

Electrochemical immunosensor for the milk allergen β -lactoglobulin based on electrografting of organic film on graphene modified screen-printed carbon electrodes

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ARTICLE INFO

Article history:

Received 14 March 2012

Received in revised form

5 June 2012

Accepted 6 June 2012

Keywords:

Graphene

Electrografting

β -lactoglobulin

Electrochemical

Immunosensor

ABSTRACT

A novel label-free voltammetric immunosensor for sensitive detection of β -lactoglobulin using graphene modified screen printed electrodes has been developed. The derivatization of the graphene electrode surface was achieved by electrochemical reduction of in situ generated 4-nitrophenyl diazonium cations in aqueous acidic solution, followed by electrochemical reduction of the terminal nitro groups to amines. The electrochemical modification protocol was optimized in order to generate monolayer of nitrophenyl groups on the graphene surface without complete passivation of the electrode. Unlike the reported method for graphene functionalization, we demonstrated here the ability of the electrografting of aryl diazonium salt to attach an organic film to the graphene surface in a controlled manner by choosing the suitable grafting protocol. Next, the amine groups on the graphene surface were activated using glutaraldehyde and used for the covalent immobilization of β -lactoglobulin antibodies. Cyclic and differential pulse voltammetry carried out in an aqueous solution containing $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair have been used for the immunosensor characterization. The results demonstrated that the DPV reduction peak current of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ decreased linearly with increasing the concentration of β -lactoglobulin due to the formation of antibody–antigen complex on the modified electrode surface. The immunosensor obtained using this novel approach enabled a detection limit of 0.85 pg mL^{-1} and a dynamic range from 1 pg mL^{-1} to 100 ng mL^{-1} of β -lactoglobulin in PBS buffer. In addition, the immunosensor evaluated in different samples including cake, cheese snacks, a sweet biscuit, showing excellent correlation with the results obtained from commercially enzyme-linked immunosorbent assay (ELISA) method.

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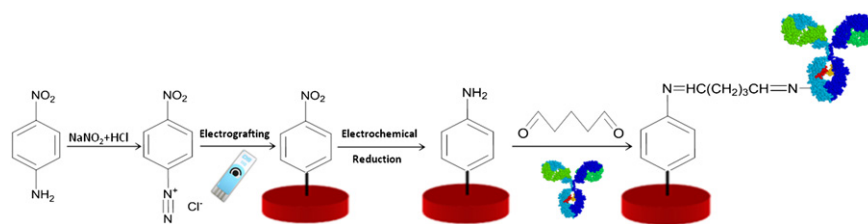
1. Introduction

Milk is one of the most common causes of food allergies (Host, 2002; Host and Halken, 1990), affecting between 2% and 3% of infants (LA and AW, 2006). β -lactoglobulin which represents 10% of the cow's milk proteins is considered the most important milk allergen especially for children. Although milk is one of the food components which should be listed on the label of the food products, most of the allergenic reactions in sensitive consumers occur due to unexpected exposure of milk proteins in a product where milk is not included in the ingredient list. Therefore, reliable detection methods are needed for regulatory agencies and the food industry to protect allergic consumers. Several analytical methods have been developed for the detection of β -lactoglobulin such as enzyme-linked immunosorbent assays (ELISA) (Luis et al., 2009), HPLC (Rena et al., 2010) and CE methods with laser-induced fluorescence (LIF) detection

(Pelaez-Lorenzo et al., 2009), enzyme linked immunoaffinity chromatography (ELIAC) (Puerta et al., 2005), and size-exclusion chromatography (Dijk and Smit, 2000). Among these methods, ELISA is the commercially available and routinely used method for the detecting β -lactoglobulin. Optical biosensors based on the resonance enhanced absorption (REA) effect (Hohensinner et al., 2007) and surface plasmon resonance (Billakanti et al., 2010) have been also reported. However, there is still an urgent need to develop alternative methods with lower cost, higher sensitivity, selectivity and faster response. Electrochemical biosensors have found wide applications in various biomedical and environmental fields in the recent years because of their low cost, fast response, sensitivity as well as their capability for automation and miniaturization. To the best of our knowledge no electrochemical biosensors has been reported before for the detection of β -lactoglobulin. Therefore, the novel electrochemical immunosensor presented herein is expected to have potential role in food safety and consumer protection.

Graphene, the recent member of the carbon nanomaterial family, has received great attention in the recent years. It exhibited various fascinating properties such as large surface area (Park and

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Scheme 1. Functionalization of the graphene electrode surface and the covalent immobilization of antibodies.

Ruoff, 2009), high mechanical strength (Lee et al., 2008), high elasticity, very high room temperature electron mobility (Novoselov et al., 2004), and thermal conductivity (Balandin et al., 2008). Graphene has shown several interesting applications in different fields including, solar cells, fuel cells, supercapacitors, batteries, electronics, as well as biosensor and bionanotechnology. Among these applications, the development of electrochemical sensors and biosensors based on graphene has been widely studied. Graphene has competed with carbon nanotubes in several electrochemical applications, showing two major advantages over carbon nanotubes, lower production cost and higher purity as it does not contain metallic impurities as carbon nanotubes (Pumera, 2009). The large surface area of graphene plays a significant role in the higher sensitivity of the graphene-based electrochemical biosensors as it provides faster rate of electron transfer for the redox probe and also more binding sites by increasing the density of the immobilized biomolecules on the surface.

The immobilization methods of the bioreceptors on transducers have a potential role in the performance of the biosensors devices. Various strategies for the covalent modification of solid electrodes which enable the subsequent attachment of biomolecules are known such as self-assembly of thiols on gold (Lahiri et al., 1999; Patolsky et al., 1999), silane on glass or indium tin oxide (Popovich et al., 2003; Ruckenstein and Li, 2005) as well as electrografting of organic layer on various conducting substrates using different reagents such as alcohols, amines, carboxylates, Grignard reagents, diazoniums, other onium salts and halides (Belangera and Pinson, 2011). Among these modification protocols, the electrochemical reduction of aryl diazonium salt has appeared as a promising alternative to conventional electrode modification schemes and used to modify different electrode materials such as carbon, metals, semiconductors, oxides and polymers (Belangera and Pinson, 2011). The versatility of this approach to modify the surface with a wide range of function groups, along with the ease of preparation and the stability of the covalently attached organic layer on the surface, have made it the method of choice for the immobilization of biomolecules in several reported biosensors.

