NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY POLITEHNICA BUCHAREST

FACULTY OF CHEMICAL ENGINEERING AND BIOTECHNOLOGIES DOCTORAL SCHOOL

DOCTORAL THESIS

Innovative methods for the determination of substances of therapeutic interest from marine sources

PhD Student: Sandu (cas.Dorneanu) Andreea-Elena
Coordinator: Prof. Dr. habil Raluca-Ioana van Staden

2026

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Innovative Methods for Determining Substances of Therapeutic Interest from Marine Sources

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Acknowledgments

At the end this academic journey, I would like to express my deep gratitude to all those who have stood by me and contributed, directly or indirectly, to the completion of this doctoral dissertation.

First and foremost, I thank God for giving me the strength, wisdom, and hope to press on during difficult times and for guiding my steps throughout this journey.

I express my deepest gratitude to my supervisor, Prof. Dr. Habil. Raluca-Ioana van Staden, for her exceptional academic guidance, patience, high standards, availability, and support throughout my doctoral studies. Her advice, experience, and trust have made an essential contribution to my professional development and the completion of this research.

I extend my sincere thanks to Professor univ. Stanciu Gabriela for the trust she has placed in me, for her constant encouragement, and for the inspiration she has provided me over the years.

I would also like to thank my colleagues at the PATLAB Laboratory for their support, their exceptional collaboration, their assistance with the experimental activities, and the friendly and collegial atmosphere that made this experience more enjoyable and rewarding.

A special thought of gratitude goes to my husband and my parents, who have stood by me with patience, understanding, and unconditional support at every stage of this journey. Without their support and trust, many of my achievements would not have been possible.

Finally, and above all, I dedicate this achievement to my daughter, Daria. She has been my greatest source of motivation and the reason I never wanted to give up. I hope that by setting this example, I can show her that perseverance, hard work, faith, and courage can turn any dream into reality.

To everyone who contributed, in one way or another, to this achievement, I extend my sincere thanks and my deepest gratitude.

INTRODUCTION

This abstract summarizes the original contributions of the doctoral thesis titled “Innovative Methods for the Determination of Substances of Therapeutic Interest from Marine Sources,” prepared within the Doctoral School of the Faculty of Chemical Engineering and Biotechnology at the National University of Science and Technology “Politehnica” Bucharest.
The research was conducted under the supervision of Prof. Dr. Habil. Raluca-Ioana van Staden at the PATLAB Laboratory of the National Research Institute of Electrochemistry and Condensed Matter.

The sapropelic mud and water from Lake Techirghiol, as well as the sapropelic mud from the Dead Sea, are natural resources with recognized therapeutic properties that have been used in balneotherapy and cosmetology for centuries.
Their complex chemical composition, rich in humic and fulvic acids and minerals, is the basis for their therapeutic effects on the human body.

Given the increasing anthropogenic pressure on aquatic ecosystems, which has led to the gradual accumulation of pollutants, particularly heavy metals and persistent organic pollutants, whose presence can compromise the therapeutic value of these resources and have adverse effects on patients health, monitoring these pollutants, as well as their fast identification and quantification at very low concentrations using screening methods, will make it possible to use therapeutic sapropel and water free of toxicological risks.

Although water and sapropel are used in therapeutic centers in Romania and Israel, there is a need to extract the active ingredients for natural medicines or to use sapropel patches for therapeutic purposes outside the cities of Techirghiol and the Dead Sea as well.

The main objective of this thesis is to identify innovative methods for determining substances with therapeutic effects from marine sources. Therefore, new methods based on stochastic and

electrochemical sensors were optimized, characterized, and validated for the determination of substances of therapeutic interest, such as humic acid, fulvic acid, and estradiol in lake water and sapropel. To determine substances with toxic potential, such as heavy metals (Pb(II), Cd(II)) and persistent organic pollutants (such as polychlorinated bisphenols) in sapropel and water, methods based on stochastic sensors and gas chromatography were proposed. The proposed methods and instruments allow for the determination of these substances at concentration levels ranging from extremely low to several mg/L, with high selectivity and sensitivity. The use of the stochastic analysis method ensured high reliability in identifying these substances in complex matrices, while their concentrations could be determined at very low levels in sapropel and water samples.

The results of the thesis were all published in ISI journals, the novelty and high quality of the work being recognized by the reviewers of the journals.

CHAPTER 1

Sapropel Mud and Water from Lake Techirghiol and Dead Sea—Sources of Health

1.1.Introduction

This study aims to analyze the benefits of the salt sapropel from Lake Techirghiol and the Dead Sea. Sapropel treats inflammatory or degenerative rheumatic conditions, post-traumatic sequelae (affecting muscles, skin, and bones), disorders of the central and peripheral nervous systems (ranging from neuralgia to hemiplegia, hemiparesis, multiple sclerosis, and Parkinson's disease), gynecological conditions (adnexitis, cervicitis, endometritis, secondary infertility, functional disorders), dermatological conditions (psoriasis, seborrheic syndrome, allergic dermatitis), and peripheral vascular conditions (ischemia, circulatory insufficiency).

Sapropel is a substance with a complex composition, formed as a result of an extremely lengthy biological process (called peloidogenesis) under the specific conditions of Lake Techirghiol. Black in color, glossy, and creamy, the sapropel has a characteristic odor (hydrogen sulfide) and possesses physical properties that make it extremely useful in treating various conditions.

Although the beneficial effects of sapropel have been known since ancient times, others have been discovered and studied relatively recently and some are still in the preliminary research stage.

Scientific research aims to gather as many explanations as possible regarding the therapeutic effects of sapropel and to derive maximum economic benefit from sapropel extracts that can be incorporated into creams and lotions used for therapeutic or cosmetic purposes. There are numerous articles focusing on therapeutic sapropel, but, unfortunately, they all remain at the research stage.

1.2. Geographical Location

1.2.1. Lake Techirghiol

The Techirghiol city is located in southern Romania, in Constanta County, on the Black Sea coast, on the northwestern shore of Lake Techirghiol, a fluvial-marine estuary separated from the sea by a sandy ridge, 16 km from the city of Constanța, 3 km from the Black Sea, 2 km from Eforie Nord, and 4 km from Movilița.

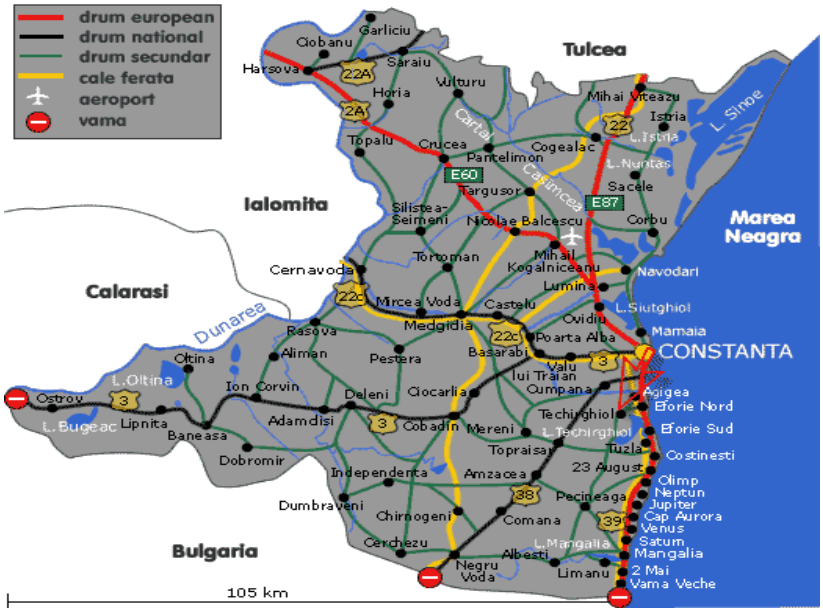


Figure 1.3. Map of Dobruja and the Romanian coast

Source: https://www.litoralsud.ro/

A general analysis of the lake's chemical composition indicates that in the central portion, which includes the resorts of Techirghiol, Eforie Nord, and Eforie Sud, mineralization and total salinity are homogeneous, both horizontally and vertically (to a depth of 3 m), at around 85.5 g per ‰.

In terms of its origin, Lake Techirghiol is a former Black Sea lagoon that is now completely separated from the sea; the complete severing of its connection to the sea is a relatively recent phenomenon. The lagoon lies 1.5 m below the level of the Black Sea; its depth varies between 0.5 m and 5 m, reaching up to 9–10 m.

1.2.2. The Dead Sea

The Dead Sea is an inland sea, fed by water from precipitation and the Jordan River, whose waters flow into it on its northern side. The Jordan River, along with the Dead Sea, forms the natural border between Israel and Jordan.

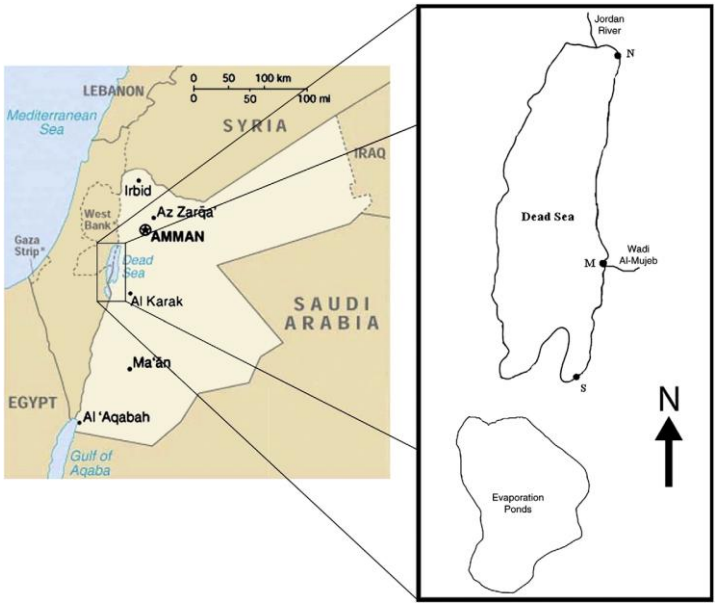


Fig. 1.4. The Dead Sea and water intake points

The Dead Sea covers an area of 600 square kilometers and is one of the lowest places on Earth.

The Dead Sea is located in the southeastern part of the Holy Land and can be reached from Jerusalem.

The Dead Sea is a natural wonder situated in the center of the vast Syrian-African desert of the Rift Valley, between Israel and the Kingdom of Jordan. The Dead Sea is, in fact, a large lake, 76 km long, up to 18 km wide, and 400 meters deep. Its maximum depth is 417 meters below sea level.

1.3. COMPOSITION OF SAPROPEL

1.3.1. Characterization of sapropel

From a physicochemical standpoint, sapropel is a heterogeneous system consisting of a liquid phase containing water and water-soluble mineral salts, a solid phase containing minerals and organic substances, and a gaseous phase containing hydrogen sulfide, carbon dioxide, hydrocarbons, etc.

The ionic balance of the sapropel is also reflected in pH. In general, sapropels are alkaline, with a pH of 8–10.

The therapeutic effect of sapropel is determined by a combination of its physical and chemical properties.

Quality indicators include:

✓ Macroscopic properties —in particular, the reference color and physical consistency; particle texture, which refers to the dispersion state of their solid phase, as well as to particle size

and proportion, may undergo changes during the processing; these must contain a low percentage of particles that may be larger than 0.25 mm, depending on the sapropel opacity;

✓ The total content of organic substances in the dry matter varies depending on the nature of the minerals (inorganic or organic) and on whether their percentage is below or above the 10% threshold. Quantitative differences among the main groups of organic substances have an impact on the quality of tertiary solids.

✓ The mineralization and ionic composition of the peloid-impregnated solution, as a factor in its interaction with the human body at the level of the skin, are followed by the chemical characterization of the mineral acids.

✓ The content of hydrogen sulfide and sulfides, particularly iron sulfide, is another defining factor for the quality of these peloids.

1.3.2. Global physicochemical characterization

Physical characteristics of sapropel

- ✓ hydropermeability
- ✓ Plasticity
- ✓ Dispersion
- ✓ thermopexy

It is worth noting that thermoextrusion is more significant for organic molecules than for mineral ones.

The physical principles of interest in the sapropel processing stage are as follows:

- ✓ Color and odor provide the first indications of the sapropel's condition.

Well-aerated and well-dispersed sapropel has a faint odor, similar to that of humus;

- ✓ Moisture content varies significantly depending on the nature of the sapropel (mineral or organic) and the type of treatment process from which it originates (primary, secondary, tertiary, etc.).
- ✓ The specific gravity of sapropel depends largely on the specific gravity of the solids it contains, their moisture content, and the source of the sapropel from the wastewater treatment plant;
- ✓ Filterability is an important property of sapropel and refers to its ability to be filtered; it is quantitatively expressed by the specific filtration resistance (r, in cm/g) and the compressibility coefficient (s).
- ✓ The calorific value of sapropel varies depending on its organic matter content (volatile substances).

1.3.3. Chemical Characterization

- ✓ The pH value varies during the anaerobic digestion of sapropel, but must be maintained between 7 and 7.5; this is a slightly alkaline process.
- ✓ Total solids—sapropel contains, on average, approximately 90% water, with the remainder consisting of solids that, from a chemical standpoint, can be minerals and organic (volatile) substances.
- ✓ The mineral and volatile fractions in the dry matter serve as criteria for classifying sapropel, therefore:

  - organic sapropel has an M/V ratio < 1;
  - inorganic sapropel has an M/V ratio greater than 1.

This criterion forms the basis for selecting treatment processes, since organic sapropel is biodegradable and its stabilization is achieved primarily through biological methods (aerobic or anaerobic fermentation), while inorganic sapropel undergoes processing (solidification, extraction of useful components, etc.).

- ✓ Fermentability

It is estimated that the organic matter in dried sapropel accounts for between 60% and 80% of the total dry matter, which poses certain challenges in the sapropel drying process.

From a chemical standpoint, sapropel primarily consists of hydrocarbons, fats, and proteins.

Gas production is expressed in kilograms of organic matter and reaches its highest levels when the organic matter is rich in organic fats.

The volatile organic acid content should be approximately 500 mg/dm³. If the value exceeds 2,000 mg/dm³, there is a risk that methane fermentation will intensify, causing acid fermentation to become dominant and leading to the production of foul-smelling gases and sapropel that is extremely harmful to the environment.

- ✓ Heavy metals and nutrients – the nutrient content (N, P, K) is of particular importance when considering the use of sapropel as an agricultural fertilizer or soil conditioner.

1.4. General Analysis

Therapeutic sapropel (peloids) are defined by the International Society of Environmental Hydrology as substances formed under natural conditions through geological processes and which, in a finely divided state and mixed with water, are used in environmental treatment practices in the form of baths, local treatments, wraps, poultices or ointments.

The flora consists of microphytic and macrophytic algae, among which Cladophora vagabunda and Cladophora cristalina predominate; these are algae that grow exclusively in saltwater. The aquatic fauna is represented by Artemia salina, which measures 10–12 mm and is reddish in color. Through bacterial decomposition, together with the alga Cladophora cristalina, it forms sapropel.

Analyses conducted over time show that mineralization levels fluctuate and that there is a trend toward increasing water salinity; however, these fluctuations are seasonal in nature and are linked to local climatic conditions.

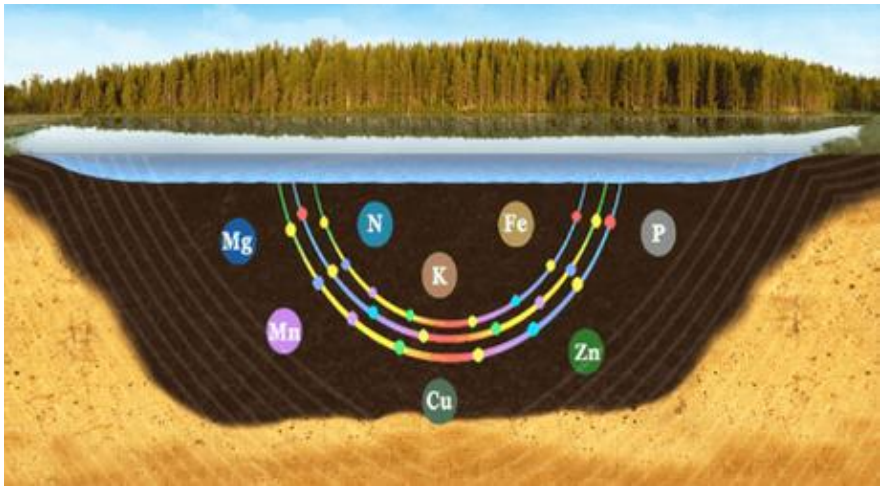


Figure 1.5. Water and Sapropel Mineralization

The general chemical properties of the lake water remain constant, corresponding to those of water containing chlorine, bromine, as well as sodium and magnesium sulfates. The dominant elements also exhibit constant values throughout the central area of the lake.

Chloride, in the form of sodium and magnesium chloride, is the most valuable indicator of mineralization, remaining at constant values; sulfates also remain within very narrow, limits: 7.5–7.6 g‰; sodium and potassium also remain within narrow ranges, around 27 g‰ and 0.8 g‰,

respectively. Magnesium, although it shows some variation, also falls within the narrow range of 2.8–3 g‰.

When analyzing the more mobile elements in the area, bicarbonates and carbonates show some variation in values due to the lability of the CO_3^{2-} / HCO_3^- system, and calcium concentrations increase with depth, likely due to the transformation of soluble carbonate into insoluble carbonate; however, the so-called equilibrium CO_2 remains in the lake.

The absence of iodine in all the samples analyzed appears to be related to the metabolic behavior of zooplankton and phytoplankton, as well as to absorption or addition processes.

The agents contributing to peloidogenesis in Lake Techirghiol are:

- ✓ Artemia salina
- ✓ Cladophora cristallina
- ✓ Oxidizable organic matter, derived from the organisms inhabiting the lake, increases from the surface toward the depths, accumulating from the trophic zone to the trofolithic zone.

