CHARLES UNIVERSITY IN PRAGUE

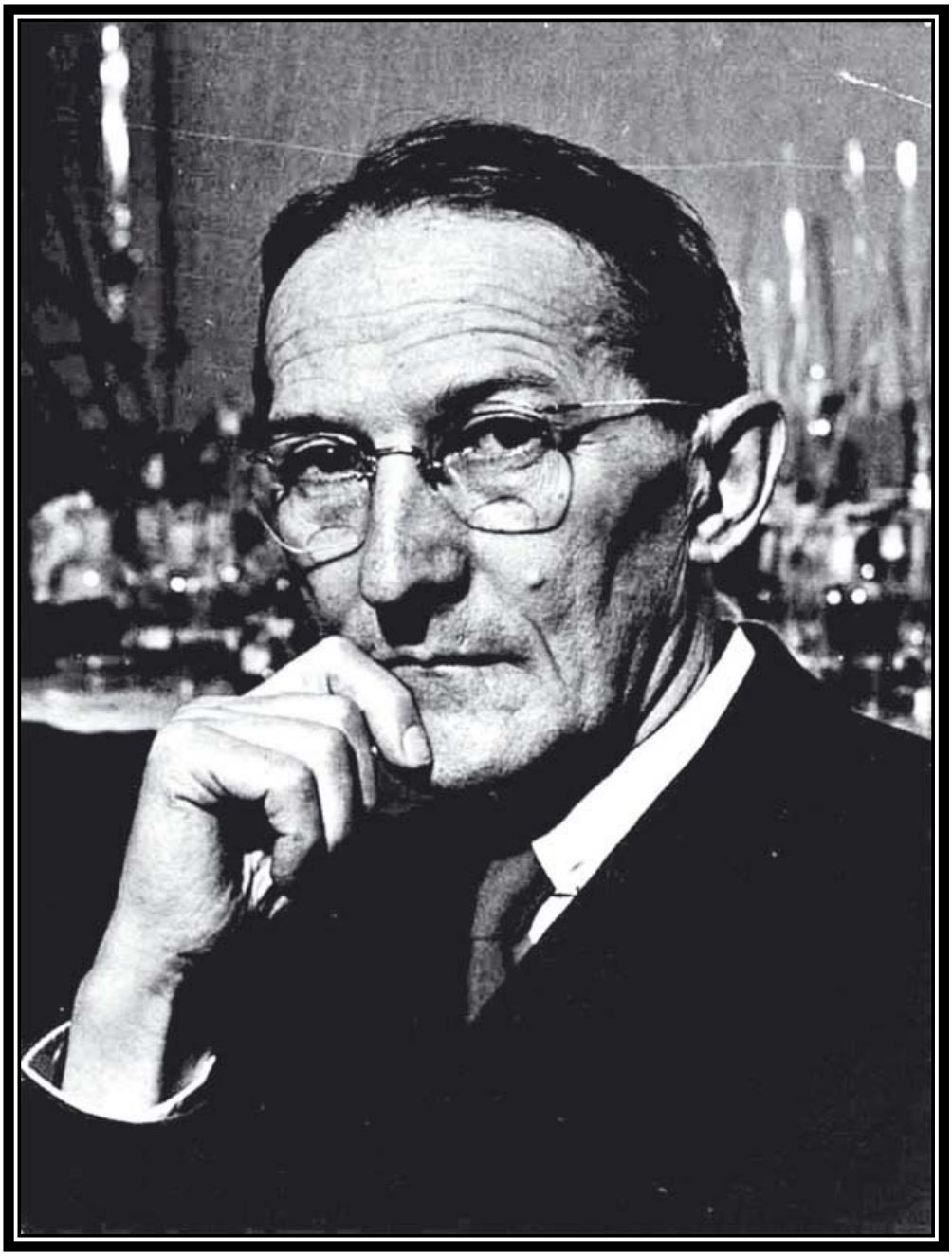
FACULTY OF SCIENCE

Department of Analytical Chemistry

NEW ELECTROCHEMICAL METHODS FOR DETERMINATION OF NITRO AND OXO DERIVATIVES OF FLUORENE

A Thesis Submitted as the Basis for the Award of the PhD Degree

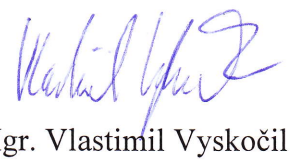
Prague 2010 **Vlastimil Vyskočil**



Dedicated to the memory of Professor Jaroslav Heyrovský
on the occasion of 50th Anniversary
of the Nobel Prize for polarography.

I declare that all the results which are used and published in this Thesis have been obtained by my own experimental work and that all the ideas taken from work of others are properly referred to in the text and the literature survey. I am conscious that the prospective use of the results, published in this Thesis, outside the Charles University in Prague is possible only with a written agreement of this university.

I also declare that neither this 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, 5. 3. 2010

Mgr. Vlastimil Vyskočil

This dissertation is based on experiments carried-out in the period from 2005 till 2009 at the Charles University in Prague, Faculty of Science, Department of Analytical Chemistry. During this period, the research visit to Professor Ján Labuda from Slovak University of Technology in Bratislava, Faculty of Chemical and Food Technology, Institute of Analytical Chemistry was completed. Further experiments were done at J. Heyrovský Institute of Physical Chemistry, v.v.i. of the Academy of Sciences of the Czech Republic.

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List of Symbols and Abbreviations

2-NF	2-nitrofluorene
2-NFN	2-nitro-9-fluorenone
2,7-DNF	2,7-dinitrofluorene
2,7-DNFN	2,7-dinitro-9-fluorenone
9-FN	9-fluorenone
α	significance level
Δn	amount of electrochemically reduced substance
A	adenine
AcB	acetate buffer
ACP	alternating current polarography
AdSCP	adsorptive stripping chronopotentiometry
AdSDCV	adsorptive stripping direct current voltammetry
AdSDPV	adsorptive stripping differential pulse voltammetry
AdSV	adsorptive stripping voltammetry
Ar	aryl
BR	Britton-Robinson
c	molar concentration
CAS	Chemical Abstract Service
CDMCPE	cyclodextrine modified carbon paste electrode
CDPA-SPE	carboxymethylated cyclodextrine condensation polymer modified carbon-based screen printed electrode
CDP-SPE	cyclodextrine condensation polymer modified carbon-based screen printed electrode
CMCPE	chemically modified carbon paste electrode
CPC	constant-potential coulometry
CPE	carbon paste electrode
CV	cyclic voltammetry
DC	direct current
DCP	direct current polarography
DCTP	direct current tast polarography
DCV	direct current voltammetry
DME	dropping mercury electrode
DNA	deoxyribonucleic acid
DNA/SPCPE	double-stranded DNA modified screen printed carbon paste electrode

DP	differential pulse
DPP	differential pulse polarography
DPV	differential pulse voltammetry
dsDNA	double-stranded deoxyribonucleic acid
DW	drinking water
$E_{½}$	half-wave potential
$E_{1,\mathrm{reg}}$ (E^{1}_{reg} or E_{ini})	more positive regeneration potential
$E_{2,\mathrm{reg}}$ (E^{2}_{reg} or E_{fin})	more negative regeneration potential
E_{acc}	accumulation potential
E_{amp}	pulse amplitude
ED	electrochemical detector
EDTA	ethylenediaminetetraacetic acid
E_{E}	electrolysis potential
E_{p}	peak potential
E_{S}	switching potential
E_{step}	potential step
F	Faraday constant
G	guanine
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GCPE	glassy carbon paste electrode
GCE	glassy carbon electrode
HMDE	hanging mercury drop electrode
HPLC	high performance liquid chromatography
HPLC-ED	high performance liquid chromatography with electrochemical detection
IARC	International Agency for Research on Cancer
I_{lim}	limiting diffusion current
I_{p}	peak current
IUPAC	International Union of Pure and Applied Chemistry
L (or l)	liter (dm^{-3})
LLE	liquid-liquid extraction
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of determination
L_{Q}	limit of quantification
LSSV	linear sweep stripping voltammetry
M	mol L^{-1} (mol dm^{-3})
m-AgSAE	mercury meniscus modified silver solid amalgam electrode

MeOH	methanol
MFE	mercury film electrode
MFE-Au	mercury film electrode at gold substrate
MFE-CG	mercury film electrode at glassy carbon substrate
MS	mass spectrometric detection
MPE	mercury pool electrode
n	number of measurements
NPAHs	nitro derivatives of polycyclic aromatic hydrocarbons
OG	8-oxoguanine
OPAHs	oxo derivatives of polycyclic aromatic hydrocarbons
oxy-NPAHs	oxygenated nitro derivatives of polycyclic aromatic hydrocarbons
oxy-PAHs	oxygenated polycyclic aromatic hydrocarbons
p-AgSACE	polished silver solid amalgam composite electrode
p-AgSAE	polished silver solid amalgam electrode
PAHs	polycyclic aromatic hydrocarbons
pH	negative of the base-10 logarithm of the activity of hydronium ions
pH^*	resulting pH of the aqueous-methanolic solution measured using combined glass electrode
phen	1,10-phenanthroline
p	peak
PBS	phosphate buffer solution
R	correlation coefficient
RSD	relative standard deviation
RW	river water
Q	electric charge
SMDE	static mercury drop electrode
SPCPE	screen printed carbon paste electrode
SPE	solid phase extraction
SWV	square wave voltammetry
t_{acc}	accumulation time
Tris	tris(hydroxymethyl)aminomethane
UNESCO	United Nations Educational, Scientific and Cultural Organization
UV	ultraviolet part of the spectrum
VIS (or Vis)	visible part of the spectrum
v	scan rate
z	number of exchanged electrons

