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Application of room temperature ionic liquids to the development of electrochemical lipase biosensing systems for water-insoluble analytes

Rasa Pauliukaite [a], Andrew P. Doherty [b], Kevin D. Murnaghan [b], Christopher M.A. Brett [a,*]

[a] Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3004-535 Coimbra, Portugal
[b] The School of Chemistry and Chemical Engineering, Queen's University of Belfast, David Keir Building, Stranmillis Road, Belfast, NI BT9 5AG, UK

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ABSTRACT

Biosensors have been prepared by modification of glassy carbon electrodes with functionalised multi-walled carbon nanotubes (MWCNT) dispersed in the room temperature ionic liquid, 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide ($BmimNTF_2$) and with lipase cross-linked with glutaraldehyde. The biosensor was applied to the determination of olive oil triglycerides by cyclic voltammetry. A phosphate buffer (pH 7.0)/$BmimNO_3$ mixture is a better electrolyte than aqueous buffer alone. The response signal in the buffer–$BmimNO_3$ mixture was found to increase with the number of cycles until a constant current was achieved. The calibration curve obtained exhibited a sigmoid shape and a four-parameter model was used to fit the data which gave a limit of detection of 0.11 $\mu g\ mL^{-1}$. Close inspection of such calibration curves showed two distinct linear regions indicating changes in the mechanism of the electrochemical response. Overall, the oxidative analytical response was found to be due to phenolic compounds present in the olive oil, released in the presence of lipase, rather than due to triglycerides per se. It was also found that there were no interferences from either cholesterol or glycerol. A possible mechanism of olive oil determination at a MWCNT–$BmimNTF_2$/Lip biosensor is proposed.

1. Introduction

Room temperature ionic liquids (RTILs), have been explored with the aim of identifying their advantages in biocatalysis [1,2] and biosensing [3]. RTILs are usually organic or mixed organic–inorganic salts that are liquid at room temperature [1,4] and because of their lack of vapour pressure and resulting ease of containment, the majority of RTILs are considered to be green solvents. Moreover, RTILs do not deactivate enzymes like other non-aqueous solvents [5].

RTILs have been recently employed in electroanalysis; one of most common applications is construction of electrochemical biosensors [3–10], where RTILs are usually used during electrode modification. Various enzymes have been applied in the development of biosensors with RTILs, such as horseradish peroxidase [6,11–13], organophosphorus hydrolase [14], and glucose oxidase [7,15–18,8]. RTIL-modified electrodes were also used for determination of enzyme activity [1,3,19], since most of them do not denature proteins [20,21] and ensure fast and easy electron transfer.

Recently, RTILs have been used for the homogenisation of carbon nanotubes (CNTs) prior to immobilisation on the surface of electrodes, in order to improve loading reproducibility since CNT are insoluble in all solvents and loading on the electrode is complicated. CNT form a gel with some RTILs and the gel polymerises to a composite at the electrode surface [3,9], which is stable and can be used for sensing without any additional mediators [9]. Moreover, RTILs decrease the capacitive current of carbon nanotubes [22] thence increasing the sensitivity of CNT-modified electrodes, as well as enabling easier enzyme immobilisation and increasing biosensor robustness [6,14]. However, a cross-linking agent is needed for enzyme immobilisation at such a composite electrode [7,15–18,8]. The improvement of CNT homogenisation in RTILs occurs due to interaction of the RTIL with CNT that leads to more uniformity and even to better nanotube mechanical properties [23]. CNT–RTIL composites have also been applied to the study of the direct electrochemistry of heme-containing proteins [10,18,24,25] or of glucose oxidase [8], which allows determination of some analytes at lower potentials without redox mediators.

Lipase is an enzyme which hydrolyses triglycerides to glycerol and fatty acids. Lipase from porcine pancreas is a triacylglycerol lipase with a sequence of 449 amino acid residues and seven disulfide bonds. Porcine pancreatic lipase has been the most extensively characterised pancreatic lipase [26]. Its activity depends on the enzyme treatment [26,27] and it can be successfully used in biosensors. It can be immobilised on top of membranes [28] or as nanowires [29] by the electrospinning with some polymers, or incorporated into a porous silicon matrix [30]. Besides these methods, lipase can also be immobilised in RTILs [31] and it preserves full catalytic activity after such immobilisation [31,32]. Nevertheless,

* Corresponding author. Tel./fax: +351 239 835295.
E-mail address: brett@ci.uc.pt (C.M.A. Brett).