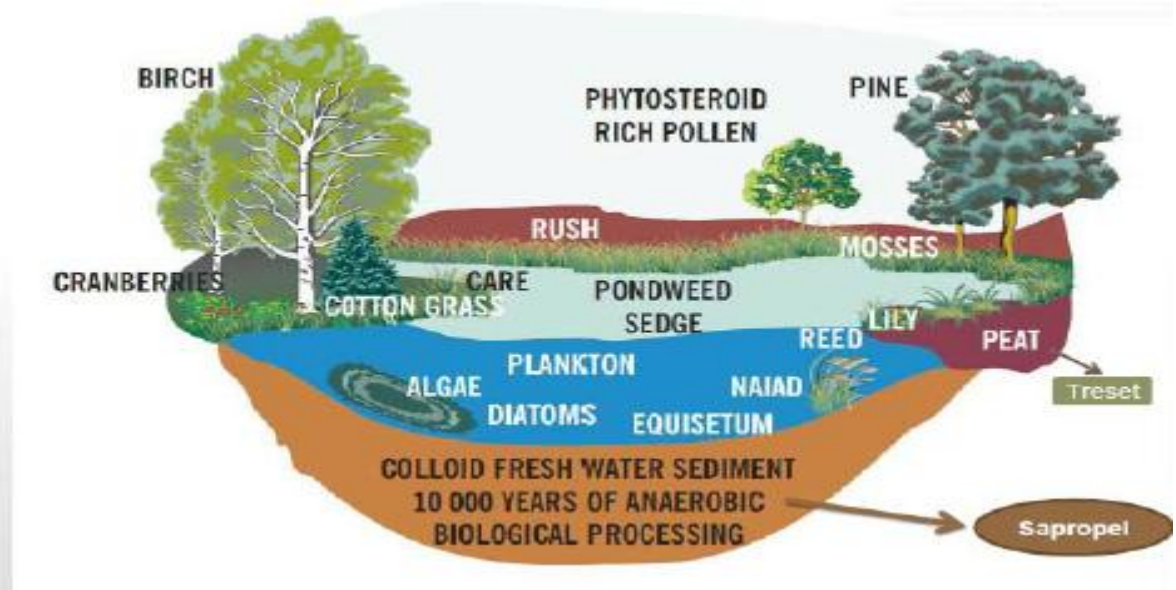


Figure 1.6. Factors involved in the natural process of sapropel formation

The sapropel from the Dead Sea is black in color (known as “black Dead Sea sapropel”) [2]. Dead Sea black sapropel is a homogeneous mixture of Dead Sea minerals and organic substances, which can be useful in treating rheumatic and dermatological conditions, as well as in cosmetics, climatotherapy, and balneology.

It is believed that Dead Sea sapropel originates from older sediments or from reddish-brown soils that are carried into the sea during the winter.

Following the investigations, isotopic measurements showed that the enrichment of the aragonite samples with heavy isotopes was primarily caused by their precipitation, which was driven by evaporation [3,4].

Some of the rheological properties of Dead Sea mud were studied (e.g., apparent viscosity, density, mixing with fresh water, and shear stress). Although this research provides valuable information for specific problems, there appears to be a complete lack of baseline data on both the physical and chemical properties of Dead Sea sapropel.

Sapropel samples from the Dead Sea are characterized by uniform mineralogical compositions: clay, dolomite, gypsum, kaolinite, pyrite, quartz, carbonates, and feldspar.

The concentration of heavy metals in the black sapropel of the Dead Sea has been studied [2,14, 15], and it has been demonstrated that black sapropel samples collected from various locations along the eastern shore of the Dead Sea exhibited low levels of heavy metals.

From the scientific literature, we know that the water of the Dead Sea is characterized by extremely high salinity (34.1%), which makes it an environment incompatible with marine life.

Its extreme density (1.24 kg/l) makes it possible for the human body to float in water without having to swim.

Contrary to expectations, certain anaerobic bacteria can live in this salty water, such as those that break down nitrate, sulfur, and cellulose. Even some plants, such as halophytes, can survive in these extreme environmental conditions.

Due to its chemical composition and content of trace elements, organic substances, amino acids, and hormones, the biological activity of sapropel is influenced, while also exhibiting a high capacity to scavenge free radicals.

Currently, its most common uses are in medicine (balneology), the chemical industry, and agriculture.

Sapropel and extracts are used in the formulation of cosmetic products due to their ability to eliminate free radicals, prevent and reduce the effects of aging, improve and regenerate the skin, and regulate the skin moisture levels.

Furthermore, due to its antibacterial properties, the sapropel is used as an active ingredient in the formulation of soaps, toning lotions, cleansing masks, shampoos, massage oils, and body creams [54].

CHAPTER 2

Tools and methods used for the determination of pollutants and active substances in sapropel and water of Lake Techirghiol and Dead Sea

To determine pollutants and active substances with therapeutic effects in the sapropel and water samples from Lake Techirghiol and the Dead Sea, the following were proposed: an electrochemical sensor, various models of stochastic sensors, and a GC method with an ECD detector.

2.1. Electrochemical Sensors and Differential Pulse Voltammetry

Electrochemical sensors are reliable instruments used to determine substances directly from samples without further processing. The design of these sensors is simple: a carbon matrix-based paste, which is the most reliable, can be used to immobilize a modifier with good electrocatalytic properties. The modifier will promote the oxidation or reduction of the substance of interest when a potential is applied.

Differential pulse voltammetry (DPV) is a highly sensitive electroanalytical technique based on the application of fixed-amplitude pulses superimposed on a linear potential ramp. The measured signal represents the difference in current recorded before and after each pulse, which allows for the elimination of capacitive charging current and improves the signal/noise ratio, enabling the precise detection of redox-active species. DPV is a pulse voltammetry method derived

from linear or step-scan voltammetry, in which a series of small voltage pulses is applied to the working electrode over a gradually increasing baseline potential. The current is measured twice for each pulse: immediately before the pulse and at the end of it. The difference between these two measurements is plotted as a function of the applied potential, which minimizes the effect of background charging currents and improves the signal/noise ratio. DPV offers several advantages over voltammetric methods, such as cyclic voltammetry: high sensitivity, improved peak resolution, and low background current, while the peak current is proportional to the analyte concentration, enabling precise calibration and quantification.

2.1.1. Response Characteristics of Voltammetric Sensors

The first parameter to be determined by cyclic voltammetry is the half-wave potential. The half-wave potential is a qualitative parameter of the voltammetric sensor, as the analyte of interest can be identified based on its value. Unfortunately, its value is influenced by the matrix composition.

The linear concentration range for voltammetric sensors is not wide, typically 5 orders of magnitude, at very low concentration levels in the nanomolar or picomolar range.

These are calculated using the IUPAC recommendation for stochastic sensors [16].

Detection limits are related to the signal recorded for the background solution [16, 17], for example, the solution containing the buffer and electrolytes. There are various methods for determining it; for example, the detection limit is the concentration at which the measured current intensity is twice that recorded for the background solution.

The selectivity of voltammetric sensors can be determined using the separate-solutions method or the mixed-solutions method [17]. For the mixed-solutions method, the equation for determining the amperometric selectivity coefficient is Equation 2.1.

In practice, the current recorded for a solution containing only the target species to be analyzed is compared with the total current obtained for a solution containing both the target species and the interfering species. The amperometric selectivity coefficient can be calculated from Equation 2.1:

$$K_{i,j}^{amp} = \left(\frac{\Delta I_t}{\Delta I_i} - 1 \right) \times \frac{c_i}{c_j} \qquad (2.1)$$

where $\Delta I_t = I_t - I_b$ and $\Delta I_i = I_i - I_b$, I_t , and I_i are the current values recorded for the mixed solution and for the solution containing only the main species, respectively, and C_i , and C_j are the concentrations of the main species and the interfering species, respectively. I_b is the current recorded for the blank solution.

In the case of the separate-solutions method, the current intensity is recorded for the solution containing only the main species and, separately, for the solution containing only the interfering species, and the values obtained are compared with each other. The following equation is proposed for determining the amperometric selectivity coefficient (Equation 2.2):

$$K_{i,j}^{amp} = \frac{\frac{\Delta I_j}{c_j}}{\frac{\Delta I_i}{c_i}} \qquad (2.2)$$

where $\Delta I_j = I_j - I_b$ and $\Delta I_i = I_i - I_b$, I_j and I_i are the recorded current values for the interfering ion/substance and the main ion/substance, respectively, and I_b is the current recorded for the blank solution.

The calibration equation is: $I = a + b \times \text{Concentration}_{analyte}$ (2.3)

where *a* is the y-intercept, and *b* is the slope of the sensor. Both terms are unreliable because they change over time; therefore, these sensors must be calibrated before each measurement of the analyte concentration in a sample.

The sensitivity of voltammetric sensors is given by the slope value recorded for the calibration equation. The minimum acceptable threshold for the sensitivity of voltammetric sensors is 100 nA per order of magnitude of concentration.

2.2. Stochastic Sensors and Methods

The most reliable configuration for stochastic sensors is based on the immobilization of a modifier on a carbon-based paste matrix. The modifier must have pores or channels capable of accommodating the analyte of interest. The matrix must be able to maintain these pores and channels in good condition to ensure adequate stability of the designed sensor. To date, the best stability has been achieved using graphene-based matrices [30]. To demonstrate that the sensor active surface is capable of producing a stochastic signal, it is essential to analyze this surface using SEM, TEM, or AFM at various stages of sensor fabrication.

2.2.1. Response Characteristics of Stochastic Sensors

Qualitative and quantitative analysis is possible using stochastic sensors. While the value t_{off} is used for qualitative determinations to identify analytes, the values t_{on} are used for all quantitative determinations.

Qualitative analysis is based on the time t_{off}, whose values are specific to each analyte and allow for its identification. This parameter is expressed in seconds.

The value of $t_{on}$is related to concentration by the equation $1/t_{on} = a + b \times$ Anal. Conc.,

where *a* is the y-intercept and *b* is the sensor slope.

The first parameter to be determined is the linear concentration range, which is also the working concentration range. Within this range, a high degree of reliability in determining the analyte concentration is possible. For this type of sensor, wide linear concentration ranges are observed, which are characteristic of stochastic sensors.

The limit of quantification is a very important response characteristic of the stochastic sensor. The subsequent IUPAC recommendation regarding the calculation of the limit of quantification proposed that the last concentration value found within the linear concentration range be considered the limit of quantification [28].

Sensitivity is given by the term *b* in the calibration equation. Its value is typically high when stochastic sensors are used.

The stability of stochastic sensors is very high, especially when no biological substances are used in their design. Such sensors can be used continuously for hundreds of measurements, which is a significant advantage.

The selectivity of the sensors is determined exclusively by the values recorded for the analyte and for possible interferences and does not depend on the ratio between the concentration of the possible interfering substance and the concentration of the analyte to be determined. Differences between values of 0.2 s or greater indicate that the sensors are selective. Selectivity is not influenced by the concentrations of the analyte and possible interfering analytes in the mixture. The high selectivity of these sensors has been demonstrated on several occasions [19–21].

2.2.2. Stochastic Method

The stochastic method is used for screening samples to identify various types of analytes, biomarkers, and hazardous substances. The stochastic sensor, together with the reference electrode (which, in many cases, is an Ag/AgCl reference electrode) and the auxiliary electrode (which can be made of Pt or C), forms the electrochemical cell that is immersed in the sample to be measured. Constant-potential chronoamperometry is chosen as the technique. The applied potential is optimized to obtain reliable readings of the t_{off}values of the analytes. After applying the potential, the curve is obtained. The analytes of interest are identified based on their signatures, and the corresponding t_{on} values are read and entered into the calibration equations to determine the analyte concentration.

2.3. GC-ECD

Gas chromatography is an analytical technique used to separate, identify, and quantify volatile compounds in a mixture using a gaseous mobile phase and a stationary phase. Gas chromatography separates compounds based on their distribution between the stationary phase and the mobile phase. The stationary phase can be a solid adsorbent (gas-solid chromatography, GSC) or a liquid applied to an inert support (gas-liquid chromatography, GLC). The mobile phase is an inert carrier gas, such as helium, nitrogen, or hydrogen, which transports the vaporized sample through the column . Separation occurs because different compounds interact differently with the stationary phase, resulting in distinct retention times for each component.

The electron capture detector (ECD) was used in the analyses in this thesis due to its high sensitivity toward electronegative analyte molecules with high electron affinity, which are capable

of capturing electrons. This sensitivity makes ECD ideal for detecting trace amounts of chemical compounds, such as halogenated compounds, in a sample. The selectivity of ECD makes it particularly useful in fields that require rigorous monitoring and analysis of trace amounts of toxic compounds, such as environmental testing and pesticide residue analysis.

ORIGINAL CONTRIBUTIONS

CHAPTER 3

2D Disposable stochastic strip for Ultrasensitive Assay of Pb(II) and Cd(II) in sapropel [104]

3.1. Introduction

It is well known that most heavy metals are carcinogenic and non-biodegradable [32, 40]. A link has been observed between exposure to cadmium (Cd) and several types of cancer, such as breast, lung, prostate, pancreatic, and kidney cancer, and the impact of Cd on heart health, which manifests as significant biochemical and physiological changes, has also been reported [35]. Pb exposure is a public health concern due to its long-term persistence, with acceptable levels set at less than 10 μmol L^{-1} [28]. Pb (II) has toxic effects on many organs, such as the ovaries (decreased ovarian mass), the brain (behavioral disorders, memory impairment, inattention, depression, anxiety), the kidneys (inflammation, glomerular lesions, alteration of renal tubular epithelial cells), and the lungs (inflammation, thrombosis, lung tumors) [28]. Cd and Pb tend to bioaccumulate in aquatic environments, as well as in aquatic sediments and sapropelic mud [22].

To date, flame atomic absorption spectroscopy [42], fluorescence chemosensors, biosensors [53], and various electrochemical methods based on electrochemical sensors have been proposed for the determination of Cd and Pb in various matrices [43, 26, 36, 29, 33, 42, 25, 39].

Although spectroscopic analytical methods are extremely reliable, they cannot be used for on-site analysis, and samples require extensive preparation before Pb and Cd can be determined, which, in some cases, reduces the accuracy of the analysis. Electrochemical analytical methods

represent a good alternative to spectroscopic methods, particularly due to minimal sample preparation and the portability of the instruments; however, matrix complexity can influence the results of amperometric or voltammetric analysis, affecting the accuracy of the results [38, 31, 41].

Stochastic sensors represent a new class of sensors capable of performing reliable qualitative and quantitative analyses [38]. Precision is improved through their use, as their response is not influenced by matrix complexity; extremely low concentrations of the analyte can be accurately determined in complex matrices.

Given the importance of on-site determination of sapropel quality, there is a real need to develop a portable device capable of monitoring Cd(II) and Pb(II) ions in the sapropel prior to its use in cosmetic and medical treatments.

In this paper, a 2D disposable stochastic strip was proposed for the simultaneous, on-site, real-time determination of Cd(II) and Pb(II) ions. The novelty of this stochastic strip compared to other graphene-based sensors [23, 24, 30, 34, 36, 37] lies in the principle of recognition of the two metals (the stochastic mode, which enables reliable molecular recognition and quantification), as well as in its design: the working electrode was fabricated by immobilizing graphene on silk (using the cold plasma technique) and modified with α-cyclodextrin, and is accompanied by a reference electrode (Ag/AgCl) and an auxiliary electrode (carbon wire). Another novelty is the simultaneous in situ determination of Cd(II) and Pb(II) in sapropel samples.

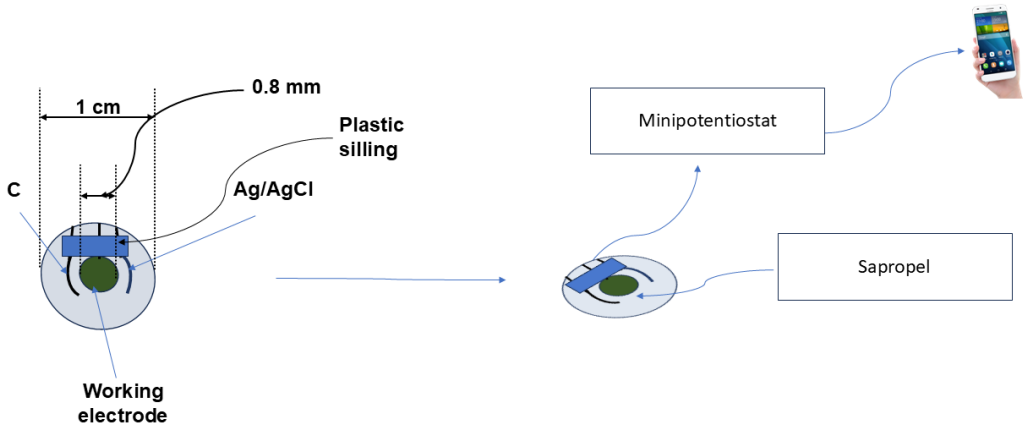


Figure 3.1. Schematic representation of the stochastic sensing strips, and the experimental set-up for the screening of sapropel using the sensing strip.

Images obtained by scanning electron microscopy (SEM) after modifying the silk with graphene (Figure 3.2.a) and after modification with α-CD (Figure 3.2.b) demonstrated that the coating and modification processes were successfully carried out.

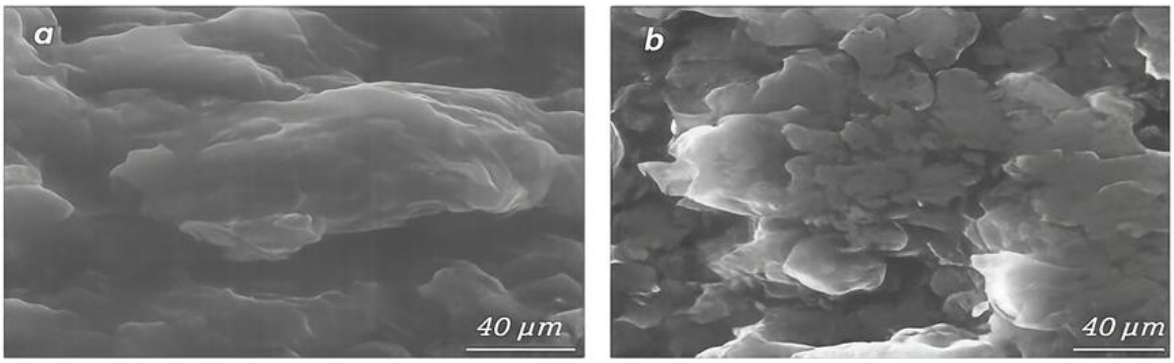


Figure 3.2. SEM morphology of a) graphene material deposied on the silk; and of b) modified graphene with α-CD

3.2. Stochastic Method

Chronoamperometry was chosen as the technique. The optimal working potential was determined so as to obtain a value for t_{off} that could be reliably read both manually and automatically. The optimal value was 0.125 V; at lower potential values (including negative values), no extraction of the analyte into the channel was observed (no signal was obtained); whereas, when potentials higher than 0.125 V were applied, the t_{off} values fell within the ns range, making them impossible to read reliably. At a potential of 0.125 V, Cd(II) and Pb(II) ions were extracted from the solution into the membrane-solution interface, then into the channel, blocking it, while the current intensity dropped to 0 (the time interval corresponding to the analyte's interaction with the channel, denoted t_{off} , can be read from the diagram and represents the parameter used for qualitative identification) (Step 1) (Figure 3.1.).

