cdot L \cdot cm^{-1}$ for HVA, VMA, and 5-HIAA, respectively, which explains higher slope of HPLC-UV dependence of 5-HIAA. Higher slope may also result from slightly different structure, specifically in the heteroaromatic region resulting in higher amperometric response.

Prior to measurements with human urine, SPE procedure was optimized and tested. In the used optimized procedure SPE columns were activated with 5 mL of methanol, washed with 1 mL of deionized water and then dried by sucking air through it for 30 seconds using vacuum. During the whole procedure flow rate was kept at 1 $mL \cdot min^{-1}$. Spiked urine samples (5 mL) consisting of 10% acetic acid and 50% of deionized water (due to added spikes of aqueous solutions of tested analytes) were loaded onto the column. The column was rinsed by 1 mL of deionized water and dried by sucking air for 30 seconds using vacuum. Analytes were eluted by 5 mL of methanol and 20 µL of thus obtained eluate were directly injected into HPLC system. SPE was used only as a preliminary separation method, it can be used for preconcentration as well, nonetheless, it is unnecessary when we consider relatively high concentration of studied analyte in urine (tens of $\mu mol \cdot L^{-1}$) combined with relatively high sensitivity of the newly developed HPLC-ED method with *LOD*s in this concentration region.

HPLC-ED and HPLC-UV chromatograms of human urine alone and after standard addition (1 mL of 1 $mmol \cdot L^{-1}$ stock solution of each analyte) into a urine sample acidified with 10% of acetic acid (final concentrations of analytes were 100 $\mu mol \cdot L^{-1}$ and total volume of the spiked urine samples was 10 mL) are depicted in Appendix V, Fig. 3. From the picture, it can be seen that 100 $\mu mol \cdot L^{-1}$ of any of the analytes can be safely detected and even 50 $\mu mol \cdot L^{-1}$ for HPLC-ED (from the inset in Fig. 3A) are distinguishable.

Solution samples for construction of calibration curves were prepared from 4 mL of urine, 1 mL of glacial acetic acid. 0.2 – 1.5 mL of the 1 $mmol \cdot L^{-1}$ stock solutions of biomarkers were pipetted into 10 mL volumetric flask and filled up with deionised

water prior to the SPE. Calibration dependences were constructed under optimum conditions in concentration range 20 to 150 $\mu mol \cdot L^{-1}$ and they were linear in this range (Appendix V, Fig. 4), with achieved *LOD*s 11.0 $\mu mol \cdot L^{-1}$ (HVA), 5.0 $\mu mol \cdot L^{-1}$ (VMA), and 8.3 $\mu mol \cdot L^{-1}$ (5-HIAA) for HPLC-ED, respectively, (Appendix V, Table 1) and 13.9 $\mu mol \cdot L^{-1}$ (HVA), 72.9 $\mu mol \cdot L^{-1}$ (VMA), and 13.1 $\mu mol \cdot L^{-1}$ (5-HIAA) for HPLC-UV, respectively (Appendix V, Table 2).

Lower concentrations of tested biomarkers (0.5 to 10 $\mu mol \cdot L^{-1}$) were difficult to evaluate due to interfering peaks of substances present in urine. Nevertheless, if necessary, SPE could be used for preconcentration to reach this concentration range. In this case it is not necessary if we consider urinary concentration of the HVA, VMA, and 5-HIAA of healthy people at concentration level of tens of $\mu mol \cdot L^{-1}$. Table 1 and Table 2 in Appendix V summarized all figures of merit from all calibration dependences. Slopes of the calibration dependences are lower than those of dependences measured using solutions in buffer alone and *LOD*s are logically higher in urine than in pure buffers because of interfering peaks. *LOD*s of HPLC-UV are higher than those of HPLC-ED due to larger and more numerous interfering peaks connected with higher selectivity of amperometric detection.

The practical applicability of the newly developed HPLC-ED method was tested by determining concentration of HVA, VMA, and 5-HIAA in spiked human urine using calibration curve method. Significant interferences were not observed in HPLC-ED and concentration found in non-spiked urine corresponds with previously published physiological urinary concentrations of HVA, VMA, and 5-HIAA (Appendix V, Table 3) **[5]**.

In the last experimental part of presented dissertation thesis, fast and sensitive method for determination of HVA, VMA, and 5-HIAA in human urine in one analysis is presented. The proposed method is cheaper due to simple SPE pre-cleaning step than previously published methods and, therefore, it can be used for easy and low-cost screening of human urine and thus help to diagnose several diseases including tumours **[5]**.

5 CONCLUSION

The presented dissertation thesis represents a contribution to the development of sensitive electrochemical methods for the determination of HVA, VMA, and 5-HIAA as biomarkers of various diseases, including tumours.

Therefore, the need of frequent monitoring of such important biomarkers should be raised to the highest priority which is supporting by increased demand for inexpensive and simple method applicable for monitoring and point-of-care testing of above mentioned biomarkers in biological fluids. Electroanalytical methods fulfil requirements on reliable, sensitive, short time of analysis, and relatively low-cost instrumentation and running cost.

Experimental work was focused on electrochemical behaviour and determination of HVA, VMA, and 5-HIAA at different electrode materials. Subsequently, the attention was paid to simultaneous determination and determination of biomarkers in human urine.

First part describes successful development of differential pulse voltammetry employing screen-printed carbon electrodes for the determination of HVA, VMA, and 5-HIAA in a batch arrangement. At voltammograms of biomarkers in BRB at pH 3.0 two peaks for HVA and for VMA and three peaks for 5-HIAA can be seen, which can be explained by their different structures and mechanism of electrochemical oxidation. Linear calibration dependences provide *LOD*s amounted to 0.24 $\mu mol \cdot L^{-1}$ for HVA, 0.06 $\mu mol \cdot L^{-1}$ for VMA, and 0.12 $\mu mol \cdot L^{-1}$ for 5-HIAA. An attempt to determine HVA and VMA in mixture was successful as well. It was possible to find peaks of HVA or VMA in the mixture where the peak height was linear function of the concentration of one biomarker at constant concentration of the other one.

For monitoring of biomarkers, flow systems have more advantages in comparison with batch arrangements, such as ability to analyse high number of samples in short time and the related low sample and reagent consumption. The second part therefore deals with flow injection determination of HVA, VMA,

and 5-HIAA at screen-printed carbon electrodes in BRB. Linear calibration dependences obtained under optimum conditions offer *LOD*s as follows: 0.07 $\mu mol \cdot L^{-1}$ for HVA, 0.05 $\mu mol \cdot L^{-1}$ for VMA, and 0.03 $\mu mol \cdot L^{-1}$ for 5-HIAA, respectively. Continuous flow in flow injection analysis at SPCE was able to eliminate passivation due to washing any potential interfering products away from the working electrode surface.

FIA appears as a suitable method for determination tumour biomarkers. Consequently, flow injection determination of HVA and VMA at BDDE was investigated. HVA and VMA were chosen due to requirements for their simultaneous determination, for example in the case of diagnosis of neuroblastoma. Method for the determination of HVA and VMA using flow injection analysis with amperometric detection in BRB at BDDE was successfully optimized. Calibration dependences were linear for HVA in the presence of VMA and vice versa. Achieved *LOD*s were 0.44 $\mu mol \cdot L^{-1}$ for HVA and 0.34 $\mu mol \cdot L^{-1}$ for VMA. This approach was studied for the simultaneous determination of HVA and VMA using flow injection analysis with multiple pulse amperometric at BDDE, SPCE, and GCE. However, the concept of FIA-MPA was not applicable for determination of HVA and VMA in the mixture, probably due to interaction of analytes and/or products of their electrochemical oxidation.

Last part of this dissertation thesis introduced HPLC with amperometric detection in human urine at GCE in lab-made wall-jet detector as a simple, sensitive and cheaper method than previously published methods for simultaneous determination of HVA, VMA, and 5-HIAA. As a simple and fast preliminary separation method solid phase extraction was used. Obtained *LOD*s were 11.0 $\mu mol \cdot L^{-1}$ for HVA, 5.0 $\mu mol \cdot L^{-1}$ for VMA, and 8.3 $\mu mol \cdot L^{-1}$ for 5-HIAA.

Methods for the determination of HVA, VMA, and 5-HIAA separately and in the mixture were presented. It was proved that they can be determined even in human urine with appropriate pre-separation. Thus, it can be concluded that amperometric and voltammetric methods are suitable for determination of tumour biomarkers, HVA, VMA, and 5-HIAA.

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7 APPENDIX I

Elektrochemické stanovení homovanilové, vanilmandlové a 5-hydroxy-3-indoloctové kyseliny jako biomarkerů nádorových onemocnění

(Electrochemical methods for the determination of homovanillic, vanillylmandelic, and 5-hydroxy-3-indoleacetic acid as cancer biomarkers)

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ELEKTROCHEMICKÉ STANOVENÍ HOMOVANILOVÉ, VANILMANDLOVÉ A 5-HYDROXY-3-INDOLOCTOVÉ KYSELINY JAKO BIOMARKERŮ NÁDOROVÝCH ONEMOCNĚNÍ

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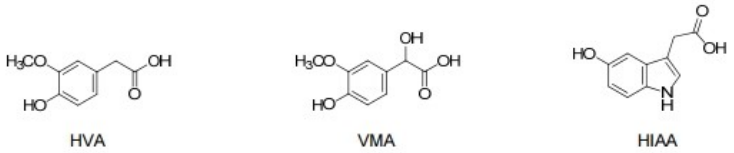
Obsah

1. Úvod

Homovanilová kyselina (HVA, 4-hydroxy-3-methoxyfenyloctová kyselina, CAS No 306-08-1) a vanilmandlová kyselina (VMA, 4-hydroxy-3-methoxymandlová kyselina, CAS No 55-10-7) jsou konečným produktem metabolismu katecholaminů[1−3]. 5-Hydroxyindol-3-octová kyselina (HIAA, CAS No 54-16-0) je produktem metabolismu neurotransmiteru serotoninu[4]. Zvýšená koncentrace HVA, VMA a HIAA v moči[5] a v krevní plasmě[2] může indikovat některá onemocnění. Koncentrace HVA a VMA v tělních tekutinách je zvýšená především u nádorů typu neuroblastomu[6,7], feochromocytomu[8,9] a karcinoidu[9,10]. Kromě zmíněných nádorů mohou HVA, VMA a HIAA sloužit k predikci schizofrenie[11], Parkinsonovy nemoci[12], autismu[13] a Tourettеova syndromu[14]. Rovněž mohou signalizovat deprese[15], posttraumatické stresové poruchy[16] a sebevražedné chování u pacientů[17]. Poměr koncentrací HVA/VMA v moči novorozenců může sloužit pro včasnou diagnosu Menkesovy nemoci[18]. Normální koncentrace výše zmíněných organických kyselin v moči jsou 8,2−41,0 μmol l^{-1} pro HVA, 11,6−28,7 μmol l^{-1} pro VMA a 17,8−58,3 μmol l^{-1} pro HIAA[19], resp. 1,3−7,6 mg l^{-1} pro všechny tyto biomarkery[20].

Pro stanovení výše zmíněných biomarkerů (obr. 1) je zapotřebí citlivých a selektivních analytických technik[21] vzhledem k jejich podobným strukturám a nízkým koncentracím v biologických tekutinách. Nejčastěji se používá HPLC s elektrochemickou detekcí (ED)[3,22−25], např. ampérometrickou[26] nebo coulometrickou[27], či s mimořádně citlivou fluorescenční detekcí (FD)[28−30]. Avšak přímá fluorescenční kvantifikace katecholaminů a jejich metabolitů v reálných vzorcích je obtížně proveditelná kvůli krátkým emisním a excitačním vlnovým délkám, a proto se často používá jejich derivatizace[1,21]. Ještě citlivější a selektivnější je chemiluminiscenční detekce (CHLD)[31] a nejcitlivější a nejselektivnější je detekce hmotnostní spektrometrií (MS)[7,32−35]. Dalšími možnými metodami pro stanovení HVA, VMA a HIAA jsou plynová chromatografie (GC)[36,37], tenkovrstvá chromatografie[38], vysokoúčinná tenkovrstvá chromatografie (HPTLC)[39], imunochemické metody[40−42] a spektrofotometrie[8,43]. Z elektromigračních metod je vhodná kapilární elektroforéza (CZE)[19,44] často spojená s ampérometrickou detekcí[44,45], micelární elektrokinetická chromatografie (MEKC)[20,46] a izotachoforéza[47].

Před samotným stanovením jsou vzorky často upravovány, nejčastěji kapalinovou extrakcí[1,9] umožňující potřebnou předběžnou separaci a prekoncentraci. Někdy lze vzorek pouze přefiltrovat a injektovat do kolony[3,34]. Popsána je i extrakce HVA a HIAA tuhou fází na bázi dutého vlákna vodivého polypyrrolu[48].



Obr. 1. **Strukturní vzorce HVA, VMA a HIAA**

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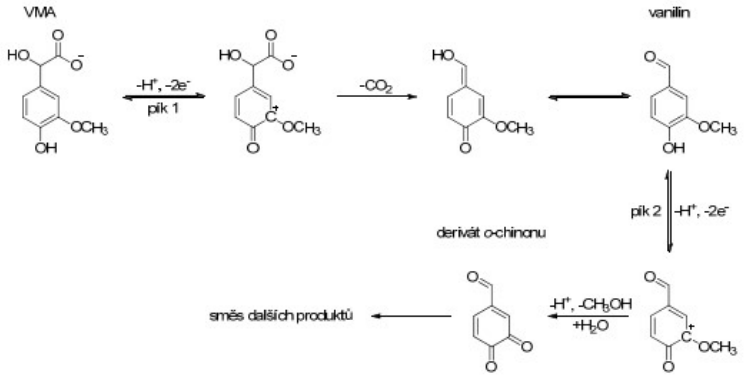
Výše uvedené separační a spektrometrické metody jsou však zpravidla finančně, instrumentálně i časově náročné. Proto je v poslední době věnována pozornost i využití moderních elektroanalytických metod, které jsou v řadě případů dostatečně citlivé i selektivní a přitom jsou rychlejší, jednodušší a levnější z hlediska pořizovacích i provozních nákladů. Navíc jsou snadno miniaturizovatelné a tudíž kompatibilní s přístupem označovaným jako „point-of-care analysis", kterému je dnes věnována zvýšená pozornost. Proto je následující přehled věnován právě využití moderních elektroanalytických metod v této oblasti, přičemž pozornost je zaměřena na nejčastěji používané voltametrické a ampérometrické metody.

2. Elektrochemická oxidace studovaných biomarkerů

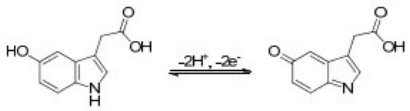
Díky –OH skupině na aromatickém systému jsou uvedené biomarkery elektrochemicky aktivní a lze je relativně snadno oxidovat na nejrůznějších pevných elektrodách (viz tab. I)[49]. Navzdory strukturní podobnosti HVA a VMA vykazují odlišné chování např. při voltametrii na hranami orientované (edge-plane pyrolytic graphite electrode, EPPGE) či na základní rovinou orientované (basal-plane pyrolytic graphite electrode, BPPGE) pyrolytické grafitové elektrodě nebo na elektrodě ze skelného uhlíku (GCE). VMA je zde oxidována v prvním kroku na radikál-kation za výměny dvou elektronů (první pík při diferenční pulsní voltametrii (DPV)). Ten je následně dekarboxylován na vanilin, který je dále oxidován za výměny dalších dvou elektronů na odpovídající *o*-chinon (druhý pík při DPV) podléhající další elektrochemické oxidaci za vzniku směsi různých produktů (viz obr. 2).

Naproti tomu HVA poskytuje jediný DPV pík při potenciálu odpovídajícímu prvnímu píku VMA. To je vysvětlováno podstatně nižší rychlostí dekarboxylace a rearomatizace vedoucí ke vzniku vanilinu poskytujícího druhý pík[16,49]. Při ireverzibilní elektrochemické oxidaci HVA na uhlíkové pastové elektrodě (CPE) dochází ke vzniku 4-aceto-*o*-chinonu, který tvoří reverzibilní redoxní systém s 3,4-dihydroxyfenyloctovou kyselinou[55]. Výskyt tří píků na voltamogramu u HIAA na některých elektrodových materiálech může být spojen s odlišným mechanismem elektrochemické oxidace vedoucí k chinoniminové struktuře, ve které C=O vazba (vzniklá z –OH skupiny na benzenovém jádře) je konjugována s C=N vazbou vzniklou v heterocyklické části molekuly[50] (viz obr. 3). Podle práce[52] je celkový mechanismus značně složitý a jednotlivé píky odpovídají vesměs ireverzibilním krokům s přenosem jednoho elektronu a jednoho protonu ať už výchozí látky či složité směsi vzniklých meziproduktů.

Poloha i výška píků diskutovaných biomarkerů při nejčastěji používané DPV závisí na pH, složení základního



Obr. 2. **Elektrochemická oxidace VMA** (cit.[49])



Obr. 3. **Elektrochemická oxidace HIAA** (cit.[50])

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Tabulka I
Přehled voltametrických metod stanovení HVA, VMA a HIAA

Elektroda	Technika	Analyt	Prostředí	Vzorek	Úprava vzorku	*LOD* [μmol l^{-1}]	Lit.
EPPGE[a] MWCNT-SPE[b]	DPV	VMA	fosfátový pufr (pH 6,85)	–	–	1,7 1	49
SPCE[c]	DPV	HVA, VMA, HIAA	BR pufr (0,04 mol l^{-1}, pH 3,0)	–	–	0,24 (HVA) 0,06 (VMA) 0,12 (HIAA)	51
OGCE[d]	SWV	HIAA	acetátový pufr (0,2 mol l^{-1}, pH 2)	–	–	0,92	52
GCE[e]	CV	HIAA	fosfátový pufr (0,025 mol l^{-1}, pH 2)	žaludeční šťáva	centrifugace, deproteinizace	0,08	53
CFE[f]	DPV	HVA, VMA	BR pufr (0,04 mol l^{-1}, pH 2,0)	–	–	0,09 (HVA) 0,24 (VMA)	54
CPE[g] (modifikovaná lipidem)	CV	HVA	fosfátový pufr (pH 7,4)	extra-celulární mozková tekutina	ředění základním elektrolytem	3	55
CPE (modifikovaná kationtovými tenzidy)	CV	HVA, VMA	fosfátový pufr (0,1 mol l^{-1}, pH 7,5)	–	–	neuveden	56
GCE (film Alizarinové červeně S)	DPV	HVA	fosfátový pufr (0,1 mol l^{-1}, pH 3,0)	lidská krevní plazma	naředění	17	57
GCE (film 3-amino-5--merkapto-1,2,4--triazolu)	DPV	HVA	fosfátový pufr (0,2 mol l^{-1}, pH 7,2)	lidská krevní plazma	ředění základním elektrolytem	$9{,}4 \cdot 10^{-5}$	16
SPCE (modifikovaná nanočásticemi zlata)	SWV	HIAA	fosfátový pufr (pH 7,2)	lidská krevní plazma	naředění	0,022	59
GCE (modifikovaná mědí)	DPV	HVA	fosfátový pufr (0,1 mol l^{-1}, pH 7,2)	–	–	0,01	60
GCE s polymerním filmem (MIP[h])	DPV	HVA	citrátový/HCl pufr (0,1 mol l^{-1}, pH 1,1) se 40% acetonitrilem)	–	–	0,007	61
GCE s polymerním filmem (MIP)	DPV	VMA	citrátový pufr (0,025 mol l^{-1}, pH 3,0)	–	–	96	62
BDDE[i]	DPV	HVA, VMA	fosfátový pufr (0,1 mol l^{-1}, pH 3,0)	–	–	0,57 (HVA) 0,41 (VMA)	64
CPE	DPV	HVA, VMA	BR pufr (0,04 mol l^{-1}, pH 2,0)	–	–	0,38 (HVA) 0,25 (VMA)	65

Tabulka I
pokračování

Elektroda	Technika	Analyt	Prostředí	Vzorek	Úprava vzorku	*LOD* [μmol l^{-1}]	Lit.
CPE	DPV	HVA	BR pufr (pH 2)	–	–	0,4	67
CCE[j]		VMA		–	–	5,02 (první pík) 0,92 (druhý pík)	
SPCE	DPV	HVA, VMA	BR pufr (0,04 mol l^{-1}, pH 3)	–	–	0,24 (HVA) 0,06 (VMA)	68
Pt elektroda	DPV	HVA, VMA	BR pufr (pH 3)	–	–	neuveden	69

[a] EPPGE – hranami orientovaná pyrolytická grafitová elektroda (edge-plane pyrolytic graphite electrode), [b] MWCNT-SPE – vícestěnnými uhlíkovými nanotrubičkami (multiwalled carbon nanotubes) modifikovaná sítotisková elektroda (screen-printed electrode), [c] SPCE – sítotisková uhlíková elektroda (screen-printed carbon electrode), [d] OGCE – elektroda z oxidovaného skelného uhlíku (oxidized glassy carbon electrode), [e] GCE – elektroda ze skelného uhlíku (glassy carbon electrode), [f] CFE – uhlíková filmová elektroda (carbon film electrode), [g] CPE – uhlíková pastová elektroda (carbon paste electrode), [h] MIP – molekulárně vtištěný polymer (molecular imprinted polymer), [i] BDDE – borem dopovaná diamantová elektroda, [j] CCE – uhlíková kompozitní elektroda (carbon composite electrode)

elektrolytu i na použitém elektrodovém materiálu a pohybuje se v širokém rozmezí od +1,2 V v případě VMA či +0,9 V v případě HVA na anodicky oxidované borem dopované diamantové elektrodě (BDDE) až po +0,6 V v případě VMA či +0,3 V v případě HVA na GCE. HIAA je pak ve většině případů oxidována při nižších potenciálech než VMA či HVA. Za určitých podmínek lze tedy získat dostatečně oddělené signály těchto analytů umožňující jejich simultánní stanovení i na nemodifikovaných elektrodách. Hledání těchto podmínek a případné aplikace voltametrických metod na reálné vzorky je předmětem většiny dále diskutovaných prací.

