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A.C. Amperometric Method for Lipase Activity Quantification

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The working electrode modification applying some specific modifiers results in capacitors formation not related to the charges exchange occurring and double layer formation at the electrode – solution interface. Chemical or biochemical processes involving the modification layer cause its thickness variations leading to capacitance changes resulting in AC amperometric response. An AC amperometric method for lipase activity quantification based on this approach was developed and characterized. A thin layer of nanocomposite (SiO_2 nanoparticles charged olive oil) deposited on the working electrode served as both: sensitive layer toward the lipase and dielectric layer of an electrolytic capacitor formed on the electrode – solution interface. The nanocomposite enzymatic degradation by the lipase causes its thickness decrease proportional to the lipase activity and a corresponding rise of the AC current is registered as analytical response by AC amperometry at fixed small AC amplitude (100 mV p.p.) and frequency of 240 Hz. The developed method was characterized in terms of: LOD, sensitivity, linear quantification range, precision, response time and reproducibility, as well as was validated by spiked samples determination using the standardized titrimetric method as reference.

Keywords: AC amperometry, lipases activity quantification, olive oil/SiO_2 nanocomposite

1. INTRODUCTION

The ion exchange occurring at the electrode – solution interface forms a double layer of electric charges possessing capacity of a few dozen microfarads per centimeter [1]. However, an electrode surface modification, an approach widely applied in the electrochemistry also may results in capacitor formation not involving ion exchange if appropriate modifiers are employed. In such cases the

modification layer may serve not only as a capacitor dielectric but also as a sensitive layer toward a specific analyte as well. Its thickness variations resulting from some specific reaction in which it is involved can be registered by some electrochemical measuring technique application. Thus, an electrode modification by a very thin high resistive layer serving as a capacitor dielectric results in an electrolytic capacitor formation where the modified electrode and the conductive sample solution serve as capacitor plates. The dielectric layer degradation by some biochemical processes such as enzyme catalyzed reaction will cause capacitance increase and hence phase sensitive AC amperometric response augmentation resulted from a small amplitude AC potential E_{AC} application with a fixed frequency ω.

The increased industrial application of the enzymes belonging to the lipases group and the highly automated technological processes require simple, rapid, precise and cost effective methods for automatic quantification of their enzymatic activity. A great variety of methods have been developed for this purpose so far detailed reviewed by Jensen [2], Thomson [3], Beisson [4], Gupta [5], Starodub [6], Hasan [7], B. Andualema [8] and Stoytcheva [9]. Most of the reported and applied methods are based on titrimetry, chromatography, spectrophotometry UV-VIS or IR, turbidimetry, quartz crystal microbalance, immunoassay, etc. [10–18]. Among the electrochemical methods the conductometry, a versatile and simple electrochemical technique [19-25] was the most applied in lipase activity quantification. Conductometric methods for lipase activity quantification were reported by Reyes [26], Ballot [27-29]. The Electrochemical Impedance Spectroscopy (EIS) also was applied for this purpose by Zlatev [30]. Each of the existing methods possesses its advantages, limitations and drawbacks, but the common disadvantages of almost all of them are their complexity requiring manual application by qualified personnel, the long analytical response and high cost of the analysis, not allowing their application in automated industrial analysis.

The development and the characterization of an AC amperometric method based on the approach described above meeting the industrial requirements is the aim of the present paper. A thin layer of a high resistive lipase substrate was deposited on the disposable/regenerable working electrode resulting in a plate electrolytic capacitor formation where the substrate layer serves as both: a capacitor's dielectric and a sensitive layer. Its enzymatic degradation by the lipase causes its thickness decrease resulting in a corresponding augmentation of the capacitance ΔC, according to the well-known equation: $\Delta C = \varepsilon A/\Delta d$ (eqn. 1). Here, the $\boldsymbol{\varepsilon}$ is the substrate dielectric permittivity (dielectric constant), **A** is the surface area of the dielectric layer covering the working electrode and **d** is its thickness. The capacitance increase ΔC proportional to the lipase activity causes a corresponding augmentation of the AC amperometric response serving as a measure of the lipase activity.

