NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY POLITEHNICA BUCHAREST

FACULTY OF CHEMICAL ENGINEERING AND BIOTECHNOLOGIES
DOCTORAL SCHOOL

DOCTORAL THESIS

Modern methods of analysis of marine extracts used in cosmetic and pharmaceutical products

PhD Student: Drăgoi (cas. Brînza) Andreea
Coordinator: Prof. Dr. habil Raluca-Ioana van Staden

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This PhD thesis contains: 106 pages, 175 references, 21 tables and 22 figures.

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INTRODUCTION

Utilization of drugs with active substances extracted from marine sources became an excellent practice in the past years. All these drugs based on marine sources proved to have better effects on health, and reduced side effects. Marine algae, jellyfish, stingray are valuable sources for fatty acids, proteins, hyaluronic acid, venom, heparin and they must be exploited for the content on these substances.

Of main importance is determination of these compounds directly from these marine sources, especially because the quantities may vary between the analyzed algae, jellyfish, stingray. One should have reliable methods of determination for these compounds which can be used for on-site analysis in real time, so that one can decide if the marine source it is or it is not a good source for extraction of a specific substance which can be function as active substance in the drugs.

Another extremely important issue which must be taken into account is the pollution of water, and the capacity of algae, jellyfish, and stingray to accumulate heavy metals from the sea water. Determination in real time, on site of heavy metals from algae, jellyfish and stingray will indicate any contamination of these marine sources, and will allow first the water decontamination as well as the cleaning of the marine sources using biological clean methods, before any extraction of active compounds is taking place.

The main objective of the thesis was to design new methods of analysis for marine extracts and marine sources, able to determine different types of active substances that can be extracted from these sources in order to design new drugs which will be more efficient, and with lower side effects. While heavy methods were identified in these sources using inductive coupled plasma (ICP) spectroscopic methods in these marine sources, the secondary extremely important objective was to determine from these sources the heavy metals using fast ultrasensitive on site methods of analysis.

The proposed objectives were fulfilled because reliable new tools and screening methods were designed for the determination of eicosapentaenoic acid and docosahexaenoic acid in the liver and meat of stingray as well as in the algae, determination of heparin in jellyfish, and determination of hyaluronic acid in the stingray liver. The ICP method was used for assay of heavy metals from different marine sources, while a sensing strip was developed for fast assessment of Cu (II), and Zn (II) in different marine sources in order to determine in real time if they are clean or not, if they can or they cannot be used for the extraction of the proposed active substances.

Different designs of stochastic sensors were proposed so that high sensitivities and selectivities were achieved, while the fast real-time measurements were done on-site.

CHAPTER 1.
MARINE ALGAE, STINGRAY AND JELLYFISH AS MARINE SOURCES OF ACTIVE COMPOUNDS FOR DRUGS AND COSMETICS

Marine sources are having a high potential for providing different active compounds. From old times, people used from the oceans and seas extracts for treatment of different skin diseases, and not only [1].



Figure 1.1. Marine algae

The increasing of the capacity of analysis of different algae, and fish samples, from optical methods to high resolution spectroscopy, and different chromatographic and extraction methods accompanied by detectors like mass spectrometers, made possible the identification of the substances of pharmaceutical and cosmetic interest which are found in algae, stingray, and jelly fish.

From far, algae are the most studied of the marine sources for pharmaceutical and cosmetic compounds. To date, natural antioxidants which were used in pharmaceutical, food, and cosmetics were extracted from the algae. The algae are rich reservoirs of polyphenols, carotenoids, vitamins, phycobiliproteins, as well as of polysaccharides. Anti-inflammatory compounds found in algae are able to modulate several inflammatory mediators.

The algae are also a great source of long-chain polyunsaturated fatty acids like eicosapentaenoic acid ethyl ester (EPA), and docosahexaenoic acid ethyl ester (DHA) [5,6].

The benefits of EPA and DHA for cardiovascular health were first reported in the 70s. Since then, research has shown benefits in many other conditions, including metabolic, inflammatory and neuropsychiatric disorders, based on their anti-inflammatory and antioxidant effects.

Stingray is also an excellent source for different active compounds used in pharmaceuticals and cosmetics. EPA and DHA were also found in stingray meat and liver, and therefore it become a valuable extraction source for EPA and DHA. Hyaluronic acid used in many cosmetic products is found in variable quantities in stingray [7].

Jelly fish synthesize three main groups of biomolecules: proteins (e.g., collagen), fatty acids, and crude venom presented in the stinging cells (nematocites). Jelly fish is also a good source of heparin. Determination of heparin in jelly fish is essential before its extraction from jelly fish.

Mapping of algae, stingray, and jelly fish cleanness is essential before any other determination of substances of pharmaceutical and cosmetics interest. Marine sources are usually considered the cleanest source of natural active compounds. Assay of the pollutants and microplastics in sea water may be of real interest before any harvesting of marine samples.

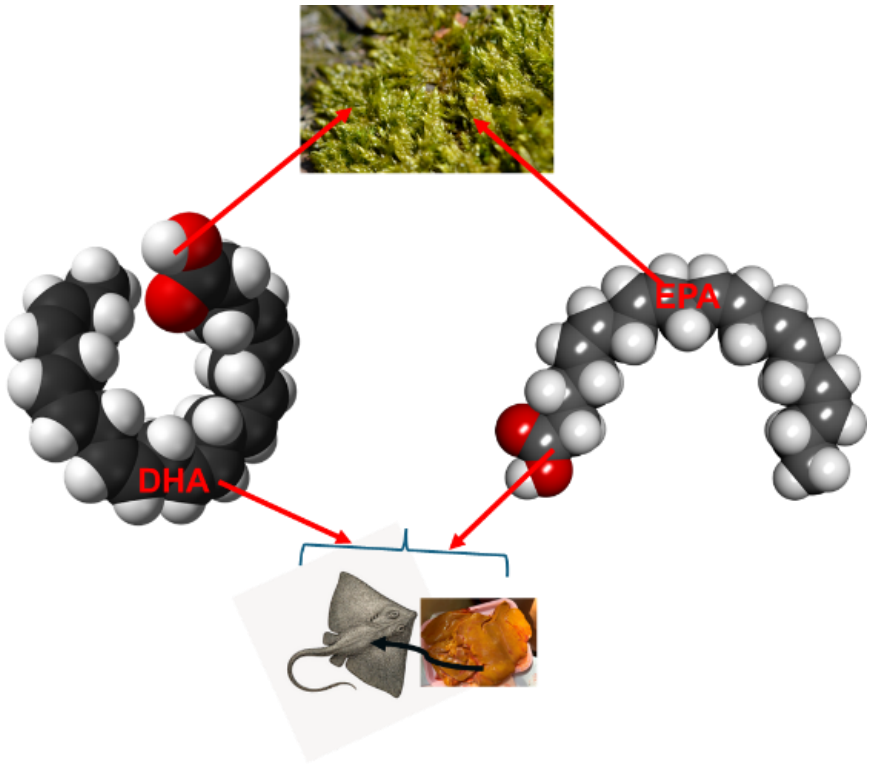


Figure 1.5. The structures of EPA and DHA.

EPA and DHA were found in both algae and stingray liver and meat, following that both algae and stingray are valuable sources for these bioactive compounds, that can be extracted and used for further formulation in drugs. It is also essential to have methods of analysis of each of them in part, but also methods that can analyse both of them simultaneously. Hyaluronic acid was found in stingray liver while the heparin was found in the jellyfish.

The sea is an excellent source of bioactive compounds that can be extracted and used in the cosmetics and pharmaceutical compounds. They are used for skin treatment, for cardiovascular diseases treatment, and for diabetes mellitus treatment. They can also be used for the formulation of cosmetic products.

CHAPTER 2
METHODS USED FOR THE DETERMINATION OF SUBSTANCES OF PHARMACEUTICAL AND CLINICAL IMPORTANCE IN MARINE SOURCES

2.1. Introduction

The determination of different substances from marine sources is very challenging. The matrices are very complex, and the concentrations of these substances may vary within the same species analysed. Therefore, the proposed methods of determination must be highly sensitive, selective, and reliable [10]. Validation of these methods is a must in order to make sure that the results are reflecting the exact amounts of the substance determined in the marine source.

Pollution is to be considered very seriously in the case of the Black Sea, from where these marine sources were picked up. Algae as well as fishes are accumulating especially the heavy metals from water. Therefore, a water analysis followed by the analysis of heavy metals in the marine sources is very important. Reliable methods of analysis are needed for the determination of heavy metals.

To improve the results, and the greenness of the methods used for the determination of substances of pharmaceutical and cosmetic interest in marine sources, one should consider on-site analysis using electrochemical sensors [10]. Electrochemical sensors can be included into measuring platforms or can be connected to portable instrumentation. Different types of electrochemical sensors can be used for the measurements: potentiometric sensors like ion-selective sensors, amperometric or voltammetric sensors. The ion-selective sensors are working only in concentration ranges comprising high level of concentrations, limits of determination recorded being of µg/mL level [11–13]. Amperometric and voltammetric sensors can be used at very low concentration levels, limits of determination of pg/mL being recorded [14].

To improve the reliability of the determinations of analytes in complex matrices, using electrochemical sensors, a new class of sensors was developed: the stochastic sensors. A reliable qualitative and quantitative analysis is possible using these sensors [15–28].

2.2. Stochastic sensors

Inspired by biology, stochastic sensors working principle is based on channel conductivity. The active side of these sensors must contain a nanochannel or a nanopore. The substances composing the solution to be analysed are getting in and out of the nanopore/nanochannel in an order governed by their size, geometry, velocity of unfolding, and the velocity of entering into the nanopore/nanochannel.

The time needed for each molecule to enter the channel is called t_{off} and, because there are many parameters contributing to its value, being specific for the molecule when the same sensor is used, it is called the signature of the analyte. The unique value of the signature, which does not change with the composition of the matrix, made possible a very reliable qualitative analysis of any analyte in a complex matrix.

The linear concentration range is a very important parameter for the stochastic sensors. Ranges between 0.001 fg/mL to 0.001 g/mL were recorded, proving that these sensors can be used for determination of both very low concentrations, but also of higher concentrations. Selectivity of the sensors is only connected with the signatures of the analytes and not with their concentrations.

2.3. Atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS) is a precise analytical technique used to measure the concentration of specific metal elements in a sample by detecting the absorption of light at characteristic wavelengths. Developed in the 1950s by Sir Alan Walsh, AAS is based on the principle that free atoms in the ground state absorb light of specific wavelengths corresponding to electronic transitions from the ground state to an excited state. Each element has a unique absorption spectrum, allowing for selective identification and quantification.

To perform AAS, the analyte is first atomized, typically by introducing the sample into a flame or graphite furnace, converting it into free atoms. A light source, often a hollow cathode lamp (HCL) containing the target element, emits radiation at the element's characteristic wavelength. The light passes through the atomized sample, and a detector measures the intensity of transmitted light. Comparing this to known standards allows precise determination of the element's concentration.

CHAPTER 3
DETERMINATION OF HEAVY METALS IN SEAFOOD [29]

3.3. Results and discussion

The study's objective was to quantify the levels of three hazardous metals in several seafood samples (mussels, shrimp, octopus, and squid) that are accessible to consumers in Romania, utilizing different AAS techniques. Hg, being volatile, was determined using the CVAAS hydride generation technique, Cd by FAAS flame atomic absorption and Pb by both FAAS flame atomic absorption and atomic absorption after atomization in the more sensitive GFAAS graphite furnace.

Table 3.2 displays the concentration limits permitted by European regulations, which are also applicable in Romania.

