Biologically friendly Room Temperature Ionic Liquids and Nanomaterials for the development of innovative enzymatic biosensors

D. Zappi, R. Caminiti, G.M. Ingo, C. Sadun, C. Tortolini, M.L. Antonelli

www.elsevier.com/locate/talanta

PII: S0039-9140(17)30805-6
DOI: http://dx.doi.org/10.1016/j.talanta.2017.07.081
Reference: TAL17780

To appear in: *Talanta*

Received date: 5 June 2017
Revised date: 26 July 2017
Accepted date: 27 July 2017

Cite this article as: D. Zappi, R. Caminiti, G.M. Ingo, C. Sadun, C. Tortolini and M.L. Antonelli, Biologically friendly Room Temperature Ionic Liquids and Nanomaterials for the development of innovative enzymatic biosensors, *Talanta* http://dx.doi.org/10.1016/j.talanta.2017.07.081

Biologically friendly Room Temperature Ionic Liquids and Nanomaterials for the development of innovative enzymatic biosensors

D. Zappi[a*], R.Caminiti[a], G. M. Ingo[c], C. Sadun[a], C. Tortolini[b] and M.L. Antonelli[a]

[a]Department of Chemistry, University of Rome "La Sapienza" – p.le A. Moro, 5 – 00185 Rome - Italy
[b]Department of Chemistry and Drug Technologies, University of Rome "La Sapienza" Rome - Italy
[c]Istituto Studio Materiali Nanostrutturati , CNR, via Salaria Km 29,300-00015 - Monterotondo Staz. (RM) - Italy

*Corresponding Author: daniele.zappi@uniroma1.it

ABSTRACT

Main purpose of the work is assembling, testing and optimizing new disposable amperometric biosensors to analyze substances in different application fields as agribusiness, clinical chemistry and environment protection.

Many kinds of modified electrodes have been prepared and tested to build portable devices to analyze quickly many analytes, in a simple and cost-effective manner.

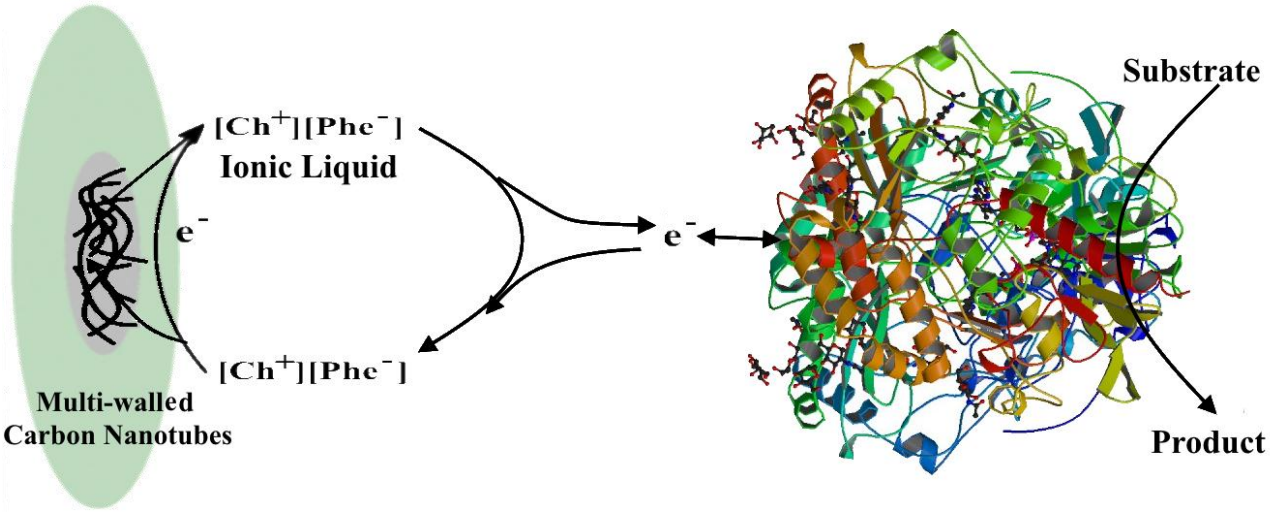
Bare electrodes of the screen-printed type, with silver as reference, have been used for modification.

The glassy carbon electrodes with multi-walled carbon nanotubes or graphene or gold nanoparticles depositions were modified with generation IV ionic liquids. Choline as cation and amino acids, such as glycine, serine, phenylalanine and histidine, as anions have been employed for these ionic liquids.

The presence of nanostructured materials on the electrode brings an increased contact surface between analytes and receptor and, consequently, an amplification of the amperometric signal and a better sensibility. Moreover the use of new ionic liquids of generation IV, biologically friendly and water soluble, improves the electronic transfer, facilitating and strengthening the redox reaction nearby the electrode.

By immobilizing the proper enzymes onto the modified electrode surface, different compounds of analytical interest can be determined by means of sensitive, properly designed amperometric biosensors.

Analytes such as antioxidant components in extra-virgin olive oils, alcohols in beverages and glucose in food matrices have been tested, using a suitable enzyme: microbial lipase, alcohol dehydrogenase and glucose oxidase, respectively.



Graphical abstract

Abbreviations:

SPE, Screen Printed Electrodes; GC, Glassy Carbon; GNP, Gold Nano-Particles; GPH, Graphene; MWCNT, Multi-Walled Carbon Nanotubes; RTILS, Room Temperature Ionic Liquids; A_{ea}, Electroactive area; EVOO, Extra-Virgin Olive Oil; GOx, Glucose oxidase; ADH, Alcohol dehydrogenase

KEYWORDS

Enzymatic biosensor; Ionic liquid; Nanostructured electrode; Glucose oxidase; Alcohol dehydrogenase; Lipase

1. Introduction

Biosensors have been object of numerous researches in recent years. Integrating biological components in known analytical instruments allow a higher degree of selectivity and simplicity compared to traditional analytical procedures. Nowadays biosensors find use in numerous fields such as biomedicine [1] (glucose sensors for diabetic patients, screening tests in hospitals and more), agribusiness [2-4] (detection of crops parasites, pesticides residue in food, analysis of substances identifying Protected Designation of Origin food production) and environment protection [5, 6].

Particular attention has been dedicated to the miniaturization of the developed biosensors for use in field analysis, without the need of voluminous laboratory equipment. Such biosensors are often mass-produced, inexpensive and used by non-specialists.

Aim of this work is the construction, testing and optimization of new disposable enzymatic electrochemical biosensors, useful to quantify various analytes of interest in different research fields.