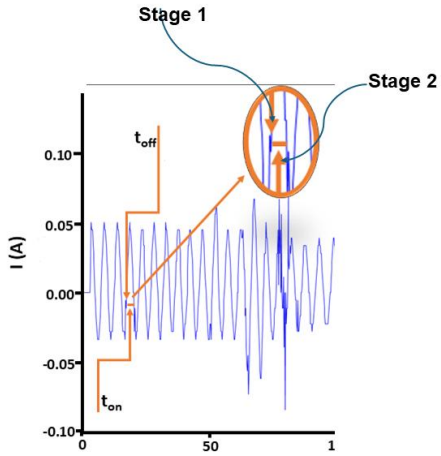


Figure 3.1. The mechanism of current generation in stochastic mode.

In stage 2 (Figure 3.1), redox processes occur inside the channel over a certain period of time denoted by t_{on}, measured on the graph between two consecutive values t_{off}; Linear regression was used to determine the parameters *a* and *b* of the calibration equation, and this equation was used to determine the concentrations of Cd(II) and Pb(II) ions in the sapropel.

The response characteristics of the proposed stochastic detection strip are presented in Table 3.1.

Table 3.1. Response characteristics of the stochastic sensing strip for the assay of Cd^{2+} and Pb^{2+}, when standard solutions, and sapropel were used.

Solution type	**Working concentration range (mol L^{-1})**	**Calibration equation and r***	**Signature t_{off} (s)**	**Sensitivity (s^{-1}/mol L^{-1})')**	**Limit of quantification (mol L^{-1})**
Cd^{2+}					
Standard solutions	1.0×10^{-17} – 1.0×10^{-6}	$1/t_{on} = 0.12 + 2.96\times 10^{6}$ C; r = 0.9997	1.1	2.96×10^{6}	1.0×10^{-17}
Sapropel	1.0×10^{-17} -1.0×10^{-6}	$1/t_{on} = 0.13 + 3.00\times 10^{6}$ C; r=0.9996	1.1	3.00×10^{6}	1.0×10^{-17}
Pb^{2+}					
Standard solutions	1.0×10^{-17} -1.0×10^{-6}	$1/t_{on} = 0.10 + 6.80\times 10^{8}$ C; r=0.9541	0.6	6.80×10^{8}	1.0×10^{-17}
Sapropel	1.0×10^{-17} -1.0×10^{-6}	$1/t_{on} = 0.11 + 6.74\times 10^{8}$ C; r=0.9544	0.6	6.74×10^{8}	1.0×10^{-17}

* $<t_{on}> =$ s; $<C_{ion}> =$ mol L^{-1} .

Different signatures were obtained for the two ions despite if the calibration is done using standard solutions or sapropel. The working concentration range as well as the limits of determination are the same for both ions, making possible their analysis in sapropel from ultra-trace levels to μmol L^{-1} levels. High sensitivities were recorded for both ions. No significative

differences between calibrations done using standard solutions and sapropel were recorded for the equations of calibration and for the sensitivity

While other sensors reported to date have detection limits of nmol L^{-1} [9–16], in this study, Cd(II) and Pb(II) can be reliably analyzed down to concentrations of 0.01 fmol L^{-1}. This very low detection limit is characteristic of stochastic sensors and represents an advantage of this class of sensors [38].

Ten stochastic detection bands were designed and used to calibrate the proposed sensor using standard solutions and sapropel. The sensitivities obtained for each of the ten stochastic bands were calculated, and the variations between these sensitivities and the average sensitivity were 0.04% and 0.05% for the determination of Cd(II) and Pb(II), respectively, proving that the design of the stochastic detection bands is reliable. The calibrations were continued for another 30 days, and the obtained sensitivities were compared; variations of 0.07% and 0.05% were obtained for the determination of Cd(II) and Pb(II), respectively, demonstrating the long-term stability of the proposed strips. Two batches of ten detection strips each were stored at room temperature in plastic boxes. Over a period of 10 weeks, one detection strip was removed from each box, and its sensitivity was determined. The sensitivities, obtained were compared after 8 weeks, when variations of 0.03% and 0.04% were recorded for the determination of Cd(II) and Pb(II), demonstrating that the detection strips are stable over time.

3.3. Selectivity of the stochastic sensor

The selectivity of stochastic sensors is determined by the differences between the t_{off} values recorded for different analytes in a mixture; a difference of at least 0.2 s indicates

selectivity. Ions such as Cr^{3+}, Fe^{3+}, Hg_2^{2+}, Cu^{2+}, and Zn^{2+} were tested as potential sources of interference.

Table 3.2. Selectivity of stochastic detection bands (N=10).

Signature, s						
Pb^{2+}	Cd^{2+}	Cr^{3+}	Fe^{3+}	Hg_2^{2+}	Cu^{2+}	Zn^{2+}
0.6±0.1	1.1±0.1	1.8±0.1	1.5 ± 0.1	2.4 ± 0.1	2.0 ± 0.1	2.7 ± 0.1

The results presented in Table 3.2 demonstrated that there is no interference from Cr^{3+}, Fe^{3+}, Hg_2^{2+}, $Cu^{(2+)}$ and Zn^{2+} on the determination of Cd^{2+} and Pb^{2+} ions.

3.4. Validation of the ultrasensitive stochastic detection strips used to determine $Cd^{2+}$and Pb^{2+}ions in sapropel

All samples were analyzed on-site using the designed sensor, the portable minipotentiostat, and a smartphone. Cd and Pb ions were identified in the diagram obtained after scanning the sapropel sample, based on the t_{offf} (Figure 3.3, Table 3.1), and subsequently, the t_{on} values required for quantitative determination were read and substituted into the calibration equations to obtain the concentrations of Cd and Pb ions (Figure 3.3).

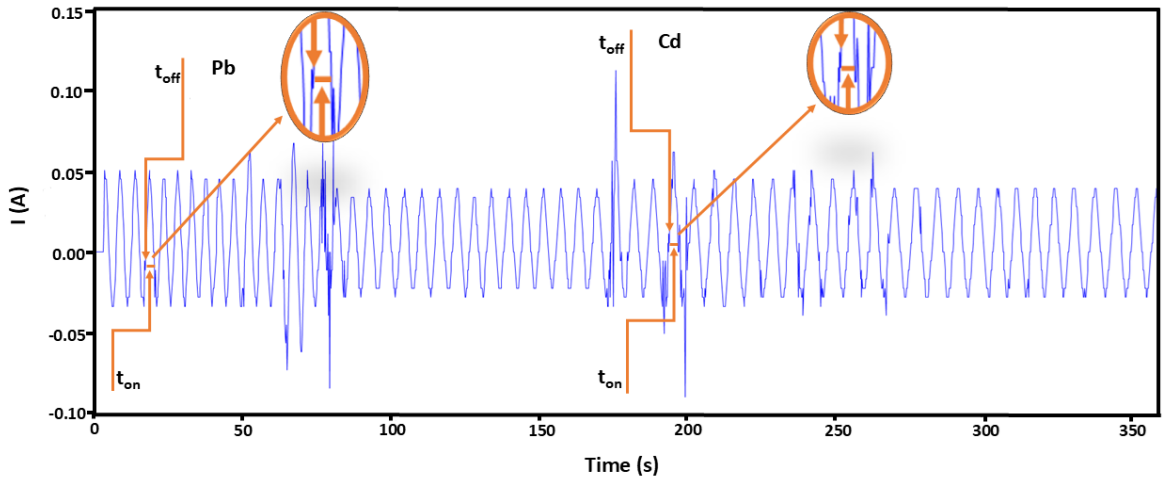


Figure 3.3. Diagram obtained for the screening of sapropel using the 2D disposable stochastic strip. Identification of the signatures of Pb(II), and Cd(II) based on the t_{off} values, and reading of t_{on} values used for the determination of Pb(II) and Cd(II) concentrations.

The results obtained for the determination of Cd and Pb ions in sapropel samples collected from Lake Techirghiol, using stochastic detection strip, were compared with those obtained by the standard reference method, ICP-MS (Table 3). ICP-MS is a multi-element method with a wide analytical range and very low detection limits.

Table 3.3. Determination of Cd(II) and Pb(II) ions from in sapropel (N=10).

Content	ng Cd/g of sample		Student's t-test
	Stochastic detection bands	ICP-MS	
Sapropel	127.05 ± 0.08	127.00 ± 0.15	1.76
	ng Pb/g of sample		
Sapropel	147.46 ± 0.05	145.90 ± 0.12	2.20

The results were compared using the paired t-test (Student t-test), applied to the Cd (II) and Pb (II) concentration values obtained by the proposed method and the standard reference method (ICP-MS), at a confidence level of 99.00%, for which the theoretical t-value from the tables is 4.032. The calculated values (Table 3.3) are lower than the theoretical value in the table, which demonstrates that there is no statistically significant difference between the results obtained by the two methods.

Tests were also conducted to recover Cd (II) and Pb (II) ions from sapropel in order to confirm the validation of the method. Known amounts of Cd (II) and Pb (II) were added to the sapropel samples, and the concentrations determined for the two ions were compared with the theoretical concentrations. The results of the recovery tests are presented in Table 3.4.

Table 3.4. Recovery of Cd(II) and Pb(II) ions from sapropel (N=10).

Sample	%, Recovery of	
	Cd(II)	Pb(II)
Sapropel	99.15 ± 0.07	99.93 ± 0.06

High recovery rates were obtained for Cd(II) and Pb(II) ions from sapropel, demonstrating the accuracy of the screening test for determining these two ions in sapropel.

CHAPTER 4

Preliminary Screening of Persistent Organic Pollutants in Water and Sapropel of Techirghiol Lake Using GC-ECD [105]

4.1. Introduction

Lake Techirghiol, located on the Black Sea coast, is the largest saltwater lake in Romania, with a total length of 7,500 m, a maximum depth of 9 m, and a salinity of over 80 mg/L^{-1} .[64] Contamination of the lake can occur through the illegal discharge of pesticide-contaminated water from agricultural areas surrounding the lake.

Persistent organic pollutants (POPs), a category that includes organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs), are characterized by resistance to biotic and abiotic degradation, high lipophilicity, volatility, and the ability to be transported over long distances by air and water currents. Their accumulation in aquatic ecosystems and sediments can lead to concentrations that exceed health risk thresholds, causing hepatotoxic, neurotoxic, endocrine-disrupting, cardiovascular, and carcinogenic effects.

The main problem is that these substances accumulate to levels that can pose problems for both marine and lake environments, as well as for the health of people who use water or sediment from these sources.

Organochlorine pesticides are among the first effective means of controlling plant pests and pathogens, but they are prone to bioaccumulation and bioconcentration. [44]

The best-known and most widely used pesticide in the world is dichlorodiphenyltrichloroethane, which has frequently been detected in certain waters and in sapropelic sediment samples at various depths. [44]

Sapropel is regularly used in various therapeutic procedures [45–49]; therefore, it is important that it be free of pesticides, which can cause undesirable effects during and after treatment.

This paper aims to present the results of a preliminary analysis of the levels of polychlorinated bisphenolic compounds and organochlorine pesticides in the water and sapropel of Lake Techirghiol, using gas chromatography with an electron capture detector (ECD). The electron capture detector was used in the analysis due to its high sensitivity to electronegative analytes, which are capable of capturing electrons. This sensitivity makes the ECD ideal for detecting trace amounts of chemical compounds, such as halogenated compounds, in a sample.

To achieve the objectives of this study, sapropel samples were collected along with water from the area adjacent to the Techirghiol Lake mining site and processed to separate and determine persistent organic compounds using a GC- ECD system .

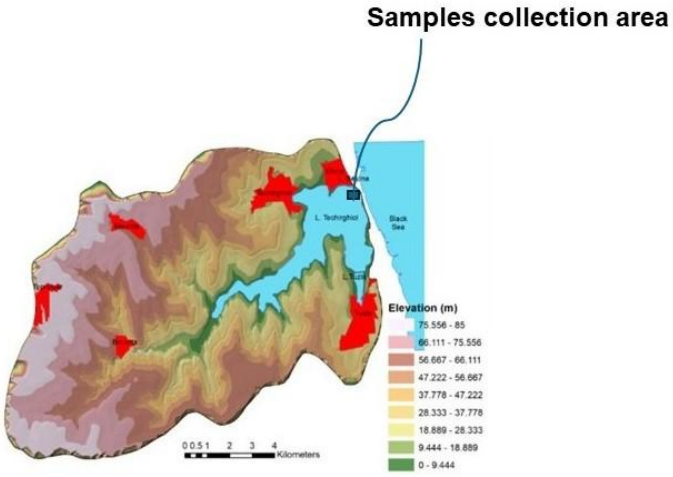


Figure 4.1. Map of Lake Techirghiol showing the sampling locations.

4.1.1. Determination of Organochlorine Pesticides and Polychlorinated Bisphenols

To determine these compounds, a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer, Inc., Shelton, CT, USA) coupled with an electron capture detection system was used.

Table 4.1. Detection limits determined for the pollutants analyzed in the water of Lake Techirghiol.

No.	Compound	LOD (µg L^{-1})
1	HCB	0.0002
2	Lindane	0.0003
3	Heptachlor	0.0001
4	Aldrin	0.0002
5	p,p'DDE	0.0001
6	Dieldrin	0.0001
7	Endrin	0.0002

8	p,p'DDD	0.0002
9	p,p'DDT	0.0001
10	PCB28	0.0004
11	PCB52	0.0003
12	PCB101	0.0005
13	PCB118	0.0003
14	PCB153	0.0005
15	PCB138	0.0007
16	PCB180	0.0002

The limit of detection (LOD) for the pollutants analyzed in the sapropel from Lake Techirghiol ranged from 0.0001 to 0.0003 µg/kg of freeze-dried sapropel for OCPs, and for PCBs, the values ranged from 0.0002 to 0.0007 µg of freeze-dried sapropel (Table 4.2.).

Table 4.2. Detection limits established for the pollutants analyzed in the sapropel from Lake Techirghiol.

No.	Compound	LOD µg/kg of freeze-dried sapropel
1	HCB	0.0002
2	Lindane	0.0003
3	Heptachlor	0.0001
4	Aldrin	0.0002
5	p,p'DDE	0.0001
6	Dieldrin	0.0001
7	Endrin	0.0002
8	p,p'DDD	0.0002
9	p,p'DDT	0.0001
10	PCB28	0.0004

11	PCB52	0.0003
12	PCB101	0.0005
13	PCB118	0.0003
14	PCB153	0.0005
15	PCB138	0.0007
16	PCB180	0.0002

Validation of the method included the evaluation of retention time (RT), linearity (R^2), and limits of detection (LOD) for each compound. Calibration curves were plotted for all analytes, which exhibited excellent linearity (R^2> 0.9587 for OCPs, R^2> 0.9734 for PCBs). To ensure accuracy and precision, procedure controls, spiked samples, and internal standards were included. Recoveries ranged from 85 to 110%, indicating acceptable method performance.

Table 4.3. Determination of organochlorine pesticides in water samples collected near the sediment in Lake Techirghiol (N=3).

Sample No.	Compound	Concentration, PCB µg/kg of freeze-dried sediment
1	PCB28	0.050±0.006
2	PCB52	0.040±0.008
3	PCB101	0.0003 ± 0.0002
4	PCB118	0.0010 ± 0.0006
5	PCB153	0.0010 ± 0.0008
6	PCB138	0.0010 ± 0.0005
7	PCB180	0.0003 ± 0.0001

Tables 4.3 and 4.4 present the results obtained using gas chromatography to determine organochlorine pesticides and polychlorinated bisphenols in water samples collected from Lake Techirghiol.

Table 4.4. Determination of polychlorinated bisphenols in water samples collected from the area adjacent to the sediment in Lake Techirghiol (N=3).

Sample No.	Compound	OPC concentration, µg L^{-1}
1	Hexachlorobenzene	0.42 ± 0.14
2	Lindane	9.06 ± 0.32
3	Heptachlor	11.71 ± 0.21
4	Aldrin	13.85±0.11
5	p,p'-dichlorodiphenyldichloroethylene	4.65 ± 0.22
6	Dieldrin	10.73 ± 0.17
7	Endrin	6.98 ± 0.10
8	p,p'-dichlorodiphenyldichloroethane	34.95 ± 0.18
9	p,p'-dichlorodiphenyltrichloroethane	14.02 ± 0.22

The concentrations of organochlorine pesticides, representing the average of three measurements, ranged from 0.42 to 34.95 µg/L^{-1}, which may be cause for concern, given that the permissible limit in marine waters according to national regulations is 0.1 µg/L for each compound. The standard deviations from the mean are presented in the table.

The average concentrations of polychlorinated bisphenols are well below 1 µg/$L^{(-1)}$, ranging from 0.003 to 0.02 µg/L^{-1}. The standard deviations from the mean are presented in the table. The chromatograms obtained for the separation and determination of polychlorinated bisphenols in the water from Lake Techirghiol and, respectively, in the control sample, are presented in Figures 4.1. and 4.2.

The peaks obtained for the analyzed compounds in the water sample (Figure 4.1) and in the control samples (Figure 4.2) were compared at the same retention time to determine the e concentration of the pollutants in the water and sediment of Lake Techirghiol. Very good separation of these compounds was observed using the proposed chromatographic analysis method, as all peaks are well-defined and good resolution was achieved.

Table 4.5 presents the results obtained for the determination of polychlorinated bisphenol compounds in the sapropel sample collected from Lake Techirghiol (N=3).

No.	Compound	Concentration, PCB µg/kg of freeze-dried sapropel
1	PCB28	0.050±0.006
2	PCB52	0.040±0.008
3	PCB101	0.0003±0.0002
4	PCB118	0.0010 ± 0.0006
5	PCB153	0.0010±0.0008
6	PCB138	0.0010±0.0005
7	PCB180	0.0003±0.0001

Table 4.5. Determination of the content of polychlorinated bisphenols in sapropel samples collected from Lake Techirghiol (N=3).

Very low PCB concentrations were recorded: in the sapropel samples, the average values of the three determinations ranged from 0.0003 to 0.05 µg/kg of freeze-dried sapropel. The standard deviations were very small. Although the lake water contains slightly higher amounts of PCBs, it appears that the PCBs did not have time to accumulate in the sapropel.