3. Voltametrické stanovení studovaných biomarkerů

K těmto účelům lze využít řadu nemodifikovaných i různým způsobem modifikovaných elektrod. Příkladem využití nemodifikovaných elektrod je stanovení HVA, VMA a HIAA na sítotiskových uhlíkových elektrodách (SPCE, screen-printed carbon electrode) pomocí DPV (cit.[51]) s *LOD* 0,24 μmol l^{-1} (HVA), 0,06 μmol l^{-1} (VMA) a 0,12 μmol l^{-1} (HIAA). Uvedenou metodou je možné stanovit každou kyselinu zvlášť, HVA a VMA i simultánně ve směsi. Square wave voltametrie (SWV, voltametrie čtvercových vln) na GCE při stanovení HIAA poskytuje *LOD* 0,9 μmol l^{-1} i za přítomnosti některých interferentů, přičemž značná pozornost byla věnována i mechanismu elektrodového děje[52]. Cyklická voltametrie (CV) na GCE byla použita i ke stanovení HIAA v lidské žaludeční šťávě[53] s *LOD* 80 nmol l^{-1}. Simultánní stanovení HVA a VMA pomocí DPV na kompozitní uhlíkové elektrodě[54] poskytlo *LOD* 0,3 μmol l^{-1} pro HVA a 0,8 μmol l^{-1} (1. pík) a 1,4 μmol l^{-1} (2. pík) pro VMA. CV na nemodifikované CPE byla použita ke sledování HVA v mozkové extracelulární tekutině. Při rychlosti skenování 0,2 V s^{-1} a vhodně zvolených podmínkách umožňuje sledování HVA v 10s intervalech s *LOD* 3 μmol l^{-1} (cit.[55]).

Voltametrické stanovení HVA a VMA na CPE modifikovaných *in situ* povrchově aktivními látkami (konkrétně kationtovými tenzidy)[56] vedlo k výraznému zvýšení citlivosti ve srovnání s nemodifikovanými CPE, což bylo vysvětleno na základě elektrostatických a hydrofobních interakcí. Za určitých podmínek povrchově aktivní látky stabilizují některé z elektrochemických produktů, což vysvětluje rozdílné voltametrické chování HVA a VMA a odráží se i v různých mechanismech jejich elektrochemické oxidace[56].

DPV na GCE s polymerním filmem Alizarinové červeně S při stanovení levodopy (L-DOPA) a HVA v přítomnosti askorbové kyseliny poskytla *LOD* 6, 17 a 30 nmol l^{-1} pro L-DOPA, HVA a askorbovou kyselinu[57]. GCE potažená filmem 3-amino-5-merkapto-1,2,4-triazolu byla použita k úspěšnému simultánnímu stanovení kyseliny 3,4-dihydroxyfenyloctové (DOPAC) a HVA, s *LOD* 66 pmol l^{-1} pro DOPAC a 94 pmol l^{-1} pro HVA (cit.[16]). Toto simultánní stanovení na nemodifikované GCE nebylo možné. Voltametrické stanovení HVA a VMA na anodicky oxidované BDDE, nemodifikované GCE a GCE modifikované Nafionem (Nafion/GCE) a Poly(neutralní červeni) (PNR/GCE) poskytlo *LOD* 0,6; 0,9; 0,8 a 1,2 μmol l^{-1} pro HVA a 0,4; 1,5; 2,4 a 1,1 μmol l^{-1} pro VMA na BDDE, nemodifikované GCE, Nafion/GCE a PNR/GCE (cit.[58]). SPCE na bázi grafitu modifikovaného nanočásticemi zlata poskytla při SWV stanovení dopaminu a HIAA ve vzorcích moči *LOD* 8 nmol l^{-1} pro dopamin a 22 nmol l^{-1} pro HIAA (cit.[59]).

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Voltametrický senzor pro stanovení močové kyseliny a HVA v přítomnosti askorbové kyseliny připravený nanesením vrstvy mědi na GCE (cit.[60]) umožňuje stanovení 10 nmol l^{-1} HVA a kyseliny močové v přítomnosti 100 nmol l^{-1} kyseliny askorbové.

Molekulárně vtištěné polymery (MIP) jsou další možností pro stanovení biomarkerů rakoviny. Molekulární imprinting je užitečná metoda pro přípravu polymerních porézních materiálů s vysokou selektivitou pro molekulu (templát) díky paměťovému efektu. Metoda založená na porovnání stabilizačních energií předpolymerizačních aduktů mezi templátem a různými funkčními monomery[61] umožnila racionální výběr nejvhodnějšího monomeru a rozpouštědla. Takto připravený voltametrický senzor pro stanovení HVA s použitím MIP jako rozpoznávacího prvku připraveného polymerací methakrylové kyseliny v prostředí toluenu poskytl *LOD* 7 nmol l^{-1}. Vývoj voltametrického senzoru na bázi elektrod modifikovaných akrylovými MIP pro stanovení VMA popsali Blanco-López a spol.[62]. Tenké vrstvy MIP byly připraveny rotačním nanášením směsi monomerů (templátu, methakrylové kyseliny, zesíťovacího činidla a rozpouštědla) na povrch GCE a následnou fotopolymerací. Vlastní měření bylo provedeno ve vodném citrátovém pufru (0,025 mol l^{-1}, pH 3) s 10 % (v/v) acetonitrilu zajišťujícím dobrou smáčivost polymeru. *LOD* se pohyboval kolem 95 µmol l^{-1}.

Stanovení HVA, VMA a HIAA pomocí voltametrie i pomocí později diskutované ampérometrie jsou věnovány i bakalářské a diplomové práce studentů z naší laboratoře. Jako příklad lze uvést stanovení HVA pomocí HPLC-ED (cit.[63]) na koloně Phenomenex Gemini C18 s coulometrickou detekcí (ESA, 0 V, +0,075 V, +0,45 V) a mobilní fází fosfáto-acetátový pufr, acetonitril a methanol o pH 5,35 s *LOD* 0,13 µmol l^{-1} nebo simultánní stanovení HVA a VMA ve směsi pomocí DPV s anodicky oxidovanou BDDE[64] ve fosfátovém pufru (0,1 mol l^{-1}, pH 3,0) s výslednými *LOD* 0,57 µmol l^{-1} pro HVA a 0,41 µmol l^{-1} pro VMA. HVA a VMA byly dále stanoveny pomocí DPV a HPLCE-ED na CPE (cit.[65]). DPV v Brittonově-Robinsonově (BR) pufru poskytla *LOD* 0,38 µmol l^{-1} pro HVA a 0,25 µmol l^{-1} pro VMA. HPLC s ampérometrickou detekcí („wall-jet" detektor, +1,1 V) s mobilní fází acetonitril-fosfáto-acetátový pufr (25:75) o pH 2,5 poskytla *LOD* 0,7 µmol l^{-1} pro HVA a 0,2 µmol l^{-1} pro VMA. Metodou standardního přídavku byly sledované biomarkery stanoveny ve zředěném vzorku moči s *LOD* 0,8 µmol l^{-1} pro HVA a 0,5 µmol l^{-1} pro VMA. Dosažené výsledky jsou zároveň shrnuty v článku[66]. Uhlíková filmová elektroda (CFE) a GCE byly použity v dizertační práci[67] pro stanovení HVA a VMA pomocí CV, DPV, voltametrie s lineárním nárůstem potenciálu (LSV, linear staircase voltammetry) a elektrochemické impedanční spektroskopie (EIS). Bylo popsáno i stanovení HVA a VMA pomocí DPV na SPCE[68] v BR pufru s *LOD* 0,24 µmol l^{-1} pro HVA a 0,06 µmol l^{-1} pro VMA. Výsledky byly spolu s dalším analytem HIAA (*LOD* 0,12 µmol l^{-1}) publikovány ve výše zmíněném článku[51]. V další závěrečné práci[69] je pro stanovení HVA a VMA využita diferenční pulsní ampérometrie (+0,6 V, platinový tubulární detektor) s platinovou elektrodou v BR pufru a rovněž HPLC s ampérometrickou detekcí a UV detekcí (254 nm) v mobilní fázi acetonitril-BR pufr (pH 2,5).

4. HPLC stanovení studovaných biomarkerů s elektrochemickou detekcí

HPLC-ED kombinuje výhody separačních metod (zejména vyšší selektivitu) a metod elektrochemických (vysokou citlivost a skutečnost, že neruší elektrochemicky neaktivní látky). Mez detekce se zpravidla pohybuje v submikromolárním koncentračním rozmezí a při analýze biologických materiálů se tato technika často kombinuje s vhodnou metodou předběžné separace a prekoncentrace, nejčastěji pomocí kapalinové extrakce (LLE) nebo extrakce tuhou fází (SPE, pozor na stejnou zkratku používanou běžně v odborné literatuře pro sítotiskové elektrody). Nejčastěji se používá ampérometrická detekce při konstantním potenciálu vloženém na vhodnou pracovní elektrodu. Nejběžnější upořádání je tenkovrstvé (thin-layer), wall-jet či tubulární. Rozhodující zde je vhodná volba podmínek pro separaci a pro detekci, které je nutné vhodným způsobem sladit, a dále vhodná volba použitého materiálu detekční elektrody. V poslední době se stále častěji používají metody s rychlou změnou potenciálu pracovní elektrody umožňující záznam řady voltamogramů v průběhu jediného chromatografického píku a tak zvyšující výpovědní hodnotu tohoto způsobu detekce. Totéž platí o elektrochemických detektorech využívajících různé typy elektrodových polí umožňujících současný záznam chromatogramů při různých hodnotách vloženého potenciálu. Pulsní ampérometrie pak umožňuje využití různých potenciálových pulsů nejen k detekci sledovaného analytu, ale i k čištění pracovní elektrody eliminujícímu případné problémy s její pasivací. Pravděpodobně nejcitlivější je coulometrická detekce pomocí porézních elektrod či jejich polí, která však vyžaduje podstatně dražší instrumentaci. Přehled metod stanovení studovaných biomarkerů pomocí různých metod HPLC s elektrochemickou detekcí je uveden v tab. II. Z lékařského hlediska je nejzajímavější práce italských vědců[6], kteří sledovali biomarkery neuroblastomu (HVA a VMA) pomocí HPLC-ED s využitím komerčně dostupné metody (HVA-VMA s HPLC, Bio Rad Laboratories, Milán, Itálie) po dobu sedmi let. Práce podrobně analyzuje diagnostické výsledky HVA/Cr a VMA/Cr (hodnoty HVA a VMA vztažené na množství vyloučeného kreatininu v moči) v různých stádiích onemocnění a věkových podskupinách a poskytuje mezní hodnoty, které mohou určit pacienty s neuroblastomem i pacienty s podezřením na neuroblastom a pomoci tak při vyhodnocování lékařských vyšetření.

Tabulka II
HPLC-ED stanovení HVA, VMA a HIAA

Detekce (typ detekce, typ detektoru, parametry)	Analyt	Kolona (typ, (zrnitost v μm; rozměry v mm))	Vzorek	Úprava vzorku	*LOD* [μmol l^{-1}]	Lit.
coul[a], ESA, +0,4 V, +0,2 V, –0,3 V	HVA,VMA, HIAA	ODS (150 × 4,6)	moč	filtrace	$(4,3–13,1)\cdot10^{-3}$	3
coul[a], ESA, +0,1 V, +0,65 V, +0,7 V	HVA,VMA, HIAA	Zorbax SP-150 C8	moč	extrakce ethyl-acetátem	2,7 (HVA) 0,5 (VMA) 5,2 (HIAA)	22
amp[b], TL-5 průtoková cela, +0,67 V	HVA, VMA, HIAA	ODS (5; 150 × 4,6)	moč	extrakce ethyl-acetátem	neuveden	23
amp, průtoková cela, +0,6 V (HVA, VMA), +0,45 V (5-HIAA)	HVA, VMA, HIAA	Yanapak ODS-T (10; 250 × 4)	moč	centrifugace	neuveden	70
amp, LC-12 průtoková cela, +0,72 V	HVA, VMA, HIAA	Hypersil ODS (5; 150 × 4,6)	moč	extrakce ethyl-acetátem	neuveden	71
coul, ESA, +0,35 V, +1 V, 0 V	HVA, VMA, HIAA	Lichrospher C18 (5; 125 × 4)	moč	extrakce etherem, centrifugace	0,08 (HVA) 0,15 (VMA) 0,03 (HIAA)	9
coul, detektorové pole, +0,2 V, +0,3 V, +0,35 V, +0,4 V, +0,45 V, +0,5 V, +0,55 V, +0,6 V	HVA, VMA, HIAA	C18/iontově výměnná (150 × 4,6)	moč	centrifugace	$9,3\cdot10^{-3}$ (HVA) $1,21\cdot10^{-2}$ (VMA) $1,31\cdot10^{-2}$ (HIAA)	24
coul, ESA, +0,1 V, +0,22 V	HVA, VMA	Inertsil-ODS 2 (250 × 4,6)	moč	analyty eluovány z filtračního papíru, zředění	0,03 (HVA, VMA)	72
amp, LC-4B průtoková cela, +0,85 V	HVA, VMA	Hitachi Gel No. 3013-0 (250 × 4)	moč	analyty eluovány z filtračního papíru, extrakce ethyl-acetátem	neuveden	73
amp, LC-4B, +0,9 V	HVA, VMA	Hitachi Gel 3013-0 (5; 250 × 4)	moč	extrakce z filtračního papíru	0,055 (HVA) 0,025 (VMA)	74
coul, ESA, +0,05 V, –0,175 V	HVA, VMA	Spherisorb ODS 2 (5; 250 × 4)	plasma	SPE[c], centrifugace	$1,7\cdot10^{-4}$ (HVA) $3,8\cdot10^{-4}$ (VMA)	27
coul, ESA, +0,45 V, +0,4 V, +0,18 V	HVA, VMA	Discovery HS C18 (5; 250 × 4)	moč	SPE	0,8 (HVA) 0,4 (VMA)	75
amp, LC-17 průtoková cela, +0,8 V	VMA, HIAA	Waters 5 μm C18 Nova Pak	moč	extrakce ethyl-acetátem	10,1 (VMA) 5,2 (HIAA)	76
amp, LC-4 průtoková cela, +0,7 V	HVA, HIAA	C18 RP (300 × 3,9)	moč	hydrolýza	$1,4\cdot10^{-3}$ (HVA) $1,3\cdot10^{-3}$ (HIAA)	77
amp, LC-4A průtoková cela, +0,85 V	HVA, HIAA	Ultrasphere ODS (5; 150 × 4,6)	myší mozková tkáň	homogenizace, centrifugace	$1,37\cdot10^{-2}$ (HVA) $5,2\cdot10^{-3}$ (HIAA)	78
coul, ESA, +0,1 V, –0,2 V, +0,4 V	HVA, HIAA	C18 Hypersil (5; 150 × 4,6)	vysušené krevní skvrny a plasma	SPE	$6,6\cdot10^{-4}$ (HVA) $2,6\cdot10^{-4}$ (HIAA)	79

Tabulka II
pokračování

Detekce (typ detekce, typ detektoru, parametry)	Analyt	Kolona (typ, (zrnitost v μm; rozměry v mm))	Vzorek	Úprava vzorku	*LOD* [μmol l^{-1}]	Lit.
amp, LC-3 průtoková cela, +0,8 V	HVA, HIAA	C18 (300 × 3,9)	lidská a myší mozková tkáň	centrifugace	$1{,}1 \cdot 10^{-2}$ (HVA) $1{,}1 \cdot 10^{-3}$ (HIAA)	80
amp, tenkovrstvá průtoková cela, +0,7 V	HVA, HIAA	Luna C18 (5; 250 × 4,6)	mozkomíšní mok	mikrodialýza	$1{,}3 \cdot 10^{-3}$ (HVA) $6 \cdot 10^{-4}$ (HIAA)	12
amp, detekce pomocí rotující diskové elektrody při +0,7 V	HVA, HIAA	Polygosil C8 (5; 100 × 3)	myší mozková tkáň	homogenizace, centrifugace	neuveden	81
amp, tenkovrstvá průtoková cela, +0,7 V	HVA	Shim-pack VP-ODS (5; 150 × 4,6)	moč	centrifugace	$7 \cdot 10^{-4}$	26
coul, ESA, +0,3 V	HVA	C18 RP (5; 250 × 4,6)	myší mozková tkáň po mikro-dialýze	–	$3 \cdot 10^{-3}$	82
coul, ESA, detektorové pole, +0,05 V na elektro-dě 1, +0,15 V na elektrodě 2, +0,25 V na elektrodě 3, +0,35 V na elektrodě 4, +0,55 V na elektrodě 5, +0,9 V na elektrodě 6, +1 V na elektrodě 7	HVA	C18 ESA MD-150 (3; 150 × 3)	myší mozková tkáň	homogenizace	$4{,}7 \cdot 10^{-3}$	83
amp, TV II tenkovrstvý, +0,8 V	HVA	Polygosil C8 (5; 100 × 3)	myší mozková tkáň	homogenizace, centrifugace	neuveden	84
amp, průtoková cela, +0,8 V	HVA	C8 (5; 150 × 4,6)	plasma	SPE	$1{,}1 \cdot 10^{-3}$	85
coul, ESA, +0,1 V, –0,2 V, +0,5 V	HVA	C8 (5; 250 × 4,6)	plasma	SPE	$5{,}5 \cdot 10^{-4}$	86
coul, ESA, 0 V, 0,075 V, +0,45 V	HVA	Phenomenex Gemini C18 (150 × 4,6)	myší mozková tkáň[d]	homogenizace, filtrace	0,13	63
coul, ESA, +0,45 V, –0,2 V	VMA	Spherisorb ODS2 (5; 100); C18 (10; 20)	moč	extrakce do vázané fáze na oxidu křemičitém	$1{,}25 \cdot 10^{-2}$	87
amp, TL-8A průtoková cela, +1,3 V	VMA	Lichrosorb RP 18 (10; 250 × 4,5)	moč	extrakce ethyl-acetátem, centrifugace	neuveden	88
amp, LC-3 průtoková cela, +0,6 V	HIAA	Nucleosil C18 RP (3; 75 × 4) Nucleosil C18 RP (5; 150 × 4,6)	moč	–	0,5	89
amp, LC-4B průtoková cela, +0,55 V	HIAA	Biophase ODS C18 (5; 250 × 4,6)	moč	SPE	6,28	90

Tabulka II
pokračování

Detekce (typ detekce, typ detektoru, parametry)	Analyt	Kolona (typ, (zrnitost v μm; rozměry v mm))	Vzorek	Úprava vzorku	*LOD* [μmol l^{-1}]	Lit.
amp, LC-4A průtoková cela, +0,5 V	HIAA	Bondapak C18 (10; 240 × 4,6)	mozkomíšní mok, plasma, moč	extrakce ethyl-acetátem, centrifugace	$4 \cdot 10^{-3}$	91
amp, průtoková cela, +0,4 V	HIAA	Luna C18 (5; 250 × 4,6)	moč	centrifugace, filtrace	$5 \cdot 10^{-4}$	92
amp, TL-8A průtoková cela, +1 V	HIAA	Ultrasphere C18 (5; 150 × 4,5)	moč	centrifugace	neuveden	93
coul, ESA, +0,25 V; +0,55 V	HVA, VMA, HIAA	Hypersil C18 (5; 250 × 4,6)	moč	–	2 (HVA) 0,3 (VMA) 1 (HIAA)	10

[a] coul – coulometrická detekce, [b] amp – ampérometrická detekce, [c] SPE – extrakce tuhou fází (solid phase extraction), [d] pokus o použití této techniky v myší mozkové tkáni po mikrodialýze byl neúspěšný

5. Další elektrochemické metody

Sem lze zařadit např. některé velmi zajímavé studie možností potenciometrického stanovení těchto biomarkerů[48,94] a dále studie jejich stanovení pomocí průtokové injekční analýzy s ampérometrickou detekcí (FIA-AD)[95].

Potenciometrie představuje jednu z nejlevnějších, nejrychlejších a tudíž v praxi nejoblíbenějších metod. Velmi zajímavou možnost detekce diskutovaných protinádorových biomarkerů nabízí diplomová práce[94] popisující přípravu potenciometrických biosenzorů na bázi vodivých polymerů a PVC membrán. Na předupravený povrch elektrod na bázi vodivých polymerů byla kovalentně navázaná merkaptofenylboronová kyselina a povrch PVC membrán na bázi anexu byl modifikován chemicky generovaným polyanilinem (PANI). Takto připravená elektroda umožňuje rychlé a jednoduché přímé potenciometrické stanovení HVA, VMA i HIAA s *LOD* kolem 2 mmol l^{-1}. FIA umožňuje výrazné zrychlení analýz a navíc v řadě případů eliminuje problémy s pasivací pracovní elektrody, neboť produkty elektrodové reakce jsou účinně odstraňovány od povrchu elektrody tokem nosné kapaliny. Využitím FIA-AD ke stanovení studovaných biomarkerů se zabývá studie[95]. Biomarkery byly stanoveny na SPCE v BR pufru (0,04 mol l^{-1}, pH 2,0) v komerčně dostupné průtokové cele. Kalibrační závislosti byly proměřeny v rozsahu 0,05 až 100 μmol l^{-1}. Závislosti byly lineární a dosažené *LOD* byly 0,065 μmol l^{-1} pro HVA (při +0,6 V), 0,053 μmol l^{-1} pro VMA (při +0,8 V) a 0,033 μmol l^{-1} pro HIAA (při +0,8 V) (počítáno z vyhodnocených výšek píků) a 0,024 μmol l^{-1} pro HVA, 0,020 μmol l^{-1} pro VMA a 0,012 μmol l^{-1} pro HIAA (počítáno z vyhodnocených ploch píků). Tato metoda je považována za slibnou z hlediska rychlosti analýzy, snadné automatizace a eliminace pasivace elektrod. Vzhledem k blízkým hodnotám detekčních potenciálů nelze všechny tři biomarkery stanovit simultánně v jednom experimentu. K tomuto účelu se nabízí dosud nezkoumaná možnost využití fast scan voltametrie, pole elektrod či vhodných matematických metod používaných k rozlišení elektrochemicky podobných látek.