The proposed sensors employ screen-printed electrodes (SPE), suitable to construct single-use miniaturized devices aimed at analyzing extremely low amounts [7]. Moreover, these features make them easy to use on the field, without fouling risk of the enzyme component arising from reuse. SPE are easily mass-produced with different nanomaterial modification of the working electrode. The SPE electrodes used in this work are composed of a glassy carbon (GC) working electrode opportunely modified with different nanomaterials, such as gold nano particles (GNP), graphene (GPH) and multi-walled carbon nanotubes (MWCNT). Additional optimization of the electrode was performed by adding room temperature ionic liquids (RTILs) of generation IV. The generation IV RTILs, formed by the cation choline (Ch) and four different amino acid as anions (glycine (Gly), serine (Ser), phenylalanine (Phe) and histidine (His)), have been prepared and characterized as reported in a previous work [8].

The electrochemical performances of all the nanomaterial/RTIL combinations were tested. The effect of the two considered elements interactions on the electron transfer phenomenon has been studied. For each prepared electrode, the electrochemically active area (A_{ea}) was calculated by measuring the current intensities of the potassium hexacyanoferrate (III) redox process at different potential scan speeds [9].

Many papers describe the use of nanomaterials to modify working electrodes for amperometric sensors assembling [10-12]. The RTILs employ is also reported [13, 14], but the generation IV ionic liquids have never been considered before.

Electrochemical biosensors, using SPE modified with nanostructures combined with green RTILs, are here presented. The best nanostructure/RTIL arrangement found was: glassy carbon electrode with multi-walled carbon nanotubes and choline-phenylalanine RTIL (GC/MWCNT/[Ch][Phe]).

In order to study the modified SPE usefulness in constructing reliable analytical tools, three different biosensors have been assembled and tested on real samples using three well-known enzymes: glucose oxidase (GOx) [15], lipase [16] and alcohol dehydrogenase (ADH) [17].

2. Materials and methods

2.1. Materials

Potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen phosphate (Na_2HPO_4) were obtained from Merck (Kenilworth, NJ, USA).

Potassium chloride, D-(+)-glucose monohydrate, potassium permanganate ($KMnO_4$), hydrochloric acid and nitric acid were purchased from Carlo Erba (Cornaredo, MI, Italy). Standard olive oil and ethylic alcohol were supplied from Sigma-Aldrich (Buchs, Switzerland).

Fungal lipase from *Candida rugosa* (EC 3.1.1.3, activity: 4320 U mg^{-1}), stored at 4°C, lipase from *Porcine pancreas* (EC 3.1.1.3, activity: 42727 U mg^{-1}), glucose oxidase (GOx) from *Aspergillus niger* (EC 1.1.3.4, activity: 174,9 U mg^{-1}), alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* (EC 1.10.3.2, activity: 331 U mg^{-1}) were from Sigma-Aldrich (Buchs, Switzerland) and stored at -18°C.

2-Hydroxy-N,N,N-trimethylethan-1-aminium hydroxide (Choline hydroxide) and the four amino acids (glycine, serine, phenylalanine and histidine sodium salts) were purchased respectively from Fluka and Sigma Aldrich (Buchs, Switzerland). All choline-amino acid room temperature ionic liquids (RTILs): choline-glycine [Ch][Gly], choline-serine [Ch][Ser], choline-phenylalanine [Ch][Phe] and choline-histidine [Ch][His] were synthesized following the titrimetric methodology reported by Masci et al. [8].

All the solutions have been prepared by means of high purity deionized water (Resistance = 18.2 MΩ × cm at 25 °C; TOC < 10 μg L^{-1}), obtained from Millipore Direct-Q UV3 device (Molsheim, France).

2.2. Instrumentation

FEG-SEM (Field Emission Gun Scanning Electron Microscope) images of the bare and modified glassy carbon screen-printed electrodes were obtained with a SEM High Brillance LEO 1530 Field Emission Scanning Electron Microscope (FESEM) with a field emission gun, equipped with an EDS X-ray spectrometer INCA 450, as detector.

All electrochemical experiments have been performed by means of a PalmSens Electrochemical Sensor Interface from PalmSens BV (Utrecht, The Netherlands), using Screen Printed electrodes

(SPEs), both glassy carbon and functionalized with nanomaterials, from DropSens (Llanera, Asturias, Spain).

2.3. Modification of the electrodes with RTILs and enzymes

In order to modify the working electrode surface with RTILs, a solution of the chosen RTIL was prepared in aqueous medium to facilitate the deposition: 50 μL of RTIL were diluted in 75 μL of appropriate buffer (pH 7.4 PBS buffer for Lipases, pH 5.1 phthalate buffer for GOx, pH 8.0 PBS for ADH). 20 μL of this solution were deposited on the working electrode and dried in a desiccator overnight.

Enzyme immobilization was performed by dissolving the proper enzyme in the above-described solution, 20 μL of this solution were then dropped on the working electrode surface. This resulted in the entanglement of the enzyme on the electrode surface due to the strong interactions with RTIL ions. In particular, for the GOx application, several immobilizing agents have been tested for comparison with the generation IV ionic liquids.

2.4. FEG-SEM characterization

The modified electrodes were grounded with a thin carbon film coated on the electrical contacts surface; then they were subjected to SEM imaging at different magnifications.

2.5. Electrochemical measurements of biosensors

Commercial SPE with different working electrodes were employed, in particular: bare glassy carbon (GC) and GC modified with graphene (GPH), multi-walled carbon nanotubes (MWCNTs) or gold nanoparticles (GNP), with a working electrode diameter of 4 mm. Reference and counter electrodes were silver and graphite respectively. These nanostructured electrodes have been further modified with different generation IV RTILs, performing all the possible combinations.

The measurements for calculating the electrochemically active areas (A_{ea}) by cyclic voltammetry (CV) at variable scan rate (5-200 mVs^{-1}) were carried out, in the presence of 1 mmol L^{-1} $K_3Fe(CN)_6$ in 100 mmol L^{-1} phosphate buffer (PBS), with 100 mmol L^{-1} KCl as electrolyte (Figure 1).