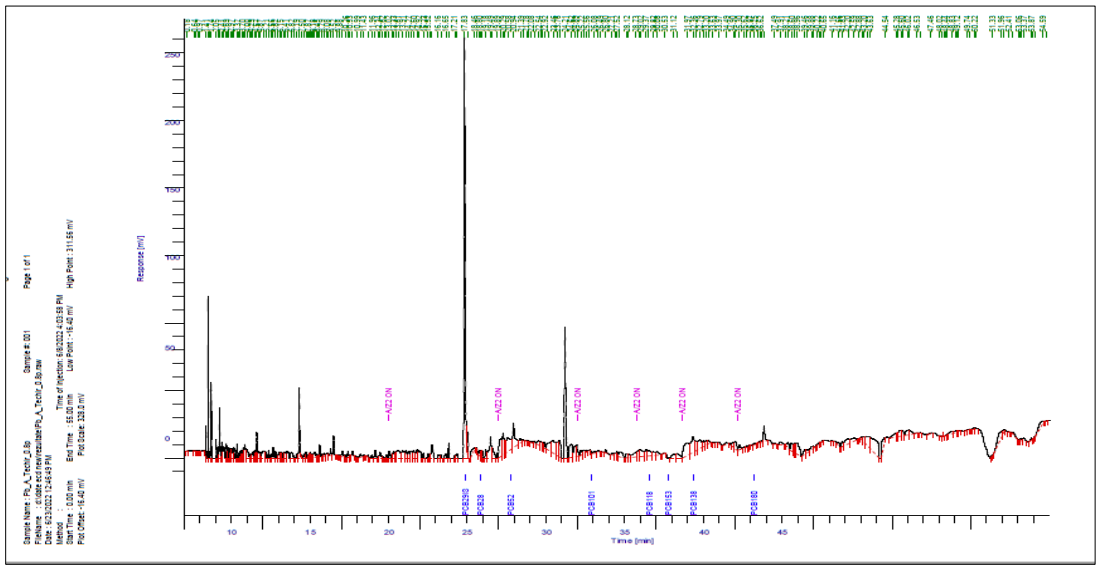


Figure 4.1. Chromatogram illustrating the separation and determination of polychlorinated bisphenols in water from the vicinity of the sapropel in Lake Techirghiol.

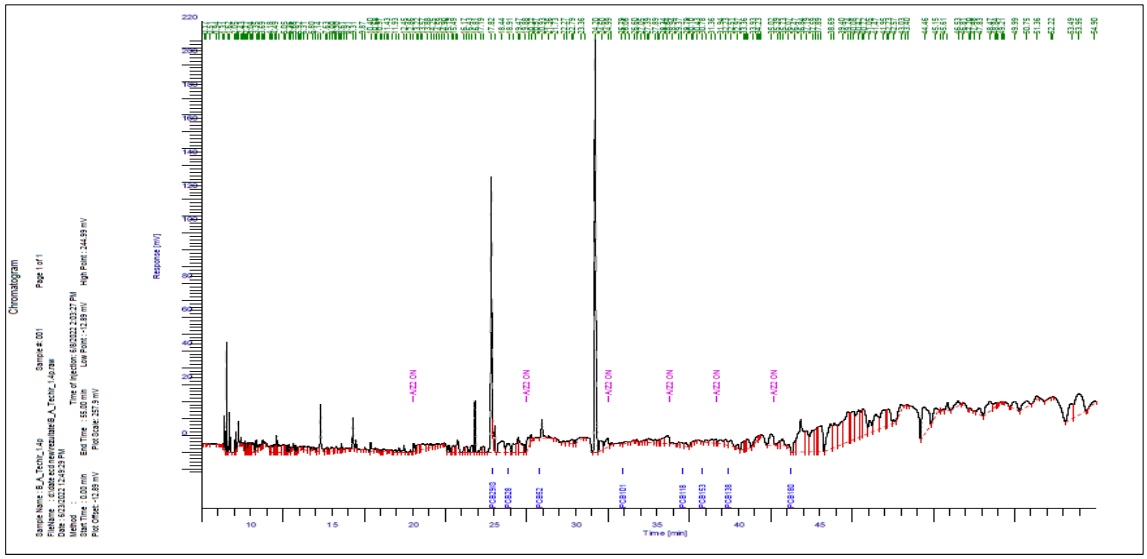


Figure 4.2. Chromatogram illustrating the separation and determination of polychlorinated bisphenols for the control samples.

Table 4.6 presents the concentration of organochlorine pesticides in the sapropel samples, determined by gas chromatography coupled with an electron capture detector.

The sapropel samples were found to contain organochlorine pesticides in concentrations ranging from 0.0002 to 6.35 μg/kg of freeze-dried sapropel, with the limits in seawater and marine sediment being 1 μg $L^{-1}$and 1 μg/kg of freeze-dried sapropel, respectively, for each compound. The concentrations of polychlorinated biphenyls (PCBs) in the sapropel are lower than those in the water of Lake Techirghiol, which demonstrates that these compounds do not accumulate in the sapropel either.

A slight contamination with polychlorinated bisphenol compounds is observed in both the lake water and the sapropel; these compounds may be harmful to human health, although they fall below the limit set by national authorities. With regard to organochlorine pesticides, significant concentrations were found in the water of Lake Techirghiol, while their concentrations in the sediment fell within the limits set by national authorities (concentrations below 0.1 μg/kg are permitted [50]), with the exception of lindane and aldrin, which exceed these limits.

Table 4.6. Determination of organochlorine pesticide content in sapropel samples collected from Lake Techirghiol (N=3).

Sample No.	Compound	OCP concentration, μg/kg of freeze-dried sapropel
1	Hexachlorobenzene	0.0003 ± 0.0001
2	Lindane	6.35 ± 0.21
3	Heptachlor	0.0002 ± 0.0001
4	Aldrin	1.12 ± 0.11
5	p,p'-dichlorodiphenyldichloroethylene	0.0002 ± 0.0001
6	Dieldrin	0.0002 ± 0.0001
7	Endrin	0.0003 ± 0.0001
8	p,p'-dichlorodiphenyldichloroethane	0.030 ± 0.009
9	p,p'-dichlorodiphenyltrichloroethane	0.0002 ± 0.0001

The pesticides identified in this study were previously reported by Oros et al. [50], as well as by Lazar et al. [51] in the Black Sea and the surrounding area, demonstrating that the coastal area is facing diffuse pollution originating from agricultural areas where pesticides have been used; another significant source is the flooding in recent years in the watersheds of the Valea Biruința and Valea Urlichioi streams. In practice, the quality of the water and sediment in Lake Techirghiol is strongly influenced by human activities, such as agriculture and hydrotechnical works carried out in its watershed [52, 53].

CHAPTER 5

Determination of humic acid in sapropel and water from Techirghiol Lake and Dead Sea

5.1. Fast Analysis of Humic Acid in Sapropel and Water Samples [106]

5.1.1. Introduction

Humic acid is one of the most important components of sapropel, possessing a broad spectrum of pharmacological activity. To date, a spectrophotometric analysis method has been proposed for determining humic acid in sapropel samples, while a liquid chromatography method has been developed for determining humic acid in fertilizers. Both the spectrophotometric and liquid chromatographic methods require a lengthy and labor-intensive sample preparation step prior to humic acid determination, involving derivatization in the case of liquid chromatography, as well as consumables of high analytical purity, while the analysis takes a long time and the working concentration range is limited, with samples requiring preconcentration steps in most cases.

There is a need to develop rapid and reliable analytical methods for determining humic acid in sapropel samples, given its therapeutic effects. Electroanalysis is an extremely reliable method based on the use of various types of sensors, which are easy to use for the direct analysis of different analytes in matrices ranging from simple to complex. Stochastic sensors represent a modern approach in the electroanalysis of organic and inorganic substances. Due to their high

sensitivity and reliability, they can first determine the presence of substances—and subsequently quantify them—in matrices ranging from simple to complex, at very low concentration levels. Therefore, a stochastic sensor was proposed for determining humic acid in sapropel samples. The novelty of this work lies in the design of the 2D disposable stochastic sensor used to determine humic acid in sapropel, as well as in the electroanalysis of humic acid in sapropel samples collected from Lake Techirghiol and the Dead Sea, and in water samples collected from Lake Techirghiol. A solution of heptakis (2,3,6-tri-O-methyl)-beta-cyclodextrin ($1x10^{-3}$mol L^{-1}) was used to modify the surface of a screen-printed electrode, whose active surface was coated with single-walled carbon nanotubes (SWCNTs). The use of cyclodextrin facilitated the formation of highly stable channels, which are necessary for stochastic measurements.

5.1.2. Design of the 2D stochastic sensor

On the top of the screen-printed electrodes – which has the surface covered with SWCNT, it was poured one drop of a 10^{-3} mol L^{-1} solution of heptakis(2,3,6-tri-O-methyl)-beta-cyclodextrin. The design of the screen-printed electrode is shown in Figure 1. The electrode was deposited under a glass to dry for 2h. When not in use, the electrode was stored at room temperature.

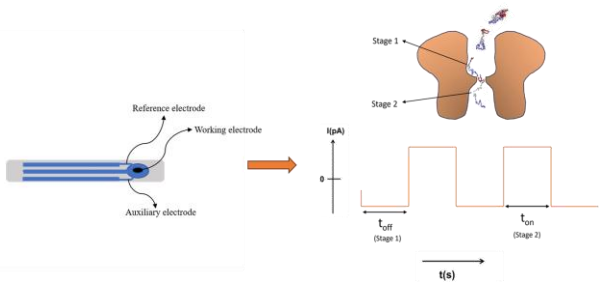


Figure 5.1. Schematic representation of the 2D stochastic sensor.

5.1.3. Stochastic method

A chronoamperometric technique was selected for stochastic sensing, measurements of t_{on} and t_{off} values were made at a constant potential of 100 mV versus Ag/AgCl. A drop of the solution containing humic acid was added to the surface of the 2D stochastic electrode cell. The diagrams were recorded for different solutions containing humic acid, and the values of the signature of humic acid (t_{off} value) and of t_{on} were determined (Figure 5.1). Equations of calibration were determined based on linear regression method results. Samples of saproepl and water were added to the surface of the 2D stochastic electrode cell. The signature of humic acid was used for its identification in the diagram. The unknown concentrations of humic acid were determined by introducing the values of $1/t_{on}$ obtained after the measurements of samples in the equation of calibration. The signal is taken using a smartphone from via Bluetooth from the measurement instrument facilitating its fast analysis and fast determination of the humic acid concentration.

5.1.4. Response characteristics of the 2D stochastic sensor used to determine humic acid

When a potential of 100 mV was applied the humic acid enter the channel, and blocked him until it gets inside (the current drops to 0A) – the time spent for this step is called t_{off} and it is assigned to its signature value. Inside the channel redox processes take place and the time spend for this step is assigned to t_{on} value used for quantitative assay of humic acid (Figure 5.1).

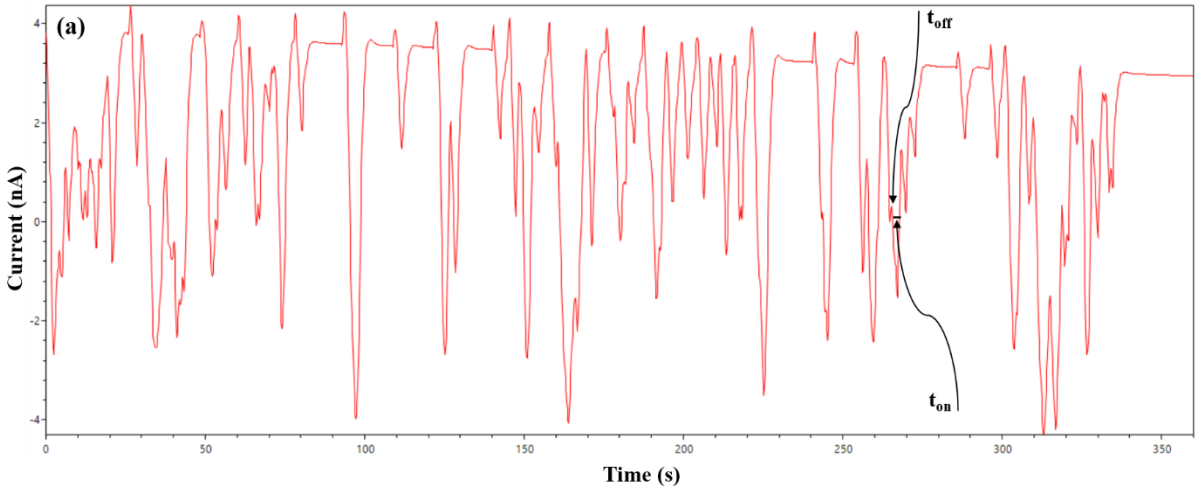
The proposed stochastic sensor was used on a linear concentration range from $1x10^{-18}$g mL^{-1} to $1x10^{-2}$ g mL^{-1}, with a sensitivity of $1.61x10^{9}$ $s^{-1}g^{-1}mL$, and a limit of quantification of $1x10^{-18}$g mL^{-1}. The wide linear concentration range facilitates determination of humic acid in samples containing very low concentrations of humic acid as well as in samples with high content

in humic acid; pre-concentration will not be needed if the proposed sensor is used. The equation of calibration was:

$1/t_{on} = 0.05(\pm0.02) + 1.61(\pm0.03)\ x10^{9}\ C_{humic\ acid}$, and a r value of 0.9999, when 10 measurements were performed. The t_{on} value was read in s, and the concentration, C in g /mL^{-1}. The signature of the humic acid was 0.9s.

5.1.5. Electroanalysis of humic acid in sapropel and water samples

The sapropel and water samples were used as picked up from the Techirghiol lake and the Dead Sea. Measurements were also done on-site in the Techirghiol lake and Dead Sea. Diagrams were recorded (**Figure 5.2.**), and readings of t_{off} values were done to identify the humic acid in the diagram.



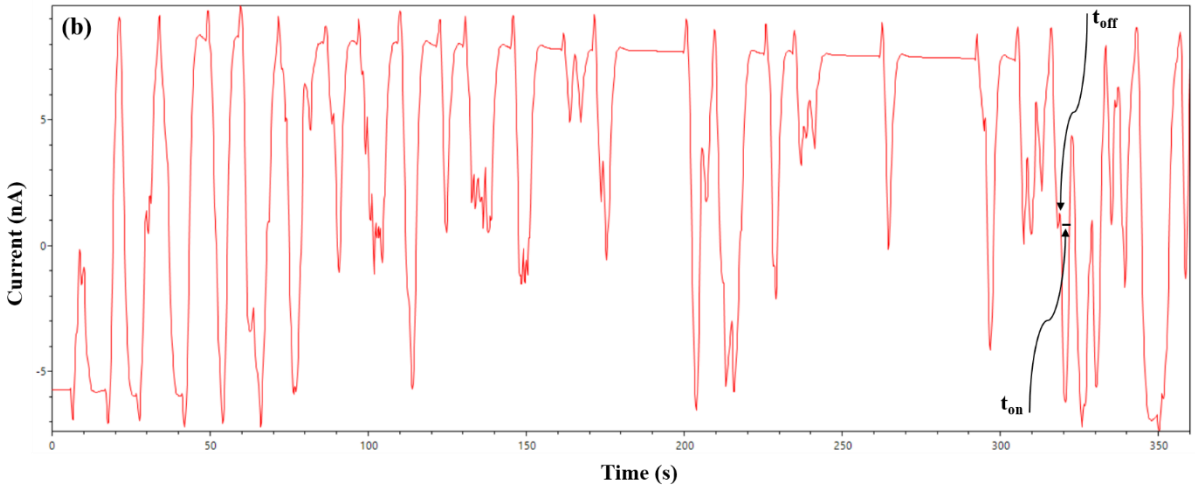


Figure 5.2. Examples of diagrams obtained when the 2D stochastic sensor was used for the screening of a) mud, and b) water samples.

Using the readings of t_{on} values inserted in the equation of calibration, there were determined the concentrations of the humic acid in the mud and water samples. Table 1 shown the results obtained using the proposed stochastic method as well as an HPLC method of analysis [4].

Table 5.1. Determination of humic acid in mud and water samples (N=10).

Sample	**Humic acid**	
	2D stochastic SWCNT/TRIMEB	HPLC [4]
Water from Techirghiol lake*	32.54±0.02	31.97±1.32
Mud from Techirghiol lake**	483.43±0.05	484.02±1.76
Mud from Dead Sea**	741.91±0.04	743.20±1.95

*pg/mL; **pg humic acid/g mud

A very good correlation between the results obtained using both methods of analysis of humic acid was observed. These correlations were proved also using the Student t-test at 99% confidence level, for each type of sample analysed. At this confidence level, the tabulated value was 4.13. The

value obtained for sapropel samples was 3.20, and for the water sample 1.93. All recorded values were lower than the tabulated value proving that there is no significant difference between the results obtained using the two methods: stochastic method and HPLC, when used for the assay of humic acid in the mud and water samples.

Recovery of humic acid in the mud and water samples was done as a second validation step. Therefore, well known amounts of humic acid were added to 10 samples of mud, and 10 samples of water, and determinations of the concentrations before and after addition of humic acid were done. The results are shown in Table 5.2.

Table 5.2. Recovery of humic acid in mud and water samples (N=10).

Sample	**%, Recovery, Humic acid**
Water from Techirghiol lake	99.98±0.01
Sapropel from Techirghiol lake	98.93±0.03
Sapropel from Dead Sea	98.20±0.03

High recovery values and very low RSD (%) values were obtained, demonstrating the accuracy and reliability of the proposed electroanalytical method, based on the use of a 2D stochastic electrode for the determination of humic acid in sapropel and water samples.

The proposed method, used to determine humic acid in sapropel and water samples, was also evaluated in terms of its environmental soundness, practicability, and applicability. The

environmental soundness indicators were determined using the AGREE approach and software, following the 12 criteria set forth in this approach.

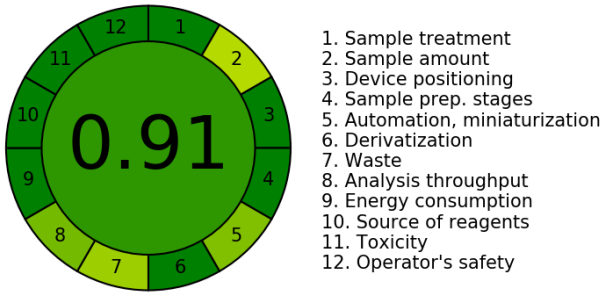


Figure 5.3. Results of the AGREE analysis for the electroanalysis of humic acid in sapropel and water samples, assuming an equal contribution from all criteria.

To evaluate the method in terms of its practicality and applicability, the Blue Applicability Index (BAGI) and a software program were used. The ten criteria considered in this method are: type of analysis, number of analytes determined simultaneously, analytical technique and required analytical instruments, number of samples that can be processed simultaneously, sample preparation, the number of samples that can be analyzed per hour, the type of reagents and materials used in the analytical method, the need for preconcentration, the degree of automation, and the sample quantity. The BAGI index results are presented in Figure 5.4.