As well-known, the capacitive reactance X_C can be presented by the equation: $X_C = 1/\omega C$ and applying the Ohm law one can obtain for the AC current passing through a capacitor: $\Delta I_{AC} = E_{AC}/X_C$ or $\Delta I_{AC} = E_{AC}\,\omega(\Delta C)$ (eqn. 2). The combination of the equations (1) and (2) yields: $\Delta I_{AC} = E_{AC}\,\omega\,\varepsilon\,A\,/\,\Delta d$ (eqn. 3) which can be presented as follows: $\Delta I_{AC} = K\,/\,\Delta d$ (eqn. 4) after uniting all the constant parameters. The equation (4) ignores the active AC current which can be neglect because of the very high active resistance of the nanocomposite modification layer (olive oil/SiO_2 nanoparticles).

The use this nonlinear equation as response function it can be divided in parts within small **Δd** intervals for which the curve can be assumed lineal if fulfils the condition: $r^2 > 0.95$. The width of

these intervals will determine the linear quantification ranges while its initial values will determines the method sensitivity. These parameters can be adjusted according to the analytical task by choosing the appropriate initial sensitive/dielectric layer thickness. Thinner layer provides higher sensitivity but shorter linear quantification range while the thick layers provides long linear quantification range but lower sensitivity, as shown below.

The low cost, the high resistivity of about 10^7 ohms m [31-32] and the relative dielectric constant of 3.2 [33] of the olive oil (which contains up to 30% triolein which is a very specific lipase substrate [34]) make it a very suitable to be applied as working electrode modifier (dielectric/sensitive layer) in the proposed amperometric method for lipase activity determination. According to equation (3) a high substrate dielectric constant will results in high sensitivity due to the magnification of the capacitance variations caused by same dielectric layer thickness d decrease. The only way to increase the olive oil dielectric constant is its modification with a material possessing higher dielectric constant ε. Very suitable for this purpose is SiO_2 in form of nanoparticles due to two reasons: first, these nanoparticles formed a nanocomposite gel with the olive oil possessing excellent adhesion properties [35] sticking together the nanoparticles and the nanocomposite layer to the electrode surface and second its higher relative dielectric constant than the olive oil (**ε** = 3.9) [36, 37].

Based on these assumptions a rapid, simple and cost effective AC amperometric method for lipase activity quantification suitable for automatic application employing disposable/regenerable modified working electrodes was developed and characterized in terms of LOD, sensitivity, linear quantification range, precision, response time and reproducibility. Finally the method was validated by real samples in comparison with the standardized titrimetric method of Pinsirodom [38].

2. EXPERIMENTAL

2.1. Instrumentation

Model CompactStat.h 20250, Ivium Technologies, Netherlands running IviumSoft software was employed for the AC amperometric essays. The modified working and the auxiliary electrodes were connected to the potentiostat in two electrodes configuration. All the experiments were performed in modified EG&G PARC, model K0264 cell stand equipped with Pt wire counter electrode and a magnetic stirrer. Model UP 800 Ultrasonic Processor (ChromTech) was used in the nanocomposite preparation. The nanocomposite structure was characterized by a Scanning Electron Microscope, model JEOL JSM-840.

2.2. Reagents and materials

A stock lipase solution used in all the experiments was prepared from (25.1 U.S.P. U. mg^{-1}) lipase, from Sigma. A phosphate buffer with pH 8 was prepared by appropriate amounts of analytical grades K_2HPO_4 and KH_2PO_4 dissolved in deionized water and used as supporting electrolyte in all the experiments. The buffer solution served as supporting electrolyte in all the lipase assays as well as for the enzyme standard solutions preparation according to the lipase producer recommendations. SiO_2 nanoparticles (NPs) (99.8%, 10–20 nm, SkySpring Nanomaterials, Inc., USA, product # 6862HN) and

$CHCl_3$ (PA grade from Fermont, USA) and commercial extra virgin olive oil were used for the nanocomposite preparation.