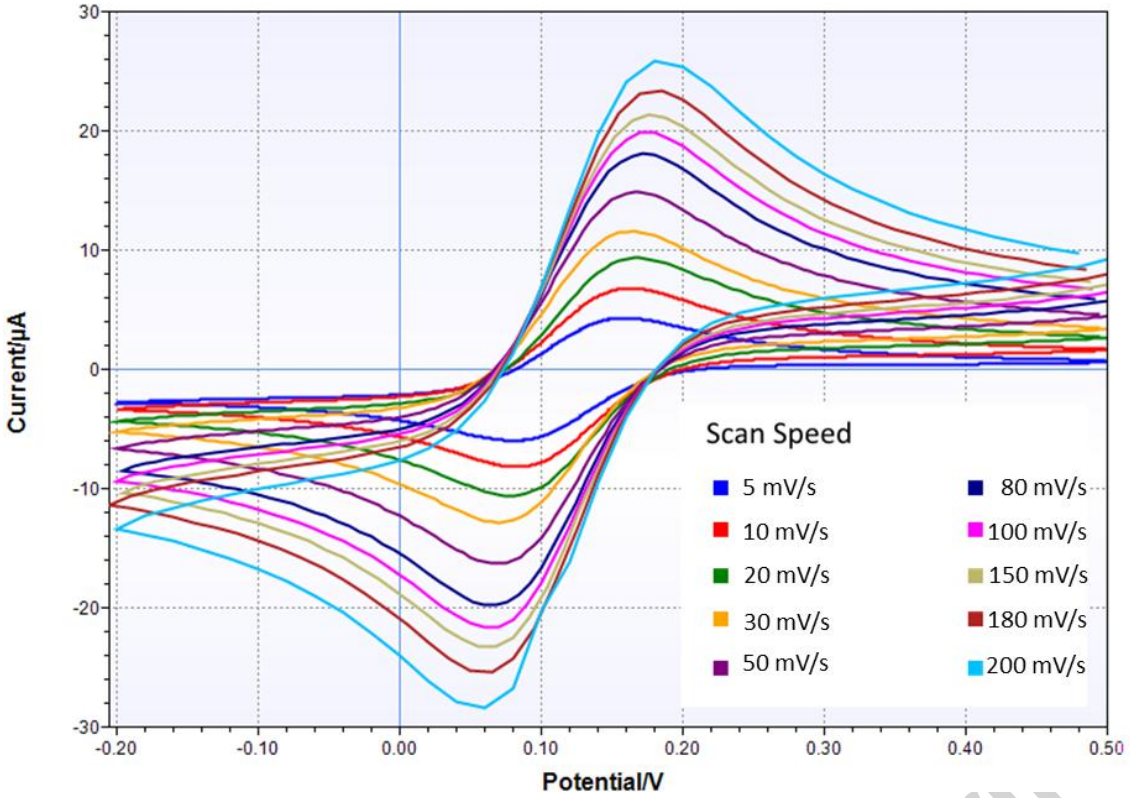


Figure 1 Cyclic voltammetry measurement at different scan speeds of a glassy carbon electrode modified with multi-walled carbon nanotubes

3. Results and discussion

3.1. Influence of nanomaterials and RTILs on the electrode surfaces

A morphological study by FEG-SEM analysis was performed, as reported in Figure 2.

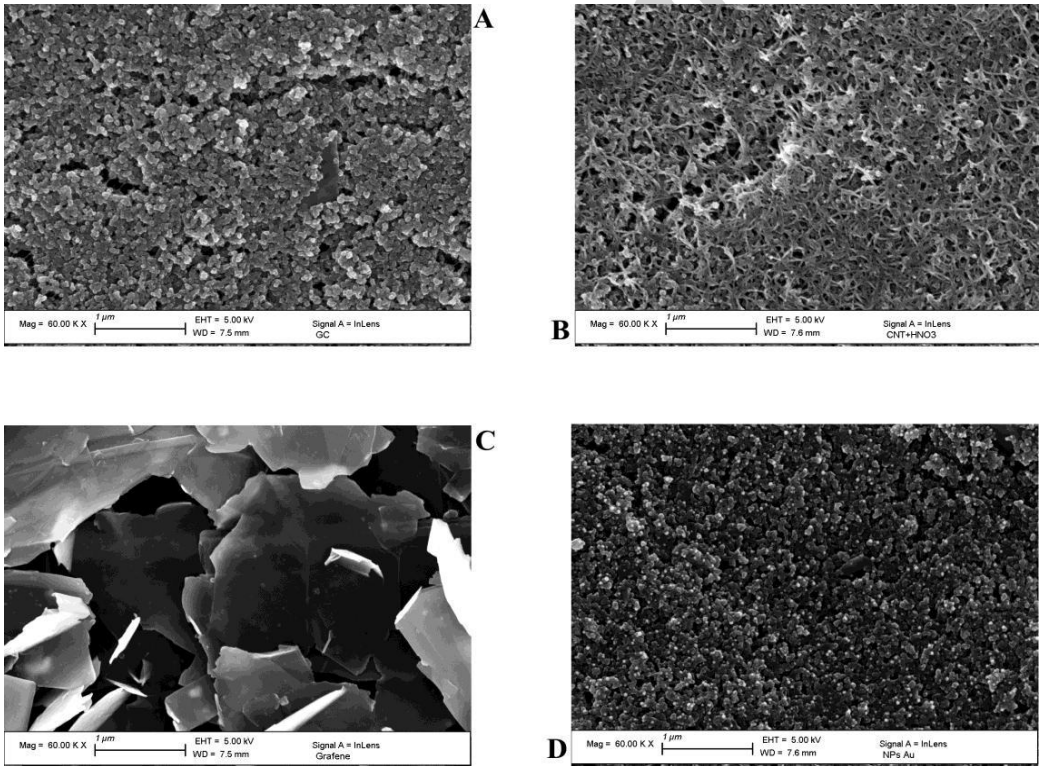


Figure 2. FEG-SEM images at 80000 magnifications of the screen-printed electrodes used: A) Glassy carbon (GC); B) GC/multi-walled carbon nanotubes; C) GC/graphene; D) GC/gold nanoparticles

As it can be seen in Figure 1 C, the GPH electrode surface is not useful to our purposes owing to its sheet-like morphology. Both MWCNT and GNP show an increased surface area with respect to bare electrode (Figure 1 B and D), but multi walled carbon nanotubes form a sort of web-like structure on the electrode surface, which is very useful to trap other compounds, such as RTILs and/or enzymes.

In order to obtain the best biosensor platform, the nanomaterials influence on the electrodes properties, particularly the electrochemically active area, has been investigated. The obtained data (Figure 3) show that nanomaterials sharply increase the A_{ea}, with respect to the bare electrode, as well known in the literature [18, 19].