Table 3.2. Concentration limits of toxic metals studied according to Regulation EC1881/2006 as amended by Regulation EC629/2008 [52]

Sample	Metal concentration, mg/kg wet weight		
	Cd	Hg	Pb
Crustaceans	0.5	0.5	0.5
Bivalve molluscs	1		1.5
Cephalopod	1		1

Tables 3.3. and 3.4. present the amounts of the heavy metals detected using AAS in samples of seafood.

Table 3.3. Cd, Hg, Pb concentration in mussels in Romania (mg/kg wet weight)

Sample no.	Month	Cd	Hg	Pb
1.	March	0.09 ± 0.01	-	<LOQ (0.10)
2.	June	-	-	<LOD (0.03)
3.	July	0.18 ± 0.02	<LOD	0.13 ± 0.01

			(0.027)	
4.	September	0.10 ± 0.01	<LOD 0.027)	-
5.	October	0.16 ± 0.02	-	0.13 ± 0.02
6.	November	-	-	0.12 ± 0.02
7.	December	0.1 ± 0.01	-	<LOD (0.03)

Table 3.4. Cd, Hg, Pb concentration in imported seafood (mg/kg wet weight)

No.	Seafood, provenance	Moon	Cd	Hg	Pb
1.	Caracatite, India	April	0.53±0.05	<LOD (0.03)	<LOD (0.01)*
2.	Squid, China	February	0.2±0.02	<LOD (0.03)	<LOQ (0.02)*
3.	Prawns, India	February	<LOQ (0.04)	<LOD (0.03)	<LOD (0.03)
4.	Prawns Vietnam	April	<LOQ (0.04)	<LOD (0.03)	<LOQ (0.01)
5.	Prawns Vietnam	July	-	-	<LOD(0.03)
6.	Shrimps Netherlands	Sept	-	-	<LOD (0.03)

* Pb determination were done using the GFAAS method;

LOQ - limit of quantification; LOD - limit of detection

Comparing the values shown in Tables 3.2 and 3.3, it can be seen that the measured concentration values do not exceed the regulated values and are often below the limit of quantification or detection of the determination methods. The results are similar to those reported by other researchers [50]. As a result the seafood is not contaminated with the three heavy metals, and can be consumed.

CHAPTER 4
SILK-BASED SENSING STRIPS FOR FAST ASSESSMENT OF ZINC AND COPPER IN MARINE ALGAE AND STINGRAY [53]

4.3. Results and discussions

4.3.1. Response characteristics of the sensing strips

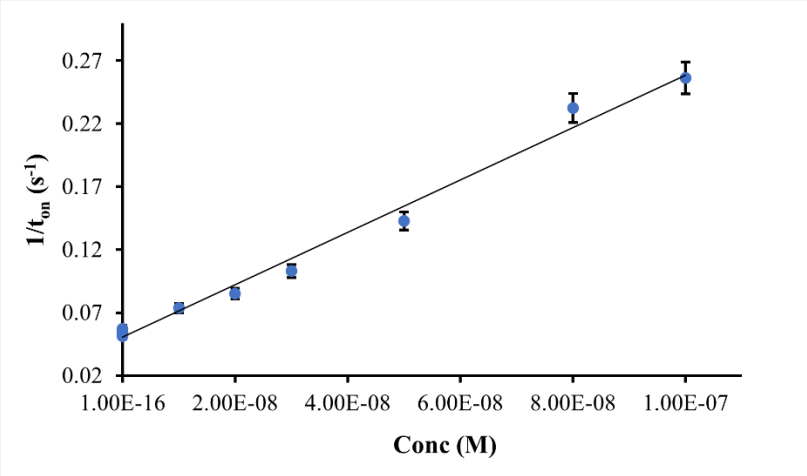
Table 4.1. shown the response characteristics obtained using the stochastic mode for the determination of Cu(II) and Zn(II) ions.

Table 4.1. Response characteristics of the sensing strips (N=10).

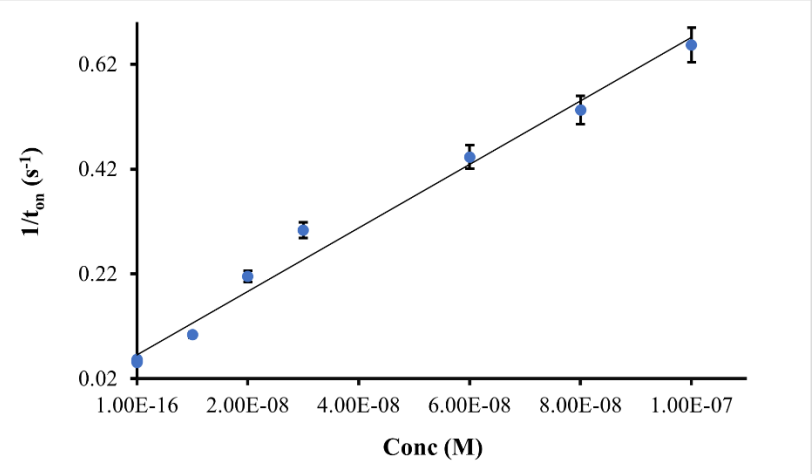
Ion	Signature (t_{off}), s	Equation of calibration*	Working concentration range, mol L^{-1}	Sensitivity, s^{-1} $mol^{-1}L$	Limit of quantification, mol L^{-1}
Cu(II)	1.5±0.1	$1/t_{on} = 0.04 + 6.44\times10^{7}C_{Cu}^{2+}$, r=0.9496	1×10^{-16} - 1×10^{-3}	$6.44(\pm0.12)\times10^{7}$	1×10^{-16}
Zn(II)	1.9±0.1	$1/t_{on} = 0.04 + 1.08\times10^{9}C_{Zn}^{2+}$, r=0.9899	1×10^{-16} - 1×10^{-4}	$1.08(\pm0.10)\times10^{9}$	1×10^{-16}

*<t_{on}>=s; <C>=mol L^{-1}

Different signatures for the ions of Cu(II) and Zn(II) were recorded, facilitating their simultaneous assay in stingray and algae. The wide linear concentration range (Figure 4.2) made possible the assay of the two ions from mmol L^{-1} to ultratrace concentrations, facilitating the determinations of these ions immediately after the algae and stingray were contaminated.



(a)



(b)

Figure 4.2. Calibration graphs of the stochastic sensing strip, when used for (a) determination of Cu(II), and (b) determination of Zn(II).

High levels of Cu(II) and Zn(II) in algae and stingrays can cause their death and may indicate early marine water contamination. The proposed sensing strips and instrumentation facilitates not only the determination of these ions, but also the sending of the results in real time, to specialized data bases in order to take fast action for water decontamination. High sensitivities were recorded for the determination of Cu(II) and Zn(II) ions. The limits of

determination for both Cu(II), and Zn(II) ions were 2.5×10^{-17}mol L^{-1} and were determined experimentally; the limits of determination is the first lower concentration of the analyte for which the $1/t_{on}$ value is less than $0.001s^{-1}$.

Comparing the response characteristics obtained using the sensing strips and other methods of analysis of the two ions [57-63, 69], lower limits of quantification (determined accordingly with the IUPAC recommendations, as the lower concentration of the metals, found on the linear concentration range) [70] were obtained, wider working concentration range are available, and also the method can be used for on-site analysis of algae and stingray, without their processing in any way.

Table 4.2. Instruments and methods used for the determination of Cu(II) and Zn(II) ions.

Tool/method	Limit of quantification	Reference
Cu(II)		
Portable biofunctional kits/fluorescence	6.13 nmol L^{-1}	[57]
R-phycoerythrim – Ag – nanoparticles/fluorescence	100.00 nmol L^{-1}	[69]
Patch-type integrated electrode modified with gold nanoparticles/electrochemical	700 pmol L^{-1}	[58]
This work	0.10 fmol L^{-1}	-
Zn(II)		
Electrochemical sensor based on multi walled carbon nanotubes/electrochemical	200.00 fmol L^{-1}	[59]
Flame atomic absorption spectrometry	7.64 nmol L^{-1}	[60]
UV/Vis spectrometry	2.30 μmol L^{-1}	[61]
This work	0.10 fmol L^{-1}	-

Table 4.2. shows that the limits of quantification obtained for the determination of Cu(II) and Zn(II) are far lower than those reported to date.

Studies on the sensing strips reproducibility and reliability in time were performed. For these studies, two sheets of ten sensing strips, cut from the same batch of silk, were constructed as described above, and they were calibrated using the stochastic mode; the sensitivities obtained through the calibration were compared for both Cu(II) and Zn(II) ions,

and values of 0.03%, and 0.02% were recorded for the relative standard deviation for Cu(II) and Zn(II) ions, respectively; these results proved the high reliability of the design of the sensing strips. The stability and reliability in time of the sensing strips was determined by measuring daily for one month the sensitivity of the sensing strips, when used for the determination of Cu(II) and Zn(II) ions; a relative standard deviation of 1.12% and 1.08% were recorded for Cu(II) and Zn(II) ions, respectively; the results proved the high reliability of the measurements for a period of one month. Values were measured on different strips to assess design reliability, and on the same strip over one month to evaluate measurement reliability.

4.3.2. Selectivity of the sensing strips

The selectivity of the stochastic sensing strips is given by the difference obtained between the signatures obtained for Cu(II) and Zn(II) ions, respectively, and the signatures obtained for other ions like Cr^{3+}, Fe^{3+}, Hg_2^{2+}, Pb^{2+}, Cd^{2+}. A minimum difference of 0.2s facilitates the selectivity. The signatures of the analytes in the stochastic mode are not dependent on the concentrations of the analytes; therefore, the selectivity in the stochastic mode is not influenced by the ratio between the concentrations of the main analyte and the possible interferent. Results are shown in Table 4.3.

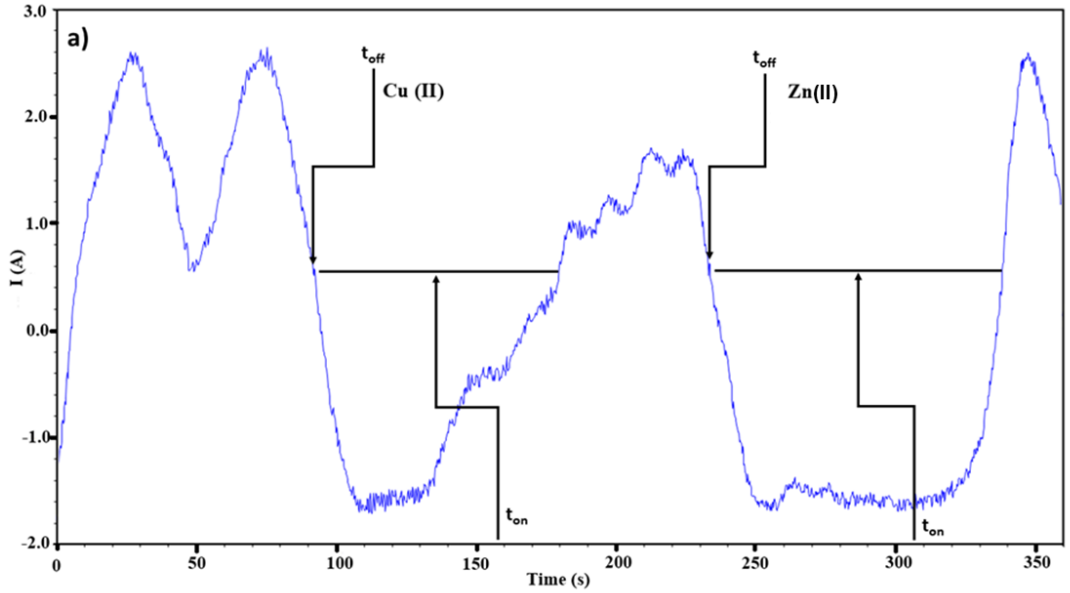
Table 4.3. The selectivity of the sensing strips (N=10).