6. Závěr

Cílem tohoto příspěvku bylo upozornit na možnosti, výhody a omezení moderních elektroanalytických metod pro stanovení vybraných nádorových biomarkerů (jmenovitě homovanilové, vanilmandlové a 5-hydroxy-3-indoloctové kyseliny) a přehledně shrnout dosud publikované voltametrické a ampérometrické metody vhodné pro stanovení zmíněných biomarkerů v biologických tekutinách. Jak již bylo uvedeno, hlavní předností elektrochemických metod je jejich rychlost, citlivost, miniaturizovatelnost a nízké pořizovací i provozní náklady. Za nevýhodu lze považovat ne vždy postačující selektivitu a problémy s pasivací pracovních elektrod ve složitých biologických matricích. A právě tímto směrem bude nutné zaměřit další výzkum. Z velkého počtu publikovaných prací o elektrochemickém stanovení diskutovaných biomarkerů lze usuzovat na jejich význam a jejich možnosti. Díky včasnému zachycení zvýšených koncentrací těchto biomarkerů v tělních tekutinách je možno léčbu nádorů zahájit včas, a zvýšit tak šanci na uzdravení pacientů.

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A. Makrlíková[a,b], J. Barek[a], V. Vyskočil[a], and T. Navrátil[b] (*[a] Charles University, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry, Prague, [b] J. Heyrovský Institute of Physical Chemistry of the AS CR*): **Electrochemical Methods for the Determination of Homovanillic, Vanillylmandelic, and 5-Hydroxy-3-indoleacetic Acid as Cancer Biomarkers**

Homovanillic (HVA), vanillylmandelic (VMA), and 5-hydroxy-3-indoleacetic acid (HIAA) are cancer biomarkers and their abnormally high urinary levels can serve as a useful tool for prediction of neuroblastoma, pheochromocytoma, and carcinoid tumors. HVA and VMA are connected with the catecholamine metabolism,

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while HIAA is related to the tryptophan metabolism. The aim of this review is to describe the electrochemical oxidation HVA, VMA, and HIAA and summarize published voltammetric and amperometric methods suitable for the determination of these biomarkers in various biological fluids.

Keywords: homovanillic acid, vanillylmandelic acid, 5-hydroxy-3-indoleacetic acid, tumor biomarkers, voltammetry, amperometry, HPLC with electrochemical detection

8 APPENDIX II

Voltammetric determination of tumor biomarkers for neuroblastoma (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) at screen-printed carbon electrodes

Anna Makrlíková, Evangelia Ktena, Anastasios Economou, Jan Fischer, Tomáš Navrátil, Jiří Barek, Vlastimil Vyskočil

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SPECIAL ISSUE

Voltammetric Determination of Tumor Biomarkers for Neuroblastoma (Homovanillic Acid, Vanillylmandelic Acid, and 5-Hydroxyindole-3-acetic Acid) at Screen-printed Carbon Electrodes

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Abstract: Three tumor biomarkers (homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindole-3-acetic acid (5-HIAA)) have been determined by differential pulse voltammetry (DPV) at screen-printed carbon electrodes (SPCEs). As an optimum medium for all three analytes, Britton-Robinson buffer (0.04 mol·L^{-1}, pH 3.0) was used. Dependencies of the peak current on the concentration of a biomarker were linear in the concentration range from 0.2 to 100 μmol·L^{-1} for all the tested compounds, with the limits of quantification 0.8 μmol·L^{-1} for homovanillic acid, 0.2 μmol·L^{-1} for vanillylmandelic acid, and 0.4 μmol·L^{-1} for 5-hydroxyindole-3-acetic acid. Moreover, it was verified that the newly developed methods can be used for simultaneous determination of HVA and VMA in mixture.

Keywords: Homovanillic acid · Vanillylmandelic acid · 5-Hydroxyindole-3-acetic acid · Differential pulse voltammetry · Screen-printed carbon electrodes

1 Introduction

Homovanillic acid (HVA, 4-Hydroxy-3-methoxyphenylacetic acid) and vanillylmandelic acid (VMA, DL-4-Hydroxy-3-methoxymandelic acid) are structurally closely related and they are the major end products of catecholamine metabolism. 5-Hydroxyindole-3-acetic acid (5-HIAA) is a breakdown product of serotonin (5-hydroxytryptamine) [1,2]. Their structural formulas are given in Table 1. The phenolic structure suggests that they can be oxidized at carbon electrodes, which can be used for their determination [3,4]. Determination of urinary levels of HVA, VMA, and 5-HIAA can be a useful tool for the prediction of neuroblastic and carcinoid tumors [2]. Urinary production of adrenal hormones (norepinephrine, epinephrine, and dopamine) and their metabolites (nor-metanephrine, metanephrine, methoxydopamine, HVA, and VMA) is increased in the case of neuroblastoma (this term refers to a spectrum of neuroblastic tumors including neuroblastoma, ganglioneuroblastoma, and ganglioneuroma). These kinds of tumors are connected with metabolism of tryptophan, with the release of large amount of serotonin, and lead to clinical syndromes like flushing, hepatomegaly, diarrhea, bronchospasm, and right-sided heart failure [5]. Measuring 5-HIAA in biological fluids for clinical purposes can be useful for the diagnosis not only of neuroblastoma or carcinoid syndrome but also of essential hypertension, depression, migraine, and Tourette syndrome [6]. The urinary concentrations for the above mentioned organic acids described as normal are from 8.2 to 41.0 μmol·L^{-1} for HVA, from 11.6 to 28.7 μmol·L^{-1} for VMA, and from 17.8 to 58.3 μmol·L^{-1} for 5-HIAA [7], i.e. between 1.3 and 7.6 mg·L^{-1} of those biomarkers [8].

So far mainly chromatographic methods were used for the determination of these biomarkers. Comparison of three direct HPLC methods for quantification of urinary HVA, VMA, and 5-HIAA (two with spectrofluorometric detection with discontinuous gradients and one with coulometric detection with linear gradient) can be found in paper [2]. In the first method with fluorescence detection [9], the method of the external standard [10] was used. The second method with fluorescence detection was performed according to [11], except for the column protection procedure based on C18 pre-column cartridges. The third method with electrochemical detection combines methods described in papers [12,13] using a dual-cell coulometric detector Coulochem type 5100 (ESA, Bedford,

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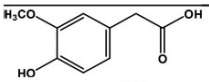
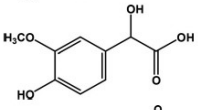
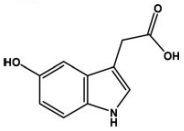
Table 1. Basic information about the studied analytes.

Structure of analyte	Common name	CAS Number
	Homovanillic acid	306-08-1
	Vanillylmandelic acid	55-10-7
	5-Hydroxyindole-3-acetic acid	54-16-0

MA, USA). The first cell was operated at 0.25 V, which was optimized for 5-HIAA, and the second cell was operated at 0.55 V for detecting HVA and VMA. Limits of detection (*LOD*s) for HVA were 1.0, 2.0, and 2.0 $\mu mol \cdot L^{-1}$ (first, second, and third method), 0.5, 0.5, and 0.3 $\mu mol \cdot L^{-1}$ for VMA (first, second, and third method), and 1.0 $\mu mol \cdot L^{-1}$ for 5-HIAA (all three methods). All the above mentioned analytical methods were proved to be suitable for the direct simultaneous determination of these analytes in human urine [2]. Yoshitake et al. [6] proposed an improved HPLC method for the determination of 5-HIAA in human urine based on automated pre-column derivatization of 5-HIAA with benzylamine followed by fluorescence detection (345/480 nm) providing *LOD* 5 $nmol \cdot L^{-1}$ without any sample purification. Another possible determination of 5-HIAA [14] is based on combination of adsorption chromatography with UV detection providing *LOD* 0.52 $\mu mol \cdot L^{-1}$.

Due to the low concentrations of these analytes, sample pre-treatment procedure is frequently used. Solid phase extraction (SPE) seems to be a suitable technique to obtain good sample purification and high extraction yields [15]. Determination of HVA in human plasma by HPLC was successfully performed using a reversed-phase column and coulometric detection after anion-exchange SPE with *LOD* around 1 $nmol \cdot L^{-1}$ [16]. Determination of HVA in human urine using SPE at weak anion-exchange hypercrosslinked polymer resin followed by HPLC with UV detection at 280 nm gave *LOD* 2.47 $\mu mol \cdot L^{-1}$ [17]. Solid phase microextraction (SPME) combined with gas chromatography quadrupole mass spectrometry after preliminary derivatization with ethyl chloroformate/ethanol was described in [18]. Combination of column and partition chromatography (GC-MS) was used in [19] to isolate and quantify biological organic acids in human urine and cerebrospinal fluid (including HVA, VMA, and 5-HIAA) after their conversion to trimethylsilyl derivatives. Simultaneous determination of HVA and 5-HIAA by HPLC-ED after liquid-liquid extraction of acidified urine with ether followed by coulometric detection at +350 mV gave *LOD* 0.08 $\mu mol \cdot L^{-1}$ for HVA and 0.03 $\mu mol \cdot L^{-1}$ for 5-HIAA [20]. An enzyme immunoassay for urinary VMA using a polyclonal antiserum and a VMA–acetylcholinesterase conjugate as an enzymatic tracer in the concentration range 0.2-2.25 $\mu mol \cdot L^{-1}$ gave cross-reactivity with HVA 0.8% and less than 0.4% with other structurally related catecholamine metabolites [21].

Differential pulse voltammetry (DPV) of VMA at an edge-plane pyrolytic graphite electrode (EPPGE) and at a commercially available multiwalled carbon nanotubes (MWCNTs) screen-printed electrodes (MWCNT-SPEs) gave *LOD*s 1.7 and 1.0 $\mu mol \cdot L^{-1}$, respectively (in phosphate buffer solution, pH 6.85) [4]. It was found that the oxidation of VMA at an EPPGE leads to the formation of vanillin via molecule decarboxylation, which may be further oxidized at a high potential, leading to two distinct voltammetric peaks. On other forms of carbon, these two voltammetric peaks are not so well defined. HVA undergoes a similar oxidation pathway but due to the lack of an -OH group, rearomatization after decarboxylation is a slower process requiring the attack of water, in order to form vanillic alcohol, and as a result only one peak is observed. The resolution of HVA and VMA can be further improved by modification of the electrode with a porous layer of MWCNTs to change the mass transport to that of a thin layer system [4]. Voltammetric behavior of HVA and VMA was also investigated at carbon paste electrodes (CPEs) modified in situ with cationic surfactants which stabilize some of the electrochemical reaction intermediates resulting in different voltammetric behavior of HVA and VMA on unmodified and modified electrodes [22]. Similarly, modification of CPEs with submicellar concentrations of surfactants results in improved reversibility and sensitivity [23]. Cyclic voltammetric determination of 5-HIAA in human gastric juice at a glassy carbon electrode (GCE) is possible in the concentration range from 0.2 to 20 $\mu mol \cdot L^{-1}$ with

LOD 80 nmol·L^{-1} [24]. An array of carbon composite film electrodes (CFEs) based on graphitic conductive microparticles and nonconductive polystyrene binder embedded in a 96-well microtitration plate was applied to DPV determination with *LOD* 0.3 µmol·L^{-1} for HVA and 0.8 µmol·L^{-1} for VMA [3]. Square wave voltammetry (SWV) of 5-HIAA at an oxidized glassy carbon electrode (OGCE) gave *LOD* 0.9 µmol·L^{-1} with minimal interference of usual urine components [31]. A voltammetric sensor for HVA was developed using different molecularly imprinted polymers (MIPs) as recognition elements [25]. This sensor gave a linear response for concentrations of HVA between 0.05 and 10 µmol·L^{-1} with *LOD* 7 nmol·L^{-1}. The comparison of existing methods is given in Table 2.

The presented paper is focused on the development of a simple, inexpensive, fast, and user-friendly DPV method for the determination of the three above mentioned tumor biomarkers in Britton-Robinson buffer (BRB) using easily commercially available screen-printed carbon electrodes (SPCEs) which are a suitable tool for the determination of various organic compounds in biological fluids [27,28]. There is no problem with their passivation because they are disposable and thus used just for one measurement. They are relatively inexpensive because of their mass production. Their application can circumvent sometimes complicated modification and/or fabrication of less common electrodes. Moreover, the newly developed methods are compatible with portable instrumentation, suitable for further miniaturization and thus compatible with the point-of-care concept.

2 Experimental

2.1 Apparatus

The voltammetric measurements were performed with a computer controlled Eco-Tribo Polarograph with Polar Pro software, version 5.1 for Windows 95/98/Me/2000/XP (Polaro-Sensors, Czech Republic). The software worked under the operational system Microsoft Windows XP Professional (Microsoft Corporation, USA). Differential pulse voltammetry (DPV) was performed using SPCEs (type DRP 110, DropSens, Spain) with three electrode system comprising a carbon working electrode (4 mm diameter), a carbon counter electrode, and a silver reference electrode. A new SPCE was used for each measurement to avoid cross-contamination and passivation and to comply with usual requirements for clinical analyses. However, it was verified that up to 5 measurements can be done at a single SPCE at micromolar concentrations of the tested biomarkers. At higher concentrations, the passivation of SPCE is observable. Therefore, it is better to use a new SPCE for each measurement for the highest concentrations of these analytes.

2.2 Reagents and Procedures

The stock solutions (1 mmol·L^{-1}) of the three tumor biomarkers were prepared by dissolving a required amount of the pure substance in 25 mL of deionized water (HVA: 4.67 mg; VMA: 4.95 mg; 5-HIAA: 4.78 mg). It follows from UV spectrophotometric monitoring of the stability

Table 2. Analytical methods for the determination of HVA, VMA, and 5-HIAA.

Analyte	Method	Detection	*LOD* [µmol L^{-1}]	References
HVA, VMA	GC	MS	0.27 (both)	[26]
HVA, VMA, 5-HIAA	HPLC	MS/MS	0.27 (HVA) 0.0050 (VMA) 0.078 (5-HIAA)	[5]
HVA, VMA, 5-HIAA	HPLC	Fluorimetry	1.0 (HVA) 0.5 (VMA) 1.0 (5-HIAA)	[2]
		Fluorimetry	2.0 (HVA) 0.5 (VMA) 1.0 (5-HIAA)	
		Coulometry	2.0 (HVA) 0.3 (VMA) 1.0 (5-HIAA)	
5-HIAA	HPLC	Fluorimetry	0.005	[6]
	HPLC	UV	0.52	[14]
VMA	Immunoassays	Enzymatic	0.2	[21]
HVA	Voltammetry	DPV (MIPs)	0.007	[25]
VMA	Voltammetry	DPV (EPPGE) DPV (MWCNT-SPEs)	1.7 1.0	[4]
VMA, HVA	Voltammetry	DPV (CFEs)	0.09 (HVA) 0.24 (VMA)	[3]
5-HIAA	Voltammetry	SWV (OGCE)	0.9	[31]
HVA, VMA, 5-HIAA	Voltammetry	DPV (SPCEs)	0.24 (HVA) 0.06 (VMA) 0.06 (5-HIAA)	This work

of those stock solutions that their absorbance at corresponding maximum wavelengths (279 nm for HVA and VMA, and 276 nm for 5-HIAA, respectively) was constant for 3 months with RSD lower than 2%, thus confirming their sufficient stability. All measurements were performed in Britton-Robinson buffer (BRB) prepared in a usual way, i.e. by mixing a solution of 0.04 mol·L^{-1} in phosphoric acid, 0.04 mol·L^{-1} in acetic acid, and 0.04 mol·L^{-1} in boric acid with the appropriate amount of 0.2 mol·L^{-1} sodium hydroxide solution. Appropriate amount of the analyte stock solution was placed into a 25 mL volumetric flask and filled up to the mark with BRB of the required pH. Afterwards, 5 mL of thus prepared solution were transferred into a voltammetric vessel and the voltammograms were recorded in five repetitions using DPV from −200 to +1400 (or 1600) mV, scan rate 20 mV·s^{-1}, pulse height +50 mV, pulse width 100 ms, and current range 100 μA. The DPV peak heights were evaluated from the straight lines connecting the minima before and after the peak (the minimum after the second peak was used when the first and the second peak were not well separated). All the experiments were performed at room temperature. For calculating calibration curve parameters and graphic expressions of results, Microsoft Office Excel 2010 (Microsoft Corporation, USA) and OriginPro 8.0 (OriginLab Corporation, USA) were used. The limit of quantification (LOQ) was calculated as $LOQ = 10\ s/a$, where s is the standard deviation of 10 repetitive measurements of the lowest measurable concentration and a is the slope of the calibration curve [29]. The limit of detection (LOD) was calculated as $LOD = 3s/a$.

3 Results and Discussions

BRB was chosen as a base electrolyte. At first, DP voltammograms of HVA in BRB of different pH were measured (see Fig. 1). The optimum pH for the determination of HVA was found to be 3.0. At pH 3.0, two well-developed peaks of HVA are formed; the more positive peak decreases with increasing pH (see Fig. 1B).

Due to similarity of structures of VMA and HVA, the pH dependence of VMA was also similar and the optimum pH for the determination of HVA was found to be 3.0 as well (see Fig. 2). For 5-HIAA, the pH dependence was measured from pH 2.0 to pH 9.0. The peak of 5-HIAA was not evaluable at pH > 9.0 (see Fig. 2B). The optimum pH for the determination of 5-HIAA was found to be 3.0, too. In acidic pH, there are two well-separated peaks of 5-HIAA.

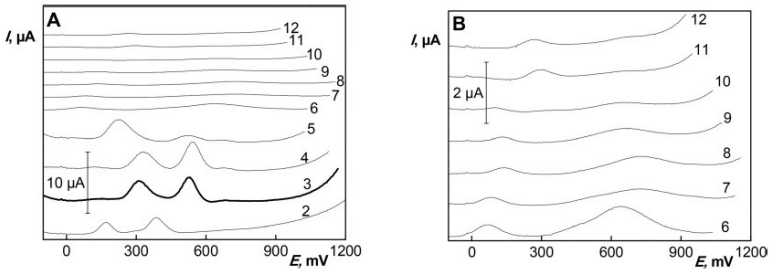


Fig. 1. (A) DP voltammograms of HVA (0.1 mmol·L^{-1}) measured at SPCE in BRB at different pH from 2.0 to 12.0; (B) enlarged DP voltammograms of HVA (0.1 mmol·L^{-1}) measured at SPCE in BRB at different pH from 6.0 to 12.0; pH is given above the curves; the bold curve corresponds to optimum pH.

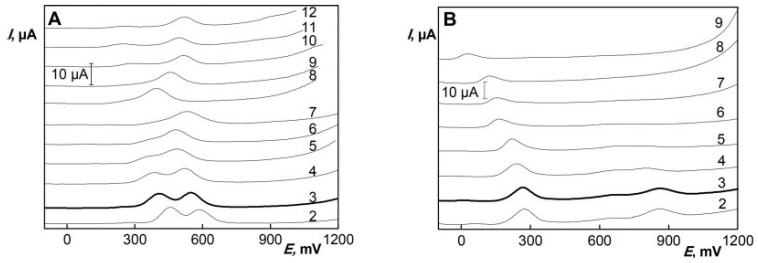


Fig. 2. DP voltammograms of VMA (A) and 5-HIAA (B), both 0.1 mmol·L^{-1}, measured at SPCE in BRB at different pH given above the curves; the bold curves correspond to optimum pH.

The existence of two peaks suggests the mechanism proceeding through the decarboxylation of VMA to form vanillin, which is further oxidized resulting in the second peak. At low pH, the electrochemical oxidation of this functionality probably follows a "$-2e^-$, $-2H^+$" mechanism leading to the formation of a carbocation [4]. This mechanism is similar to that found for the oxidation of catechol [22]. Oxidative voltammetric responses of VMA and HVA at SPCEs are similar, contrary to the situation at an EPPGE and MWCNT-SPEs [4] where different number of voltammetric peaks was observed. The appearance of three peaks in the case of 5-HIIA can be connected with a slightly different mechanism of its electrochemical oxidation resulting in quinoneimine structure in which the C=O double bond formed from the –OH group on the benzene ring is conjugated with the C=N double bond formed in the heterocyclic (pyrrole) part of the molecule [30].

Afterwards, the dependencies of the peak current on the analyte concentration were measured in BRB at pH 3.0 in the concentration range from 0.1 to 100 $\mu mol \cdot L^{-1}$. All dependencies were linear in this range. DP voltammograms of the analytes in the concentration range from 10 to 100 $\mu mol \cdot L^{-1}$ are shown in Fig. 3, from 1 to 10 $\mu mol \cdot L^{-1}$ in Fig. 4, and from 0.1 to 1 $\mu mol \cdot L^{-1}$ in Fig. 5. For HVA and VMA, both peaks are significant, well-developed, and useful for analytical purposes. For 5-HIAA, three peaks were observed. However, only the peak at the potential around +270 mV was evaluated for the calibration dependence because evaluation of the other two peaks is more difficult and leads to less precise results. The obtained *LOQ*s were: 1.0 $\mu mol \cdot L^{-1}$ (1st peak) and 0.8 $\mu mol \cdot L^{-1}$ (2nd peak) for HVA, 0.4 $\mu mol \cdot L^{-1}$ (1st peak) and 0.2 $\mu mol \cdot L^{-1}$ (2nd peak) for VMA, and 0.2 $\mu mol \cdot L^{-1}$ for 5-HIAA (1st peak), respectively (see Table 3).

An attempt to determine HVA and VMA in mixture is documented by Figs. 6 and 7. It can be seen that it is possible to find the peaks of HVA and of VMA in the mixture whose height is linear function of the concentration of one of the biomarkers at constant concentration of the other one. Therefore, DPV at SPCE can be used for the determination of the two biomarkers in their mixture both in the 10^{-5} and 10^{-6} $mol \cdot L^{-1}$ concentration ranges using the standard addition of these analytes.