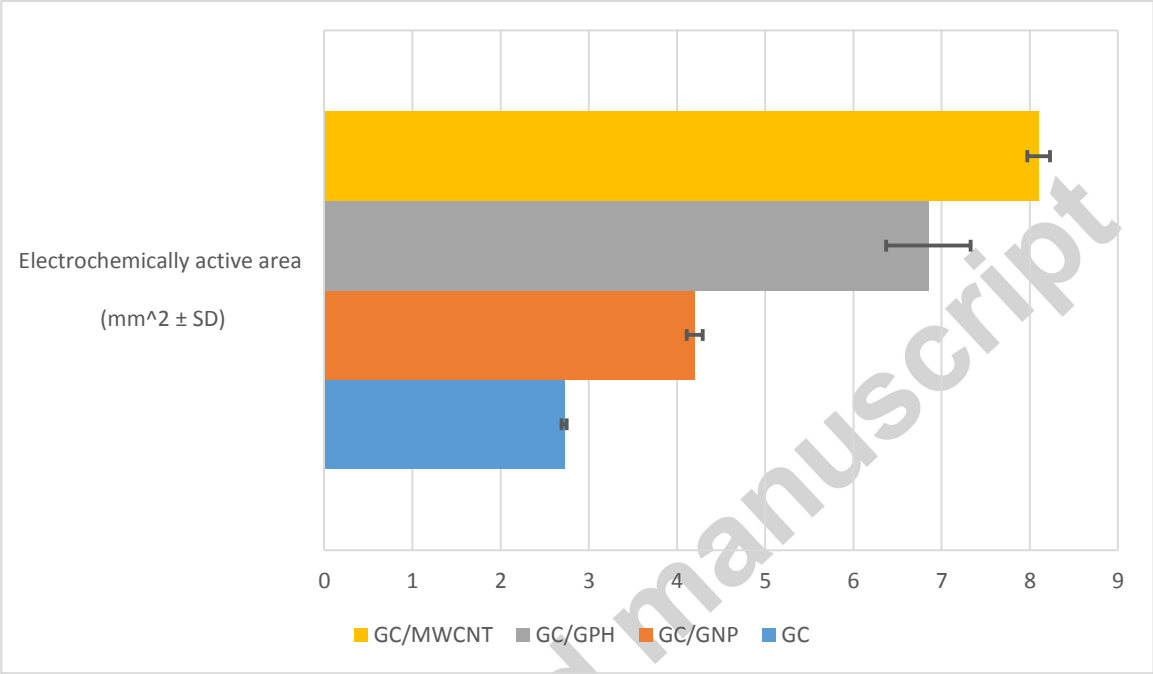


Figure 3. Effects of nanomaterials (MWCNT: multi-walled carbon nanotubes; GPH: graphene, GNP: gold nanoparticles, GC: glassy carbon) on the electrochemically active areas of the screen-printed electrodes used

Measures with different carbon based screen-printed electrodes modified with different combinations of nanomaterials and RTILs have been carried out, the respective electrochemically active areas have been calculated.

Table 1. Electrochemically active areas of screen-printed electrodes modified with different nanomaterials and room temperature ionic liquids (Ch: choline, Gly; glycine, Ser: serine, Phe: phenylalanine, His: histidine, GC: glassy carbon, GNP: gold nanoparticles, GPH: graphene, MWCNT: multi-walled carbon nanotubes)

Nanomaterial modification	**[Ch][Gly]** (mm^2 ± SD)	**[Ch][Ser]** (mm^2 ± SD)	**[Ch][Phe]** (mm^2 ± SD)	**[Ch][His]** (mm^2 ± SD)
GC/GNP	6.97 ± 0.05	4.85 ± 0.33	3.14 ± 0.33	6.85 ± 0.92
GC/GPH	6.23 ± 0.16	7.13 ± 0.09	5.32 ± 0.62	6.13 ± 1.15
GC/MWCNT	4.39 ± 0.07	4.99 ± 0.01	9.94 ± 0.04	6.35 ± 0.46

The results reported in Table 1 show the different behaviors of the considered ionic liquids with the examined nanomaterials on the SPE surface. As it can be seen, the greatest electrochemically active area was achieved with the GC/MWCNT/[Ch][Phe] electrode. Therefore, this modified electrode

has been chosen in all the subsequent measurements as platform for enzymatic biosensors assembling.

3.2. Analyses of oils by lipase biosensor

Lipase enzymes act on triglyceride molecules hydrolyzing the ester bond between glycerol and carboxylic acid; related to their origins, products of the reaction are glycerol and free carboxylic acids or a monoglyceride and two free carboxylic acids. All the lipase enzymes perform their catalytic activity at the water/oil interface [20]. It has been shown [3] that the oxidation of antioxidant compounds in oil is closely related to triglycerides hydrolysis by lipase. All the oxidative reactions, occurring at the working electrode, are due to the antioxidant compounds released from the oil matrix after the lipase hydrolytic action.

The enzyme lipase was immobilized on the surface of MWCNT-SPE by means of ionic liquid [Ch][Phe] to obtain the lipase biosensor. All the considered RTILs were tested, as solvent medium for oils in buffer solution (PBS pH 7.4). [Ch][Ser]/buffer/oil emulsion (40/20/40 % respectively) resulted particularly stable, increasing the substrate/enzyme contact surface and providing reproducible measurements. 40 μL of [Ch][Ser]/PBS/oil sample emulsion were deposited on the biosensor. After 30 min incubation time, cyclic voltammetry measurements were carried out.

Two lipases of different origin (from *Candida rugosa* and *Porcine pancreas*) have been used to find the best electrochemical response. Olive oils and different seed oils have been tested with the two enzymes. The two enzyme did not show appreciably different results, hence, the microbial enzyme has been used, being less expensive.

Table 2. Results comparison of different origin oils in terms of potential and peak intensity. (Working Electrode: GC/MWCNT/[Ch][Phe]/lipase from Candida rugosa, Reference Electrode: silver, Counter Electrode: graphite)

Sample	**Potential** *(anodic peak)* **(V± SD)**	**Current intensity** *(anodic peak)* **(μA± SD)**
Standard Fluka olive oil	0.300 ± 0.002	5.369 ± 0.005
Extra virgin olive oil 1	0.385 ± 0.001	4.171 ± 0.002
Extra virgin olive oil 2	0.409 ± 0.001	6.191 ± 0.002
Peanut oil	0.429 ± 0.001	4.005 ± 0.003
Sunflower oil	0.373 ± 0.002	7.498 ± 0.002

In Table 2, the results of only two of the examined extra virgin olive oils (EVOOs) are reported together with standard Fluka olive oil and two other seed oils. From these results, it is not possible to discriminate the olive oil samples from oils obtained from seeds.

By matching all EVOOs examined data, a new classification could be achieved based on the plants geographic origin (Figure 4).