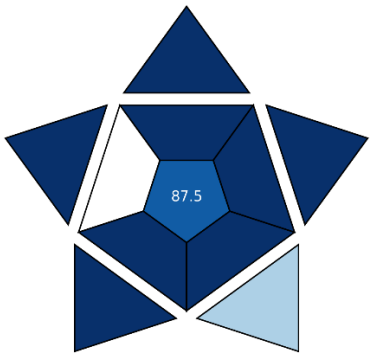


Figure 5.4. The BAGI index icon obtained when electroanalysis was used to determine humic acid in sapropel and water samples.

According to BAGI, 10 points—which is the maximum number of points—correspond to dark blue, light blue corresponds to 5 points, and white corresponds to 2.5 points. The icon (Figure 5.4.) shows that 8 of the 10 criteria were rated 10 points: the test is qualitative and confirmatory; a smartphone and a portable device were used for the test; samples can be measured simultaneously; no sample preparation is required; more than 10 samples can be analyzed per hour; all reagents are available, no preconcentration is required, and the sample volume required for analysis is less than 100 µL.

5.2. Disposable Electrochemical Sensor for Determination of Humic Acid in Sapropel and Water Samples [107]

5.2.1. Introduction

Sapropel from Lake Techirghiol, as well as sapropel from the Dead Sea, are well known for their cosmetic and therapeutic properties, most of which are associated with the presence of

humic acid in their composition. Determining the humic acid content in sapropel and in humic acid containing waters is necessary before using them as therapeutic agents, as pollutants can affect its concentration; a decrease in humic acid concentration has been observed in samples of polluted sapropelic mud and water [58–60].

To date, spectrometric analysis methods [57, 61, 62], qualitative electrochemical analysis methods [62], a stochastic analysis method [63], and chromatographic analysis methods [64] have been proposed for determining humic acid in sapropel and water samples. With the exception of the stochastic analytical method, all other methods proposed to date have required laborious sample preparation prior to analysis. Improving the limits of quantification and detection, as well as sensitivity and selectivity, is necessary for the reliable determination of humic acid in sapropel and water samples. The use of electrochemical sensors with an active surface based on nanomaterials can help lower the limits of determination and quantification, as well as increase sensitivity [63, 65–67].

The novelty of this study lies in the determination of humic acid using an electrochemical sensor based on the modification of single-walled carbon nanotubes with heptakis (2,3,6-tri-O-methyl)-beta-cyclodextrin, which increased the sensitivity of the measurements.

5.2.2. Design of the Electrochemical Sensor

Screen-printed sensors DRP-110 SWCNT were modified with a solution of heptakis(2,3,6-tri-O-methyl)-beta-cyclodextrin (1.00×10^{-3} mol L^{-1}). 20µL of this solution was dropped on the surface of the DRP-110 SWCNT and left for 3h at room temperature until utilization for

measurements. Modified sensors were able to be kept at room temperature for more than one month.

5.2.3. Response Characteristics of the Electrochemical Sensor

DPV was used for all measurements performed using the proposed disposable electrochemical sensor. The response characteristics of the sensors are shown in Table 5.1.

Table 5.3. The response characteristics of the disposable electrochemical sensor

Working concentration range (g mL^{-1})	1.00×10^{-14} - 1.00×10^{-8}
Calibration equation (<I>=μA, <C_{HA}>=g mL^{-1})	$I = 110.08 + 5.31\times10^{-11} \times C_{HA}$
Correlation coefficient	0.9976
Sensitivity (μA g^{-1} mL)	5.31×10^{-11}
Quantification limit (g mL^{-1})	1.00×10^{-14}
Determination limit (g mL^{-1})	3.00×10^{-15}
Number of data points	7

A wide working concentration range (Fig. 5.1) was recorded for the assay of humic acid, making possible its assay without a preconcentration step. The limit of quantification was determined accordingly with IUPAC recommendations [102], as the lower concentration from the working concentration range; the quantification limit is 10fg mL^{-1} – lower than those reported earlier [89-96]. The determined limit of determination was 3fg mL^{-1} – far lower than the reported limits of detection [109-115] to date.

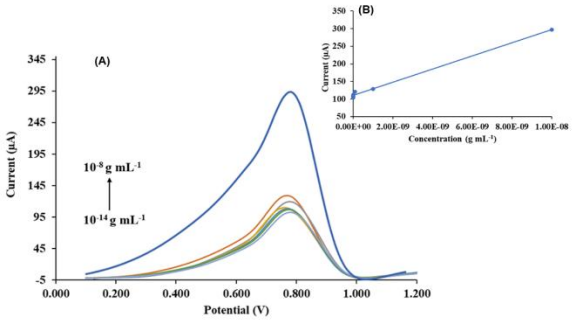


Fig. 5.5.. (**A**) Differential pulse voltammograms obtained for the calibration of the sensor (scan rate of 0.05 V s^{-1}); (**B**) calibration graph of the disposable sensor used for the assay of humic acid

Reproducibility of the sensors' design was determined as follows: ten sensors were manufactured according to the procedure described above; for each of the sensors was determined the sensitivity and the relative standard deviation vs. the average value was reported. A relative standard deviation of 1.74% was recorded, proving the good reproducibility of the design of the sensor. For its stability in time, the sensitivity was measured for 30 days, when the relative standard deviation of 2.15% was recorded, proving the good stability in the time of the proposed sensor.

5.2.4. Selectivity of the electrochemical sensor

The selectivity of the proposed disposable electrochemical sensor was determined using the mixed solution method: measurements of solutions containing both the humic acid and the possible interferent will be compared with those obtained for a solution containing the humic acid; in both solutions, humic acid will have the same concentration, while the concentration of the interferent will be ten times higher than the concentration of humic acid. Estradiol, NH_4^+, and Fe^{2+}

were studied as possible interferents. Amperometric selectivity constants were determined; their values are given in Table 5.4.

Table 5.4. Amperometric selectivity coefficients

K_{sel}^{amp}		
Estradiol	NH_4^+	Fe^{2+}
1.20×10^{-4}	1.00×10^{-4}	1.03×10^{-4}

All values recorded for the amperometric selectivity coefficient were of 10^{-4} magnitude order, proving that the proposed disposable sensor is selective versus the tested substances.

5.3. Validation of the electrochemical sensor for determining of humic acid in sapropel and water samples

Sapropel and water samples from Techirghiol Lake and the Dead Sea were used for the method validation. No preparation of the samples was done before the measurements. The results obtained for the content of humic acid in the samples were compared with those obtained using the HPLC method proposed earlier by Brondi et al. [93] (Table 5.5).

Table 5.5. Determination of humic acid in sapropel and water samples (N=10)

Sample	**Humic acid**	
	Disposable sensor	HPLC [11]
Water from Lake Techirghiol*	31.12 ± 0.13	31.97 ± 1.32
Sediment from Lake Techirghiol**	484.97 ± 0.21	484.02 ± 1.76
Sediment from the Dead Sea**	742.98 ± 0.35	743.20 ± 1.95

*pg/mL; **pg of humic acid/g of sapropel

DPV voltammograms obtained for the assay of humic acid in sapropel and water samples are shown in Figure 5.6.

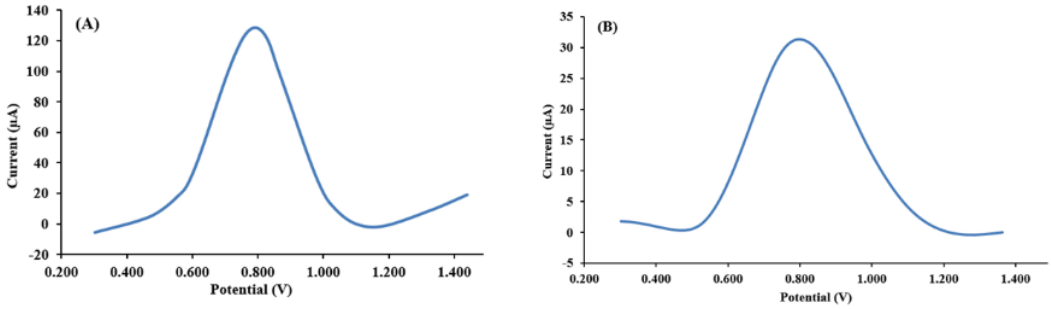


Figure 5.6. Differential pulse voltammograms obtained for the **(A)** water samples and **(B)** sapropel

There is a good correlation between the results obtained using the proposed method, based on the utilization of a disposable electrochemical sensor, and the HPLC method [64].

Stochastic and electrochemical sensors were used to determine humic acid in sapropel samples collected from Lake Techirghiol and the Dead Sea, as well as from the water of Lake Techirghiol. The sensors proved to be extremely reliable for determining humic acid in the aforementioned samples. Due to the portability of the instruments, they can be used to determine the concentration of humic acid in the sapropel and water of seas and lakes, in order to assess their quality and determine whether they meet the conditions for treatments prescribed by physicians.

CHAPTER 6

Ultrasensitive and Fast Determination of Fulvic Acid in Sapropel and water samples from Lake Techirghiol [64]

6.1. Introduction

Fulvic acid is an organic humic compound with antioxidant and anti-inflammatory properties, used in the treatment of neurodegenerative diseases [65]. Fulvic acid also has significant therapeutic potential in chronic inflammatory diseases, such as cardiovascular diseases and colitis, as well as in diabetes [66]. Sapropel [69] and the water of Lake Techirghiol are important sources of natural fulvic acid; their therapeutic effects have been studied since ancient times. Although clinics have been built around the lakes and sites where sapropel has formed, it is essential to monitor the quality of the water and mud with regard to the components that contribute to patients health [70].

To date, the following methods have been proposed for quantifying fulvic acid in various matrices: chromatographic methods [71, 72], extraction/preconcentration at the point of turbidity using nonionic and cationic surfactants with UV detection via flow injection [73], spectrophotometric methods [74, 75], and a potentiometric method based on an ion-selective electrode [75].

These methods are costly and cannot be used for on-site determination of fulvic acid. Furthermore, their limits of quantification are higher than the concentration of fulvic acid in various marine samples, such as sapropelic sediment, seawater, etc.

An electrochemical analytical method based on the use of a screen-printed graphene oxide electrode has been proposed for the determination of fulvic acid [76]. Electrochemical sensors can be used in screening tests of marine samples with much greater reliability, as well as with higher sensitivities, lower limits of quantification, and greater selectivity. Unfortunately, the only electrochemical method proposed to date [78] had a high limit of quantification (0.27 μg L^{-1}). Electroanalysis, in terms of cost, sample preparation, speed, and suitability for on-site analysis, remains the primary method for rapid and inexpensive on-site analysis of substances in the marine environment [77, 78]. Stochastic sensors represent a new class of sensors capable of performing both qualitative and quantitative analyses [79, 80]; the matrix does not influence the response of stochastic sensors, and no sample preparation is required, which facilitates real-time, on-site analysis of fulvic acid.

This paper proposes a stochastic sensor for determining fulvic acid in the sapropel and water of Lake Techirghiol; the sensor's sensitivity, limit of quantification, and selectivity will be investigated, as well as its reproducibility when used to determine fulvic acid in the sapropel and water of Lake Techirghiol. The novelty of this work lies in the use of a stochastic sensor based on reduced graphene oxide paste, decorated with gold and palladium nanoparticles and modified with quinine, for the ultrasensitive, rapid, and on-site determination of fulvic acid in the samples to be analyzed. Its use will facilitate real-time quality control of these two natural resources, and since the results can be transmitted in real time to a specialized database using the smartphone involved in data acquisition and processing, a map of its concentration can be generated, enabling immediate decisions regarding the suitability of water and sapropel for therapeutic purposes or their collection for fulvic acid extraction in specialized laboratories.

While reduced graphene oxide is an excellent matrix capable of maintaining the channels involved in stochastic measurements in a stable form, gold and palladium nanoparticles contribute to graphene conductivity, and quinine provides the channels necessary for the current development of stochastic sensors. Graphene oxide plays an important role in nanotechnology due to its tunable physicochemical properties and interdisciplinary applications in biomedical analysis; it is also used for the development of wearable sensors for continuous biomarker monitoring. Both palladium and gold nanoparticles have been shown to improve the conductivity of graphene matrices. Quinine (QN) is a new modifier proposed for the design of electrochemical sensors [85, 86]. The V-shape of quinine also promotes the formation of the channels necessary for generating the stochastic signal.

6.2. Design of the stochastic sensor

50 mg of reduced graphene oxide@Pd powder and 10 μL of Au nanoparticles were mixed with 20 μL of paraffin oil to form a paste to which 50 μL of quinine solution (1×10^{-3} mol L^{-1}) was added to provide the modified paste. The contact between the modified paste and external circuit was done using an Ag wire. The paste was placed in the 3D plastic-printed tube (Scheme 1); the plastic material of the tube is non-conductive and non-porous. The internal diameter of the working electrode was 5 μm.

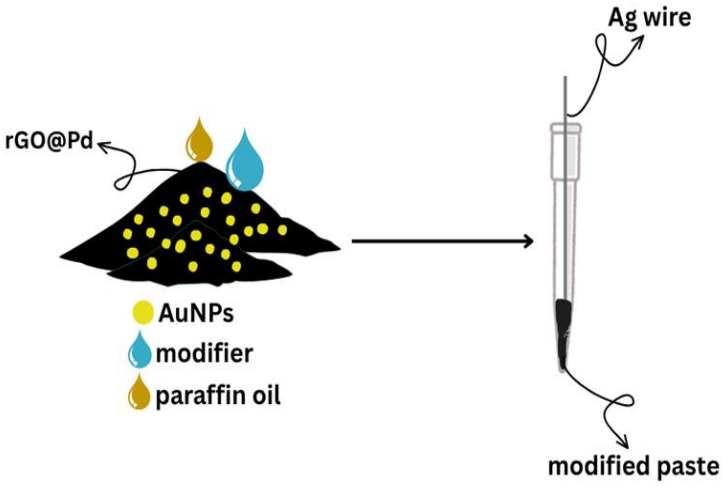


Figure 6.1. Design of the stochastic sensor.

For all measurements, a Sensit/BT minipotentiostat (PalmSens, Houten, Netherlands) was connected to a smartphone capable of transmitting the graphs and processed measurement results wirelessly to a specialized database.

Chronoamperometry was chosen as the technique. The optimal working potential was determined so as to obtain a value for t_{off} that could be reliably read both manually and automatically. The optimal value was 125 mV; at lower potential values (including negative values), no extraction of the analyte into the channel was observed (no signal was obtained); whereas, when values higher than 125 mV were applied, the t_{off} values fell within the ns range, making them impossible to read accurately. At a potential of 125 mV, fulvic acid was extracted from the solution into the channel, blocking it, while the current intensity dropped to 0 (the time required to enter the channel is called the fulvic acid signature and is read on the graph as t_{off}) (stage 1, Figure 6.2). In the second stage, redox processes take place inside the channel over a

specific period of time called t_{on}, measured on the graph between two consecutive values of t_{off} (Figure 6.2); this value is related to the concentration of fulvic acid by the following equation:

$$1/t_{on} = 0.27 + 1.97 \times 10^{8}\, C_{Fulvic\ acid}\ (r=0.9991),\ <t_{on}> = s,\ <C_{Fulvic\ acid}> = g\ mL^{-1}.$$

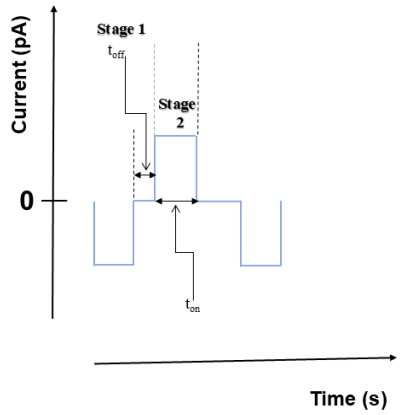


Figure 6.2. Mechanism of current generation in stochastic sensors.

Linear regression was used to determine the parameters of the calibration equation above, and this equation was used to determine the concentration of fulvic acid in the sapropel and in the water of Lake Techirghiol.

The electrochemical behaviour of the electrochemical characterisation was conducted utilizing two approaches, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) on both bare (rGO@Pd-AuNPs) and modified (QN/rGO@Pd-AuNPs) electrodes. The CV method was utilized to evaluate the electrochemical response of the QN/rGO@Pd-AuNPs. The cyclic voltammograms presented in Figure 1a were obtained in a solution of 5.0×10^{-3} mol $L^{-1}K_3[Fe(CN)_6]$ (0.1 mol L^{-1} KCl) across a potential range of -0.6 to 1.0 V, at a scan rate of 0.1 V s^{-1}, utilizing rGO@Pd-AuNPs and QN/rGO@Pd-AuNPs as working electrodes. The modified sensor demonstrates higher conductivity compared to the unmodified sensor. The findings

indicate that modifications with QN enhance the electrochemical response. Consequently, it may be assumed that the modification was successfully implemented, resulting in an improved electrochemical result.