Contents

1. INTRODUCTION

This dissertation has been submitted as a contribution to the ever growing efforts of environmental analysis. It was elaborated under the framework of a long term research at UNESCO Laboratory of Environmental Electrochemistry in Prague to develop highly sensitive and selective methods for determination of polycyclic aromatic hydrocarbons (PAHs) and their nitro, amino, oxo, and hydroxy derivatives. **The Thesis presented is based on following six scientific publications [1-6] which are attached as Appendix parts I – VI (Chapters 6 – 11). To distinguish the references referring to these publications in entire text of this Thesis, corresponding numbers in square brackets are in bold and underlined.**

PAHs and their derivatives constitute a class of biologically active organic pollutants generated in a variety of chemical processes. PAHs are ubiquitous in the environment, their higher occurrence in urban and industrial areas is given by the fact that they are formed during incomplete combustion, thus they are present in automobile exhausts and industrial exhalations [7,8]. They are released to atmosphere preferably associated with submicron-size particles. When released into the atmosphere, PAHs are exposed to a variety of gaseous compounds, including stable molecules and highly reactive intermediates as free radicals or electronically excited species, originated from absorption of radiation. These interactions result in an introduction of functional groups to the aromatic system of the parent molecule. The polar fractions of the aerosols were found to be highly complex, containing several hundred PAH derivatives, including compounds with hydroxy, nitro, oxo, ketone, aldehyde, and carboxylic acid substituents [9]. These derivatives may have even higher mutagenicity compared to non-substituted PAHs, thus the analysis of atmospheric aerosols and other environmental samples is of great concern [10].

Fluorene (it has a violet fluorescence, hence its name) is one of the simplest and most volatile of all PAHs, worldwide present in various part of environment (air, soil, water) and in

a variety of workplaces [11-14]. Fluorene has posed a great problem for environmental and occupational medicine, because significant carcinogenic and genotoxic effects have been associated with fluorene exposure [15-17]. To evaluate the exposure of humans to fluorene, the urinary concentrations of its metabolites – hydroxyfluorenes – in the general population and in subjects occupationally exposed to fluorene are monitored [18,19]. Nitrofluorenes, fluorenones and nitrofluorenones are the most abundant nitro/oxo derivatives of PAHs in urban air, their health risk is causatively connected to the generation of active species binding to DNA, which are responsible for their mutagenicity [20].

Owing to the extremely wide range of matrices, where PAHs and their derivatives can be present, the often small amounts of individual compounds and a myriad of interfering substances present in, analytical methods are required which are not only extremely sensitive, but can also provide great selectivity [21,22].

With respect to above mentioned facts, the aim of the Thesis was the development of sensitive electroanalytical methods for the determination of selected genotoxic nitro and oxo derivatives of fluorene, namely **2-nitrofluorene** (2-NF), **2,7-dinitrofluorene** (2,7-DNF), **9-fluorenone** (9-FN), **2-nitro-9-fluorenone** (2-NFN), and **2,7-dinitro-9-fluorenone** (2,7-DNFN). The main attention was paid to nitrofluorenes and nitrofluorenones, because these compounds are polarographically reducible, thus a variety of electroanalytical methods at classical electrode materials as mercury and non-traditional materials as silver solid amalgams can be used for their determination [**1,2**]. The applicability of different electroanalytical detection techniques was studied in order to obtain low limits of quantification for studied derivatives [**3-5**]; they are widely extended in the environment, thus the development of new methods for their sensitive determination is of great concern. The mechanisms of electrode reactions were investigated to provide overall information about electrochemical behavior of studied compounds at mercury and silver solid amalgam electrodes [**3-5**]. Interactions of calf thymus double-stranded DNA with 2-NF and 2,7-DNF at the double-stranded DNA modified screen printed carbon paste electrodes (DNA/SPCPEs) were investigated and DNA damages have been studied [**6**]; this study represents simple electroanalytical methodology and shows the potential of the disposable DNA/SPCPE biosensor for the investigation of genotoxic effects of chemical compounds of environmental and health interest.

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2. NITRO AND OXO DERIVATIVES OF FLUORENE IN ENVIRONMENT

2.1 Sources, Formation, Occurrence and Biological Effects

Polycyclic aromatic hydrocarbons (PAHs) are well-known environmental chemical carcinogens [1]. These contaminants may be discharged on to soil and water bodies thereby leading to various deleterious consequences on the natural ecosystem. It is more than two decades since it was discovered that PAHs could undergo atmospheric reactions with nitrogen oxides and/or ozone to form nitro and/or oxo derivatives [2,3].

Nitrated polycyclic aromatic hydrocarbons (NPAHs), which are causally connected with increasing risk of cancer [4], are among frequently monitored environmental pollutants. Their mutagenicity and carcinogenity was firstly reported in 1977 [5] and, one year later, Jäger [3] and Pitts [6] independently discovered that they are formed during incomplete combustion processes by reaction of PAHs with atmospheric nitrogen oxides or formed from their parent PAHs by atmospheric OH or NO_X radical initiated reactions [7]. The increasing quantity of NPAHs in atmosphere, soils and waters is thus connected mostly with emissions of gasoline and diesel engines [7]. Oxygenated PAH compounds (oxy-PAHs) such as aromatic ketones, aromatic aldheydes, quinones, and carboxylic acids have also been identified in various environmental samples [8-12]. Oxygenated NPAHs (oxy-NPAHs) are mainly emitted from combustion processes as well. However, they are also produced by heterogeneous reactions of particulate associated NPAHs with ozone [11] or as the metabolites of PAHs and/or NPAHs bacterial and fungal degradation [13,14]. The presence of NPAHs, oxy-PAHs and oxy-NPAHs in environment threatens natural biological functions of ecosystem and healthy organism growth [15].

Two nitro derivatives of fluorene belonging to the group of NPAHs, namely **2-nitrofluorene** (2-NF) and **2,7-dinitrofluorene** (2,7-DNF), two oxy-NPAHs derived from

9-fluorenone, namely **2-nitro-9-fluorenone** (2-NFN) and **2,7-dinitro-9-fluorenone** (2,7-DNFN), and oxy-PAH **9-fluorenone** (9-FN) (see Fig. 2-1) are investigated in this Thesis.

It has been shown that mononitro and dinitro PAHs and/or oxy-NPAHs can be many times more mutagenic and/or carcinogenic than their parent PAHs [7]. In the eighties and the nineties of the last century, Möller studied a negative influence of NPAHs on living organisms and their DNA [16]. Carcinogenic 2-NF was chosen as a NPAHs model marker [17] and lately, the genotoxic effect of structurally similar compounds, e.g. of 2,7-DNF, was studied as well [18,19]. Nowadays, 2-NF is proven to be carcinogenic to rats and thus possibly carcinogenic to humans (IARC class 2B) [20], genotoxic and tumorigenic 2,7-DNF is known as a potential carcinogen [21,22]. Remaining two genotoxic oxy-NPAHs (i.e., 2-NFN and 2,7-DNFN) act in living environment like unique mutagens formed in the photo-oxidation of their amino analogues [23,24]. Finally, 9-FN has been shown to be toxic in several bioassays [25-30] and has carcinogenic [31] and mutagenic potential [32].

