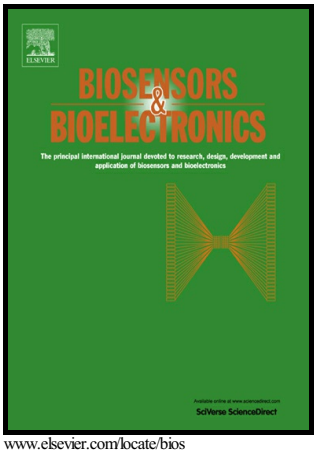

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PII: S0956-5663(15)30459-0
DOI: http://dx.doi.org/10.1016/j.bios.2015.09.064
Reference: BIOS8031

To appear in: *Biosensors and Bioelectronic*

Received date: 24 July 2015
Revised date: 14 September 2015
Accepted date: 27 September 2015

Cite this article as: Stéphanie Patris, Marie Vandeput, Gersonie Momo Kenfack Dominique Mertens, Bieke Dejaegher and Jean-Michel Kauffmann, An experimental design approach to optimize an amperometric immunoassay on screen printed electrode for *clostridium tetani* antibody determination, *Biosensor and Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2015.09.064

An experimental design approach to optimize an amperometric immunoassay on a screen printed electrode for *Clostridium tetani* antibody determination

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Highlights

- An innovative amperometric immunosensor with the antigen directly attached onto a screen printed electrode for the sensitive and rapid detection of *Clostridium tetani* antibody
- A response surface methodology using a central composite design permitted a limited number of experiments for the optimization of four parameters affecting the immunoassay response
- Minimum immunosensor and sample handling for possible point of care assays.

Abstract

An immunoassay for the determination of anti-*tetani* antibodies has been developed using a screen printed electrode (SPE) as solid support for toxoid (antigen) immobilization. The assay was performed in guinea pig serum. The immunoreaction and the subsequent amperometric detection occurred directly onto the SPE surface. The assay consisted of spiking the anti-*tetani* sample directly onto the toxoid modified SPE, and then a second antibody, i.e. a HRP-labeled anti-immunoglobulin G, was deposited onto the biosensor. Subsequent amperometric detection was realized by spiking 10 µL of a hydroquinone (HQ) solution into 40 µL of buffer solution containing hydrogen peroxide. An experimental design approach was implemented for the optimization of the immunoassay. The variables of interest, such as bovine serum albumin (BSA) concentration, incubation times and labeled antibody dilution, were optimized with the aid of the response surface methodology using a circumscribed central composite design (CCCD). It was observed that two factors exhibited the greatest impact on the response, i.e. the anti-*tetani* incubation time and the dilution factor of the labeled antibody. It was discovered that in order to maximize the response, the dilution factor should be small,

while the anti-*tetani* antibody incubation time should be long. The BSA concentration and the HRP-anti-IgG incubation had very limited influence. Under the optimized conditions, the immunoassay had a limit of detection of 0.011 IU/mL and a limit of quantification of 0.012 IU/mL. These values were below the protective human antibody limit of 0.06 IU/mL.

Keywords: *Clostridium tetani*, Immunoassay, Central composite design, Screen printed electrode, Immunosensor

1. Introduction

The development of a rapid and sensitive analytical strategy to determine the patients' immunological state is an important issue. The status of antibodies level in a patient permits to estimate its immunological protection against an infectious disease, provides information about allergies and allows diagnosis of auto-immune diseases (Fontaine et al., 2007; Giannetto et al., 2014; Hainz et al., 2005; Kreuzer et al., 2001). It is also important to determine the antibody level during the potency testing of vaccines (Casey et al., 2011). This can be achieved by immunoassays which allow the antibodies level determination in a sensitive and quite selective manner.

Tetanus is a fatal disease caused by a bacterium, *Clostridium tetani*, found universally in soil. It is a spore-forming and strictly anaerobic Gram-positive bacterium. This bacterium produces a 150 kDa protein, called tetanus toxin or tetanospasmin, which binds to the nerve centers and causes violent muscular contractions. The toxin can be fatal by blocking the respiratory muscles (Brostoff et al., 1993). The toxin is considered as an antigen by the immune system and causes the synthesis of antibodies, i.e. the tetanus antitoxins (anti-*tetani*). The active immunization by vaccination is effective by injecting the tetanus toxoid, i.e. the detoxified formaldehyde-treated tetanus toxin (Galazka, 1993; Million, 1997; Popoff and Poulain, 2005; WHO, 2006). Vaccination is the best way to prevent the disease. Furthermore, vaccination of the mother protects her baby during delivery (Galazka, 1993; Maral et al., 2001; Popoff and Poulain, 2005). The antibodies level ensuring full immunity against tetanus is not known. Indeed, there is no level of absolute protection. Protection is provided when the amount of antibody is sufficient to neutralize the amount of toxin produced by the bacterium (Galazka, 1993). In case of tetanus infection without previous vaccination, a passive protection is necessary. This passive protection is realized by injection of human tetanus immunoglobulin

(HTIgG). The tetanus vaccine is administered with HTIgG so the passive protection lasts long enough to protect against the disease until the patient can produce its own antibodies against tetanus (Miyagi and Shah, 2011). The vaccination has significantly reduced the incidence of tetanus. The lack of coverage by missing the vaccine booster, however, exposes patients to contamination, particularly in case of injury. Because the patients' immunological state against *tetanus* is not always known, the post-potentially-exposure prophylaxis consists of a booster vaccine administration to the patient (Collins et al., 2015). Unfortunately, this systematic toxoid injection is not without risk when the patient is already immunized, as repeated administrations of tetanus toxoid lead to a hyper-immunization which can result in allergic reactions, mainly local, including an Arthus reaction due to antibody/antigen complex deposits leading to inflammatory reactions (Facktor et al., 1973; Mayorga et al., 2003; Moylett and Hanson, 2004; Olander et al., 2009; Pollard and Selby, 1978). A more important risk to develop neurological reactions, such as polyneuropathies, in the event of multiple injections of tetanus toxoid has also been reported in the literature (Ponvert, 2006; Reinstein et al., 1982). Although tetanus vaccination is an important aspect of trauma care, particularly when traumatic injury involves an open wound, antitoxin levels should be known before determining the need for a subsequent booster (Ponvert, 2006).