Signature, s						
Cu^{2+}	Zn^{2+}	Cr^{3+}	Fe^{3+}	Hg_2^{2+}	Pb^{2+}	Cd^{2+}
1.5±0.1	1.9±0.1	1.1±0.1	2.3±0.1	0.4±0.1	3.1±0.1	2.5±0.1

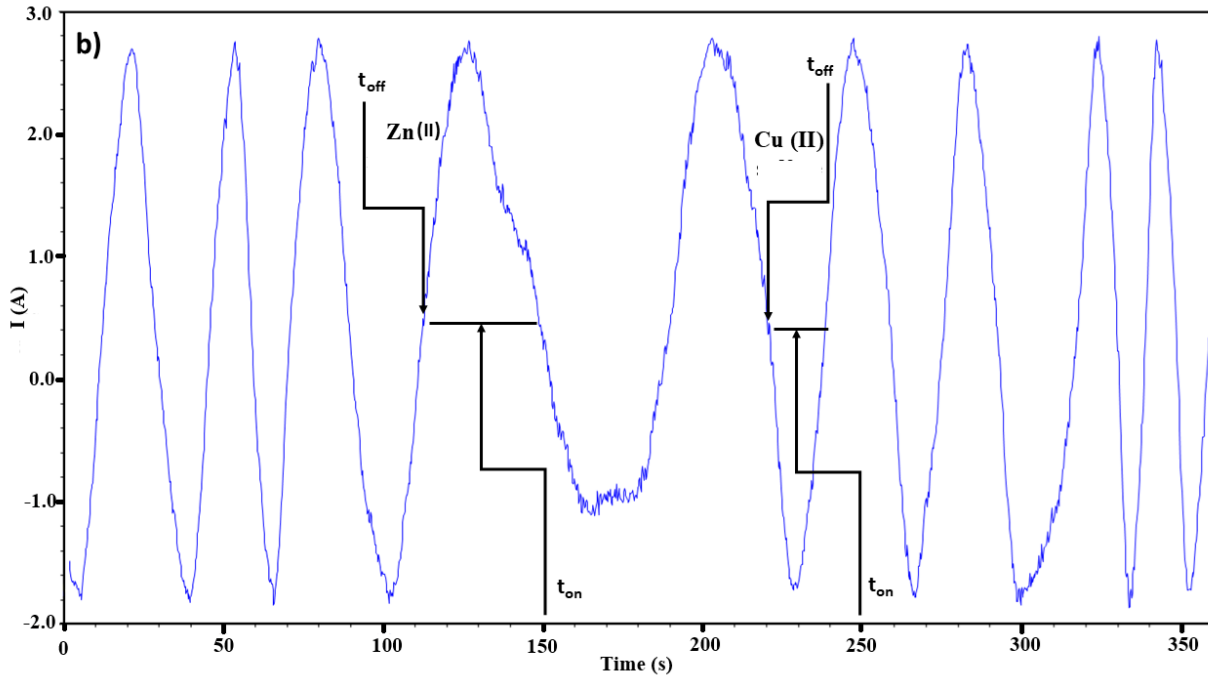
The results shown in Table 4.3 proved that the sensing strips are selective versus Cr^{3+}, Fe^{3+}, Hg_2^{2+}, Pb^{2+}, Cd^{2+} considered main possible interference for the determination of Cu(II) and Zn(II) ions in green and brown algae as well as in stingray.

4.3.3. Validation of the determination of Cu(II) and Zn(II) ions in green and brown algae and in stingray.

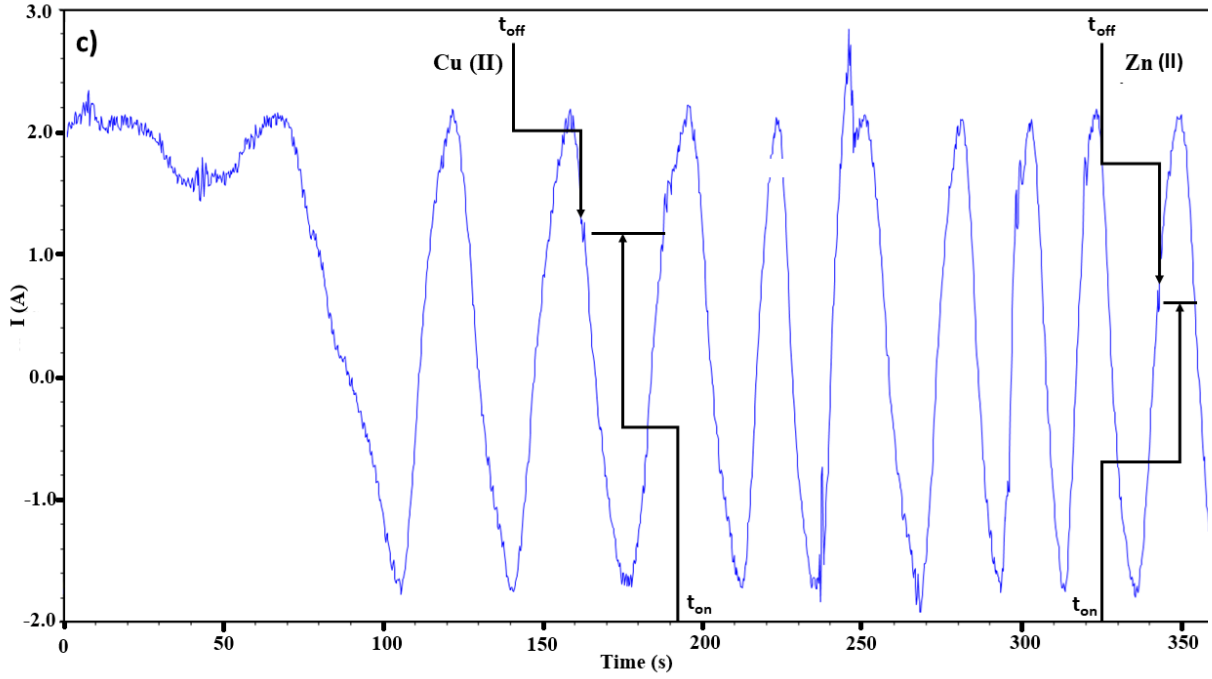
Two types of algae were analysed: brown algae and green algae. Measurements of the algae and stingray meat and liver were done without any sampling, by placing the algae or the stingray meat or liver on top of the sensing strip. The signatures of Cu(II) and Zn(II) ions were identified in the diagrams (Table 4.1.), followed by the measurements of the corresponding t_{on} values (Figure 4.3); the concentrations of Cu(II) and Zn(II) ions were determined as described in the stochastic mode, and reported to the mass of algae or stingray meat or leaver.



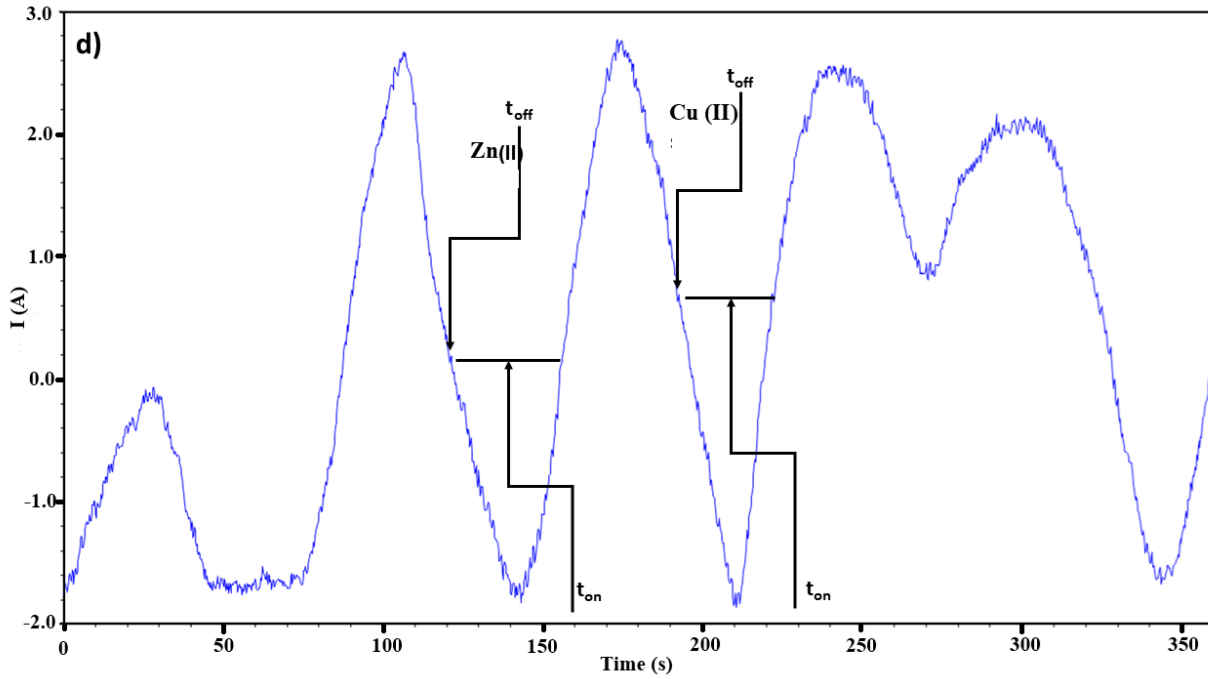
(a)


b)
t_{off}
Zn (II)
t_{on}
t_{off}
Cu (II)
t_{on}
I (A)
Time (s)
3.0
2.0
1.0
0.0
-1.0
-2.0
0
50
100
150
200
250
300
350

(b)


c)
t_{off}
Cu (II)
t_{on}
t_{off}
Zn (II)
t_{on}
I (A)
Time (s)
3.0
2.0
1.0
0.0
-1.0
-2.0
0
50
100
150
200
250
300
350

(c)



(d)

Figure 4.3. Diagrams obtained after the screening of (a) brown algae, (b) green algae, (c) meat stingray, (d) liver stingray using the sensing strips, using the proposed stochastic sensing strip.

The results were compared with those obtained using the ICP-MS method.

Table 4.4. Determination of Cu(II) and Zn(II) ions in algae and stingray (N=10).

Sample	ng Cu/g of sample		Student t-test	F-test	Bias%
	Intelligent miniplatform	ICP MS			
Green Algae	7.52 ± 0.04	7.60 ± 0.12	1.12	1.20	0.98
Brown Algae	128.37 ± 0.05	129.00 ± 0.10	1.65	1.07	0.99
Stingray meat	39.47± 0.07	39.20± 0.08	1.40	1.25	1.00
Stingray liver	22.20± 0.05	22.80± 0.12	1.32	1.09	0.97
	ng Zn/g of sample				
Green Algae	5.26± 0.03	5.50± 0.04	1.02	2.08	0.96
Brown Algae	3.04± 0.03	3.00± 0.05	1.10	1.03	1.01
Stingray	1.45± 0.08	1.38± 0.10	1.25	2.36	1.05

meat					
Stingray liver	0.42± 0.06	0.50± 0.09	1.00	1.94	0.90

Good correlations between results obtained using the sensing strips and the ICP-MS method were proved using a paired Student t-test performed at the 99.00% confidence level (with tabulated value is 4.13). Table 4.4 has shown that the Student t-test values are lower than the tabulated value at 99.00% confidence level, and accordingly, there is no significant difference between the results obtained using the sensing strips and ICP-MS.