4 Conclusions

Voltammetric determination of tumor biomarkers represents a useful approach for prediction of diseases and could help to start their treatment in time. This paper describes determination of three tumor biomarkers using DPV at SPCE. The optimum medium for the determination of HVA, VMA, and 5-HIAA was found to be BRB at pH 3.0. Calibration dependencies were linear in the whole tested concentration range from 0.2 to

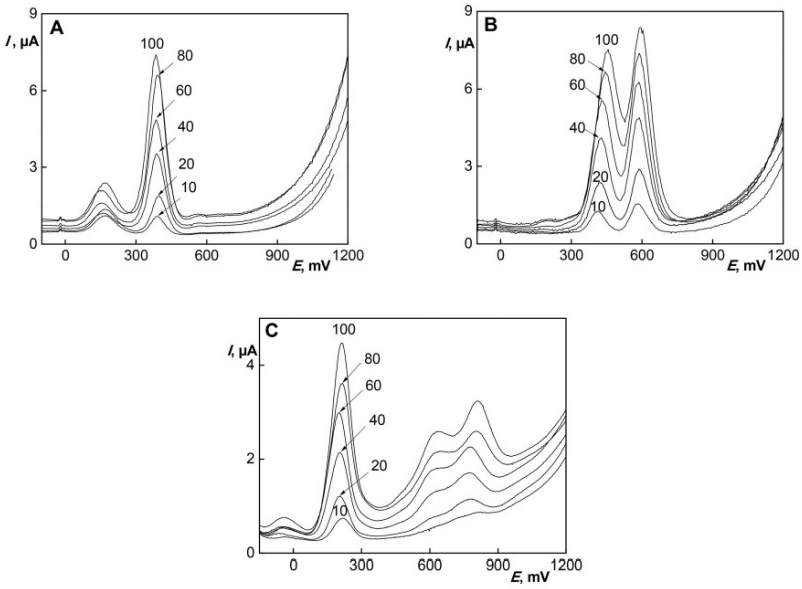


Fig. 3. DP voltammograms of HVA (A), VMA (B), and 5-HIAA (C) measured at SPCE in BRB at pH 3.0 in the concentration range from 10 to 100 $\mu mol \cdot L^{-1}$; the numbers next to the curves correspond to the analyte concentrations in $\mu mol \cdot L^{-1}$.

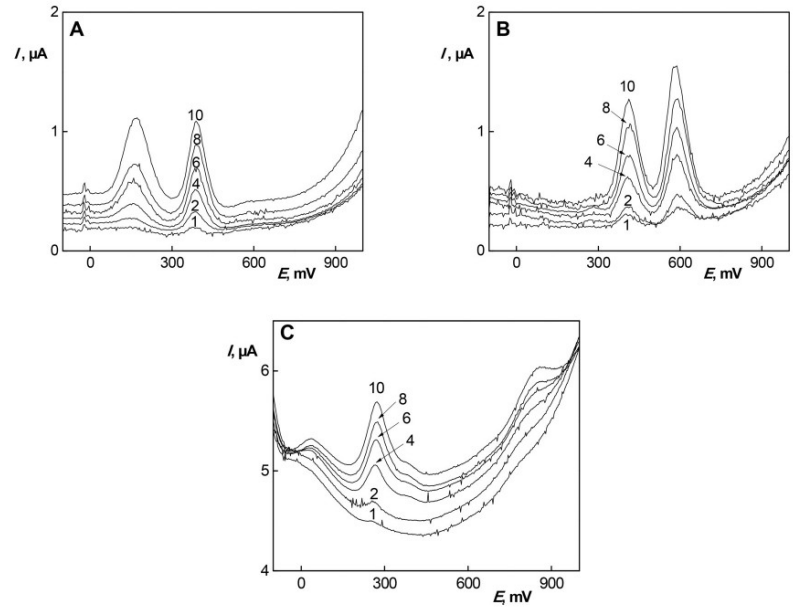


Fig. 4. DP voltammograms of HVA (A), VMA (B), and 5-HIAA (C) measured at SPCE in BRB at pH 3.0 in the concentration range from 1 to 10 μmol·L^{-1}; the numbers next to the curves correspond to the analyte concentrations in μmol·L^{-1}.

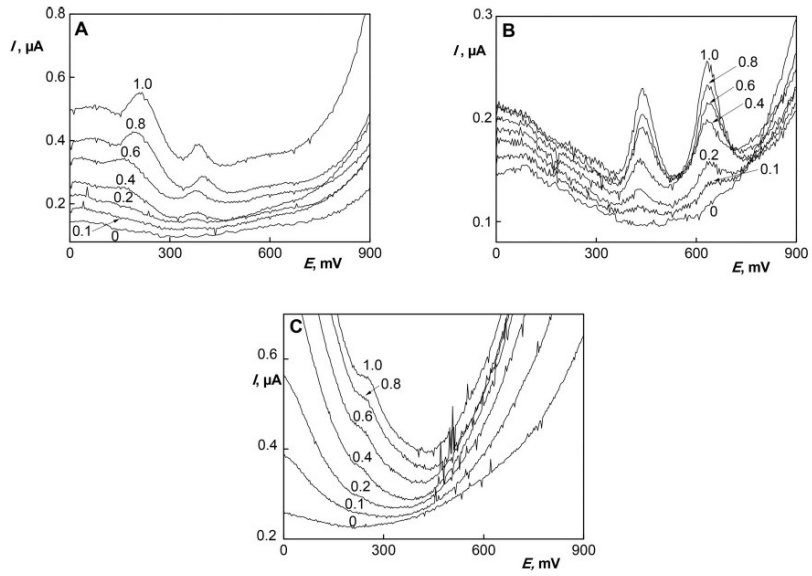


Fig. 5. DP voltammograms of HVA (A), VMA (B), and 5-HIAA (C) measured at SPCE in BRB at pH 3.0 in the concentration range from 0.1 to 1 μmol·L^{-1}; the numbers next to the curves correspond to the analyte concentrations in μmol·L^{-1}.

Table 3. Parameters of calibration straight lines (including standard deviations) for DPV determination of tested biomarkers at SPCE in BRB at pH 3.0 constructed from voltammograms measured in the concentration range from 0.1 to 100 μmol·L^{-1}.

Analyte	Slope (mA·L·mol^{-1})	Intercept (nA)	Correlation coefficient	*LOQ* (μmol·L^{-1})	*LOD* (μmol·L^{-1})
HVA (2nd peak)	63.0±0.6	41±23	0.9993	0.8	0.24
VMA (2nd peak)	78.2±2.5	170±94	0.9922	0.2	0.06
5-HIAA (1st peak)	35.8±1.2	116±45	0.9930	0.4	0.12

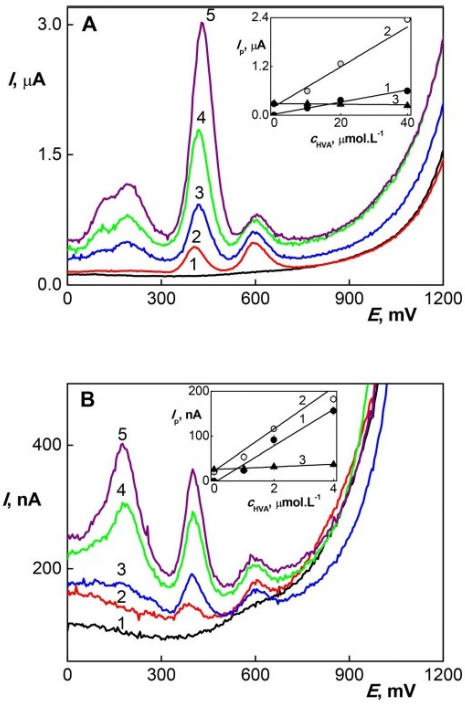


Fig. 6. DP voltammograms of HVA and VMA mixture measured at SPCE in BRB at pH 3.0. (A) 1 – supporting electrolyte, 2-5 – constant c_{VMA} 20 μmol·L^{-1}, 2-5 – increasing c_{HVA} 0, 10, 20, and 40 μmol·L^{-1}. Inset: Dependence of the first, second, and third peak current (line 1, 2, and 3) of the mixture of HVA and VMA on c_{HVA} at constant c_{VMA}. (B) 1 – supporting electrolyte, 2-5 – constant c_{VMA} 2 μmol·L^{-1}, 2-5 – increasing c_{HVA} 0, 1, 2, and 4 μmol·L^{-1}. Inset: Dependence of the first, second, and third peak current (line 1, 2, and 3) of the mixture of HVA and VMA on c_{HVA} at constant c_{VMA}.

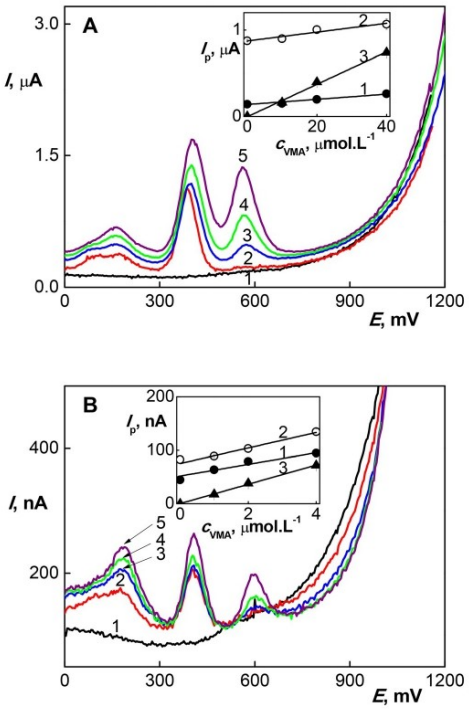


Fig. 7. DP voltammograms of HVA and VMA mixture measured at SPCE in BRB at pH 3.0. (A) 1 – supporting electrolyte, 2-5 – constant c_{HVA} 20 μmol·L^{-1}, 2-5 – increasing c_{VMA} 0, 10, 20, and 40 μmol·L^{-1}. Inset: Dependence of the first, second, and third peak current (line 1, 2, and 3) of the mixture of HVA and VMA on c_{VMA} at constant c_{HVA}. (B) 1 – supporting electrolyte, 2-5 – constant c_{HVA} 2 μmol·L^{-1}, 2-5 – increasing c_{VMA} 0, 1, 2, and 4 μmol·L^{-1}. Inset: Dependence of the first, second, and third peak current (line 1, 2, and 3) of the mixture of HVA and VMA on c_{VMA} at constant c_{HVA}.

100 μmol·L^{-1}. *LOQ*s were as follows: 0.8 μmol·L^{-1} for HVA, 0.2 μmol·L^{-1} for VMA, and 0.4 μmol·L^{-1} for 5-HIAA. The obtained results prove that DPV at SPCE is sensitive enough for monitoring of the tested tumor markers, thus paving the way for their monitoring in various biological matrices. Moreover, it was proved that DPV at SPCE can be used for the determination of HVA and VMA in mixture.

It follows from our preliminary experiments that the application of the newly developed methods for urine samples would require some preliminary separation and preconcentration, SPE and/or various forms of membrane

separation being the most suitable candidates for this purpose, as shown in the Introduction. Therefore, our further research will be focused on the combination of these separation techniques with the newly developed DPV at SPCE determination of the tested biomarkers.

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9 APPENDIX III

Determination of three tumor biomarkers (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) using flow injection analysis with amperometric detection

Anna Němečková-Makrlíková, Frank-Michael Matysik, Tomáš Navrátil, Jiří Barek, Vlastimil Vyskočil

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Determination of three Tumor Biomarkers (Homovanillic Acid, Vanillylmandelic Acid, and 5-Hydroxyindole-3-Acetic Acid) Using Flow Injection Analysis with Amperometric Detection

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Abstract: Flow injection analysis with amperometric detection (FIA-AD) at screen-printed carbon electrodes (SPCEs) in optimum medium of Britton-Robinson buffer (0.04 mol·L^{-1}, pH 2.0) was used for the determination of three tumor biomarkers (homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindole-3-acetic acid (5-HIAA)). Dependences of the peak current on the concentration of biomarkers were linear in the whole tested concentration range from 0.05 to 100 μmol·L^{-1}, with limits of detection (*LOD*s) of 0.065 μmol·L^{-1} for HVA, 0.053 μmol·L^{-1} for VMA, and 0.033 μmol·L^{-1} for 5-HIAA (calculated from peak heights), and 0.024 μmol·L^{-1} for HVA, 0.020 μmol·L^{-1} for VMA, and 0.012 μmol·L^{-1} for 5-HIAA (calculated from peak areas), respectively.

Keywords: Screen-printed carbon electrodes · Flow injection analysis · Amperometric detection · Homovanillic acid · Vanillylmandelic acid · 5-Hydroxyindole-3-acetic acid

1 Introduction

Determination of HVA ((4-hydroxy-3-methoxyphenyl)-acetic acid), VMA (hydroxy(4-hydroxy-3-methoxyphenyl)-acetic acid), and 5-HIAA (2-(5-hydroxy-1*H*-indol-3-yl)-acetic acid) is a tool for prediction of neuroendocrine tumors: neuroblastoma, pheochromocytoma, and carcinoid tumors. Neuroblastoma is the most common tumor in childhood. It arises from primitive sympathetic ganglion cells and has wide range of symptoms [1]. Pheochromocytomas (as well as neuroblastoma) are catecholamine-secreting tumors arising from the chromaffin cells of the adrenal glands. They are rare vascular tumors with classic symptoms as a headache, palpitation, anxiety, and diaphoresis [2]. Carcinoid tumors are neuroendocrine tumors which sometimes can metastasize. Tumors are characteristic with clinical syndromes including carcinoid syndrome with symptoms including skin flushing, wheezing, and diarrhea. Carcinoid tumors are rare in the adult population [3].

HVA and VMA are structurally closely related; they are major end products of catecholamine metabolism. 5-HIAA is a breakdown product of serotonin [4,5]. Their structural formulae are shown in Table 1. They are excreted in urine; normal urinary concentrations are from 8.2 to 41.0 μmol·L^{-1} for HVA, from 11.6 to 28.7 μmol·L^{-1} for VMA, and from 17.8 to 58.3 μmol·L^{-1} for 5-HIAA [6], i.e. between 1.3 and 7.6 mg·L^{-1} of these biomarkers [7]. Their abnormally high concentrations can signalize tumors.

Most common methods for the determination of these biomarkers are HPLC, GC, and immunoassay [8] for HVA and VMA, and HPLC or GC-MS for 5-HIAA [4]. Due to phenolic structure (hydroxyl group on aromatic system) of the biomarkers, they can be electrochemically oxidized and electrochemical methods for their determination can be used [9,10].

In spite of HVA and VMA similarity, their voltammetric responses are different, see review [11]. On a glassy carbon electrode, VMA is oxidized to a cation-radical (first peak at differential pulse voltammetry (DPV)); in second step, decarboxylation leads to vanillin, which is further oxidized to a corresponding *o*-quinone derivative (second peak at DPV) [8]. On the other hand, only one DPV peak is observable for HVA at the potential corresponding to the first peak of VMA. The most likely explanation is the lower rate of decarboxylation and

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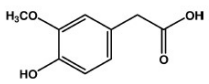
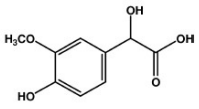
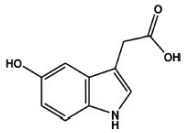
Table 1. Basic information about the studied biomarkers.

Common name	homovanillic acid	vanillylmandelic acid	5-hydroxyindole-3-acetic acid
Structure of analyte			
CAS Number	306-08-1	55-10-7	54-16-0

rearomatization leading to vanillin, resulting in the disappearance of the second peak. Mechanism of the electrochemical oxidation of 5-HIAA, which leads to a quinoneimine structure, is different from that of HVA and VMA. In the resulting product, a C=O bond (arising from an –OH group on the aromatic system) is conjugated with a C=N bond in the heterocyclic part of the molecule [8, 12].

Simple way for the determination of HVA, VMA, and 5-HIAA could be voltammetry on bare electrodes, which are user-friendly without the necessity of any other pretreatment. DPV determination of VMA on an edge plane pyrolytic graphite electrode in phosphate buffer (pH 6.85) gave *LOD* 1.7 $\mu mol \cdot L^{-1}$ [8]. HVA and VMA were determined by DPV on a carbon composite electrode in Britton-Robinson (BR) buffer pH 2 with *LOD*s 0.3 $\mu mol \cdot L^{-1}$ for HVA and 0.8 $\mu mol \cdot L^{-1}$ for VMA [9]. BR buffer pH 3.0 was used for DPV determination of HVA, VMA, and 5-HIAA at SPCEs with *LOD*s 0.24 $\mu mol \cdot L^{-1}$ (HVA), 0.06 $\mu mol \cdot L^{-1}$ (VMA), and 0.12 $\mu mol \cdot L^{-1}$ (5-HIAA) [13]. Cyclic voltammetric determination of HVA on a carbon paste electrode in brain extracellular fluid with *LOD* 3 $\mu mol \cdot L^{-1}$ [14] and square-wave voltammetric determination of 5-HIAA on an oxidized glassy carbon electrode with *LOD* 0.9 $\mu mol \cdot L^{-1}$ [15] were described.

A useful inspiration for FIA-AD determination of the above mentioned biomarkers can be found in papers devoted to HPLC with electrochemical detection. Glassy carbon [16] or carbon paste [17] are commonly used electrode materials. HVA and VMA were determined simultaneously and 5-HIAA separately in urine by HPLC-AD with a glassy carbon electrode flow cell (VMD-101, Yanaco, Japan) at +0.6 V (HVA, VMA) and +0.45 V (5-HIAA) [18]. Interesting arrangement based on extraction of analytes from filter paper treated with tartrate buffer followed by HPLC-AD with an amperometric detector LC-4B with a glassy carbon flow cell TL-5A (Bioanalytical Systems Inc., USA) at +0.9 V gave *LOD*s 0.055 $\mu mol \cdot L^{-1}$ (HVA) and 0.025 $\mu mol \cdot L^{-1}$ (VMA) in urine [19]. Simultaneous determination with an amperometric detector LC-4A with an LC-17 flow cell (Bioanalytical Systems Inc., USA) at +0.8 V in urine gave *LOD*s 10.1 $\mu mol \cdot L^{-1}$ (VMA) and 5.2 $\mu mol \cdot L^{-1}$ (5-HIAA) [20]. In the case of rat brain tissue [21], the LC-4A electrochemical detector was used and the effluent was passed through a TL-5 flow cell (Bioanalytical Systems Inc., USA) with a glassy carbon electrode at +0.85 V, giving estimated *LOD*s 13.7 $nmol \cdot L^{-1}$ for HVA and 5.2 $nmol \cdot L^{-1}$ for 5-HIAA. Rat and human brain tissues [22] were analyzed using an amperometric detector LC-3 with a carbon paste electrode flow cell TL-3 (Bioanalytical Systems Inc., USA) at +0.8 V, giving *LOD*s 11.0 $nmol \cdot L^{-1}$ for HVA and 1.1 $nmol \cdot L^{-1}$ for 5-HIAA.

Many HPLC-AD methods were used for the determination of only one of these biomarkers. For the sake of illustration, HVA was determined in different matrices, e.g. in urine [23] (self-fabricated wall-jet/thin-layer amperometric detector, glassy carbon working electrode, +0.7 V), in rat brain tissue [24] (home-made thin-layer amperometric detector with a glassy carbon working electrode, +0.8 V), or in plasma [25] (wall-jet amperometric detector (Antec Leyden BV, The Netherlands), glassy carbon electrode, +0.8 V), with *LOD*s around 1 $nmol \cdot L^{-1}$; VMA was determined in urine after an ethyl acetate extraction [26] (glassy carbon electrode cell TL-8A (Bioanalytical Systems Inc., USA), +1.3 V). 5-HIAA was monitored using an electrochemical detector LC-3 with a glassy carbon electrode (Bioanalytical Systems Inc., USA) at +0.6 V, with *LOD* 0.5 $\mu mol \cdot L^{-1}$ [27], an electrochemical detector with a glassy carbon electrode cell LC-4B (Bioanalytical Systems Inc., USA) at +0.55 V, with *LOD* 6.28 $\mu mol \cdot L^{-1}$ [28], and a glassy carbon electrode cell TL-8A (Bioanalytical Systems Inc., USA) at +1 V, with estimated *LOD* 5.23 $\mu mol \cdot L^{-1}$ [29].

FIA is a suitable method for large scale monitoring of HVA, VMA, and 5-HIAA because of high throughput, short time of analysis, and easy automation. Moreover, its combination with amperometric detection (AD) guarantees sufficient sensitivity. From the above given summary of voltammetric and amperometric monitoring of these biomarkers, it can be concluded that the application of carbon-based electrodes combined with a thin-layer arrangement is the most promising approach for FIA-AD. Therefore, this paper is devoted to the study of electrochemical behavior of the three tumor biomarkers and to their determination using FIA-AD at easily commercially available SPCEs.

Table 2. Parameters and their tested ranges for FIA-AD determination of the three tumor biomarkers at SPCE. Injected volume 20 µL was kept constant.

Analyte	Potassium ferrocyanide	HVA	VMA	5-HIAA
Flow rate [mL·min^{-1}]	0.2–4	0.5–2.5	0.5–2.5	0.5–2.5
Working electrode potential [V]	0.1–1.2	0.4–1.2	0.4–1.2	0.4–1.2
pH of carrier solution	2–10	2–8	2–8	2–8
Concentration of injected solutions [µmol·L^{-1}]	10–1000	0.05–100	0.05–100	0.05–100

2 Experimental

2.1 Apparatus

FIA-AD was performed using an apparatus consisting of a syringe pump (NE-510 L, LABICOM, s.r.o., Czech Republic), an injection valve (type 7725i, Rheodyne, USA) with a 20 µL injection loop, a flow cell (flow-cell in Teflon for screen-printed electrodes FLWCL-TEF, DropSens, Spain), and a computer-controlled potentiostat PalmSens3 (PalmSens BV, The Netherlands). A three-electrode SPCE system (type DRP-110, DropSens, Spain) comprising of a screen-printed carbon working electrode (4 mm diameter), a carbon counter electrode, and a silver reference electrode was used. (SPCEs are disposable, however, it was verified that up to 20 measurements can be repeated at a single SPCE at micromolar concentrations of the tested biomarkers without problems with passivation. Cross-contamination was not observed).