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lipase activity in RTILs can be enhanced using carbon nanotubes by immobilising them together with enzyme [33] or covalently attaching lipase to CNTs [34]. Lipase is usually used for the preparation of biosensors for triglyceride determination [30,35].

This work follows on from a previous study [36], where MWCNT–COOH were mixed with 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide ($BmimNTF_2$) in an optimised ratio, and the modified electrode was characterised electrochemically. The electroactive area obtained at the modified electrode was found to be 4.5 times higher than the geometric area. The most promising system for using MWCNT–$BmimNTF_2$ modified electrodes was found to be either in aqueous solution or in 1-butyl-1-methylpyrrolidinium bis(trifluoromethane)sulfonimide, $BpyrNTF_2$, electrolyte [36]. This study reports the application of the same CNT–RTIL composite in biosensing systems of water-insoluble samples, in particular of olive oil. Determination was performed using a MWCNT–$BmimNTF_2$ modified biosensor with immobilised lipase, determining triglycerides and phenols.

2. Experimental

2.1. Chemicals and solutions

The room temperature ionic liquids used were 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (abbreviated to bistriflimide) ($BmimNTF_2$) and 1-butyl-3-methylimidazolium nitrate ($BmimNO_3$) which were synthesised as previously described [36]. The structure of these RTILs is shown elsewhere [36,37].

Lipase (Lip) from porcine pancreas was from Fluka (Switzerland). Glyceryl tributyrate (triglyceride), bovine serum albumin (BSA), and glutaraldehyde (GA) were obtained from Sigma (Germany). Multi walled carbon nanotubes of diameter ~30 nm and length 1–5 μm were obtained from NanoLab (USA). Certified olive oil (Portugal) was used as a sample for triglyceride determination.

Solutions were prepared either in RTILs, or in Milli-Q nanopure deionised water (⩾18 Ω cm). Experiments were carried out at room temperature (25 ± 1 °C).

2.2. Methods and instrumentation

The three-electrode electrochemical cell contained the modified glassy carbon working electrode, a platinum wire counter electrode, and a saturated calomel electrode (SCE), was used as reference. Measurements were performed using a computer-controlled μ-Autolab Type II potentiostat/galvanostat with GPES 4.9 software (Eco Chemie, The Netherlands). The measurements were performed using cyclic voltammetry (CV).

2.3. Electrode preparation

A glassy carbon electrode (diameter 1.5 mm) was polished with the diamond spray with particle size of 3 and 1 μm using a polishing cloth (Kemet, UK). After each polishing the electrode was sonicated for 1 min in Milli-Q deionised water. Afterwards, the electrode was pre-treated electrochemically in neutral phosphate buffer solution by potential cycling between −1.0 and +1.0 V until a stable CV was obtained (usually ~10 cycles).

CNTs were pre-treated and functionalised with carboxylic groups in nitric acid using the procedure described elsewhere [36]. Functionalised CNTs, MWCNT–COOH, were mixed in an agate mortar with $BmimNTF_2$ in optimised proportions as described in the previous work [36]. The components were thoroughly mixed for 30 min and then left for 30 min to equilibrate and stabilise. In the best composite, which contained MWCNT–COOH and $BmimNTF_2$, the mixture prepared contained 0.3 mg of MWCNT–COOH and 6 μL of $BmimNTF_2$. The mixture was placed on the top of the GC electrode and left for 2 days to polymerise and cure.

Lipase was immobilised using glutaraldehyde cross-linking: 10 μL of 10% Lip solution (in 0.1 M phosphate buffer (PB) pH 7.0); 10 μL of 10% BSA solution (in 0.1 M PB pH 7.0); 1 μL of $BmimNTF_2$; 1 μL of 23% GA solution (in water), was used. It should be noted that $BmimNTF_2$ was added to the modifying matrix make the membrane softer and more stable, since quite often cross-linked enzyme membranes de-attach from the electrode surface. All the components were carefully mixed, and 10 μL of the mixture was applied to the surface of the MWCNT–RTIL coated electrode and left to dry, then another aliquot of 10 μL of the same mixture was placed on the top. Afterwards, the electrode was placed into phosphate buffer solution to dissolve traces of GA which did not react. The modified electrode was then left in air for 1 h to dry out. The electrode was kept in phosphate buffer at +4 °C while not in use.

