Charles University
Faculty of Science

Study programme: Analytical Chemistry

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Development of electrochemical methods for study of antibacterial compounds in small volumes

Vývoj elektrochemických metod k studiu antibakteriálních látek v malých objemech

Ph.D. thesis

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Prague 2019

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Experimental work included in this Ph.D. thesis was carried out at the Charles University, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry in the period from 2015 to 2019.

Acknowledgments

I would like to express acknowledgements to all who have supported my research efforts during my studies. Firstly, I would like to thank my supervisor prof. RNDr. Jiří Barek, CSc., Faculty of Science, Charles University for his kind leading of my doctoral program and my supervisor-consultant RNDr. Jan Fischer, Ph.D., Faculty of Science, Charles University for his extensive help and support of my research. Furthermore, I would like to thank all the colleagues from our research group and from the Department of Analytical Chemistry. I also acknowledge the excellent cooperation and help for my research topic from doc. RNDr. Miroslav Fojta, CSc., Mgr. Marie Brázdová, Ph.D. and Mgr. Zuzana Soldánová from Institute of Biophysics of the CAS, Brno. I would like to thank the research team around prof. PharmDr. Josef Jampílek, Ph.D. (Comenius University, Bratislava), namely PharmDr. Tomáš Goněc, Ph.D. (University of Veterinary and Pharmaceutical Sciences, Brno) and Mgr. Hana Michnová (Palacký University, Olomouc) for their excellent cooperation and providing me with studied compounds. I also thank Konstantina Tsami (National and Kapodistrian University, Athens) for her help with the experimental work during her internship at Charles University.

Lastly, I thank to my parents, family and all my friends for their support during my studies.

Financial support of my research was provided from: the Czech Science Foundation (projects P206/12/G151, and 17-03868S) and Specific University Research (SVV).

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KEYWORDS

Antimycobacterial activity

Electrochemical microcell

Glassy carbon electrode

Single drop analysis

Structure-activity relationship

Voltammetry

KLÍČOVÁ SLOVA

Analýza jedné kapky

Antimykobakteriální aktivita

Elektroda ze skelného uhlíku

Elektrochemická microcela

Voltametrie

Vztah struktura-aktivita

LIST OF SYMBOLS AND ABBREVIATIONS

AgSAE - silver solid amalgam electrode
AdSV - adsorptive stripping voltammetry
BR - Britton-Robinson
c - concentration
Compd - compound
CV - cyclic voltammetry
DMSO - dimethyl sulfoxide
DPV - differential pulse voltammetry
E - potential
FIA - flow injection analysis
GCE - glassy carbon electrode
HMDE - hanging mercury drop electrode
HNC - hydroxynaphthalenecarboxamides
HPLC - high performance liquid chromatography
I - current
IC_{50} - half maximal inhibitory concentration
LOQ - limit of quantification
M. - *Mycobacterium*
m-AgSAE - meniscus modified silver solid amalgam electrode
MIC - minimum inhibitory concentration
N - number of measurements
PET - photosynthetic electron transport
pK_a - acid dissociation constant
r - correlation coefficient
RSD - relative standard deviation
SWV - square wave voltammetry
TB - tuberculosis
σ - Hammett constant

ABSTRACT

Main goal of this Ph.D. thesis is to develop voltammetric methods for the electrochemical study of novel antimycobacterial compounds hydroxynaphthalene-carboxamides.

Firstly, this study was focused on the miniaturization of voltammetric methods and construction of an electrochemical microcell due to usually small volume of samples that are associated with an analysis of biologically active compounds in biological matrices. Therefore, all aspects of the voltammetric procedure were studied in a relation to miniaturization. Microcells were based on commercially available electrodes: glassy carbon electrode as a reliable electrode material with well-described characteristics and a novel silver solid amalgam electrode. This study was carried out with analytes 4-nitrophenol, pesticide difenzoquat, and 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide. Attention was paid especially to the optimization of oxygen removal procedures in the drop of a solution. Developed miniaturized methods had the same parameters for the determination of studied compounds as in bigger volumes. The proposed electrochemical microcell can be generally used for voltammetric analysis of those samples of biological or environmental origin that are usually available in very limited volumes.

Second part of the thesis was focused on the electrochemical study of novel antibiotics. A model compound, 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide, was used for the pilot study concerning investigation of reduction and oxidation of the model analyte by voltammetric methods. The model analyte was used to carry out the optimization of parameters of determination such as composition of supporting electrolyte and pH of the solution by cyclic, differential pulse, square wave and adsorptive stripping voltammetry in developed electrochemical microcell on a glassy carbon electrode. This investigation continued by a study of twenty-two ring-substituted 1-hydroxynaphthalene-2-carboxamides. These compounds were studied by cyclic voltammetry with a goal to correlate their oxidation potentials and structure via Hammett substituent constants. Furthermore, relationship between biological activity and oxidation potential of derivatives was closely investigated with a goal of finding a correlation between the electrochemistry and pharmacology that can provide relevant information about a design of novel antibiotic agents.

ABSTRAKT

Hlavním cílem této disertační práce je vývoj voltametrických metod pro elektrochemickou studii nových antimykobakteriálních látek hydroxynaftalenkarboxamidů.

Tato studie je v první řade zaměřena na miniaturizaci voltametrických metod a konstrukci elektrochemické mikrocely za účelem analýzy biologicky aktivních látek v biologických matricích, se kterou je obvykle spojené omezené množství vzorku. Všechny aspekty voltametrické analýzy musely být prostudovány s důrazem na miniaturizaci. Mikrocely byly zkonstruovány pomocí běžně komerčně dostupných elektrod: elektrody ze skelného uhlíku, jako spolehlivého a podrobně charakterizovaného materiálu, a nové stříbrné pevné amalgámové elektrody. Toto porovnávání bylo provedeno pomocí modelových analytů: 4-nitrofenol, pesticid difenzoquat a 1-hydroxy-*N*-(4-nitrofenyl)naftalen-2-karboxamid. Důraz byl kladen speciálně na optimalizaci postupů k odstranění signálu kyslíku v kapce roztoku. Byly vyvinuty miniaturizované metody, které měly stejné parametry stanovení těchto látek jako stanovení ve velkém mililitrovém objemu. Takto vyvinuté elektrochemické mikrocely mohou být obecně užívané k voltametrické analýze biologických nebo enviromentálních vzorků, kterých je k dispozici jen omezený objem.

Druhá část této práce byla zaměřena na elektrochemickou studii nově vyvinutých antibiotik. Modelový analyt, 1-hydroxy-*N*-(4-nitrofenyl)naftalen-2-karboxamid, byl použitý v pilotní studii, ve které byla voltametrickými metodami podrobně prostudována redukce a oxidace tohoto analytu. Optimalizace parametrů stanovení, mezi kterými bylo studium složení základního elektrolytu a pH roztoku probíhalo pomocí cyklické, diferenční pulsní, square wave a adsorpční rozpouštěcí voltametrie ve vyvinuté mikrocele na elektrodě ze skelného uhlíku. Tento výzkum dále pokračoval studiem dvaceti dvou substituovaných 1-hydroxynaftalen-2-karboxamidů. Ke studiu těchto látek byla využita cyklická voltametrie s cílem korelace oxidačního potenciálu a struktury pomocí Hammettových substitučních konstant. Vztah mezi biologickou aktivitou a oxidačním potenciálem studovaných derivátů byl zkoumán se zaměřením na hledání korelace mezi elektrochemií a farmakologií. Toto spojení může poskytnout relevantní informace k následnému návrhu a přípravě nových antibiotických chemoterapeutik.

1. AIMS OF THE WORK

This thesis has two major goals. The first and foremost goal is the development of an electrochemical microcell and techniques for work in micro volumes of samples that can be subsequently used for achieving the second goal, which is a qualitative and quantitative study of novel antimycobacterial agents by electrochemical methods.

The first goal involves the development of electrochemical microcells, modifications of voltammetric methods and techniques, and adjustments to sample handling and sample pre-treatment. The target is to achieve sample volumes in the range of tens of microliters. Electrochemical methods and techniques that are already used for conventional volume (around 5-10 mL) are used as the basis to achieve these goals. It is necessary to properly investigate arrangement and positioning of the electrodes and modify measuring protocols, voltammetric methods and pre-treatment of working electrodes that will eventually lead to optimal microcell design. Sample pre-treatment and its subsequent transport to the microcell must be adjusted as well. Cathodic voltammetry also requires removal of oxygen; therefore, novel methods of removing the oxygen signal from cathodic voltammograms in microcells are necessary. Functionality and design of developed microcells were assessed with glassy carbon and amalgam electrodes that were used for investigations of model analyte 4-nitrophenol in organic solvent (DMSO) and model pollutant, pesticide difenzoquat. These developed methods and microcells are employable for the determination of drugs, pharmaceuticals, or markers of illness in biological fluids, pollutants in environmental samples with minimal amount of sample necessary or with limits of detection significantly decreased by some extraction procedure to very small volumes to achieve exceptionally high preconcentration factors.

The second goal of this thesis encompasses an electrochemical study of novel antimycobacterial agents from the group of hydroxynaphthalenecarboxamides. This study must involve finding optimal parameters (including content of organic solvent, optimal pH of supporting electrolyte, etc.) for the micro volume determination of a selected model compound (1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide) in the developed microcell. The consequent investigation is focused on investigation of the relationship of electrochemical characteristics (potential of oxidation/reduction) with the structure (Hammett constant) and biological activity (antimycobacterial activity MIC value, PET inhibiting activity IC_{50} value) of more than 20 derivatives of hydroxynaphthalenecarboxamides. This is based on

a presumption, that various electrochemically non-active substituents in the structure cause an excess or lack of electrons in an electrochemically active moiety causing a shift of oxidation/reduction potential to either more positive or more negative values. Finding a positive relationship between oxidation/reduction potential and biological activity can link the change of electron density with biological activity. Additionally, it can be particularly useful in designing new and more effective derivatives of studied antibiotics. Developed and optimized method for the determination of these compounds in micro volumes of samples can be advantageous in the study of biological activity and effectiveness. Furthermore, miniaturized voltammetric methods are suitable for *in-vivo* analysis in various samples of biological origin.