In vivo assays to determine the potency of tetanus vaccine and HTIgG are described in the European Pharmacopoeia (Eur. Ph.) monographs "Assay of tetanus vaccine (adsorbed)" (20708) and "Human tetanus immunoglobulin" (0398) (European Directorate for the Quality of Medicines, 2014). The Eur. Ph. describes two alternative in vitro assays, i.e. an enzyme-linked immunosorbent assay (ELISA) and a toxoid inhibition assay (TIA) (Gross et al., 2006). The alternative in vitro assays combined with a colorimetric end point measurement are preferred over tests based on laboratory animals (in vivo) to determine the antibodies level. ELISA and TIA generally require sophisticated instruments and reaction times as long as 24 h (European Directorate for the Quality of Medicines, 2014; Niklasson et al.). Other tests, based on passive haemaglutination (Pitzurra et al., 1983) and immunochromatography (Ardelean-Jaby et al., 2002; Orimadegun et al., 2013), are also on the market and mainly used in hospitals. These tests, however, provide only a semi-quantitative response.

An alternative to the ELISAs and TIAs is the use of electro-immunosensors. Electrochemical (EC) immunosensors are integrated devices that combine the high sensitivity of an electrochemical transducer with the high selectivity of an immunochemical reaction

(Thévenot et al., 2001; Zhou et al., 2003). The biological component consists of an immobilized antigen which is selectively recognized by the antibody analyte. A SPE can be used as solid support for the immunoassay with the antigen directly immobilized onto the transducer surface (Balkenhohl and Lisdat, 2007; Díaz-González et al., 2005; Messina et al., 2007; Neves et al., 2012). A subsequent immuno-component, labeled with an enzyme, permits to generate an electroactive species that can be detected at the SPE. In the sandwich type procedure, a second antibody, labeled with an enzyme, is used and in the competitive type procedure, the unknown species competes with a labeled antibody for the binding sites.

It was found of interest to develop a handheld test to determine the anti-*Clostridium tetani* level. A satisfactory strategy for its determination using magnetic microbeads was developed by our team in 2010 (Patris et al., 2010). The use of microbeads and the need for applying several analytical steps, however, made the method unpractical for being used as point of care testing. Screen-printed electrode with immunosensing can, however, advantageously be considered for point of care applications since they are manufactured at a large scale with good reproducibility and are disposable with no need for surface renewing and no risk of cross contamination.

In the present work, an immunoassay using an antigen directly immobilized onto a SPE was developed for the detection of anti-*Clostridium tetani* antibodies. The strategy was based on a sandwich type format using horseradish peroxidase (HRP) as enzymatic label. A carbon-based SPE, modified by the tetanus toxoid, was used as transducer. Subsequent to the immunoreaction, the enzymatic substrate HQ was added in the presence of H_2O_2 in a microdroplet onto the SPE and the amperometric signal was recorded. Parameters, such as the blocking agent concentration, the incubation times and the labeled antibody dilution factor, were optimized applying a circumscribed central composite design (CCCD) (Asadollahzadeh et al., 2014; B. Dejaegher and Vander Heyden, 2009; Ghoreishi et al., 2012). The response considered was the amperometric signal. The examined experimental factors can affect the results of the method and several strategies can be used to optimize the methods. Classically in the literature, the biosensors are optimized by applying the one-variable-at-a-time (OVAT) method. Such OVAT approach varies only one factor at a time between consecutive experiments (Dejaegher and Vander Heyden, 2011; Dejaegher et al., 2013), and does not take interactions between the factors into account. The optimal conditions found will therefore depend on the starting conditions of the optimization. In addition, if the numbers of factors is

too important, the number of experiments to be realized quickly will become too large. In order to consider the possible interactions between different factors and to reduce the number of experiments, a multivariate approach using an experimental design can advantageously be envisaged (Dejaegher et al., 2010). A CCCD, the most often used response surface design, combines a two-level full factorial design (2^f experiments), a star design (2f experiments), and an often replicated center point. Applying a CCCD is a good choice because of its high efficiency with respect to the number of required experiments (Bieke Dejaegher and Vander Heyden, 2009; Dejaegher et al., 2010). Such central composite design (CCD) is frequently used during optimization of liquid chromatography assays (flow, gradient, injection volume…) (Székely et al., 2014) or capillary electrophoresis methods (Dejaegher et al., 2013). This design can be also used to optimize the electrochemical conditions (scan rate, step potential, pH…) (Ghoreishi et al., 2012; Masoum et al., 2014), the protein immobilization parameters (Alonso-Lomillo et al., 2010, 2009) or the analytes extraction procedure (solvent volume, pH, extraction time, concentrations…) (Asadollahzadeh et al., 2014). In a Design-of-Experiment approach, after the selection of the experimental factors to be evaluated and their levels, the design, and the responses, the planning and the execution of the experimental set-up are realized. Then, a polynomial model is built to relate the factors and the considered response, and a graphical and/or statistical interpretation of the model is conducted in order to determine the optimal experimental conditions (Bieke Dejaegher and Vander Heyden, 2009). To the best of our knowledge, this work is reporting for the first time a CCCD approach in order to optimize an immunosensor development.