3. Results and discussion

The MWCNT–RTIL/Lip electrochemical biosensor prepared as described in Section 2, was first tested for its response to olive oil by cyclic voltammetry, followed by sensor calibration. The components of the olive oil responsible for the biosensor response were then examined in detail and the interaction of lipase with olive oil examined.

3.1. MWCNT–RTIL/Lip biosensor for olive oil

3.1.1. Response of biosensor

Lipase (Lip) hydrolyses triglycerides to glycerol and fatty acids:

$$\begin{array}{l} CH_2{-}OOCR_1 \\ | \\ CH{-}OOCR_2 \\ | \\ CH_2{-}OOCR_3 \end{array} + 3H_2O \xrightarrow{\text{Lip}} \begin{array}{l} CH_2{-}OH \\ | \\ CH{-}OH \\ | \\ CH_2{-}OH \end{array} + \begin{array}{l} R_1COOH \\ \\ R_2COOH \\ \\ R_3COOH \end{array} \quad (1)$$

Since vegetable oils consist mostly of triglycerides, olive oil was used for testing the MWCNT–$BmimNTF_2$/Lip biosensor: A certified olive oil was chosen for the measurements as was also done in [38] since it consists of 99% of triglycerides. The MWCNT–$BmimNTF_2$/Lip biosensor was used for olive oil determination in 0.1 M phosphate buffer, pH 7.0 by cyclic voltammetry. Addition of olive oil to the buffer solution led to the appearance of a redox peak with oxidation peak at +0.19 V, which shifts to less positive potentials with increasing number of cycles and reaches a constant current after 3–5 cycles (Fig. 1). From these measurements it was observed

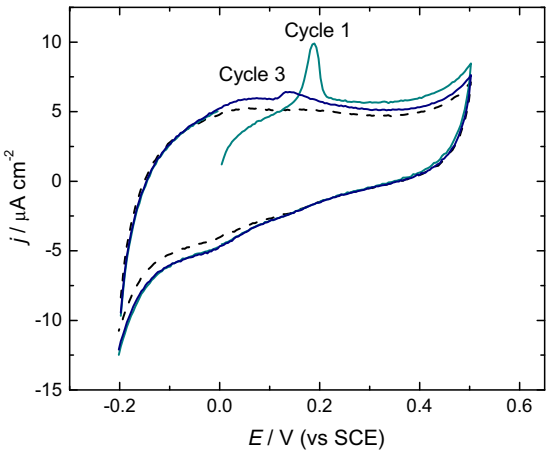

Fig. 1. Cyclic voltammograms at GC/MWCNT–$BmimNTF_2$/Lip in 0.1 M PBS, pH 7.0 in the absence (dashed line) and presence of 0.85 μg mL^{-1} (the 1st and the 3rd cycles) of olive oil (solid line). Scan rate 10 mV s^{-1}.

that the oxidation peak potential and the number of cycles necessary for stabilisation and obtaining a constant voltammetric profile depend on the olive oil concentration. The corresponding reduction peak was at −0.02 V and did not change with the number of cycles at low concentrations, but at higher concentrations it decreased.

Fig. 1 presents the response to 1 μL mL^{-1} of olive oil, which corresponds to 0.85 μg mL^{-1} of triglycerides. The decrease in signal with the number of cycles demonstrates limited access of triglycerides to the enzyme due to poor olive oil dispersion in buffer solutions. This problem was solved by pre-dissolving the olive oil in $BmimNO_3$ and adding this mixture to the PB solution, since the RTIL dissolves the olive oil easily and because of its hydrophilic nature it is homogeneously dispersed in aqueous buffer solution as mentioned above to form homogeneous solutions in which the enzyme electrode can be deployed. This RTIL was also chosen because in previous work [7] it was found that the glucose biosensor responded better in $BmimNO_3$ medium (as compared to other RTILs) since this RTIL always contains some traces of water, and some water for hydration is needed for the enzyme reactions to proceed; moreover, water is essential for the lipase catalysed hydrolysis of triglycerides (see Eq. (1)).

Initially, just $BmimNO_3$ without olive oil was injected to see if it has any influence and the reduction current increased significantly (not shown). After the second addition it did not increase but the voltammetric profile changed, there appearing a reduction wave at around 0.0 V, showing that the biosensor equilibrates in RTIL–PB solution. The CV remained unchanged after further RTIL additions. Such a behaviour occurred only when the electrode was used for the first time, and might also be due to initial activation of the enzyme.