2.3. Working electrode construction and modification procedure

Stainless electrode 1.05 x 1.05 cm placed vertically was used as working electrode connected to the potentiostat. The modification procedure of its both sides involves electrode immersing for 2 seconds in $CHCl_3$ solution of the nanocomposite: olive oil and SiO_2 nanoparticles in preliminary optimized weight ratio of the ingredients. The $CHCl_3$ function is to decrease the nanocomposite viscosity facilitating the uniform nanocomposite layer formation. It was found by SEM image that the working electrode immersion for 2 s yields 30 μm thick nanocomposite layer formations after the $CHCl_3$ evaporation and the procedure repetition doubles the layer thickness. The nanocomposite $CHCl_3$ solution was sonicated by the ultrasonic processor for 1 minute any time before electrode modification for solution homogenization. After the organic solvent evaporation at room temperature a uniform nanocomposite film was formed on the working electrode surface. The already used electrodes were regenerated applying the same procedure after the nanocomposite layer preliminary removal in pure $CHCl_3$ under sonication.

3. RESULTS AND DISCUSSION

3.1. Measuring condition optimization to maximize the sensor response

3.1.1. Typical sensor response

The AC amperometry current response along the time registered applying the nanocomposite modified working electrode has the shape of a wave. The nanocomposite layer degradation by the lipase catalyzed hydrolysis causes its thickness decrease leading to corresponding capacitance augmentation and AC current increase. This process is responsible for the rising part of the response curve presented in Figure 1 however, the progressive lipase active centers saturation along the time results in a gradually curve transition into a plateau.

In accordance with the Eqn. 4 higher lipase enzymatic activity causes higher AC current response and as seen from the Figure 1 the plateau height of the curve 3 is twice the height of the curve 2 one at double lipase activity of the first with respect to the second sample. The plateau appears in less than 30 seconds after the lipase sample addition (green line) illustrating the sensor response time which however depends on the measured lipase activity as shown below.

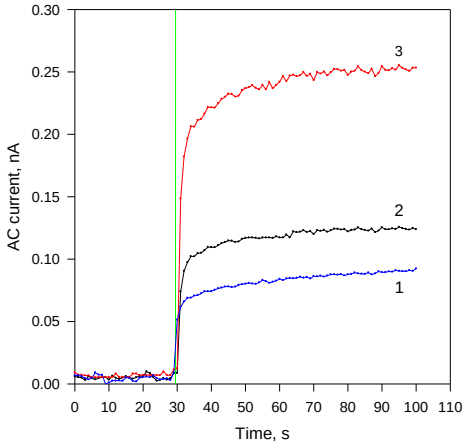


Figure 1. AC current response vs. the time (after background subtraction) to lipase activities of 0.6 USP U mL^{-1} (curve 1 y curve 2) and 1.2 USP U mL^{-1} (curve 3); Conditions: AC potential = 100 mV p.p., frequency of 240 Hz; stirrer rate = 300 rpm at pH 8 and 25 ^{o}C. Pure olive oil sensitive layer (curve 1) and SiO_2 NPs charged nanocomposite sensitive layers (20% w charged, 60 µm thick) for curves 2 and 3

3.1.2. Nanocomposite composition and stirrer rate optimization

One of the assumptions this work is based on is that the nanocomposite application as a sensitive/dielectric layer instead of pure olive oil magnifies the sensor response due to the nanoparticles higher dielectric constant. The sensor responses obtained by pure olive oil only and nanocomposite application were experimentally compared at same conditions (curves 1 and 2 in Figure 1). The curve 1 was registered using 60 µm thick sensitive layer of pure olive oil (deposited by drop-coating on the working electrode) while the curve 2 was registered employing 60 µm thick sensitive layer of nanocomposite with 20% w. nanoparticles charge deposited by the procedure described above.