2. Materials and methods

2.1. Materials

The *tetanus* toxoid (not adsorbed, 02/232) and *tetanus* antitoxin guinea pig serum (98/572) were obtained from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, United Kingdom). N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), the peroxidase labeled goat anti-guinea pig IgG (solution in 0.01 M phosphate buffered saline, pH 7.4, containing 1% BSA and 0.01% thimerosal), polysorbate 20 (tween 20), and hydroquinone were purchased from Sigma (Bornem, Belgium). BSA (fraction V) was from Fluka (Neu-Ulm, Germany). Hydrogen peroxide 30% (w/w), citric acid monohydrate, sodium carbonate anhydrous, sodium hydrogen

carbonate, potassium chloride, di-sodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate and sodium chloride were of analytical grade (VWR International Inc, Leuven, Belgium). The buffers used were, for the immunochemical reactions, a phosphate buffered saline pH 7.4 (PBS) and for the electrochemical measurements, a 0.05 M citric acid solution containing 0.1 M sodium chloride and adjusted to pH 5.5 with a solution of sodium hydroxide.

2.2. Apparatus and softwares

EC experiments were performed using a LC Epsilon computer-controlled potentiostat with an Epsilon EC software (BASi, Kenilworth, United Kingdom). The SPE and the adapted connector were purchased from DropSens (Oviedo, Spain). The SPE had the following dimensions: 3.4 x 1.0 x 0.05 cm (length x width x height). Working (4 mm diameter) and counter electrodes were made of carbon, whereas the reference electrode and the electric contacts were made of silver. Experimental data were analysed using Nemrodw software (http://www.nemrodw.com) and Excel (version 2011).

2.3. SPE modification

In order to remove the glycine which can interfere during the immobilization steps, the content of the ampoules of *tetanus* toxoid was dialyzed against PBS pH 7.4. Subsequently, the toxoid was immobilized onto the SPE as follows: (i) first, the SPE was activated in the presence of 50 µL of 2 M H_2SO_4 dropped onto the SPE by applying a voltage of + 1.600 V during 150 seconds and subsequently washed with Milli-Q water (Millipore, Molsheim, France); (ii) 10 µL of 40 mM EDC and 40 mM NHS solution in MES buffer were spiked onto the pretreated SPE surface during 1 hour; (iii) 10 µL of 150 µg/mL tetanus toxoid was spiked onto the pretreated SPE surface during 1 h at room temperature; (iv) the SPE was washed to remove protein excess and the free sites were blocked by adding 10 µL of BSA solution for 30 min at room temperature. Finally the SPE was rinsed with Milli-Q water (Fig. 1.A).

2.4. Immunoassay procedure

The antigen immobilized onto the SPE was put in contact with 10 µL of guinea pig serum containing anti-*C. tetani* antibodies (IgG). After the first incubation period, the SPE was

rinsed with Milli-Q water and 10 µL of anti-IgG labeled with HRP solution was added to quantify the first immunoreaction. This immunoassay strategy was based on a sandwich type scheme (Fig. 1.B). The SPE was washed twice with the washing buffer, and then 40 µL of citrate buffer containing 2.5 mM H_2O_2 was added for the amperometric detection. A potential of -280 mV was applied and when the current was stabilized, 10 µL HQ (10 mM) was spiked onto the electrode and the reduction current was monitored (Fig. 1.C).

PLEASE INSERT FIGURE 1

2.5 Experimental design

Before performing the experimental design, the amount of immobilized toxoid was selected preliminary. This allowed simplifying the subsequent experimental plan. The amount of toxoid immobilized onto the SPE was varied between 1 and 200 µg/mL. After modification of the SPE with the tested concentration, the immunoassay was realized with an anti-*tetani* solution of 0.2 IU/mL (other experimental conditions: BSA 1% (m/v); anti-*tetani* incubation time: 20 min; HRP-anti-IgG dilution 100 time and HRP-anti IgG incubation time: 30 min). A current of 3 µA was recorded for the immunoassay realized with a SPE modified by a toxoid solution of 150 and 200 µg/mL. No response was observed for toxoid solutions of 1 and 10 µg/mL. Finally, a concentration of 150 µg/mL was selected for all experiments.

The response surface methodology allowed exploring the relation between variables (factors) and response. The circumscribed central composite design (CCCD) was chosen to optimize the immunoassay response.

2.5.1 Selection of factors and their levels

Preliminary experiments (data not shown) led to select four factors that influence the response of the immunoassay: (1) BSA concentration, (2) anti-*tetani* incubation time, (3) HRP-anti IgG incubation time, and (4) HRP-anti IgG dilution factor.

Preliminary experiments also helped to determine the range to investigate. The BSA concentration should be sufficient to remove non-specific reactions, but should not completely block the SPE. For the incubation times, a compromise was made between a

relatively quick test and the time needed to allow the reaction. The HRP-anti IgG dilution factor should be at least 10 in order to reduce the cost of the test but it should still allow a significant response to be observed. The BSA concentration was varied between 0.5 and 2.5% (m/v), the two incubation times between 5 and 35 min, and the HRP-anti IgG dilution factor between 10 and 150 (Table 1).

PLEASE INSERT TABLE 1

2.5.2 Selection of the experimental response surface design and the response

The circumscribed central composite design (CCCD) was selected as response surface design. Since four factors were studied, a minimum of 25 experiments (2^4 + 2x4 + min 1 (center point)) had to be conducted. The experimental design was performed during seven days, and the center point was repeated once daily (7 times) in order to evaluate the variability of the model. Five test points have also been added to validate the constructed model. Therefore, a total of N=36 experiments were performed. The CCCD with coded factor levels is included in Table S1. The response selected, i.e. the amperometric signal, was qualitative.

2.5.3 Experimental protocol

In order to obtain the experimental protocol (Table 2), the coded factor levels of the CCCD (Table S1) should be replaced by the real factor levels (Table 1). Furthermore, the experiments were performed in a randomized order to exclude the effect of time on the response.