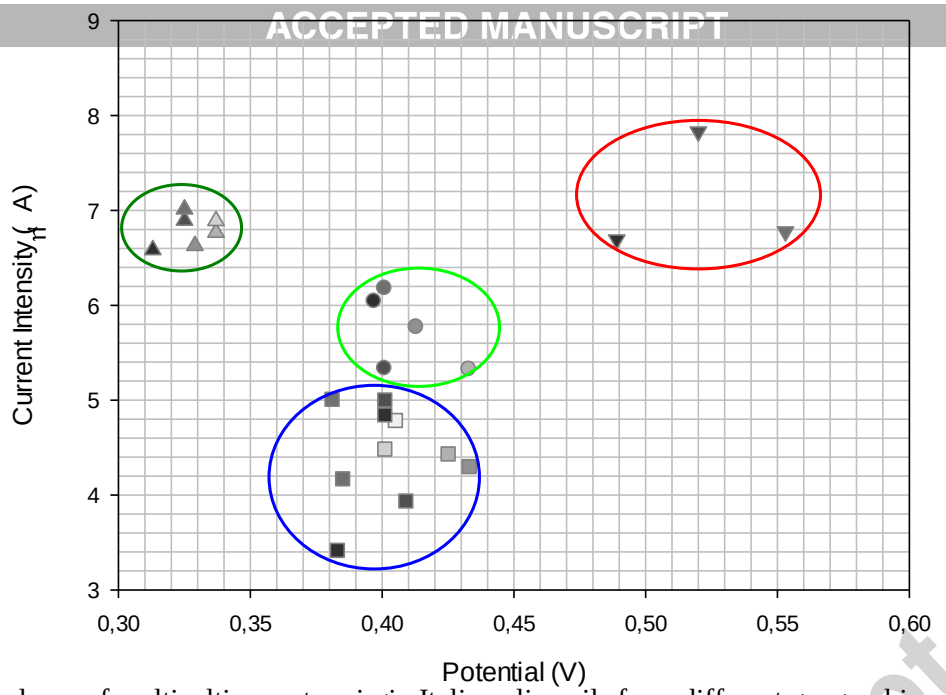


Figure 4. Analyses of multicultivar extra virgin Italian olive oils from different geographic areas of Italy. (Working Electrode: GC/MWCNT/[Ch][Phe]/lipase from Candida rugosa, Reference Electrode: silver, Counter Electrode: graphite). Points in the graph correspond to different oil samples. Each geographical zone is represented by: ▲South Italy ▼Tuscany ● Abruzzo ■ Lazio.

The large potential range values and different peak intensities obtained could be attributed to different antioxidants composition specific for each examined oil. It is to be noticed that this variability is influenced by the particular olive *cultivar*, geological nature and composition of the soils and climate were the tree grew.

As shown in Figure 4, some samples responses appear in the same part of the plot, as oils are obtained from olives of the same cultivar and/or olives originate from very similar Italian climatic zones.

3.3. Beverage analysis with glucose oxidase biosensor

To assemble an electrochemical biosensor for glucose determination, glucose oxidase enzyme (GOx) was chosen, being able to directly transfer electrons from glucose to the electrode through its active site (DET). The reaction catalyzed by glucose oxidase is the oxidation of glucose to D-glucono-δ-lactone with production of hydrogen peroxide. If direct electron transfer between the catalytic center and the electrode is permitted (for example using carbon nanotubes or other conductive nanowires), the electrons produced from the oxidation of glucose are transferred to the working electrode when a potential between 0 and 0,2 V is applied, thus preventing the formation of hydrogen peroxide. To be sure of the catalytic activity of GOx, immobilized on GC-MWCNT by means of ionic liquid [Ch] [Phe], cyclic voltammetry measurements were performed (Figure 5).

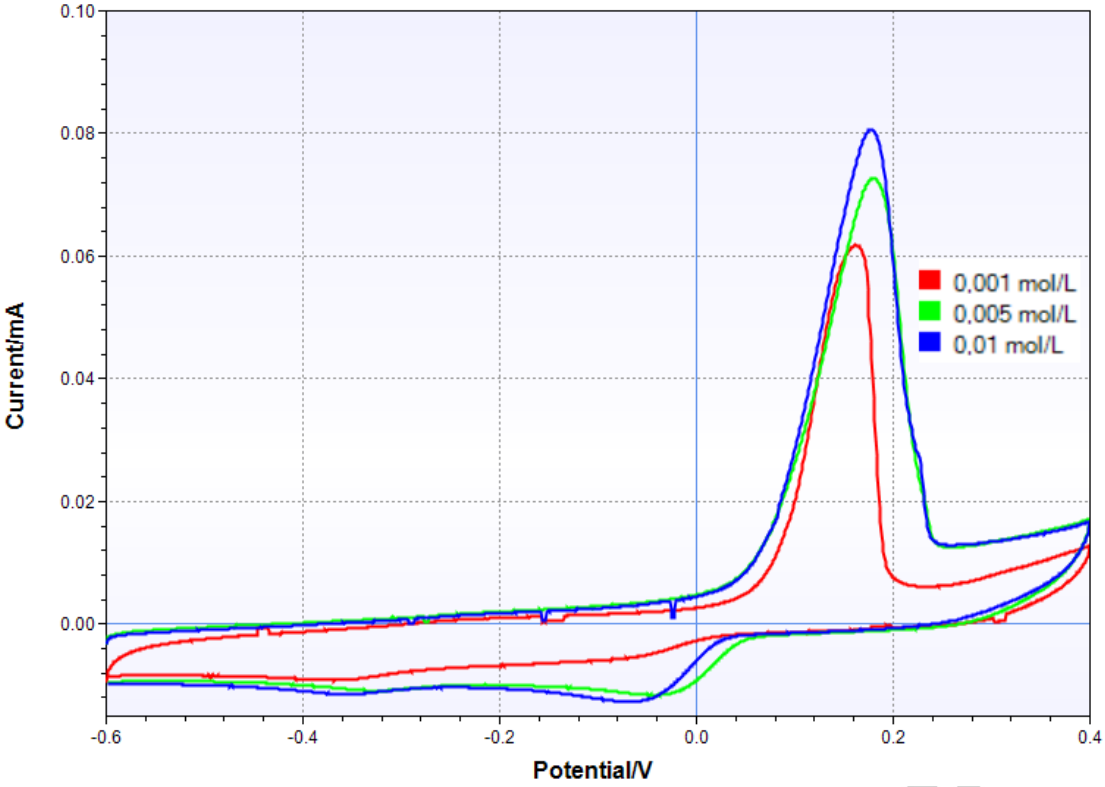


Figure 5 Cyclic voltammograms of the glassy carbon - multi-walled carbon nanotubes + [Choline][Phenylalanine] + glucose oxidase electrode in presence of different glucose concentrations

It has been observed that the reaction occurs even in the absence of enzyme, but the reaction rate is much slower. Subsequently, an investigation of the influence of different GOx immobilization methods on its catalytic efficiency was carried out. The methods used were: immobilization with a 0,1% (v/v) solution of Nafion; a two-step immobilization using glutaraldehyde 2,5% (v/v), to create a suitable base on the WE, followed by immobilization of the enzyme using Nafion 0,1%, as seen in literature [15]; a 30% solution (v/v) of [Ch][Phe] RTIL in pH 5.1 buffer solution.

Results for measurements using the three immobilization systems are presented in Table 3.

Table 3 Comparison of different immobilization systems for glucose oxidase enzyme in presence of 0.01 mol L^{-1} glucose in phthalate buffer (pH 5.1) (scan speed = 10 mV s^{-1}).

Immobilization used for GOx	Potential (V)	Oxidation peak intensity (µA)
NAFION 0,1% (v/v)	0,027 ±0,005	0,805 ±0,152
Glutaraldehyde 2,5% + NAFION 0,1% (v/v) [15]	0,027 ±0,004	1,134 ± 0,226
RTIL [Ch][Phe] 30% (v/v)	0,076 ±0,002	4,605 ±0,100

The best results in terms of response intensity were obtained by using RTIL [Ch][Phe] as immobilization agent for GOx**.** The difference in the oxidation intensities could be assigned to different causes. Polymeric initiation and propagation reactions could lead to bindings between growing polymer and sites required for the enzymatic catalysis, inhibiting it. Moreover, the

polymers employed are unable to transfer electrons, so that films and bonds formed during polymerization could hinder electron migration from the enzyme to the electrode surface, whereas the [Ch] [Phe] RTIL actually helps electron transfer by increasing the WE electrochemically active area.