Recently, the reduction of diazonium salt has been used for the chemical functionalization of epitaxial graphene (EG) (Bekyarova et al., 2009), CVD graphene (Jin et al., 2011), graphene nanoribbons (Jin et al., 2009), chemically converted graphene (CCG) (Lomeda et al., 2008), and mechanically exfoliated graphene (MEG) (Lim et al., 2010). In all the reported approaches graphene has been functionalized by spontaneous reduction of diazonium salts in organic solvent. However, this method usually takes several hours, and often needs either surfactants such as sodium dodecyl sulfate (Sharma et al., 2010) and sodium dodecylbenzenesulfonate (Lomeda et al., 2008) or highly charged electrolytes such as tetrabutylammoniumhexa fluorophosphate (Bekyarova et al., 2009) for the successful covalent functionalization, sometimes even at elevated temperature. Moreover, the reduction of the diazonium salt in organic solvent (acetonitrile), usually needs a synthesis of the salt, followed by a separation and purification step.

Different from the reported approaches for graphene functionalization, the approach presented herein is based on the electrochemical modification of graphene-modified screen printed electrode with 4-nitrophenyl diazonium cations. The diazonium cations were first prepared by the diazotisation of 4-nitroaniline. Subsequently, the in situ generated diazonium cations were covalently grafted through an electrochemical reduction step on the graphene electrode surface, followed by the electrochemical reduction of the terminal nitro groups to generate an aminated surface (Scheme 1). Moreover, the density of the deposited organic layer on graphene surface can be controlled by choosing the electrografting time and the grafting protocol. Then, the aminophenyl modified surface was activated with glutaraldehyde to give a stable intermediate derivative which is used for covalent immobilization of the β -lactoglobulin antibodies. A simple differential pulse voltammetric immunoassay has been used for the β -lactoglobulin detection based on the decrease of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ reduction peak current due to the specific binding of the β -lactoglobulin with the antibody modified electrodes. This new approach has enabled a fast and simple electrochemical detection method of β -lactoglobulin as a milk allergenic protein without the need of labeling and several incubations and washing steps.

2. Experimental

2.1. Reagents and materials

Anti- β -lactoglobulin antibody was obtained from Abcam (Cambridge, USA). β -lactoglobulin, 4-nitroaniline, potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$), potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), dipotassium hydrogen orthophosphate, potassium dihydrogen orthophosphate, sodium chloride, sodium nitrite, 50% glutaraldehyde, hydrochloric acid, bovine serum albumin, ovalbumin from chicken egg white, chicken egg lysozyme and casein, were purchased from Sigma (Ontario, Canada). β -lactoglobulin ELISA Kit was purchased from ELISA Systems (Queensland, Australia). A phosphate buffered saline PBS solution (10 mM, pH 7.4) was used for the preparation of the antibody and the β -lactoglobulin standard stock solutions and dilutions. PB buffer, (200 mM, pH 7.0, $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$) was used to dilute the glutaraldehyde solution for the activation of the amine groups. Milli-Q water was used throughout.

2.2. Instrumentation

The electrochemical experiments were carried out using Autolab PGSTAT302N (Eco Chemie, The Netherlands) potentiostat/galvanostat. Disposable graphene-modified screen printed carbon electrodes (GSPE) were purchased from Dropsens, Inc. (Spain, ref. 110GPH). The electrodes incorporate a conventional three-electrode configuration, which comprises a carbon working electrode modified with graphene, carbon counter and silver reference electrode. A sensor connector (Dropsens, Inc., Spain) allows connecting the GSPE to the Autolab potentiostat.

2.3. Procedure

2.3.1. Functionalization of the graphene electrode surface and the covalent immobilization of antibodies

The diazonium cations were synthesized *in situ* by mixing 1.0 mM sodium nitrite and 1.0 mM 4-nitroaniline in 0.5 M HCl solution. The mixture was stirred and left to react for about 5 min at room temperature. The electrochemical modification was immediately performed in the *in situ* generated 4-nitrophenyl diazonium salt (4-NPDS) by chronoamperometric (CA) step at -0.4 V for 45 s. The electrode was then thoroughly washed with water, transferred to 0.1 M KCl solution and subjected to five cyclic voltammetry (CV) scans between 0.4 and -1.4 V at 100 mV s^{-1} for reduction of the nitro groups on the surface to amine groups. Then, the 4-aminophenyl (AP) film on the electrode surface was activated by incubation with 2.5% glutaraldehyde (GA) solution in phosphate buffer PB (200 mM, pH 7.0) for 60 min. After activation the electrodes were washed with PBS buffer pH 7.4 to remove the excess GA. Then 50 μL of β -lactoglobulin antibody solution ($10\text{ }\mu\text{g mL}^{-1}$ in 10 mM PBS buffer, pH 7.4) was used to cover the working area of the electrode and incubated for 60 min at room temperature in water-saturated atmosphere. After washing with PBS buffer (10 mM, pH 7.4) the electrode surface was then blocked by the addition of 50 μL blocking buffer (0.25% of BSA in PBS buffer, pH 7.4) and incubated for 45 min to deactivate the remaining aldehyde groups and to block unreacted active sites. The modified electrode was then rinsed with PBS and used in the assay or kept at $4\text{ }^{\circ}\text{C}$ in PBS buffer pH 7.4 until further use.

2.3.2. Electrochemical measurements

For the β -lactoglobulin measurements, 50 μL of β -lactoglobulin of the required concentration in PBS buffer pH 7.4 was pipetted onto the modified electrode and incubated for 45 min in water-saturated atmosphere. Then, the immunosensor was rinsed thoroughly with PBS buffer pH 7.4 prior to electrochemical measurements. All the electrochemical measurements were performed in 10 mM PBS (pH 7.0) containing 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair using cyclic voltammetry, and differential pulse voltammetry (DPV) at room temperature. The decrease of the DPV current which was relative to the concentration of β -lactoglobulin has been used to construct the calibration curve. The parameters used for the DPV measurements: modulation amplitude 25 mV; modulation time 0.05 s; interval time 0.5 s, and step potential -5 mV .

2.3.3. X-ray photoelectron spectroscopy (XPS) measurements

The XPS measurements were performed at INRS-EMT, Varennes, Quebec, Canada, with VG Escalab220iXL instrument using Mg polychromatic source ($\text{MgK}\alpha=1253.6\text{ eV}$) at base pressure between 5×10^{-10} and 1×10^{-9} mbar. The survey spectra were recorded using 300 W of X-ray power, 100 eV pass energy and 1.0 eV step size. The high-resolution scans were run using power of 300 W, 20 eV pass energy and step size of 0.1 eV. All spectra have been calibrated according to the C1s binding energy at 284.6 eV and the peak fits consist of a mix of Lorentzian and Gaussian distributions.