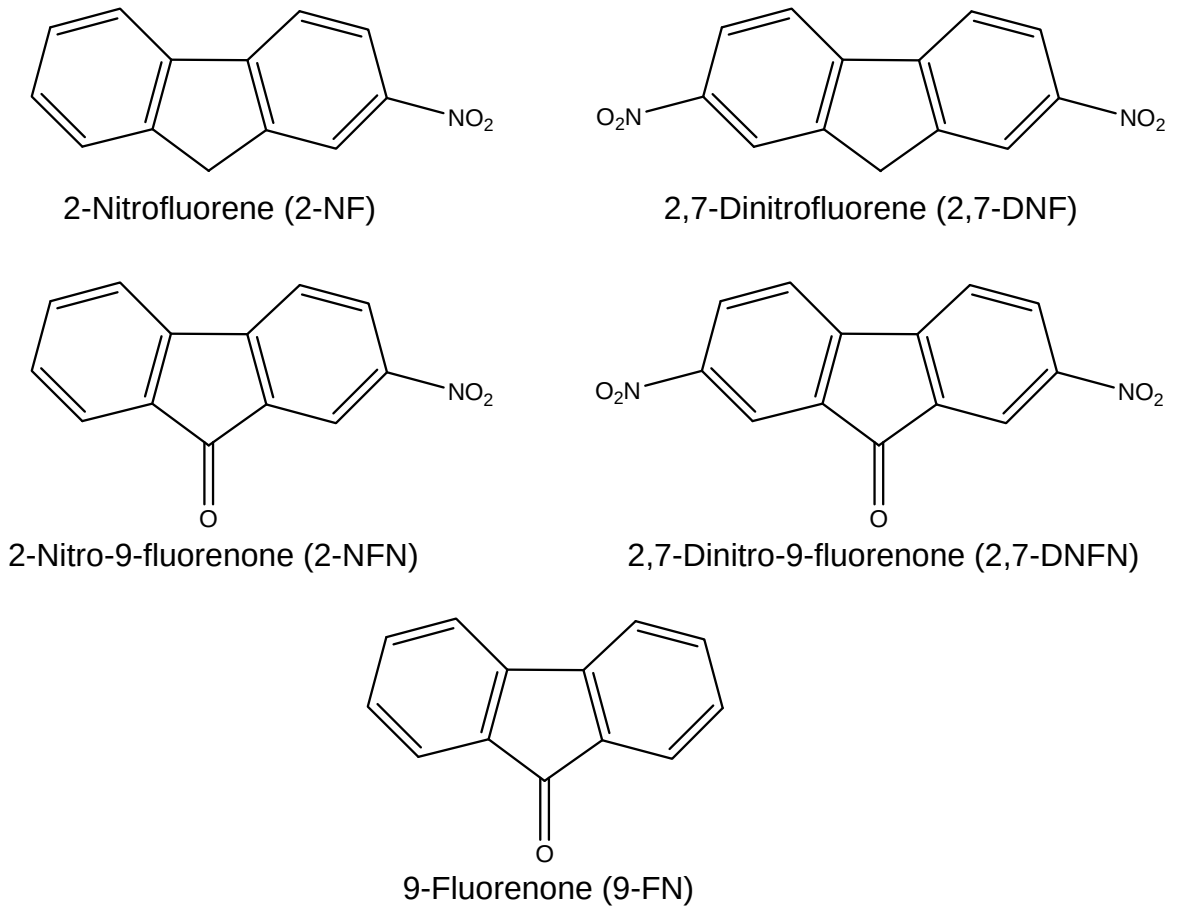

Fig. 2-1 Structural formulas of investigated compounds.

2.2 Analytical Methods for Determination of Nitro and Oxo Derivatives of Fluorene

Because of ubiquitous presence of mentioned derivatives in environment and their adverse health effects, new analytical methods are developed for their determination. Owing to the extremely wide range of matrices, low concentration of individual compounds and a myriad of interfering substances, analytical methods are required which are not only extremely sensitive, but can also provide great selectivity. Many chromatographic techniques have been brought to cope with the separation and quantification of solutes in such complex samples [7,33-35]. Although gas chromatography with selective detectors is widely used [11,13], it is rather limited to the low polarity and relatively high volatility compounds. Reversed phase HPLC with a wide range of detection techniques becomes an option to be considered for its ability to resolve lower volatile, thermally unstable, polar compounds with high sensitivity of detection [36,37].

Recent advancements in analytical equipment, in conjunction with more affordable prices, have contributed to the development of more sensitive and selective methods for analyses of nitro and oxo derivatives of fluorene in environmental samples. Although the traditional GC-MS techniques are still being used [38,39], they are being improved continuously by the introduction of more selective stationary phases for capillary columns, more sensitive and selective mass spectrometric ionization techniques, and more advanced MS analysis methods (such as tandem MS/MS). HPLC coupled to MS seems to be a particularly promising new analytical method for analysis of mentioned fluorene derivatives, since it allows for less labor-intensive clean-up of environmental samples while still providing the structural information for an unknown compound. The further development of LC-MS methods and improvement in sensitivity of the new commercial LC-MS instruments, will probably contribute to the broader application of this method in analysis of nitrofluorenes and nitrofluorenones [40,41]. Although the sensitivity of techniques such as HPLC with fluorescence [12] or chemiluminescence detection [42-44] appears to be appropriate for examination of relatively pristine environmental samples, constraints on selectivity should be considered.

Electroanalytical methods represent a useful alternative to more frequent chromatographic methods. They meet the stringent conditions on sensitivity, although the selectivity is often presented as a weak point in analysis of complex matrices. However, the selectivity can be increased by application of preliminary separation step, or it is sufficient in the case where electroanalytical methods are used to estimate the sum of analytes or as an

inexpensive screening methods monitoring the presence of derivatives of interest in a complex matrix [45,46]. Moreover, the study of polarographic and voltammetric behavior of biologically active organic compounds can provide a great amount of information about their electron-transfer reactions. This can be useful in elucidation of the mechanism of their interaction with living cells, because both electrochemical and biological reactions are essentially heterogenous processes occurring at the electrode-solution or enzyme-solution interface [47].

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3. ELECTROCHEMICAL TECHNIQUES AND WORKING ELECTRODES

3.1 Polarography/Voltammetry and Potentiostatic Coulometry

Historically, the branch of electrochemistry we now call voltammetry developed from the discovery of polarography in 1922 by the Czech chemist Jaroslav Heyrovský, for which he received the 1959 Nobel Prize in chemistry. The early voltammetric methods experienced a number of difficulties, making them less than ideal for routine analytical use. However, in the 1960s and 1970s significant advances were made in all areas of voltammetry (theory, methodology, and instrumentation), which enhanced the sensitivity and expanded the repertoire of analytical methods. The coincidence of these advances with the advent of low-cost operational amplifiers also facilitated the rapid commercial development of relatively inexpensive instrumentation.

The common characteristic of all polarographic/voltammetric techniques is that they involve the application of a potential to an electrode and the monitoring of the resulting current flowing through the electrochemical cell. They are considered active techniques (as opposed to passive techniques such as potentiometry) because the applied potential forces a change in the concentration of an electroactive species at the electrode surface by electrochemical reduction or oxidation.

The analytical advantages of the various polarographic/voltammetric techniques include excellent sensitivity with a very large useful linear concentration range for both inorganic and organic species (10^{-12} to 10^{-1} mol L^{-1}), a large number of useful solvents and electrolytes, a wide range of temperatures, rapid analysis times (seconds), simultaneous determination of several analytes, the ability to determine kinetic and mechanistic parameters, a well-developed theory and thus the ability to reasonably estimate the values of unknown parameters, and the ease with which different potential waveforms can be generated and small currents measured.

Analytical chemists routinely use polarographic/voltammetric techniques for determination of a variety of dissolved inorganic and organic substances. Inorganic, physical, and biological chemists widely use polarographic/voltammetric techniques for a variety of purposes, including fundamental studies of oxidation and reduction processes in various media, adsorption processes on surfaces, electron transfer and reaction mechanisms, kinetics of electron transfer processes, and transport, speciation, and thermodynamic properties of solvated species. Voltammetric methods are also applied to the determination of compounds of pharmaceutical interest and, when coupled with HPLC, they are effective tools for the analysis of complex mixtures [1].

The electrochemical cell, where the polarographic/voltammetric experiment is carried out, consists of a working (indicator) electrode, a reference electrode, and usually a counter (auxiliary) electrode. In general, working electrode provides the interface across which a charge can be transferred or its effects felt. Because the working electrode is where the reaction or transfer of interest is taking place, whenever we refer to the electrode, we always mean the working electrode. The reduction or oxidation of a substance at the surface of a working electrode, at the appropriate applied potential, results in the mass transport of new material to the electrode surface and the generation of a current. Even though the various types of polarographic/voltammetric techniques may appear to be very different at first glance, their fundamental principles and applications derive from the same electrochemical theory [2].

To provide general information about most frequently used polarographic (even though polarography could be considered just form of voltammetry, it differs from other voltammetric methods both because of its unique place in the history of electrochemistry and in respect to its unique working electrode, the dropping mercury electrode) and voltammetric techniques in the field of environmental analysis, the Appendix I [**3**] is attached. Following polarographic/voltammetric techniques have been used in this Thesis: **DC polarography** (DCP), **DC tast polarography** (DCTP), **differential pulse polarography** (DPP), **DC voltammetry** (DCV), **differential pulse voltammetry** (DPV), **square wave voltammetry** (SWV), **cyclic voltammetry** (CV), and **adsorptive stripping differential pulse voltammetry** (AdSDPV).

The last-mentioned technique belongs to the most sensitive and frequently used analytical methods; however, its use in environmental analysis is limited because it is less robust and more prone to interferences from surface active substances and other compounds likely to be present in environmental matrices. Thus, it should be used mainly for analysis of

relatively clean samples (e.g., of drinking water) or of samples after preliminary clean-up or separation. The Appendix II [**4**] describes our recent results regarding adsorptive stripping voltammetric (AdSV) determination of submicromolar and nanomolar concentrations of various environmentally important chemical carcinogens using both traditional (hanging mercury drop electrode, carbon paste electrode) and non-traditional types of electrodes (solid amalgam electrodes, glassy carbon paste electrodes, carbon ink film electrodes, solid composite electrodes) and concentrates on our own results in the context of the general development in the filed.