The relatively high sensor response increase (33.2%) yielded in case of the nanocomposite application in comparison with the pure olive oil probably is due not only to the higher SiO_2 dielectric constant but also to the bigger SiO_2 nanoparticles volume released compared with the olive oil one. The olive oil serves as thin “glue” film between the nanoparticles sticking them together to form the nanocomposite the same way as a thin cement layer sticks together the bricks in a wall. That is why the enzymatic degradation of the olive oil releases the nanoparticles causing much bigger thickness decrease of the nanocomposite layer than that resulted from the olive oil degradation only. Moreover, the SiO_2 nanoparticles dielectric constant is much higher than the olive oil one.

Based on this result one may suppose that very high nanoparticles percentage in the nanocomposite composition may results in a huge analytical response. Unfortunately a high nanoparticles percentage makes the nanocomposite mechanically unstable and the solution stirring during the measurement causes its continuous slow degradation resulting in a positive slope of the response curve as illustrated in Figure 2.

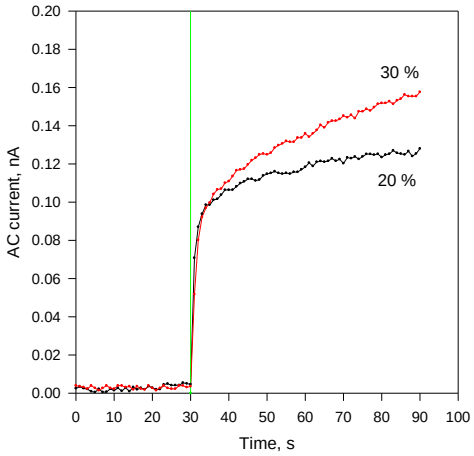
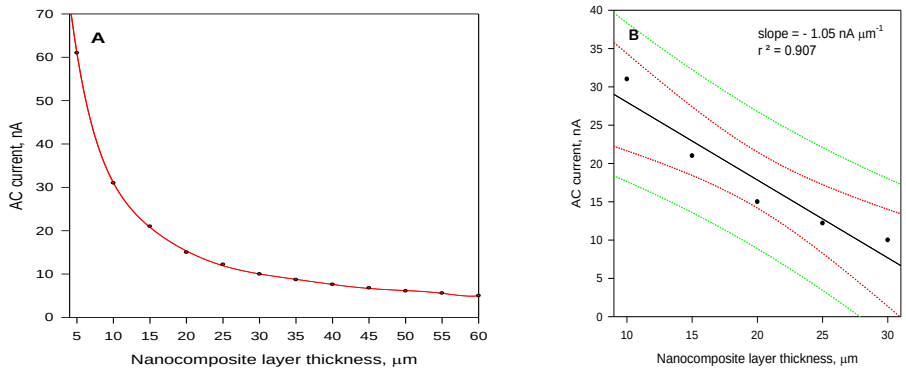


Figure 2. AC current response vs. the time (after background subtraction). Working electrodes modified with 20 and 30 % w. SiO_2 NPs charged nanocomposites at 300 rpm. Lipase activity sample: 0.7 USP U mg^{-1} at the same condition as Figure 1.

As it was found experimentally highly charged nanocomposites may stay stables at extremely low stirrer rates (as low as 50 rpm) but unfortunately the low stirrer rate degrades the sensor response time. That is why a balance between the nanoparticles charge and the stirrer rate must be established. The compromise values were found to be 300 rpm stirrer rate and 20% nanoparticles charge of the nanocomposite.

3.1.3. Nanocomposite layer initial thickness influence

The only parameter depending on the lipase activity is the nanocomposite sensitive layer thickness which determines the AC current response according to Equation (4). However, this equation graphically presented in Figure 3A for: $E_{AC} = 10^{-1}$ V; ω = 1,507 rad (240 Hz); $\varepsilon = 9\ 10^{-11}$ F·m^{-1}; A = 2.2 10^{-4} m^2 is not linear. As mentioned above, to use the AC current response as measure of the lipase activity some nanocomposite thickness sub-ranges must be found, within which the current response might be considered linear with $r^2 > 0.95$ (see Figure 3 B-E).