After injection of a solution of olive oil dissolved in $BmimNO_3$ into the buffer solution, the response increased and reached a steady-state after 2–10 cycles (a higher concentration needed a longer stabilisation time), depending on the olive concentration. This might be caused either by slow analyte diffusion to the enzyme in the membrane or maybe the membrane became solvated with olive oil making CNTs more active. The solution was stirred intensively after each injection for 2 min. Fig. 2 shows the relative response, normalised by the steady-state current, at a higher concentration, i.e. 4.3 μg mL^{-1} of olive oil. As seen, the response to olive oil at the biosensor stabilises after 12 cycles at this concentration. This fact confirms that the process is limited by either extraction of the compounds from olive oil or diffusion of analyte to the active part of the enzyme.

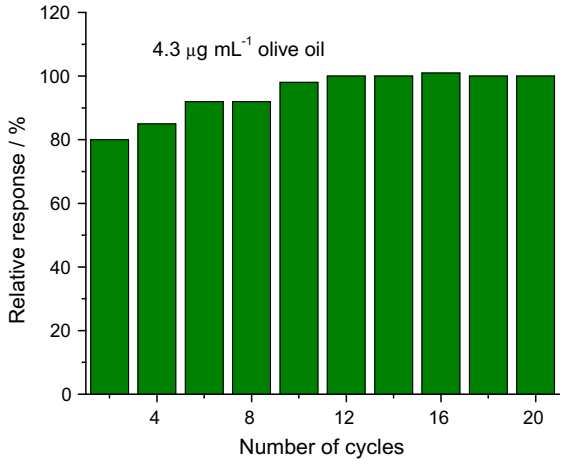

Fig. 2. Dependence of the relative response to 4.3 μg mL^{-1} olive oil dissolved in $BmimNO_3$ by cyclic voltammetry on number of cycles. All other conditions as in Fig. 1.

3.1.2. Biosensor calibration

The successive olive oil (solution in $BmimNO_3$) additions corresponded to an increase in the electrochemical response, as seen in Fig. 3. The 2-electron oxidation (at +0.1 V) and reduction (−0.05 V) peaks increase with increase in olive oil concentration up until 2.55 μg mL^{-1} and then become constant due to saturation effects. The reduction peak response is much less sensitive than the oxidation one. The separation between these peaks and their different shape leads to the conclusion that the electrochemical processes are unrelated and both are irreversible. No response was obtained when Lip was immobilised directly on the GC electrode or when the electrode was modified with just MWCNT–$BmimNTF_2$, i.e. without enzyme, demonstrating that both the enzyme and MWCNTs are necessary for an electrode response.

The dependence of the peak current on olive oil concentration (Fig. 4, inset) has a sigmoid shape and appears to have two linear ranges as presented in Table 1. This behaviour (Fig. 4) may be a result of different kinetics or reaction mechanism occurring under different concentration regimes. Nevertheless, a four-parameter logistic model [39] was used to evaluate the response for calibration purposes. This flexible calibration model can be expressed by the equation [40]:

$$y = d + \frac{a - d}{1 + (x/c)^b} \quad (2)$$

where x is the concentration, y is the current response, a is the lower asymptote, d is the higher asymptote, c is the IC_{50}, and b is the slope of the linearised curve ($\log(I)$ vs. $\log(c)$). The parameters in this particular instance were $a = 0$ μA, $b = 2.82$, $c(IC_{50}) = 13.9$ μg mL^{-1}, $d = 850$ μA ($R^2 = 0.990$). The LOD calculated using this four-parameter logistic model, Eq. (2), was 0.11 μg mL^{-1}, which is similar to that calculated from the lower linear range (Table 1).

The shape of the calibration curve shows a complex enzymatic reaction mechanism. Moreover, the redox peaks are rather sharp which rarely happens at electrochemical enzymatic biosensors. These facts demonstrate that there is some complex response from compounds present within the oil; thus, the influence of different compounds was studied.