Cyclic voltammetry is practically not used for the trace analysis (DCV derived from CV is used predominantly), nevertheless, it is widely used for the study of redox processes, for understanding reaction intermediates, and for investigation of reaction products. This technique is based on varying the applied potential at a working electrode in both forward and reverse directions (at some scan rate) while monitoring the current. For example, the initial scan could be in the negative direction to the switching potential. At that point the scan would be reversed and run in the positive direction. Depending on the analysis, one full cycle, a partial cycle, or a series of cycles can be performed.

Potentiostatic coulometry (constant-potential coulometry; CPC) involves keeping the electrochemical potential at the working electrode constant, which results in an exponential decrease in current as the reaction proceeds. Maintaining the constant potential at the working electrode allows for the component of interest to react without the involvement of other components in the sample. In CPC, the total number of coulombs flowing through the system is measured. CPC, together with CV, are thus widely used for elucidation of the mechanism of electrochemical redox reactions [1].

3.2 Working Electrodes Used

A typical electrochemical cell consists of the sample dissolved in a solvent, an ionic electrolyte, and three (or sometimes two) electrodes. The reference electrode should provide a reversible half-reaction with Nernstian behavior, keep constant potential over time, and be easy to assemble and maintain; the most commonly used reference electrodes for aqueous solutions are the calomel electrode and the silver/silver chloride electrode. Counter electrode (also called an auxiliary electrode) is used to make a connection to the electrolyte so that a current can be applied to the working electrode; the counter electrode is usually made of an

inert material, such as a noble metal or graphite, to keep it from dissolving. The working electrodes are of various geometries and materials, ranging from small mercury drops to flat platinum disks. Ideally, the electrode should provide a high signal-to-noise ratio as well as a reproducible response. Mercury is useful because it displays a wide negative potential range (because it is difficult to reduce hydrogen ion or water at the mercury surface), its surface is readily regenerated by producing a new drop or film, and many metal ions can be reversibly reduced into it. Other commonly used electrode materials are e.g. gold, platinum, and glassy carbon.

The most common polarographic working electrode is the **classical dropping mercury electrode** (DME) [5]. The DME consists of a glass capillary through which mercury flows under gravity to form a succession of mercury drops. Each new drop provides a clean surface at which the redox process takes place, giving rise to a current increase with increasing area as the drop grows, and then falling when the drop falls.

The main disadvantages of DME (high consumption of mercury and higher charging current) are eliminated by using **hanging mercury drop electrode** (HMDE) as the most frequently used mercury electrode with high reproducibility, low consumption of mercury, and the possibility of adsorptive or electrolytic accumulation of analytes on its surface. Possibilities and limitations, advantages and disadvantages, and the applications of mercury electrodes in environmental electroanalysis are critically reviewed in [**3**] (Recent developments in the use of polarography and voltammetry at mercury electrodes in environmental analysis have been reviewed and their combination with preliminary separation and preconcentration using liquid or solid phase extraction has been discussed. Attention has been focused on ecotoxic NPAHs, heterocyclic compounds, and pesticides. Advantages and limitations of mercury electrodes have been critically evaluated and some recent applications of these techniques developed in our laboratory have been given).

There is no doubt that mercury is the best electrode material because of easily renewable and atomically smooth surface and large cathodic window [**3**]. However, there is a tendency to avoid the use of mercury because of unsubstantiated fears of its toxicity and because of its low mechanical stability complicating the use of mercury electrodes in flowing systems and in portable devices. It was clearly shown that amalgam electrodes can, in many cases, successfully substitute mercury electrodes [6]. Amalgam can be liquid, paste, or solid depending on mercury-metal ratio. **Mercury meniscus modified silver solid amalgam electrode** (m-AgSAE; see Fig. 3-1) [7] represent suitable non-toxic replacement for traditional mercury electrodes; it should be stressed that mercury meniscus prepared at the surface of

solid amalgam is rapidly converted into saturated amalgam of the metal contained in electrode material. Good mechanical stability, simple handling and regeneration including an electrochemical pretreatment of electrode surface are among the main advantages of m-AgSAE. In absence of specific interactions between the analyte and silver from silver solid amalgam, the DPV peak potentials on m-AgSAE and HMDE are nearly the same [8]. By immersing a polished silver solid amalgam electrode (p-AgSAE) into liquid mercury, a mercury meniscus is formed at its surface which is not visually changed for several months. However, it is recommended to repeat the amalgamation once per week to prevent deterioration of sensitivity or reproducibility. Then, the electrode is rinsed with water and checked by means of a lens to determine whether a meniscus of mercury has been formed (see Fig. 3-1). The m-AgSAE is the most reliable and most similar to the HMDE so that most analytical applications published are using this electrode [6]. The above-described procedure for meniscus forming is simple and fast. However, it does not guarantee that the new meniscus will give the same response as the previous one, which is partially connected with possible passivation of old meniscus and partially with a different area of the new meniscus. The problem of electrode reproducibility is addressed (as with many other solid electrodes) by standard addition technique.

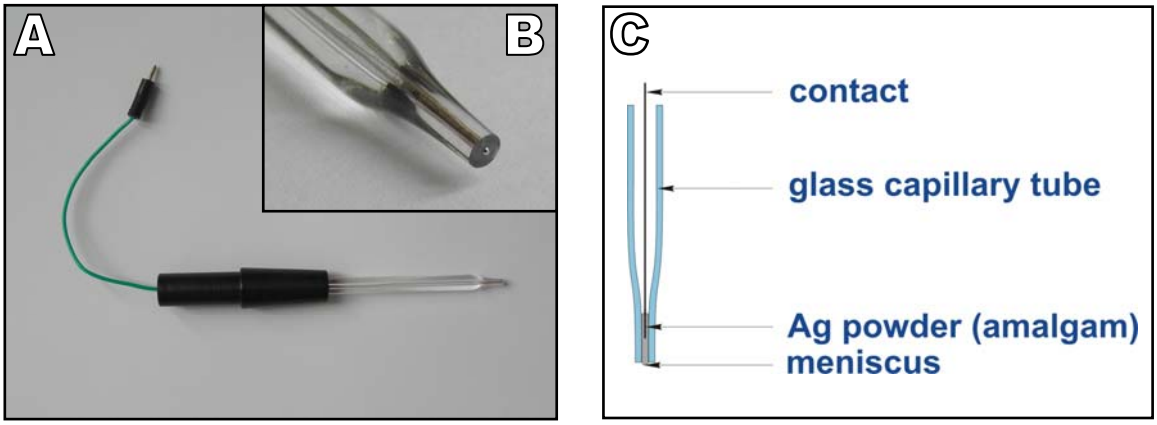

Fig. 3-1 (A) Mercury meniscus modified silver solid amalgam electrode, (B) detailed picture of meniscus and (C) scheme of electrode.

Solid electrodes based on carbon materials are commonly used in electroanalysis due to their broad potential window, low background current, rich surface chemistry, low cost, chemical inertness and suitability for various sensing and detection applications [1]. Among them, carbon paste electrodes (CPE) combine a carbon powder with a pasting liquid (an organic binder). They are characterized by a decreased residual current and a better

reproducibility of currents compared to the pure carbon material [9]. The screen printing microfabrication technology is nowadays well established for the mass production of thick film electrodes. This process implies the sequential deposition of layers of different conductive or non-conductive inks on a variety of inert substrates. Although the formulation of the printing inks is regarded by the manufacturer as proprietary information, it is known that these inks are comprised mainly of synthetic grade graphite, vinyl or epoxy-based polymeric binder and solvents. The electrochemically active graphite particles are the electrodic material, the binder is included to enhance the affinity of the ink for the substrate in terms of adhesion properties and mechanical strength and solvents are used to improve the viscosity of the ink for the printing process. Substrates most frequently used in screen printing technology are ceramic and plastic based materials, in this sense ceramic substrates allow to achieve higher curing temperatures of inks than those that can be reached at plastic substrates.