2.5.4 Model construction and determination of the optimum

After performing the experiments and determining the responses, the polynomial model describing the relationship between the response and the factors considered was built (Bieke Dejaegher and Vander Heyden, 2009). Most often, a polynomial second order model is built including the intercept (β_0), the main factor coefficients (β_i), the two-factor interaction

coefficients (β_{ij}), and the quadratic coefficients (β_{ii}). The general model for f factors can be written as follows:

$$\boldsymbol{y = \beta_0 + \sum_{i=1}^{f} \beta_i x_i + \sum_{i \leq 1 \leq j}^{f} \beta_{ij} x_i x_j + \sum_{i=1}^{f} \beta_{ii} x_i^2}$$

(Equation 1)

The model between the four examined factors (X_1, X_2, X_3, and X_4) and the considered response Y was calculated as in Equation 2, using least squares regression.

$$Y = b_0 + b_1 . X_1 + b_2 . X_2 + b_3 . X_3 + b_4.X_4 + b_{1-1} . (X_1.X_1) + b_{2-2} . (X_2.X_2) + b_{3-3} . (X_3.X_3) + b_{4-4}. (X_4.X_4) + b_{1-2} . (X_1.X_2) + b_{1-3} . (X_1.X_3) + b_{2-3} . (X_2.X_3) + b_{1-4} . (X_1.X_4) + b_{2-4} . (X_2.X_4) + b_{3-4} . (X_3.X_4)$$

(Equation 2)

3. Results and discussion

3.1 Amperometric signal

The recorded signal was the amperometric response measured at -280mV. The signal was related to the amount of HRP present onto the SPE. Indeed, the substrate HQ is enzymatically oxidized in the presence of hydrogen peroxide to quinone which in turn is reduced, at the SPE, back to its original form.

3.2 Model building

In Table 2, the experimental protocol of the CCCD, including the experiments order, the levels of the four examined factors, and the particularity of each experiment, is presented. The obtained responses for each experiment are shown.

As can be seen in Table 2, four experiments did not give any response (9, 10, 19, and 21). The labeled antibody dilution factor of 115 was probably responsible for the absence of a signal

for experiments 9 and 10. One possible reason for no response in experiments 19 and 21 could be a mistake during the modification steps of the SPE.

PLEASE INSERT TABLE 2

Before calculating the model, the experiments without response (9, 10, 19, and 21) and the test points (32-36) were removed. Then a second-order polynomial model was built, using the remaining experiments and including an intercept (b_0), the main factor coefficients (b_i), the two-factor interaction coefficients (b_{ij}), and the quadratic coefficients (b_{ii}).:

$$Y = 2.374 - 0.077X_1 + 1.065X_2 + 0.599X_3 - 1.494X_4 - 0.045(X_1.X_1) - 0.074(X_2.X_2) - 0.080(X_3.X_3) + 0.593(X_4.X_4) - 0.592(X_1.X_2) + 0.008(X_1.X_3) + 0.531(X_2.X_3) + 0.076(X_1.X_4) - 0.541(X_2.X_4) + 0.626(X_3.X_4)$$

(Equation 3)

3.3 Model evaluation

In order to evaluate the model, a residual analysis was performed in which the experimental and predicted responses for each experiment were compared. A residual is the difference between the experimental response (Yexp) and the response calculated by the model (Ycal). For the majority of data points, this difference was small, so the model was found to be reliable. Furthermore, the residues of the 5 test points were of the same order of magnitude as those for the other experiments, confirming the adequacy of the model. The 2 experiments with the highest residues were 2 of the 4 experiments that gave no response (Table S2).

3.4 Graphical evaluation and determination of the optimum

A study of the optimal path was performed to determine the conditions giving the best results, i.e. the highest response (Fig. 2). This type of graph combines information concerning the four factors. The x-axis corresponded to the distance between the experimental points and the zero level. As for 4 factors, $|\alpha| = 2$, the experimental range was from -2 to 2, the maximum distance between a point and zero was |2|. The optimal conditions, expressed in coded factor

levels, were found by projecting each factor at response maximization on the ordinate axis, and then the coded level was converted to its real value: (i) factor 1 (concentration BSA) coded level -0.25 corresponded to a real level of 1.375 % (m/v); (ii) factor 2 (anti-*tetani* incubation time) coded level +0.875 corresponded to a real level of 26 min 34 s; (iii) factor 3 (HRP-anti-IgG incubation time) coded level 0 corresponded to a real level of 20 min; (iv) factor 4 (HRP-anti-IgG dilution) coded level -1.75 corresponded to a real level of a dilution factor of 18.75.

PLEASE INSERT FIGURE 2

To determine the expected response at the optimum conditions, a second graph from the study of optimal path was performed (Fig. not shown). This graph shows the response Y calculated from the polynomial defined in Equation 3, depending on the distance between each of the four factors and the zero level. Maximizing the response is shown on the right of the graph. The ordinate of the highest point corresponding to the optimal conditions, gave the maximum expected response, i.e. approximately 8.50.

Graphically, the model can be visualized by drawing 2D contour plots or 3D response surface plots (Dejaegher and Vander Heyden, 2011). Only two factors can be shown in such plots, while the other two should be fixed to, for example, their optimum.

The two factors that have the greatest impact on the response were factors 2 and 4, i.e. the anti-*tetani* incubation time and HRP-anti-IgG dilution. To maximize the response, factor 4 (HRP-anti-IgG dilution) should be small, while factor 2 (anti-*tetani* incubation time) should be high (Fig. 3). From a response surface plot for factors 1 and 3 (not shown), it could be seen that they have very limited influence, as the response was substantially similar whatever the level of these factors. This information was already visible on the graph of the optimal path study, since the curves showing the effect of the BSA concentration and HRP-anti-IgG incubating times were very close to the x-axis. If a screening phase, during which all factors potentially influencing the method are screened in a given range (Dejaegher et al., 2010), had been performed prior to the optimization design, these two factors would probably not have been considered as significant and only the most important factors (the anti-*tetani* incubation time and HRP-anti-IgG dilution) would probably have been identified.

PLEASE INSERT FIGURE 3

3.5 Optimum verification

In order to verify the predicted optimum, the response should be determined at the defined optimal conditions. During 6 days, two tests have been repeated daily at the optimal conditions. To make sure to have at least two tests per day, 5 SPE were modified every day (one for the blank assays and 4 for the assays). The two reported results are those from the two first successful experiments. The predicted response at the defined optimum conditions was 8.5 µA.

