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Conductometric Method for Rapid Lipase Activity Quantification

Ana Luisa Reyes[1], Roumen Zlatev[1,], Margarita Stoytcheva[1], Carlos Villa[1], Rafael Villa[1], Benjamín Valdez[1], Gisela Montero[1], Lydia Toscano[2], Lorenzo Alejandro Sánchez[1], Ricardo Salinas[1], Lesle Hernández[1]*

[1] Universidad Autónoma de Baja California, Instituto de Ingeniería, Blvd. Benito Juarez s/n, 21280 Mexicali B.C., México
[2] Instituto Tecnológico de Mexicali, Tecnológico Nacional de México, Mexicali B. C., México
[*]E-mail: roumen@uabc.edu.mx

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A new approach for lipase activity quantification was proposed and applied in a simple, rapid and cost effective conductometric method based on conductance change registration along the time during the enzymatic degradation of a thin nanocomposite substrate layer (SiO_2 nanoparticles loaded olive oil) deposited onto conductometric electrodes. The sensitive layer thickness diminution along the time causes its conductance augmentation proportional to the lipase activity. The proposed method was characterized in terms of lipase activity linear quantification range and LOD, quantification time, precision and reproducibility at optimized pH 8. The relative error was found to be from 3.6% to 1.2% at the linear quantification range from $1.1x10^{-2}$ to 1.17 U mL^{-1} respectively with a LOD of $0.8x10^{-3}$ U mL^{-1}. Finally, the method was validated with spiked samples applying spectrophotometric method as reference.

Keywords: Conductometry, Lipase activity quantification, nanocomposite

1. INTRODUCTION

The conductometry is a very simple but powerful tool for solutions properties characterization widely applied in a great variety of scientific and industrial areas. Conductometric methods for organic and inorganic solutions properties studies, measurement of dissolved organic and inorganic substances concentration, complexes formation and properties studies, etc. were reported till now [1-7].

The lipases (EC 3.1.1.3 triacylglycerol acylhydrolase) are enzymes naturally produced in animal pancreas degrading the fats and oils in fatty acids and glycerol [8-10] in the digestion processes. These unique specific properties make the lipases unreplaceable catalysts for some

industries mostly those involved in detergent, leather, textile food, cosmetics, paper, and biofuel production [11–19]. The lipase activity is crucial for the industrial processes stability maintenance, as well as it serves is indicator of some pancreatic diseases in the medicine [20]. That is why many types of sensors and methods for lipase activity quantification have been developed until now based on measuring techniques as spectrophotometry, titrimetry, radioactive assay, quartz crystal microbalance, immunoassay, conductometry, chromatography, etc. [21–36].

The conductometric methods for lipase activity quantification are based on the sample conductance change measurement resulting from the enzyme catalyzed reactions. In general they can be classed as reactions: releasing strong electrolytes, releasing feebly ionizing ampholytes and releasing nonelectrolytes. For example the conductometric method reported by Ballot [32-34] is based on a reaction of the second type: enzymatic degradation of high conductive lipase substrate such as triacetin resulting in solution conductance decrease. The common drawbacks of all the mentioned methods for lipase activity determination including the conductometric ones are: complicated and long procedures (hours) requiring skilled personal, expensive reagents and sophisticated laboratory equipment.

The industrial processes control however requires simple, rapid, precise and cost effective evaluations of relative high enzymatic activities and this paper is dedicated to the development and characterization of a conductometric method suitable for industrial applications. It is based on the conductometric determination of the rate of lipase catalyzed hydrolysis of a substrate sensitive layer deposited as a thin sensitive layer with thickness ***d*** on a conductometric electrode with surface area ***A***. The sensitive layer enzymatic decomposition by the lipase causes its thickness diminution along the time proportional to the lipase enzymatic activity resulting in corresponding resistance ***R*** diminution (conductivity ***G*** increase) according to the Equation 1:

$$R = 1/G = \rho d/A \quad (1)$$

where ρ is the resistivity of the sensitive layer material.