As with conventional electrodes, the **screen printed carbon paste electrodes** (SPCPEs; see Fig. 3-2) have been developed to a great extent in comparison to other metal (gold, platinum) based electrodes. It was demonstrated that there is a relationship between the surface morphology and the electrochemical activity of the electrode [10] and therefore that the choice of the working electrode should depend upon the specific electroanalytical application and the technique used. Commonly pre-anodization treatments are chosen to increase the electrochemical activity of SPCPEs for a wide range of redox processes through the increase of surface functionalities and roughness or removing surface contaminants [11]. These activations have a stripping or renewing effect on the surface of the carbon electrodes. Due to the nature of screen printed electrodes, any mechanical manipulation (polishing) should be avoided in contrast with conventional CPEs. Other surface modification schemes

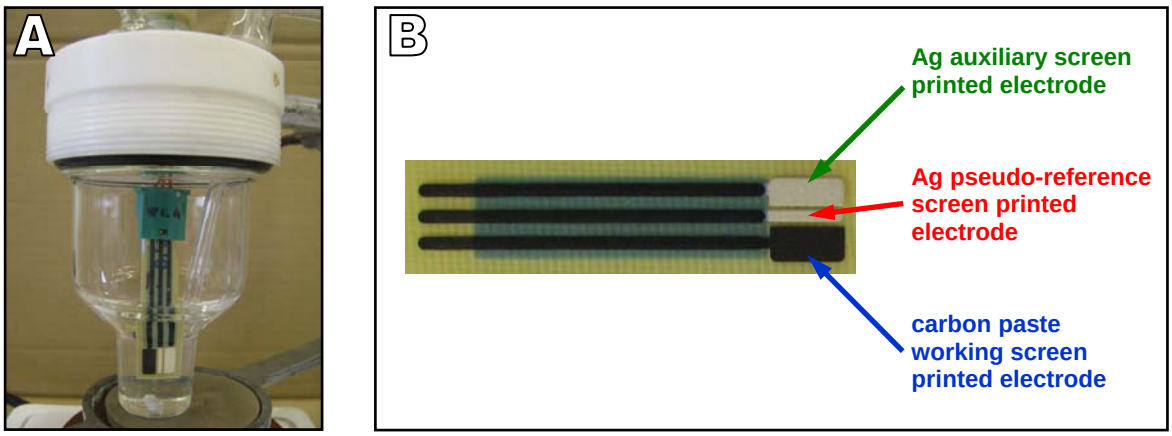

Fig. 3-2 (A) Experimental arrangement and (B) detailed picture of screen printed carbon paste electrode.

can be used to improve the performance of screen printed electrodes including doping with various catalysts [12] or modification with carbon nanotubes [13-15].

3.3 Electrochemical DNA Biosensor

A dsDNA biopolymer layer immobilized on the surface of SPCPE represents novel type of electrochemical biosensor – **DNA modified screen printed carbon paste electrode** (DNA/SPCPE) – for testing of water, food, soil and plant samples for the presence of pathogenic microorganisms and for the presence of analytes (carcinogens, drugs, mutagenic pollutants, etc.) with binding affinities for DNA [16]. The binding of small molecules to DNA and, generally, DNA damage has been described through the variation of the electrochemical signal of guanine [17,18]. Intercalation has been observed with planar aromatic molecules, and classical compounds are daunomycin, ethidium bromide, acridine dyes, etc.

Damaged segments of DNA can be detected by measuring changes in the redox signals of base residues (guanine and adenine moieties) in DNA immobilized on SPCPEs. Covalently closed circular DNA can be attached to an electrode surface to obtain a sensor that detects a single break in the DNA sugar-phosphate backbone, or for the detection of agents leaving the DNA backbone such as hydroxyl radicals, ionizing radiation or nucleases [19]. It has been shown that polypyridyl transition metal complexes can bind with DNA covalently and noncovalently, in which the noncovalent binding is more important, which includes three modes, such as external electrostatic, groove binding, and intercalative binding. The complex with planar aromatic heterocyclic ring can insert and stack with the base pairs of double helical DNA and caused its damage. This binding mode is most important since some anticancer drugs have been found to interact with DNA by intercalation [20].

This damaging intercalative DNA-analyte interaction can be expected from structurally similar compounds, mainly of priority environmental pollutants with planar aromatic molecules, such as fluorene and its derivatives. The disposable DNA/SPCPE biosensor can thus represent useful tool for investigation of genotoxic effects of chemical compounds of environmental and health interest.

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4. RESULTS AND DISCUSSION

4.1 Mechanisms of Electrochemical Reduction of Studied Nitro and Oxo Derivatives of Fluorene

The polarographic reduction of relatively few fluorene derivatives containing nitro and/or oxo groups has been reported in the literature [1-4]. Ashworth has studied the mechanism of polarographic reduction of 9-fluorenone (9-FN) at a classical dropping mercury electrode (DME) using DC polarography (DCP) in Britton-Robinson (BR) buffer – ethanol (9:1) solutions [1]. In the sixties of the last century, the polarographic reduction of the nitro and oxo groups in 9-FN, 2-nitro-9-fluorenone (2-NFN), 2,7-dinitro-9-fluorenone (2,7-DNFN), 2,5-dinitrofluorene, and 2,5-dinitro-9-fluorenone has been studied in buffered water–acetone solutions over a pH range about 1 to 13 [4]. Although overall electrochemical view was thus provided, some aspects of the reduction mechanism of nitro and oxo derivatives of fluorene at mercury electrodes have not been satisfactorily elucidated. Moreover, the reduction mechanisms of these substances have not been studied at solid electrodes at all.

A contribution to the investigation of reduction mechanisms of 9-FN, 2-nitrofluorene (2-NF), 2,7-dinitrofluorene (2,7-DNF), and 2,7-DNFN is given in this Thesis, in refs. [**5-7**].

4.1.1 Mercury Electrodes

Recently, polarographic and voltammetric determination of 2-NF and 2,7-DNF [8,**9**] and of 2-NFN [**9**] at mercury electrodes (namely DME and hanging mercury drop electrode (HMDE)) was developed. Two remaining derivatives, i.e. 9-FN and 2,7-DNFN, have thus been investigated in the Thesis to complete the whole set of investigated fluorene derivatives.

Polarographic and voltammetric behavior of genotoxic **9-fluorenone** was investigated in this Thesis by DC tast polarography (DCTP) and differential pulse polarography (DPP), both at DME, and by differential pulse voltammetry (DPV) at HMDE [**6**]. The influence of

pH on the polarographic behavior of 9-FN was investigated in a mixture of BR buffer – methanol (1:1). It has been shown that 9-FN gave in whole investigated pH range (BR buffer pH 2.0 – 12.0) one well-developed wave, the second wave was observable only in pH region of BR buffer from 8.0 to 11.0. The half-wave potential ($E_{½}$) of the first wave was shifted towards negative values with increasing pH and varied with pH in the range of BR buffer pH 2.0 – 12.0, whereas $E_{½}$ of the second wave was constant and pH independent. It was verified that the limiting current is controlled by diffusion.

Ashworth [1] has described two waves in whole pH range (1.8 to 12.0). In our case of BR buffer – methanol (1:1) medium, two waves were observed only in the alkaline region (BR buffer pH 8.0 to 11.0). The absence of the second wave in acidic medium has been explained by a coincidence of the second wave with the evolution of hydrogen starting at less negative potentials in most acidic media. Thus, the 9-FN second wave was well-observable only in alkaline region where the evolution of hydrogen started at more negative potentials. Our finding that $E_{½}$ of the first wave was pH dependent and $E_{½}$ of the second wave was pH independent was in the agreement with reduction mechanisms supposed by Ashworth [1]. The proposed mechanism of 9-FN reduction at DME in BR buffer – methanol (1:1) solutions is described in detail in ref. [**6**]. Using DPP at DME, 9-FN gave only one well-developed cathodic peak in the whole investigated pH region. This peak corresponds to the first step of 9-FN electrochemical reduction. The peak corresponding to the second step of reduction was not observable. Analogously to DPP at DME, 9-FN gave only one well-developed cathodic peak in whole investigated pH region using DPV at HMDE.

Unlike 9-FN, which has only one reduction center, **2,7-dinitro-9-fluorenone** represents the substance with three reduction centers. Therefore, more complicated reduction mechanism can be expected. The electrochemical reduction of the nitro and oxo groups in 2,7-DNFN at mercury electrodes has been studied in this Thesis in buffered water-methanolic solutions over a pH range of 2.0 to 12.0 [**5**]. The influence of pH on polarographic behavior of 2,7-DNFN was investigated using DCTP in a mixture of methanol – BR buffer (1:1). 2,7-DNFN gave two or three well-developed irreversible waves. At pH of BR buffer 2.0 and 3.0 it was possible to distinguish three waves, in pH range 4.0 – 7.0 two waves and in pH range 8.0 – 12.0 three waves. Therefore, it is supposed that the reduction of 2,7-DNFN proceeds in two or three consecutive reduction steps [**5**].

The number of exchanged electrons was determined by constant-potential coulometry (CPC) at a mercury pool electrode (MPE) in stirred acidic (pH 2.0), neutral (pH 6.0) and alkaline (pH 11.0) methanol – BR buffer (1:1) media. It has been proved that 2,7-DNFN

exchanges 8 electrons in the first wave, 4 electrons in the second wave and 2 electrons in the third wave in the acidic medium. In neutral medium, 2,7-DNFN exchanges successively 8 electrons in the first wave and 4 electrons in the second wave. In alkaline medium, 2,7-DNFN exchanges successively 4 electrons in the first wave, 4 electrons in the second wave and 6 electrons in the third wave. The ratio of limiting diffusion currents of individual waves corresponds to the presumed numbers of exchanged electrons in the corresponding steps.