2.3.4. Food samples analysis

1. Extraction: Three commercial food samples labeled as containing milk were screened for β -lactoglobulin: (i) "cake" (from local store, Canada); (ii) "Doritos" cheese snacks (from Fritolay, Canada) and (iii) "Desiree" sweet biscuit (from Hans Freitag, Germany). The food samples were blended to provide a

homogeneous mixture. To 0.1 g of each food sample, 1 mL of preheated ($60\text{ }^{\circ}\text{C}$) β -lactoglobulin ELISA Systems extraction buffer (ELISA Systems, Australia) was added and the mixtures were vortexed. The extraction was done in a water bath at $60\text{ }^{\circ}\text{C}$ with 15 min shaking. Then, the aqueous fractions were collected by centrifugation at 20,000 g for 15 min. Finally, the supernatants were recovered and divided into small aliquots (100 μL) and stored at $-20\text{ }^{\circ}\text{C}$ until used for analysis using ELISA and the developed immunosensor.

2. Enzyme-linked immunosorbent assay (ELISA) analysis: The samples have been analyzed firstly using the β -lactoglobulin ELISA Systems kit. Briefly, 100 μL of the diluted extracted samples (the cake and biscuit samples were diluted 1:10, and the snacks sample was diluted 1:100,000 in 10 mM PBS buffer) and the five standard solutions (negative, 0.1, 0.25, 0.5, 1.00 $\mu\text{g/mL}$) provided in the ELISA kit were incubated in the antibody coated test wells for 15 min. Then, the wells were washed thoroughly with the washing buffer and a 100 μL of the substrate solution were added to each well and incubated for 10 min. The reaction was finally stopped by addition of 1 M H_2SO_4 , and the absorbance was measured at 450 nm after 30 min. The analysis of both standards and samples was carried out in duplicates.
3. Immunosensor detection: The food extracts were diluted before analysis as follow: 1:100 for the biscuit, 1:150 for the cake and 1:100,000 for the snacks in 10 mM PBS buffer and incubated for 30 min at room temperature with the immunosensor and the DPV responses were recorded as previously described.

3. Results and discussion

3.1. Electrochemical functionalization

Indeed, the surface functionalization strategies is a determining factor in the performance of biosensor platforms as it greatly affects both the sensitivity and the selectivity of the biointerfaces. Eight different electrochemical modification protocols has been used for the reduction of the diazonium salt in order to study the density of the organic layer formed on the graphene surface; (1) spontaneous reaction for 180 s, (2) one CV from 0.4 to -0.6 V at 100 mV s^{-1} , (3) five CV scans from 0.4 to -0.6 V at 100 mV s^{-1} , (4) linear sweep voltammetry from 0.4 to -0.6 V at 100 mV s^{-1} , (5) 15 s duration CA, (6) 45 s duration CA, (7) 60 s duration CA, and (8) 180 s duration CA. In each case the nitro groups attached to the electrode surface after each electrografting process are reduced using five CV scans from $+0.4$ to -1.4 V in 0.1 M KCl solution and the surface coverage of the NP groups has been calculated from the area under the reduction peaks.

The consecutive cyclic voltammograms (CVs) for *in situ* generated 4-nitrobenzene diazonium cation at a GSPE are shown in Fig. 1A. The CVs are characterized by a broad irreversible reduction wave in the first cycle at -0.35 V vs. Ag reference electrode. This behavior corresponds to the electroreduction peak of the diazonium salts via one electron transfer process, resulting in the elimination of a nitrogen molecule and the production of an aryl radical which forms a covalent bond with the graphene surface (Belanger and Pinson, 2011; Pinson and Podvorica, 2005). During the second and subsequent cycles, the intensity of the reduction peak is decreased and not completely diminished. This behavior could be attributed to the fact that the electrografting step using the CV scans was not enough to cover the entire electrode surface with the grafted organic layer, therefore, preventing the complete passivation of the electrode and allowing the reduction of other diazonium cations in solution.

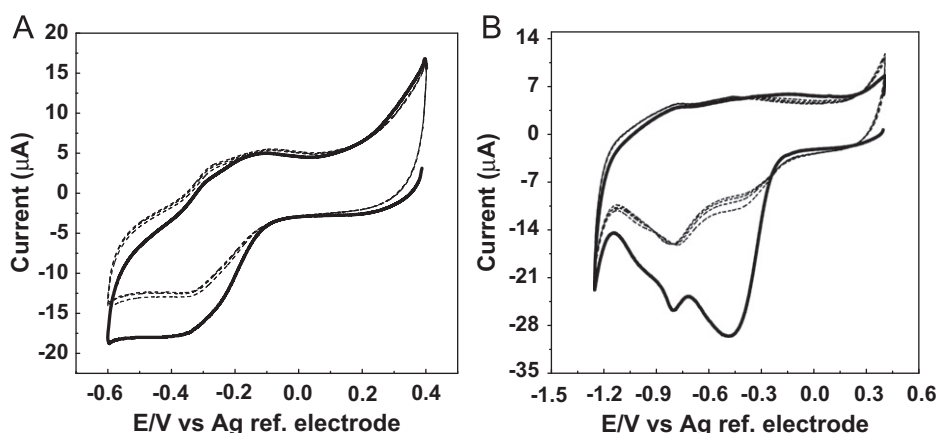


Fig. 1. (A) Cyclic voltammogram for the in situ generated 4-nitrophenyl diazonium salt (4-NPDS) in the diazotization mixture (2 mM NaNO₂ + 2 mM 4-nitroaniline in 0.5 HCl) at graphene modified screen printed electrode, (B) Successive cyclic voltammograms of the reduction of NP/GSPE in 0.1 M KCl at scan rate of 100 mV s⁻¹. The solid lines represent the first CV and the dotted lines represent the subsequent cycles.

Fig. 1B shows successive CVs for the reduction of 4-nitrophenyl modified GSPE in aqueous 0.1 M KCl solution from 0.4 to -1.4 V vs. Ag reference electrode. Two reduction peaks were observed in the voltammograms which correspond to the irreversible reduction of electrografted 4-NP groups on the graphene electrode surface. The two cathodic peaks are attributed to the reduction of the nitro groups to hydroxylamine derivatives in ($4e^-/4H^+$) process and further to the corresponding amines ($2e^-/2H^+$). Then, the reduction peaks significantly decreased in the subsequent scans which indicate almost a complete reduction of the nitro groups into amines. A similar behavior was previously reported for the reduction of nitrophenyl groups on other surfaces (Actis et al., 2008; Gui et al., 2010; Radi et al., 2009).