Results reported in this Ph.D. thesis were published (or have been submitted for publication) in following 4 papers attached as appendices:

I. **Gajdár J.**, Barek J., Fojta M., Fischer J.: Micro volume voltammetric determination of 4-nitrophenol in dimethyl sulfoxide at a glassy carbon electrode. Monatshefte für Chemie - Chemical Monthly *148*, 1639-1644 (2017).

II. **Gajdár J.**, Goněc T., Jampílek J., Brázdová M., Bábková Z., Fojta M., Barek J., Fischer J.: Voltammetry of a novel antimycobacterial agent 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide in a single drop of a solution. Electroanalysis *30*, 38-47 (2018).

III. **Gajdár J.**, Barek J., Fischer J.: Electrochemical microcell based on silver solid amalgam electrode for voltammetric determination of pesticide difenzoquat. Sensors and Actuators B: Chemical *299*, 126931 (2019).

IV. **Gajdár J.**, Tsami K., Michnová H., Goněc T., Brázdová M., Soldánová Z., Fojta M., Jampílek J., Barek J., Fischer J.: Electrochemistry of ring-substituted 1-hydroxynaphthalene-2-carboxanilides: Relation to structure and biological activity. Electrochimica Acta, *submitted* (2019).

2. INTRODUCTION

2.1. Miniaturization and electrochemistry

Miniaturization of analytical methods and development of micro analysis systems designed for sample preparation, separation and detection integrated into a single fabricated device is a goal of many studies in the field of analytical chemistry [1]. Majority of these devices are based on optical or electrochemical sensors which often complement each other. Electrochemical devices offer simplicity, high sensitivity, selectivity, uncomplicated operation procedures, simple sample treatment, and easy to manufacture low-cost instrumentation [2]. Novel electrochemical techniques and electrode materials have been utilized for determination of several biologically active organic and inorganic species even in complex samples as proved by several reviews [3-5].

Miniaturized electrochemical systems (and miniaturized systems in general) should be evaluated especially based on simplicity of design, portability, cost, uncomplicated fabrication, analytical response, sensitivity, and fouling [2]. Miniaturized electrochemical devices can be favourably based on well-functioning microelectrodes as a key component [6]. Additionally, disposable sensors based on screen printed electrodes have been used as an easily fabricated and inexpensive option [7, 8]. These devices are usually based on voltammetric, amperometric or potentiometric methods [2].

One of the advantages of these micro systems is their portability. Sampling procedures are expensive, time-consuming and involves problems with sample stability. All these problems can be eliminated by bringing a measuring device to the sample, which is favourable in the case of (real-time) environmental analysis of organic and inorganic pollutants at the point of interest. These electrochemical miniaturized systems can be used as sensors or biosensors for *in-situ* analysis, analysis of markers of illness, analysis of pharmaceuticals, or other biologically active compounds in complex matrices of biological origin, etc. [1, 2, 9]. So, in general, miniaturization is very useful in areas of clinical diagnosis, environmental monitoring, and forensic investigations [10].

Development of miniaturized sensors and techniques is also within the goals of green analytical chemistry. This approach leads to lower sample, reagents and solvents consumption, and, consequently, lower waste production. Therefore, minimal to no amounts of toxic

and hazardous materials are disposed while these methods are relying on fast, small, easy-to-use and energy-efficient electrochemical microsystems [11, 12].

2.1.1. Design of electrochemical microcells and electrode arrangements

Electrochemical microcell can be defined as a system consisting of working, auxiliary and reference electrodes, electrode compartments, devices for sample manipulation (inlet, outlet), devices for oxygen removal, etc. A term "microcell" is not necessarily related to the cell size but to the sample volume, which ranges from less than 10 μL up to hundreds of microliters. Microcells can be divided in two groups: flow-through and non-flow cells. Flow-through cells rely on the sample continuously moving through the cell in some carrier solution, for example as detectors in HPLC or FIA/SIA. Non-flow microcells are based on the sample being directly introduced into the cell with some device or pipette and are mostly used in voltammetry and stripping voltammetry. They usually have simpler design compared to flow-through microcells. There are multiple designs of electrochemical microcells designed for studies of both organic and inorganic substances. Working (micro and macro) electrodes set in their usual state from top to bottom can be used; however, electrodes set in an inverted state (from bottom to top) have been used more often because of an easier work with smaller volumes of samples [13].

Several microcell designs were summarized in a review [13] and the following are designs on which this study is based on. A temperature-controlled anaerobic microcell suitable for electrochemical studies of biomolecules or enzymes at various temperatures was constructed based on a pyrolytic graphite working electrode [14]. Another microcell based on a mercury film electrode was constructed for stripping analysis of trace amounts of metals for volumes as low as 5 μL in the absence of oxygen [15]. A simple, low-cost and easy-to-use microcell with similar design was able to provide a sensitive and reproducible determination of dopamine and chlorpromazine in 10 μL of sample, where especially depletion and evaporation effects were discussed in detail [16]. Amperometric microcell detector which is a part of a very specific enzymatic assay was devised for the determination of ascorbate in biological samples [17]. A simple design using three band electrodes that is usable even in resistive media (non-aqueous or without supporting electrolyte) because of the close proximity of the electrodes was introduced with ferrocene as a model electroactive species for volumes down to 0.05 μL [18]. Pollutant triclosan was determined in some real samples on carbon composite electrodes

embedded in microtitration plate, which, if connected to small potentiostat, can be easily used for point-of-care analysis [19]. A novel micro-volume voltammetric cell separated by an agar membrane to two compartments (small volume and large volume) with an amalgam working electrode positioned in small volume compartment was successfully tested by determining anthraquinone derivative [20]. An electrochemical multisensor based on the idea of multiple working electrodes embedded in Teflon body, that were simultaneously used for detection of nucleic bases, could significantly simplify complex electrochemical experiments [21]. Additionally, several microcells developed for mercury-based electrodes that were designed for the determination of electrochemically active compounds in very small sample volumes used hanging mercury drop [22], static mercury drop [23], mercury drop [24], and amalgam-based [25] electrodes.

Analysis of small volume samples can also be performed by using disposable screen-printed electrodes, which offer the same benefits for experiments with drops of samples as above-mentioned microcells. They are cheap, reliable, and easily modifiable single-use sensors that can be designed in accordance with their analytical purpose [7, 8]. However, most of commercially made screen-printed electrodes are unsuitable for work in organic solvents, even after some dilution because the ink used to construct electrodes can be dissolved by those solvents [26, 27]. This study relies on usage of mixed organic media due to the non-solubility of analytes in the water and therefore, screen-printed electrodes might not be optimal for this type of work.

2.1.2. Electrochemical techniques for monitoring of pharmaceuticals and drugs

Development of new drugs is tightly intertwined with detailed chemical studies concerning quality control, stability testing, clinical studies and studies of metabolites of drugs. Drug analysis is also necessary for pharmacokinetic studies and drug monitoring [28]. Therefore, there is a need for highly selective and precise analytical methods for detailed chemical studies concerning several aspects of drug development and drug manufacturing [29].

Electrochemical methods have always attracted attention in the analysis of drugs and pharmaceuticals. The most common techniques for analysis of pharmaceuticals are based on HPLC-MS, capillary electrophoresis-MS, and UV/vis spectrometry that require expensive instrumentation and special and lengthy sample pre-treatment. Analysis of samples of biological origin usually deals with small volumes of blood or urine where a low concentration

of drugs or metabolites must be determined. Electrochemical methods can be considered as an interesting alternative which offers simple, portable and inexpensive instrumentation and they additionally can be easily employed for *in vivo* analysis [28, 30].

Redox reactions observed by electrochemical methods can be similar to biological reactions and it is assumed that reaction mechanism at the electrode and in the body might share similar principles. Thus, the study of an electrode mechanism can give us an insight into the metabolic fate and pharmacological activity of drugs [30].

There has been numbers of papers dealing with analysis of pharmaceuticals in complex matrices by voltammetric or stripping techniques, with possibilities of screen-printed electrodes, microelectrodes, or chemically modified electrodes, and a movement towards miniaturization of methods is clearly pronounced [30-32].

2.1.3. Supressing the response of dissolved oxygen

Dissolved oxygen is usually considered to be interfering with electrochemical studies due to its pronounced response in cathodic part of potential window. The concentration of oxygen in aqueous solutions open to the atmosphere is approximately 1 mmol L^{-1} and it provides up to two signals depending on experimental conditions. Oxygen is at first reduced to peroxide, followed by its reduction to oxides/water [33]. In organic solvents like DMSO; oxygen is present in concentrations around 0.1 mmol L^{-1}. Oxygen gives several signals in DMSO. In the first step, one-electron reversible reduction to stable superoxide radical (O_2^{-}) can be observed followed by the formation of peroxide anions at more negative potentials [34-36]. There are several ways how to address the issue of eliminating the oxygen response (with focus on miniaturization).

First approach is to remove dissolved oxygen from a solution. This can be achieved either physically or chemically. Physical removal is performed by the introduction of an inert gas: most commonly nitrogen or argon. This is carried out by introducing the gas stream into a solution for some time (use of wash bottle is advisable when working with volatile solvents) [33]; however, this procedure cannot be easily performed with microvolumes of samples. Nevertheless, multiple procedures of removing oxygen have been investigated including a remarkable method of flowing a thin sample film in a capillary filled with nitrogen [23],

creating a special enclosed microcell [14], or just introducing nitrogen stream into the microcell isolated from the outer atmosphere to some extent [15, 22].

Chemical removal can be carried out by the reducing agent sodium sulphite which is directly added to the solution and reacts with oxygen [33]. Use of sulphite is restricted to neutral and alkaline pH. Sodium sulphite has been used in a miniaturized electrode system for determination of aminofluorenone [25]. However, it must be noted that, as it is a reducing agent, it can also reduce the analyte [37]. Therefore, physical removal is preferred because an inert gas like nitrogen or argon is not expected to interact with solution components.