Cyclic voltammetric (CV) measurements of 2,7-DNFN at HMDE were used to obtain an overall view of electrochemical behavior of the test substance. Three consecutive cathodic/anodic CV scans of 2,7-DNFN ($c = 1\times10^{-5}$ mol L^{-1}) at HMDE in acidic, neutral and alkaline medium of methanol – BR buffer (1:1) were measured to observe reduction/oxidation behavior of 2,7-DNFN and its intermediates. The proposed overall mechanism of 2,7-DNFN reduction at mercury electrodes in BR buffer – methanol (1:1) solutions is described in detail in this Thesis in ref. [**5**].

4.1.2 Mercury Meniscus Modified Silver Solid Amalgam Electrode

For comparison of electrochemical behavior at mercury electrodes [4,**5,6**], the electrochemical behavior of 9-FN, 2-NF, 2,7-DNF, 2-NFN, and 2,7-DNFN at a mercury meniscus modified silver solid amalgam electrode (m-AgSAE) has been investigated in this Thesis using DC voltammetry (DCV) and DPV [**6,10**]. The influence of the pH on the DCV and DPV curves of 1×10^{-4} mol L^{-1} 2-NF and of 1×10^{-5} mol L^{-1} 9-FN, 2,7-DNF, 2-NFN and 2,7-DNFN at m-AgSAE has been investigated first in mixture of methanol and the BR buffer with pH values of 2.0 to 12.0 (1:1); the lower concentration of 9-FN, 2,7-DNF, 2-NFN and 2,7-DNFN was used because of their limited solubilities in methanol.

DC voltammograms indicated that **9-fluorenone** gave one well-developed irreversible voltammetric peak in the whole investigated pH range (the signal has a sigmoid shape similar to wave in strong acidic media). It was confirmed that the electrochemical reduction of 9-FN at m-AgSAE is also controlled by diffusion. The peak potential (E_p) of 9-FN moved towards negative values with increasing pH, but the dependence of E_p on pH value was not linear. The observed break indicates a possible change in the mechanism of electrochemical reduction. This is in good agreement with the reduction mechanism previously investigated at DME [**6**].

The test nitrofluorenes and nitrofluorenones yielded one to three DC or DP voltammetric peaks at m-AgSAE over the whole pH region, similar to their behavior at mercury electrodes [4,**5,6**]. **2-Nitrofluorene** gave rise to only one irreversible voltammetric

peak in acidic, neutral and alkaline media. **2,7-Dinitrofluorene** yielded two cathodic peaks over the whole pH region. The presence of the oxo group in **2-nitro-9-fluorenone** did not change the mechanism of the nitro group reduction at the m-AgSAE, only the second more negative peak was observed corresponding to the two-electron reduction of the oxo group to the hydroxy group [**6**]. In the case of **2,7-dinitro-9-fluorenone** containing three reduction centers, three irreversible voltammetric peaks were observed. The E_p values of all the observed peaks shifted towards more negative potentials with increasing pH. This shift has been explained (as well as in all previous cases described above) by preliminary protonation of the test substances leading to a decrease in the electron density at both nitrogen and oxygen atoms and resulting in easier electron acceptance at low pH values.

Detail description of reduction processes at m-AgSAE and corresponding DC and DP voltammograms are presented in ref. [**10**].

4.1.3 Screen Printed Carbon Paste Electrodes

At screen printed carbon paste electrodes (SPCPEs), CV measurements have been used in this Thesis to obtain an overall view of electrochemical behavior of 2-NF and 2,7-DNF [**7**]. The voltammetric behavior of these nitrofluorenes has been investigated over the pH range from 3.0 to 7.0, i.e. from weak acidic to neutral medium. This narrow pH range was used because of the tendency to investigate the electrochemical behavior of both nitrofluorenes in media compatible with conditions in living cells. BR buffer, 0.25 mol L^{-1} acetate buffer (AcB) and 0.1 mol L^{-1} phosphate buffer (PBS) were used for the preparation of solutions of appropriate pH values.

The voltammetric behavior of **2-nitrofluorene** and **2,7-dinitrofluorene** at SPCPE and at a double-stranded DNA modified SPCPE (DNA/SPCPE) in AcB (pH 4.75) – methanol (1:1) medium is described in this Thesis [**7**]. Usual reduction and follow-up oxidation and electron transfer reactions characteristic for this class of compounds were found. 2-NF gave rise to only one irreversible voltammetric peak over the whole pH region investigated, 2,7-DNF yielded one irreversible voltammetric peak and a hint of a second, more negative, peak corresponding to reduction of the second nitro group. The E_p of obtained responses of 2-NF and 2,7-DNF was shifted towards negative values with increasing pH, as it is well known from similar reduction processes depending on pH [11].

Comparison of the CVs obtained at DNA/SPCPE and SPCPE indicates the same redox mechanism of 2-NF or 2,7-DNF reduction with some changes in the peaks height. An

enhancement of the analyte response at the DNA/SPCPE was observed which indicated its accumulation within the DNA layer during the electrode incubation for 5 minutes in analyzed solution.

4.2 Polarographic and Voltammetric Determination of 9-Fluorenone and 2,7-Dinitro-9-fluorenone at Mercury Electrodes

As mentioned in Chapter 4.1.1, determinations of 2-NF, 2,7-DNF, and 2-NFN at DME and HMDE have been described recently. In this Thesis, newly developed methods of determination of the two remaining fluorene derivatives – 9-FN and 2,7-DNFN – at mercury electrodes can thus be compared wih above mentioned substances.

The optimum conditions for polarographic and voltammetric determination of **9-fluorenone** at DME and HMDE have been investigated in BR buffered aqueous-methanolic solutions (1:1) [**6**]. Optimum conditions for the determination of 9-FN were found to be BR buffer pH 10.0 – methanol (1:1) medium for DCTP at DME, BR buffer pH 7.0 – methanol (1:1) medium for DPP at DME, and BR buffer pH 3.0 – methanol (1:1) medium for DPV at HMDE. Under these conditions, the calibration dependences of 9-FN were measured within concentration ranges of 2×10^{-6} – 1×10^{-5} mol L^{-1} (for DCTP at DME, with limit of quantification (L_Q) 3×10^{-6} mol L^{-1}), 2×10^{-7} – 1×10^{-5} mol L^{-1} (for DPP at DME, $L_Q = 5\times10^{-7}$ mol L^{-1}), 2×10^{-8} – 1×10^{-5} mol L^{-1} (for DPV at HMDE, $L_Q = 2\times10^{-8}$ mol L^{-1}) [**6**]. An attempt to increase the sensitivity using adsorptive stripping voltammetry (AdSV) at HMDE was not successful. The possible application of the newly developed method of DPV determination of 9-FN at HMDE for environmental samples is under further investigation.

Optimum conditions for the determination of **2,7-dinitro-9-fluorenone** at mercury electrodes have been found in this Thesis [**5**]. Because of a different polarographic behavior of 2,7-DNFN in the acidic, neutral and alkaline pH region was observed (see Chapter 4.1.1), BR buffer – methanol (1:1) with resulting pH 2.7, 7.0 and 11.2 was used for the construction of calibration curves in the concentration ranges from 2×10^{-6} to 1×10^{-5} mol L^{-1} (for DCTP at DME, $L_Q = 1\times10^{-6}$ mol L^{-1}) and from 2×10^{-7} to 1×10^{-5} mol L^{-1} (for DPP at DME, $L_Q = 2\times10^{-7}$ mol L^{-1}). Under optimum conditions of BR buffer pH 11.0 – methanol (1:1 for DPV at HMDE and 9:1 for adsorptive stripping differential pulse voltammetry (AdSDPV) at HMDE), calibration curves in the concentration ranges 2×10^{-8} to 1×10^{-5} mol L^{-1} (for DPV at HMDE, $L_Q = 2\times10^{-8}$ mol L^{-1}) and from 2×10^{-9} to 1×10^{-7} mol L^{-1} (for AdSDPV at HMDE at

optimum potential of accumulation (E_{acc}) –300 mV and time of accumulation (t_{acc}) 60 s; $L_Q = 4\times10^{-8}$ mol L^{-1}) were constructed [**5**].

The optimum conditions found above for DPV determination of 2,7-DNFN were used for direct determination of the substance in model samples of drinking water from the public water line in Prague. The BR buffer pH 11.0 was replaced by 0.001 mol L^{-1} NaOH for simplification. It was found that the calibration curve is linear (in the concentration range $2\times10^{-8} - 1\times10^{-7}$ mol l^{-1}, $L_Q = 2\times10^{-8}$ mol L^{-1}) [**5**].