As shown in Supplementary Table 1 all the values of the surface coverage of the nitrophenyl groups grafted using electrochemical method are significantly higher than that grafted using spontaneous electron transfer reaction by immersing the electrode in the diazonium salt solution for 180 s without applying potential. Furthermore, the results demonstrated that both monolayers and multilayers can be obtained by the reduction of diazonium salts as described in details in the Supplementary Information, which is a known behavior observed on other materials (Combella et al., 2009; Laforgue et al., 2005).

The purpose of the present work was to modify in a reproducible way the graphene electrodes with a well ordered monolayer of aminophenyl groups enabling maximum antibody immobilization, but at the same time avoiding a complete surface passivation. Thus, the chronoamperometry reduction at -0.4 V for 45 s was selected as the optimum condition for the graphene modification.

3.2. X-ray photoelectron spectroscopy (XPS) surface characterization

XPS is one of the widely used techniques for the surface characterization. The 4-nitrophenyl modified graphene electrode and the reduced form (4-aminophenyl film) prepared as described in the experimental section were examined by XPS. Fig. S1A shows the typical XPS survey spectra of the bare GSPE. A strong C1s peak and a weak O1s peak were observed. The oxygen peak could be attributed to some oxygen atoms at the graphene edges. Fig. 2A shows the high resolution of the C1s spectra of the bare GSPE. Two peaks were observed at a binding energy of 284.55 and 283.76 eV. The former peak at 284.55 eV is assigned to the sp² hybridized carbon atoms of the graphene, however the exact source of the peak at 283.76 eV is unknown. The survey spectra after the nitrophenyl modification of the graphene electrodes is shown in

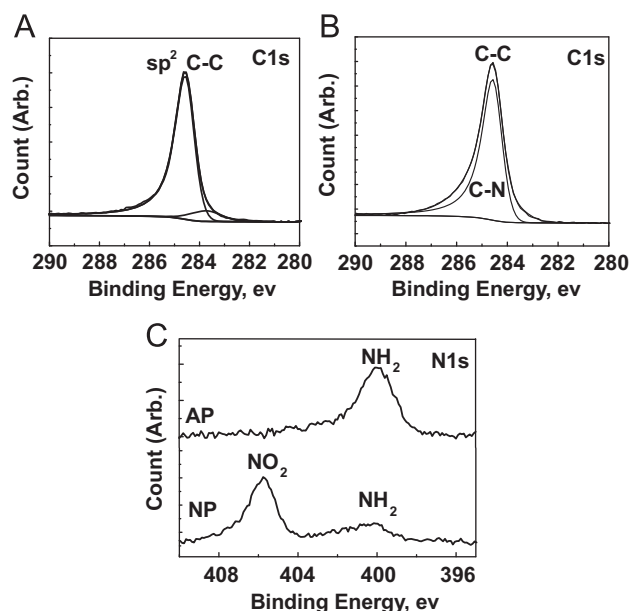


Fig. 2. XPS C1s core level spectra for bare GSPE and NP-GSPE (A,B) and N1s spectra of NP-GSPE and AP-GSPE (C).

Fig. S1B. An additional N1s peak is observed. The high resolution of the C1s spectra of the nitro phenyl modified graphene screen printed electrode (NP-GSPE) (Fig. 2B) shows a peak at 285.23 eV assigned to C-N. Moreover, the high resolution of the N1s spectrum of the NP sample (Fig. 2C) shows two peaks at 405.85 and 400.04 eV. The peak at higher binding energy is due to the nitro groups (NO₂) and confirms the presence of the nitrophenyl film on the graphene surface. The other peak at lower binding energy (400.04 eV) is assigned to the amino (NH₂) groups, which is most probably due to the reduction of some nitro groups in the XPS analysis chamber during the measurements as reported elsewhere (Baranatan and Belanger, 2005; Bekyarova et al., 2009; Gui et al., 2010). The electrochemical reduction of the nitrophenyl groups to aminophenyl groups was further confirmed by the XPS measurements of the aminophenyl graphene modified screen printed electrodes (AP-GSPE). Fig. 2C shows the high resolution of the N1s spectrum of the AP-GSPE compared with NP-GSPE. A single peak at 400.04 eV was observed for the aminophenyl modified electrode assigned to the amino groups which confirm the conversion of the nitro groups to amines and also demonstrate

the robust nature of the aryl modified graphene (Bekyarova et al., 2009).

3.3. Differential pulse voltammetric characterization of the immunosensor stages

Differential pulse voltammetry has been used in order to characterize the stepwise modification of immunosensors. Fig. 3 shows the DPVs of 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ probe in PBS, pH 7.4 for the bare GSPE (a); 4-NP/GSPE modified electrode (b); 4-AP/GSPE modified electrode (c); GA/4-AP/GSPE (d); Ab/4-AP/GSPE modified electrode (e); BSA/Ab/4-AP/GSPE modified electrode (f) and after incubation with 100 ng mL⁻¹ β -lactoglobulin solution for 45 min (g). It is clearly observed a well-defined reduction peak for a bare GSPE. The electrochemical grafting modification using chronoamperometry at -0.4 V for 45 s, the produced 4-NP film on the GSPE electrodes, and led to further reduction of the intensity of the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ reduction peak. This is due to the deposition of the organic layer which partially blocks the surface but in the same time the electron transfer still takes place. After the reduction of the nitro groups on the surface to amine groups the peak current increased again. This could be attributed to the ionization of some amino groups on the surface, which leads to an electrostatic attraction with the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ anions in solution as reported by Covaci et al. (2010). The reduction peak current of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ was further decreased after the reaction of the amino groups with glutaraldehyde, due to the displacement of the amine groups on the surface with the aldehyde groups. In fact, the reaction of glutaraldehyde and the aromatic amine is expected to be as presented in Scheme 1 following the previous reports (Covaci et al., 2010; Radi et al., 2009; Radulescu et al., 2010; Cortina-Puig et al., 2010). However, the exact reaction mechanism is yet to be confirmed. Then after the antibody immobilization and blocking the free active sites on the electrodes surface with BSA, the peak current of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ was further decreased due to the blocking effect of these proteins. The formation of the immunochemical reaction of β -lactoglobulin molecules with the antibody modified electrodes was also accompanied by a decrease in the reduction peak current of the redox probe, showing that the electron-transfer kinetics of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ is