The repeatability (s_r^2), the between-days variance ($s^2_{between-days}$), and time-different intermediate precision ($s^2_{I(t)}$) of the obtained experimental responses at the defined optimal conditions can be derived from an analysis of variance (ANOVA) (Table 3 and Equation 4).

PLEASE INSERT TABLE 3

$$s^2_{I(t)} = s^2_r + s^2_{between-days}$$

(Equation 4)

The repeatability and the time-different intermediate precision were investigated and expressed as variance (s^2), standard deviation (s), and percentage relative standard deviation (%RSD). Also the between-days variance ($s^2_{between-days}$) was determined. The repeatability s_r^2 was 0.45, the between-days variance $s^2_{between-days}$ was 0.28, and time-different intermediate precision $s^2_{I(t)}$ was 0.74. The repeatability and the time-different intermediate precision %RSD are similar (respectively 10.7 and 13.6), meaning that the time had little effect on the response.

3.6 Analytical performance

With the above-optimized conditions, the response was directly proportional to the logarithm of the anti-*tetani* concentration between 0.01 IU/mL and 0.25 IU/mL (r^2= 0.82), with a limit of detection (LOD) of 0.011 IU/mL and a limit of quantification (LOQ) of 0.012 IU/mL. The LOD and the LOQ were calculated from the signal given by the blank (the signal obtained without tetanus antibodies). The mean and the standard deviation of the blank were calculated from 3 assays (0.07 ± 0.02 µA). Then the LOD and the LOQ were estimated from the mean of the blank plus respectively three and ten times the standard deviation of the blank (Shrivastava and Gupta, 2011).

These values were lower than the protective human antibody limit of 0.06 IU/mL, established by an ELISA test (Galazka, 1993). The immunoassay using magnetic microbeads, however, was more sensitive since the LOD and LOQ were 0.0046 IU/mL and 0.0057 IU/mL, respectively (Patris et al., 2010). The LOQ of an ELISA method was 0,004 IU/mL (Genway, 2013). However, the calculation method was different, therefore both LOQ's cannot be compared.

4. Conclusions

This work demonstrated that it is possible to determine the anti-*tetani* level with tetanus toxoid immobilized directly onto SPE. The immunoassay was performed onto the SPE and the amperometric detection was not affected by the presence of the immobilized biocomponents. The immunoassay took less than 50 min and was easier to be implemented compared to the immunoassay using magnetic microbeads. It could be considered for point of care applications.

The use of a response surface experimental design, i.e. a circumscribed central composite design, allowed the optimization of four parameters of the immunoassay response with a limited number of experiments.

It was demonstrated that the anti-*tetani* incubation time and the dilution factor of the labeled antibody had the greatest impact on the response. On the contrary, the concentration of BSA used as blocking agent, as well as the incubation time of the labeled antibody had a limited influence on the amperometric response.

References

Alonso-Lomillo, M.A., Domínguez-Renedo, O., Matos, P., Arcos-Martínez, M.J., 2010. Disposable biosensors for determination of biogenic amines. Anal. Chim. Acta 665, 26–31.

Alonso-Lomillo, M.A., Yardimci, C., Domínguez-Renedo, O., Arcos-Martínez, M.J., 2009. CYP450 2B4 covalently attached to carbon and gold screen printed electrodes by diazonium salt and thiols monolayers. Anal. Chim. Acta 633, 51–6.

Ardelean-Jaby, D., Kaddari-Himeur, F., Nkana-Tameze, K., Paulin, C., Sancho, J., Cailliez, M., 2002. Évaluation du test sanguin « Tetanos Quick Stick » (TQS) en situation d'urgence. Immuno-analyse Biol. Spécialisée 17, 330–335.

Asadollahzadeh, M., Tavakoli, H., Torab-Mostaedi, M., Hosseini, G., Hemmati, A., 2014. Response surface methodology based on central composite design as a chemometric tool for optimization of dispersive-solidification liquid-liquid microextraction for speciation of inorganic arsenic in environmental water samples. Talanta 123, 25–31.

Balkenhohl, T., Lisdat, F., 2007. Screen-printed electrodes as impedimetric immunosensors for the detection of anti-transglutaminase antibodies in human sera. Anal. Chim. Acta 597, 50–57.

Brostoff, J., Scadding, G.K., Male, M., Roitt, I.M., 1993. Immunologie Clinique, De Boeck U. ed. De Boeck Université, Bruxelles.

Casey, W., Schmitt, M., McFarland, R., Isbrucker, R., Levis, R., Arciniega, J., Descamps, J., Finn, T., Hendriksen, C., Horiuchi, Y., Keller, J., Kojima, H., Sesardic, D., Stickings, P., Johnson, N.W., Lipscomb, E., Allen, D., 2011. Improving animal welfare and reducing animal use for human vaccine potency testing: state of the science and future directions. Procedia Vaccinol. 5, 33–46.

Collins, S., White, J., Ramsay, M., Amirthalingam, G., 2015. The importance of tetanus risk assessment during wound management. IDCases 2, 3–5.

Dejaegher, B., Durand, A., Vander Heyden, Y., Hanrahan, G., Gomez, F.A., 2010. Experimental design in method optimization an robustness testing. John Wiley & Sons, Inc., New Jersey.

Dejaegher, B., Mangelings, D., Vander Heyden, Y., 2013. Experimental design methodologies in the optimization of chiral CE or CEC separations: an overview. Methods Mol. Biol. 970, 409–27.

Dejaegher, B., Vander Heyden, Y., 2009. The use of experimental design in separation science. Acta Chromatogr. 21, 161–201.

Dejaegher, B., Vander Heyden, Y., 2009. The use of experimental design in separation science. Acta Chromatogr. 21, 161–201.