After several chrono-amperometric measurements, optimal experimental conditions have been reached and, in Figure 4, the calibration plot is reported.

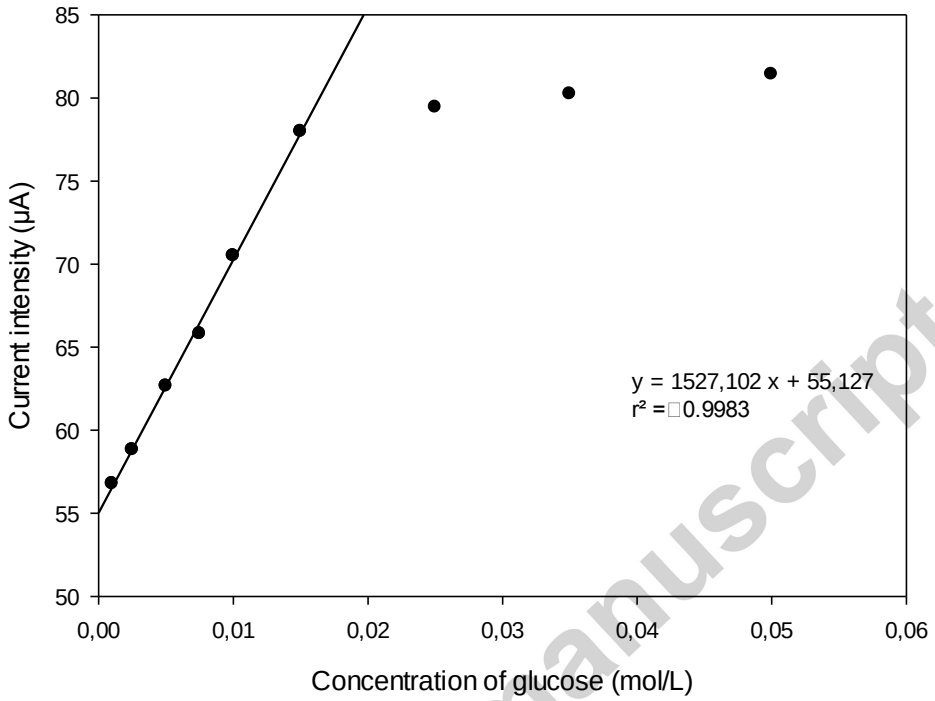


Figure 6 Calibration curve for glucose analysis with the glucose oxidase biosensor. (Working Electrode: GC/MWCNT/[Ch][Phe]/ glucose oxidase from *Aspergillus niger*, Reference Electrode: silver, Counter Electrode: graphite)

As shown in Figure 6, the assembled biosensor presents good linear range between 0.001-0.015 mol L^{-1} and optimum correlation coefficient (R^2=0.9983), indexes of reliability of the proposed apparatus.

To further demonstrate the increase in performances due to the MWCNT/[Ch][Phe] assay, in Table 4 is reported a comparison against electrodes prepared without RTIL or MWCNT respectively.

Table 4 Comparison of the performances for different modifications of the working electrode using glucose oxidase enzyme. For each different modification, analytical performances (R^2, LOD and LOQ) are reported.

Working electrode modification	Slope of the calibration curve	R^2 value	Limit of Detection (mol L^{-1})	Limit of Quantification (mol L^{-1})
GC-MWCNT+GOx	*No anodic or cathodic peak*	/	/	/

GC+[Ch][Phe]+GOx	2631.686	0.9778	0.001947	0.006490
GC-MWCNT+[Ch][Phe]+GOx	1527,102	0,9983	0,000335	0,001014

The reported biosensor has been tested for glucose analysis on a commercial energy drink. 1 mL of the energy drink was diluted up to 100 mL using pH 5.1 buffer solution in order to have both pH and glucose concentration of the solution fit the operative parameters of the biosensor. The measured glucose concentration was 0.79 ± 0.07 mol L^{-1}, in good agreement with the labelled value. Our method was compared with a standard titration one based on the reaction between hydrogen peroxide, produced by the GOx action on glucose, and potassium permanganate [21]: the energy drink was first treated with activated carbon in order to decolorize it. 40 mL of the decolorized solution were put in a titration flask along with 60 mL of pH 5.1 buffer and 5 mg of GOx. The titration flask was then corked and put in a thermostated bath at 25 °C for 30 minutes to allow the enzymatic reaction to proceed. Once the reaction time expired, 100 mL of diluted (10%) sulfuric acid were added to the solution which was then titrated with $KMnO_4$ 0,5 mol L^{-1}, stopping when a faint pink color appeared. Applying this method to the same sample of energy drink, we obtained a glucose concentration of 0.70 ± 0.06 mol L^{-1}. The relatively small difference in the results obtained with the two methods could be attributed to the spontaneous decomposition of small quantities of hydrogen peroxide during the titration process.

3.4. Beverage analysis with alcohol dehydrogenase biosensor

Alcohol dehydrogenase enzymes catalyze the dehydrogenation of primary alcohols to aldehydes. Alcohol dehydrogenase from *Saccharomyces cerevisiae* in particular shows selectivity towards ethanol as its substrate, which is thus transformed in acetaldehyde. The enzymatic reaction involves the transfer of two electrons to NAD^+ coenzyme, which is reduced to NADH. Since there is a 1:1 proportion between ethanol transformed in acetaldehyde and NADH produced, the latter can be oxidized in an electrochemical biosensor to measure the concentration of ethanol.

The alcohol dehydrogenase (ADH) enzyme was immobilized by ionic liquid [Ch][Phe] on the surface of GC/MWCNT. The biosensor was characterized by linear sweeps scans in presence of its substrate, ethyl alcohol, at different concentrations (Figure 7).