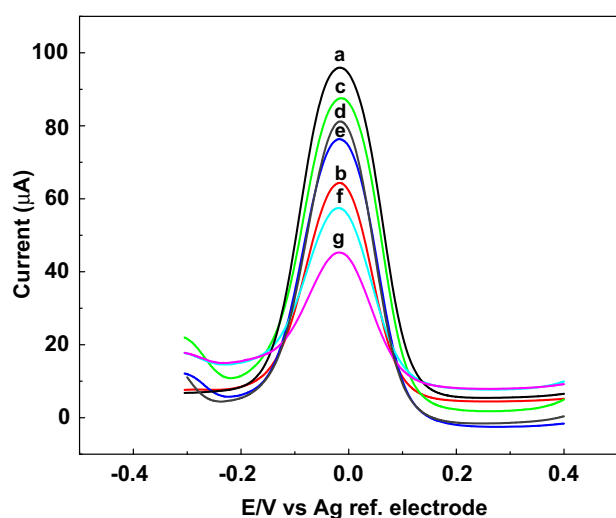


Fig. 3. DPVs of 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ probe in PBS, pH 7.4 for bare graphene modified screen printed electrode (GSPE) (a); 4-nitrophenyl/GSPE modified electrode (b); 4-aminophenyl/GSPE modified electrode (c); GA/4-AP/GSPE (d); Ab/4-AP/GSPE modified electrode (e); after blocking with 0.25% BSA for 45 min (f), and after incubation with 100 ng mL⁻¹ of β -lactoglobulin solution for 45 min (g). The parameters of DPV: modulation amplitude, 25 mV; modulation time, 0.05 s; interval time 0.5 s, step potential -5 mV.

decreased. This current decrease after binding is not only due to the blocking effect from the β -lactoglobulin protein but could also be attributed to a number of factors. For example, the isoelectric point of the β -lactoglobulin equals 5.2 (McKenzie, 1971), which in turn offers a negative charge at pH 7.4, acting as an electrostatic barrier for the electron transfer. It could be also partly due to conformational changes of the antibody after binding with the protein (Nezlin, 1998).

3.4. Dose response of the β -lactoglobulin immunosensor

To evaluate the immunochemical reaction between the antibody and β -lactoglobulin, the immunosensor was exposed to various concentrations of β -lactoglobulin (0, 0.001, 0.01, 0.1, 1.0, 10 and 100 ng mL⁻¹). Fig. 4A shows the decrease in the differential pulse voltammetric reduction peak of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ with the increment of β -lactoglobulin. The inset in Fig. 4A shows a good linear relationship between the change of the cathodic peak currents obtained by DPV and the logarithm of β -lactoglobulin concentrations from 1.0 pg mL⁻¹ to 100 ng mL⁻¹. Each data point in the calibration plot represents three independent measurements and the error bars show the standard deviations of measurements. The relative standard deviations were from 1.6% to 6.0% which indicates good reproducibility of the immunosensor. The linear regression equation was $I (\mu\text{A}) = 7.88 + 1.56 \times \log C$ [ng mL⁻¹], $R = 0.998$, with a detection limit (LOD) of 0.85 pg mL⁻¹ ($S/N = 3$). The results indicated that the developed immunosensor

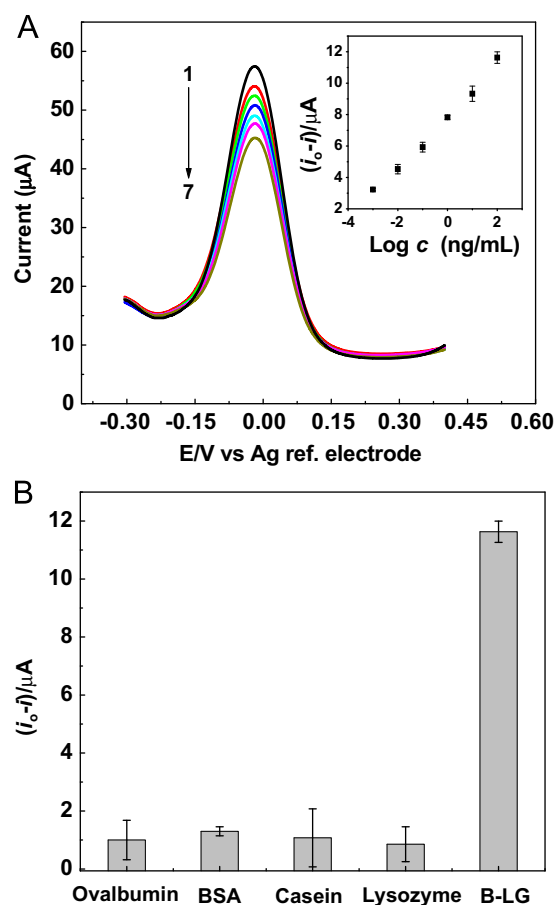


Fig. 4. (A) DPVs of the immunosensor incubated with different concentrations of β -lactoglobulin (1–7): the concentrations of β -LG are 0.000, 0.001, 0.01, 0.1, 1.0, 10 and 100 ng mL⁻¹. The inset is the calibration curve based on the change of the DPV peak currents vs. the logarithm of the concentrations. (B) Comparison of response of GSPE voltammetric immunosensors to 1000 ng mL⁻¹ Ovalbumin, BSA, Casein, Lysozyme, and 100 ng mL⁻¹ β -lactoglobulin.

Table 1Comparison of β -lactoglobulin detection in real samples using the proposed immunosensor and ELISA.

Sample	ELISA ($\mu\text{g/mL}$)	Immunosensor ($\mu\text{g/mL}$)
"Cake" (from local store, Canada)	9.30 ± 0.71	7.50 ± 0.16
"Desiree" sweet biscuit (from Hans Freitag, Germany)	7.70 ± 0.50	7.47 ± 0.25
"Doritos" cheese snacks (from Fritolay, Canada)	92300 ± 6461	87330 ± 3000

in this study exhibited lower detection limit compared with other reported methods (Billakanti et al., 2010; Hohensinner et al. 2007; Maier et al., 2009) without any amplification steps. A comparison of the detection methods is shown in Supplementary Table 2. This sensitivity is attributed to the success of the functionalization method, to develop a highly oriented monolayer, which enabled the immobilization of higher amount of β -lactoglobulin antibody, and also the excellent higher conductivity of graphene which enhanced the charge transfer of the redox probe in solution.

3.5. Selectivity of the immunosensor

It was very important to study the selectivity of the immunosensor to confirm that the immunosensor response is due to specific interaction between the β -lactoglobulin and the antibody immobilized on the electrode. A control experiment has been performed by incubating the immunosensor with four different non-specific proteins which may present in the food: ovalbumin, BSA, egg lysozyme and casein. Fig. 4B shows non-significant change in reduction peak current for the antibody modified electrodes incubated with $1.0 \mu\text{g mL}^{-1}$ of each protein for 45 min compared with another immunosensor prepared using identical protocol and incubated with 100 ng mL^{-1} of β -lactoglobulin. These results confirm that the immunosensor displayed good selectivity for the determination of β -lactoglobulin.