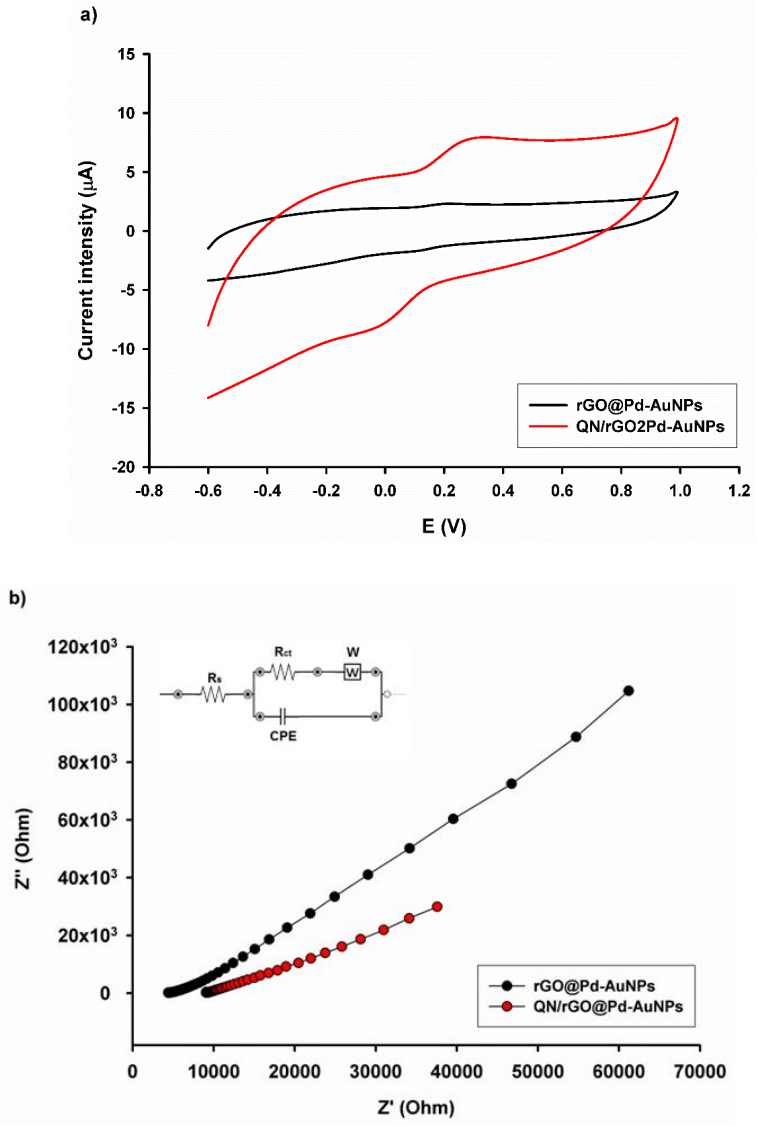
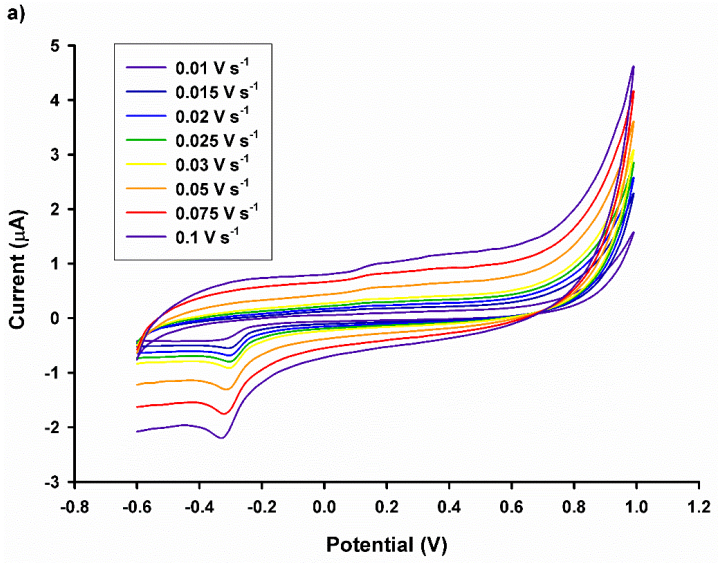


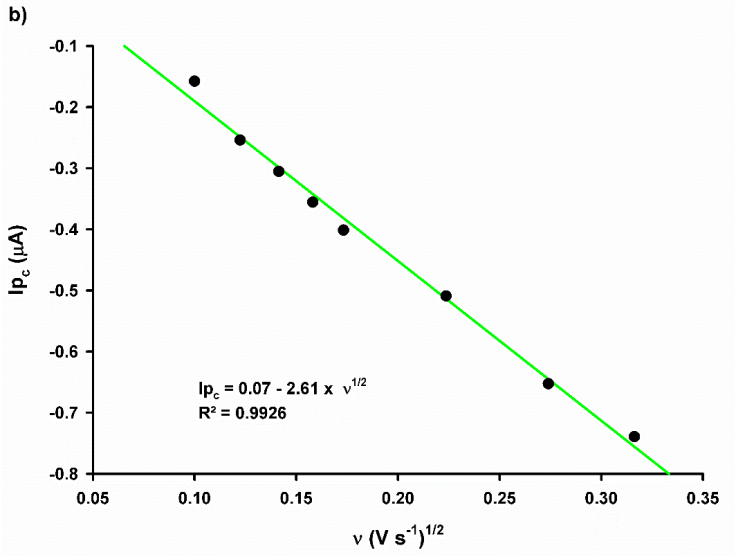
Figure 6.1. a) CVs recorded using rGO@Pd-AuNPs (black line) and QN/rGO@Pd-AuNPs (red line) in a 5.0×10^{-3} mol L^{-1} $K_3[Fe(CN)_6]$ (0.1 mol L^{-1} KCl) solution (working conditions: step potential 0.025 V; scan rate 0.1 V s^{-1}); **b)** EIS recorded for rGO@Pd-AuNPs (black line) and

QN/rGO@Pd-AuNPs (red line) in a 5.0×10^{-3} mol L^{-1} $K_3[Fe(CN)_6]$ (0.1 mol L^{-1} KCl) solution (working conditions: frequency range: 10^5 - 10^{-1} Hz). Inset: Randles equivalent circuit.

The evaluation of the interfaces of bare and modified electrodes using the EIS method was conducted within a frequency range of 10^5 to 10^{-1} Hz. The studies were conducted in a solution of 5.0×10^{-3} mol L^{-1} of $K_3[Fe(CN)_6]$ and 0.1 mol L^{-1} of KCl. Figure 6.1b illustrates the Nyquist plots for rGO@Pd-AuNPs (depicted in black) and QN/rGO@Pd-AuNPs (depicted in red). The Randles equivalent circuit, depicted in the inset of Fig. 6.2b, was selected to fit the EIS data and to determine the electron charge transfer resistance (R_{ct}) values. The W denotes Warburg impedance, the CPE signifies the constant phase element, and R_s represents the resistance of the electrolyte. The Nyquist plot reveals significant differences particularly in the processes of diffusion and electron transport. The examination of the EIS data revealed that the measured value of R_{ct} for the unmodified electrode rGO@Pd-AuNPs was equal to 2202 Ω. Following the modification of rGO@Pd-AuNPs with QN, the R_{ct} value disminished to 567.4 Ω, attributed to the higher conductivity of the material, resulting in an improved electron transfer rate at the QN/rGO@Pd-AuNPs interfaces. The EIS results correlate appropriately with the CV values.

The electrochemical behaviour of fulvic acid was investigated in a 5×10^{-3} g mL^{-1} fulvic acid solution including PBS pH 7.4, using CV with QN/rGO@Pd-AuNPs at different scan rates ranging from 0.01 V s^{-1} to 0.1 V s^{-1}, as shown in Figure 2a. An irreversible reduction reaction of fulvic acid is observed at the QN/rGO@Pd-AuNPs-modified electrode. The CV displays a reduction peak with a recorded current intensity of 0.5 μA, at about -0.29 V.


a)
0.01 V s-1
0.015 V s-1
0.02 V s-1
0.025 V s-1
0.03 V s-1
0.05 V s-1
0.075 V s-1
0.1 V s-1
Current (μA)
Potential (V)


b)
Ipc (μA)
Ipc = 0.07 - 2.61 x ν1/2
R² = 0.9926
ν (V s-1)1/2

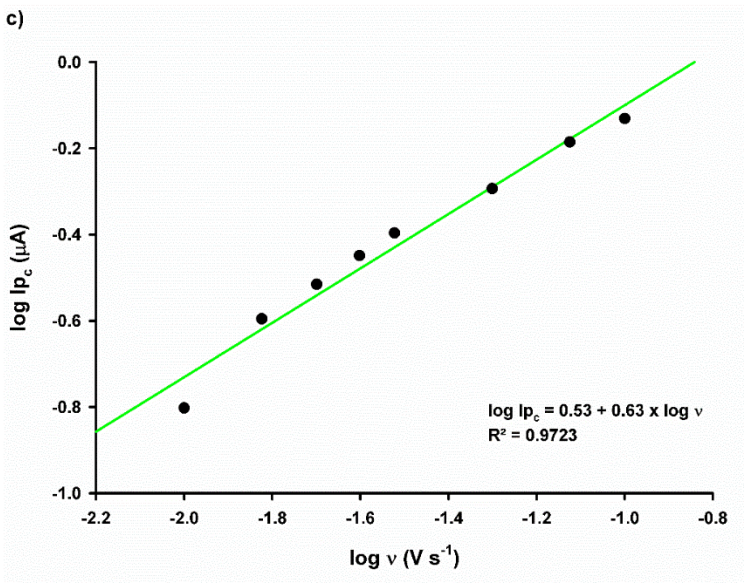


Figure 6.2. a) CVs obtained at various scan rates starting from 0.01 V/s of a solution of 5.0×10^{-3} g mL^{-1} fulvic acid in phosphate buffer saline pH 7.4 utilizing the QN/rGO@Pd-AuNPs electrode; **b)** the linear dependence of cathodic peak current vs square root of the *v* (Ip_c vs $v^{1/2}$); **c)** the linear dependence of log of cathodic peak current vs log *v* (log Ip_c vs log *v*).

We closely examined the correlations between the cathodic peak current (Ipc) and the square root of the scan rate (ν1/2), as well as between the logarithm of the cathodic peak current (log Ipc) and the logarithm of the scan rate (log ν). The regression equation demonstrates that the cathodic peak currents display a linear correlation with the square root of the scan rate: $Ip_c = 0.07 - 2.61 \times v^{1/2}$, $R^2 = 0.9926$ (Figure 6.2.b). The regression equation derived from the log Ip_c versus log ν graph is as follows: $\log Ip_c = 0.53 + 0.63 \times \log v$, $R^2 = 0.9723$. The value of the slope is 0.63, which is above the theoretical value of 0.5, indicating a mixed diffusion-adsorbtion process occuring at the electrode surface [143] (Figure 6.2.c).

6.2.1. SEM Characterization of the Active Surface of the Stochastic Sensor

Scanning electron microscopy (SEM) was employed to evaluate the morphology of the stochastic sensors (Figure 6.3). Figure 6.3a displays a heterogeneous and coarse surface texture, characteristic of the irregular distribution of graphite particles within the paste matrix. The SEM image of the quinine-modified grapheme paste, shown in Figure 6.3b, exhibits a significant modification in surface morphology compared to the unmodified one. Figure 6.3b shown a surface exhibiting a dense and entangled arrangement of quinine, forming a porous and interconnected conductive framework, on which well defined channels can be seen.

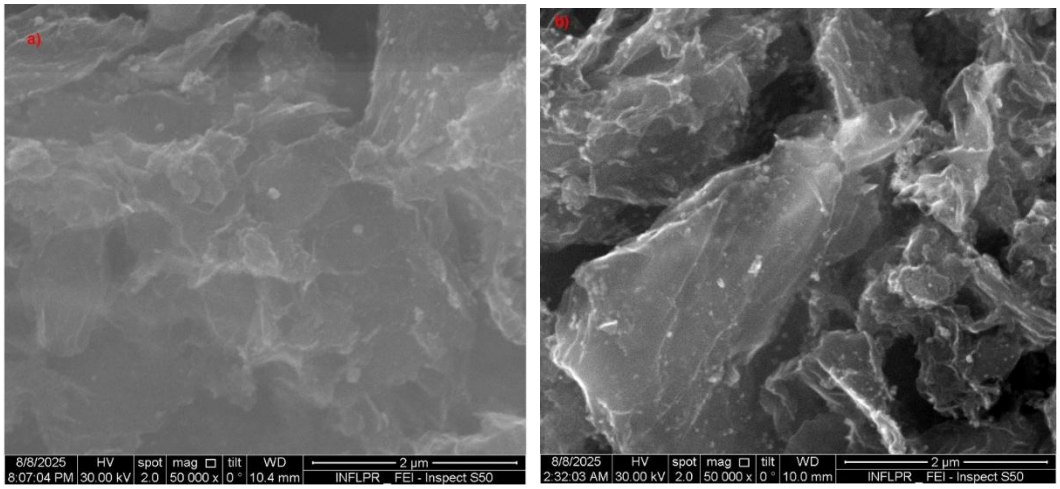


Figure 6.3. SEM images of (a) rGO@Pd-AuNPs, (b) QN/rGO@Pd-AuNPs.

6.3.Response Characteristics of the Stochastic Sensor

The response characteristics of the proposed stochastic sensor are presented in Table 6.1.

Table 6.1. Response characteristics of the stochastic sensor used to determine fulvic acid, both when using standard solutions and when using sapropel and water from Lake Techirghiol.

Type of solution	**Working concentration range (g mL^{-1})**	**Calibration equation and r***	**Signature t_{off} (s)**	**Sensitivity (s^{-1}/ g mL^{-1})**	**Limit of determination (g mL^{-1})**
Standard solutions	5.0×10^{-15}-5.0×10^{-6}	$1/t_{on} = 0.27 + 1.97 \times 10^{8}$ C; r=0.9991	0.4	1.97×10^{8}	5.0×10^{-15}
Sapropel	5.0×10^{-15}-5.0×10^{-6}	$1/t_{on} = 0.30 + 1.95 \times 10^{8}$ C; r=0.9991	0.4	1.95×10^{8}	5.0×10^{-15}
Techirghiol Lake water	5.0×10^{-15}-5.0×10^{-6}	$1/t_{on} = 0.26 + 1.97 \times 10^{8}$ C; r=0.9991	0.4	1.97×10^{8}	5.0×10^{-15}

* $<t_{on}> =$ s; $<C_{fulvic\ acid}> =$ g mL^{-1} .

The signature value determined for fulvic acid is 0.4 s. By changing the type of solution used for the calibration, small non-significant changes were recorded for the free term of the equation of calibration and also for the sensitivity when the calibration was performed using sapropel samples. A high sensitivity was recorded for a wide linear concentration range: 5.0×10^{-15}-5.0×10^{-6} g mL^{-1}. A limit of determination of 5fg mL^{-1} was determined, and its value was not influenced by the type of solution used for the calibration of the proposed stochastic sensor.

Table 6.2. Other methods used for the determination of fulvic acid.

Method/Instrument	**Limit of quantification (g/mL)**	**Reference**
Electroanalysis/Screen-printed graphene oxide electrode	0.27 μg L^{-1}	[9]
Alkaline extraction	15.3 mg mL^{-1}	[16]
Turbidimetric extraction/preconcentration by flow injection—UV detection method	5 ng/mL	[5]
Spectrophotometric analysis method	135 μg/mL	[7]
Stochastic method	5.0×10^{-15}g/mL	This paper

The lower limits of quantification were obtained using the proposed stochastic sensors, compared to previously reported limits of quantification (Table 6.2).

Ten stochastic sensors were designed and used to calibrate the proposed sensor using standard solutions, sapropel, and water from Lake Techirghiol. The variations in the sensitivities obtained were calculated, and a value of 0.02% demonstrated that the design is accurate. Calibrations were performed over the course of the remaining 29 days, and the sensitivities were

compared; a variation of 0.09% was calculated—and this value demonstrated that the proposed stochastic sensor is stable over time.

6.4. Selectivity of the stochastic sensor

The selectivity of stochastic sensors is determined by the differences between the signature values recorded for different analytes in a mixture; a difference of at least 0.2 s indicates selectivity. Humic acid, estradiol, and heparin were chosen as the main substances that may interfere. Their signature values are: humic acid (1.0 s), estradiol (2.5 s), and heparin (1.8 s). Differences greater than 0.2 s were recorded between the signatures of fulvic acid and those of humic acid, estradiol, and heparin, demonstrating that the proposed sensor is selective toward these compounds.

6.5. Validation of the ultrasensitive stochastic sensor when used for the determination of fulvic acid in sapropel and Techirghiol Lake water

All samples were analyzed on-site using the designed sensor, the portable minipotentiostat, and a smartphone. Fulvic acid was identified in the plots based on the t_{off}value (Figure 6.4, Table 6.1.), and subsequently, the t_{on}value was read and substituted into the calibration equation to obtain the fulvic acid concentration (Figure 6.3).

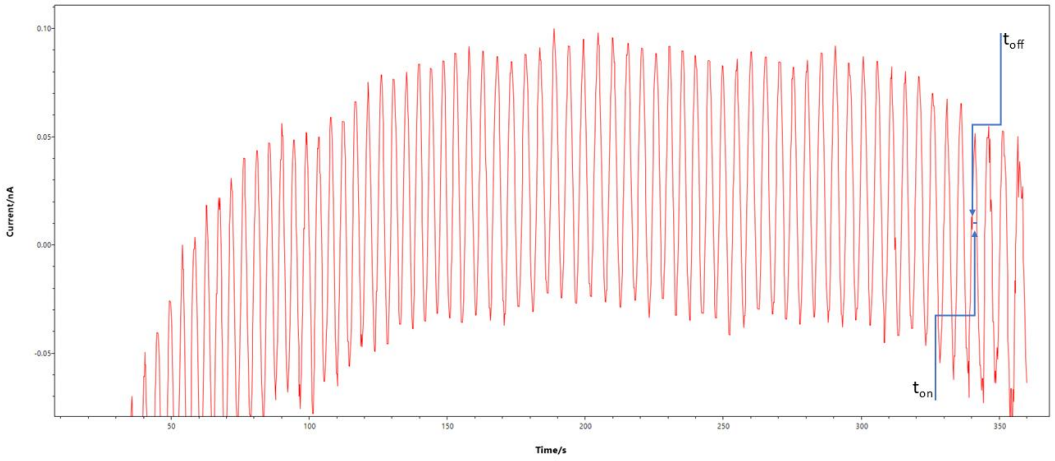


Figure 6.4. Plot obtained for sapropel screening.

The results obtained for the three samples analyzed (sediment from the Dead Sea and Lake Techirghiol, as well as water from Lake Techirghiol) were compared with those obtained using the chromatographic method proposed by Aoyama [70] (Table 6.3).

Table 6.3. Determination of fulvic acid in sapropel from the Dead Sea and Lake Techirghiol, and in water from Lake Techirghiol, using the stochastic sensor and reverse-phase HPLC (N=10).

Sample	**Stochastic method/HPLC**	**Fulvic acid ng mL^{-1}**
Sapropel from the Dead Sea	Stochastic sensor	2.07 ± 0.03
	HPLC	1.76 ± 0.32
Sapropel from Lake Techirghiol	Stochastic sensor	3.28 ± 0.04
	HPLC	3.00 ± 0.43
Water from Lake Techirghiol	Stochastic sensor	2.96 ± 0.02
	HPLC	2.20 ± 0.55
t-test for samples		3.021

Student paired t-tests were used to compare the results obtained using the proposed method for the determination of fulvic acid and the HPLC method proposed earlier by Aoyama [122] at a 99.00% confidence level, for which the tabulated theoretical t-value is 4.032. The calculated value was 3.021 (lower than the tabulated theoretical value), proving that there is no statistically significant difference in the results obtained using the two methods.

Recovery tests were also performed in order to confirm the validation of the method. Known quantities of fulvic acid were added to the three samples, and the determined concentration of fulvic acid was compared with the theoretical concentration of fulvic acid. The results of the recovery tests are shown in Table 6.4.

Table 6.4. Recovery tests for the determination of fulvic acid in sapropel from the Dead Sea, Lake Techirghiol, and water from Lake Techirghiol (N=10).

Sample	%, Fulvic Acid Recovery
Sapropel from the Dead Sea	99.96±0.03
Sapropel from Lake Techirghiol	99.95±0.03
Water from Lake Techirghiol	99.98 ± 0.02

High values were obtained for the recovery of fulvic acid in the three samples, proving again that the fulvic acid can be determined reliably in the three types of samples.