The high resistivity of the olive oil which is a lipase substrate ($\rho = 10^{7}$ Ohms m [37]) makes it a suitable substance to be used as conductometric electrode modifier serving as a sensitive layer. On the other hand the olive oil loading by nanoparticles made by a material possessing higher electrical resistivity such as SiO_2 (possessing resistivity $\rho = 10^{17}$ Ohms m [38]), a very high resistance nanocomposite will be formed. In this case a network of silica percolating throughout the oil matrix is formed possessing a good adhesion with solid surfaces, as reported by Adelmann [39]. Similar type of nanocomposite was already applied as a sensitive layer by the authors earlier in a photometric and a QCM method for lipase activity determination [22-24, 27] and it was proved that its decomposition rate is proportional to the lipase activity rate.

Thus, the method subject of the present work is based on the two following assumptions: i) the high resistance of the sensitive layer (olive oil/SiO_2 nanocomposite) will determine the total resistance of the interface: conductometric electrodes modified by nanocomposite/lipase containing buffer solution. Taking into account the Equation (1) it is clear that the interface conductance (resistance) will be proportional to the sensitive layer thickness which in turn depends along the time on the lipase

activity. ii) the enzymatic degradation of the olive oil which sticks together the SiO_2 nanoparticles in the nanocomposite will provoke their release from the sensitive layer into the lipase containing solution. As a result much larger sensitive layer resistance change will occur compared with bare olive oil only application as a sensitive layer due to the nanoparticles higher resistivity. This will result in sensitivity amplification of the proposed conductometric method.

Based on these assumptions the development of a rapid, simple, precise and cost effective method for lipase activity quantification is the purpose of the present work together with its characterization in terms of linear quantification range, LOD, precision, response time and reproducibility. The conductometric response is registered during the enzymatic degradation by the lipase of a thin nanocomposite sensitive layer deposited on the conductometric electrodes.

2. EXPERIMENTAL

2.1. Reagents

Lipase, (25.1 U mg^{-1}) from Sigma was used for the stock lipase solution preparation employed in all the experiments. A phosphate buffer (pH 8) was prepared by appropriate amounts of analytical grade K_2HPO_4 and KH_2PO_4 dissolution in deionized water produced by a MilliQ Water Purification System (Millipore). The buffer was applied for the lipase standard solutions preparation according to the producer recommendations as well as supporting solution for the lipase samples. Commercial extra virgin olive oil and SiO_2 nanoparticles modified by single layer organic chains giving them super-hydrophobic and oleophilic properties (99.8%, 10–20 nm, SkySpring Nanomaterials, Inc., USA, product # 6864HN) were used for nanocomposite preparation. $CHCl_3$ of PA purity (Fermont, USA) was applied as nanocomposite solvent facilitating its deposition on the electrode surface.

2.2. Instrumentation

USB powered potentiostat Model CompactStat.h 20250, Ivium Technologies, Netherlands running IviumStat software in mode AC Detection and YSI 3200 Conductivity Instrument were employed for the basic and the additional conductometric measurements respectively. Two electrodes configuration of the potentiostat was used employing two identical modified by nanocomposite conductometric electrodes. All the measurements were carried out in Model K0264 (EG&G PARC) micro cell electrode stand equipped with a magnetic stirrer. The IviumStat software running in AC Detection mode produces the following output data along the time: electrode/solution interface resistance R and capacitance C as well as the AC current phase angle shift φ resulted from constant AC voltage amplitude and frequency application. The registered resistance R vs. the time plots were converted in conductance G vs. the time ones applying the equation $G = 1/R$. In fact, these plots represent kinetic curves of the substrate layer hydrolysis catalyzed by the lipase.

Model UP 800 Ultrasonic Processor, ChromTech was used for the nanocomposite $CHCl_3$ solution preparation. Scanning Electron Microscope, model JEOL JSM-840 was employed for characterization of the modified by nanocomposite electrode surface.

2.3. Conductometric electrode construction

The common and very low cost PCB technology was applied for 1 x 5 cm stick conductometric electrodes elaboration employing 0.9 mm thick FR-4 PCB fiberglass substrate laminated by 35 μm thick Cu foil. The conductometric electrode cross section is presented in Figure 1. The Cu foil (2) laminated on the epoxy support (1) served as electric conductor between the electrode terminal (2) and the nanocomposite sensitive layer (4) deposited on a Cu disk 8 mm in diameter with a surface area of 0.5 cm^2. The electrode terminal and the Cu disc are situated at the two ends of the stick conductometric electrode. The rest of the Cu foil between them was insulated by the common epoxy solder mask (3) used in the PBC technology.