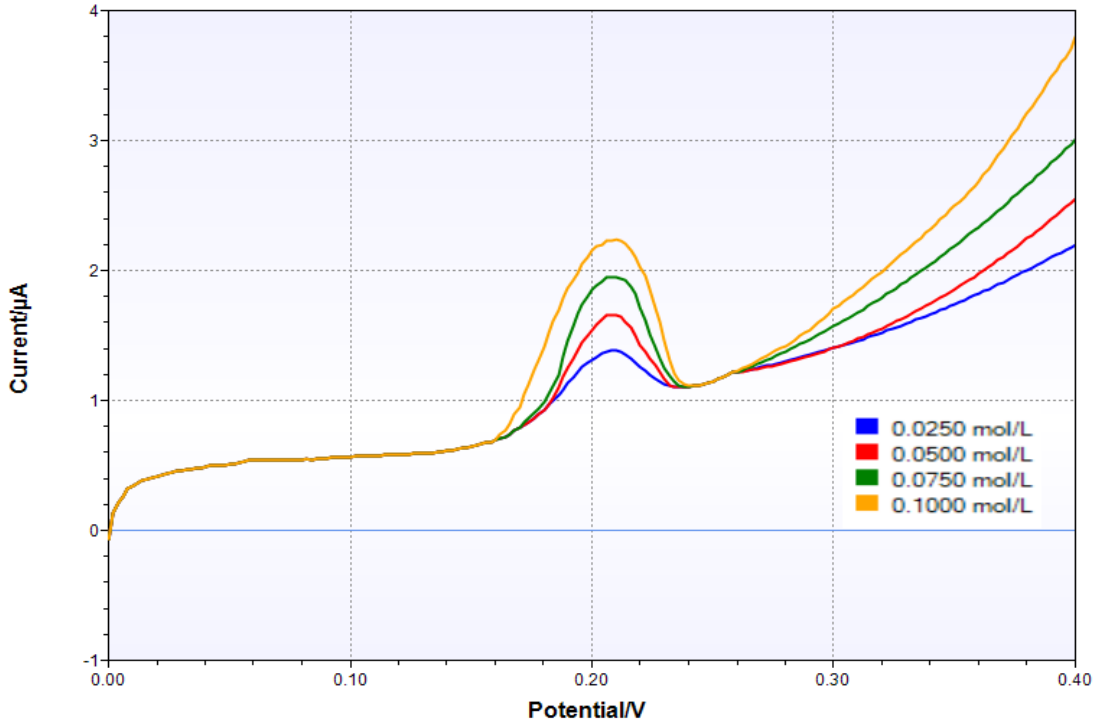


Figure 7 Linear sweep scans of the glassy carbon - multi-walled carbon nanotubes electrode modified with [Choline][Phenylalanine] RTIL and alcohol dehydrogenase with different ethyl alcohol concentrations in PBS (pH 8.0)

It was observed an anodic peak at 0.206 V with intensity proportional to the concentration of substrate in solution. Afterwards, to verify the linear dependence between anodic peak currents and ethyl alcohol concentration, chrono-amperometric measurements have been carried out.

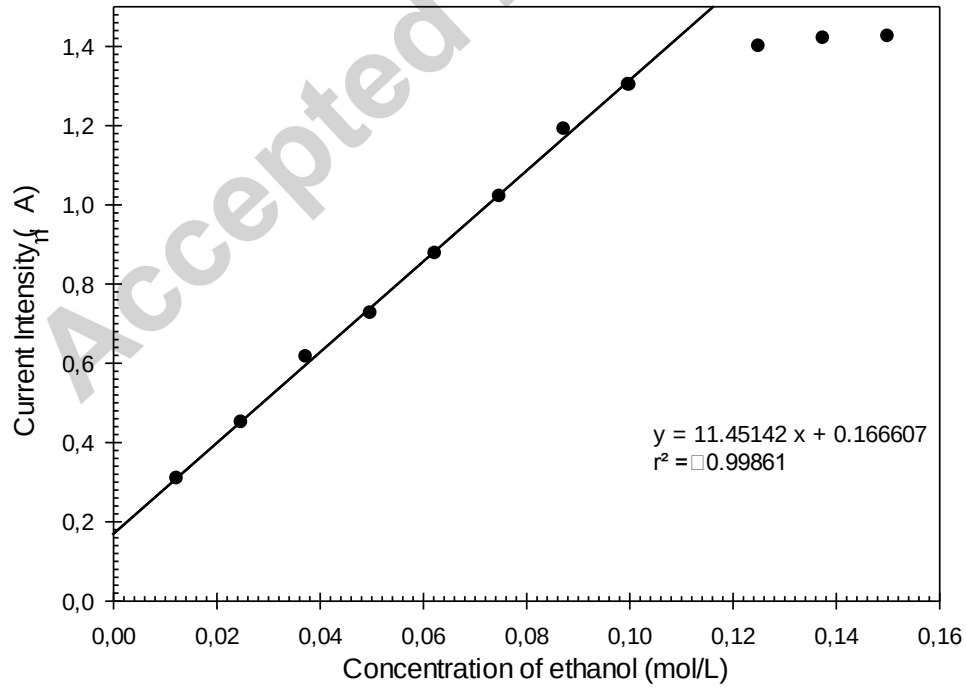


Figure 8. Calibration curve for ethyl alcohol analysis with the alcohol dehydrogenase (ADH) biosensor. (Working Electrode: GC/MWCNT/[Ch][Phe]/ADH from *Saccharomyces cerevisiae*, Reference Electrode: silver, Counter Electrode: graphite)

As can be seen in Figure 8, the ADH biosensor shows a wide linear range (12.5 –100 mmol L^{-1}) and a good correlation coefficient (R^2=0.99861).

Once again, to demonstrate the increase in performances due to the proposed assay, a comparison against electrodes prepared without RTIL or MWCNT respectively is reported in Table 5.

Table 5 Comparison of the performances for different modifications of the working electrode, using alcohol dehydrogenase enzyme. For each different modification, analytical performances (R^2, LOD and LOQ) are reported.

Working electrode modification	Slope of the calibration curve	R^2 value	Limit of Detection (mol L^{-1})	Limit of Quantification (mol L^{-1})
GC-MWCNT+ADH	0,60035	0,98212	0,0241	0,0731
GC+[Ch][Phe]+ADH	1,32291	0,99804	0,0073	0,0222
GC-MWCNT+[Ch][Phe]+ADH	11,45142	0,99861	0,0037	0,0111

An important feature of the enzyme ADH from *Saccharomyces cerevisiae* is its high selectivity towards ethyl alcohol. To confirm whether this selectivity was maintained by the proposed biosensor, measurements were carried out using different substrates as ethyl, methyl and isopropyl alcohols. These two last have been chosen because they are very toxic for humans, methyl alcohol in particular is metabolized to formic acid, which has a minimal lethal dose in humans of 1 g/kg of body weight in people not having simultaneously consumed ethanol [22].

Table 6 Selectivity of Alcohol dehydrogenase biosensor in respect to different alcohols as substrates. (Working Electrode: GC/MWCNT/[Ch][Phe]/ADH from Saccharomyces cerevisiae, Reference Electrode: silver, Counter Electrode: graphite)

Substrate	**Concentration** (mol L^{-1})	**Anodic current intensity (μA)**
Ethyl alcohol	0.025	0,450 ± 0,002
Methyl alcohol	0,025	0,029 ± 0,001
Isopropyl alcohol	0,025	Not detected

As it can be seen in Table 6, the proposed ADH biosensor is very selective towards the ethyl alcohol substrate. This specificity makes the developed biosensor an interesting tool for alcohol-test.