An F-test was also carried out at the 95% confidence level for comparing the results obtained using ICP-MS and the results obtained using stochastic mode. The values obtained using the F-test (Table 4.4) are lower than the tabulated value (3.18), that means the null hypothesis is accepted, meaning that any difference in the sample standard deviations is due to random error.

Bias% values (Table 4.4.) indicate good accuracy for the proposed screening method using stochastic strips for assay of Cu(II), and Zn(II) ions in algae and stingray meat and liver.

For a second validation step, recovery tests of Cu(II) and Zn(II) ions in algae and stingray were performed. The samples were treated with known amounts of Cu(II) and Zn(II) ions, and these quantities were compared with the determined quantities of Cu(II) and Zn(II) ions. The results are shown in Table 4.5.

Table 4.5. Recovery of Cu(II) and Zn(II) ions in algae and stingray (N=10).

Sample	Cu(II)			Zn(II)		
	Added, ng	Average recovered value, ng	%, Recovery	Added, ng	Average recovered value, ng	%, Recovery
Green Algae	256.34	256.00	99.87± 0.04	315.25	315.09	99.95± 0.03
Brown Algae	234.00	233.86	99.94± 0.03	320.00	319.78	99.93± 0.05
Stingray meat	235.59	235.38	99.91± 0.08	197.00	196.72	99.86± 0.07
Stingray liver	230.00	229.65	99.85± 0.07	230.00	229.61	99.83± 0.05

Recovery values shown that both ions can be reliably determined in algae and stingray using the proposed silk-based sensing strips.

CHAPTER 5
FAST ON-SITE ELECTROANALYSIS OF EICOSAPENTAENOIC ACID ETHYL ESTER IN THE LIVER AND MEAT OF STINGRAY USING A STOCHASTIC MINIPLATFORM BASED ON A 3D STOCHASTIC MICROSENSOR [71]

5.3. Results and discussions

5.3.1. Analytical performances of the stochastic miniplatform based on a 3D stochastic microsensor

Stochastic detection is a two-step analysis process on which the first step is called the recognition step (it is the step when the signature, t_{off} value is recorded); this step is followed by the quantification step, when the t_{on} is recorded, and its value is used for the assay of the concentration of the eicosapentaenoic acid ethyl ester.

A signature of 0.6s (t_{off} value) was recorded for the eicosapentaenoic acid ethyl ester. This signature serves for its recognition in the diagrams obtained after the screening of meat and liver from stingray.

The equation of calibration of the 3D stochastic platform is the following:

$1/t_{on} = 0.03(\pm0.01) + 2.55(\pm0.07)\times10^{4}C$, where $<t_{on}> = s$, $<C> = g\ mL^{-1}$; with a correlation coefficient of 0.9914.

A sensitivity of $2.55(\pm0.07)\times10^{4}\ s^{-1}g^{-1}mL$ was recorded for a working concentration range between 5×10^{-15} and $5\times10^{-6}\ g\ mL^{-1}$. The limit of determination was $5\times10^{-15}\ g\ mL^{-1}$.

5.3.2. Selectivity of the 3D stochastic platform

The selectivity was checked versus docosahexaenoic acid ethyl ester, omega 3, and omega 6. The only parameter responsible for the selectivity is the signature of the analyte. The following signatures were obtained for the checked substances: 1.2s for docosahexaenoic acid ethyl ester, 1.8s for omega 3, and 2.3s for omega 6.

5.3.3. Reproducibility and robustness of the proposed stochastic miniplatform based on a 3D stochastic microsensor

The reproducibility and robustness studies were done using 10 miniplatforms designed as described above, and determining the variation of their sensitivities in time. Each of the 10 miniplatforms was evaluated in the same way, and the sensitivities were determined and compared when immersed in the solutions of eicosapentaenoic acid ethyl ester. The RSD (%) value recorded for the variation of the sensitivities was 1.02%, proving the reproducibility of the design of the miniplatforms. The robustness of each miniplatform designed was checked as following: 10 miniplatforms were stored for one month. Each miniplaform was taken from the storage space and they were immersed in the solutions containing eicosapentaenoic acid ethyl ester of different concentrations; the sensitivities of each measurement were retained for comparison after the whole lot of sensors was consumed, in 30 days. The results recorded at the end of this period showed the high stability of the electrodes in time because the variation of the sensitivities in time were less than 0.37%.

5.3.4. Validation of the stochastic miniplatform based on a 3D stochastic microsensor

The determination of eicosapentaenoic acid ethyl ester in the stingray liver and meat was done directly, without any processing of the liver or meat and also using the procedure described in the Samples section. The results obtained using the stochastic miniplatform were compared with those obtained using the LC-MS/MS method (Table 5.1).

Table 5.1. Determination of eicosapentaenoic acid ethyl ester in biological samples (N=10).

Sample	μg eicosapentaenoic acid ethyl ester/g of sample		Student t-test
	Stochastic miniplatform	LC-MS/MS[6]	
Stingray liver	0.59 ± 0.11	0.60 ± 0.18	1.96
Stingray meat	1.20 ± 0.19	1.18 ± 0.56	1.45

A paired Student t-test performed at the 99.00% confidence level (for which the tabulated value is 4.13) was performed. The values obtained for the t-test were lower than the

tabulated value, proving that there is no significant difference between the results obtained using the developed stochastic miniplatform and the method using the LC-MS/MS described by Viswanathan et al. [77].

A recovery test of eicosapentaenoic acid ethyl ester in the stingray liver and meat was performed. An average recovery of the eicosapentaenoic acid ethyl ester of 99.95±0.02% (N=10) was determined (N=10) in the leaver of the stingray, while in the meat of the stingray the recovery of the eicosapentaenoic acid ethyl ester was 99.98±0.02% (N=10). Recovery values are better than those reported previously when LC-MS/MS[6] was used, for which also the limit of determination was on the ng mL^{-1} magnitude order[6] compared with the fg mL^{-1} obtained using the proposed stochastic miniplatform.

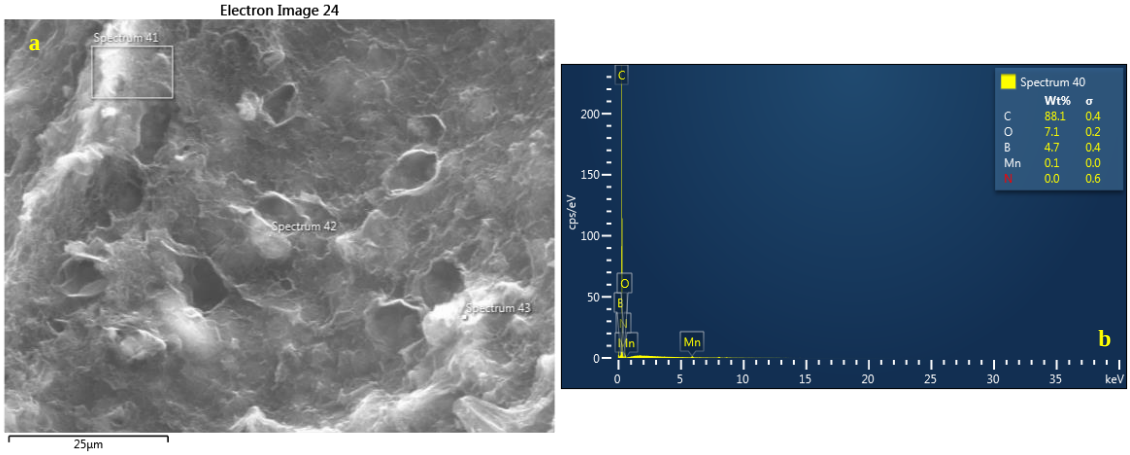
CHAPTER 6

FAST MOLECULAR RECOGNITION OF DOCOSAHEXAENOIC ACID ETHYL ESTER IN THE LIVER AND MEAT OF STINGRAY USING A MINIPLATFORM BASED ON A 3D STOCHASTIC MICROSENSOR [93]

6.3. Results and discussions

6.3.1. Characterisation of the active side of the sensor

The characterization of the active surface of the sensor was done using scanning electron microscopy (SEM) as well as using X-ray dispersion spectroscopy (Figure 6.3). Figure 6.3.a indicates that there are channels in the membrane that can make possible the development of the stochastic signal. Figure 6.3.b indicates that Boron (B) is found in a percentage of 4.7% while the Nitrogen (N) is found in a very low percentage (% N < 0.1%).



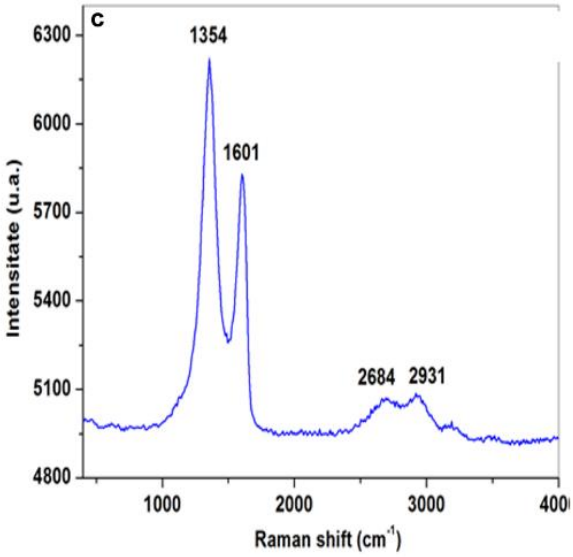


Figure 6.3. (a) SEM image of the active surface of the sensor; (b) Spectrum obtained using the X-ray dispersion method, obtained for the active surface of the sensor; (c) Raman spectrum.

Accordingly with the Raman spectrum, the intensity of the D band is greater than the relative intensity of the G band, indicating the presence of B and N atoms, accordingly with Kaykilarli et al. [100].

6.3.2. Analytical parameters obtained for the miniplatform based on a 3D stochastic microsensor.

The t_{off} value (signature) of DHA was 0.7s, and was further used for its qualitative determination in the graphs obtained after the screening of stingray samples. The equation of calibration obtained using the linear regression method was:

$1/t_{on} = 0.06(\pm0.02) + 1.65(\pm0.03)\times10^5 C$, ($\langle t_{on} \rangle$ = s, $\langle C \rangle$ = g mL^{-1}). The correlation coefficient was 0.9995 (N=10). The calibration graph is shown in Figure 6.4.

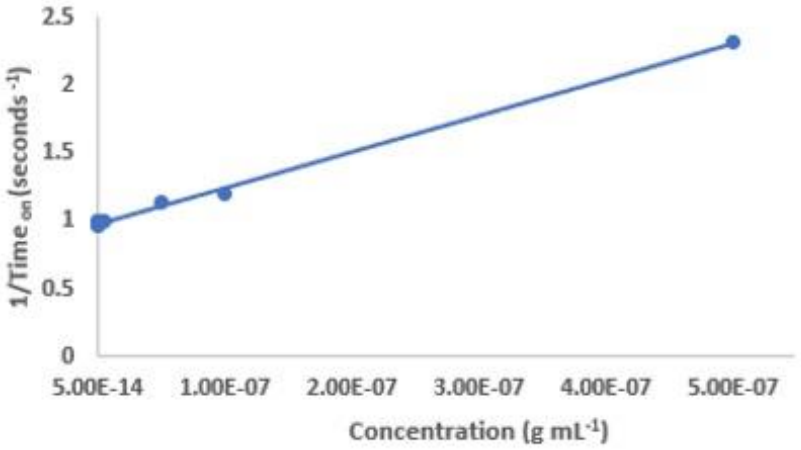


Figure 6.4 Calibration graph of the 3D stochastic microsensor when used for the assay of docosahexaenoic acid ethyl ester.