3.6. Real sample analysis

The developed immunosensor was then applied for the detection of β -lactoglobulin in commercial milk-containing food products. As can be seen in Table 1, a good correlation was obtained between the immunosensor and the ELISA, which indicated that the developed immunosensor might be applied for the determination of β -lactoglobulin for routine use. Moreover, the low detection limit enabled the successful application of the immunosensor in the highly diluted real extracts which significantly minimized the matrix effect.

4. Conclusions

A sensitive label-free voltammetric immunosensor for the detection of β -lactoglobulin has been developed. A controllable electrodeposition method of organic film based on the reduction of diazonium salt on graphene-modified screen printed electrodes was successfully achieved. This enabled a suitable, simple, and versatile platform for the immobilization of antibodies and the immunosensor fabrication. The immunosensor developed using this approach offers a linear range of $0.001\text{--}100 \text{ ng mL}^{-1}$ of β -lactoglobulin and was able to reproducibly detect β -lactoglobulin at the pg mL^{-1} level without any amplification step. The immunosensor was successfully applied for the detection of β -lactoglobulin in food samples, and the results were correlated to those of the commercial ELISA method offering a trustful analytical screening tool. Thus, the present sensing platform

appears to be very promising and is expected to open the door for extended biosensing applications of graphene.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.06.008>.

References

- Actis, P., Caulliez, G., Shul, G., Opallo, M., Mermoux, M., Marcus, B., Boukherroub, R., Szunerits, S., 2008. *Langmuir* 24, 6327–6333.
- Balandin, A.A., Ghosh, S., Bao, W.Z., Calizo, I., Teweldebrhan, D., Miao, F., Lau, C.N., 2008. *Nano Letters* 8, 902–907.
- Baranaton, S., Belanger, D., 2005. *Journal of Physical Chemistry B* 109, 24401–24410.
- Belanger, D., Pinson, J., 2011. *Chemical Society Reviews* 40, 3995–4048.
- Bekyarova, E., Itkis, M.E., Ramesh, P., Berger, C., Sprinkle, M., de Heer, W.A., Haddon, R.C., 2009. *Journal of the American Chemical Society* 131, 1336–1337.
- Billakanti, J.M., Fee, C.J., Lane, F.R., Kash, A.S., Fredericks, R., 2010. *International Dairy Journal* 20, 96–105.
- Combella, C., Jiang, D.-e., Kanoufi, F., Pinson, J., Podvorica, F.I., 2009. *Langmuir* 25, 286–293.
- Covaci, O., Bucur, B., Bucur, M., Radu, G., 2010. *Microchimica Acta* 169, 335–343.
- Dijk, J.A.P.v., Smit, J.A.M., 2000. *Journal of Chromatography A* 867, 105–112.
- Gui, A.L., Liu, G., Chockalingam, M., Le Saux, G., Luais, E., Harper, J.B., Gooding, J.J., 2010. *Electroanalysis* 22, 1824–1830.
- Hohensinner, V., Maier, I., Pittner, F., 2007. *Journal of Biotechnology* 130, 385–388.
- Host, A., 2002. *Annals of Allergy, Asthma & Immunology* 89, 33–37.
- Host, A., Halken, S., 1990. *Allergy* 45, 587–596.
- Jin, Z., Lomeda, J.R., Price, B.K., Lu, W., Zhu, Y., Tour, J.M., 2009. *Chemistry of Materials* 21, 3045–3047.
- Jin, Z., McNicholas, T.P., Shih, C.-J., Wang, Q.H., Paulus, G.L.C., Hilmer, A.J., Shimizu, S., Strano, M.S., 2011. *Chemistry of Materials* 23, 3362–3370.
- Lee, L.A., Burkes, A.W., 2006. *Annual Review of Nutrition* 26, 539–565.
- Laforge, A., Addou, T., Belanger, D., 2005. *Langmuir* 21, 6855–6865.
- Lahiri, J., Ostuni, E., Whitesides, G.M., 1999. *Langmuir* 15, 2055–2060.
- Lee, C., Wei, X.D., Kysar, J.W., Hone, J., 2008. *Science* 321, 385–388.
- Lim, H., Lee, J.S., Shin, H.-J., Shin, H.S., Choi, H.C., 2010. *Langmuir*, 12278–12284.
- Lomeda, J.R., Doyle, C.D., Kosynkin, D.V., Hwang, W.-F., Tour, J.M., 2008. *Journal of the American Chemical Society* 130, 16201–16206.
- Luis, R.d., Lavilla, M., Sánchez, L., Calvo, M., Pérez, M.D., 2009. *Food Control* 20, 643–647.
- Maier, I., Lindner, W., Pittner, F., 2009. *Monatshefte für Chemie* 140, 921–929.
- McKenzie, H.A. (Ed.), 1971. *Milk Proteins: Chemistry and Molecular Biology*. Academic Press, New York.
- Cortina-Puig, M., Muñoz-Berbel, X., Calas-Blanchard, C., Marty, J.-L., 2010. *Microchimica Acta* 171, 187–193.
- Nezlin, R. (Ed.), 1998. *The Immunoglobulins: Structure and Function*. Academic Press, New York.
- Novoselov, K.S., Geim, A.K., Morozov, S.V., Jiang, D., Zhang, Y., Dubonos, S.V., Grigorieva, I.V., Firsov, A.A., 2004. *Science* 306, 666–669.
- Park, S., Ruoff, R.S., 2009. *Nature Nanotechnology* 4, 217–224.
- Patolsky, F., Katz, E., Bardea, A., Willner, I., 1999. *Langmuir* 15, 3703–3706.
- Pelaez-Lorenzo, C., Díez-Masaa, J.C., Vasallo, I., Frutos, M.d., 2009. *Analytica Chimica Acta* 649, 202–210.
- Pinson, J., Podvorica, F., 2005. *Chemical Society Reviews* 34, 429–439.
- Popovich, N.D., Yen, B.K., Wong, S.S., 2003. *Langmuir* 19, 1324–1329.
- Puerta, A., Díez-Masa, J.C., Frutos, M.d., 2005. *Analytica Chimica Acta* 537, 69–80.
- Pumera, M., 2009. *Chemistry—A European Journal* 15, 4970–4978.
- Radi, A.-E., Muñoz-Berbel, X., Cortina-Puig, M., Marty, J.-L., 2009. *Electrochimica Acta* 54 (8), 2180–2184.
- Radulescu, M.C., Chira, A., Radulescu, M., Bucur, B., Bucur, M.P., Radu, G.L., 2010. *Sensors* 10 (12), 11340–11351.
- Rena, Y., Hanb, Z., Chuc, X., Zhangb, J., Caia, Z., Wub, Y., 2010. *Analytica Chimica Acta* 667, 96–102.
- Ruckenstein, E., Li, Z.F., 2005. *Advances in Colloid and Interface Science* 113, 43–63.
- Sharma, R., Baik, J.H., Perera, C.J., Strano, M.S., 2010. *Nano Letters* 10, 398–405.