Dejaegher, B., Vander Heyden, Y., 2011. Experimental designs and their recent advances in set-up, data interpretation, and analytical applications. J. Pharm. Biomed. Anal. 56, 141–58.

Díaz-González, M., Hernández-Santos, D., González-García, M.B., Costa-García, A., 2005. Development of an immunosensor for the determination of rabbit IgG using streptavidin modified screen-printed carbon electrodes. Talanta 65, 565–573.

European Directorate for the Quality of Medicines, 2014. European Pharmacopoeia. Strasbourg.

Facktor, M.A., Bernstein, R.A., Fireman, P., 1973. Hypersensitivity to tetanus toxoid. J. Allergy Clin. Immunol. 52, 1–12.

Fontaine, C., Mayorga, C., Bousquet, P.J., Arnoux, B., Torres, M.-J., Blanca, M., Demoly, P., 2007. Relevance of the determination of serum-specific IgE antibodies in the diagnosis of immediate beta-lactam allergy. Allergy 62, 47–52.

Galazka, A.M., 1993. Immunological basis for immunization module 3: Tetanus (WHO/EPI/GEN/93.4). Genève.

Genway, 2013. Tetanus Toxoid IgG ELISA Instruction Manual.

Ghoreishi, S.M., Behpour, M., Khoobi, A., 2012. Central composite rotatable design in the development of a new method for optimization, voltammetric determination and electrochemical behavior of betaxolol in the presence of acetaminophen based on a gold nanoparticle modified electrode. Anal. Methods 4, 2475–2485.

Giannetto, M., Mattarozzi, M., Umiltà, E., Manfredi, A., Quaglia, S., Careri, M., 2014. An amperometric immunosensor for diagnosis of celiac disease based on covalent immobilization of open conformation tissue transglutaminase for determination of anti-tTG antibodies in human serum. Biosens. Bioelectron. 62, 325–30.

Gross, S., Volkers, P., Eckert-Ziem, M., Kuschel, S., Schäffner, G., 2006. Validation of in vitro potency assays for tetanus immunoglobulin. Pharmeuropa Bio 2006, 1–6.

Hainz, U., Jenewein, B., Asch, E., Pfeiffer, K.-P., Berger, P., Grubeck-Loebenstein, B., 2005. Insufficient protection for healthy elderly adults by tetanus and TBE vaccines. Vaccine 23, 3232–5.

Kreuzer, M.., O'Sullivan, C.., Pravda, M., Guilbault, G.., 2001. Development of an immunosensor for the determination of allergy antibody (IgE) in blood samples. Anal. Chim. Acta 442, 45–53.

Maral, I., Cirak, M., Aksakal, F.N., Baykan, Z., Kayikcioglu, F., Bumin, M. a, 2001. Tetanus immunization in pregnant women. Serum levels of antitetanus antibodies at time of delivery. Eur. J. Epidemiol. 17, 661–665.

Masoum, S., Behpour, M., Azimi, F., Motaghedifard, M.H., 2014. Potentiality of chemometric approaches for the determination of (+)-catechin in green tea leaves at the

surface of multiwalled carbon nanotube paste electrode. Sensors Actuators B Chem. 193, 582–591.

Mayorga, C., Torres, M.J., Corzo, J.L., Alvarez, J., CornejoGarcía, J.A., Rodríguez, C.A., Blanca, M., Jurado, A., 2003. Immediate allergy to tetanus toxoid vaccine: determination of immunoglobulin E and immunoglobulin G antibodies to allergenic proteins. Ann. Allergy, Asthma Immunol. 90, 238–243.

Messina, G.A., De Vito, I.E., Raba, J., 2007. Screen-printed immunosensor for quantification of human serum IgG antibodies to Helicobacter pylori. Sensors Actuators B Chem. 128, 23–30.

Million, A., 1997. Diagnosis, treatment, and prevention of tetanus. Prim. Care Update Ob. Gyns. 4, 75–79.

Miyagi, K., Shah, A.K., 2011. Tetanus prophylaxis in the management of patients with acute wounds. J. Plast. Reconstr. Aesthet. Surg. 64, e267–9.

Moylett, E.H., Hanson, I.C., 2004. Mechanistic actions of the risks and adverse events associated with vaccine administration. J. Allergy Clin. Immunol. 114, 1010–1020.

Neves, M.M.P.S., González-García, M.B., Nouws, H.P.A., Costa-García, A., 2012. Celiac disease detection using a transglutaminase electrochemical immunosensor fabricated on nanohybrid screen-printed carbon electrodes. Biosens. Bioelectron. 31, 95–100.

Niklasson, B., Tkachenko, E., Ivanov, A.P., van der Groen, G., Wiger, D., Andersen, H.K., LeDuc, J., Kjelsson, T., Nyström, K.,. Haemorrhagic fever with renal syndrome: evaluation of ELISA for detection of Puumala-virus-specific IgG and IgM. Res. Virol. 141, 637–648.

Olander, R.-M., Auranen, K., Härkänen, T., Leino, T., 2009. High tetanus and diphtheria antitoxin concentrations in Finnish adults-time for new booster recommendations? Vaccine 27, 5295–5298.

Orimadegun, A.E., Orimadegun, B.E., Adepoju, A.A., 2013. Immunity Against Tetanus Infection, Risk Factors for Non-Protection, and Validation of a Rapid Immunotest Kit among Hospitalized Children in Nigeria. Front. Neurol. 4, 142–149.

Patris, S., De Vriese, C., Prohoroff, F., Calvo, E.B., Martínez, J.A., Kauffmann, J.-M., 2010. Anti-Clostridium tetani Antibody Determination in Serum Samples by Amperometric Immunosensing. Electroanalysis 22, 41–48.

Pitzurra, L., Bistoni, F., Pitzurra, M., Bastianini, L., Perito, S., Vecchiarelli, A., Marconi, P., 1983. Comparison of passive hemagglutination with Turkey erythrocyte assay, enzyme-linked immunosorbent assay, and counterimmunoelectrophoresis assay for serological evaluation of tetanus immunity. J. Clin. Microbiol. 17, 432–435.