Second approach is to keep oxygen dissolved in a solution and resolve this issue by multiple possible procedures. In some cases, an oxygen signal can be considered as just another electroactive species in a solution, and therefore, an analyte with adequate reduction potential can be simultaneously determined in non-degassed solution. However, products of oxygen reduction (peroxide) can react with the analyte and they can also alter pH near the electrode, so caution must be paid in these cases [38].

Furthermore, high frequency methods like AC polarography or square wave voltammetry can be used to discriminate against irreversible electrode processes (like oxygen reduction in aqueous media). These techniques can be used for analytical studies of reversible processes that, on the other hand, are pronounced [38-40]. The determination of metals by stripping methods can be easily achieved even with oxygen present in a solution. By carrying out fast scanning method like square wave voltammetry, there is no oxygen present near the electrode immediately after the stripping step [41, 42]. This approach was also applied for analysis of metals in small volumes [43]. Another similar method is subtractive stripping voltammetry, in which a signal of background (supporting electrolyte with oxygen) is simply subtracted from analytical signal [44].

2.2. Working electrodes

Two types of working electrodes were used in this study: a well-established glassy carbon electrode and a novel silver solid amalgam electrode.

2.2.1. Glassy carbon electrodes

Glassy carbon (GC) has been used as an electrode material since its introduction in 1960s [45] because of its excellent mechanical and electrical properties, wide potential window, chemical inertness, reproducible results and low cost [46]. The potential window of a glassy carbon electrode ranges over approx. ±1 V and it is even larger in non-aqueous solvents [45, 47]. GC is prepared by slowly heating polymeric resin (often polyacrylonitrile) in inert atmosphere until all heteroatoms evaporate and only carbon is left [48, 49]. GCEs can be used for voltammetric, coulometric and amperometric methods, in flow-through detectors, and even in non-aqueous media. They are used as a substrate electrode for numbers of various surface modifications or film depositions that can be then used for biosensing applications [50, 51]. As any other solid electrode, GCEs also suffer from electrode fouling. To obtain reproducible response of the electrode an optimal pre-treatment procedure must be developed based on several aspects, such as characteristics of studied system, cost and duration. Pre-treatment can include polishing of the electrode with silicon carbide papers, followed by fine polishing on alumina slurry or diamond pastes. Additionally, this can be followed by an ultrasonic cleaning in various solvents or some electrochemical pre-treatment (or anodic/cathodic activation) [46, 47, 49]. GCEs and modified GCEs have been used for determination of many pharmaceuticals and drugs as proved by these reviews [28, 30, 31].

2.2.2. Amalgam electrodes

Silver solid amalgam electrodes were introduced in 2000 by Novotny and Yosypchuk as a well-performing alternative to dropping mercury electrode [52]. Mercury is one of the best electrode materials (e.g. dropping mercury electrode, DME; hanging mercury drop electrode, HMDE) for determination of traces of metals and reducible organic substances. Almost a hundred years (DME was introduced in 1922) of polarographic and voltammetric investigations of thousands of organic compounds on mercury electrodes makes them a foundation for any electrochemical research [53-55]. However, because of restrictions on the use of mercury non-toxic solid amalgam electrodes were developed [52]. They share same qualities as DME/HMDE: e.g. sensitivity, reproducibility, wide cathodic potential window; and additionally, offer good mechanical stability, simple handling and, therefore, they widen the fields of application for mercury-based electrodes. Amalgams can be prepared from several metals (Cu, Ag, Au, Bi, Ir, Tl), however, electrodes based on silver solid amalgam exhibited

the most favourable characteristics for analysis [56]. Furthermore, amalgams can be prepared as a liquid, paste or solid and all of these forms of amalgam have been intensively studied for voltammetric applications. Their surface can even be further modified to improve sensitivity for given analyte [57, 58]. Amalgam electrodes require a pre-treatment consisting of polishing or amalgamation (dipping in mercury) followed by activation (application of negative potential in KCl solution) and regeneration [59]. These electrodes have been used for electrochemical studies of various organic compounds, insecticides, herbicides, pollutants and pharmaceuticals [59-66].

2.3. Relation of electrochemical potentials, structure, and biological activity

Since the beginnings of polarography, there has been an effort to establish a relationship between electrochemical characteristics of organic compounds and their structure. In the most basic approach, the change of oxidation/reduction potential can be explained by a shifting of electrons influenced by the substituent. Inductive effect represents a shift of σ bond electrons and mesomeric effect represents a shift of π bond electrons. The final effect of the substituent is a combination of these two effects. This was a foundation for a Hammett equation; the first approach to study potential-structure relationship [67, 68]. Since then, a number of more accurate computational methods were developed based on calculation of ionization potential [69], HOMO-LUMO gap [70], substituent charge [71], and based on quantum chemistry [72]. However, even today, Hammett correlation is used for its simple approach to study fundamental structure-potential relationship [69-78].

2.3.1. Hammett correlations

Hammett equation is a relation that can be used for a study of any physico-chemical parameter and it expresses an effect of the substituent in side-chain reactions of benzene derivatives. In its simplest form, an equation describing the change of the half-wave potential or oxidation/reduction $\Delta E_{1/2}$ can be expressed as:

$$\Delta E_{1/2} = \sigma \times \rho$$

where σ denotes a substituent constant that depends solely on the nature and position of a substituent and it is in principle independent on the nature of the reaction. Reaction constant

ρ depends on the nature of the reaction (supporting electrolyte, solvent, temperature) of an electroactive moiety and it is independent on the substituent. Most commonly studied compounds are substituted aromatic compounds. This approach is only applicable for *meta*- and *para*- positions of substituents. *Ortho*- position, because of its close proximity to the electroactive moiety, must have σ values corrected due to steric effects [68, 79, 80]. Hammett constants for a number of substituents were collected by Jaffé [80] and Zuman [68].

Requirements for constructing Hammett correlations are following: Reduction/oxidation of all compounds in a series must be driven by the same electrode mechanism; the reaction must be diffusion controlled; it must involve the same number of electrons and the same degree of reversibility. It is essential to keep the same experimental conditions. The studied reaction should not depend on pH so a study in aprotic solvents is favourable; however, the same slope of d$E_{1/2}$/dpH for all compounds might be sufficient. For correctly determining the potential, it is crucial to use a reliable reference electrode, or some internal standard (ferrocene, Tl^+, etc.). The construction of a correlation should start with an unsubstituted compound and its Hammett constant σ is established as zero [68].

2.3.2 Relation of biological activity and redox potential

This investigation presents convergence of electrochemistry and medicinal chemistry. Many biological processes are based on redox reactions and studying electron transfer mechanism of those reactions by electrochemical methods can be useful for observing and predicting biological phenomena since these processes are based on the same principles. Electrochemical methods provide clean and easy-to-control chemical system which allows for more accurate evaluation of drug behaviour. Adjusting parameters and composition of the studied electrochemical system closer to real system can provide more precise information concerning drug investigation. Prediction based on the substituent effect can possibly aid designing more powerful derivatives. However, biological processes are highly complex so to expect an absolute correlation with electrochemistry is not reasonable and numbers of other parameters must be considered such as stereochemistry, lipophilicity, diffusion, solubility, metabolism, membrane permeability, bioavailability, enzyme interactions, etc. [81-83]. Nevertheless, there are many studies that investigated relationship between electrochemical parameters and biochemical data [78, 84-88]. The main electrochemical parameter discussed in

these studies is the potential of reduction/oxidation that gives a quantitative parameter related to the ease of the reaction [81-83].

Relationship of reduction potential, structure and antituberculotic activity of quinoxalines di-*N*-oxides was reported and it suggested a bio-reduction mechanism based on electrochemical experiments [84]. Inconclusive relationship was obtained in the study of cytostatic activity of similar compounds in aprotic solvent and only some tentative trends could be drawn for certain subsets [85]. On the other hand, a statistically significant correlation between biological activity and reduction potential was obtained in the study of antitumor mitomycin derivatives [86], antineoplastic furanquinones [87], and in series of antitumor pyridoacridones [88].

2.4. Analytes

Main objects of this study are novel antimycobacterial agents from a group of hydroxynaphthalenecarboxamides. Additionally, 4-nitrophenol and difenzoquat were used as model analytes in investigations concerning the development of the microcell and miniaturization of voltammetric methods. 4-nitrophenol is a compound extensively studied by voltammetry due to its well-described electrode mechanism making it an ideal model analyte [89-93]. Difenzoquat is a pesticide that was detected only on mercury electrodes as a result of its very negative reduction potential [94-97].

2.4.1. Hydroxynaphthalenecarboxamides: Antimycobacterial chemotherapeutics and tuberculosis

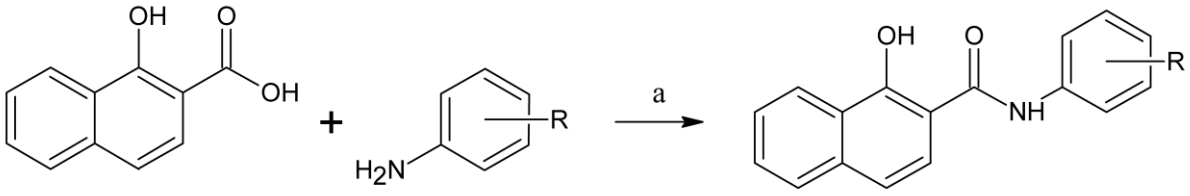
Tuberculosis (TB) is an infectious disease caused by the pathogen *Mycobacterium tuberculosis* that primarily affects lungs. The disease is spread by the air, for example by coughing. An estimated 2 billion people have a latent TB infection, however, only a small portion of infected people (5-10%) will develop TB in their life. Probability of developing TB is much higher among people with HIV and it is additionally influenced by risk factors such as under-nutrition, diabetes, smoking, and alcohol consumption. TB causes ill-health for approximately 10 million people a year. TB is the ninth leading cause of death worldwide and it is a leading cause of death caused by a single infectious agent [98, 99]. In 2017, global

mortality rates were 17 per 100 000 population among HIV-negative people and 21 (per 100 000 population) when HIV-positive people were included [98]. The treatment of common drug-susceptible TB is an administration of four first-line drugs: isoniazid, rifampicin, ethambutol, and pyrazinamide, that lasts for 6 months [98, 100]. Furthermore, other *Mycobacteria*, also called nontuberculous *Mycobacteria*, are associated with a number of human diseases, including pulmonary diseases, skin and soft tissue diseases, gastrointestinal and skeletal infections that can be especially dangerous for immunocompromised patients [101, 102].