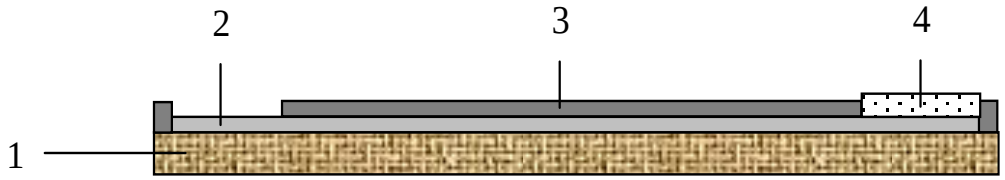

Figure 1. Cross section of the nanocomposite modified stick conductometric electrode with: 1 – sensor epoxy support, 2- Cu laminate, 3- epoxy insulation layer, 4 – nanocomposite sensitive layer

2.4. Sensitive layer composition and preparation

In spite that triolein is the most specific lipase substrate [27] a commercial extra virgin olive oil was used as lipase substrate in all the experiments because of its low cost, moreover it contain up to 30% triolein [35]. The procedure proposed by the authors earlier [22-24, 27] for a nanocomposite preparation and deposition was modified and applied in this work too. The nanoparticles interaction with the olive oil explained by Adelmann [39] allowed uniform nanocomposite layer formation on the Cu electrode due to the gelation causing a good adhesion to solid surfaces. The nanocomposite composition: the olive oil to the SiO_2 nanoparticles ratio was optimized experimentally to obtain the shortest response time and the highest sensitivity (see the Results and Discussion section).

The conductometric electrodes were modified by deposition from 5 to 10 μL of the nanocomposite chloroform solution on the Cu disc electrode surface by a micropipette. After the organic solvent evaporation a silk-wise white sensitive layer appears. The electrode can be easily regenerated by a new sensitive layer deposition after the lipase activity measurement. For this purpose the sensitive layer is removed by a soft serviette and a new one is deposited by a micropipette. The reproducibility was found to be satisfactory (see the Result section) which allows a great number of pre-deposited electrodes to be prepared and used as disposable due to their extremely low cost.

3. RESULTS AND DISCUSSION

3.1. Nanocomposite sensitive layer characterization by SEM

The modified by nanocomposite electrode surface was characterized by scanning electron microscopy (SEM), see Figure 2. The organic solvent evaporation resulted in SiO_2 nanoparticles agglomeration and clusters formation of about 400 nm in diameter. The satisfactory reproducibility of the results obtained by a great number of electrodes application at same conditions (see Results and Discussion section) showed that the clusters formation does not affect the analytical characteristics.

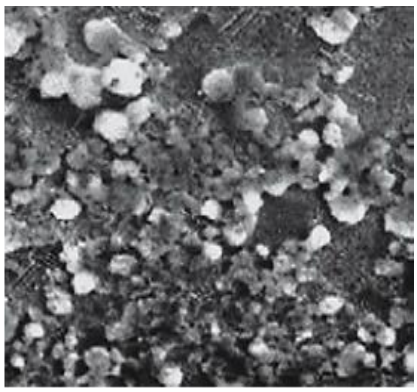

Figure 2. SEM micrograph of the nanocomposite sensitive layer (20% SiO_2 nanoparticles in olive oil) deposited on the conductometric electrode surface. Scale: 1000 nm cm^{-1}.

3.2. Typical sensor response and the nanoparticles influence

The main assumption this work is based on is that the enzymatic degradation rate of a thin nanocomposite sensitive layer deposited on the conductometric electrode depends on the measured lipase activity. The sensitive layer enzymatic degradation results in its thickness decrease which can be evaluated measuring the sensitive layer resistance (conductance) according to Eqn. 1. Thus, the conductance can serve as a measure of the lipase activity. The sensitive layer enzymatic degradation continues up to the saturation of the lipase active centers which determine the wave shaped curve in coordinates: resistance / conductance vs. the time, presented in Figures 3 and 4 respectively.