2.2 Reagents

The stock solutions (100 µmol·L^{-1}) of the three tumor biomarkers (all Sigma Aldrich, Germany) were prepared by dissolving an appropriate amount of the substance in 100 mL of deionized water. Potassium ferrocyanide was obtained from Lachema, Czech Republic. All measurements were performed in Britton-Robinson (BR) buffer prepared in a usual way, i.e. by mixing a solution of 0.04 mol·L^{-1} in phosphoric acid, 0.04 mol·L^{-1} in acetic acid, and 0.04 mol·L^{-1} in boric acid with the appropriate amount of 0.2 mol·L^{-1} sodium hydroxide solution. Deionized water was produced by a Millipore Milli-Q system (Millipore, USA).

2.3 Procedures

An SPCE was inserted into the flow cell and the syringe pump was filled with BR buffer of an appropriate pH. Then, the pump was set to an appropriate flow rate. The potentiostat was computer-controlled for an easy change of any parameter of AD. Samples were injected through an injection valve at regular time intervals. All experiments were performed at room temperature. For evaluation of electrochemical signals, PSTrace software for PalmSens3 (PalmSens BV, The Netherlands) was used. For calculating calibration curve parameters and graphic expressions of results, Microsoft Office Excel 2010 (Microsoft Corporation, USA) and OriginPro 8 (OriginLab Corporation, USA) were used. *LOD*s were calculated as $LOD = 3 \times s/a$, where s is the standard deviation of 10 repetitive measurements of the lowest measurable concentration and a is the slope of the calibration curve [30].

3 Results and Discussion

To obtain the best results for the determination of tested biomarkers, the following parameters were optimized: flow rate of carrier solution, potential of detection, and pH of BR buffer used as a carrier solution. At first, pilot experiments with potassium ferrocyanide were performed to optimize possible flow rate and to obtain general information about behavior of a combined (assembled) FIA apparatus. Afterwards, the experiments were repeated with the tested biomarkers. Tested ranges of individual FIA parameters are summarized in Table 2.

Optimum pH for potassium ferrocyanide (pH 3) was slightly different than that for the tested tumor biomarkers, detection potential was +0.5 V, and calibration dependences were tested in the range from 10 to 1000 µmol·L^{-1}. Injected volume (20 µL) and optimum flow rate (1 mL·min^{-1}) were the same for both potassium ferrocyanide and the tested biomarkers.

Signals of tumor biomarkers were evaluated from peak heights and from peak areas. For the sake of illustration and brevity, only dependences for the peak heights are shown in this paper. Dependences of the peak heights on the potential of detection for each analyte are depicted in Figure 1. For HVA and VMA, optimum detection potentials were +0.6 V and +0.8 V, respectively. For 5-HIAA, the highest peaks were obtained for detection potentials from +1.0 to +1.2 V. However, the repeatability of determination was somewhat worse than at lower detection potentials. For different potentials (+0.8 V, +0.9 V, and +1 V), the determination was repeated ten times and the relative standard deviations (RSDs) were evaluated. RSDs calculated from the peak heights were 0.42% for potential +0.8 V, 1.47% for +0.9 V, and 0.87% for +1.0 V. The potential of detection with the lowest RSD (+0.8 V) was thus chosen as the optimum one for 5-HIAA.

The tested range of flow rates was 0.5–2.5 mL·min^{-1} for each biomarker (see Figure 2). Around flow rate 1 mL·min^{-1}, peaks were reasonably high. Therefore, testing of repeatability (same procedure as for detection potential) was carried out. Higher flow rates (above

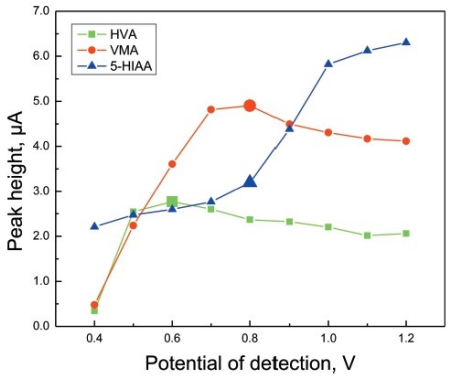


Fig. 1. Dependence of the peak height (FIA-AD at SPCE) on the potential of detection for (■) HVA in BR buffer pH 3, (●) VMA in BR buffer pH 2, and (▲) 5-HIAA in BR buffer pH 2; concentration of each injected analyte solution 100 µmol·L^{-1}, flow rate 1 mL·min^{-1}, three repeated measurements, current range 1–100 µA, injected volume 20 µL. Optimum values for each analyte are magnified.

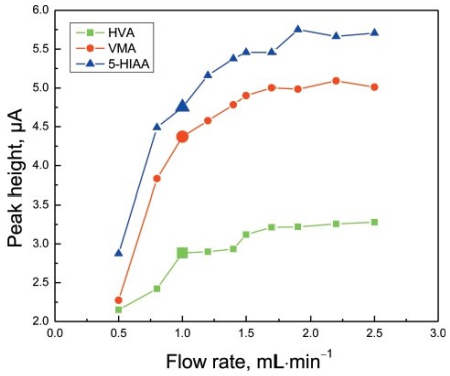


Fig. 2. Dependence of the peak height (FIA-AD at SPCE) on the flow rate for (■) HVA in BR buffer pH 3, potential of detection +0.6 V, (●) VMA in BR buffer pH 2, potential of detection +0.8 V, and (▲) 5-HIAA in BR buffer pH 2, potential of detection +0.8 V; concentration of each injected analyte solution 100 µmol·L^{-1}, three repeated measurements, current range 1–100 µA, injected volume 20 µL. Optimum values for each analyte are magnified.

2 mL·min^{-1}) deformed a syringe pump and repeatability was worse. For different flow rates around 1 mL·min^{-1} (1, 1.2, 1.5, and 2 mL·min^{-1}), RSDs were evaluated and the lowest values were obtained for flow rate 1 mL·min^{-1} for all analytes. Therefore, the flow rate 1 mL·min^{-1} was selected as optimum.

From previous voltammetric studies of the tested biomarkers [13], optimum pH was expected to be in acidic region. This assumption was also confirmed for FIA-AD. Figure 3 shows the decreasing peak heights with the increasing values of pH. Therefore, for all the studied biomarkers, pH 2 was chosen as optimum.

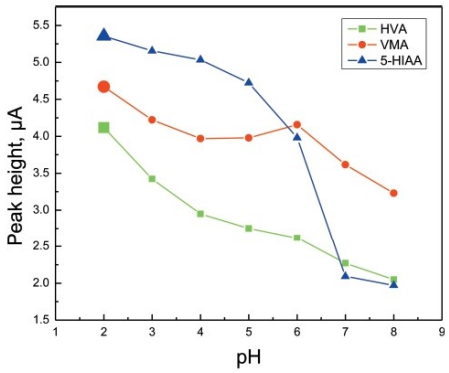


Fig. 3. Dependence of the peak height (FIA-AD at SPCE) on the pH for (■) HVA, potential of detection +0.6 V, (●) VMA, potential of detection +0.8 V, and (▲) 5-HIAA, potential of detection +0.8 V; concentration of each injected analyte solution 100 µmol·L^{-1}, BR buffer, flow rate 1 mL·min^{-1}, three repeated measurements, current range 1–100 µA, injected volume 20 µL. Optimum values for each analyte are magnified.

Three measurements were performed for each optimized value (potential of detection, flow rate, and pH). Obtained RSDs for the tested potentials of detection were between 0.05% and 8.25%, in one outlying case 17% (potential of detection +0.4 V for HVA); from 0.06% to 5.67% for the tested flow rates; and from 0.21% to 2.89% for the tested pH values.

Under optimum conditions for each biomarker (see Table 3), calibration dependences were measured (see Figure 4 which depicts FIA recordings of 5-HIAA in the concentration range of 0.05–100 µmol·L^{-1} for the sake of illustration). Three measurements were performed at each concentration and averages were evaluated. FIA recordings for HVA and VMA look similarly (not shown). Used optimum parameters, parameters of calibration straight lines, and found *LOD*s are summarized in Table 3.

All concentration dependences were linear in the whole tested concentration range. *LOD*s were calculated both from peak heights and from peak areas. All figures of merit of the calibration straight lines are summarized in Table 3.

To the authors' knowledge, this work is the first one that deals with determination of HVA, VMA, and 5-HIAA using FIA-AD. The paper [31] combines FIA with monolithic columns and chemiluminescence detec-

Table 3. Parameters of calibration straight lines for FIA-AD at SPCE determination of the tested biomarkers in the concentration range from 0.05 to 100 μmol·L^{-1}, BR buffer pH 2, detection potential +0.6 V (HVA) or +0.8 V (VMA and 5-HIAA), flow rate 1 mL·min^{-1}, three repeated measurements, current range 1–100 μA, injected volume 20 μL.

Analyte	Evaluated signal	Slope (μA·L·μmol^{-1} or μA·s·L·μmol^{-1} *)	Intercept (μA or μA·s *)	Correlation coefficient	*LOD* (μmol·L^{-1})
HVA	peak height	0.0365 ± 0.0001	−0.005 ± 0.003	0.9999	0.065
	peak area*	0.0979 ± 0.0007	−0.001 ± 0.025	0.9996	0.024
VMA	peak height	0.0420 ± 0.0002	−0.008 ± 0.008	0.9997	0.053
	peak area*	0.1127 ± 0.0003	+0.003 ± 0.011	0.9999	0.020
5-HIAA	peak height	0.0525 ± 0.0002	−0.004 ± 0.007	0.9999	0.033
	peak area*	0.1480 ± 0.0006	−0.033 ± 0.020	0.9999	0.012

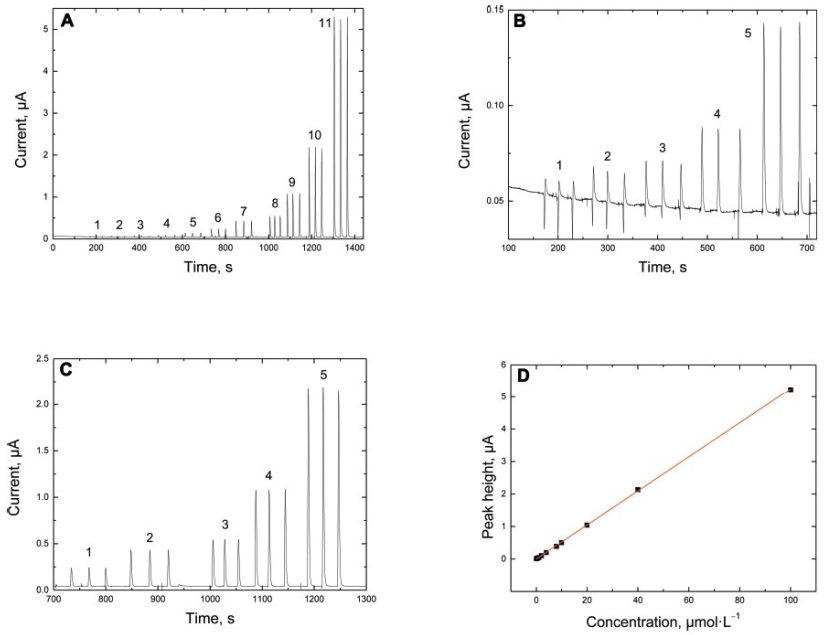


Fig. 4. (A) FIA-AD at SPCE recordings of 5-HIAA: (1) 0.05 μmol·L^{-1}, (2) 0.1 μmol·L^{-1}, (3) 0.4 μmol·L^{-1}, (4) 1 μmol·L^{-1}, (5) 2 μmol·L^{-1}, (6) 4 μmol·L^{-1}, (7) 8 μmol·L^{-1}, (8) 10 μmol·L^{-1}, (9) 20 μmol·L^{-1}, (10) 40 μmol·L^{-1}, and (11) 100 μmol·L^{-1} in BR buffer pH 2, potential of detection +0.8 V, flow rate 1 mL·min^{-1}, current range 1–100 μA, injected volume 20 μL. (B) Magnified FIA-AD at SPCE recordings from (A): (1) 0.05 μmol·L^{-1}, (2) 0.1 μmol·L^{-1}, (3) 0.4 μmol·L^{-1}, (4) 1 μmol·L^{-1}, and (5) 2 μmol·L^{-1}. (C) Magnified FIA-AD at SPCE recordings from (A): (1) 4 μmol·L^{-1}, (2) 8 μmol·L^{-1}, (3) 10 μmol·L^{-1}, (4) 20 μmol·L^{-1}, and (5) 40 μmol·L^{-1}. (D) Calibration dependence from (A) for FIA-AD determination of the tested biomarkers at SPCE in the concentration range from 0.05 to 100 μmol·L^{-1}.

tion and describes determination of HVA, VMA, 5-HIAA, and serotonin in human urine with *LOD*s 0.3, 0.5, and 0.1 μmol·L^{-1} for HVA, VMA, and 5-HIAA, respectively.

4 Conclusion

It was confirmed that FIA-AD at SPCE represents a suitable technique for the determination of HVA, VMA, and 5-HIAA as frequently monitored tumor biomarkers. Under found optimum conditions (BR buffer pH 2, detection potential +0.6 V for HVA and +0.8 V for VMA and 5-HIAA, flow rate 1 mL·min^{-1}, current range 1–100 μA,

injected volume 20 µL), all of calibration dependences were linear in the concentration range 0.05–100 µmol·L^{-1}, with *LOD*s 0.065 µmol·L^{-1} for HVA, 0.053 µmol·L^{-1} for VMA, and 0.033 µmol·L^{-1} for 5-HIAA (calculated from peak heights), and 0.024 µmol·L^{-1} for HVA, 0.020 µmol·L^{-1} for VMA, and 0.012 µmol·L^{-1} for 5-HIAA (calculated from peak areas). Using FIA-AD, there is no problem with passivation of the used SPCEs because reaction products are washed away from the working electrode surface, thus minimizing the risk of its passivation. The determination is fast and could be easily automated. Moreover, coupling of FIA-AD with preliminary separation and/or preconcentration can be envisaged.

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10 APPENDIX IV

Determination of tumour biomarkers homovanillic and vanillylmandelic acid using flow injection analysis with amperometric detection at a boron doped diamond electrode

Anna Němečková-Makrlíková, Tomáš Navrátil, Jiří Barek, Pavla Štenclová, Alexander Kromka, Vlastimil Vyskočil

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Determination of tumour biomarkers homovanillic and vanillylmandelic acid using flow injection analysis with amperometric detection at a boron doped diamond electrode

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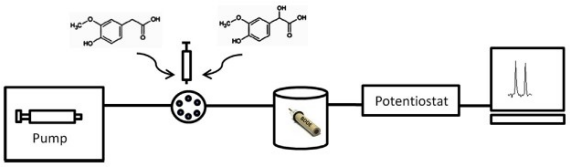
HIGHLIGHTS

- FIA at boron doped diamond electrode was used for the first time for determination of tumour biomarkers HVA and VMA in mixture.
- Electrode passivation was successfully eliminated by imposing suitable cleaning pulses on the working electrode.
- The limit of detection for both studied analytes is in the submicromolar concentration range.

GRAPHICAL ABSTRACT



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ABSTRACT

A new method for the simultaneous determination of two tumour biomarkers, homovanillic (HVA) and vanillylmandelic acid (VMA), using flow injection analysis (FIA) with amperometric detection (AD) at a commercially available boron doped diamond electrode (BDDE) was developed. It was found that this method is suitable for the determination of HVA (in the presence of VMA) and VMA (in the presence of HVA) in optimum medium of Britton-Robinson buffer (0.04 mol L^{-1}, pH 3.0). Calibration dependences consist of two linear parts for both biomarkers, the first one being in the concentration range from 1 to 10 μmol L^{-1} and the second one from 10 to 100 μmol L^{-1} (with obtained *LODs* 0.44 μmol L^{-1} for HVA and 0.34 μmol L^{-1} for VMA, respectively). To minimize any negative effects related to the passivation of the working electrode, suitable cleaning pulses (+2.4 V for 30 s) were imposed on the working electrode after each measurement. An attempt to use FIA with multiple pulse amperometric detection to determine both analytes in one run was not successful. Changing potentials in short intervals in multiple pulse detection probably results in mutual interaction of analytes and/or products of their electrochemical oxidation, thus preventing the application of this approach.

1. Introduction

Homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid, CAS Number: 306-08-1) and vanillylmandelic acid (VMA,

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D,L-4-hydroxy-3-methoxymandelic acid, CAS Number: 55-10-7) are products of catecholamine metabolism with similar structure (see Fig. 1). They can be used for diagnosis and monitoring of neuroendocrine catecholamine-producing tumours: neuroblastoma and pheochromocytoma [1]. Neuroblastoma is malignant tumour of sympathetic ganglion cells with wide range of symptoms; it typically manifests in childhood up to 10 years with frequent metastases [1,2]. Pheochromocytoma is benign (malignancy is rare) tumour arising from the chromaffin cells of the adrenal gland; metastases could be in the lungs, liver, bones, and lymph nodes. Pheochromocytoma occurs most often between 30 and 40 years of age [1,3]. HVA and VMA are excreted in urine. Normal concentrations in urine vary from 8.2 to 41.0 μmol L^{-1} for HVA and from 11.6 to 28.7 μmol L^{-1} for VMA [4]. Their several times higher urinary concentration can predict the above mentioned tumours.

The determination of HVA and VMA requires selective and sensitive analytical methods. The most frequently used methods are HPLC with electrochemical detection [5–9], GC-MS [10,11], capillary electrophoresis [4,12], immunoassays [13,14], and voltammetric methods (see review [9] and original paper [15]). Routine analysis and possible screening in infants result in a great interest in the development of fast, inexpensive, selective, and sensitive electrochemical methods compatible with a point-of-care approach. HVA and VMA are electroactive compounds and thus can be electrochemically oxidized because of hydroxyl group on aromatic system [16,17]. Among a large family of electrode materials, boron doped diamond electrode (BDDE) provides unique combination of intrinsic properties, e.g. chemical, electrochemical, and mechanical stability, wide potential window, low capacitive background current, low noise, and resistance to passivation [18–21]. BDDE was used in batch arrangement for the determination of HVA and VMA by DC voltammetry (DCV) and differential pulse voltammetry (DPV), both separately and in the mixture [22]. VMA was determined by DPV at BDDE combined with hollow fibre based microextraction in model urine samples [23]. Possibilities of the use of BDDE in flow systems are discussed in review [24]. So far only disposable screen-printed carbon electrodes were used for FIA determination of HVA and VMA [25]. FIA at BDDE with high sampling rate, fast response time, low cost instrumentation, and acceptable precision presenting an alternative to traditional batch analysis was not used so far for the determination of HVA and VMA. Therefore, this paper is devoted to simultaneous FIA determination of HVA and VMA at BDDE making use of its well-known above mentioned advantages and simplicity, low cost, and high speed of this approach. Moreover, BDDE can be used many times in comparison with previously used disposable screen-printed carbon electrodes and thus it is better compatible with principles of green analytical chemistry. Another envisaged advantage of BDDE is lower danger of passivation by high molecular interferents, e.g. proteins, present in biological samples. The main aim of the paper is to verify the applicability of BDDE for FIA AD determination of HVA and VMA and to verify the functionality of the proposed FIA-BDDE system. Therefore, FIA was performed with a constant potential amperometric detection (AD) resulting in oxidation peaks of HVA and VMA. Published studies employing multiple pulse amperometric detection (MPA) for simultaneous determination of drugs [26,27], sugars [28], antioxidants [29], and synthetic colorants [30] as well as a novel internal standard method in FIA system based on the application of sequential potential pulses to the working electrode in an electrochemical flow cell [31] prompted us to investigate this approach for the analysis of the mixture of HVA and VMA using BDDE as well.

2. Experimental

2.1. Reagents and solutions

Homovanillic acid (HVA, CAS Number: 306-08-1) and vanillylmandelic acid (VMA, CAS Number: 55-10-7) were supplied by Sigma-Aldrich, USA. Their stock solutions (1 mmol L^{-1}) were prepared by dissolving the exact amount of the substance in 100 mL of deionized water and kept in a fridge. Diluted solutions were prepared by measuring appropriate amount of the stock solution into a 10 mL volumetric flask and filling up to the mark with Britton-Robinson (BR) buffer of the required pH. BR buffer was prepared by mixing a solution of 0.04 mol L^{-1} in phosphoric acid, 0.04 mol L^{-1} in acetic acid, and 0.04 mol L^{-1} in boric acid with the proper amount of 0.2 mol L^{-1} sodium hydroxide solution. All chemicals used for BR buffer preparation were of analytical grade purity, p.a., Lach-Ner, Czech Republic. Deionized water was produced by Millipore Milli-Q system (Millipore, USA). Stability of HVA and VMA stock solutions was confirmed spectrophotometrically at 279 nm; solutions were stable at least for three months.

2.2. Apparatus

The amperometric measurements were carried out using a potentiostat/galvanostat Autolab PGSTAT101 controlled by NOVA version 1.11.2 (Metrohm, Switzerland) working under Windows XP Professional version 2002 (Microsoft Corporation, USA) and at a three electrode system in "wall-jet" arrangement with a working BDDE (D-517-SA, 3 mm in diameter, Windsor Scientific, UK), a reference Ag/AgCl (3 mol L^{-1} KCl) electrode (Elektrochemicke Detektory, Czech Republic), and a platinum wire counter electrode (0.5 mm in diameter, Monokrystaly, Czech Republic). High pressure pump HPP 5001 (Laboratorni Pristroje, Czech Republic) was used to provide a flow of the carrier solution together with six-way injection valve Injector D UNI (Ecom, Czech Republic) for injection of samples (20 μL injection loop). pH was measured with pH-meter Jenway 3510 (Jenway, UK) with a combined glass electrode calibrated using standard aqueous buffers at room temperature.