The proposed stochastic sensor can be reliably used for the determination of fulvic acid in sapropel from the Dead Sea, from Techirghiol Lake, and from water from Techirghiol Lake. The response characteristics do not change significantly when the calibration is done in different solutions (standard solutions, sapropel, water from Techirghiol Lake), proving that the matrix does not influence the determinations.

CHAPTER 7

Determination of Estradiol in Surface Waters and Related Sapropel Samples [108]

7.1. Introduction

Estradiol (E2) is known as a key bioactive regulator in female physiological processes, including ovulation, growth, the reproductive cycle, mental health, and metabolism [88]. There is evidence that E2-positive receptors in breast cancer proliferate in response to estrogen [89]; furthermore, a reduction in E2 therapy has been recommended for women undergoing bilateral oophorectomy for benign indications. Glutamatergic synapses mediate rapid synaptic transmission in the central nervous system, and these processes are influenced by the presence of E2. Furthermore, an E2 receptor has been identified, and gene expression has been analyzed [89]. E2 contributes to improving women's health, particularly during early menopause, by preventing cardiovascular diseases that may develop [90]. Furthermore, E2 is used to induce puberty in individuals with Turner syndrome [91].

Sapropel and associated waters from lakes and the sea can be a good source of E2 (E2 is typically found in the order of ng/g for water samples and in the order of ng/g for sediment samples) ; therefore, the presence and concentrations of E2 must be determined accurately. For the determination of E2, recent analytical methods include electrochemical methods [88, 94, 95], optical methods [88, 95, 96], and a method based on surface plasmon resonance (SPR) [97]. The

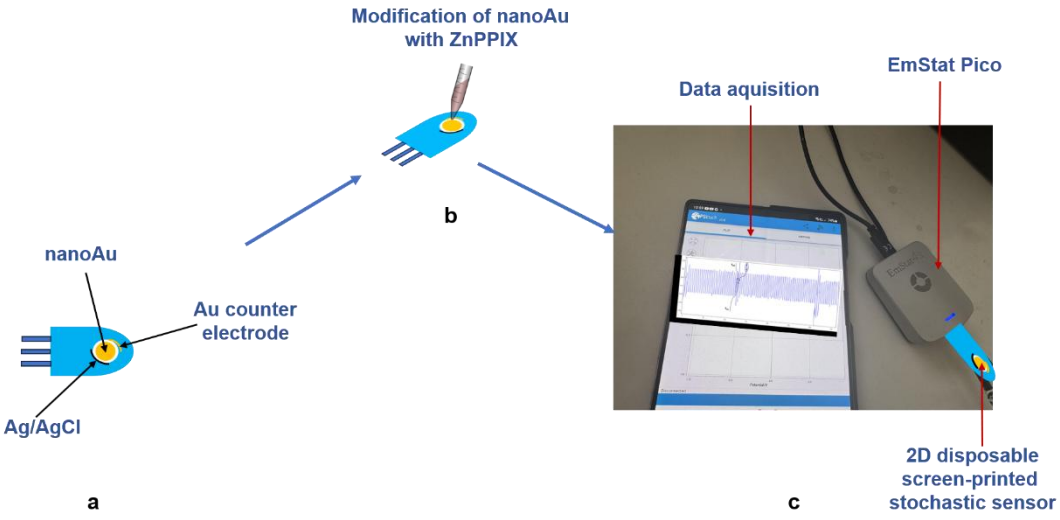
voltammetric sensor based on Au nanoparticles exhibited a detection limit of 500 nmol L^{-1}, whereas, using SPR, a detection limit of 40 nmol $L^{-1}$was achieved. Optical-fiber-based sensors [96], as well as chemiluminescence-based sensors [95], have reduced detection limits to the pmol L^{-1} level.

The use of electrochemical sensors has facilitated the achievement of low detection limits and rapid analysis at a low cost per analysis [98–102]. Furthermore, the introduction of stochastic sensors as new tools for analyzing samples such as water and sapropel has made it possible to achieve even lower detection limits and broader working concentration ranges, facilitating the determination of very low concentrations in complex matrices [103]; they are also cost-effective, facilitating the determination of one or more compounds in complex matrices without any prior sample preparation.

This paper proposes a stochastic sensor for the determination of E2 in sapropel and in associated lake/sea samples. The novelty of this work lies in the use of a stochastic sensor for the determination of E2, as well as in the development of a single-use two-dimensional stochastic sensor, which is based on the deposition of gold nanoparticles onto a ceramic material, followed by its modification with a solution containing Zn(II) protoporphyrin IX (ZnPPIX). The role of ZnPPIX was to provide the nanochannels necessary for obtaining the stochastic signal, since ZnPPIX molecules form molecular aggregates that exhibit nanochannels.

7.2. Design of the screen-printed 2D stochastic sensor

The 2D screen printed electrode were printed in the laboratory and comprises the active surface - a circle with a diameter of 4 mm covered with Au nanoparticles (deposited using cold plasma technology), a semicircle comprises of Au – as counter electrode, and a semicircle comprises of Ag/AgCl ink as reference electrode. On the top of the active surface (nanogold side) of the screen-printed electrode were poured 20µL of a 10^{-3} mol L^{-1} solution of ZnPPIX (obtained by dissolution of ZnPPIX in dimethylformamide) (Scheme 7.1).



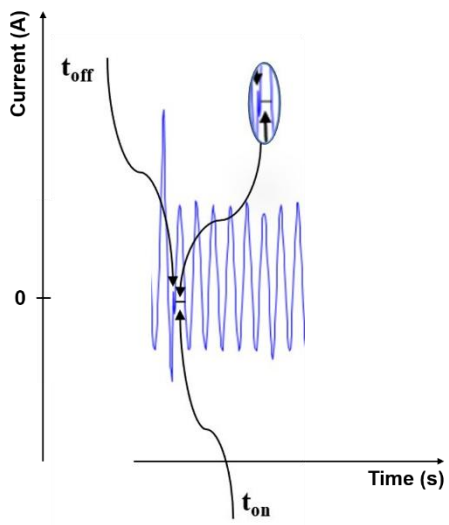
Scheme 7.1. a. 2D screen-printed electrode; b. Modification of the 2D screen-printed electrode; c. Set-up measurements using the 2D disposable screen-printed stochastic sensor.

For the adsorbtion of ZnPPIX onto the surface of the sensor, after the addition of the ZnPPIX solution, the sensor was left for 3h at room temperature, covered with Al paper (the covering with Al paper was done due to the light sensitivity of ZnPPIX). The thickness of the

ZnPPIX layer was determined to be 3nm. After that, the sensor was coupled to the EmStat Pico, and the data acquisition was done using a Samsung fold smartphone (Scheme 7.1).

7.3. Stochastic Method

A potential of 125 mV vs Ag/AgCl (reference electrode) was applied on a chronoamperometric technique. This value of potential was optimesed: lower values than 125mV for the applied potential will give no stochastic signal (molecules cannot be extracted into the channels), while for hugher values than 125mV, the signatures were lower than 0.01s, making the reading not reliable. The current shape was analysed, and the values t_{on} and t_{off} were determined. The current development for stochastic sensors is based on the nanochannel conductivity: by application of the 125 mV potential vs Ag/AgCl, the molecules from the solution are extracted one by one from the solution inside the nanochannel – on the first stage, the molecules may block partial or total the nanochannel, making the current value to drop to zero until the molecule is getting inside the nanochannel (the time needed to get inside the channel is called the signature of the molecule, and is measured while the current is having the constant value of zero or very closed with zero; this parameter is marked on the diagram as t_{off}); inside the channel there are redox processes taking place, the molecule may change its charge and oxidation state (the time needed for this process is marked in the diagram as t_{on}, and it is connected with the concentration of the molecule in the solution) (Scheme 7.2).



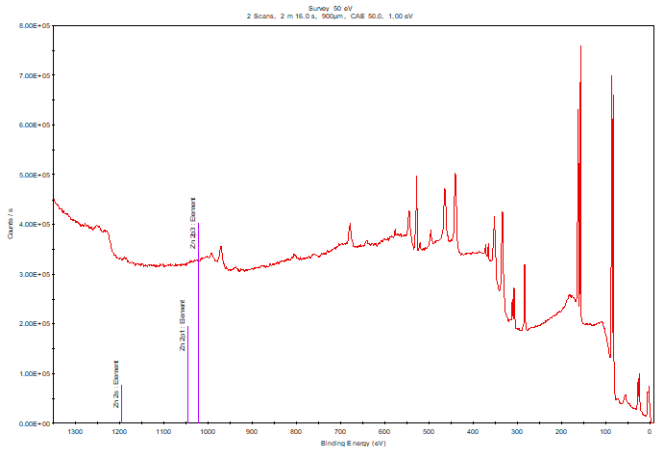
Scheme 7.2. Reading of t_{off} and t_{on} values on the recorded diagram obtained using the 2D disposable screen-printed stochastic sensor.

Linear regression method was used to find the parameters of the calibration equation: $1/t_{on} = f(C_{E2})$. Samples of mud and water were pored onto the surface of the 2D disposable screen-printed stochastic sensor. The signature of E2 was used for its identification into the diagram (Scheme 2), and the immediate t_{on} value was read and substituted into the calibration equation to obtain the E2 concentration. All data acquisition was done using a Samsung fold smartphone able to automatically measure the signature values and compare it with the signature of E2, and also able to automatically measure the t_{on} value and convert it into the concentration of E2. All measurements were done at 25°C; no modification of sensors' parameters (sensitivity, intercept) was obtained on a temperature range between 18°C and 32°C.

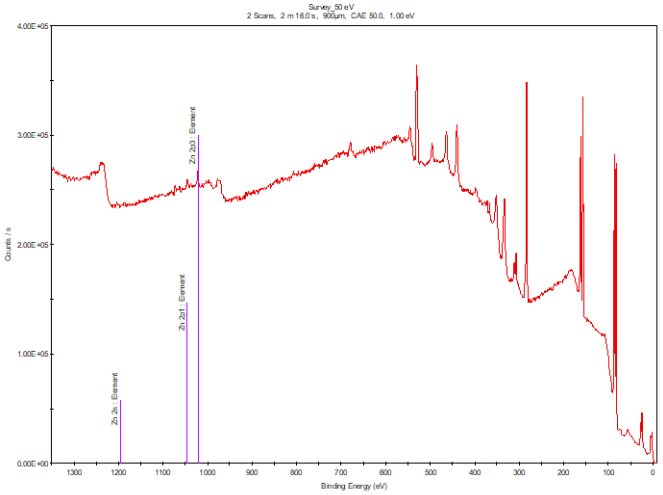
7.4. Surface characterisation for the 2D disposable screen-printed stochastic sensor

Surface characterisation was performed using the following surface analysis tools: X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM) and SEM-EDS mapping.

Figure 7.1. **a**) and **b**) present the wide-scan spectra of bare and modified sensors, respectively. The appearance of a peak at 1022 eV in the spectrum of the modified sensor suggests the presence of a Zn compound, thus suggesting modification of the sensor with ZnPPIX.



(a)

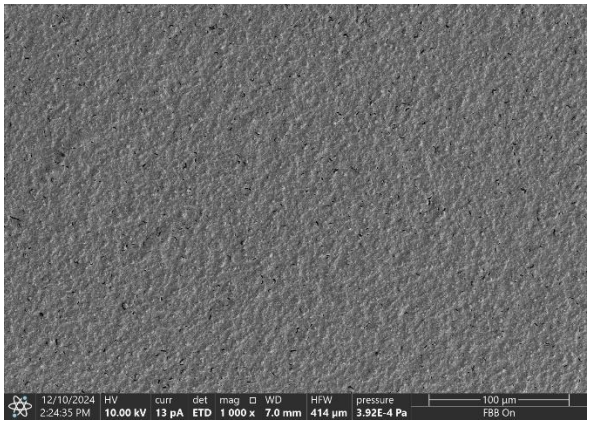


(b)

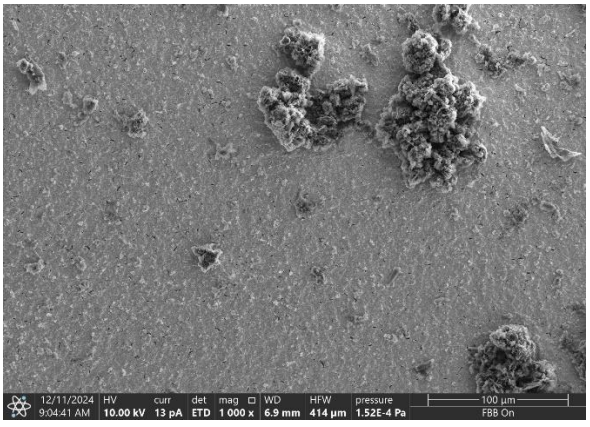
Figure 7.1. Wide-scan spectra of the unprocessed (**a**) and modified (**b**) screen-printed 2D electrode.

By comparing also, the high resolution XPS spectra of bare and modified sensors, the presence of Zn and N peaks in the modified sensor is observed along with a significant increase of C1s counts compared to the bare one. As these atoms are constituents of ZnPPIX ($C_{34}H_{32}N_4O_4Zn$), these experimental results confirm the presence of zinc protoporphyrin on the modified sensor. Concerning the modified sensor, the lower counts of gold (coming from the underlying electrical contact) are due to attenuation of the XPS signal passing through the ZnPPIX layer.

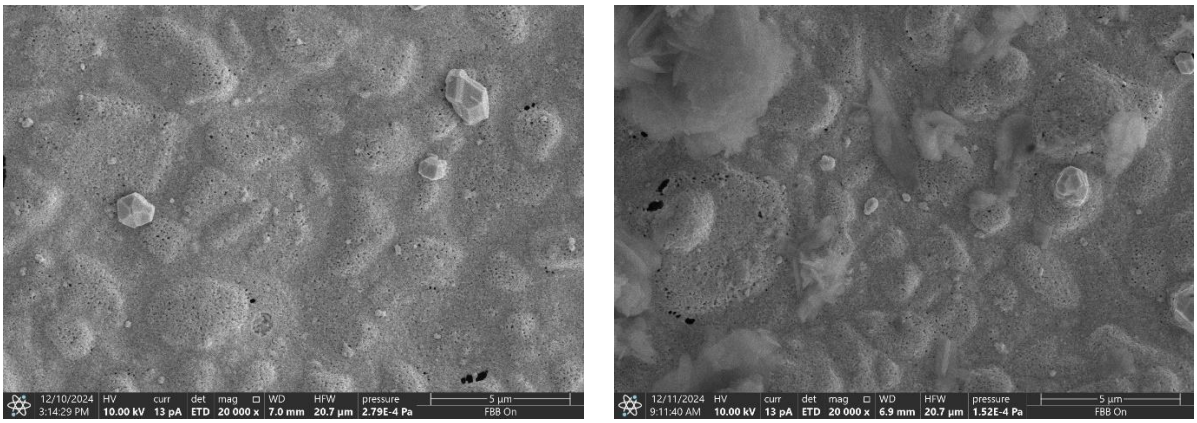
Figure 7.2. displays the SEM images of the bare (**a**), (**b**) and modified (**c**), (**d**) electrodes. When comparing the two, the unmodified electrode has a mostly plane gold surface that also contains a few larger gold particles, while the modified electrode has in addition numerous molecular aggregates of ZnPPIX molecules; the nanochannels are also visible in Figure 2 (**c**).



(a)



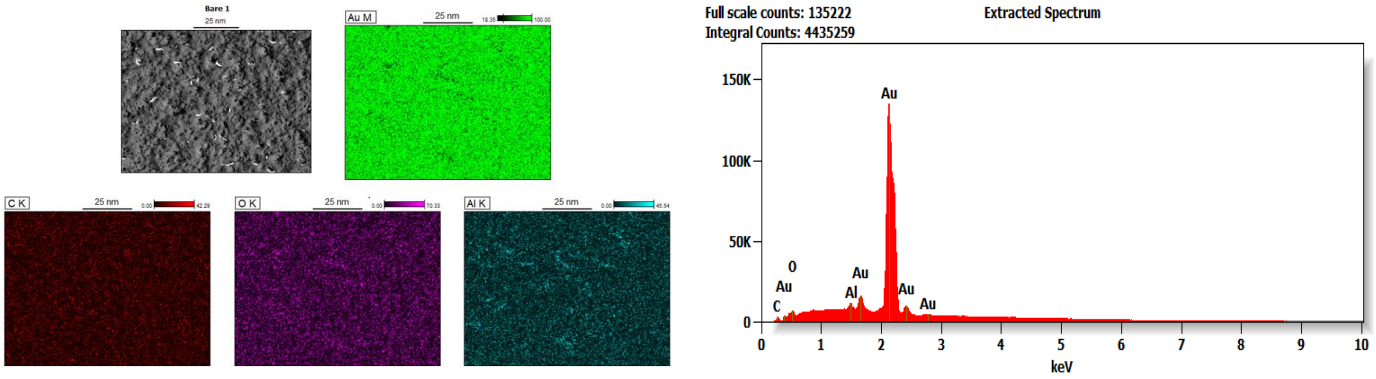
(c)



(b) **(d)**

Figure 7.2. SEM images for the bare electrode at 1000× (**a**) and 20000× (**b**) magnifications, and for the modified electrode at 1000× (**c**) and 20000× (**d**) magnifications.

The chemical composition for the active surface of the sensor was carried out by SEM-EDS analysis on the same system as the SEM microscopy. Figure 7.3 shows a typical EDS map (a) and spectrum (b) for the initial, unmodified, nanogold surface, while the measured chemical composition is presented in Table 7.1. It can be seen from both that, as expected, the surface comprised of a single, homogenous mass of gold, with only small amounts of other contaminants being detected.