A sensitivity of $1.65(\pm0.03)\times10^{5}$ $s^{-1}g^{-1}mL$ was recorded for a wide linear concentration range determine to be between 5×10^{-14} and 5×10^{-7} g mL^{-1}. The limit of determination (the limit of determination is given by the lowest concentration found in the linear concentration range accordingly with the new IUPAC recommendation [99] was 5×10^{-14} g mL^{-1}. By comparison with the method proposed by Viswanathan et al (Viswanathan et al, 2017), which shows the assay of DHA using a method based on LC-MS/MS (limit of quantification 4.06×10^{-9} g mL^{-1}, and linear concentration range 4.06×10^{-9} – 3.28×10^{-6} g mL^{-1}), the limit of quantification of the proposed method is lower and the linear concentration range is wider.

6.3.3. Selectivity of the miniplatform

Selectivity tests were performed versus eicosapentaenoic acid ethyl ester, as well as versus omega 3, and omega 6. The parameter used to determine the selectivity of this type of sensor is the signature of the DHA versus the signatures of the supposed interferences; differences of at least 0.2 s between the signature of DHA and the signatures recorded for the other supposed interferences shown that the miniplatform is selective. The following signatures were obtained for the supposed interferences: 1.0 s for eicosapentaenoic acid ethyl

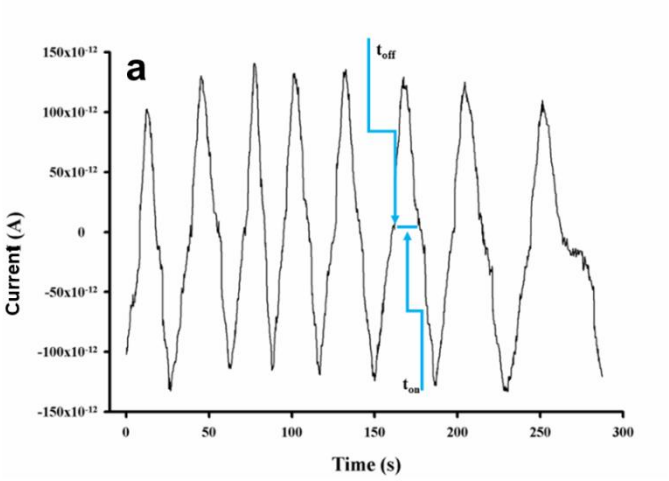
ester, 3.2 s for omega 3, and 3.5 s for omega 6 versus the signature of the DHA which is 0.7 s. While difference higher than 0.2 s were recorded, one can say that the designed platform is selective vs the tested compounds.

6.3.4. Reproducibility and robustness of the miniplatform when used for the assay of DHA

10 miniplatforms were constructed as described above, and use for the tests of reproducibility and robustness. In order to determine these parameters, the sensitivity of each of the platform was measured every day during a 30 days period of time. Every day comparison of the selectivities determined for each miniplatform resulted in a relative standard deviation of 0.04 % which certified the reproducibility of the design. By comparison of the sensitivities measured during a period of time of 30 days, the relative standard deviation was 0.12 % proving the robustness of each miniplatform designed during 30days period of time.

6.3.5. Validation of the miniplatform designed

No sample preparation was done before the assay of DHA in the stingray liver and meat. Measurements were performed using the proposed miniplatform, data processing and acquisition was done with a smartphone which had a program able to identify in the diagrams the signature of DHA, and also to read the corresponding t_{on} value which was substituted into the equation of calibration in order to obtain the concentration of DHA (Figure 6.5).



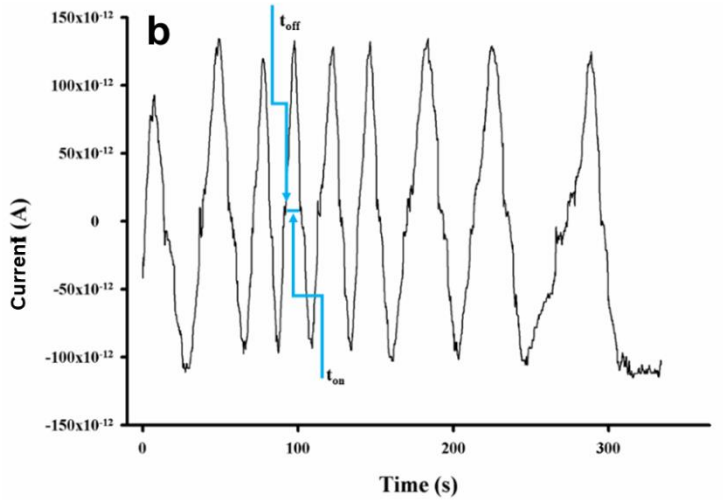


Figure 6.5. Graphs recorded for the screening of the a. liver, and b. meat of stingray**.**

The results of the screening obtained using the miniplatform are given in Table 6.1. Also, results obtained using the LC-MS/MS method were shown in Table 6.1.

Table 6.1 Determination of docosahexaenoic acid ethyl ester in stingray liver and meat (N=10).

Sample	µg DHA/g of sample		Student t-test
	Stochastic miniplatform	LC-MS/MS (Viswanathan et al., 2017)	

Stingray liver	0.38 ± 0.14	0.35 ± 0.21	2.12
Stingray meat	0.54 ± 0.19	0.50 ± 0.22	3.15

A paired Student t-test conducted at the 99.00% confidence level (tabulated value 4.13) was done versus . The values obtained for the t-test were lower than the tabulated value, proving that there is no significant difference between the results obtained using the developed miniplatform and the LC-MS/MS method described by Viswanathan et al. [111].

A recovery test of DHA in the stingray liver and meat was performed, when the concentrations of DHA were read before and after the addition of a well - known amount. An average recovery of the DHA of 99.27±0.03 % (N=10) was determined in the leaver of the stingray, while in the meat of the stingray the recovery of the DHA was 99.87±0.02 % (N=10).

The paired student t-test as well as the recovery test demonstrated that the proposed miniplatform based on a 3D stochastic microsensor is able to reliably perform a screening test of liver and meat of the stingray in order to determin the DHA concentration.

CHAPTER 7
FAST ON-SITE ASSESSMENT OF THE CONTENT OF EICOSAPENTAENOIC ACID ETHYL ESTER AND OF DOCOSAHEXAENOIC ACID ETHYL ESTER IN MARINE ALGAE USING AN INTELLIGENT MINIPLATFORM

7.3. Results and discussions

7.3.1. Response characteristics of the intelligent miniplatform used for the determination of eicosapentaenoic acid ethyl ester and docosahexaenoic acid ethyl ester

Table 7.1 shown the response characteristics obtained using the stochastic mode for the determination of EPA and DHA.

Table 7.1 Response characteristics of the intelligent miniplatform (N=10).

Analyte	Signature, s	Equation of calibration*	Working concentration range, g mL^{-1}	Sensitivity, s^{-1} $g^{-1}mL$	Limit of quantification, g mL^{-1}
EPA	1.9±0.1	$1/t_{on} = 0.03 + 1.19\times10^{9}C_{EPA}$, R=0.9954	5×10^{-18} - 5×10^{-1}	$1.19(\pm0.10)\times10^{9}$	5×10^{-18}
DHA	1.6±0.1	$1/t_{on} = 0.04 + 6.58\times10^{7}C_{DHA}$, R=0.9954	5×10^{-18} - 5×10^{-1}	$6.58(\pm0.08)\times10^{7}$	5×10^{-18}

*$<t_{on}>$=s; <C>=g mL^{-1}

Different signatures for EPA and DHA facilitates their simultaneous assay in algae. The wide linear concentration range made possible the assay of EPA and DHA not only in algae, but also in animal products, and in human biological samples. A high sensitivity was obtained for the determination of both EPA and DHA.

Comparing the response characteristics obtained using the proposed intelligent miniplatform with the chromatographic methods reported earlier [136, 137], lower limits of quantification were obtained, wider working concentration range are available, and also the method can be used for on-site analysis of algae, without their processing in any way.

Studies on the miniplatform reproducibility and reliability in time were performed. For these studies, ten miniplatforms were constructed as described above, and they were calibrated using the stochastic mode; the sensitivities obtained through the calibration were compared for both analytes EPA and DHA, and values of 0.04%, and 0.05% were recorded for the relative standard deviation for EPA, and DHA, respectively; these results proved the high reliability of the developed intelligent miniplatform. The stability and reliability in time was done by measuring daily the sensitivity of the miniplatform, when %, relative standard deviation of 1.05% and 1.00% were recorded for EPA, and DHA, respectively; the results proved the high reliability of the designed miniplatform in time.

7.3.2. Selectivity of the intelligent miniplatform used for the determination of eicosapentaenoic acid ethyl ester and docosahexaenoic acid ethyl ester

The selectivity of the miniplatform based on the 3D stochastic microsensor is given by the difference obtained between the signatures obtained for EPA and DHA, and the signatures obtained for the supposed interferences like omega 3, and omega 6. Results are shown in Table 7.2.

Table 7.2 The selectivity of the intelligent miniplatform (N=10).

Signature, s			
EPA	DHA	Omega 3	Omega 6
1.9±0.1	1.6±0.1	3.2±0.1	2.7±0.1

The results shown in Table 2 proved that the proposed miniplatform is selective versus omega 3 and omega 6 - as main possible interferences in the determination of EPA, and DHA in green and brown algae.

7.3.3. Validation of the on-site determination of eicosapentaenoic acid ethyl ester and docosahexaenoic acid ethyl ester in algae

Two types of algae were analysed: brown algae and green algae. Measurements of the algae were done without any sampling, by inserting the tip of the combined sensor on the algae. The signatures of EPA and DHA were first identified in the diagrams (Table 7.1), followed by the measurements of the corresponding t_{on} values (Figure 7.1); the concentrations of EPA and DHA were determined as described in the stochastic mode, and reported to the mass of algae.

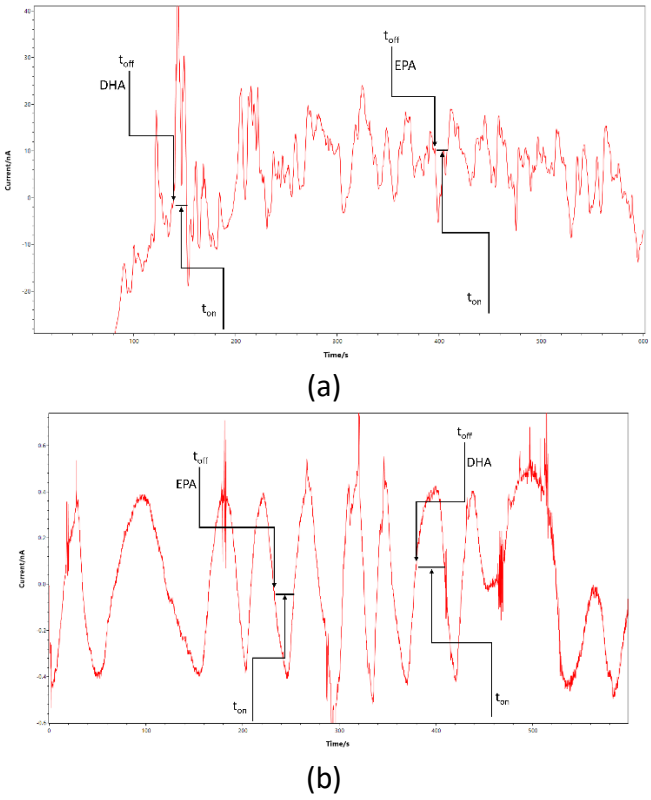


Figure 7.1. Diagrams obtained after the screening of (a) brown algae, and (b) green algae using the intelligent miniplatform.