That is why the wave height corresponds to the maximal sensitive layer thickness change able to be provoked by the measured lipase activity. Thus, the wave height can serve as a quantitative measure of the lipase activity as shown below in sub-section 3.4 and this parameter was chosen as analytical response. The slope of the curve can also serve as analytical response but the wave height was preferred because of the better precision and reproducibility of the results.

The second assumption this work is based on is that the SiO_2 nanoparticles loaded olive oil (a nanocomposite) application as a sensitive layer will cause sensor response amplification due to the higher resistivity of the SiO_2 (possessing $\rho = 10^{17}$ Ohms m) compared with that of the bare olive oil ($\rho = 10^7$ Ohms m) used as lipase substrate. This assumption was experimentally tested applying two types of experiments: static and dynamic and the results are presented below.

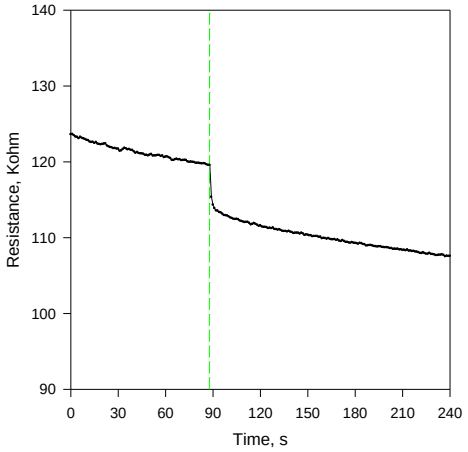

Figure 3. Resistance of the interface: nanocomposite modified conductometric electrode/lipase containing solution possessing activity of 0.03 U mL^{-1}. Stirring rate = 300 rpm, AC voltage amplitude = 50 mV at 120 Hz, pH = 8 (phosphate buffer)

For the purpose of the static experiments (without lipase addition) the conductivity of 5 mL deionized water containing 120 µL phosphate buffer with pH 8 was measured employing: a) a couple of 8 mm in diameter bare Cu conductometric electrodes, b) the same electrodes modified by 50 µm thick olive oil and c) the same electrodes modified by 50 µm thick nanocomposite (olive oil + 20% SiO_2 NPs). The average of 10 measurements obtained by the YSI Model 3200 Conductivity Instrument application at 1 cm distance between the electrodes are presented in Table 1.

Table 1. Electrode modification influence on the resistance of the electrode/phosphate buffer interface

Cu electrode modifier	No modified	50 µm thick olive oil	50 µm thick nanocomposite
Conductance, µS cm^{-1}	1139	17.6	7.7

The sensitive layers thicknesses values presented in the table were calculated taking into account the disk electrode diameter (8 mm), the volume (5 µL) of the deposited chloroform solution as well as the nanocomposite to chloroform volumes ratio. As seen from the table the electrodes modification with olive oil causes 64.7 times conductivity decrease compared with that obtained by the bare metal electrodes while the electrodes modification by nanocomposite (20% wt. SiO_2 NPs) provokes 147.9 times conductivity decrease respectively. Thus the static results confirmed the viability of the second assumption this work is based on.

The nanoparticles influence on the sensor analytical response was also verified by a dynamic experiment: resistance (conductance) vs. the time curves were registered in presence of lipase applying conductometric electrodes modified by bare olive oil and by nanocomposite at same lipase activity and same experimental conditions. The Conductance vs. the Time curves registered by the Ivium CompactStat potentiostat in AC detection mode are presented in Figure 4. The enzymatic degradation of the olive oil sticking together the high resistivity SiO_2 nanoparticles in the nanocomposite mass

causes the sensitive layer total thickness diminution accompanied with nanoparticles release into the lipase containing solution. As a result the wave height of curve B registered with nanocomposite application as electrodes modifier was found to be 1.86 times higher than those in curve A obtained at same lipase activity but with bare olive oil application as electrode modifier, both possessing the same initial thickness of 50 µm. Figure 4 presenting the dynamic experiments results also proves the viability of the second assumption this work is based on.

The shape of the conductance vs. the time curves presented in Figure 4 is similar to those registered by the QCM method application [22] reported by the authors earlier also based on enzymatic degradation of nanocomposite sensitive layer, which however was deposited on a QCM crystal. The QCM curve in coordinates: frequency vs. the time is related to the sensitive layer mass (thickness) decrease resulted from its enzymatic degradation. The similarity of the curves shape of the two methods proves the main assumption this work is based on: the enzymatic degradation of the nanocomposite layer by the lipase causes its thickness diminution proportional to the lipase activity.