3.2. Electrode response to other analytes

First, the response to triglycerides was evaluated. Addition of pure triglyceride dissolved in $BmimNO_3$ decreased the reduction

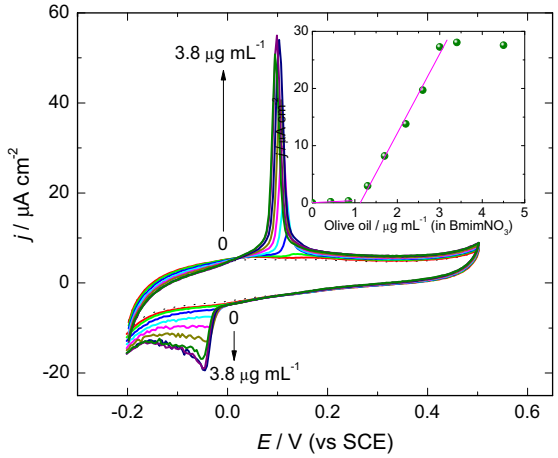

Fig. 3. Cyclic voltammograms at GC/MWCNT–$BmimNTF_2$/Lip in 0.1 M PB, pH 7.0 in the absence (dashed line) and presence of different concentrations: 0.43, 0.85, 1.28, 1.70, 2.13, 2.55, 2.98, 3.40, and 3.83 μg mL^{-1} (the steady-state cycles) of olive oil dissolved in $BmimNO_3$. Scan rate 10 mV s^{-1}. Inset shows calibration curve calculated from oxidation peak currents.

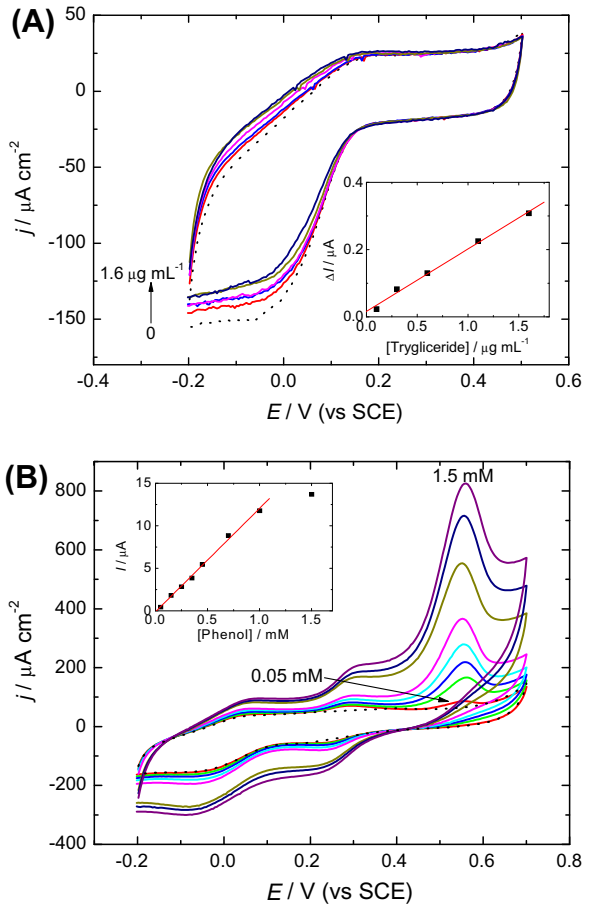

Fig. 4. Cyclic voltammograms at GC/MWCNT–BmimNTF$_2$/Lip in 0.1 M PB, pH 7.0 after addition of glyceryl tributyrate of (μg mL^{-1}): 0; 0.1; 0.3; 0.6; 1.1; 1.6 (A) and phenol of (mM): 0; 0.05; 0.15; 0.25; 0.35; 045; 0.7; 1.0; 1.5 (B) dissolved in $BmimNO_3$. Scan rate 10 mV s^{-1}. Insets of (A) and (B) show the calibration curves.

current at 0.0 V after each injection, but no other peaks appeared for triglyceride oxidation (Fig. 4A). The reduction response can be attributed to oxygen reduction at the electrode which decreases when O_2 is consumed during the oxidative rancidity of hydrolysed olive oil or triglycerides [41]. Lipase hydrolyses fats to glycerol and fatty acids (see Eq. (1)) and these products in the presence of oxygen and applied potential form free radicals that react with O_2 and form peroxide radicals [42]:

$$RH \rightarrow R^{\bullet} + H^{\bullet} \quad (3)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (4)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad (5)$$

Reaction (3) occurs under the influence of light, applied potential, at metals, and possibly also at CNT; reaction (5) is slow. In fact, and usually for triglyceride analysis, bi- or tri-enzymatic sensors are used with glycerol-degrading enzymes [35,42,43], although for direct triglyceride detection a potentiometric biosensor has been reported using just lipase enzyme [30]. However, our results obtained with pure triglyceride show that the oxidative response obtained in olive oil (see Fig. 3) was not from triglycerides since the reduction peak in olive oil had a different profile and behaviour. This means that the response at the MWCNT–BmimNTF$_2$/Lip biosensor was due to another electroactive molecule present in olive oil.