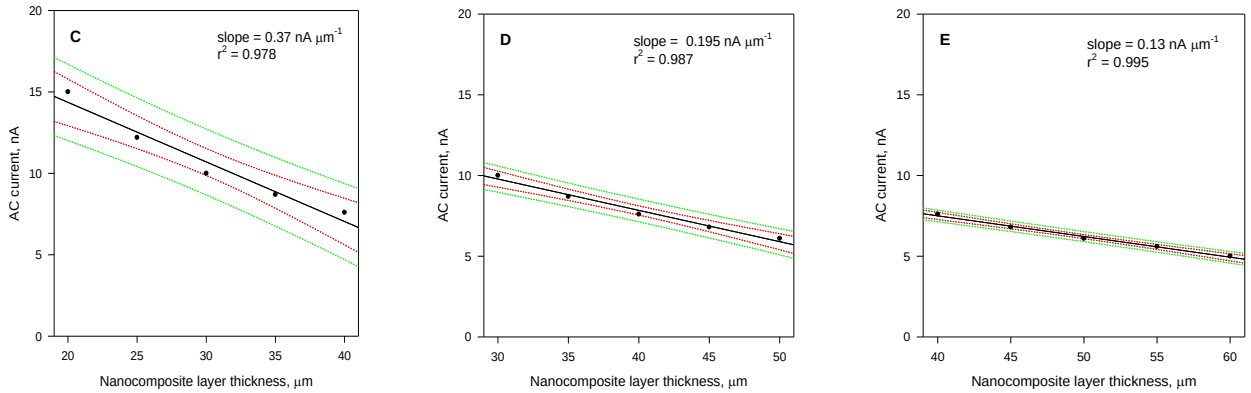


Figure 3. Curve A: Graphic presentation of Equation (1) for nanocomosite double 1.5 x 1.5 cm sensitive layer (20% nanoparticles) in thicknesses range from 5 to 60 µm; Curves B - E: Equation (1) 20 µm wide sub-ranges with initial nanocomposite thicknesses from 30 to 60 µm together with the 95% confidence and prediction intervals (red and green lines respectively).

The sub-range width defines the highest measurable lipase activity (the linear quantification range upper end), while its initial nanocomposite thickness defined the lowest measurable lipase activity (the lower end of the quantification range, the sensitivity and the LOD). For example it was experimentally determined by SEM that 1.2 U.S.P. U mg^{-1} lipase activity degrades about 20 µm of the nanocomposite layer thickness. Each of the 4 sub-ranges with different initial nanocomposite layer thicknesses presented in Figure 3 provides different sensitivities from 0.13 nA U.S.P. U mg^{-1} μm^{-1} (at 60 µm initial thickness) up to 1.05 nA U.S.P. U mg^{-1} μm^{-1} (at 30 µm initial thickness). As seen in the Figure 3 thicker nanocomposite layer provides lower sensitivity but more precise results.

3.2. Analytical characterization of the AC amperometric method for lipase activity quantification

3.2.1. Precision and reproducibility evaluation

It is clear that the nanocomposite thickness reproducibility during the working electrode modification determines the results reproducibility and precision. These parameters evaluation was done employing a series of 10 modified working electrodes applying the measuring procedure described in the Experimental part.

Same lipase artificial solution containing 2.5 10^{-1} U.S.P. U mg^{-1} lying in the middle of the linear quantification range (corresponding to 60 µm sensitivity/dielectric layer thickness) was determined by each of these 10 electrodes and the relative deviations in respect to the average were calculated and presented in Figure 4. The maximal deviation found do not exceed 3.31 % which includes all the errors.