The four-drug combination treatment was introduced in 1970s and it has not been changed since. This paved a way for an emergence of multi-drug-resistant strains that make current treatment options ineffective. The absence of effective treatment combined with ineffective public health system in resource-poor countries fuels further growth of drug-resistant forms of TB [99]. In 2017 globally, there were approximately 558 000 new cases of drug-resistant TB (resistant to a first-line drug, rifampicin) [98]. Therefore, there is an urgent need for new TB drugs that would shorten and simplify treatment, that are effective against drug-resistant strains and have lower dosing frequency. Nowadays, this treatment consists of combination of 8 to 10 second- or third-line drugs that lasts up to 24 months. In addition, these drugs have much more serious adverse effects than first-line drugs [100, 103]. Second- and third-line drugs such as fluoroquinolones, ethionamide, thioacetazone, linezolid, bedaquilline, delamanid, etc. are currently in use [100].

2.4.2. Hydroxynaphthalenecarboxanilides: preparation, characterization and biological properties

A research into a novel design of antituberculotic agents has led to the preparation and characterization of hydroxynaphthalenecarboxanilides (HNCs). All studied compounds **1**-**8c** are listed in **Table 3.1**. These compounds were prepared according to the **Scheme 2.1** from commercially available compounds.



Scheme 2.1 Synthesis of ring-substituted 1-hydroxynaphthalene-2-carboxanilides **1-8c**: R = H (**1**), OCH_3 (**2a** (*ortho*-), **b** (*meta*-), **c** (*para*-)), CH_3 (**3a-c**), F (**4a-c**), Cl (**5a-c**), Br (**6a-c**), CF_3 (**7a-c**), NO_2 (**8a-c**); (a) PCl_3, chlorobenzene, microwave (adapted from [104]).

HNCs can be considered as the ring analogues of salicylanilides (*N*-substituted hydroxybenzamides) which are used as drugs for treatment of TB (e.g. niclosamide) [105]. HNCs are compounds with wide range of pharmacological activities; they were identified with anti-infectious [104-111] and antiproliferative properties [112, 113]. It is presumed that carboxamide moiety (-CONH-) is mimicking a peptide bond and via this bond compounds are bound to their targets. Analogously to salycilanilides, HNCs can be considered as multi-target compounds, meaning that they can affect various targets in different microbial pathogens [114-117]. Activity of HNCs depends on the mutual position of carboxamide and phenolic moieties that is demonstrated by different biological spectrum and activity for variously substituted compounds [104-106, 108, 111].

This thesis is focused on the subgroup of 1-hydroxynaphthalene-2-carboxanilides characterized by Gonec et al. [104]. Some of the studied compounds in the series exhibited higher activity against *Mycobacteria* strains than isoniazid (a drug commonly used for treatment of TB). Their biological activities were discussed in relation to their structure, lipophilicity, and calculated values of Hammett's σ parameters and partition coefficients [104].

HNCs have been also studied for their ability to inhibit photosynthetic electron transport (PET) in spinach chloroplasts. The amide moiety that is also present in HNCs is present in many herbicides acting as PET inhibitors [114, 118]. It has been observed that antimycobacterial activity correlates with PET inhibiting activity [104-110, 114, 119-121]. Pharmaceuticals and herbicides are often overlapped by targeting similar functions for both purposes and good correlation can be found between antimicrobial activity and herbicidal effects [122].

2.4.3. Electrochemistry of similar compounds

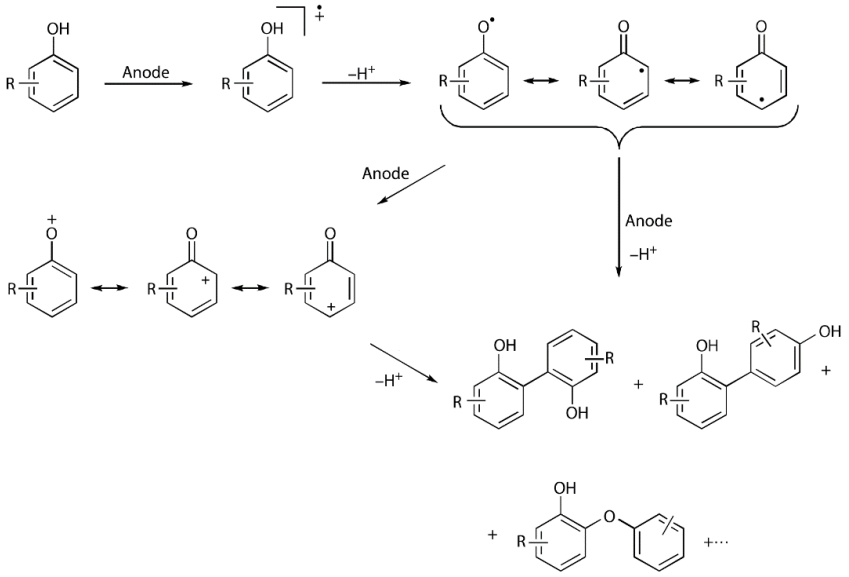
HNCs have not been yet studied by electrochemical methods. Therefore, an investigation into the compounds with similar structures or with similar moieties was used as a baseline for this work with a presumption that electrochemical reactions will be analogical. From the structural formula, it is obvious that electrochemically interesting group is hydroxyl/phenolic group on naphthalene moiety which can be readily oxidized. Additionally, compounds **8a**-**c** also contain nitro group on benzene nucleus that can be readily reduced. A model compound **8c** was used for a pilot study because it can be electrochemically both oxidized and reduced. No other substituent gave electrochemical signal in studied media. Therefore, literature concerning electrochemical reduction of nitro moiety and oxidation of hydroxyl/phenolic group was closely studied.

2.4.3.1. Oxidation of phenols/naphthols

Redox behaviour of phenolic and naphtholic compounds has been studied on multiple electrodes e.g. GCE [123-125] or boron doped diamond electrodes [126, 127].

Generally, oxidation of phenols can be described according to **Scheme 2.2**. First step involves one electron one proton oxidation to phenoxyl radical, which can then undergo several possible pathways. Radicals can either attack other molecules present in a solution and give various polymerization products which are then often adsorbed at the electrode contributing to the passivation, or in dependence on conditions, radicals can be further oxidized at more positive potentials to phenoxonium intermediate, which can then be hydrolysed to quinone-like structures. To summarize, oxidation of phenols usually involves a number of by-products [123, 128-130]. For monophenols the most probable products of oxidations are dimers, based mostly on the stability of intermediate phenoxyl radicals [131]. First step of the reaction is influenced by pH of the medium. At pH > pK_a, the deprotonation step is not involved in the electrode reaction as anionic form of phenol is already present in the solution. In very acidic conditions, a stable radical cation is formed. Phenol dissociation influences dependency of oxidation potential on pH as seen in **Fig. 2.1**, where pK_a value is clearly indicated [124, 132]. This effect can be used for the determination of pK_a values as proved by a study of more than 100 substituted phenols at graphite electrode [132]. Investigation of niclosamide, a structure similar to HNCs, proposed intra-molecular reaction taking place. After the oxidation to phenoxyl

radical, another one electron oxidation on the electrode creates bi-radical molecule that reacts with itself to produce a cyclic compound [133].



Scheme 2.2 Possible oxidation pathways for phenolic compounds (adapted from [129]).

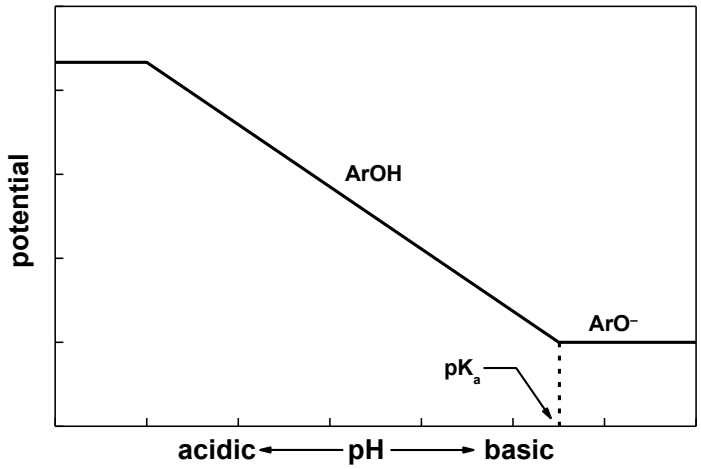
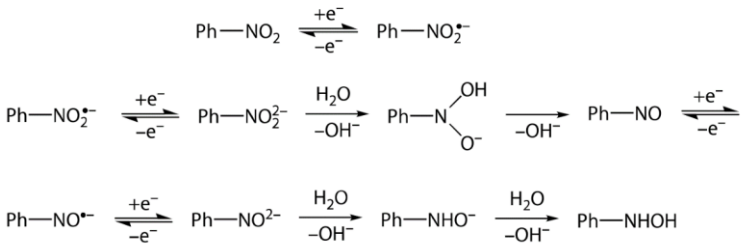


Fig. 2.1 Dependency of the peak potential on pH for phenolic compounds. Prevalent form of phenol in a solution and pK_a value are indicated (adapted from [132]).