Supplementary Information

Electrochemical immunosensor for the milk allergen β -lactoglobulin based on electrografting of organic film on graphene modified screen-printed carbon electrodes

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1- Effect of electrochemical grafting protocol

Table 1. Active surface coverage concentration (Γ) of nitrophenyl groups on the GSPE formed using different protocols.

Method	Surface coverage
	$\times 10^{-10}$ (mol. cm ⁻²)
Spontaneous reaction 180 s	0.80
One cyclic voltammetry	4.00
Five cyclic voltammetry	4.10
Linear weep voltammetry	4.80
Chronoamperometry 15 s	5.70
Chronoamperometry 45 s	7.35
Chronoamperometry 60 s	13.0
Chronoamperometry 180 s	19.9

As shown in Table 1 all the values of the surface coverage of the nitrophenyl groups grafted using electrochemical method are significantly higher than that grafted using spontaneous electron transfer reaction by immersing the electrode in the diazonium salt solution for 180 s without applying potential. Furthermore, the surface coverage values of the electrografted NP groups on the graphene surface for 1 min. or less were ranging from 4.00×10^{-10} to 13.0×10^{-10} , which is in comparable with the value reported previously for the chemically grafted NP groups on epitaxial graphene (Bekyarova et al., 2009) as well as other carbon

surfaces (Allongue et al., 1997; Saby et al., 1997; Zhong et al., 2008). It was also noticed that the electrodeposition using cyclic voltammetry scans produced the lowest NP coverage which confirm that the grafted film was not very compact as it was explained above. The linear sweep voltammetric method gave higher coverage than the cyclic voltammetric procedure. However, the chronoamperometric method gave the highest surface coverage indicating more compact layer even with 15 s duration and the density of the grafted nitrophenyl groups was increased by increasing the time from 15 to 180 s.

The results demonstrated that both monolayers and multilayers can be obtained by the reduction of diazonium salts which is a known behaviour observed on other materials (Combellas et al., 2009; Laforgue et al., 2005). The use of chronoamperometry step at -0.4 for 45 s or 60 s for the grafting has achieved the best agreement with the theoretical surface coverage of nitrophenyl groups (12.5×10^{-10} mol/cm²) which is based on ideal highly ordered closed packed monolayer (Liu and McCreery, 1995). By increasing the time of the electrochemical grafting process the surface coverage increases, indicating the continuous growth of multilayer and higher film thickness. The formation of multilayer of 4-nitrophenyl (NP) is not desirable as it sterically hindered the access of the nitro groups on the layers closer to the electrode and also causes disorder of the grafted film preventing access to some nitro groups on the surface.

The purpose of the present work was to modify in a reproducible way the graphene electrodes with a well ordered monolayer of aminophenyl groups enabling maximum antibody immobilization, but at the same time avoiding a complete surface passivation. Thus, the chronoamperometry reduction at -0.4 V for 45 s was selected as the optimum condition for the graphene modification. However, an easier electron transfer to the electrode surface would be an advantage for electrodes modified by cyclic and linear sweep voltammetry where non compact

layer is formed. This could also find applications in some cases as enzyme-based amperometric biosensors in which the maximum electric communication between the enzyme and the electrode surface is required as previously reported elsewhere (Radi et al., 2009).

2. XPS survey spectra before and after modification

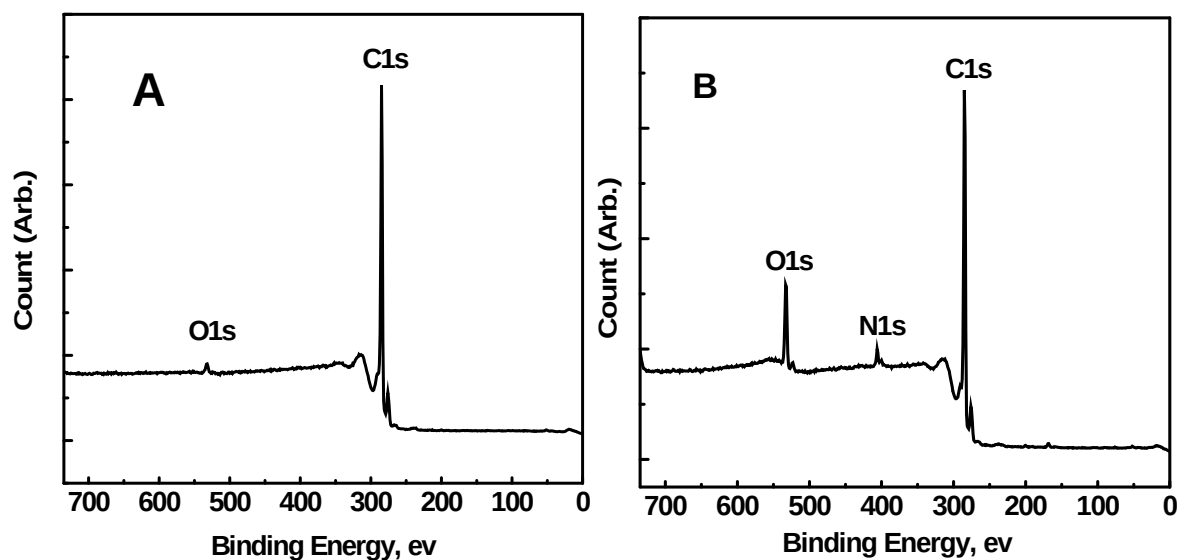


Figure S1. XPS survey spectra for bare GSPE (A) and NP-GSPE (B).

3-Cyclic voltammetric characterization of the mechanism of the electrochemical process on the modified graphene electrodes:

The relationship between the peak current and the square root of the scan rate was studied in order to gain insight into the electrochemical mechanism taking place on the immunosensor, . The CVs of the resulting immunosensor in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at different scan rates were investigated in the range of 10-200 mVs^{-1} (Fig. S2.). It is clear that both the anodic and cathodic peak currents increased linearly with the square root of scan rate, with correlation coefficients of 0.998 and 0.990 respectively, indicating diffusion-controlled process over the studied range of the scan rate.