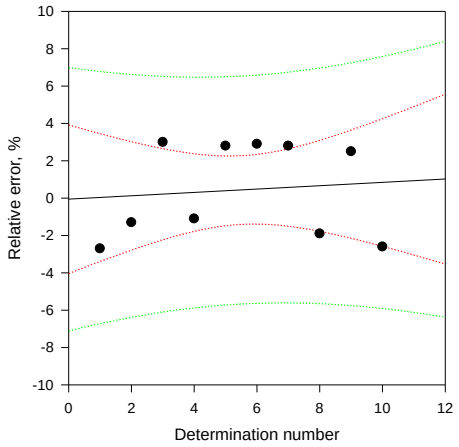


Figure 4. The relative errors of AC current plateau heights at $1x10^{-1}$ U.S.P. U mg^{-1} obtained employing 10 modified working electrodes with 95 confidence (red) and prediction (green line) intervals. 20% charged 60 μm thick nanocomposite, 300 rpm, 100 mv AC potential, 240 Hz, 25 ^{o}C.

3.2.2. Calibration plot building, sensitivity and linear quantification range determination

The calibration plot presented in Figure 5 and can be described by following equation: H = -0.022 + 2.171 A_{lipase}, with H, the plateau height in nA and A_{lipase} the lipase activity in U.S.P. U mg^{-1}. The correlation coefficient was calculated to be: r^2 = 0.962. The calibration plot was build applying working electrodes modified by 60 μm thick nanocomposite layer employing one electrode per point. Since the electrode's nanocomposite sensitive/dielectric layer thickness (determining the sensitivity) diminishes during the measurement, precise successive measurements cannot be done.

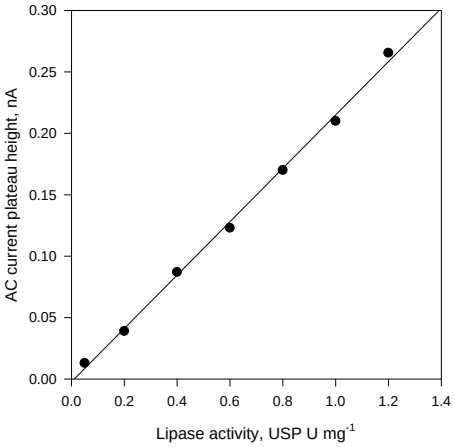


Figure 5. Calibration plot of lipase activities in the full linear quantification range: from 0.06 up to 1.2 U.S.P. U mg^{-1} at 60 μm 20% charged nanocomposite layer thicknesses and stirrer rate of 300 rpm; AC voltage amplitude = 100 mv p.p. frequency = 240 Hz; pH =8 and temperature of 25^{o}C

The lower end of the linear quantification range was found to be 0.06 U.S.P. U mg^{-1} while it upper end was as high as 1.2 U.S.P. U mg^{-1}. The sensitivity was determined to be 2.171 nA (U.S.P. U mg^{-1})$^{-1}$.

3.2.3. Response time evaluation

In can be expected that higher lipase activities will provoke more rapid enzymatic reactions resulted in shorter response time as presented in Table 1.

Table 1. Response time as a function of the determined lipase activity

Lipase activity, U.S.P. mL^{-1}	6 10^{-2}	1.2 10^{-2}	2.5 10^{-1}	6 10^{-1}	1.2
Response time, s	78.4	60.4	48.2	36.9	17.1

The response time determined as the time period from the lipase sample addition to the plateau establishment was evaluated for lipase activities within the entire linear quantification range and it was found that it does not exceeded 90 s.

3.2.4. LOD evaluation

The limit of the detection (LOD) was found to be 0.19 10^{-2} U.S.P. mg^{-1} lipase activity applying the 3 sigma rule at optimized nanocomposite ingredients ratio (20% SiO2 nanoparticles charge), layer thickness of 60 μm, 300 rpm stirrer rate, 100 mV AC potential at 240 Hz and pH 8.