Pollard, J.D., Selby, G., 1978. Relapsing neuropathy due to tetanus toxoid. J. Neurol. Sci. 37, 113–125.

Ponvert, C., 2006. Les réactions allergiques et pseudo-allergiques aux vaccins. Immuno-analyse Biol. Spécialisée 21, 99–104.

Popoff, M.R., Poulain, B., 2005. Tétanos : physiopathologie, épidémiologie, formes cliniques, traitements et vaccination. Antibiotiques 7, 23–41.

Reinstein, L., Pargament, J.M., Goodman, J.S., 1982. Peripheral neuropathy after multiple tetanus toxoid injections. Arch. Phys. Med. Rehabil. 63, 332–334.

Shrivastava, A., Gupta, V., 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles Young Sci. 2, 21.

Székely, G., Henriques, B., Gil, M., Alvarez, C., 2014. Experimental design for the optimization and robustness testing of a liquid chromatography tandem mass spectrometry method for the trace analysis of the potentially genotoxic 1,3-diisopropylurea. Drug Test. Anal. 6, 898–908.

Thévenot, D.R., Toth, K., Durst, R.A., Wilson, G.S., 2001. Electrochemical biosensors: recommended definitions and classification. Biosens. Bioelectron. 16, 121–131.

WHO, 2006. Tetanus vaccine. Wkly. Epidemiol. Rec. 20, 198–208.

Zhou, Y.-M., Hu, S.-Q., Shen, G.-L., Yu, R.-Q., 2003. An amperometric immunosensor based on an electrochemically pretreated carbon-paraffin electrode for complement III (C3) assay. Biosens. Bioelectron. 18, 473–481.

FIGURES CAPTIONS

Fig. 1: Representation of the developed amperometric immunoassay. A: SPE modification procedure. B: Immunoassay. C: Amperometric detection

Fig. 2: Study of optimal path: (1) BSA: optimal coded level -0.25; (2) anti-*tetani* incubation: optimal coded level 0.875; (3) HRP-anti-IgG incubation: optimal coded level 0; (4) HRP-anti-IgG dilution: optimal coded level -1.75

Fig. 3: 2D contour plots of factor 2 and factor 4 (anti-*tetani* incubation time and HRP-anti-IgG dilution). The factors 1 and 3 are fixed at their optima: factor 1: -0.25 (BSA concentration: 1.375% (m/v)), factor 3: 0 (HRP-anti-IgG incubation time: 20 min).