2.3. Procedures

Prior to the first measurement, BDDE surface was activated by applying positive potential +2.4 V for 20 min in H_2SO_4

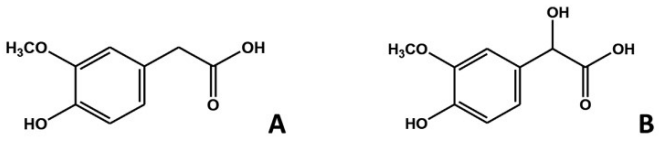

Fig. 1. Structural formulae of homovanillic acid (A) and vanillylmandelic acid (B).

($0.5\ \mu mol\ L^{-1}$) and then by cyclic voltammetry (CV) in BR buffer at pH 7.0 in potential range between −0.8 V and +2.3 V at scan rate 100 mV s^{-1}. CV scans were repeated until CV curve was stable. Before every single measurement, a high positive potential (+2.4 V for 30 s) was applied on BDDE to eliminate passivation. Amperometric detection was carried out at a constant potential which was (together with pH) optimized by recording hydrodynamic voltammograms of HVA and VMA from +0.5 to +1.7 V in the pH range of 2.0–7.0. Under the optimized conditions, the calibration dependences were constructed for each analyte alone and in the presence of the other one. All measurements were made in triplicate at laboratory temperature, and peak heights were evaluated from the amperometric FIA AD curves. Limits of detection (*LODs*) were calculated as a threefold of a standard deviation of ten consequent measurements at the lowest measurable concentration divided by the slope of the calibration curve [32].

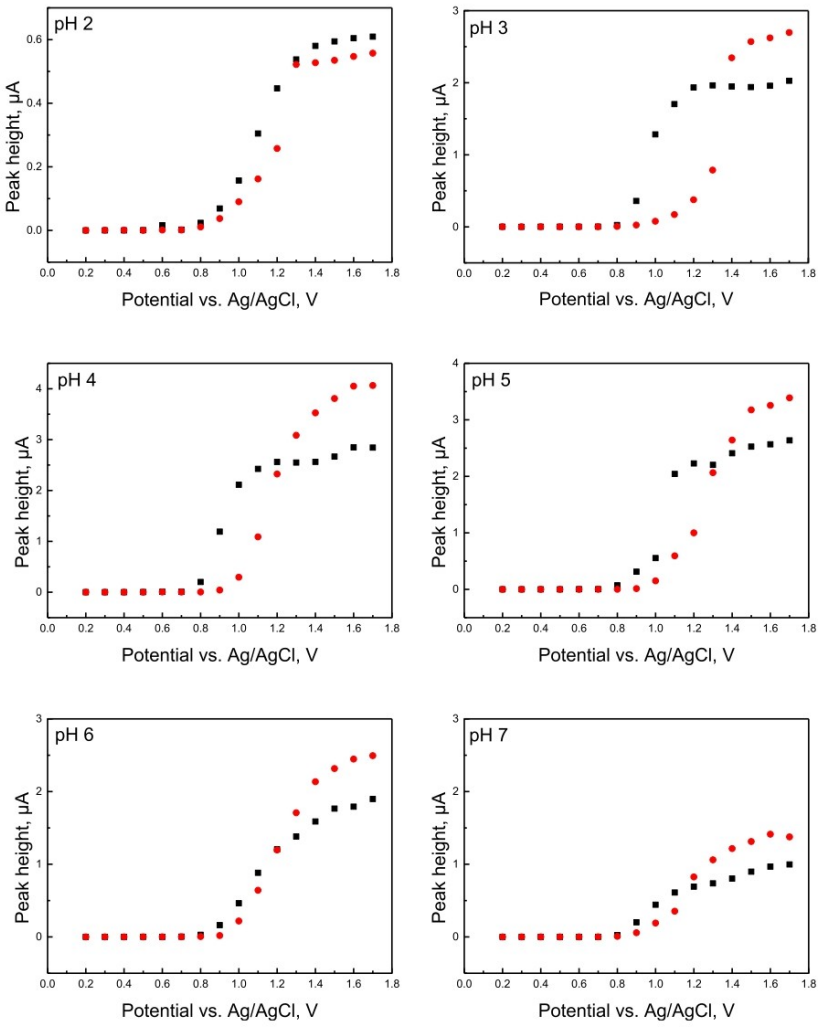

Fig. 2. Hydrodynamic voltammograms (i.e. FIA AD peak heights as a function of applied detection potential) of HVA (■) and VMA (●) (both $100\ \mu mol\ L^{-1}$). Carrier solution: BR buffer ($0.04\ mol\ L^{-1}$, at appropriate pH), injected volume 20 μL, flow rate $1\ mL\ min^{-1}$.

3. Results and discussion

3.1. Optimization of parameters for FIA AD determination of HVA and VMA

At first, hydrodynamic voltammograms were recorded to find out the optimum pH and applied potential. Tested pH varied from 2.0 to 7.0 and applied potential from +0.5 to +1.7 V. Each voltammogram was recorded three times, and then peak heights were evaluated. Selected hydrodynamic voltammograms are depicted in Fig. 2. For successful determination of HVA and VMA using FIA AD, their potentials have to be sufficiently different. The best developed, the best repeatable, and the most stable peaks were obtained at pH 3.0 which was thus selected as an optimum medium for further measurements. (Acidic pH is recommended for HPLC-ED determination of these biomarkers as well [5–9]). FIA MPA is based on the assumption that if at the potential of the first pulse only first analyte (HVA) is oxidized, and at the potential of the second pulse both analytes (HVA and VMA) are oxidized, the FIA MPA response of VMA can be calculated by subtraction of HVA signal from total signal using a correction factor (ratio of FIA MPA peak responses of HVA obtained at the first pulse potential (lower) and at the second pulse potential (higher) [33]). Optimum pulse potentials were found to be +1.1 V (first pulse potential) for HVA and +1.5 V (second pulse potential) for VMA at pH 3.0 (see Fig. 2). Analogously, the constant potential +1.1 V was found to be optimum for FIA AD calibration dependences of HVA and +1.5 V for FIA AD calibration dependences of VMA, both for individual analytes alone and for their mixture. Even though passivation at FIA is less frequent than at batch analysis, in this case passivation of BDDE was observed. It was probably caused by adsorption of electrode reaction products on BDDE surface. In batch DPV determination of HVA and VMA [34], passivation was eliminated by inserting cleaning step at +2.4 V for 30 s. To keep the FIA AD signal stable, the same cleaning step was used (i.e. +2.4 V for 30 s). This cleaning step was inserted prior to each measurement. (Tested lower applied potentials +1.6 V, +1.8 V, +2.0 V, and +2.2 V were not sufficient enough for FIA AD to keep the signal stable).

After finding optimum pH and applied detection potential, injection volume and flow rate were optimized. Injection volume was tested from 20 to 100 µL. Due to better elimination of passivation, sufficiently high signal, and lower sample consumption, 20 µL injection volume was selected as optimum for further measurements. Optimum flow rate was set to 1 mL min^{-1}, based on previous results with FIA AD determination of HVA and VMA at screen-printed electrodes [25]. Under these conditions, 25 consequent injections gave amperometric responses with relative standard deviations (RSDs) lower than 4% for both analytes at concentration 100 µmol L^{-1}, confirming that problems with electrode passivation were successfully eliminated by this approach.

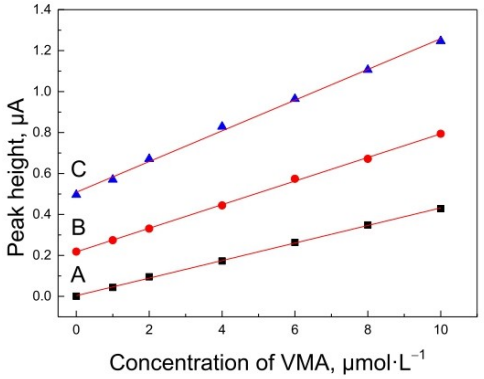

Fig. 4. The calibration dependence for FIA AD determination of VMA in the concentration range 1–10 µmol L^{-1}: (A) alone; (B) in the presence of 5 µmol L^{-1} of HVA; (C) in the presence of 10 µmol L^{-1} of HVA. Carrier solution: BR buffer (0.04 mol L^{-1}, pH 3.0), injected volume 20 µL, flow rate 1 mL min^{-1}. Peak height of VMA measured at optimum detection potential +1.5 V.

3.2. Concentration dependences

Under the optimized conditions, FIA AD calibration curves for

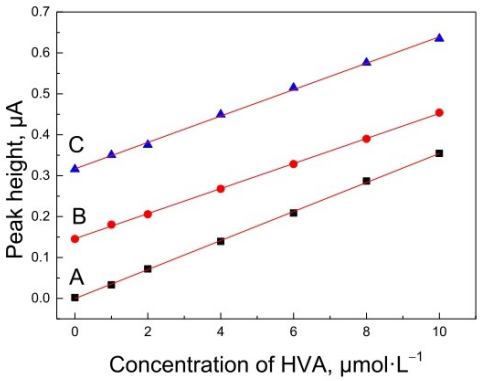

Fig. 3. The calibration dependence for FIA AD determination of HVA in the concentration range 1–10 µmol L^{-1}: (A) alone; (B) in the presence of 5 µmol L^{-1} of VMA; (C) in the presence of 10 µmol L^{-1} of VMA. Carrier solution: BR buffer (0.04 mol L^{-1}, pH 3.0), injected volume 20 µL, flow rate 1 mL min^{-1}. Peak height of HVA measured at optimum detection potential +1.1 V.

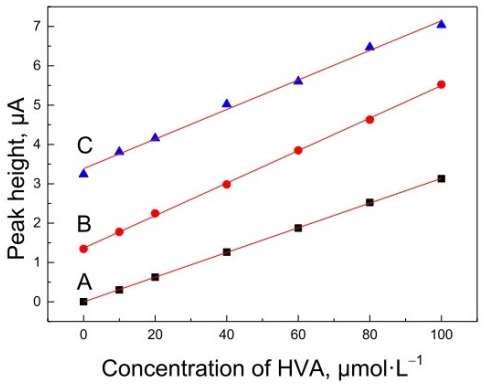

Fig. 5. The calibration dependence for FIA AD determination of HVA in the concentration range 10–100 µmol L^{-1}: (A) alone; (B) in the presence of 50 µmol L^{-1} of VMA; (C) in the presence of 100 µmol L^{-1} of VMA. Carrier solution: BR buffer (0.04 mol L^{-1}, pH 3.0), injected volume 20 µL, flow rate 1 mL min^{-1}. Peak height of HVA measured at optimum detection potential +1.1 V.

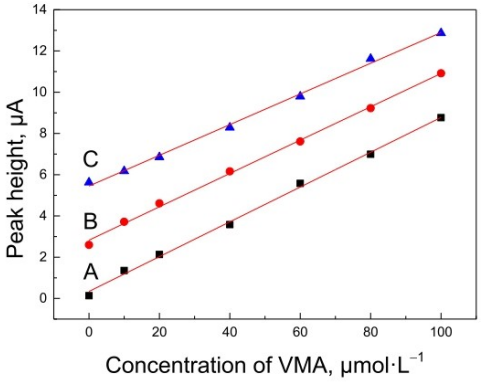

Fig. 6. The calibration dependence for FIA AD determination of VMA in the concentration range 10–100 μmol L^{-1}: (A) alone; (B) in the presence of 50 μmol L^{-1} of HVA; (C) in the presence of 100 μmol L^{-1} of HVA. Carrier solution: BR buffer (0.04 mol L^{-1}, pH 3.0), injected volume 20 μL, flow rate 1 mL min^{-1}. Peak height of VMA measured at optimum detection potential +1.5 V.

HVA and VMA were constructed using varying concentrations of HVA (VMA) at constant concentration of VMA (HVA) in concentration ranges 1–10 μmol L^{-1} (see Figs. 3 and 4, constant concentrations of the other analyte were 5 and 10 μmol L^{-1}), and 10–100 μmol L^{-1} (see Figs. 5 and 6, constant concentrations of the other analyte were 50 and 100 μmol L^{-1}). All obtained calibration dependences were linear with acceptable correlation coefficients and with *LOD*s 0.44 μmol L^{-1} for HVA and 0.34 μmol L^{-1} for VMA, respectively. Figs. 3–6 depict a gradual linear increase in intercepts of the calibration dependences of HVA (VMA) after addition of constant concentration of VMA (HVA) (see Fig. 7). All figures of merit of the calibration dependences are summarized in Table 1.

3.3. Multiple pulse flow injection analysis

FIA MPA generally enables simultaneous determination of several compounds with sufficiently different oxidation potentials. Principle of FIA MPA is based on pulses with alternating optimum potentials selected for each analyte. Pulse potentials (+1.1 V for HVA and +1.5 V for VMA) were selected from hydrodynamic voltammograms. The FIA MPA signal of HVA at pulse potential +1.1 V is high enough with a small contribution of VMA while FIA MPA signal of VMA at pulse potential +1.5 V is at its maximum level. Other conditions were the same as in amperometric detection: BR buffer (0.04 mol L^{-1}, pH 3.0), injected volume 20 μL, flow rate 1 mL min^{-1}. The pulse width for FIA MPA at BDDE was tested in the range from 50 to 150 ms and no significant differences were observed. Therefore, the pulse width 100 ms was chosen as an optimum. Apart from alternating pulses of +1.1 V and +1.5 V, cleaning pulse at +2.2 V was added. Contrary to FIA AD, cleaning pulse at +2.2 V was sufficient, probably because of lower passivation in the case of FIA MPA connected with shorter time for electrode reaction given by short pulses in comparison with much longer time in the case of FIA AD. Varying concentration of HVA or VMA (10, 20, 40, 60, 80, and 100 μmol L^{-1}) and 100 μmol L^{-1} of the other compound were used. In Fig. 8, FIA MPA calibration dependence of HVA is depicted in the presence of 100 μmol L^{-1} of VMA in each solution. Peaks of HVA (■) should be linearly increasing with increasing HVA concentration, and peaks of VMA (●) should be constant because of constant VMA concentration. Moreover, peaks of VMA should be reproducible to prove they are not influenced by changing concentration of HVA. However, contrary to the expectations, VMA at concentration level 100 μmol L^{-1} provides different FIA MPA signals with increasing trend, and FIA MPA calibration dependence of HVA is not linear. Therefore, another experiment was performed under the same conditions, where FIA MPA calibration dependence of HVA was constructed without addition of VMA at concentration level of 100 μmol L^{-1}. In that case, the calibration dependence of HVA was linear. Moreover, FIA MPA signal of VMA at concentration 100 μmol L^{-1} was measured separately in the absence of HVA in multiple pulse system, and this signal was constant and reproducible. Thus, it can be concluded that FIA MPA cannot be used for the determination of HVA and VMA in a mixture simultaneously, probably due to an interaction of products of their electrochemical oxidation with each other or with the parent compounds. If VMA and HVA are determined separately in multiple pulse arrangement, they exhibit standard behaviour. This conclusion is supported by experiments with electrodes made of other electrode materials, namely commercially available screen-printed carbon electrodes (DropSens, Spain) and a glassy carbon electrode (3 mm in diameter, Metrohm, Switzerland), giving similar negative results for FIA MPA. Moreover, a stronger passivation at both carbon electrodes was observed in comparison with BDDE and several attempts to eliminate it were not successful. Thus, we were not able to find conditions for simultaneous FIA MPA determination of HVA and VMA.

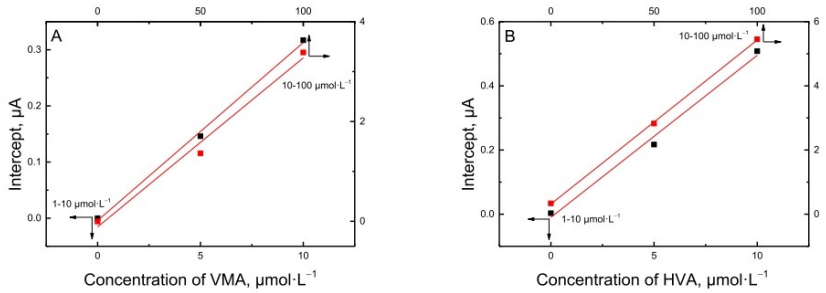

Fig. 7. Dependence of the FIA AD calibration straight line intercepts for: (A) HVA in the concentration ranges (1–10) and (10–100) μmol L^{-1} measured at +1.1 V on the concentration of added VMA; (B) VMA in the concentration ranges (1–10) and (10–100) μmol L^{-1} measured at +1.5 V on the concentration of added HVA.

Table 1
Parameters of the calibration curves for FIA AD determination of HVA and VMA at BDDE. BR buffer at pH 3.0 as a carrier solution, detection potentials +1.1 V (HVA) and +1.5 V (VMA), injected volume 20 μL, flow rate 1 mL min^{-1}.

First analyte and its concentration range (μmol L^{-1})	Second analyte and its added concentration (μmol L^{-1})	Slope ± SD (μA L ·mol^{-1})	Intercept ± SD (μA)	Correlation coefficient	*LOD* (μmol L^{-1})
HVA (1–10)	VMA (0)	0.0355 ± 0.0003	0.000 ± 0.002[a]	0.9995	0.20
HVA (1–10)	VMA (5)	0.0306 ± 0.0002	0.146 ± 0.001	0.9997	0.37
HVA (1–10)	VMA (10)	0.0323 ± 0.0005	0.317 ± 0.003	0.9987	0.43
HVA (10–100)	VMA (0)	0.0314 ± 0.0001	–0.003 ± 0.007[a]	0.9999	0.30
HVA (10–100)	VMA (50)	0.0413 ± 0.0004	1.364 ± 0.024	0.9994	0.52
HVA (10–100)	VMA (100)	0.0376 ± 0.0012	3.384 ± 0.067	0.9939	0.80
VMA (1–10)	HVA (0)	0.0429 ± 0.0005	0.003 ± 0.003[a]	0.9993	0.19
VMA (1–10)	HVA (5)	0.0576 ± 0.0007	0.217 ± 0.004	0.9992	0.24
VMA (1–10)	HVA (10)	0.0750 ± 0.0016	0.508 ± 0.009	0.9972	0.37
VMA (10–100)	HVA (0)	0.0844 ± 0.0018	0.34 ± 0.10	0.9972	0.19
VMA (10–100)	HVA (50)	0.0808 ± 0.0016	2.826 ± 0.089	0.9977	0.43
VMA (10–100)	HVA (100)	0.0744 ± 0.0017	5.451 ± 0.095	0.9969	0.60

[a] Denoted values are not statistically significantly different from zero.

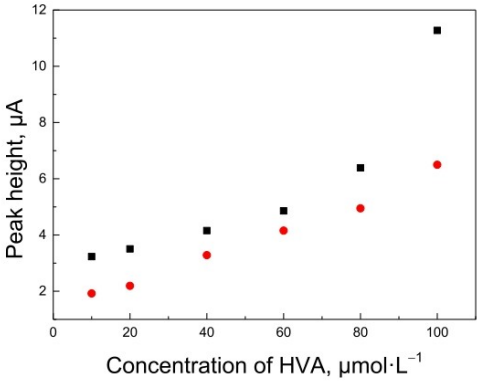

Fig. 8. The calibration dependence for FIA MPA determination of HVA (■) (10, 20, 40, 60, 80, and 100 μmol L^{-1}), each sample with addition of VMA (●) (at constant concentration level 100 μmol L^{-1}). Carrier solution: BR buffer (0.04 mol L^{-1}, at pH 3.0), applied potential pulses +1.1 V (HVA), +1.5 V (VMA), pulse width 100 m s, cleaning pulse +2.2 V, injected volume 20 μL, flow rate 1 mL min^{-1}.