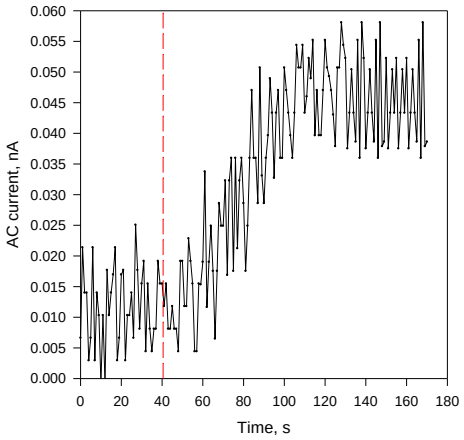


Figure 6. AC amperometric response to lipase activity of 0.19 10^{-2} U.S.P. mg^{-1}

The LOD and the sensitivity can be improved using very thin nanocomposite modification layer deposited on the working electrode. This however, may degrade the results precision as shown in Figure 3.

3.3. Results validation

The validation experiments were performed applying the Pinsirodom [39] standardized titrimetric method with milk whey (lipase free) samples taken from the dairy industry. Three lipase containing spiked samples were prepared belonging to the lowest (0.1 U.S.P. mL^{-1}), the middle (0.5 U.S.P. mL^{-1}), and the highest (1 U.S.P. mL^{-1}), parts of the linear quantification range. Each sample was measured with 3 different working electrodes, modified with 20% nanoparticles charged 60 μm nanocomposite at pH = 8, stirrer rate of 300 rpm and 25 ^{o}C and the average was taken. The recovery percentage was found to be higher than 96.4%.

Table 2. Real samples analysis

Sample	Found level, this paper method (average of 3) U.S.P. mL^{-1}	Found level, Pinsirodom method (average of 3) U.S.P. mL^{-1}	Recovery %
low	0.098	0.102	96.5
middle	0.462	0.47	98.3
high	1.00	0.99	101.2

3.4. Comparison of the AC amperometric method presented here with other electrochemical methods

According to the best of our knowledge no other AC amperometric method for lipase activity quantification has been reported in the literature so far. That is why the analytical characteristics of the described here AC amperometric method were compared with those of some other electrochemical methods: conductometric and impedimetric [30, 31]. The comparison is presented in Table 3.

Table 3. Comparison of the AC amperometric method presented here with other electrochemical methods

Method	LOD, U.S.P. U mg^{-1}	Sensitivity $(U.S.P.\ U\ mg^{-1})^{-1}$	Linear quantification range U.S.P. U mg^{-1}	Rel. error % max	Response time, s
Conductometric	0.008	2.29 μS	0.011 up to 1.17	3.60	86
Impedimetric	0.080	13.4 deg	0.099 up to 1.68	3.75	80
This paper	0.019	2.17 nA	0.06 up to 1.2	3.31	90

As seen from the Table 3 the analytical characteristics of the method described in this paper are similar to those of the conductometric method [31] in respect to LOD, response time and sensitivity and superior to those of the impedimetric method [30]. The main advantage of the proposed method is its simplicity not requiring qualified personal and the ability to be applied automatically in the industry.

4. CONCLUSION

A simple, rapid and cost effective AC amperometric method for lipase activity quantification, applicable in the automated industrial processes was developed and characterized. It is based on the following simple approach: a nanocomposite (SiO_2 nanoparticles loaded olive oil) modified electrode was used as working electrode in AC amperometry at fixed AC small amplitude (100 mV p.p.) and low frequency (240 Hz) applied on the electrode. The sensitive nanocomposite layer enzymatic degradation by the lipase catalyzed hydrolysis causes AC current increase forming a wave shaped curve which height is proportional to the lipase activity.

The proposed method was characterized in terms of LOD which was found to be as low as 0.19 10^{-2} U.S.P.U mg^{-1}, sensitivity of 2.171 nA (U.S.P. U $mg^{-1})^{-1}$, relative error not exceeded 3.31 % $_{rel}$ for all the linear quantification range of 0.06 up to 1.2 U.S.P. U mg^{-1}, maximal quantification time of 90 s in entire linear quantification range. Finally, the method was validated with milk whey spiked samples applying the standardized titrimetric method as reference. The recovery percentage was found to be higher than 96.4%.

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