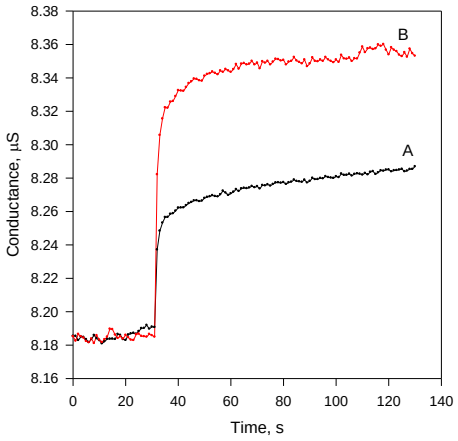

Figure 4. Sensor responses to 0.06 U mL^{-1} lipase at 300 rpm, AC voltage amplitude = 50 mV at 120 Hz. 50 µm bare olive oil (curve A) and 50 µm nanocomposite (curve B) sensitivity layers

3.3. Response amplitude optimization

3.3.1. Stirrer rate and pH optimization

The stirring of the lipase containing buffer solution intensify the contact of the lipase with the nanocomposite sensitive layer deposited on the conductometric electrode and serving as substrate.

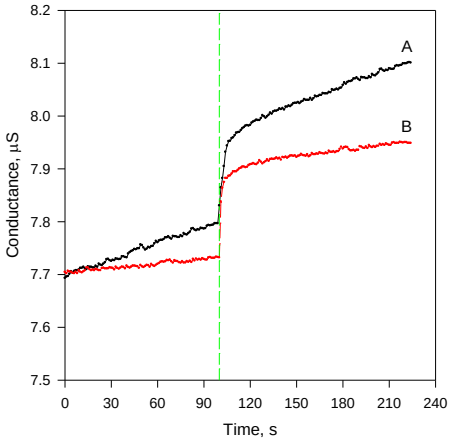

Figure 5. A typical conductance vs. time response to 0.08 U mg^{-1} lipase at pH = 8. Stirring rates: 600 and 300 rpm for curves A and B respectively; AC voltage amplitude = 50 mV at 120 Hz.

Conductance vs. the time curves were registered in the stirring rates range from 100 to 1000 rpm to evaluate its influence. The results showed that the nanocomposite layer gradually loses its stability at more than 500 rpm causing a negative slope appearance of the entire curve due to a slow mechanical degradation of the nanocomposite sensitive layer (see curve A in Figure 5). To obtain stable results in all the experiments were applied a stirrer rate of 300 rpm.

According to the data already reported in the literature about the maximal lipase enzymatic activity the optimal pH value lies in the range between 7 and 10 [40, 41]. As mentioned above all the experiments were carried out at pH 8 (phosphate buffer) allowing the maximal enzymatic activity according to the enzyme producer recommendation.

3.3.2. Sensitive layer thickness optimisation

The sensitive layer thickness was optimized by registering conductance vs. the time curves for same lipase activity at same AC voltage amplitude and frequency but different nanocomposite layer thickness: 50 and 100 µm. For this purpose volumes of 5 and 10 µL of the dissolved in chloroform nanocomposite were deposited on the electrode surface yielding layers thicknesses of about 50 and 100 µm correspondingly after the organic solvent evaporation. Volumes less than 5 µL were not enough to cover the entire electrode surface, while ones larger than 10 µL formed irregular layer thickness.

The sensor responses obtained with 50 and 100 µm sensitive nanocomposite layer thicknesses are shown in Figure 6. The resistance of a thicker sensitive layer is higher and as a result lower initial conductance was registered. The enzymatic degradation of a thicker sensitive layer causes smaller sensor response (wave heights) resulting in a lower sensitivity compared with the thinner one. That is why the minimal possible thickness of 50 µm was chosen for the nanocomposite layer thicknesses.