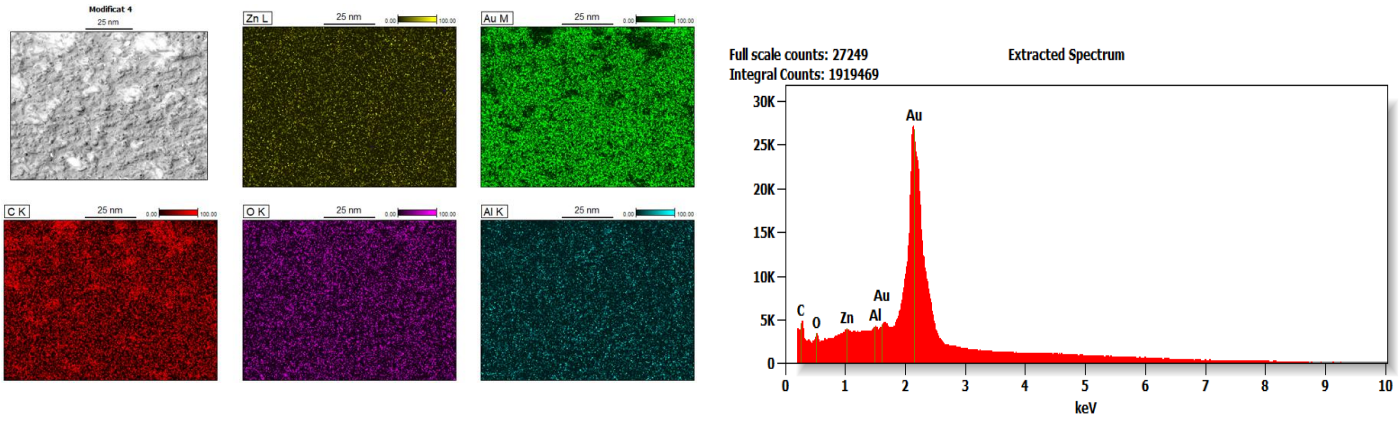
(a) **(b)**

Figure 7.3. The EDS map (**a**) and spectra (**b**) of the unmodified nanoAu surface.

Table 7.1. Chemical composition of the unmodified nanoAu surface.

Element	Line type	Weight (%)	Atomic (%)	Normalized weight (%)
C	K	0.30	4.07	0.30
O	K	1.06	10.96	1.06
Al	K	0.42	2.56	0.42
Au	M	98.23	82.41	98.23

Meanwhile, Figure 7.4 contains a typical EDS map (**a**) and spectrum (**b**) for the modified, nanoAu surface, after the ZnPPIX deposition. The measured chemical composition is also shown in Table 7.2. Same as before, the surface comprised a single, homogenous mass of gold, along with small amounts of contaminants; however, the presence of Zn as well as the detected increase of C atoms, confirms the successful deposition of the ZnPPIX solution.



(a) **(b)**

Figure 7.4. EDS map (**a**) and spectra (**b**) of the modified nanoAu surface with ZnPPIX.

Table 7.2. Chemical composition of the nanoAu surface after its modification with ZnPPIX.

Element	Line type	Weight (%)	Atomic (%)	Normalized weight (%)
C	K	4.91	41.66	4.91
O	K	0.99	6.29	0.99
Al	K	0.63	2.38	0.63
Zn	L	1.20	1.88	1.20
Au	M	92.27	47.79	92.27

7.5. Response characteristics of the 2D disposable screen-printed stochastic sensor when used for the assay of estradiol

The signature of E2 was 0.5s. The sensor can be used on a wide working concentration range, from 5×10^{-18} to 5×10^{-2} g mL^{-1}, with a sensitivity of 3.01×10^{8} $s^{-1}g^{-1}mL$, and a limit of determination

(which accordingly with IUPAC guidelines is the lowest concentration from the working concentration range) of 5×10^{-18} g mL^{-1}.

The equation of calibration was:

$1/t_{on} = 0.50(\pm0.03) + 3.01(\pm0.02)x10^{8}C_{E2}$, and a r value of 0.9998, when 10 measurements were performed.

The selectivity of the proposed sensor was determined versus other possible ions/compounds present in the mud and associated water samples, like: Fe^{3+}, Ca^{2+}, Mg^{2+}, humic acid, fulvic acid. Because, the selectivity criterium for the stochastic sensors is a difference of 0.2s between the signature of the E3 and the signature recorded for the possible interferences, the signatures of Fe^{3+}, Ca^{2+}, Mg^{2+}, humic acid, fulvic acid, estriol, ethinylestradiol (Table 7.3).

Table 7.3. Signatures obtained for the estradiol and possible interferences.

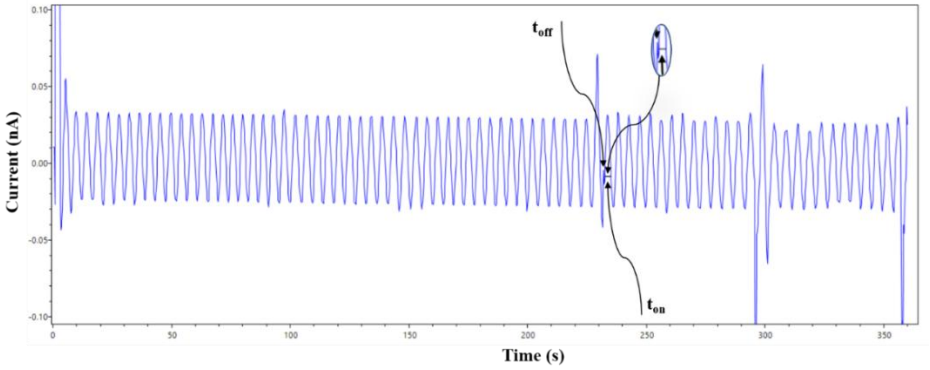
Analyte	Signature(s)
Estradiol	0.5
Fe^{3+}	0.9
Ca^{2+}	1.3
Mg^{2+}	1.1
Humic acid	2.3
Fulvic acid	1.7
Estriol	2.7
Ethinyl estradiol	3.2

The results obtained for the signatures of Fe^{3+}, Ca^{2+}, Mg^{2+}, humic acid, fulvic acid, estriol, ethinylestradiol proved that the proposed 2D disposable screen-printed stochastic sensor is selective.

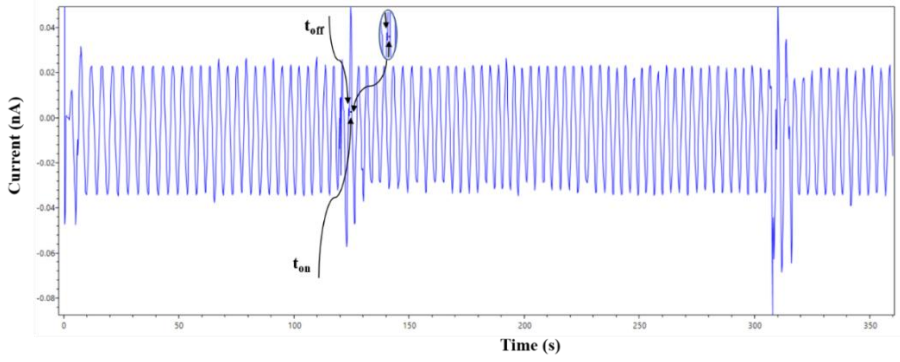
By comparing these results with those obtained using other electrochemical and optical sensors proposed earlier for the assay of E2 [146,154-156], a lower limit of determination was recorded when the 2D disposable screen-printed stochastic sensor was used, also the working concentration range was wider, and the sensitivity of the measurements higher. A higher selectivity was recorded by using the 2D disposable screen-printed stochastic sensor.

7.5.1. Determination of Estradiol in Surface Water Samples and Related Mud Samples

The mud and water samples were used as picked up from the Techirghiol lake and the Dead Sea. Measurements were done on-site in the Techirghiol lake and Dead Sea. Diagrams were recorded (**Figure 7.4**), and readings of t_{off} values were done to identify the E2.



(a)



(b)

Figure 7.4. Diagrams obtained when the 2D disposable screen-printed stochastic sensor was used for the screening of **a**) water, and **b**) mud samples.

The results obtained using the 2D disposable screen-printed stochastic sensor for the assay of E2 in the Techirghiol water and mud and Dead Sea mud were compared with those obtained using an ELISA kit (Table 7.4).

Table 7.4. Determination of estradiol in sapropel and water samples (N=10).

Sample	**Estradiol**	
	Single-use, screen-printed 2D stochastic sensor	ELISA kit
Water from Lake Techirghiol*	2.51 ± 0.01	2.00 ± 0.50
Sediment from Lake Techirghiol**	3.11 ± 0.02	3.00 ± 0.20
Sediment from the Dead Sea**	2.25 ± 0.03	2.10±0.25

*ng/mL; **ng E2/g sapropel

A Student t-test at 99% confidence level was performed (tabulated value 4.13); for the mud samples a value of 1.05 was obtained, while for the water sample a value of 1.02 was obtained. All recorded values were lower than the tabulated value proving that there is no significant difference between the results obtained using the two methods: stochastic method and ELISA, when used for the assay of E2 in the mud and water samples.

Recovery of E2 in the mud and the associated water samples was done as a second validation step. Different known amounts of E2 were added to 10 samples of mud, and 10 samples of water, and determinations of the concentrations before and after addition of humic acid were done.

The results are shown in Table 7.5.

Table 7.5. Recovery of estradiol in mud and water samples (N=10).

Sample	**%, Recovery, Estradiol**
Water from Lake Techirghiol	99.98 ± 0.02
Sapropel from Lake Techirghiol	99.95 ± 0.02
Sediment from the Dead Sea	99.02 ± 0.05

The results presented in Table 7.5 demonstrated that E2 was recovered with values close to 100.00% and with relative standard deviations of less than 0.10%.

Both the first and second validation stages demonstrated that the single-use, screen-printed 2D stochastic sensor can be reliably used to determine E2 in sapropel samples and associated water samples.

The screen-printed 2D stochastic sensor was designed, characterized, and validated for the determination of estradiol in sapropel samples collected from Lake Techirghiol and the Dead Sea, as well as in water from Lake Techirghiol.

Conclusions

General conclusions

Ultrasensitive and ultrafast electrochemical and stochastic sensors were proposed for the determination of hazardous substances like heavy metals in water and sapropel, before their utilization for any type of therapeutic purpose, as well as for the determination of few active compounds like humic acid, estradiol, and fulvic acid. A prescreening of water and mud for persistent organic pollution was also developed in order to establish their presence in mud and water samples. All tools and methods were characterized and validated using real samples collected from Techirghiol Lake, and Dead Sea.

Stochastic sensing strips were used reliably for on-site screening test of sapropel in order to assess its pollution with Cd and Pb. The response characteristics do not change significantly when the calibration is done using standard solutions, and sapropel. The main advantages of the sensing strip are the on-site analysis of Cd and Pb in sapropel, no sample processing of sapropel is needed, and Cd and Pb can be reliable determined at ultra trace levels in sapropel. The main feature of the proposed stochastic sensing strip is its utilisation as an ultrasensitive tool for the on-site screening of sapropel from the Techirghiol Lake, helping the authorities to assess if the sapropel is contaminated or not, and if it can be used for cosmetics and therapeutics procedures.

A screening for persistent organic pollutants (polychlorinated biphenyls and organochlorine pesticides) in the water and sludge of Techirghiol Lake was performed using gas chromatography with an electron capture detector to determine a possible contamination of the lake and sludge with persistent organic pollutants. The findings must warn the authorities regarding the presence of

these contaminants, as they can influence the health of the population using them for therapeutics. Therefore, a regular control of the quality of water and sludge in Techirghiol Lake must be done using such a screening test to avoid the accumulation of contaminants and to clean the water and sludge before their utilization for therapeutics. Also, further determinations using GC-MS must be done to confirm the presence of pesticides.

Stochastic and electrochemical sensors were used for the determination of humic acid in mud samples picked up from Techirghiol lake and from the Dead Sea, and also from the water of Techirghiol lake. The sensors proved to be used highly reliable for the determination of humic acid in the specified samples. Due to instrumentation portability, its feature is to be used for the assay of the concentration of humic acid in the mud and water of sea and lakes in order to determine the quality of them, and if they qualify for the treatments prescribed by the medical doctors.

A stochastic sensor was reliably used for the determination of fulvic acid in sapropel from the Dead Sea, from Techirghiol Lake, and from water from Techirghiol Lake. The response characteristics do not change significantly when the calibration is done in different solutions (standard solutions, sapropel, water from Techirghiol Lake), proving that the matrix does not influence the determinations. The main feature of the proposed stochastic sensor is its utilisation as an ultrasensitive tool for the on-site screening of sapropel from the Dead Sea and Techirghiol Lake and of the water from Techirghiol Lake to find in real time the concentration of fulvic acid; this will help the authorities to know if the sapropel and water have the required characteristics needed in the therapeutics. Furthermore, given the possibility of the stochastic sensor being used for more than one analyte analysis, the sensor can be calibrated for more substances of therapeutic interest found in sapropel and Techirghiol Lake, making possible a full quality control.

A 2D disposable screen-printed stochastic sensor was designed, characterised and validated for the determination of estradiol in mud samples picked up from Techirghiol lake and from the Dead Sea, and also from the water of Techirghiol lake. The instrument used for all measurements as well as the smartphone allowed the on-site determination of estradiol in mud and associated water samples. Therefore, its features are its utilisation for estradiol determination in mud and sea/lake waters in order to establish the content of estradiol, and prevent utilisation of mud and lake/sea water where therapeutic effects may affect the health of population.

Original contributions

The novelties of the combined 2D disposable stochastic strip for the on-site simultaneous assay in real time of Cd(II) and Pb(II) ions versus other sensors based on graphene material are the recognition principle of the two metals (stochastic mode which allow reliable molecular recognition and quantification), as well as its design - the working electrode was designed using graphene immobilisation on silk (using cold plasma technique), and modified with α-cyclodextrin, and it was accompanied by a reference electrode (Ag/AgCl), and an auxiliary electrode (C wire). Novelty is given also by the on-site simultaneous assay of Cd(II) and Pb(II) in sapropel. The graphene was modified with α-cyclodextrin (α-CD), because α-CD has the necessary channel for stochastic determinations. The graphene has the role of the matrix for the designed working electrode on the sensing strip, and it can keep the channel of α-cyclodextrin in a good shape.

The novelty of the prescreening test used for the determination of persistent organic pollutants is given by the sampling performed for the water and sludge analysis as well as by their determination from water and sludge from a salty lake that are used for medical treatments.

A stochastic sensor was proposed for the determination of humic acid in mud samples. The novelty is the design of the 2D stochastic sensor used for the assay of humic acid in the mud as well as the electroanalysis of humic acid in the mud samples picked up from Techirghiol lake and from the Dead Sea, and in the water taken from Techirghiol lake. Heptakis(2,3,6-tri-O-methyl)-beta-cyclodextrin solution ($1x10^{-3}$mol L^{-1}) was used to modify a surface of a screen printed electrode of which the active surface was covered with single walled carbon nanotubes (SWCNT). The utilisation of the cyclodextrin facilitated very stable channels needed for the stochastic measurements.

The novelty of the electrochemical disposable sensor used for the determination of humic acid it its design that used single-walled carbon nanotubes modified with a new cyclodextrin: heptakis(2,3,6-tri-O-methyl)-beta-cyclodextrin which increased the sensitivity of the measurements bringing the detection limit for the assay of humic acid to fg mL^{-1}.

The novelty of the stochastic sensor designed for sapropel is its design: the sensor is based on reduced graphene oxide paste decorated with gold and palladium nanoparticles and modified with quinine for the ultrasensitive, fast, on-site determination of fulvic acid in sapropel and Techirghiol Lake water. Its utilisation will facilitate the real-time quality control of sapropel and water, and taking into account that the results can be sent immediately to a specialised database using the smartphone involved in data acquisition and processing, one can have a map regarding its concentration, making possible the immediate decision-taking regarding the possibility of using the water and sapropel for therapeutics or picking it up for the extraction of fulvic acid in specialised laboratories.

The novelty of the stochastic sensor proposed for estradiol assay in mud and associated lake/sea samples is related to the contruction of the 2D disposable stochastic sensor, which is based on deposition of gold nanoparticles on a ceramic material followed by its modification with a solution containing Zn (II)protoporphyrin IX (ZnPPIX). The role of ZnPPIX was to provide the necessary nanochannels needed to obtain the stochastic signal, as the molecules of ZnPPIX are forming molecular aggregates presenting nanochannels.

Future perspectives

The new devices developed in this thesis were validated for the assay of two heavy metals, persistent organic pollutants and three very important active substances: fulvic acid, humic acid,

and estradiol. First of all their application for on-site analysis will benefit the therapeutics because they will allow in real time to find if the mud and water are clean, and if they have the required concentrations of fulvic acid, humic acid, and estradiol.

An extension of measurements using the sensing strips of more heavy metals will facilitate building a map in real time regarding the quality of mud and water. Also, it is a real need to have sensing strips developed for persistent organic pollutants, too. An extention of the list to other substances of therapeutic importance is needed, and will done further using the sensing strips concept for stochastic sensors design.

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Annex1 Participation in national and international conferences

1. Rosca Adrian Comin, **Andreea-Elena Dorneanu**, Recent Biopharmaceutical Aspects of Transdermal Drug Delivery - **poster**, MED-FARM LAB Galati, Octombrie 06-08.
2. **Andreea-Elena Dorneanu**, Raluca-Ioana Stefan-van Staden, Catalina Cioates Negut, Gabriela Stanciu, Determination of Estradiol in Sapropel and Water using a New Stochastic Microsensor, - **poster,** 23rd Romanian International Conference on Chemistry and Chemical Engineering Constanța - Mamaia, ROMANIA, September 4 – 7, 2024.
3. **Andreea-Elena Dorneanu**, Raluca-Ioana van Staden, Catalina Cioates Negut, Gabriela Stanciu, Determination of humic acid in sapropel using a new stochastic sensor – **poster** PN8, INTERNATIONAL CONFERENCE ″CHIMIA″ 2024, NOMARES Workshop, 30th of May – 1st of June 2024, Constanta, Romania, ISSN 2360-3941, p. 139.
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1. Fast analysis of humic acid in mud and water samples
   RI Stefan-van Staden, **AE Sandu Dorneanu**, C Cioates Negut, G Stanciu
   J. Electrochem.Soc., 171, 117515, 2024 **Q2, FI = 3.3**

2. Disposable electrochemical sensor for determination of humic acid in sapropel and in water samples
   AE Sandu Dorneanu, RI Stefan-van Staden, C Cioates Negut
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3. Determination of estradiol in surface waters and related mud samples
   AE Sandu Dorneanu, RI Stefan-van Staden, C Cioates Negut, G Stanciu, A Dan
   Microchem. J., 214, 114067, 2025 **Q1, FI = 5.1**

4. Ultrasensitive and fast determination of fulvic acid in sapropel and in the Techirghiol Lake water
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5. 2D Disposable stochastic strip for ultrasensitive assay of Pb(II) and Cd(II) in sapropel
   AE Sandu Dorneanu, RI Stefan-van Staden, DC Gheorghe
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6. Preliminary Screening of Persistent Organic Pollutants in the Water and Sludge of Techirghiol Lake Using GC-ECD
   AE Sandu Dorneanu, RI Stefan-van Staden, G Stanciu, D Danilov, HY Aboul-Enein
   Journal of Environmental Science and Health, Part B, 00, 000, 2026; https://doi.org/10.1080/03601234.2026.2685438 **Q4, FI = 1.8**

Cite score: 21.1