2.4.3.2. Reduction of nitrocompounds

Reduction of compounds containing nitro moiety has been extensively studied by polarography [53, 134-136] and voltammetry on GCE [91, 137-139], AgSAE [89, 140], HMDE [53], or other metallic electrodes [93, 141].

Nitro-substituted compounds in this thesis were studied in aqueous and non-aqueous media, therefore, electrode reactions in both protic and aprotic solvents are discussed in this chapter. Nitroaromatic compounds are reduced at first by 1-electron reversible reduction to radical anion, according to **Scheme 2.3**. The radical-anion is stable in aprotic solvents and very basic aqueous solutions (pH>13). The radical-anion is then reduced in second 3-electron reduction to form a hydroxylamine compound. In aqueous media, this reaction is observed in one step as a four-electron irreversible reduction. A 2-electron reversible redox reaction can be later observed on the electrode as a hydroxylamine is oxidized back to nitroso compound [134, 137, 142]. In very acidic solutions, a two electron reduction of hydroxylamine to aniline occurs at more negative potentials [142, 143]. Reduction and final products in aprotic solvents are highly influenced by residual water content. Determination and control of water content is therefore crucial for accurate study of the electrode mechanism [142]. Polarographic studies of nitrobenzanilides in aqueous and mixed media [135, 136] and niclosamide on GCE in aqueous media [139] are an example of structurally similar compounds to nitro-substituted HNCs which can be presumably reduced by analogous pathways.



Scheme 2.3 Reduction pathway for nitro-substituted aromatic compounds (adapted from [142]).

3. RESULTS AND DISCUSSION

3.1. Development of the microcell and microvolume voltammetric procedures

First part of this thesis is dedicated to the development of the electrochemical microcell. Several aspects of the miniaturization of voltammetric methods were discussed (**Appx. I-III**) [144-146]. The main goal of the development of the microcell was to minimize the sample volume necessary for one voltammetric measurement. Throughout this study several designs of microcell were introduced with several modifications that were performed to eliminate all the observed problems which are further discussed in the detail in next chapters.

3.1.1. Investigation of the electrode arrangement and construction of the microcell

Development and construction of the electrochemical microcell were evolving based on problems encountered during experiments. The microcell was designed with working electrode in an inverted position. The electrode was always held by a clamp in a stable position. Development of designs with photos of used constructions is shown in (**Appx. I**; **Fig. 1**) [145].

The simplest design is using a working electrode with only two wires (silver and platinum) introduced into the drop of a solution. Silver wire was used as a pseudo-reference electrode and platinum wire was used as an auxiliary electrode. Both wires were held by clips in a stable position. The fundamental issue with is setup is the instability of the potential of a silver wire pseudo-reference electrode. However, in most of analytical applications, it did not present a problem. This construction had to be used for the determination of model compound **8c** in a drop of solution due to the adsorption of the analyte in the fritted glass of the commonly used Ag|AgCl reference electrode [144]. The depth of the submersion of wires was studied closely. It was found out that the submersion of platinum wire as an auxiliary electrode had no effect on measured cyclic voltammograms. However, the depth of silver wire used as a pseudo-reference electrode had obvious effect observed as an offset of the potential window. No shortening of potential window was evident, but edges of the potential window were shifted in the same direction with additional submersion of the silver wire. To conclude, if it is necessary to precisely measure the electrochemical potential, then a pseudo-reference electrode is inapplicable.

A standard glass reference electrode with frit at the end was dominantly used in microcells [144-146]. This electrode was positioned in the upright position opposite of the working electrode as seen in **Fig. 3.1**. Two types of reference electrodes were used: a Ag|AgCl|3M KCl reference electrode for the work in aqueous media [144, 146], and Ag|0.01M $AgNO_3$, 0.1M Bu_4NBF_4 (all in DMSO) reference electrode for the work in aprotic (DMSO) media [145]. The arrangement containing only working, reference and auxiliary electrode is satisfactory for voltammetric experiments in the anodic area. Measurements in cathodic area require all the components included in **Fig. 3.1**.

Optimization of sample volume was carried out by evaluating the difficulty of sample application. Minimum volume was limited by the amount necessary to fill the space between electrodes and creating an electrical contact between them. Another limitation of minimal volume was due to the distance between the electrodes. In the study of difenzoquat on m-AgSAE, a distance of 1 mm and less between the electrodes gave lower peak heights [146]. Maximum volume was limited exclusively by the surface tension of the applied drop of a solution. Therefore, sample volumes in the range approx. 10 (m-AgSAE) / 20 – 50 μL (GCE) can be used with this microcell (depending on the size of the working electrode). Variations of sample volumes or distance between reference and working electrode in this range have no significant effect on observed parameters (peak potential, peak height) [145, 146].

An effort was put to replace the common glass reference electrode with a Luggin capillary which could have provided some decrease of the sample volume. However, some periodic oscillations were observed (**Appx. I**, **Fig. 2**) that were probably caused by a very close distance between the working electrode and the end of capillary according to earlier studies into this matter [147-150]. Unfortunately, attempts to design a microcell with Luggin capillary were unsuccessful [145].

The problem of oxygen removal was solved by modifying the construction as shown in **Fig. 3.1** and in (**Appx. I-III**) [144-146]. All three of the electrodes were enclosed in a plastic tube manufactured from a plastic pipette tip. The platinum wire was folded in a shape of a half-circle and taped tightly to the reference electrode with parafilm. Nitrogen was input by a small hole in the tube and thus it created a slight overpressure inside the microcell. This tube could be freely moved up and down for sample manipulation. Oxygen can also be "pre-removed" from the sample in a separate vial.

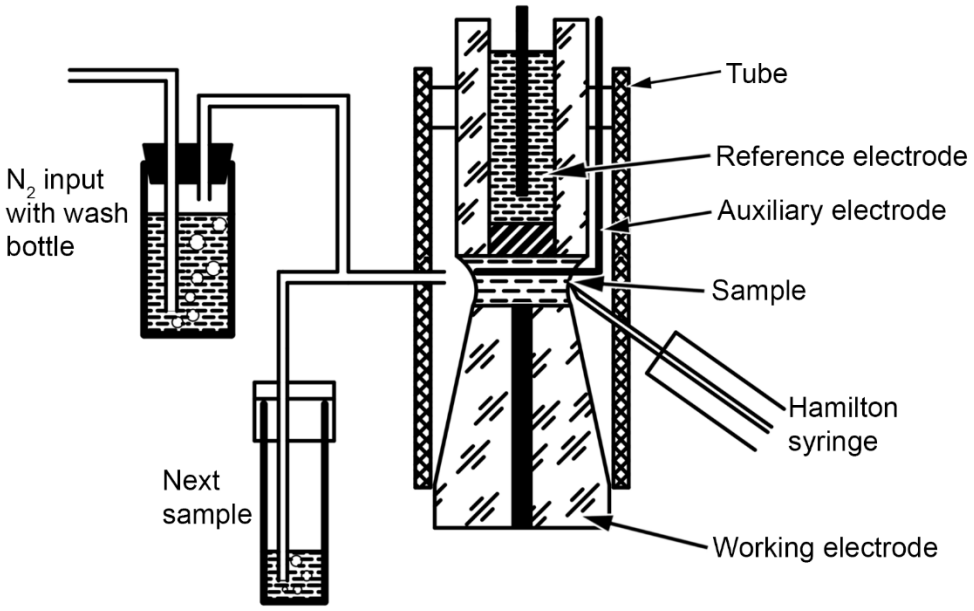


Fig. 3.1 Final general arrangement of designed microcell suitable for samples with volume as low as 10 μL.

3.1.2. Microcell procedures

Voltammetric measurements in an anodic area required much simpler procedures. After the assembly of the microcell, given sample was carefully pipetted between the electrodes. After the measurement, the microcell was washed, dried and disassembled if working electrode had to be polished [144].

Voltammetry in cathodic area had more complicated procedure due to the oxygen removal. Sample was at first purged in a vial. It is crucial to use a wash bottle with the same composition as the measured medium to minimize evaporation of samples which can be significant in a work with small volumes of solutions. The drop of sample then had to be transferred by Hamilton syringe to the assembled microcell (**Fig. 3.1**) with nitrogen atmosphere inside. After the measurement, the plastic tube was moved up and the microcell was washed and dried. In the meantime, another sample was purged with nitrogen and it was ready for measurements immediately after experiments with first sample were completed [146].

3.1.3. Procedures of oxygen removal

Removal of oxygen turned out to be a major issue in relation to miniaturization. Oxygen gave three responses in DMSO. The first response was a reversible one-electron reduction to superoxide anion radical at −0.9 V, followed by an irreversible reduction to a peroxide anion (wide signal at approx. −1.6 V) and finally the peroxide anion was reduced at −2.6 V to oxides (**Appx. 1**, **Fig. 2**) [145]. Oxygen in buffered aqueous solutions (pH around 7) on GCE gave only one very large and wide peak at approx. −0.4 V corresponding to the reduction to a peroxide [144]. Two signals assigned to oxygen were detected on m-AgSAE: oxygen (approx. −0.6 V) and then peroxide reduction (approx. −1.1 V) were observed in BR buffer pH 12 [146]. Generally, removal of oxygen from DMSO is a lengthy process that takes up to 10 mins. On the contrary, 5-minute purging with nitrogen is sufficient in aqueous solutions.

A chemical way of removing oxygen by sodium sulphite was considered; however, it was unsuccessful because reduction of the studied compound **8c** by the sulphite was occurring [144]. Therefore, a physical removal of oxygen was exclusively used. This could be carried out by two modified procedures.

The simpler but more time-consuming method of removing oxygen was to pipette the drop of a solution between electrodes in **Fig. 3.1** and then isolate the microcell from the atmosphere by sliding the tube over the drop. Oxygen was then slowly removed depending on the medium (10 mins for aqueous media, 20 mins for DMSO) as illustrated by amperometric recordings of oxygen in **Fig. 3.2**. The drop was not stirred in any way; therefore, oxygen was presumably only removed from the surface of the drop and the transport of oxygen from the middle of the drop was provided only by slow diffusion processes [144, 145]. Furthermore, the drop of the sample is resting between electrodes for a considerable time. It was observed that analyte was diffusing and adsorbing on the frit of the reference electrode, which was then contaminating the next measurement. This was overcome by using silver wire pseudo-reference electrode, for which no adsorption was observed [144].