The results were compared with those obtained using the LC-MS/MS method [137] reported earlier.

Table 7.3 Determination of eicosapentaenoic acid ethyl ester and of docosahexaenoic acid ethyl ester in algae (N=10).

Algae	mg EPA/g of algae		Student t-test
	Intelligent miniplatform	LC-MS/MS [23]	
Green	2.75 ± 0.04	2.20 ± 0.47	2.11
Brown	9.09 ± 0.01	8.76 ± 0.40	1.90
	mg DHA/g of algae		
Green	15.10± 0.04	14.98± 0.52	1.98
Brown	1.20± 0.05	1.00± 0.21	1.78

Good correlations between results obtained using the intelligent miniplatform and the LC-MS/MS method were proved using a paired Student t-test performed at the 99.00% confidence level (with tabulated value is 4.13). Table 7.3 results shown that the Student t-test values are lower than the 4.13, and accordingly, there is no significant difference between the results obtained using the intelligent miniplatform and the LC-MS/MS [136].

For a second validation step, recovery tests of eicosapentaenoic acid ethyl ester and of docosahexaenoic acid ethyl ester in algae were performed. The algae were treated with known amount of EPA and DHA, and these quantities were compared with the determined quantities of EPA and DHA. The results are shown in Table 7.4.

Table 7.4 Recovery of eicosapentaenoic acid ethyl ester and of docosahexaenoic acid ethyl ester in algae (N=10).

Algae	%, Recovery of	
	EPA	DHA
Green	98.87± 0.03	99.95± 0.02
Brown	98.84± 0.02	99.97± 0.01

Recovery values shown that both EPA and DHA can be reliably determined in algae using the proposed intelligent miniplatform based on a 3D stochastic microsensor.

CHAPTER 8
2D DISPOSABLE STOCHASTIC SENSOR FOR MOLECULAR RECOGNITION AND DETERMINATION OF HEPARIN FROM JELLYFISH

8.3.Results and Discussion

8.3.1. Response characteristics of the 2D disposable stochastic sensor

Stochastic mode was used to determine the qualitative and the quantitative parameters of the 2D disposable stochastic sensor. Table 8.1. shows the parameters of the sensor.

Table 8.1. Response characteristics of the 2D disposable stochastic sensor when used for the determination of heparin (N=10).

Signature, t_{off} (s)	Equation of calibration[a] r	Sensitivity (s^{-1} U^{-1} mL)	Limit of quantification (U mL^{-1})	Working concentration range (U mL^{-1})
0.4±0.1	$1/t_{on}$ = 0.30 (±0.08) + 5.33(±0.03) × 10^{-3} $C_{heparin}$ r=0.9998	5.33(±0.08) ×10^{-3}	1.2×10^{-20}	1.2×10^{-20}-1250

[a] $\langle 1/t_{on} \rangle = s^{-1}$; $\langle C \rangle$ = U mL^{-1}

The signature of heparin is 0.4 s; this value is used in the diagrams (Figure 1) for the molecular recognition of heparin – in the qualitative analysis step.

The linear concentration range is very high, allowing the determination of heparin not only in jellyfish but also in whole blood or in samples where its concentration is very low. The limit of quantification, defined by the latest IUPAC recommendations as the lowest concentration in the linear concentration range is very low 1.2×10^{-20} U mL^{-1}. A good value was obtained for the selectivity of the method.

Table 8.2 highlights limits of quantification obtained by different methods of heparin determination.

Table 8.2. Determination of heparin using different methods of analysis.

Method	Limit of quantification	Reference

HPLC/resonance light scattering detection	0.2 ng mL^{-1}	[149]
HPLC/circular dichroism detection	0.22 mg mL^{-1}	[150]
Fluorescence based sensor	60 pmol mL^{-1}	[151]
Electrochemical platform	1.5×10^{-9} mmol mL^{-1}	[154]
This method	1.2×10^{-20}U mL^{-1}	-

The lowest limit of quantification was obtained when the proposed method was used for the determination of heparin (Table 8.2). The proposed method is faster and easier to use for the determination of heparin.

Robustness of the 2D disposable stochastic sensor was measured using stability tests performed for 20 consecutive days. Calibration of the sensor was done every day, and the sensitivity was calculated using the linear regression method. By comparing the sensitivities' values obtained in this period of time, a deviation from the mean of 0.03% was obtained, proving that the sensor is stable and reliable.

8.3.2. Selectivity tests

Selectivity tests involved the determination of the signatures of heparin and the signatures of the possible interferences like collagen, green fluorescent protein, and coelenterazine. Differences higher or equal to 0.2s between the signatures of the analytes proves the selectivity of the sensor.

Table 8.3. Results of the selectivity tests.

Heparin	Collagen	Green fluorescent protein	Coelenterazine
Signature, t_{off} value (s)			
0.4±0.1	2.6±0.1	3.1±0.1	1.8±0.1

The values of the signatures shown in Table 8.3 proved that higher differences than 0.2 s were recorded between the signature of heparin and the signatures of the collagen, green fluorescent protein, and coelenterazine. Accordingly, the proposed sensor is selective versus these compounds.

8.3.3. Validation of the screening method for the molecular recognition and quantification of the heparin in jellyfish

The jellyfish was screened on-site, at the seaside, using the portable instrumentation. The first step on the diagram reading was to identify the signature of heparin (Figure 8.1), followed by the reading of the t_{on} value in between two t_{off} values (Figure 8.1). The stochastic mode procedure was applied further for the determination of the concentration of heparin in jellyfish.

For the HPLC method [149], the jellyfish was taken into the laboratory for processing and determination.

Results of the validation of the screening nethod are shown in Table 8.4.

Table 8.4. Determination of heparin in jellyfish (N=10).

Samples no.	HPLC[7] (U mL^{-1})	Stochastic mode (pg mL^{-1})
1	61.40±0.20	61.32±0.03
2	151.93±0.32	152.18±0.02

Sample 1 is the blue jellyfish from the Black Sea; Sample 2 is the common jellyfish from the Black Sea.

Student t-tests were employed for the validation of the method. The results of the proposed screening method based on the 2D stochastic sensor were compare with those obtained using HPLC method [149]. The student-t-test was performed at 99.00% confidence level. The value obtained for the test was 2.32; while this value is lower than 4.18 (tabulated value), it means that there is no significant difference between HPLC and the stochastic methods based on 2D stochastic sensors.

An F-test was also carried out at the 95% confidence level, when a value of 0.74 was obtained by comparing the results obtained using HPLC [149] and the results obtained using the stochastic mode. The value obtained using the F-test is lower than the tabulated value

(3.18); that means the null hypothesis is accepted, meaning that any difference in the sample standard deviations is due to random error.

Recovery tests were also performed by adding a known amount of heparin to jellyfish samples and determining (after the screening of the sample using the 2D disposable stochastic sensor) using the stochastic mode the concentration of heparin added. By comparing the determined concentration with the added concentration of heparin, a recovery of heparin in jellyfish of 99.85% with a relative standard deviation of 0.04% was obtained (a number of ten measurements were performed).

The tests performed for the validation of the proposed method and sensor proved that the stochastic method and the 2D disposable stochastic sensor can be reliably used for the on-site determination of heparin from jellyfish.

CHAPTER 9
ULTRASENSITIVE AND RAPID DETERMINATION OF HYALURONIC ACID IN THE LIVER OF STINGRAY [16]

9.3. Results and discussions

9.3.1. Characterisation of the stochastic sensor based on GMN/SPGE

9.3.1.1. Electrochemical characterization of the GMN/SPGE based sensor

The electrochemical behavior of the electrodes was studied in a 5.0 mmol L^{-1} $K_3[Fe(CN)_6]$ solution containing 0.1 mol L^{-1} KCl as the redox probe. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) experiments were conducted to investigate the electroactive surface area and interfacial processes.

Figure 9.2(A) depicts the cyclic voltammograms of bare SPGE and GMN/SPGE and reveals higher current intensity peaks corresponding to the modified electrode, thus suggesting an increase in the electrochemical performance after the modification of SPGE with galactomannan.

EIS measurements were performed in 5.0 mmol L^{-1} $K_3[Fe(CN)_6]$ solution containing 0.1 mol L^{-1} KCl, over a frequency range from 0.1 Hz to 100 kHz. The corresponding Nyquist plots revealed a well-defined semicircle in the high-frequency region, followed by a linear Warburg tail at lower frequencies, indicating a diffusion-controlled process. The experimental data were fitted using a Randles equivalent circuit. The charge transfer resistance (R_{ct}) decreased from 238.1 Ω (bare SPGE) to 146.2 Ω (GMN/SPGE), as shown in Figure 9.2(B). The result shows facilitated electron transfer kinetics at the electrode-electrolyte interface, attributed to the galactomannan layer.

The electroactive surface area of the bare and modified electrode was computed using the Randles-Sevcik equation by plotting the peak current intensities vs. sweep rate square root (Figures 9.2(B), and 9.2(C)). The results indicated an increase in the apparent electroactive surface area, from 0.099 (bare SPGE) to 0.112 cm^2 (GMN/SPGE), suggesting successful modification of the SPGE electrode with galactomannan. This is in good

agreement with EIS experiments, indicating an enhanced active surface area and improved electron-transfer kinetics due to the presence of the galactomannan layer.