4. Conclusions

Four generation IV RTILs (room temperature ionic liquids), Choline-Gly, Choline-Ser, Choline-Phe, Choline-His, and three nanomaterials (graphene, gold nanoparticles, multi-walled carbon nanotubes) have been employed to modify screen-printed electrodes. All the RTILs tested are

biologically friendly in opposition to those of previous generations. Among all the combinations studied, the best modification resulted glassy carbon-multiwalled carbon nanotubes/Choline-Phe, in relation to the electrochemically active area obtained. This modification of the working electrode has been used to construct different biosensing platforms, by immobilizing three different enzymes: lipase from *Candida rugosa*, glucose oxidase from *Aspergillus niger* and alcohol dehydrogenase from *Saccharomyces cerevisiae*.

All the developed biosensors show good reliabilities; in particular, their analytical parameters such as sensitivity, limit of detection and linearity range, are acceptable for food analysis applications and are in good agreement with other reference methods [21].

The proposed biosensing platforms also show better analytical performances compared to the biosensors modified with nanomaterials or RTILs alone.

The developed biosensors could be miniaturized to obtain really portable, user-friendly and cheap devices through engineering knowledge.

The future goal of this work will be constructing and optimizing a lab-on-chip platform, useful for multi-analytes quantitation.

Funding

This work was supported by the Italian Education Ministry (MIUR) and the IMT srl Company (Via Cipriano Facchinetti, 67 - 00159 Roma – ITALY).

Conflicts of interest: none.

References

[1] A. Hasan, Md Nurunnabi, M. Morshed, et al., Recent Advances in Application of Biosensors in Tissue Engineering, BioMed Research International 2014 (2014) Article ID 307519, 18 pages, doi:10.1155/2014/307519.

[2] R. Pauliukaite, Andrew P. Doherty, Kevin D. Murnaghan, and Christopher M.A.Brett, Application of Some Room Temperature Ionic Liquids in the Development of Biosensors at Carbon Film Electrodes. Electroanalysis 20 (2008) 485–490.

[3] R. Pauliukaite, A.P. Doherty, K.D. Murnaghan, C.M.A. Brett, Application of room temperature ionic liquids to the development of electrochemical lipase biosensing systems for water-insoluble analytes, Journal of Electroanalytical Chemistry 656 (2011) 96-101.

[4] K.K. Subraya, A. Diggs, D. M. Porterfield, Amperometric Biosensor Approaches for Quantification of Indole 3-Acetic Acid in Plant Stress Responses, Communications in Soil Science and Plant Analysis 44 (2013) 1749-1763.

[5] J. Bao, C. Hou, M. Chen, J. Li, D. Huo, M. Yang, X. Luo, and Y. Lei, Plant Esterase–Chitosan/Gold Nanoparticles–Graphene Nanosheet Composite-Based Biosensor for the Ultrasensitive Detection of Organophosphate Pesticides, J. Agric. Food Chem. 63 (2015) 10319−10326.

[6] C. Tortolini, P. Bollella, M.L. Antonelli, R. Antiochia, F. Mazzei, G. Favero, DNA-based biosensors for Hg^{2+} determination by polythymine-methylene blue modified electrodes, Biosens. Bioelectron., 67 (2015) 524-531.

[7] C. Lanzellotto, G. Favero, M.L. Antonelli, C. Tortolini, S. Cannistraro, E. Coppari, F. Mazzei, Nanostructured enzymatic biosensor based on fullerene and gold nanoparticles: Preparation, characterization and analytical applications, Biosen. Bioelectron. 12 (2014), 430-437.

[8] S. De Santis, G. Masci, F. Casciotta, et al., Cholinium-amino acid based ionic liquids: a new method of synthesis and physico-chemical characterization, Phys. Chem. Chem. Phys. 17 (2015) 20687-20698.

[9] D. Salinas-Torres, F. Huerta, F. Montilla, E. Morallón, Study on electroactive and electrocatalytic surfaces of single walled carbon nanotube-modified electrodes, Electrochimica Acta 56 (2011), 2464-2470.

[10] J. Wang, M. Musameh, Y. Lin, Solubilization of Carbon Nanotubes by Nafion toward the Preparation of Amperometric Biosensors, J. Am. Chem. Soc. 125 (2003) 2408-2409.

[11] M.D. Rubianes, G.A. Rivas, Enzymatic biosensors based on carbon nanotubes paste electrodes, Electroanalysis 17 (2005), 73-78.

[12] J. Wang, M. Musameh, Reagentless amperometric alcohol biosensor based on carbon-nanotube/Teflon composite electrodes, Anal. Lett. 36 (2003) 2041-2048.

[13] C. Xiang, Y. Zou, L-X. Sun, F. Xu, Direct electron transfer of cytochrome c and its biosensor based on gold nanoparticles/room temperature ionic liquid/carbon nanotubes composite film, Electrochem. Commun. 10 (2008) 38-41.

[14] A.C. Franzoi, J. Dupont, A. Spinelli, I.C. Vieira, Biosensor based on laccase and an ionic liquid for determination of rosmarinic acid in plant extracts, Talanta 77 (2009) 1322-1327.

[15] M.L. Antonelli, F. Arduini, A. Laganà, D. Moscone, V. Siliprandi, Construction, assembling and application of a trehalase-GOD enzyme electrode system, Biosens. and Bioelectron. 24 (2009) 1382-1388.

[16] J.C. Escamilla, J.A. Rodriguez, G.A. Alvarez, C.A. Galan, Monoenzymatic Lipase Potentiometric Biosensor for the Food Analysis Based on a pH Sensitive Graphite-epoxy Composite as Transducer, J. Mex. Chem. Soc. 59 (2015) 19-23.

[17] F. Salimi, M. Negahdary, G. Mazaheri, H Akbari-dastjerdi, Y. Ghanbari-kakavandi, S. Javadi, S.H. Inanloo, M. Mirhashemi-route M.h Shokoohnia, A. Sayad, A novel Alcohol Biosensor Based on Alcohol Dehydrogenase and Modified Electrode with ZrO_2 Nanoparticles, Int. J. Electrochem. Sci. 7 (2012) 7225-34.

[18] M. Pumera, Graphene-based nanomaterials and their electrochemistry, Chem. Soc. Rev. 39 (2010) 4146-4157.

[19] M. Pumera, The Electrochemistry of Carbon Nanotubes: Fundamentals and Applications, Chem. Eur. J. 15 (2009) 4970-4978.

[20] P. Desnuelle, The Lipases , in: P.D.Boyer, The Enzymes, Elsevier B.V., Amsterdam, 1972, pp. 575-616

[21] Interox Chemicals Ltd., A Bleachers Handbook, Widnes, Cheshire, UK, 1980.

[22] O. Roe, Species differences in methanol poisoning, CRC Crit. Rev. Toxicol. 10 (1982): 275–286.

Highlights:

- GC screen-printed electrodes modified with nanostructures and green ionic liquids
- Multi-walled CNT, graphene, gold nanoparticles used as nanomaterials
- Generation IV room temperature ionic liquids formed by choline and amino acids use
- Analysis of olive oils, glucose and ethyl alcohol
- Realization of three enzymatic biosensors with good analytical parameters