Furthermore, the preconcentration of 2,7-DNFN using solid phase extraction (SPE) have been tested. Recovery parameters were measured using samples of spiked deionized water and methanol as eluent. Recoveries of SPE of 2,7-DNFN from 50 mL ($c = 1\times10^{-7}$ mol L^{-1}) and 500 mL ($c = 1\times10^{-8}$ mol L^{-1}) of deionized water were evaluated using DPV at HMDE. The values were 97 % (about 10-fold preconcentration) and 95 % (about 100-fold preconcentration), respectively. 2,7-DNFN was then extracted from 500 mL of spiked drinking water ($c = 1\times10^{-8}$ mol L^{-1}) with recovery 95 % (extraction from 50 mL of drinking water was not tested due to the possibility of using direct determination in this concentration range), from 50 mL of spiked river water from the Vltava river in Prague ($c = 1 \times 10^{-7}$ mol L^{-1}) with recovery 85 % and from 500 mL of spiked river water ($c = 1 \times 10^{-8}$ mol L^{-1}) with recovery 82 %.

After SPE, DPV calibration dependences of 2,7-DNFN model water samples were measured in concentration ranges of $(2 - 10)\times10^{-8}$ mol L^{-1} (for SPE from 50 mL of spiked deionized and river water with L_Qs 1×10^{-8} mol L^{-1} and 3×10^{-8} mol L^{-1}, respectively) and $(2 - 10)\times10^{-9}$ mol L^{-1} (for SPE from 500 mL of spiked deionized, drinking, and river water with L_Qs 2×10^{-9} mol L^{-1}, 2×10^{-9} mol L^{-1}, and 4×10^{-9} mol L^{-1}, respectively) of 2,7-DNFN spiked into appropriate water sample [**5**].

4.3 Voltammetric Determination of Studied Nitro and Oxo Derivatives of Fluorene at a Mercury Meniscus Modified Silver Solid Amalgam Electrode

The development of DC and DP voltammetric methods for the determination of studied compounds at m-AgSAE in this Thesis (described in refs. [**6,10**]) included optimization of the pH and composition of the supporting electrolyte and further characterization of the analytical parameters (repeatability, linear dynamic range, and L_Q).

The influence of pH upon the DC and DP voltammetric behavior of **9-fluorenone** at m-AgSAE has been investigated in a mixture of BR buffer – methanol (1:1). The highest and best developed DCV peaks were obtained in BR buffer pH 8.0 – methanol (1:1) medium and DPV peaks in BR buffer pH 10.0 – methanol (1:1). Furthermore, optimum regeneration potentials were found. Without regeneration step prior to each measurement, RSD of 20 consecutive DCV and DPV determinations of 9-FN (c = 4×10^{-5} mol L^{-1}) was found to be 11.9 % and 15.0 %, respectively. By using the regeneration step with regeneration potentials $E_{1,reg}$ = –300 mV and $E_{2,reg}$ = –1300 mV (for DCV at m-AgSAE), and $E_{1,reg}$ = –200 mV and $E_{2,reg}$ = –1500 mV (for DPV at m-AgSAE), the RSD of DCV and DPV determinations (n = 20) for the same 9-FN concentration decreased to 1.0 % and 1.9 %, respectively. The range of measured concentrations of 9-FN was from 8×10^{-7} mol L^{-1} to 4×10^{-5} mol L^{-1} for DCV at m-AgSAE (L_Q = 9×10^{-7} mol L^{-1}) and from 8×10^{-7} mol L^{-1} to 2×10^{-5} mol L^{-1} (L_Q = 5×10^{-7} mol L^{-1}) for DPV at m-AgSAE (measurement of concentrations higher than 4×10^{-5} mol L^{-1} was limited by the solubility of 9-FN) [**6**].

Similarly to HMDE, the attempt to increase the sensitivity using AdSV at m-AgSAE was not successful and the possible practical application of the newly developed method of DPV determination of 9-FN at m-AgSAE is also under further investigation. In comparison with DPV at HMDE, SPE preconcentration, however, will probably be necessary to reach the requested sensitivity and L_Q of DPV determination of 9-FN at m-AgSAE in environmental samples.

Voltammetric behavior of studied genotoxic nitro compounds (**2-nitrofluorene**, **2,7-dinitrofluorene**, **2-nitro-9-fluorenone**, and **2,7-dinitro-9-fluorenone**) has also been investigated using DCV and DPV at m-AgSAE. Moreover, the attempt at increasing the sensitivity using AdSDPV at m-AgSAE was successful for 2-NF [**10**].

The optimum conditions have been found for their determination in a 1:1 mixture of methanol and aqueous BR buffer solution. The highest and best developed DCV and DPV peaks were obtained in BR buffer pH 10.0 – methanol (1:1) medium for 2-NF ($E_{1,reg}$ = –200 mV, $E_{2,reg}$ = –1500 mV), in BR buffer pH 8.0 – methanol (1:1) medium for 2,7-DNF ($E_{1,reg}$ = –200 mV, $E_{2,reg}$ = –1100 mV), and in BR buffer pH 4.0 – methanol (1:1) medium for 2,7-DNFN ($E_{1,reg}$ = –100 mV, $E_{2,reg}$ = –900 mV). 2-NFN gave best responses in medium of BR buffer pH 8.0 – methanol (1:1) and BR buffer pH 9.0 – methanol (1:1) for DCV and DPV at m-AgSAE (both $E_{1,reg}$ = –300 mV, $E_{2,reg}$ = –1000 mV), respectively [**10**].

Under these optimum conditions, the calibration dependences of studied compounds were measured in concentration ranges of 2×10^{-6} – 1×10^{-4} mol L^{-1} (for DCV of 2-NF,

$L_Q = 2\times10^{-6}$ mol L^{-1}), $2\times10^{-7} - 1\times10^{-4}$ mol L^{-1} (for DPV of 2-NF, $L_Q = 2\times10^{-7}$ mol L^{-1}), and of $2\times10^{-7} - 1\times10^{-5}$ mol L^{-1} (for DCV of 2,7-DNF, 2-NFN, and 2,7-DNFN with L_Qs 3×10^{-7}, 5×10^{-7}, and 5×10^{-7} mol L^{-1}, respectively, and for DPV of 2,7-DNF, 2-NFN, and 2,7-DNFN with L_Qs 2×10^{-7}, 4×10^{-7}, and 2×10^{-7} mol L^{-1}, respectively) [**10**].

The achieved L_Qs are consistent with L_Qs achieved using DCV and DPV at amalgam electrodes for a number of organic analytes with reducible nitro, nitroso or azo groups [12]. A further increase in the sensitivity of the determination of **2-nitrofluorene** has been achieved by adsorptive accumulation of the test substance on the m-AgSAE surface [**13**]. It has been proven that only 2-NF gave a significantly increased voltammetric response in dependence on the t_{acc}. Because methanol is also adsorbed on the electrode surface, its content in the supporting electrolyte was decreased to 10 %. Moreover, the BR buffer of pH 10.0 contained impurities interfering with the analyte response and thus it was replaced with 1×10^{-4} mol L^{-1} LiOH. The t_{acc} value 30 s was selected (the peak was well-developed, longer accumulation times were not useful) and the optimum E_{acc} was found to be –200 mV for the whole concentration range measured ($1\times10^{-9} - 1\times10^{-7}$ mol L^{-1}; $L_Q = 2\times10^{-9}$ mol L^{-1}) [**10**]. Thus for 2-NF, even lower L_Q was reached using AdSDPV at m-AgSAE in comparison to HMDE [**9**].

In order to verify practical applicability of the newly developed DPV methods, the determination of 2-NF, 2,7-DNF, 2-NFN and 2,7-DNFN was carried out in model samples of drinking and river waters in a submicromolar concentration range under optimum conditions. Since the AdSDPV technique is less robust and more prone to interferences from surface active substances and other compounds likely to be present in river or surface waters, DPV at the m-AgSAE was used for this determination of 2-NF instead of AdSDPV.

Calibration curves were measured using a mixture of 9.0 mL of a spiked model water sample (drinking water from the public water line in Prague or the river water from the Vltava river in Prague) and 1.0 mL of a BR buffer of appropriate pH in concentration range $(2 - 10)\times10^{-7}$ mol L^{-1} for all studied nitro derivatives of fluorene and 9-FN. Reached L_Qs of DPV determination of 2-NF, 2,7-DNF, 2-NFN and 2,7-DNFN in model samples were 2×10^{-7}, 3×10^{-7}, 4×10^{-7}, and 3×10^{-7} mol L^{-1} (for spiked drinking water) and 4×10^{-7}, 5×10^{-7}, 5×10^{-7}, and 4×10^{-7} mol L^{-1} (for spiked river water), respectively [**10**].

These results confirm the possible application of the proposed methods for both drinking and river water.

4.4 Voltammetric Detection of Damage to DNA Caused by Nitro Derivatives of Fluorene Using an Electrochemical DNA Biosensor

Disposable electrochemical DNA-based biosensors are often used for the determination of low-molecular weight compounds with affinity for nucleic acids and for the detection of the hybridisation reaction [14]. An interesting application of a DNA biosensor is the testing of water, food, soil, and plant samples for the presence of analytes (carcinogens, drugs, mutagenic pollutants, etc.) with binding affinities for the structure of DNA [15]. In this Thesis, an electrochemical DNA biosensor based on the SPCPE with immobilized calf thymus double-stranded DNA (dsDNA) layer has been used for *in vitro* investigation of the interaction between genotoxic nitro derivatives of fluorene (namely **2-nitrofluorene** and **2,7-dinitrofluorene**) and DNA [**7**].