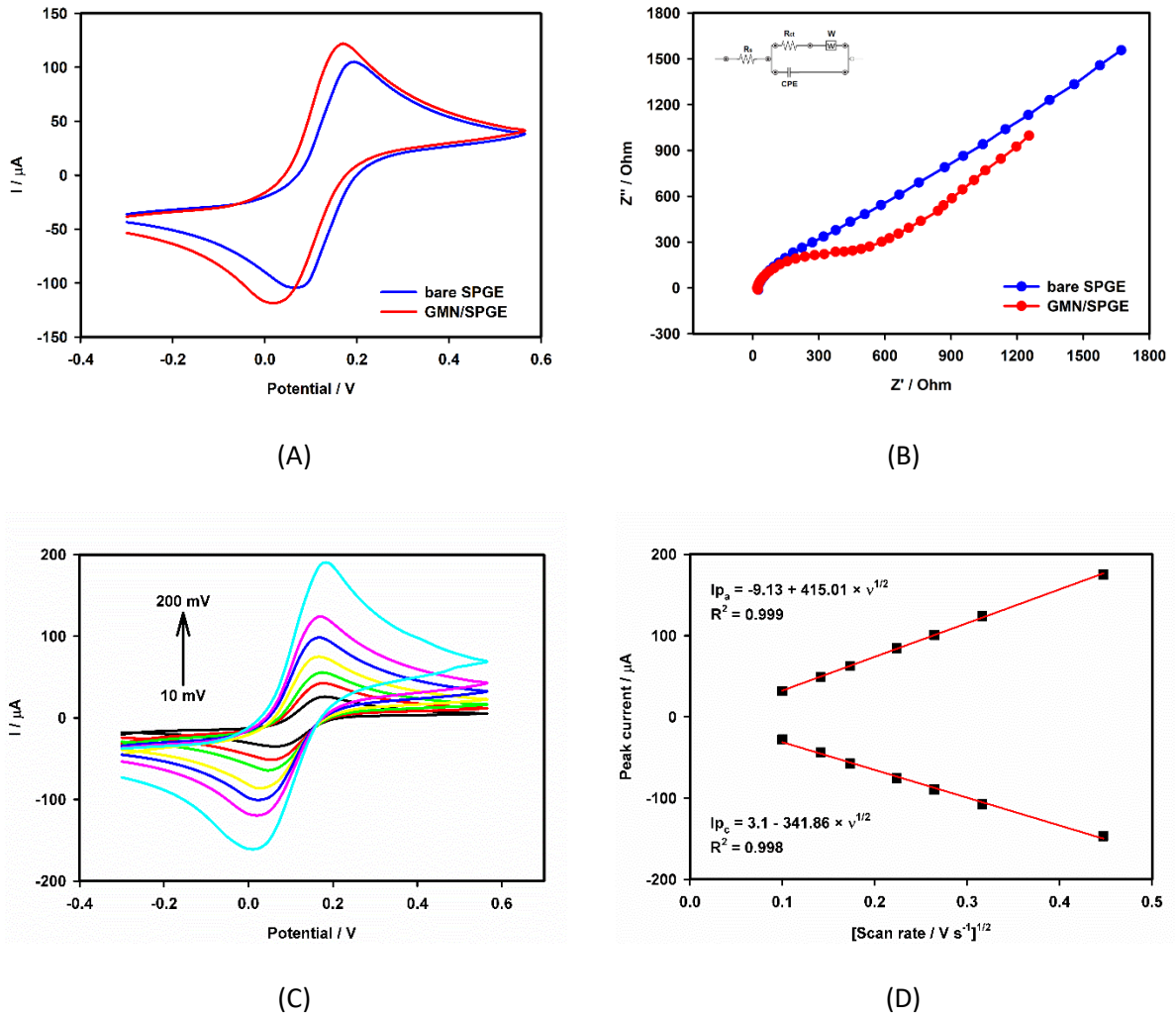


Figure 9.2 (A) cyclic voltammograms of bare SPGE and GMN/ SPGE recorded in a potential range from -0.3 to 0.6 V at a scan rate of 100 mV s-1; (B) Nyquist plots of bare SPGE and GMN/ SPGE recorded in a frequency range from 0.1 Hz to 100 kHz, Eac 10 mV, Edc = 200 mV (inset: Randles equivalent circuit); (C) cyclic voltammograms of GMN/SPGE recorded at increasing sweep rates (10 to 200 mV s-1); (D) peak current intensity vs. square root of sweep rate.

9.3.1.2. Influence of galactomannan solution volume on electrochemical performance of the stochastic sensor based on GMN/SPGE

To optimize the electrochemical performance of the modified electrode, the casting volume of the galactomannan solution was systematically varied. Modified electrodes were prepared by drop-casting 2, 4, 6, and 8 µL of 0.05% galactomannan hydroalcoholic solution. The electroactive surface area and interfacial properties corresponding to each casting volume were evaluated by CV and EIS in 5.0 mmol L^{-1} $K_3[Fe(CN)_6]$ containing 0.1 mol L^{-1} KCl. Based on the results presented in Figure 9.3, 4 µL yielded the largest electroactive surface area and the lowest charge-transfer resistance (R_{ct}), indicating the most favorable electrochemical performance. Therefore, this volume was selected as optimal for subsequent studies.

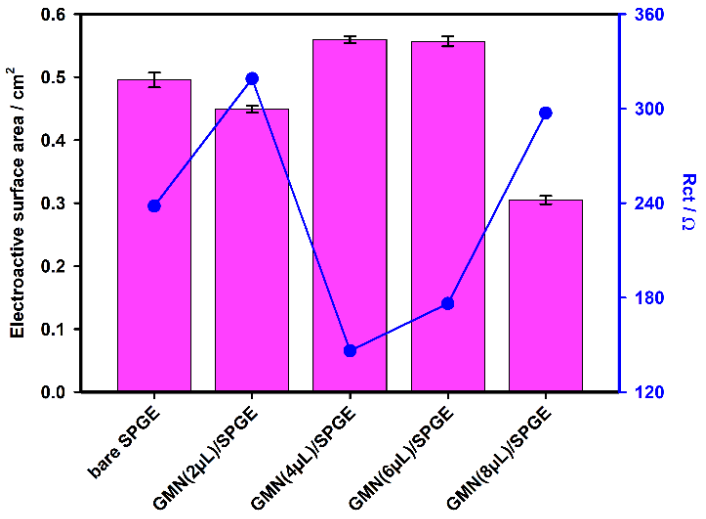


Figure 9.3. Influence of galactomannan casting volume on the electroactive surface area and interfacial electron-transfer properties.

9.3.1.3. Response characteristics of the stochastic sensor based on GMN/SPGE

The proposed stochastic sensor was evaluated using the standard solutions prepared as shown above. The response characteristics are given in Table 9.1.

Table 9.1. Response characteristics of the stochastic sensor used for the determination of hyaluronic acid, when standard solutions were used.

Working concentration range (g mL^{-1})	**Calibration equation and r***	**Signature t_{off} (s)**	**Sensitivity (s^{-1}/ g mL^{-1})**	**Limit of quantification (g mL^{-1})**
3.0×10^{-15}-3.0×10^{-11}	$1/t_{on} = 0.38 + 1.32 \times 10^{7} C_{HA}$; r=0.9995	1.2	1.32×10^{7}	3.0×10^{-15}

* $<t_{on}>$ = s; $<C_{HA}>$ = mg mL^{-1}.

The signature determined for HA has the value of 1.2 s. A high sensitivity was recorded, while the working concentration range is wide. The limit of quantification determined accordingly with IUPAC regulations as the lower concentration found on the linear concentration range was 3 fg mL^{-1}.

Table 9.2. Other methods used for the determination of hyaluronic acid.

Method/Tool	**Limit of quantification**	**Reference**
Electroanalysis/Immunosensor	156 pg mL^{-1}	[169]
Electroanalysis/Gold nanoparticles based sensor	115 ng mL^{-1}	[174]
Electroanalysis/molecularly imprinted conducting paper based sensorpaper	32 ng mL^{-1}	[168]
HPLC-MS/MS	491 pg mL^{-1}	[166]
Stochastic method	3.0 fg mL^{-1}	This work

A lower limit of quantification versus those obtained using other methods of analysis (Table 9.2) was obtained when the proposed stochastic sensor was used for HA determination.

The reliability of the design was determined by constructing ten stochastic sensors using the procedure described and optimised earlier; variations of sensitivities obtained were calculated, and a value of 0.14% proved that the design is reliable. The calibrations of the ten sensors were made for another 30 days, and the sensitivities were compared; a variation of

0.21% was calculated – and the value proved that the proposed stochastic sensor is stable in time.

9.3.2. The selectivity of the stochastic sensor based on GMN/SPGE

The selectivity of the stochastic sensors is given by the differences between the signatures' values recorded for different analytes from a mixture. A 0.2 s difference means that the stochastic sensor is selectiv. Eicosapentaenoic acid ethyl ester, docosahexaenoic acid ethyl ester, omega 3, and omega 6 where checked as possible interferences. The values obtained for their signatures were: 4.2 s for eicosapentaenoic acid ethyl ester, 3.8 s for docosahexaenoic acid ethyl ester, 2.8 s for omega 3, and 3.2 s for omega 6. The values are higher than the signature recorded for the HA (1.2 s). Accordingly with these values, the proposed stochastic sensor is selective versus these compounds.

9.3.3. Validation of the ultrasensitive stochastic sensor based on GMN/SPGE when used for the determination of hyaluronic acid in the liver of stingray.

All samples were determined on-site using the designed sensor, the portable minipotentiostat and the smartphone using the set-up shown in Figure 9.4.

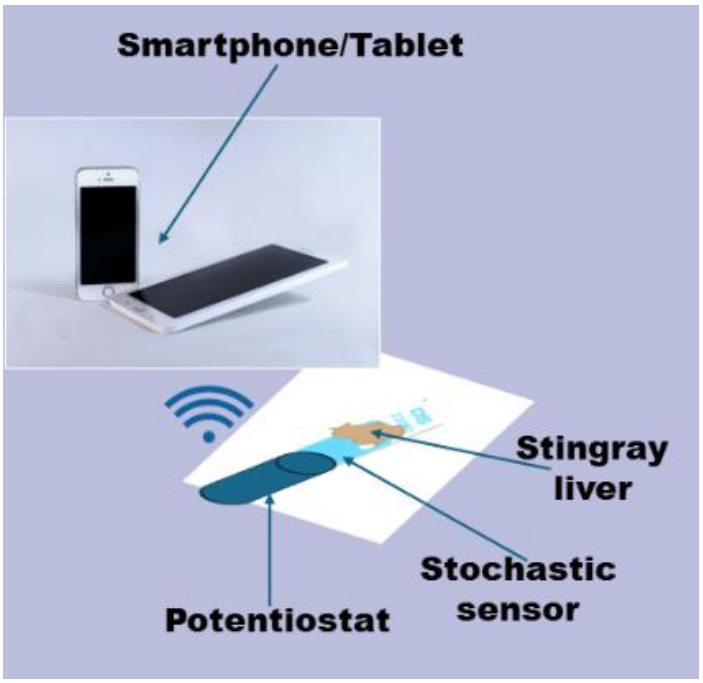


Figure 9.4 Set-up of the determination of hyaluronic acid in the liver of stingray

The HA was identified in the diagrams based on the value of its signature (Figure 9.1, Table 9.1), and further, the t_{on} value was read and substituted in the equation of calibration to obtain the concentration of HA (Figure 9.1).

The results obtained for the determination of HA in the liver of stingray using the proposed stochastic sensor and the HPLC/UV are shown in Table 9.3.

Table 9.3. Determination of hyaluronic acid in the liver of stingray, using the stochastic sensor and the HPLC/UV [165] method (N=10).

Method	**Hyaluronic acid, ng/g stingray**
Stochastic Sensor	348.63±0.11
HPLC	346.97±0.41
Student t-test	2.54

Student paired t-test was used to compare the results obtained using the proposed method for the determination of HA and the HPLC method proposed earlier [165] at a 99.00% confidence level, for which the tabulated theoretical t-value is 4.032. The calculated value was 2.54 (lower than the tabulated theoretical value), proving that there is no statistically significant difference in the results obtained using the two methods.

The recovery test was also employed for the validation of the proposed sensor and method. A certain amount of HA was added to the stingray - as prepared for the chromatography, and the determined amount was compared with the added amount of HA. A value of recovery of 97.12% was obtained as a mean of 10 consecutive determinations, with a standard deviation of 0.09%.

Both tests performed for the validation of the sensor and method proved that they can be successful used for the on-site determination of HA in the liver of stingray.

CONCLUSIONS

The concentration of Cd, Hg, and Pb was determined with AAS. The concentrations found are within the permissible limits (0.5 -1.5 mg/kg) and are similar to those reported before. Following the determinations carried out, it was observed that the seafood studied does not contain concentrations of toxic metals above the normal regulated limits, which means that they can be consumed without presenting a danger to the health of human and animal organisms, but as a result of the gradual build-up of heavy metals in the body it is advisable to consume seafood with moderation: they should not be consumed in excess and should not become the main food of a diet. This was an important screening test in order to determine which of the heavy metals are found in the Black Sea waters, and will be also found in fish, and algae.

The proposed sensing strips were reliable used for determination of Cu(II) and Zn(II) ions in algae and stingray. The ability to send the data to a smartphone/tablet/laptop or database, can be used to assess these ions in real time, and alert immediately the authorities about the algae and stingray contamination with metals that may have a negative impact on the quality of the marine ecosystem. The screening method is fast, easier to be used than other methods like ICP-MS, and cost-effective. The sheet of silk can be recovered after the measurements, and reused for the new deposition for at least three times using cold plasma of working sensor, auxiliary sensor and reference sensor; tests conducted on reused silk strips gave sensitivities with variations less than 1.00% versus initial values. The main feature of the screening test is its utilization for on-site assay of the quality of algae and stingray.