A faster but slightly more complicated method of removing oxygen has been described in the previous **Chapter 3.1.2**. The sample was purged with nitrogen in a vial and then appropriate amount of a solution (10-50 μL) was taken with Hamilton syringe, quickly transferred to the microcell, and immediately measured as necessary. The transport with regular pipette quickly reintroduced oxygen in the drop. It was necessary to create nitrogen atmosphere inside the microcell by inputting nitrogen for 1-2 mins after any manipulation with

the microcell. Determination of samples that are of limited volume (< 100 μL) can be carried out in two ways. Oxygen removal can be carried out in a vial before measurement; however, the stream of nitrogen must be only blowing over the sample due to its very small volume. On the other hand, oxygen removal can be performed directly in the microcell with all disadvantages carefully considered [146] (**Appx. III**).

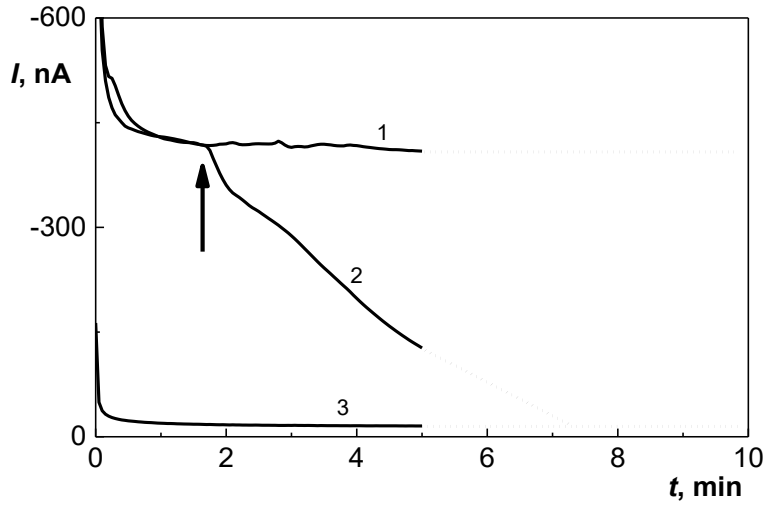


Fig. 3.2 Amperometric recordings of dissolved oxygen obtained in the microcell in 10 μL of a solution on m-AgSAE in BR buffer (pH 12). Potential −0.8 V was applied for 300 s. Dotted lines represent linear extrapolation of measured curves. Curve 1 corresponds to the solution with dissolved oxygen. Curve 2 corresponds to the experiment in which a nitrogen stream was started (represented by the arrow). Curve 3 corresponds to the solution with oxygen removed.

3.1.3.1. Removal of oxygen signal by square wave voltammetry

Oxygen does not need to be physically removed before the voltammetric measurement. Properly optimized SWV method was utilized to lower the response of oxygen in favour of the studied analyte. In this study, SWV was suitable only for the determination in the presence of oxygen in DMSO medium, in which signals of the analyte and oxygen did not overlap significantly [145, 151]. The signal of oxygen in aqueous and mixed media could not be distinguished from the signal of the analyte **8c** by SWV [144].

The most important parameter in SWV optimizing was frequency. It significantly influenced ratio of heights of peaks of analyte (4-nitrophenol and compound **8c**) and oxygen.

Maximum of this ratio was observed at the optimal frequency 100 Hz (from the range 25-300 Hz) for determinations of both 4-nitrophenol and compound **8c**. More noise and higher background were negatively influencing measurements at higher frequencies. Improving signal ratio of analyte and oxygen was not the only effect of SWV. Another effect was a significant decrease of a wide second oxygen signal (superoxide → peroxide) due to the low sensitivity of SWV to irreversible reactions. A fact that removing oxygen was not necessary resulted in the least time-consuming method in addition to SWV scan being very fast (in the order of seconds). Generally, determination by DPV after the removal of oxygen proved to be a bit more sensitive than SWV [144, 145, 151].

Determination of 4-nitrophenol in the drop of a solution in DMSO in the presence of oxygen by SWV significantly improved *LOQ* compared to DPV. *LOQ* was lowered from 20 to 6.0 μmol L^{-1}. However, *LOQ* could be further lowered to 2.4 μmol L^{-1} by using DPV in the microcell after the physical removal of oxygen (**Appx. I**) [145].

Same comparison was carried out for the determination of the compound **8c** in DMSO. Optimized methods of SWV and DPV were compared between themselves in DMSO medium in 10 mL and in a drop of a solution (20 μL) to find out the best conditions for the determination of the compound **8c**. This comparison is illustrated in **Fig. 3.3**. As mentioned before, the signal of oxygen was significantly decreased by using SWV (curve 1) compared to DPV (curve 2) in relation to the analyte. Additionally, this can be illustrated by comparing *LOQ* which is decreased from 60 (DPV) to 7.3 (SWV) μmol L^{-1}. Further decrease to 5.0 μmol L^{-1} can be achieved by DPV in the microcell after physical removal of oxygen [151].

To conclude, the main advantage of using SWV in the microcell is that it is significantly less time-consuming. Main disadvantage is lower sensitivity and higher *LOQ*, and it is also important to mention that SWV calibration dependencies were not linear in the whole studied range [144, 145, 151].

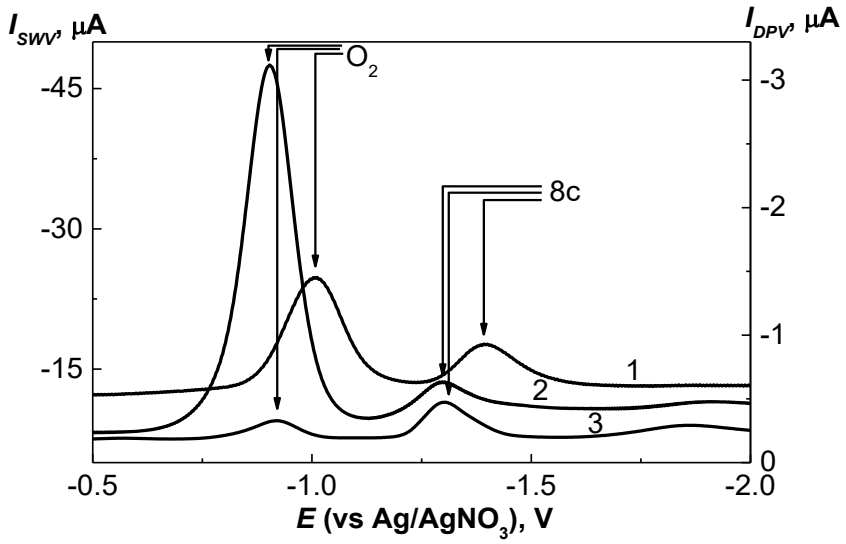


Fig. 3.3 Comparison of SW (1) and DP (2, 3) voltammograms of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide (**8c**) (c = 50 µmol L^{-1}) obtained on GCE in 20 µL of 0.1 mol L^{-1} Bu_4NBF_4 in DMSO. Curves 1 and 2 were measured in the presence of oxygen, curve 3 was measured after 15 mins in the nitrogen atmosphere.

3.1.4. Depletion of analyte in the drop of a solution

Conversion of the studied analyte to products is considered negligible in most of voltammetric experiments with volumes in the range of millilitres. This statement must be reconsidered in relation to the miniaturization. As a result of relatively large electrode surface area (2 mm diameter) that was used for the determination of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide (**8c)**, a significant amount of the analyte was reduced in the drop volume of 20 µL during one voltammetric scan. It was calculated that about 4-5 % of the analyte is irreversibly reduced in the drop of a solution during one CV scan (scan rate 100 mV s^{-1}), therefore, one scan had a significant impact on the concentration in the bulk. The adsorption of products and the passivation of the electrode surface also contributed to the decrease of the response. The percentage of the reduced analyte goes up to approx. 10 % at slower DPV scan (scan rate 20 mV s^{-1}). This was observed in consecutive measurements with the compound **8c** on GCE as a rapid decrease of the peak height in the drop of the solution, as seen in **Fig. 3.4**. Because of the depletion of the analyte, that was the most apparent with low concentrations, a new drop of the solution had to be applied to the microcell before every single measurement [144].

On the contrary, no significant decrease of the signal of the pesticide difenzoquat was observed with the smaller amalgam electrode m-AgSAE (0.5 mm diameter). Therefore, 5 consequent measurements could be performed without any significant decrease of the signal height that were necessary for the concentration dependency [146].

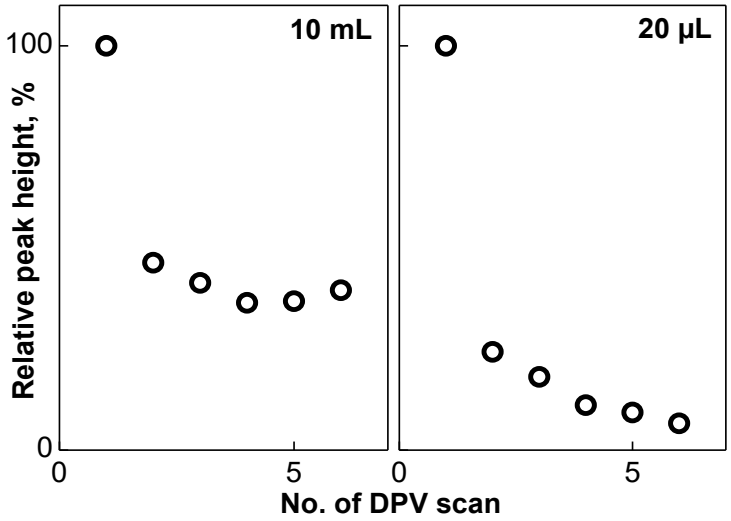


Fig. 3.4 Comparison of relative peak heights (1st measurement is defined as 100%) of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide (c = 2 µmol L^{-1}) on the number of consecutive scans obtained on GCE in 10 mL and 20 µL of BR buffer pH 7 – DMSO (9:1) medium by DPV.