4. Conclusions

Optimum conditions were found for the determination of tumour biomarkers homovanillic and vanillylmandelic acid using flow injection analysis with amperometric detection at boron doped diamond electrode. The main advantages of the newly developed method are low cost and short time of analysis, sufficient precision and sensitivity, low consumption of samples and reagents, and linear calibration dependences in broad concentration range with average *LOD*s 0.44 μmol L^{-1} and 0.34 μmol L^{-1} for homovanillic and vanillylmandelic acid, respectively. The possible interference of some electrochemically active urine components or adsorbable non-specific proteins and other large molecular urine components can be partially compensated by sample dilutions with the buffer used or by a preliminary separation using solid-phase extraction, which is currently under further investigation. Flow injection analysis with multipulse detection is not applicable for the determination of the mixture of the two tumour biomarkers, probably because of mutual interaction of products of electrochemical reaction and initial analytes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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11 APPENDIX V

Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection

Anna Němečková-Makrlíková, Jiří Barek, Tomáš Navrátil, Jan Fischer, Vlastimil Vyskočil, Hana Dejmková

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Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection

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Abstract:	HPLC with amperometric detection (HPLC-ED) was used for the simultaneous determination of tumour biomarkers homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindole-3-acetic acid (5-HIAA) in one run in human urine using inexpensive lab-made glassy carbon wall-jet detector. Simple and fast solid-phase extraction was used instead of earlier used more time consuming liquid-liquid extraction. Simultaneous determination of the three analytes in one run is fast with limits of detection at micromolar concentrations; amperometric detection is more sensitive than the spectrophotometric one. Calibration dependences are linear in the concentration range from 20 to 150 μmol·L–1. Interferences of other compounds present in urine were not observed for HPLC-ED. Concentrations of tumour biomarkers in urine of healthy volunteer found by HPLC-ED correspond with previously published normal physiological concentrations.
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Cover Letter

Prague, May 31, 2020

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Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection

by authors **Anna Němečková-Makrlíková, Jiří Barek, Tomáš Navrátil, Jan Fischer, Vlastimil Vyskočil, Hana Dejmková**

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Many thanks for your considering the publication. Yours sincerely,

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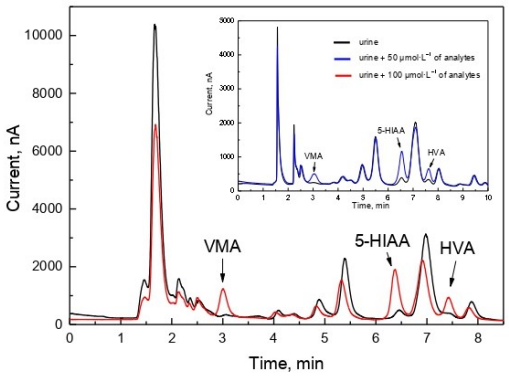
Graphical Abstract

Graphical abstract



Research Highlights

Highlights

- Simultaneous determination of HVA, VMA, and 5-HIAA was successfully performed by HPLC-ED using inexpensive lab-made wall-jet glassy carbon detector
- Simple SPE of urine samples was used for sample clean-up before injecting into HPLC system instead of time consuming liquid-liquid extraction
- The newly developed method was verified for fast, simple and inexpensive screening of human urine

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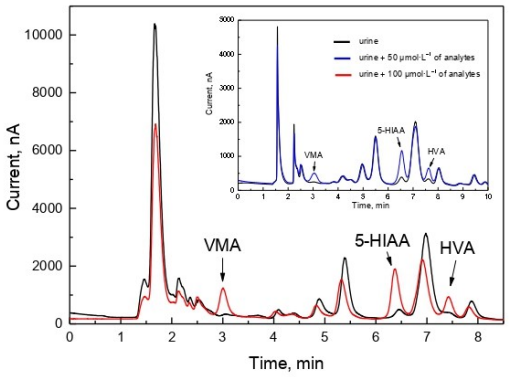

Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection

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A B S T R A C T

HPLC with amperometric detection (HPLC-ED) was used for the simultaneous determination of tumour biomarkers homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindole-3-acetic acid (5-HIAA) in one run in human urine using inexpensive lab-made glassy carbon wall-jet detector. Simple and fast solid-phase extraction was used instead of earlier used more time consuming liquid-liquid extraction. Simultaneous determination of the three analytes in one run is fast with limits of detection at micromolar concentrations; amperometric detection is more sensitive than the spectrophotometric one. Calibration dependences are linear in the concentration range from 20 to 150 $\mu mol \cdot L^{-1}$. Interferences of other compounds present in urine were not observed for HPLC-ED. Concentrations of tumour biomarkers in urine of healthy volunteer found by HPLC-ED correspond with previously published normal physiological concentrations.

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1. Introduction

Homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid), vanillylmandelic acid (VMA, D,L-4-hydroxy-3-methoxymandelic acid), and 5-hydroxyindole-3-acetic acid (5-HIAA, 5-hydroxyindole-3-acetic acid) are compounds excreted in urine. HVA and VMA are catecholamine metabolites; 5-HIAA is a product of serotonin metabolism. Published normal physiological urinary concentrations of these biomarkers are from 8.2 to 41 μmol L^{-1} for HVA, from 11.6 to 28.7 μmol·L^{-1} for VMA, and from 17.8 to 58.3 μmol·L^{-1} for 5-HIAA [1].

Their higher secretion can predict neuroblastoma, pheochromocytoma and carcinoid tumours [1,2]. HVA and VMA are associated with the presence of neuroblastoma and pheochromocytoma. Neuroblastoma is a malignant childhood tumour of sympathetic nervous system with a wide range of clinical presentation and frequent metastases. It typically occurs in childhood up to 10 years, median age being 17 months. [3,4]. Unlike neuroblastoma, pheochromocytoma is in most cases benign tumour with manifestation between 30 and 40 years of age [3,5]. The tumour arises from the chromaffin cells of the adrenal medulla and it can metastasize [6]. Carcinoid tumour is a rare asymptomatic tumour. Some tumours can be benign. Larger ones, especially with metastatic spread with clinical manifestation into carcinoid syndrome, are usually malignant. Carcinoid syndrome manifests itself by skin flushing, wheezing, and diarrhoea [7]. For all above mentioned tumours, an early diagnosis is important to prevent malignancy and mortality in metastatic or recurrent stage of the disease [3,8].

Apart from tumours, an unusual secretion level of HVA, VMA, and 5-HIAA indicates other diseases. HVA and VMA are associated with Parkinson disease [9], schizophrenia [10], suicide attempts [11], and posttraumatic stress disorder [12]. Diagnosis of hypertension [13], depression [14], migraine [15], and Tourette syndrome [16] is related to 5-HIAA and serotonin, respectively.

Despite HVA and VMA structural similarities (see Fig 1), their electrochemical behaviour is different [17]. Oxidation of VMA at an edge-plane pyrolytic graphite electrode (EPPGE) using differential pulse voltammetry (DPV) occurs in two steps resulting in two voltammetric peaks. In the first step, vanillin is formed through molecule decarboxylation and in the second step, vanillin is oxidized at a higher potential. HVA oxidation is similar, but slower process of rearomatization after decarboxylation to form vanillyl alcohol results only in one oxidation

peak [17]. 5-HIAA has different oxidation mechanism (due to quinoneimine structure in which C=O bond is conjugated with C=N bond) resulting in three voltammetric peaks [18].

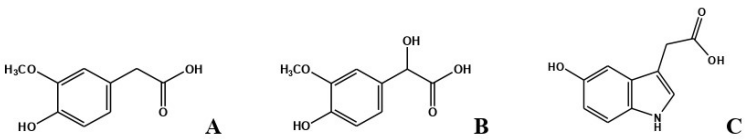


Fig. 1. Structures of homovanillic acid (A), vanillylmandelic acid (B), and 5-hydroxyindole-3-acetic acid (C).

Determination of the above mentioned biomarkers in human urine can be performed only with selective and sensitive methods. HPLC with amperometric [19] and coulometric [20] detection, more sensitive fluorescence [21] or chemiluminescence detection [22], and the most sensitive MS detection [23,24] have been used for these analytes. Moreover, GC [25,26], TLC [27], HPTLC [28], immunoassays [29,30], spectrophotometric methods [31], capillary electrophoresis [1,32], micellar electrokinetic chromatography [33], and isotachophoresis [34] have been described. A thorough and inspiring overview of the recent trends in the quantification of biogenic amines (e.g. serotonin or dopamine, which are metabolized into HVA, VMA, and 5-HIAA) as biomarkers in biofluids can be found in review [35].

Even though HPLC is a common method for the determination of HVA, VMA, and 5-HIAA in urine in clinical laboratories, it usually requires expensive instrumentation and complicated sample pre-treatment. In review [36] on electrochemical methods for the determination of HVA, VMA, and 5-HIAA, many published articles about HPLC-ED of these analytes in urine can be found, but only few of them allow simultaneous determination of all three mentioned biomarkers in one analysis. Moreover, a complex sample pre-treatment is necessary in all cases and thus analyses are quite time-consuming. Papers [37–39] describe HPLC with amperometric detection of HVA, VMA, and 5-HIAA using glassy carbon [37, 38] and carbon paste electrode [39]. In paper [38] HVA and VMA were detected simultaneously, but 5-HIAA was detected separately. Sample pre-treatment involved time-consuming extraction with ethyl acetate followed by re-extraction into buffer or acidic aqueous solution [37,38,39]. Retention times of analytes were within 16 minutes [37], 25 minutes [38], and 27 minutes [39], respectively.

HPLC with coulometric detection using sophisticated but rather expensive ESA Coulochem II Multi-Electrode electrochemical detector was used for determination of HVA and 5-HIAA in

[40], where extraction with ether and centrifugation as a sample pre-treatment were used and analysis took 15 minutes. However, VMA was not included in this study. In paper [41] after a sample clean-up the analysis took over 30 minutes but it required sophisticated but relatively expensive eight-channel electrode electrochemical detector. In paper [42] analysis took also over 30 minutes but extraction with ethyl acetate was necessary and expensive ESA Coulochem II Multi-Electrode electrochemical detector was again used. In paper [43] the analysis of slightly different set of analytes (3,4-dihydroxymandelic acid, VMA, 3,4-dihydroxyphenyl-glycol, and 4-hydroxy-3-methoxyphenylglycol took even 70 minutes with extraction, centrifugation and filtration as a sample pre-treatment. Other mentioned papers in review [36] deal with determination of only one or two analytes out of these three biomarkers (HVA, VMA, 5-HIAA). The analysed samples were not only urine, but also plasma, rat (human) brain tissue, and cerebrospinal fluid in which case a sample pre-treatment is even more difficult (solid phase extraction, homogenization, hydrolysis, and microdialysis).

Overall, these studies highlight the need for fast and simple HPLC method for the simultaneous determination of HVA, VMA, and 5-HIAA. Therefore, the aim of this work was to develop reliable, fast and inexpensive analytical method employing HPLC with amperometric detection using an inexpensive commercially available glassy carbon electrode in a simple lab-made wall-jet arrangement for simultaneous determination of all three cancer biomarkers, i.e. HVA, VMA, and 5-HIAA in human urine in one run without complicated and time-consuming sample pre-treatment and without the need for expensive ESA Coulochem II Multi-Electrode electrochemical detector and thus decreasing investment cost for this important determination.

2. Experimental section

2.1. Materials and reagents

Homovanillic acid (HVA, CAS Number 306-08-1, ≥ 99 %), vanillylmandelic acid (VMA, CAS Number 55-10-7, ≥ 98 %), and 5-hydroxyindole-3-acetic acid (5-HIAA, CAS Number 54-16-0, ≥ 98 %) were purchased from Sigma-Aldrich, USA. Their stock solutions (c = 1 mmol·L^{-1}) were prepared by dissolving appropriate amount of pure substances in 25 mL of deionized water. Based on UV spectrophotometric monitoring at 279 nm it was proved that those stock solutions are stable for at least 3 months. Acetate-phosphate buffer was prepared in a usual way by mixing solution A (2.84 mL of glacial acetic acid and 3.37 mL of concentrated phosphoric acid (both analytical grade purity, Lach-Ner, Czech Republic) in 1 L of deionized water) and

solution B (8 g of sodium hydroxide (analytical grade purity, Lach-Ner, Czech Republic) in 1L of deionized water). Acetonitrile and methanol (both HPLC grade, Merck, Germany) and deionized water (Millipore, USA) were used.

2.2. Instrumentation

HPLC with amperometric detection was performed in a "wall-jet" arrangement [46], however, with easily commercially available glassy carbon working electrode (3 mm diameter, Metrohm, Switzerland), a reference Ag | AgCl electrode (3 mol·L^{-1} KCl, Monokrystaly, Czech Republic), and a platinum wire auxiliary electrode (Monokrystaly, Czech Republic). The surface of the working electrode was mechanically polished after each measurement with aqueous slurry of alumina powder (1.1 μm particle size, Elektrochemicke Detektory, Czech Republic). For the sake of comparison with electrochemical detection, spectrophotometric detection was carried out.

HPLC-ED/UV apparatus consisted of a degasser DG 4014, a high pressure pump Beta 10, an injection valve with a 20 μL loop, a UV/VIS detector Sapphire 800 (all Ecom, Czech Republic), and an amperometric detector ADLC 2 (Laboratorni Pristroje, Czech Republic). Column Kromasil Eternity-5-PhenylHexyl 4.6×150 mm, 5 μm (AkzoNobel, The Netherlands) was used. The mobile phase was a mixture of acetate-phosphate buffer at pH 2.5 and acetonitrile (content of acetonitrile linearly increased from 5 to 25% in 10 minutes). The HPLC-ED/UV system was controlled via Clarity 2.3 software (DataApex, Czech Republic).

pH was measured with a pH meter Jenway 3510 (Jenway, UK) with a combined glass electrode. For calculation of calibration parameters and graphic expressions of results, Microsoft Office Excel 2010 (Microsoft, USA) and OriginPro 8.0 (Origin-Lab, USA) were used. Limits of detection (*LOD*s) were calculated as the analyte concentration corresponding to a threefold of a standard deviation s of ten repetitive measurements at the lowest measurable concentration divided by the slope a of the calibration curve ($LOD = 3s/a$) [44].

2.3. Solid phase extraction

As the urine sample pretreatment, solid phase extraction (SPE) was performed before injecting the samples into HPLC system. This approach is much faster and more „green" then previously used liquid-liquid extraction. For that purpose, commercially available poly(styrene-divinylbenzene) based SPE columns (LiChrolut EN 200 mg 3 mL standard PP-tubes; Merck Millipore, Germany) were used. SPE columns were activated with 5 mL of methanol, rinsed with 1 mL of deionized water and then dried by sucking air for 30 seconds using vacuum, flow

rate being kept at 1 mL·min^{-1} during the whole procedure. Spiked urine samples (5 mL) containing 10% acetic acid and 50% of deionized water (due to added spikes of aqueous solutions of tested analytes) were loaded onto the column, the column was washed by 1 mL of deionized water and then dried by sucking air through it for 30 seconds using vacuum. Afterwards, the analytes were eluted by 5 mL of methanol and 20 μL of thus obtained eluate were directly injected into HPLC system. SPE was thus used only as a preliminary separation method. Optionally, it can be used for preconcentration as well. However, relatively high concentrations of HVA, VMA, and 5-HIAA in urine (tens of μmol·L^{-1}) combined with relatively high sensitivity of the newly developed HPLC-ED method with *LOD*s in this concentration region (tens of μmol·L^{-1}) makes the time consuming preconcentration step unnecessary.

3. Results and discussion

3.1. HPLC separation of HVA, VMA and 5-HIAA

Chromatographic conditions were partly adapted from [45], where HPLC-ED was successfully used for the determination of HVA and VMA alone. The mobile phase consisted of a mixture of acetate-phosphate buffer at pH 2.5 using gradient with linearly increasing content of acetonitrile from 5 to 25% in 10 minutes. Optimum flow rate was 1 mL·min^{-1}, detection potential +1.1 V, and injected volume 20 μL. Spectrophotometric detection was performed at 279 nm. Under these conditions all three analytes were well separated in less than 10 minutes (see Fig. 2) and the calculated *LODs* were is submicromolar concentration regions (see Table 1).

Concentration dependences obtained under the above given conditions for monitored biomarkers dissolved in acetate-phosphate buffer pH 2.5 were linear in the concentration range from 0.5 to 10 μmol·L^{-1} (see Table 1 and 2 and Fig. 2.). Molar absorptivity at 279 nm wavelength is 2900, 3100, and 7000 mol^{-1} L cm^{-1} for HVA, VMA, and 5-HIAA, respectively, which explains higher slope of HPLC-UV dependence of 5-HIAA. Similarly, higher slope for 5-HIAA in HPLC-ED can be due to its slightly different structure especially in the heteroaromatic region resulting in higher amperometric response.

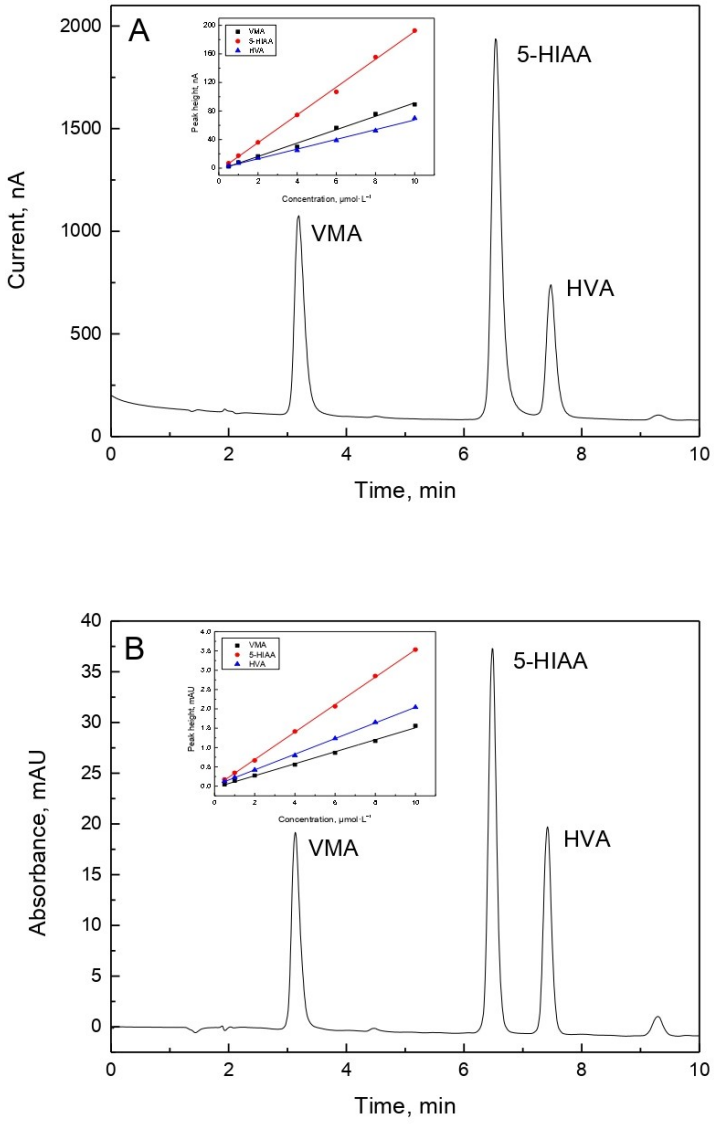


Fig. 2. HPLC-ED (A) and HPLC-UV (B) chromatograms of mixture of the tested biomarkers (100 μmol·L^{-1} each) in acetate-phosphate buffer pH 2.5. Gradient elution (acetate-phosphate

buffer at pH 2.5:acetonitrile (from 5 to 25 % acetonitrile in 10 minutes)), flow rate 1 mL·min^{-1}, injection loop 20 μL, injected sample in acetate-phosphate buffer pH 2.5, lab-made wall-jet detector with glassy carbon working electrode (3 mm diameter), detection potential +1.1 V (for HPLC-ED), detection wavelength 279 nm (for HPLC-UV). Calibration dependences in the concentration range from 0.5 to 10 μmol·L^{-1} and evaluated from peak heights are in insets.

3.2. Solid phase extraction

Most of biological matrices including urine require appropriate sample pre-treatment to avoid HPLC column damage due to many organic compounds present in the sample. In the case of relatively polar compounds, including the studied analytes, partial sample clean-up improves the HPLC part of the determination. Most of the so-far used sample pre-treatment steps for tested biomarkers were rather tedious. Therefore, SPE at commercially available columns was chosen as a simple and straightforward technique. Two different solvents, acetonitrile and methanol, were used both for SPE column activation and for elution of analytes. Best results were obtained with methanol giving recoveries 96–98% for all three analytes. Afterwards, the following SPE parameters were optimized: volume of solvent for SPE column conditioning, volume and flow rate of deionized water for washing the column and of solvent for elution of tested analytes, and volume of used acidified urine sample. The optimized SPE procedure is described in Paragraph 2.3. Due to the sufficient sensitivity of the determination methods, it was not necessary to use the SPE for pre-concentration of analytes (which could be easily done by eluate evaporation and re-dissolution in a smaller volume of methanol) and no other sample pre-treatment was necessary.

3.3. HPLC-ED determination of tested biomarkers in human urine

HPLC-ED chromatograms of human urine alone and after standard addition (1 mL of stock solution of each analyte (1 mmol·L^{-1}) into a urine sample acidified with 10% of acetic acid (final concentration of all analytes was 100 μmol·L^{-1} and total volume of the spiked urine sample subjected to SPE was 10 mL) are shown in Fig. 3 together with HPLC-UV chromatograms for the sake of comparison. It can be seen from this figure that the electrochemical detection is more selective than UV detection, considering interfering peaks in unspiked urine samples. HPLC-ED chromatogram (Fig. 3A) shows that 100 μmol·L^{-1} of any of the analytes can be safely detected. Moreover, it is obvious from the inset in Fig. 3A that even 50 μmol·L^{-1} spiked concentrations are easily distinguishable. In the case of HPLC-UV (Fig. 3B) it is not the case for VMA and HVA because of higher interfering peaks.