FIGURES

Fig. 1

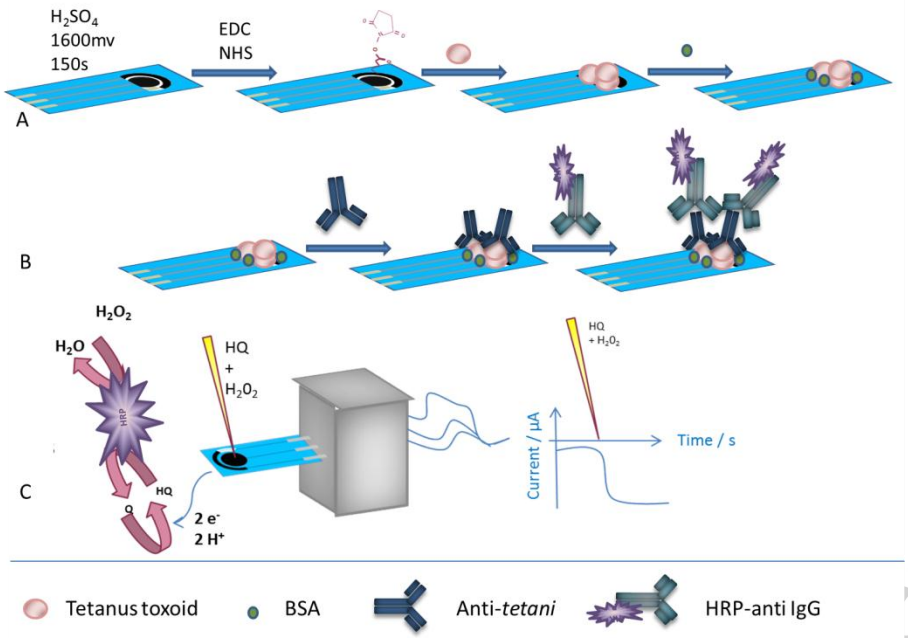


Fig. 2

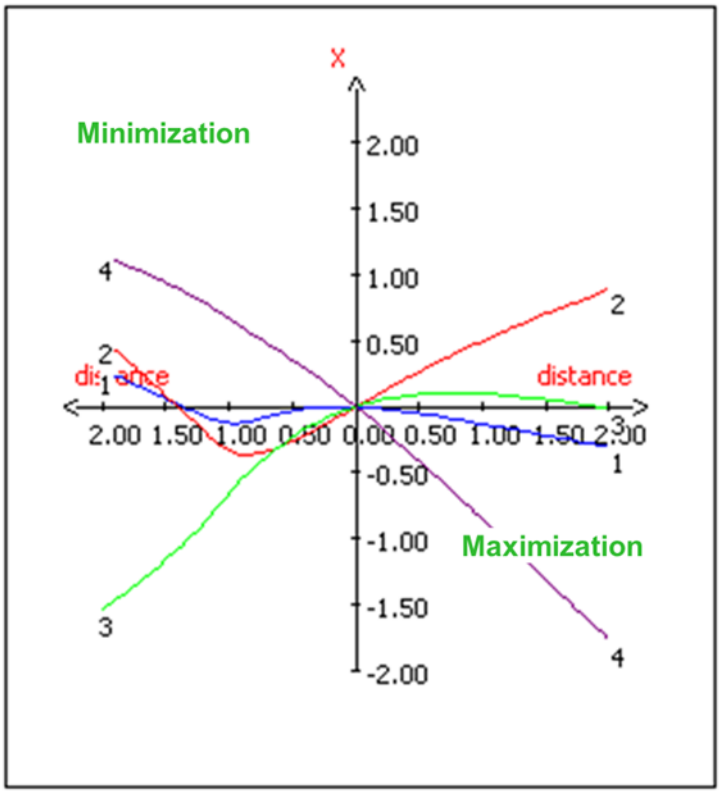
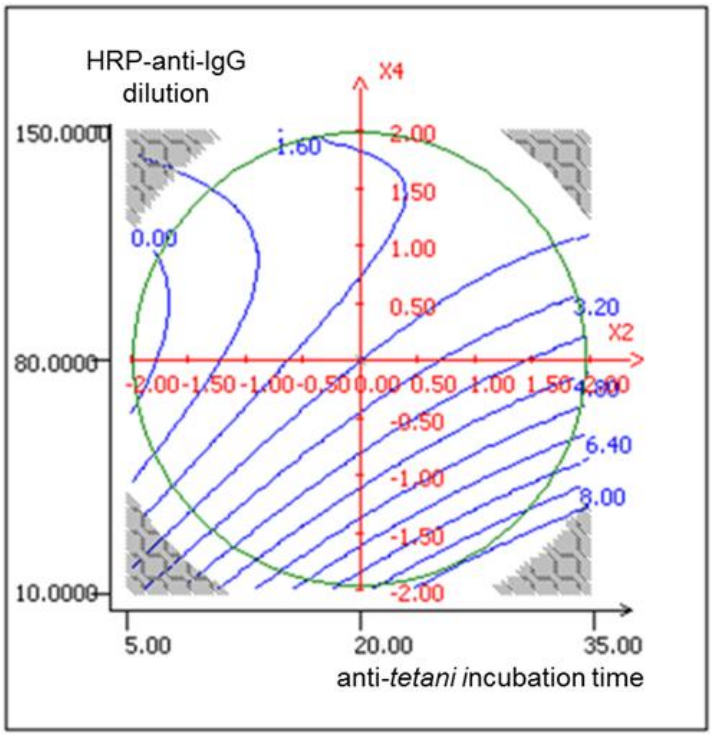


Fig. 3



TABLES CAPTIONS

Table 1: Selected factors and levels for the optimization

Table 2: CCCD with the order of experiments, the real factor levels, the experimental results, and the particularity of each response. The response was the amperometric signal (µA) to an anti-*tetani* concentration of 0.2 IU/mL.

Table 3: ANOVA n=2, p=6

Table S1: Matrix of experiments with coded levels of the factors (X1: BSA concentration; X2: anti-*tetani* incubation time; X3: HRP-anti-IgG incubation time; X4: HRP-anti-IgG dilution)

Table S2: Residual analysis, with Yexp: experimental response and Ycal: response calculated by the model

TABLES

Table 1

Factor	**X**	**Unit**	**- α (-2)**	**- 1**	**0 (center)**	**1**	**+ α (+2)**
BSA concentration	X_1	% (m/v)	0.5	1	1.5	2	2.5
Anti-*tetani* incubation time	X_2	min	5	12.5	20	27.5	35
HRP-anti-IgG incubation time	X_3	min	5	12 .5	20	27.5	35
HRP-anti-IgG dilution factor	X_4	/	10	45	80	115	150

Table 2

N° Exp	Order	X_1	X_2	X_3	X_4	Amperometric signal (µA)	Particularity
1	14	1	12.5	12.5	45	2.593	experimental point
2	23	2	12.5	12.5	45	4.154	experimental point
3	20	1	27.5	12.5	45	6.443	experimental point
4	19	2	27.5	12.5	45	3.528	experimental point
5	2	1	12.5	27.5	45	0.585	experimental point
6	10	2	12.5	27.5	45	1.962	experimental point
7	11	1	27.5	27.5	45	6.918	experimental point
8	26	2	27.5	27.5	45	5.492	experimental point
9	29	1	12.5	12.5	115	/	experimental point
10	32	2	12.5	12.5	115	/	experimental point
11	7	1	27.5	12.5	115	0.408	experimental point
12	3	2	27.5	12.5	115	0.918	experimental point
13	27	1	12.5	27.5	115	1.078	experimental point
14	8	2	12.5	27.5	115	2.108	experimental point
15	9	1	27.5	27.5	115	3.916	experimental point
16	1	2	27.5	27.5	115	2.129	experimental point
17	22	0.5	20	20	80	2.305	experimental point
18	16	2.5	20	20	80	2.333	experimental point
19	35	1.5	5	20	80	/	experimental point
20	18	1.5	35	20	80	4.633	experimental point
21	36	1.5	20	5	80	/	experimental point
22	15	1.5	20	35	80	4.81	experimental point
23	5	1.5	20	20	10	8.571	experimental point
24	25	1.5	20	20	150	1.598	experimental point
25	17	1.5	20	20	80	0.637	center point

26	31	1.5	20	20	80	3.519	center point
27	33	1.5	20	20	80	4.005	center point
28	34	1.5	20	20	80	1.751	center point
29	28	1.5	20	20	80	2.061	center point
30	4	1.5	20	20	80	3.383	center point
31	6	1.5	20	20	80	1.404	center point
32	24	1.1	16.577	17.58	71.25	2.991	test point
33	13	1.9	16.577	17.58	71.25	2.94	test point
34	21	1.5	26.8468	17.58	71.25	3.281	test point
35	30	1.5	20	27.26	71.25	2.171	test point
36	12	1.5	20	20	115	0.794	test point

Table 3

Source of variations	Sum of squares (SS)	Degrees of freedom (df)	Mean Squares (MS)	Expected Mean Squares (EMS)
Inter-day	5.13	5	1.08	$\sigma_r^2 + n\sigma_{r\ \text{between-days}}^2$
Intra-day	2.75	6	0.75	σ_r^2
Total	7.88	11		