3.1.5. Determination of model compounds difenzoquat and 4-nitrophenol in the microcell

4-nitrophenol was determined as a model compound for the evaluation of the electrochemical microcell. Non-aqueous conditions were based on 0.1M Bu_4NBF_4 solution in DMSO. 4-nitrophenol gave two peaks in this medium, one at −0.95 V and second at −1.9 V which was evaluated for calibration dependencies. 4-nitrophenol was determined by DPV and SWV in the microcell with and without oxygen. Calibration curves are shown in **Appx. I** (**Fig. 4, 5** and **Table 1**) [145].

A pesticide difenzoquat was determined at a novel m-AgSAE which were proved to have similar characteristics to mercury electrodes. Difenzoquat was reduced at a very negative potential at −1.39 V. It was possible to detect difenzoquat at m-AgSAE similarly to mercury electrodes [94-97] thanks to the wide potential window of mercury electrodes. Very low *LOQ*

of approx. 0.4 μmol L^{-1} was obtained in BR buffer pH 12 medium. Similar *LOQ* was also achieved in samples of river water (**Appx. III**) [146]. Much lower *LOQ* was reached in this study compared to the only other study dedicated to the voltammetric determination of DFQ with *LOQ* = 3.2 μmol L^{-1} by HPLC with the voltammetric detection [97]. Additionally, this was the only work where a linear calibration curve was obtained in the purely aqueous medium as the result of the addition of surface-active compound (gelatine). Other works had to use media containing organic solvents [94, 96].

3.2. Electrochemical study of 1-hydroxynaphthalene-2-carboxanilides

Developed electrochemical microcell and miniaturized voltammetric procedures were applied for the study of the model compound **8c** [144] which was a pilot investigation that continued with an electrochemical study of all compounds **1-8c** and their relationship of electrochemical potential, structure and biological activity [152].

3.2.1 Mechanisms of oxidation / reduction

Electrode mechanism was investigated in aprotic solvent DMSO and in the mixed aqueous media consisting of buffer and DMSO (9:1, v/v). Organic solvent had to be always used to some extent due to the insolubility of HNCs in the water. Additionally, DMSO was mostly used in biological activity testing for its low toxicity at low concentrations [104-109, 119, 153, 154]. Reduction and oxidation mechanisms of compound **8c** were studied on GCE in these media.

3.2.1.1 Mechanism of oxidation of phenolic moiety

Mechanism of oxidation was closely studied for the model compound **8c** and unsubstituted compound **1** in BR buffer pH 7.0 and DMSO (9:1) medium. One irreversible diffusion-controlled anodic wave Ia was observed at +0.35 V on GCE. This response should correspond to a process in which a radical is created (**Appx. IV**, **Scheme 2**). In the reverse scan, the peak Ic appeared at +0.22 V corresponding to adsorption processes connected with products of oxidation (**Appx. III**, **Fig. 3**). The potential of oxidation was dependent on pH. More alkaline conditions caused a peak shift towards 0 V until pK_a value of given derivative at which point

the potential of oxidation remained constant. The slope of the pH dependency on the potential was approx. −59 mV pH^{-1}. Clearly, a deprotonation step is a part of the oxidation mechanism and at pH > pK_a all molecules are already in the deprotonated form in a solution. Given this phenomenon, it was possible to experimentally determine pK_a value of compound **8c** as pK_a = 9.7. Additionally, generated radicals can react with components of a solution or with themselves creating polymeric films which contribute to the passivation of the electrode. It was concluded that all the compounds are oxidized by the same mechanism and their oxidation potentials are summarized in **Table 3.1** (**Appx. III**, **IV**) [144, 152].

Table 3.1 Anodic potentials of signals Ia and Ic obtained from CV of compounds **1**-**8c** on GCE in phosphate buffer pH 7.2 and DMSO (9:1, v/v) medium (**Appx. IV**) [152].

Compd	R-	E^a, mV		Compd	R-	E^a, mV	
		Ia	Ic			Ia	Ic
1	H	279	43	**5b**	3-Cl	180	44
2a	2-OCH_3	224	−11	**5c**	4-Cl	143	47
2b	3-OCH_3	240	28	**6a**	2-Br	192	6
2c	4-OCH_3	199	40	**6b**	3-Br	147	29
3a	2-CH_3	207	−7	**6c**	4-Br	185	30
3b	3-CH_3	241	28	**7a**	2-CF_3	104	26
3c	4-CH_3	241	27	**7b**	3-CF_3	195	39
4a	2-F	197	−6	**7c**	4-CF_3	145	55
4b	3-F	196	44	**8a**	2-NO_2	132	33
4c	4-F	182	43	**8b**	3-NO_2	147	44
5a	2-Cl	186	−7	**8c**	4-NO_2	117	57

[a]- Potentials reported vs $[Fe^{II}(CN)_6]^{4-}$

3.2.1.2 Mechanism of reduction of nitro group

Compound **8c** was presumably reduced in two steps in non-aqueous DMSO by similar mechanism as found in the literature (**Chapter 2.4.3.2**). It was at first reversibly reduced at −1.3 V to anion-radical which was then further irreversibly reduced at −2.3 V. It was crucial to

dry the solvent as much as possible because significant amount of water introduced additional peaks in CV [151].

Reduction in aqueous solution was also analogous to the findings in the literature (**Chapter 2.4.3.2**). Mechanism was closely studied in BR buffer pH 7.0 and DMSO (9:1) medium. One irreversible peak IIIc at −0.59 V was observed corresponding to the four-electron reduction to the hydroxylamine (**Appx. III**, **Fig. 3**). This diffusion-controlled process depended on pH. More alkaline medium caused a shift of the peak to more negative values. Additionally, a reversible pair of peaks at approx. −0.10 V appeared in second CV scan which corresponded to the hydroxylamine/nitroso reversible redox process (IVa/IVc). It was presumed that the same redox process was occurring for all nitro-substituted compounds **8a-c** as summarized in the **Table 3.2** (**Appx. III**, **IV**) [144, 152].

Table 3.2 Cathodic potentials of signals IIIc and IVa/IVb obtained from CV of compounds **8a-c** on GCE in phosphate buffer pH 7.2 and DMSO (9:1, v/v) medium (**Appx. IV**) [152].

Compd	R-	E^a, mV	
		IIIc	IVa/IVc
8a	2-NO2	−690	−268
8b	3-NO2	−721	−242
8c	4-NO2	−755	−292

[a]- Potentials reported vs $[Fe^{II}(CN)_6]^{4-}$

3.2.2. Determination of model analyte 8c

Model compound **8c** was determined in mixed media consisting of buffer and DMSO. Dependency of the current response on the concentration was studied for both cathodic and anodic peaks, corresponding to reduction and oxidation of the analyte (**Appx. III**) [144].

Passivation of the surface of GCE was observed as is common in the case of solid electrodes. Electrode gave stable cathodic peaks of reduction of compound **8c** for only about 5 scans (RSD = 7 %, c = 10 μmol L^{-1}). However, electrode had to be polished before every single measurement because of significant drop in the anodic peak current of oxidation of compound **8c** (RSD = 7 %, N = 10, c = 10 μmol L^{-1}). The optimal pre-treatment of GCE was

polishing with aqueous slurry of alumina powder (1.1 μm) on a polishing pad followed by sonication for 30 s in methanol and deionised water.

Determination of HNN was at first optimized in a volume of 10 mL. Optimal conditions for determination of compound **8c** were in a BR buffer (pH 7) – DMSO (9:1) medium. Additionally, this medium was also used for the reason that pH value is similar to most of biological samples which usually have pH around 7. Compound **8c** gave a cathodic DPV peak at −0.55 V and anodic DPV peak at +0.26 V at optimal conditions. *LOQ* = 0.22 μmol L^{-1} was achieved by DPV in the cathodic area and *LOQ* = 1.1 μmol L^{-1} was achieved by DPV in anodic area. Obtained concentration dependencies were linear up to 10 μmol L^{-1}; higher amounts of compound **8c** were already not dissolved in this medium. Significantly lower detection limits were accomplished by applying method of adsorptive stripping voltammetry (AdSV) and decreasing DMSO content down to 1 %. Optimized AdSV gave lowest *LOQ* = 11 nmol L^{-1} using 20 min accumulation time. However, 60-second accumulation time already provided approx. 10-times lower *LOQ*. AdSV was also used to find out the solubility of the compound **8c** in deionized water at 22 (±3) nmol L^{-1}. Application of the method was successfully carried out in bacterial growth medium – DMSO (9:1) (used for photosynthetic electron transport (PET) testing) with *LOQ* 0.23 (cathodic peak) and 2.0 (anodic peak) μmol L^{-1} which is well within the expected concentration range (0.1 – 1000 μmol L^{-1}) (**Appx. III**, **Table 1**).

Voltammetric methods optimized in 10mL volume were used as a baseline for the consequent miniaturization. Miniaturized techniques and microcell were previously described in **Chapter 3.1.** and only specific matters involving the determination of the compound **8c** will be further discussed. The determination of the compound **8c** was carried out in the electrochemical microcell using developed procedures. The removal of oxygen was performed by leaving the drop in the microcell in nitrogen atmosphere for approx. 10 mins during which an adsorption of the analyte on the frit of reference electrode was occurring as described in **Chapter 3.1.3**. Silver wire pseudo-reference electrode was therefore used in the microcell for the determination of the cathodic peak current. The compound **8c** was successfully determined in a drop of sample (20 μL) with *LOQ*s similar to those obtained in 10 mL of a solution (**Appx. III**, **Table 2**). The voltammetric method was also applied for the determination of the compound **8c** in the 20 μL of the bacterial growth medium. Additional problem with miniaturization was encountered in a work with this real matrix. A splitting of a single anodic peak only observed in a drop of a sample was caused by surface-active compounds. Therefore, great attention must be paid to the sample manipulation as even traces

of surface-active compounds from the instrumentation or the environment can influence measurements in a drop of solution which is apparently especially sensitive to these influences (**Appx. III**) [144].