Besides triglycerides, olive oil also contains some phenolic compounds and polyphenols [44–46] that may interfere with the triglyceride signal. Thus, the response of simple phenol, dissolved in $BmimNO_3$, was also investigated. Furthermore, lipase can catalyse the hydrolysis of some phenolic compounds [47]. Fig. 4B shows the response to different concentrations of phenol in PB solution, pH 7.0, under the same conditions as with olive oil. Two redox waves, at 0.00/−0.07 V, and at 0.30/0.23 V vs. SCE are clearly seen, oxidation and reduction respectively, as well as an irreversible oxidation peak at ~0.55 V.

Calibration data for both triglyceride and phenol are presented in Table 2. The calibration data for triglycerides in Table 2 have been recalculated to molar concentrations in order to compare them with phenol concentrations; in mass/volume units the sensitivity was 0.016 μA cm^{-2} μg^{-1} mL and limit of detection 0.016 μg mL^{-1}, demonstrating a higher sensitivity and lower detection limit than in olive oil. This happens because olive oil contains many more compounds than the model solutions of glyceryl butyrate or phenol, and the sensitivity in olive oil might be lower due to matrix effects.

Most probably the oxidative response in olive oil is from one of the phenolic compounds present in this oil. These include hydroxytyrosol, tyrosol, elenolic acid, deacetoxy oleuropein aglycon, (+)-pinoresinol, (+)-1-acetoxy-pinoresinol, oleuropein aglycon, ligstroside aglycon [47], ferulic acid, syringic acid, caffeic acid, p-coumaric acid, p-OH benzoic acid, vanillic acid, and protocatecuic acid [45]. Most of them are electrochemically active and respond at a higher potential than simple phenol (Fig. 4B); however, hydroxytyrosol oxidation occurs at ~0.2 V vs. Ag/AgCl [45]. Moreover, extracts of virgin olive oil were found to give oxidation waves at low potentials using hydrodynamic voltammetry [45]. Thus, the oxidative response in olive oil, exemplified by the voltammograms in Figs. 1 and 3, is probably due to hydroxytyrosol oxidation.

Table 1
Calibration data for olive oil at MWCNT–BmimNTF$_2$/Lip electrode, calculated from cyclic voltammetry in Fig. 3.

Concentration range	Linear range (μg mL^{-1})	Sensitivity (μA cm^{-2} μg^{-1} mL)	Correlation coefficient (R)	LOD (μg mL^{-1})
Low concentrations	Up to 0.9	0.012	0.994	0.12
Higher concentrations	1.2–3.8	0.43	0.995	0.24
Whole range[a]	Up to 3.8	–	0.990	0.11

[a] Data calculated using four-parameter logistic model [42].

Table 2
Calibration data for triglyceride and phenol at MWCNT–BmimNTF$_2$/Lip electrode, calculated from cyclic voltammetry in Fig. 4A and B.

Compound	Linear range (mM)	Sensitivity (μA cm^{-2} mM^{-1})	Correlation coefficient (R)	LOD (μM)
Glyceryl tributyrate	Up to 5.3	0.056	0.997	0.05
Phenol	Up to 1.0	12.1	0.998	7