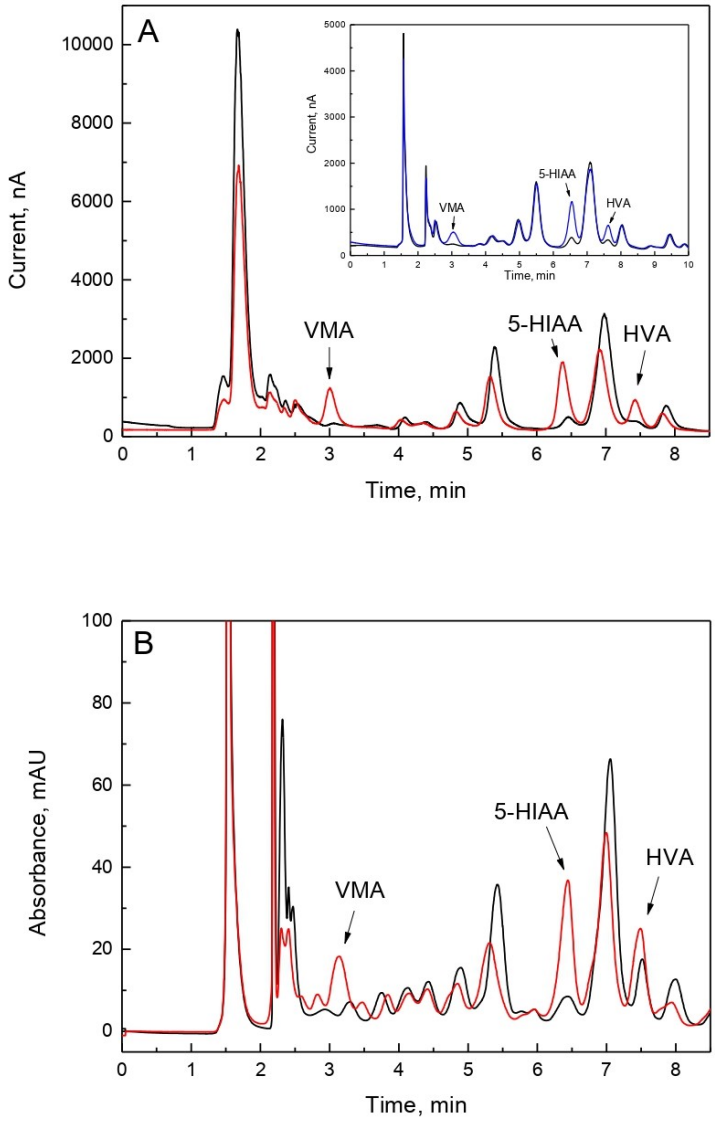
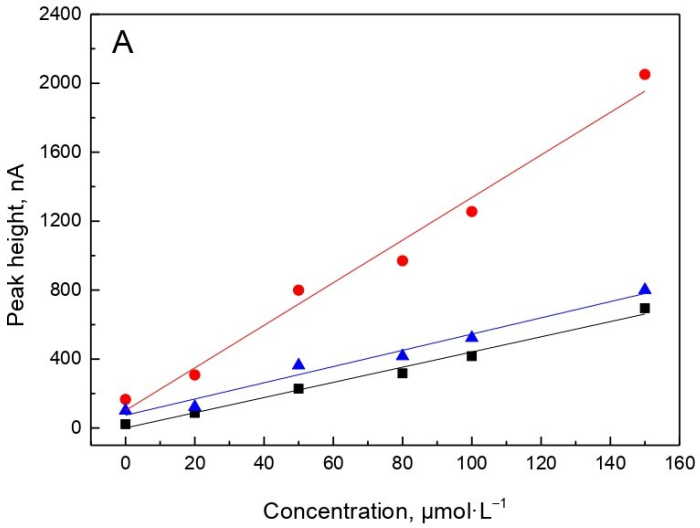


Fig. 3. HPLC-ED (A) and HPLC-UV (B) chromatograms of urine acidified with 10% of acetic acid after SPE (**black line**) and acidified urine after SPE with addition of HVA, VMA, and 5-

HIAA, each at concentration 100 μmol·L^{-1} (**red line**). Gradient elution (acetate-phosphate buffer at pH 2.5:acetonitrile (from 5 to 25 % acetonitrile in 10 minutes)), flow rate 1 mL·min^{-1}, injected volume 20 μL, glassy carbon working electrode, detection potential +1.1 V (for HPLC-ED), detection wavelength 279 nm (for HPLC-UV). Inset in (A) corresponds to addition of HVA, VMA, and 5-HIAA, each at concentration 50 μmol·L^{-1} (**blue line**).

For the construction of calibration curves in urine, 4.00 mL of urine, 1 mL of glacial acetic acid, and 0.2–1.5 mL of the stock solution of tested biomarkers (1 mmol·L^{-1}) was measured into 10 mL volumetric flask and made up to the mark with deionised water prior to the SPE procedure. Calibration dependences in human urine were then measured under optimum conditions (Fig. 4). All measurements were repeated five times. They were linear in the whole tested concentration range from 20 to 150 μmol·L^{-1} for all three biomarkers. The calibration curves in lower concentration range from 0.5 to 10 μmol·L^{-1} were difficult to evaluate because of interfering peaks of compounds present in urine. If necessary, SPE could be used for preconcentration to reach this concentration range. However, from practical purposes it is not necessary taking into account the fact that usual normal physiological concentration of the tested biomarkers in urine of healthy people is tens of μmol·L^{-1}.

All figures of merit from of all calibration dependences are summarized in Table 1 (for HPLC-ED) and in Table 2 (for HPLC-UV). The dependences are linear with the correlation coefficients close to one, although their slopes are lower than those of dependences measured using solutions in buffer alone. *LOD*s are logically higher in urine than in pure buffers due to interfering peaks. Moreover, the *LOD*s of electrochemical detection are lower than those of UV detection due to larger and more numerous interfering peaks in UV detection connected with higher selectivity of amperometric detection. This results in worse linearity, higher intercepts and higher *LOD*s of HPLC-UV.


A
Peak height, nA
Concentration, μmol·L^{-1}
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400
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1200
1600
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2400
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120
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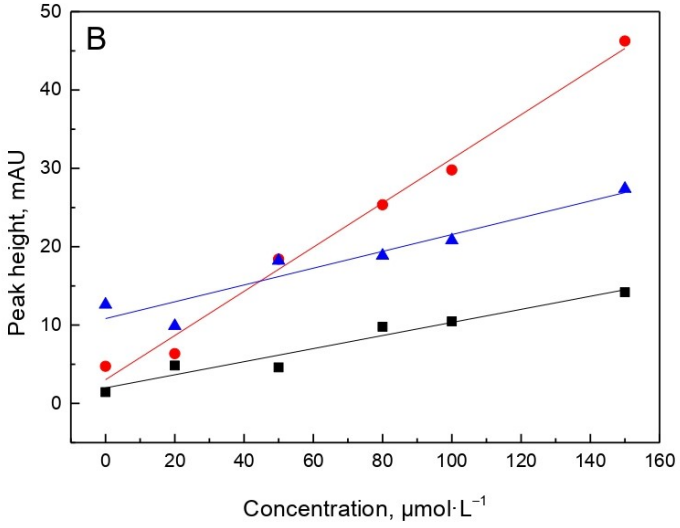
160


B
Peak height, mAU
Concentration, μmol·L^{-1}
0
10
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20
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80
100
120
140
160

12

Fig. 4. Calibration dependences for HPLC-ED (A) a HPLC-UV (B) of HVA (▲), VMA (■), and 5-HIAA (●) in the concentration range from 20 to 150 μmol·L^{-1} in urine sample. Gradient elution (acetate-phosphate buffer at pH 2.5:acetonitrile (from 5 to 25 % in 10 minutes), flow rate 1 mL·min^{-1}, injected volume 20 μL, lab-made wall-jet detector with glassy carbon working electrode, detection potential +1.1 V (for HPLC-ED), detection wavelength 279 nm (for HPLC-UV).

Table 1. Parameters of calibration dependences for HPLC-ED determination of tested biomarkers in the concentration range from 0.5 to 10 μmol·L^{-1} in buffer samples and from 20 to 150 μmol·L^{-1} in urine samples after SPE. Gradient elution (acetate-phosphate buffer at pH 2.5:acetonitrile (from 5 to 25 % in 10 minutes), flow rate 1 mL·min^{-1}, injected volume 20 μL, glassy carbon electrode, detection potential +1.1 V.

Analyte	Matrix	Slope ± *SD* (nA·L·μmol^{-1})	Intercept ± *SD* (nA)	Correlation coefficient	*LOD* (μmol·L^{-1})
HVA	buffer	6.8 ± 0.2	–0.5 ± 1.1*	0.9949	0.2
VMA	buffer	9.3 ± 0.5	–2.3 ± 2.0	0.9914	0.3
5-HIAA	buffer	19.6 ± 0.4	–3.3 ± 2.1	0.9977	0.3
HVA	urine	4.7 ± 0.4	74 ± 30	0.9717	11
VMA	urine	4.4 ± 0.2	0.5 ± 20*	0.9853	5.0
5-HIAA	urine	12.4 ± 0.8	101 ± 40*	0.9774	8.3

* Insignificant on the level of significance $\alpha = 0.05$.

Table 2. Parameters of calibration dependences for HPLC-UV determination of tested biomarkers in the concentration range from 0.5 to 10 μmol·L^{-1} in buffer samples and from 20 to 150 μmol·L^{-1} in urine samples after SPE. Gradient elution (acetate-phosphate buffer at pH 2.5: acetonitrile (from 5 to 25 % in 10 minutes), flow rate 1 mL·min^{-1}, injected volume 20 μL, detection wavelength 279 nm.

Analyte	Matrix	Slope ± SD (mAU·L·μmol^{-1})	Intercept ± SD (mAU)	Correlation coefficient	*LOD* (μmol·L^{-1})
HVA	buffer	0.202 ± 0.002	0.017 ± 0.011	0.9897	0.2
VMA	buffer	0.155 ± 0.004	0.041 ± 0.021	0.9898	0.6

5-HIAA	buffer	0.355 ± 0.003	0.020 ± 0.018	0.9923	0.3
HVA	urine	0.107 ± 0.017	10.8 ± 1.4	0.8840	13.9
VMA	urine	0.084 ± 0.010	2.0 ± 0.8	0.9381	72.9
5-HIAA	urine	0.282 ± 0.015	3.0 ± 1.2	0.9867	13.1

The practical applicability of the newly developed HPLC-ED method was tested by determining concentration of HVA, VMA, and 5-HIAA in spiked human urine using calibration curve method. Substantial interferences were not observed in HPLC-ED, where concentrations found in unspiked human urine correspond with published normal concentrations in urine [1]. Despite relatively high standard deviation (SD) it is obvious that HPLC-ED can confirm increased concentrations of tested biomarkers in urine.

Table 3. HPLC-ED determination of HVA, VMA, and 5-HIAA in urine samples. A – added concentration, F – found concentration, R – recovery (calculated from the found concentration after subtracting the concentration in the sample before standard addition).

Analyt	A $\mu mol \cdot L^{-1}$	F ± SD[a] $\mu mol \cdot L^{-1}$	A $\mu mol \cdot L^{-1}$	F ± SD $\mu mol \cdot L^{-1}$	R ± SD %	A $\mu mol \cdot L^{-1}$	F ± SD $\mu mol \cdot L^{-1}$	R ± SD %
HVA	0	24 ± 7	50	73 ± 14	98 ± 28	100	117 ± 16	92 ± 16
VMA	0	6 ± 4	50	53 ± 21	93 ± 42	100	112 ± 19	99 ± 19
5-HIAA	0	13 ± 4	50	60 ± 8	92 ± 16	100	108 ± 16	95 ± 16

[a] These values are in good agreement with the published normal physiological concentration range of healthy people, namely 8.2 – 41 $\mu mol \cdot L^{-1}$ for HVA, 11.6 – 28.7 $\mu mol \cdot L^{-1}$ for VMA and 17.8– 58.3 $\mu mol \cdot L^{-1}$ for 5-HIAA [1].

4. Conclusions

Simultaneous determination of HVA, VMA, and 5-HIAA in human urine was successfully performed using HPLC-ED with inexpensive lab-made wall-jet detector with easily commercially available glassy carbon electrode coupled with SPE on LiChrolut EN columns used as a pre-cleaning step after column activation with methanol. (5 mL of acidified urine samples were loaded onto SPE column and eluted by 5 mL of methanol). Obtained calibration dependences in urine samples were linear in the concentration range from 20 to 150 $\mu mol \cdot L^{-1}$

and *LOD*s were at micromolar concentrations, the amperometric detection providing somewhat lower *LOD*s than spectrophotometric one. The practical applicability of the newly developed method was verified by the determination of HVA, VMA, and 5-HIAA in spiked urine samples. It can be concluded that the presented HPLC-ED method for simultaneous determination of the three tumour biomarkers is fast, simple and sufficiently sensitive and much cheaper the previously published methods using sophisticated and expensive electrochemical detectors and it could be used for simple low-cost screening of human urine and thus help to diagnose tumours and other diseases.

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Declaration of Interest Statement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

12 APPENDIX VI

Confirmation of participation

[1] **A. Makrlíková**, J. Barek, V. Vyskočil, T. Navrátil, Electrochemical methods for the determination of homovanillic, vanillylmandelic, and 5-hydroxy-3-indoleacetic acid as cancer biomarkers (in Czech), *Chemické Listy* 112 (2018) 605–615.

Impact factor: **0.311** (2018)
Percentage of participation of RNDr. Anna Němečková (Makrlíková) ~ **80 %**.

[2] **A. Makrlíková**, E. Ktena, A. Economou, J. Fischer, T. Navrátil, J. Barek, V.Vyskočil, Voltammetric determination of tumor biomarkers for neuroblastoma (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) at screen-printed carbon electrodes, *Electroanalysis* 29 (2017) 146–153. doi:10.1002/elan.201600534.

Impact factor: **2.691** (2018)
Percentage of participation of RNDr. Anna Němečková (Makrlíková) ~ **40 %**.

[3] **A. Němečková-Makrlíková**, F.-M. Matysik, T. Navrátil, J. Barek, V. Vyskočil, Determination of three tumor biomarkers (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) using flow injection analysis with amperometric detection, *Electroanalysis* 31 (2019) 303–308. doi:10.1002/elan.201800540.

Impact factor: **2.691** (2018)
Percentage of participation of RNDr. Anna Němečková (Makrlíková) ~ **75 %**.

[4] **A. Němečková-Makrlíková**, T. Navrátil, J. Barek, P. Štenclová, A. Kromka, V. Vyskočil, Determination of tumour biomarkers homovanillic and vanillylmandelic acid using flow injection analysis with amperometric detection at a boron doped diamond electrode, *Analytica Chimica Acta* 1087 (2019) 44–50. doi:10.1016/j.aca.2019.08.062.

Impact factor: **5.256** (2018)
Percentage of participation of RNDr. Anna Němečková (Makrlíková) ~ **70 %**.

[5] **A. Němečková-Makrlíková**, J. Barek, T. Navrátil, J. Fischer, V. Vyskočil, H. Dejmková, Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection – *submitted to Journal of Electroanalytical Chemistry* (2020).

Impact factor: **3.218** (2018)
Percentage of participation of RNDr. Anna Němečková (Makrlíková) ~ **65 %**.

I declare that the percentage of participation of RNDr. Anna Němečková (Makrlíková) at the above given papers corresponds to above given numbers.

Prague, 10th June 2020

prof. RNDr. Jiří Barek, CSc.

13 APPENDIX VII

List of publications, oral and poster presentations, internship, and grants

A) List of publications

1. **A. Makrlíková**, F. Opekar, P. Tůma, Pressure-assisted introduction of urine samples into a short capillary for electrophoretic separation with contactless conductivity and UV spectrometry detection, *Electrophoresis* 36 (2015) 1962–1968. doi:10.1002/elps.201400613.

2. **A. Makrlíková**, E. Ktena, A. Economou, J. Fischer, T. Navrátil, J. Barek, V. Vyskočil, Voltammetric determination of tumor biomarkers for neuroblastoma (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) at screen-printed carbon electrodes, *Electroanalysis* 29 (2017) 146–153. doi:10.1002/elan.201600534.

3. **A. Makrlíková**, J. Barek, V. Vyskočil, T. Navrátil, Electrochemical methods for the determination of homovanillic, vanillylmandelic, and 5-hydroxy-3-indoleacetic acid as cancer biomarkers (in Czech), *Chemické Listy* 112 (2018) 605–615.

4. **A. Němečková-Makrlíková**, F.-M. Matysik, T. Navrátil, J. Barek, V. Vyskočil, Determination of three tumor biomarkers (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) using flow injection analysis with amperometric detection, *Electroanalysis* 31 (2019) 303–308. doi:10.1002/elan.201800540.

5. **A. Němečková-Makrlíková**, T. Navrátil, J. Barek, P. Štenclová, A. Kromka, V. Vyskočil, Determination of tumour biomarkers homovanillic and vanillylmandelic acid using flow injection analysis with amperometric detection at a boron doped diamond electrode, *Analytica Chimica Acta* 1087 (2019) 44–50. doi:10.1016/j.aca.2019.08.062.

6. **A. Němečková-Makrlíková**, J. Barek, T. Navrátil, J. Fischer, V. Vyskočil, H. Dejmková, Determination of tumour biomarkers homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid in human urine using HPLC with electrochemical detection – *submitted to Journal of Electroanalytical Chemistry* (2020).

B) Oral presentations

1. **A. Makrlíková**, F. Opekar, P. Tůma, Determination of selected components of human urine by electrophoresis in short capillary with hydrodynamic sampling controlled by pressure pulse, *XXXV. Modern Electrochemical Methods* – Jetřichovice u Děčína, Czech Republic (18. – 22. 5. 2015).

2. **A. Makrlíková**, F. Opekar, P. Tůma, Determination of selected components of human urine by electrophoresis in a short capillary with pressure-assisted sampling, *11th International Students Conference Modern Analytical Chemistry* – Prague, Czech Republic (22. – 23. 9. 2015).

3. **A. Makrlíková**, T. Navrátil, Determination of tumor markers using screen-printed electrodes, *Seminar of Students of JHI 2016* – Liblice, Czech Republic (10. – 11. 5. 2016).

4. **A. Makrlíková**, E. Ktena, J. Fischer, T. Navrátil, V. Vyskočil, Electrochemical methods for determination of three tumor biomarkers for neuroblastoma: homovanillic acid, vanillylmandelic acid, and 5-hydroxyindoleacetic acid, *XXXVI. Modern Electrochemical Methods* – Jetřichovice u Děčína, Czech Republic (23. – 27. 5. 2016).

5. **A. Makrlíková**, T. Navrátil, J. Barek, V. Vyskočil, Voltammetric determination of cancer biomarker 5-hydroxyindole-3-acetic acid at screen-printed carbon electrodes, *12th International Students Conference Modern Analytical Chemistry* – Prague, Czech Republic (22. – 23. 9. 2016).

6. **A. Makrlíková**, F.-M. Matysik, T. Navrátil, J. Barek, V. Vyskočil, Determination of 5-hydroxyindole-3-acetic acid using flow injection analysis with electrochemical detection, *XXXVII. Modern Electrochemical Methods* – Jetřichovice u Děčína, Czech Republic (15. – 19. 5. 2017).

7. **A. Makrlíková**, T. Navrátil, Optimization of conditions for voltammetric detection of tumor biomarker 5-hydroxyindole-3-acetic acid using flow injection analysis, *Seminar of Students of JHI 2017* – Liblice, Czech Republic (9. – 10. 5. 2017).

8. **A. Makrlíková**, F.-M. Matysik, T. Navrátil, J. Barek, V. Vyskočil, Determination of vanillylmandelic acid using flow injection analysis with amperometric detection, *XXII. Meeting of the Portuguese Electrochemical Society* – Ponta Delgada, Azores, Portugal (19. – 22. 6. 2017).

9. **A. Makrlíková**, F.-M. Matysik, T. Navrátil, J. Barek, V. Vyskočil, Voltammetric determination of tumor biomarkers using flow injection analysis with amperometric detection, *13th International Students Conference Modern Analytical Chemistry* – Prague, Czech Republic (21. – 22. 9. 2017).

10. **A. Makrlíková**, F.-M. Matysik, J. Barek, V. Vyskočil, T. Navrátil, Electrochemical methods for determination of tumor biomarkers in human urine, *1st Cross-Border Seminar on Electroanalytical Chemistry*, Furth im Wald, Germany (4. – 6. 4. 2018).

11. **A. Makrlíková**, H. Dejmková, T. Navrátil, J. Barek a V. Vyskočil, HPLC-ED/UV with solid phase extraction for the determination of 5-hydroxyindole-3-acetic acid, *XXXVIII. Modern Electrochemical Methods* – Jetřichovice u Děčína, Czech Republic (21. – 25. 5. 2018).

12. **A. Makrlíková**, T. Navrátil, Electrochemical methods for determination of homovanillic, vanillylmandelic, and 5-hydroxyindole-3-acetic acids in human urine, *Seminar of Students of JHI 2018* – Prague, Czech Republic (12. – 13. 6. 2018).

13. **A. Němečková-Makrlíková**, H. Dejmková, T. Navrátil, J. Barek, V. Vyskočil, HPLC-ED/UV for determination of vanillylmandelic acid in human urine after solid phase extraction, *14th International Students Conference Modern Analytical Chemistry* – Prague, Czech Republic (20. – 21. 9. 2018).

14. **A. Němečková-Makrlíková**, J. Barek, V. Vyskočil, T. Navrátil, Determination of homovanillic and vanillylmandelic acid using flow injection analysis with amperometric detection at boron doped diamond electrode, *2nd Cross-Border Seminar on Electroanalytical Chemistry* – České Budějovice, Czech Republic (10. – 12. 4. 2019).

15. **A. Makrlíková**, T. Navrátil, Determination of tumor biomarkers using flow injection analysis with amperometric detection at boron doped diamond electrode, *Seminar of Students of JHI 2019* – Liblice, Czech Republic (29. – 30. 4. 2019).

Lectures within the seminars of J. Heyrovský Institute of Physical Chemistry of the Czech Academy of Sciences:

16. **A. Makrlíková**, Voltametrické a amperometrické stanovení kyseliny homovanilové jako biomarkeru neuroblastomu (Voltammetric and amperometric determination of homovanillic acid as tumour biomarker), Prague, Czech Republic (30. 1. 2017).

17. **A. Němečková-Makrlíková**, Elektrochemické metody pro stanovení biomarkerů rakoviny (Electrochemical methods for determination of tumour biomarkers), Prague, Czech Republic (7. 6. 2019).

C) Poster presentations

1. **A. Makrlíková**, E. Ktena, A. Economou, J. Fischer, V. Vyskočil, Electrochemical determination of selected tumor biomarkers using screen-printed electrodes, *16th International Conference on Electroanalysis ESEAC* – Bath, United Kingdom (12. – 16. 6. 2016).

2. **A. Makrlíková**, T. Navrátil, J. Barek, V. Vyskočil, H. Dejmková, Determination of homovanillic acid in human urine using HPLC-ED/UV with solid phase extraction, *17th International Conference on Electroanalysis* – Rhodos, Greece (3. – 6. 6. 2018).

D) Internship

Long-term foreign internship in laboratory of Prof. Dr. Frank-Michael Matysik, University of Regensburg, Faculty of Chemistry and Pharmacy, Institute of Analytical Chemistry, Chemo- and Biosensors, Regensburg, Germany (1. 10. 2016 – 31. 12. 2016).

E) Grants

Principal investigator

Determination of selected biomarkers in human urine by flow methods with electrochemical and spectrophotometric detection. Grant Agency of Charles University. Project 243-734216, 2016 – 2018.

Team member

New approaches to monitoring of agrochemicals, food additives and contaminants and their transformation processes in environmental and food matrices. Grant Agency of the Czech Republic (the Czech Science Foundation). Project 17-03868S, 2017 – 2019.