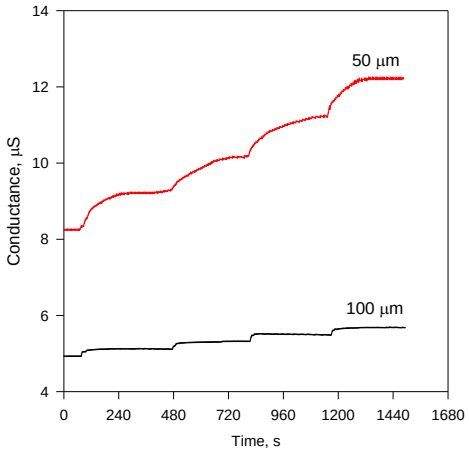

Figure 6. Sensor response of 0.4 U mL^{-1} lipase at 50 and 100 µm nanocomposite layer thicknesses. Stirrer rate = 300 rpm; A.C. voltage amplitude = 50 mv at 120 Hz frequency

All the reported conductometric methods for lipase activity quantification as those developed by Ballot [32-34] are based on substrate solution conductance measurements during its enzymatic degradation by the lipase. That is why they suffer from the main problem of the conventional conductometry: strong dependence on the temperature. The achievement of reliable results applying the Ballot conductometric method requires very precise temperature maintenance within 0.01ºC because of the temperature influence on the conductometric results of about 2% deg^{-1} [32]. In contrast no temperature control was applied to achieve the precision of the method subject of the present work presented in subsection 3.4.4.

3.4. Analytical characterization of the conductometric method

3.4.1. Linear quantification range determination and calibration plot building

The linear quantification range was experimentally determined to be from $1.1x10^{-2}$ to 1.17 U mL^{1} lipase activity at the optimized experimental condition: 300 rpm, 50 mV/120 Hz, 50 µm thick nanocomposite layer, pH 8. The calibration plot is presented in Figure 7 right, described by the equation: G = -0.026 + 2.31LA, where G is the registered conductance change and LA is the lipase activity in U mL^{1}. The corresponding conductance vs. the time curves used for the calibration plot building are presented in Figure 7 left and Figure 9.

The precision of the determination within the linear quantification range was found to be in the range from 3.86 to 1.22 % for the lipase activity range from $1.1x10^{-2}$ to 1.17 U.S.P. mL^{1} respectively. The reported linear quantification range of the Ballot conductometric method [32] lies in the range from 20 to 600 U in 4 mL sample volume (equal to 3 decades), while the sensor subject of the present work reaches 17 decades of lipase's activity within a maximal relative error of 3.86%.

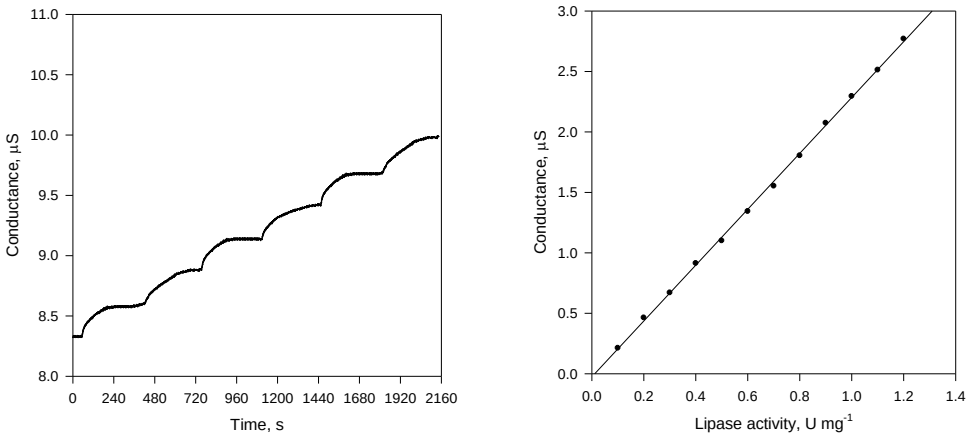

Figure 7. Conductance vs. the time curve for successive addition of 0.2 U mg^{-1} of lipase (left) and the corresponding calibration plot, see Figure 9 also. Nanocomposite sensitive layer thicknesses = 50 µm, stirrer rate = 300 rpm, A.C. voltage amplitude = 50 mv at 120 Hz

3.4.2. LOD evaluation

The limit of the detection (LOD) of the proposed method was determined applying the 3 σ rule and it was found to be $0.8x10^{-3}$ U mg^{-1} at optimized nanocomposite ingredients ratio (20% SiO2 nanoparticles in olive oil) and experimental conditions: layer thickness of 50 µm, 300 rpm stirring rate, 50 mV amplitude of the AC voltage at 120 Hz frequency and pH 8. This LOD value was calculated as average of 10 determinations after successive appropriate dilution to obtain the minimal lipase activity able to be detected. The curve presented in Figure 8 is the raw one without any smoothing.