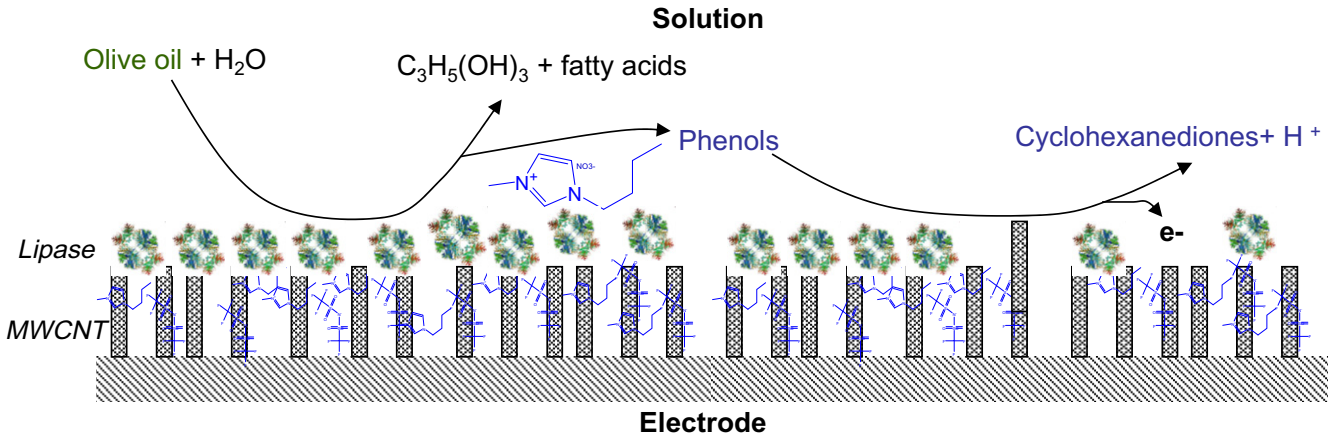

Fig. 5. Scheme of the biosensor mechanism: lipase hydrolyses triglycerides in olive oil to glycerol and fatty acids and, with $BmimNO_3$, aids extraction of phenols that are oxidised at the electrode modified with CNTs. $BmimNO_3$ is shown intercalated within the CNTs.

Interference tests were carried out. Some lipases not only catalyse the hydrolysis of triglycerides but also the transesterification of other compounds, such as ibuprofen and other phenolic compounds, chromenes, cholesterol or sometimes even glycerol in ionic liquids [21,31,48]. Some of these interferents, in particular glycerol and cholesterol, were investigated and no response to these compounds was obtained under the same experimental conditions or even for up to 10 times higher concentrations than in olive oil.

3.3. Lipase interaction with olive oil at MWCNT–RTIL/Lip biosensor

According to the results obtained: shape and position of the peaks, shape of the calibration curve, response to triglyceride and phenol, it appears that phenols are detected at the MWCNT–$BmimNTF_2$/Lip biosensor. Since there was no response either without lipase or without carbon nanotubes, the facts suggest that lipase is involved in the release of phenols, presumably via hydrolysis, and nanotubes catalyse their oxidation.

On the other hand, the redox signal obtained for olive oil at the biosensor stabilised slowly, possibly due to slow extraction with $BmimNO_3$ of the compounds from olive oil. It also could be caused by lipase kinetics, but usually its activity decreases in time [49]; thus, the effect of increase of the signal with the number of cycles should not be determined by enzyme kinetics.

The following mechanism can be suggested from the results obtained, see Fig. 5. Lipase hydrolyses triglycerides to glycerol and fatty acids (Eq. (1)), at the same time as, due to the $BmimNO_3$, it aids extraction of phenols which are oxidised at the electrode surface. Either this extraction is a slow process or depends on the rate of the enzymatic reaction but the phenol concentration increases in time until a steady-state is reached when the signal is proportional to the olive oil (triglyceride) concentration.

4. Conclusions

A MWCNT–$BmimNTF_2$/Lip biosensor, to exemplify the potential applications of RTILs in the preparation and use of electrochemical biosensing systems for water-insoluble analytes, has been constructed and applied to the indirect determination of olive oil by cyclic voltammetry. Certified olive oil was dissolved in the hydrophilic ionic liquid $BmimNO_3$, which dispersed homogeneously in phosphate buffer at pH 7. The response signal increases with the number of cycles until a steady-state is reached, depending on the amount of olive oil due to slow extraction of some phenolic compounds from olive oil by the RTIL. The sigmoid-shape calibration curve was indicative of a complex reaction mechanism. The limit of detection calculated by a four-parameter logistic model and from the first linear range was ~0.1 μg mL^{-1}. There was no interference from cholesterol and glycerol. The possible mechanism of olive oil determination at a MWCNT–$BmimNTF_2$/Lip biosensor is proposed, involving phenolic compounds extracted from olive oil during the enzymatic reaction of lipase with triglycerides.

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