A miniplatform having as detection tool a 3D stochastic microsensor was designed and used for the assay of eicosapentaenoic acid ethyl ester from stingray liver and meat. Natural monocrystalline diamond paste was modified with a solution of heptakis(2,3,6-tri-O-methyl)-beta-cyclodextrin to obtain the modified diamond paste used in the 3D stochastic microsensor. Wide working concentration range ($5.0 \times 10^{-15} - 5.0 \times 10^{-8}$) and a very low limit of quantitation (5.0×10^{-15}) were attained. The proposed miniplatform was successfully used for the assay of eicosapentaenoic acid ethyl ester from stingray liver and meat with recoveries higher than 99.90%, and % RSD values lower than 0.05 (N=10).

Docosahexaenoic acid ethyl ester has an important role in the prevention of the neuropsychiatric and neurodegenerative diseases. Its extraction from fish like stingray as oil will create an essential pharmaceutical formulation for this kind of diseases. Therefore, it is essential to develop fast molecular recognition methods able to perform both qualitative and quantitative analyses for the determination of docosahexaenoic in stingray (meat and the liver). A miniplatform incorporating a 3D stochastic microsensor was designed and validated for the molecular recognition of docosahexaenoic acid ethyl ester from stingray (liver and meat). The design of the 3D stochastic microsensor was based on the modification of a graphene decorated with N (nitrogen) and B (boron) paste (NB-graphene paste) with a 10^{-3}mol L^{-1} solution of alpha-cyclodextrin to obtain the modified NB-graphene paste. A wide linear concentration range ($5.0 \times 10^{-14} - 5.0 \times 10^{-7}$ g mL^{-1}) with a low limit of quantitation (5.0×10^{-14} g mL^{-1}) were attained. High recovery values were determined when the miniplatform was used for the assay of docosahexaenoic acid ethyl ester from stingray liver and meat with recoveries higher than 99.20%, and % relative standard deviation values lower than 0.05 (N=10).

Marine algae are an excellent vegetarian source for eicosapentaenoic acid ethyl ester as well as for docosahexaenoic acid ethyl ester. Fast on-site assessment of their concentration in algae is essential. An intelligent miniplatform based on a 3D stochastic microsensor was developed. The design of the 3D stochastic microsensor is based on modification of a graphene matrix with 4-tert-butyl-calix[4]arene. Wide working concentration ranges ($5.0 \times 10^{-18} - 5.0 \times 10^{-1}$ g mL^{-1}) and a low limit of quantitation (5.0×10^{-18} g mL^{-1}) were obtained. The proposed miniplatform can be used for simultaneous assay of eicosapentaenoic acid ethyl ester and docosahexaenoic acid ethyl ester, with recoveries exceeding 99.80%, and to send via Bluetooth to specialized databases the results.

Jellyfish represents a valuable source of heparin. A 2D disposable stochastic sensor was proposed for the direct assay of heparin in jellyfish. The sensor can be used on a wide linear concentration range: 1.14×10^{-21} to 1120 U mL^{-1}, with a sensitivity of 5.33×10^{-3} s^{-1} U^{-1} mL and a limit of quantification of 1.14×10^{-21} U mL^{-1}. The recovery of heparin in jellyfish was 99.85% with a relative standard deviation of 0.04 %. The sensor and stochastic method were validated versus a chromatographic method - HPLC coupled with light scattering detection. Both the student's t-test (performed at a 99.00% confidence level) and the F-test (performed at a 95.00% confidence level) proved that the method based on the stochastic sensor is a good alternative to the chromatographic method of analysis.

Stingrey liver is one of the natural sources of hyaluronic acid. Its on-site determination is necessary for futher development of pharmaceuticals or even cosmetics containing hyaluronic acid as active compound. An ultrasensitive stochastic sensor based on immobilisation of galactomannan on a screen-printed gold electrode was used for rapid determination of hyaluronic acid in stingray's liver. The sensor diplayed a wide working concentration range (3.00 fg mL^{-1} to 30.00 pg mL^{-1}, with a high sensitivity (1.32×10^{7} s^{-1} g^{-1} mL). High recovery values (> 97.00%) were recorded for the determination of hyaluronic acid in the stingray's liver. Validation of the stochastic sensor and screening method for hyaluronic acid was done versus an HPLC method. The on-site measurements with the ultrasensitive stochastic sensor of the hyaluronic acid in stingray liver will help the pharmaceutical and cosmetics industry to assess if they can obtain the necessary hyaluronic acid out of its extraction from stingray liver.

Original contributions

The first novelty is related to the utilization of silk-based sensing strips for fast, on-site assessment of Zinc and Copper in marine algae and stingray. The sensing strips are combined stochastic sensors connected to a portable instrument, which are able to send in real time the results to cell, tablet, laptop, or specialized data base; they were developed for the first time in our laboratory as a subclass of the stochastic sensors class. Also, the design of the stochastic sensor (the working electrode in the sensing strip) is new: the working electrode from the sensing strip is based on a thin nanolayer of nanographene modified with a maltodextrin of dextrose equivalent between 4 and 7. Graphene was selected as matrix because it can keep the pore in its initial shape; by using nanographene the active surface of the sensor was increased facilitating an increase on the sensing strip's sensitivity. Maltodextrin with the lowest dextrose equivalent was selected due to its shape which provides a natural pore needed for the stochastic sensing. Utilisation of the sensing strip for on-site real-time simultaneous assay of Cu(II) and Zn(II) ions directly from the algae and stingray, without any processing of algae or stingray it is a new approach of this type of analysis essential for a clean environment.

The novelty of the miniplatform based on a 3D stochastic microsensor for the assay of eicosapentaenoic acid ethyl ester in the liver and meat of the stingray. Stochastic sensors' advantages for such analysis are: the sample does not need any processing before its analysis, a qualitative analysis of the eicosapentaenoic acid ethyl ester is fast and reliable done, before its quantitative analysis, the matrix does not influence the analysis of eicosapentaenoic acid ethyl ester[7-9]. Inclusion of the proposed sensor in the miniplatform facilitated automatic assay and submission of data to a data base, or user.

A novel miniplatform was developed for the assay of DHA in stingray (liver and meat). Graphene decorated with N (nitrogen) and B (boron) paste (NB-graphene paste) was modified with a 10^{-3} mol L^{-1} solution of α-cyclodextrin (α-CD). The advantage of utilization of the NB - graphene is the keeping of the internal cavity of α-CD in its initial shape, while N and B atoms improved the conductivity of the graphene (N and B are creating hales in the graphene structure, making from it a good semiconductor) favoring determination of DHA at very low concentration levels, and with high sensitivities.

Due to the importance of establishing on-site the EPA and DHA content of marine algae, there is very important to develop a fast, and reliable method of analysis for the on-site

determination of EPA and DHA. The novelty is the development of an intelligent miniplatform based on a 3D stochastic microsensor for fast, on-site simultaneous determination of EPA and DHA in algae. The design of the 3D stochastic microsensor is new; 4-tert-butyl-calix[4]arene was used for the modification of a reduced graphene oxide matrix.

The novelty of this manuscript is given by the utilisation of a 2D disposable carbon stochastic sensor with a special channel design for the on-site determination of heparin from jellyfish. The advantages of using the stochastic sensor versus other types of sensors and versus chromatographic methods are that no sample preparation is needed before the measurements, the determinations are done in less than 6 minutes, there is the possibility of performing the determination on-site with the results sent to a specialised database in real time, the response of the stochastic sensor does not depend on the type and nature of the matrix, and both qualitative and quantitative analyses can be performed reliably.

The novelty of the determination of hyaluronic acid is the utilisation of galactomannan as modifier for the design of the gold-based screen-printed electrode. The novelty is also given by the on-site determination of hyaluronic acid in liver stingray without any processing of the liver.

Future work

The miniplatform will be validated at the Black Sea in order to increase the TRL to higher one, up to 8. Also, there will be checked more heavy metals and pesticides in order to first check how clean is the water, as well as the fish, and algae. More bioactive substances of pharmaceutical and cosmetic interest will be determined using the proposed intelligent miniplatforms.

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Annex 1 Participation in national and international conferences

1. **Drăgoi Brînza Andreea,** Raluca-Ioana Stefan-van Staden, Damaris Cristina Gheorghe, Gabriela Stanciu, Validation of a new method for the assay of docosahexaenoic acid ethyl ester in the liver of stingray, poster, p. 49, Eurachem Workshop Complex Matrices: Applications, Laboratory standards and Accreditation, May 26 – 27, 2025, Library Building of National University of Science and Technology POLITEHNICA Bucharest, Romania

2. **Drăgoi Brînza Andreea,** Stefan-van Staden Raluca-Ioana, Gheorghe Damaris-Cristina, Stanciu Gabriela, Poster: "Determination of eicosapentanoic acid ethyl ester in the liver of stingray – poster PN7", International Conference ″CHIMIA″ 2024, NOMARES Workshop A Constanța, 30 May 2024-01 June 2024, ISSN 2360-3941, p. 138

3. **Drăgoi Brînza Andreea**, Stefan van Staden Raluca-Ioana, Gheorghe Damaris-Cristina, Stanciu Gabriela, Determination of docosahexaenoic acid ethyl ester in the liver of stingray, S1 –P– ID 248, The 23rd Romanian International Conference on Chemistry and Chemical Engineering (RICCCE), 4 - 7 September 2024, Mamaia - Constanța, Romania.

Annex 2 Papers published in ISI Journals

Fast on-site electroanalysis of eicosapentaenoic acid ethyl ester in the liver and meat of stingray using a stochastic miniplatform based on a 3D stochastic microsensor RI Stefan-van Staden, **A Drăgoi Brînza**, DC Gheorghe, G Stanciu **J Electrochem Soc**, 171, 0875120, 2024 **Q2 IF=3.3**

Fast molecular recognition of docosahexaenoic acid ethyl ester in the liver and meat of stingray using a miniplatform based on a 3D stochastic microsensor RI Stefan-van Staden, **A Drăgoi Brînza**, DC Gheorghe, G Stanciu **Anal.Lett.**, 58(15), 2569-2577, 2025 **Q2 IF=1.8**

Determination of heavy metals in seafood **A. Drăgoi-Brînza**, RI Stefan-van Staden, G Stanciu, C Cioates Negut **UPB Sci Bull**, 87(2), 15-22, 2025 **Q4, FI=0.147**

2D Disposable stochastic sensor for determination of heparin from jellyfish **A. Drăgoi Brînza**, R.I. Stefan-van Staden, D.C. Gheorghe **Submitted to journal**

Intelligent miniplatform based on 3D stochastic microsensor and its application for fast on-site molecular recognition of eicosapentaenoic acid ethyl ester and of docosahexaenoic acid ethyl ester in marine algae **A Drăgoi Brînza**, RI Stefan-van Staden, RM Ilie-Mihai, G Stanciu **Submitted to journal**

Silk-based sensing strips for fast assessment of Zinc and Copper in marine algae and stingray **A Drăgoi Brînza**, RI Stefan-van Staden, RM Ilie-Mihai **J Electrochem Soc.**, 173(5), 057501, 2026 **Q2 IF=3.3**

Ultrasensitive and fast on-site determination of hyaluronic acid in the liver of stingray **A Drăgoi Brînza**, RI Stefan-van Staden, BM Tuchiu-Stanca, IR Comnea-Stancu **J Electrochem Soc**, 173(13), 137501, 2026. **Q2 IF=3.3**