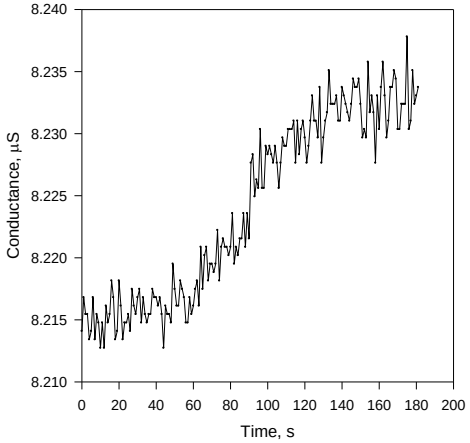

Figure 8. Conductance vs. the time raw unsmoothed curve for $0.8x10^{-3}$ U mg^{-1} lipase activity at 300 rpm stirring rate, 50 mV / 120 Hz A.C voltage and 50 µm nanocomposite layer thickness

The LOD of the proposed method comparison with those obtained by the standardized titrimetric method using olive oil as substrate [42] the colorimetric assay using the copper soap as substrate [43] and the conductometric assay using triaceine as substrate [32] is presented in Table 2.

Table 2. Limit of detection (LOD) of the key methods applied for lipase activity determination

Method/sensor for lipase activity quantification	LOD (U mg^{-1})	Reference
Titrimetric assay using olive oil as substrate	10^{-2}	[42]
Colorimetric assay using the copper soap as substrate	10^{-1}	[42]
Conductometric assay using triaceine as substrate	10^{-2}	[32]
Conductometric assay using nanocomposite sensitive layer	$0.8x10^{-3}$	This paper

3.4.3. Response time evaluation

As expected it was found that the sensor response time depends on the measured lipase activity: higher the activity, shorter the response time, due to the increased rate of the enzymatic reaction (see Figure 9). The time to reach the wave plateau in the conductance vs. the time curve was evaluated for lipase activities within the linear quantification range using the results taken from Figures 7 and 9 (see Table 3).

Table 3. Response time as a function of the determined lipase activity

Lipase activity, U mL^{-1}	$1.1\ 10^{-2}$	$6\ 10^{-2}$	$1.5\ 10^{-1}$	$6\ 10^{-1}$	1.5
Response time, s	86	57.2	39.1	17.8	6.5

The average response time for the lowest measurable lipase activity was found to be 86 s while for the highest activity it was as short as 6.5 s. According to the reported data the shortest response time achieved by the conductometric method application for lipase activity determination developed by Ballot [32-34] was as long as 30 minutes.