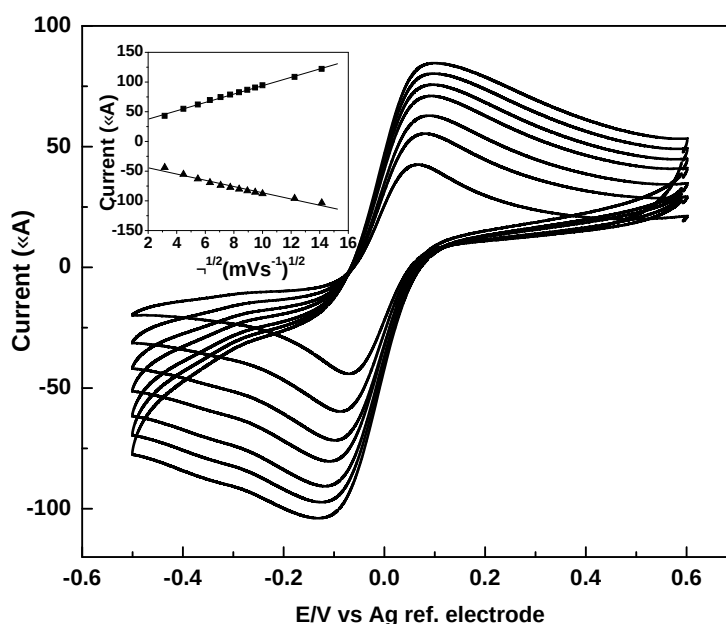


Figure S2 Cyclic voltammograms of the modified electrode at different scan rates (from inner to outer): 10, 20, 30, 40, 50, 60, and 70 mVs^{-1} in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ + 10 mM PBS solution (pH

7.4). The inset shows the dependence of the oxidation and reduction peak currents on the square root of scan rates.

4-Stability and reproducibility of the immunosensor

The stability of the immunosensor was studied after storage at 4 °C for 15-day period. The immunosensor was used to detect 1 ng/mL β -lactoglobulin. The difference in the peak current was 7.88 μ A before and 7.99 μ A after (less than 2 % current change), demonstrating that the immunosensor had good stability. The reproducibility of the immunosensor was examined by performing three independent measurements for each concentration in the calibration plot. The relative standard deviations were from 1.6 % to 6.0 % indicates good reproducibility of the immunosensor.

5. Comparison of performance of the different detection methods for β -lactoglobulin.

Table 1. Comparison of performance of the different detection methods for β -lactoglobulin.

Method	Limit of detection	Analytical range	Reference
Voltammetric immunosensor	0.85 pg mL ⁻¹	1 pg mL ⁻¹ - 100 ng mL ⁻¹	This work
Optical near field biosensor	n.a.	100 ng mL ⁻¹ -100 μ g mL ⁻¹	Hohensinner et al., 2007
Colourimetric immunosensor	1 ng mL ⁻¹	10 ng mL ⁻¹ -1 mg mL ⁻¹	Maier et al., 2009
ELISA	9.7 $\times$ 10 ⁻¹³	3.5 $\times$ 10 ⁻¹¹ - 4.6 $\times$ 10 ⁻⁸ M	Pelaez-Lornezo et al., 2010

6. Real samples Analysis

Table 2. F-test and t-test calculations for the three food samples analysis

Parameter	Sweet biscuit sample		Cake		Cheese snacks	
	Immunosensor	ELISA	Immunosensor	ELISA	Immunosensor	ELISA
n	3	3	3	3	3	3
$\bar{X}$	7.47	7.70	7.50	9.30	87330	92300
S.D.	0.25	0.50	0.16	0.71	3000	6461
R.S.D. (%)	3.35	6.49	2.13	7.63	3.44	7.00
t-test	0.71	{t(P = 0.05)} = 2.78	4.28	{t(P = 0.05)} = 2.78	1.20	{t(P = 0.05)} = 2.78
F-test	4.00	{F(P = 0.05)} = 19.0	19.69	{F(P = 0.05)} = 19.0	4.64	{F(P = 0.05)} = 19.0

As shown in Table 2. the F-test and t-test calculations have been done for the three food samples.

The results of the student's t-test and variance ratio F-test of the sweet biscuit and the cheese snacks show that there are no significant differences between the techniques with regard to accuracy and precision. However, the results of the cake sample showed more difference which could be attributed to the effect of the matrix of the cake extract in the ELISA detection which can cause overestimation of the β -lactoglobulin level, while the higher dilution of the same sample (1:150) for the detection by the immunosensor had significantly minimized the matrix effect. Moreover, the lower RSD of the proposed immunosensor (2.13 %) compared with ELISA (7.63 %) indicates more precision of the developed immunosensor.

7. References

- Allongue, P., Delamar, M., Desbat, B., Fagebaume, O., Hitmi, R., Pinson, J., Savéant, J.-M., 1997. *J. Am. Chem. Soc.* 119, 201-207.
- Billakanti, J.M., Fee, C.J., Lane, F.R., Kash, A.S., Fredericks, R., 2010. *Int. Dairy J.* 20, 96–105.
- Combellas, C., Jiang, D.-e., Kanoufi, F., Pinson, J., Podvorica, F.I., 2009. *Langmuir* 25, 286.
- Hohensinner, V., Maier, I., Pittner, F., 2007. *J. biotechnol.* 130, 385–388.
- Laforge, A., addou, T., Belanger, D., 2005. *Langmuir* 21, 6855.
- Liu, Y.-C., McCreery, R.L., 1995. *J. Am. Chem. Soc.* 117, 11254-11259.
- Maier, I., Lindner, W., Pittner, F., 2009. *Monatsh. Chem.* 140, 921–929.
- Pelaez-Lorenzo, C., Diez-Masaa, J.C., Vasallo, I., Frutos, M.d., 2009. *Anal. Chim. Acta* 649, 202–210.
- Radi, A.-E., Muñoz-Berbel, X., Cortina-Puig, M., Marty, J.-L., 2009. *Electrochim. Acta* 54(8), 2180-2184.
- Saby, C., Ortiz, B., Champagne, G.Y., Bélanger, D., 1997. *Langmuir* 13, 6805-6813.
- Zhong, Y.L., Loh, K.P., Midya, A., Chen, Z.-K., 2008. *Chem. Mater.* 20, 3137-3144.