The 2-NF–DNA and 2,7-DNF–DNA interaction has been investigated using the DNA-biosensor prepared by immobilizing DNA onto the SPCPE surface. The DNA gel on the electrode surface enabled the accumulation of the analyte on the biopolymer matrix (see Chapter 4.1.3). General protocol for the investigation of the damage caused to DNA by the direct interaction with 2-NF or 2,7-DNF consists of five main steps: pretreatment, DNA immobilization, blank or sample interaction, flushing, and measurement. After pretreatment of SPCPE by applying a potential of +1600 mV for 120 s and +1800 mV for 60 s in 10 mL of AcB, under stirred conditions, the baseline control scan was performed to monitor the cleaning stage of the screen printed carbon paste working electrode. Then, the biosensor was prepared by immobilizing DNA (5 μL of 0.1 mg mL^{-1} calf thymus dsDNA) onto the screen printed electrode surface. The square wave voltammetric (SWV) scan of immobilized DNA was performed for a few electrodes to evaluate the peak height corresponding to the oxidation of guanine ($I_{p,G}$) and adenine ($I_{p,A}$) (at about +1000 mV and +1300 mV vs. Ag pseudo-reference screen printed electrode for guanine and adenine, respectively) as a blank (relative value of $I_{p,G}$ and $I_{p,A}$ ~ 100.0 %). For the rest of the electrodes an interaction step was performed by immersing the DNA/SPCPE in a stirred solution containing 2-NF or 2,7-DNF (AcB – methanol (99:1) medium). After 2 minutes of incubation, the sensor was washed by immersing the electrode in a clean AcB solution under open-circuit condition for 10 s and after that SWV scan was carried out in fresh AcB.

Obtained results have shown the decrease of $I_{p,G}$ and $I_{p,A}$ from 100.0 % in average (n = 3) to 77 % and 52 % (in the case of direct interaction of DNA with 2-NF in concentration 5×10^{-6} mol L^{-1}), and to 60 % and 41 % (for 2-NF in concentration 1×10^{-5} mol L^{-1}),

respectively. 2,7-DNF caused the decrease of $I_{p,G}$ and $I_{p,A}$ to 88 % and 76 % (in the case of direct interaction of DNA with 2,7-DNF in concentration 1×10^{-6} mol L^{-1}), and to 80 % and 65 % (for 2,7-DNF in concentration 5×10^{-6} mol L^{-1}), respectively.

From these facts it can be concluded that 2-NF and 2,7-DNF interacts with DNA, immobilized at SPCPE surface, concentration-dependently. The type of interaction was investigated voltammetrically (using DPV) by binding reactions of copper(II) and cobalt(III) complexes of 1,10-phenanthroline with calf thymus dsDNA, previously described in ref. [16]. Obtained results, described in detail in ref. [**7**], confirm: (i) accumulation of the complex indicators because of their association with dsDNA and (ii) the transition in the interaction mode from dominantly electrostatic to dominantly intercalative with increasing ionic strength of the PBS medium. It was also found that under *in vitro* conditions both nitrofluorenes interact with dsDNA by intercalation.

The electrochemical reduction of 2-NF and 2,7-DNF (during the first four-electron reduction step of nitro group reduction to hydroxylamino group) can generate short-lived radicals that interact with DNA causing damage [17]. This interaction can be detected by electrochemical sensing of the oxidation of the DNA purine bases [18] using DPV. Two ways of reduction of 2-NF and 2,7-DNF (both in concentration 1×10^{-5} mol L^{-1}) have been tested (in AcB – methanol (99:1) medium for 2-NF and AcB – methanol (9:1) medium for 2,7-DNF): (i) successive cathodic/anodic cycling, from 0 to –1000 mV (15 scans), and (ii) reduction at fixed potential of –900 mV (10 minutes). The solution was stirred or unstirred during the reduction, and the solution was tempered at room temperature 25 °C or at human body temperature 36 °C. However, under these conditions only (stirred medium tempered at 36 °C and using reduction by successive cathodic/anodic cycling, from 0 to –1000 mV (15 scans)), the appearance of guanine (E_p ~ +800 mV) and adenine moieties oxidation peaks (E_p ~ +1100 mV) demonstrated clearly that the damage by the radicals of the $Ar–NO_2H^{\bullet}$ type caused the distortion of the double helix and exposure of the bases that can be oxidized. The oxidation peak of 8-oxoguanine, a product of guanine moiety oxidation (E_p ~ +450 mV) [18], could also be identified.

The results lead to the proposal that the toxicity of the compounds under study can be caused by above described intercalative interaction and by their redox activation. The complex study, reported in this Thesis [**7**], thus represents simple electroanalytical methodology and shows the potential of the disposable DNA/SPCPE biosensor for the investigation of genotoxic effects of chemical compounds of environmental and health interest.

4.5 References

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5. CONCLUSION

The presented Thesis describes development of new electroanalytical methods for the determination of toxic derivatives of fluorene. This compound and its nitro, oxo, amino, and hydroxy derivatives are drawing attention as highly hazardous substances. This trend is on raise in last years, because it was proved that fluorene is a possible human carcinogen. Despite this fact it is still used in chemical industry as intermediate for the production of a wide spectrum of products. Fluorene and other PAHs are emitted to the environment as a result of incomplete combustion of organic materials, during industrial processes, and other human activities and may undergo further photochemical atmospheric reactions when a number of other more polar products are formed. Between them, NPAHs and oxy-NPAHs represent a considerable health risk to humans due to their genotoxicity and/or carcinogenicity. Taking these facts into account, large scale monitoring of environmental pollutants has become more and more important together with the studies of their biological impact on humans and other organisms. This requires development of independent, sensitive and selective detection techniques.

This Thesis represents a contribution to the search for new analytical methods applicable on environmental and biological samples. The current state-of-art concerning the formation, occurrence, and biological activity of fluorene and its nitro and oxo derivatives is summarized in Chapter 2. Further experimental work was focused on assessment of new approaches in the detection of the studied compounds. Attention was paid mainly to nitrofluorenes, fluorenone and nitrofluorenones, namely 9-fluorenone, 2-nitrofluorene, 2,7-dinitrofluorene, 2-nitro-9-fluorenone, and 2,7-dinitro-9-fluorenone and the possibilities of their determination using polarographic and voltammetric methods.

Interactions of calf thymus dsDNA with 2-nitrofluorene and 2,7-dinitrofluorene at the DNA/SPCPE were investigated and subtle DNA damage under conditions of direct DNA-analyte interaction at room temperature and damage to DNA bases under condition of

electrogeneration of short-lived radicals of nitrofluorenes at human body temperature were found.

The obtained results can be summarized as follows:

- Modern polarographic and voltammetric methods at mercury electrodes developed for determination of trace amounts of 9-fluorenone and 2,7-dinitro-9-fluorenone (and other NPAHs generally) in concentration ranges from 2×10^{-8} to 1×10^{-5} mol L^{-1} (for 9-fluorenone) and from 2×10^{-9} to 1×10^{-5} mol L^{-1} (for 2,7-dinitro-9-fluorenone) offer a sensitive, inexpensive, independent, and reliable alternative to more frequently used chromatographic methods.
- Solid phase extraction can be successfully used for preliminary separation and preconcentration of 2,7-dinitro-9-fluorenone from drinking and river water samples with over 82 % extraction efficiency.
- m-AgSAE in combination with modern voltammetric techniques is a suitable sensor for the determination of submicromolar concentrations of 2-nitrofluorene, 2,7-dinitrofluorene, 9-fluorenone, 2-nitro-9-fluorenone, and 2,7-dinitro-9-fluorenone. It provides high stability and reproducibility, although the achieved L_Qs are about one order of magnitude higher compared to DPV at HMDE. However, amalgam electrodes can replace mercury electrodes in cases where higher robustness and easy operation is required or unsubstantiated fear of "toxic" mercury disables its use as electrode material. For 2-nitrofluorene, even lower L_Q ($\sim 2\times10^{-9}$ mol L^{-1}) was reached using AdSDPV at m-AgSAE in comparison to HMDE. The m-AgSAE in combination with DPV offers verified possibility of determination of tested nitro derivatives of fluorene and 9-fluorenone in model samples of drinking and river water.
- Proposed mechanism of redox electrode reactions of studied compounds at different electrode types provides overall view of their electrochemical behavior. That can contribute to better understanding of the mechanism of their interaction with living systems.
- Voltammetric detection of damage to DNA using DNA/SPCPE biosensor represents simple electroanalytical methodology and shows the potential of this disposable biosensor for the investigation of genotoxic effects of chemical compounds of environmental and health interest. The type of association of 2-nitrofluorene and 2,7-dinitrofluorene with the surface attached dsDNA under *in vitro* conditions is the intercalative association. The toxicity of the compounds under study can be caused by this interaction and by their redox activation.