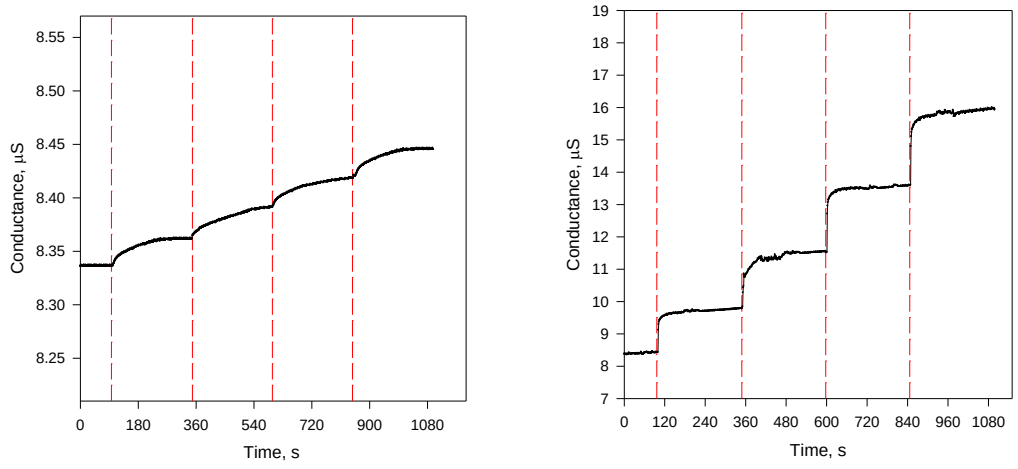

Figure 9. Sensor responses for the lowest (left) and the highest (right) lipase activities within the linear quantification range. Stirring rate = 300 rpm, A.C. voltage amplitude = 50 mV at 120 Hz and nanocomposite layer thickness = 50 µm

3.4.4. Reproducibility evaluation

The low cost of the conductometric stick electrodes allows their use as disposable and regenerable as well. In both cases the reproducibility of the modified electrode surface area is very important to obtain reliable and reproducible results. The reproducibility was evaluated by series of 10 electrodes modified by 50 µm nanocomposite containing 20% nanoparticles.

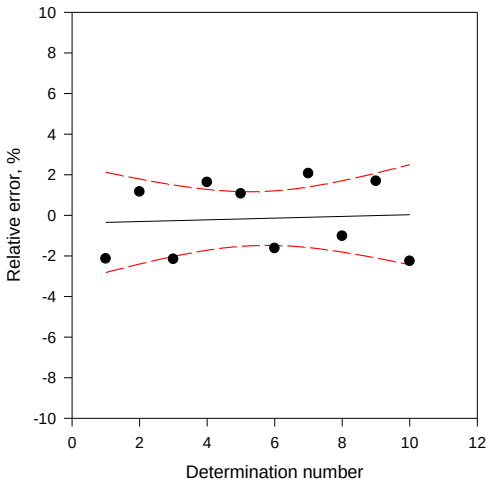

Figure 10. Relative errors of the determinations of $1x10^{-1}$ U mg^{-1} using 10 nanocomposite modified stick electrodes (50 µm) at 300 rpm, 50 mv A.C. voltage amplitude at 120 Hz

A lipase solution with 10^{-1} U mg^{-1} activity (a value in the middle of the linear quantification range) was determined by each of these 10 electrodes and the relative deviations in respect to the average are presented in Figure 10. As seen in the figure the maximal deviation of 2.3% not exceeded the relative error corresponding to the applied lipase activity.

3.5. Application to real samples and results validation

Validation experiments were performed applying the titrimetric method described by Pinsirodom [42] possessing same LOD as the method subject of the present work. Five spiked samples in lipase free milk whey from the cheese production industry were prepared by addition of lipase solutions to achieve final activity of 0.5 U mL^{-1}. Each of the 5 samples was determined 3 times and the average results were taken to be presented as A_1 to A_5 in Table 4. As seen there the relative error belongs to the relative error range corresponding to the linear quantification range confirming this way the satisfactory accuracy of the proposed method subject of the present work.

Table 4. Results obtained for 0.5 U mL^{-1} lipase by titrimetry [42] and the proposed method

Measurement number	**A_1**	**A_2**	**A_3**	**A_4**	**A_5**	**Average**
Titrimetric method, U mL^{-1}	0.52	0.52	0.53	0.52	0.51	0.520
Reported sensor, U mL^{-1}	0.50	0.51	0.51	0.50	0.49	0.502
Relative error %	-	-	-	-	-	1.8

4. CONCLUSION

A new approach for the lipase activity quantification was proposed and applied in a simple, rapid and cost effective conductometric method suitable for industrial application. The method is based on the conductance change registration along the time during the enzymatic degradation of a thin nanocomposite (SiO_2 nanoparticles loaded olive oil) substrate layer deposited on the conductometric electrodes. The sensitive layer thickness diminution along the time causes its conductance augmentation proportional to the lipase activity.

The proposed method was characterized in terms of lipase activity linear quantification range and LOD, precision, quantification time and reproducibility at optimized pH = 8. The relative error was found to be from 3.6% to 1.2% at the linear quantification range of $1.1x10^{-2}$ to 1.17 U mL^{-1} respectively with a LOD of $0.8x10^{-3}$ U mL^{-1}. Finally, the method was validated with spiked samples applying a spectrophotometric method as reference one.

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