3.2.3. Structure – potential relation

Twenty-two monosubstituted compounds **1**-**8c** listed in **Table 3.1** were investigated by CV on GCE in the phosphate buffer pH 7.2 – DMSO (9:1) medium. Potentials of peaks Ia and Ic were evaluated from cyclic voltammograms. The oxidation peak Ia was used in comparison studies as the peak Ic did not provide good correlations. All the compounds gave an anodic response in a range from ca. +200 to +400 mV. Presumably, all compounds were oxidized by the same mechanism as no obvious irregularities were observed. Substituents (apart from nitro group) did not provide any additional redox reaction under these conditions. All of the requirements for correct construction of Hammett correlation were successfully fulfilled. The studied process Ia was a diffusion-controlled process and a reasonable assumption was made that the slope $dE_{1/2}/dpH$ of the dependency is constant for all studied derivatives. A standard redox marker, $[Fe^{II}(CN)_6]^{4-}$, was additionally used with a Ag|AgCl reference electrode for precise evaluation of the oxidation potential.

It was possible to establish a correlation between structure (Hammett σ substituent constants) and the oxidation potential of peak Ia. Obtained dependencies of the anodic potential and substituent constants can be seen in **Appx. IV**, **Fig. 2**. Compounds **1**-**8c** were divided into three subgroups (*ortho*-, *meta*-, *para*-) based on the position of substituent on the benzene ring. Substituents influenced the oxidation potential of naphtholic moiety via their electron-withdrawing (-NO_2, -CF_3, -Br, -Cl, -F groups) or electron-donating effects (-CH_3, -OCH_3 groups) as is illustrated on **Fig. 3.5**. Electron-donating groups were causing a shift of the oxidation potential to more positive values. On the other hand, electron-withdrawing groups were causing a shift to 0 V. A pronounced shift towards 0 V was observed with strong electron-withdrawing groups e.g. -NO_2, -CF_3. Based on these findings, it was confirmed that electron-withdrawing and donating effects at benzene ring are transmitted through –CO–NH– bridge to naphthalene ring. All of compounds **1**-**8c** gave good correlations ($r \sim 0.88$) in their respective series (*ortho*-, *meta*-, *para*-). Unsubstituted compound **1** gave the most positive oxidation potential and it had to be excluded from *ortho*- and *para*- series as an outlier. This can be

attributed to steric and adsorption effects that can cause some deviations from an ideal correlation (**Appx. IV**) [152].

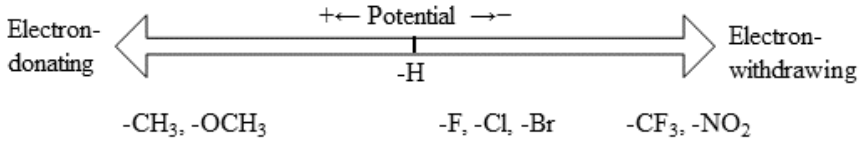


Fig. 3.5 General theoretical dependency of electron-donating/withdrawing effect on the oxidation potential of derivatives **1**-**8c**.

Reduction of three nitro-substituted compounds **8a**-**c** was facilitated in a series *para* → *meta* → *ortho*. *Para*-substituted compound **8c** gave the most negative reduction potential. Unfortunately, compounds **8a**-**c** did not show any antimycobacterial activity and therefore the relation between the reduction potential and the biological activity could not have been investigated (**Appx. IV**) [152].

3.2.4. Biological activity – potential relation

The *in vitro* antimycobacterial activity of compounds **1**-**8c** was investigated against *Mycobacterium marinum* CAMP 5644, *M. kansasii* DSM 44162, *M. smegmatis* ATCC 700084 in Gonec et al. [104], and *M. tuberculosis* ATCC 25177/H37Ra in **Appx. IV** [152]. Values of antimycobacterial activity were expressed as minimum inhibitory concentrations (MIC) which is the lowest concentration at which no visible bacterial growth is observed. Studied compounds were also evaluated for their ability to inhibit photosynthetic electron transport (PET test) in spinach (*Spinacia oleracea* L.) chloroplasts and were expressed as IC_{50} values [104]. All these data were collected in **Appx. IV**, **Table 1**.

Strain *M. tuberculosis* ATCC 25177/H37Ra has a similar pathology to *M. tuberculosis* affecting humans [155]. This strain of *M. tuberculosis* and strain of *M. marinum* are commonly used pathogens in basic laboratory screening [104]. *M. kansasii* is a representative of non-tuberculous mycobacteria causing lung infections [156]. These 3 strains are considered slow growing. Therefore, *M. smegmatis* was used as a representative of fast-growing non-pathogenic bacteria that can be used for an investigation of basic cellular processes [104, 156].

Physicochemical properties and binding strength of the test compounds to the potential target is presumably influenced via the anilide part of the molecule. Furthermore, an increase or decrease of electron density in the amide bond, caused by variously substituted anilide rings, has an effect on acidity/oxidizability of a spatially close phenolic moiety. This results in the wide spectrum of activity and potency of HNCs. Electrochemical methods can be therefore used to investigate these fine changes of electron density or oxidizability of phenolic moiety. A change of an oxidation potential could indicate a different structure and possibly even a different potency and activity (**Appx. IV**) [152].

Investigation of relationships between antimycobacterial activities and anodic potentials was only limited to active compounds (MIC < 100 μmol L^{-1}). Dependencies of the antimycobacterial activity against *Mycobacteria* strains (expressed as log(1/MIC)) and PET inhibition (expressed as $\log(1/IC_{50})$) on the oxidation potential can be seen in **Appx. IV**, **Fig. 3-5**. Accordingly, it was possible to identify quasi-parabolic and linear correlations with acceptable correlation coefficients ($r \sim 0.7 - 0.8$) for some subsets of studied compounds **1-8c**. These fits had a maximum in a range approx. +180 – +195 mV. Additionally, the oxidation potential for the most active compounds **3b** (R = 3-CH_3) and **4b** (R = 3-F) was slightly more positive (+196 mV and +241 mV, respectively). In conclusion, antimycobacterial activity should be positively influenced by higher oxidation potential. It was proved that the oxidation potential can be used as one of many parameters to help design and investigate novel HNCs. However, as mentioned earlier, a lot of other factors and physicochemical properties (e.g. lipophilicity [104]) are crucial for the drug effectivity (**Appx. IV**) [152].

4. CONCLUSIONS

Submitted Ph.D. thesis is a contribution to the efforts of miniaturization of analytical methods and a contribution to the connection of electrochemistry and biochemistry / pharmacology.

The novel electrochemical microcell, sample manipulation procedures and modified voltammetric procedures were introduced. Sample volumes as low as 10 μL were necessary for one analysis. The construction of the microcell was carried out with three electrodes: working electrode (GCE, m-AgSAE), reference electrode (non-aqueous Ag|0.01M $AgNO_3$, 0.1M Bu_4NBF_4 in DMSO; aqueous Ag|AgCl|3M KCl; silver wire pseudo-reference electrode) and auxiliary electrode (Pt wire). The microcell consisting only from these electrodes was sufficient for measurements in an anodic area. Voltammetry in a cathodic area required special procedures for the oxygen removal. This can be achieved either by a physical removal of oxygen by displacing it with nitrogen in a modified microcell that is enclosed from outside atmosphere, or optimized method of SWV can be used even in the presence of oxygen. SWV offers much faster analysis, however, it decreases sensitivity and increases *LOQ*. It is also important to note that particular conditions must be met to use SWV for this purpose as was proved by unsuccessful testing of SWV in aqueous media. It is also crucial to keep in mind that working with a large electrode in small volume can cause a significant depletion of an analyte in a bulk of a solution. The proposed microcell was used to determine 4-nitrophenol in the pilot study. Furthermore, the microcell based on the amalgam sensor was used to determine pesticide difenzoquat. Low *LOQ* = 0.4 μmol L^{-1} was achieved even in the model river water sample determination. This was a significant improvement over other electroanalytical methods used for the determination of the pesticide which also always used media with organic solvents. Main advantages of the proposed electrochemical microcell are:

a) significant decrease of chemicals and solvent consumption that is within the framework of green analytical chemistry.
b) possibility of work with biological or environmental samples that cannot be obtained in big quantities.
c) possibility to significantly improve preliminary separation and preconcentration by extraction into microliter volumes.

Electrochemical study of HNCs was initiated by an investigation of the model analyte **8c**. This compound contained reducible nitro group and oxidizable hydroxyl group. The analyte was determined in buffer – DMSO solutions with pH around 7. Mechanisms of electrode redox processes were proposed based on the literature and experimentally confirmed. Compound **8c** gave one cathodic and one anodic response on GCE in the studied medium. DPV provided *LOQ* as low as 0.22 μmol L^{-1} that can be even more decreased to 11 nmol L^{-1} by utilizing AdSV. Voltammetric determination was applied in a bacterial growth medium as a real matrix. Determination in the proposed electrochemical microcell in a drop of a solution gave approximately the same parameters (sensitivity, *LOQ*s).

Subsequently, twenty-two ring-substituted HNCs were measured by CV on GCE. All of the compounds contain an oxidizable phenolic moiety and they gave similar CVs. The only difference was a change of value of the oxidation potential caused by the nature and the position of the substituent. Correlations of the structure (Hammett σ substituent constant) and oxidation potentials were constructed for subsets based on the position of the substituent. Obtained fits gave satisfactory correlations ($r \sim 0.88$). Correlations of antimycobacterial and PET inhibiting activity on the oxidation potential were more difficult to evaluate. It was possible to observe some trends in the experimental data for subsets. These trends must be interpreted with caution as a direct correlation between electrochemical and pharmacological data must not be expected.

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