



Biotin determination in food supplements by an electrochemical magneto biosensor

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ABSTRACT

An electrochemical magneto biosensor for the rapid determination of biotin in food samples is reported. The affinity reaction was performed on streptavidin-modified magnetic microbeads as a solid support in a direct competitive format. The biotinylated horseradish peroxidase enzyme (biotin-HRP) competes with free biotin in the sample for the binding sites of streptavidin on the magnetic microbeads. The modified magnetic beads were then easily captured by a magneto graphite-epoxy composite electrode and the electrochemical signal was based on the enzymatic activity of the HRP enzyme under the addition of H_2O_2 as the substrate and o-phenylenediamine as cosubstrate. The response was electrochemically detected by square wave voltammetry. The limit of detection was $8.4 \times 10^{-8} \text{ mol L}^{-1}$ of biotin ($20 \mu\text{g L}^{-1}$) with a dynamic range from 0.94 to $2.4 \times 10^{-7} \text{ mol L}^{-1}$. Biotin-fortified commercial dietary supplement and infant formula samples were evaluated obtaining good performances in the results. Total time of analysis was 40 min per 20 assays.

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1. Introduction

Biotin (also called vitamin B8, B7 or H) is a water-soluble vitamin and an essential co-factor for five biotin-dependent carboxylase enzymes. It is synthesized in a wide variety of bacteria and plants. However, several microorganisms as well as higher animals are not able to synthesize it and their needs in this vitamin are met by dietary intake [1]. Besides the typical clinical features, recent evidence indicates that the pregnant women develop biotin deficiency during normal pregnancy [2,3].

Although a maximum level of biotin in food is not specified, $1.5 \mu\text{g}$ in 100 kcal is recommended by the current regulation in infant formula in substitution of breast-milk to satisfy the nutritional requirements of infants during the first months of life [4].

Consequently, the diagnosis of the biotin deficiency and the monitoring of its levels in patients receiving biotin treatment are important issues, as well as the determination in foods and food supplement products. For this reason, analytical methods were developed, in order to determine biotin in biological fluids, as well as in food products and pharmaceutical preparations. The analytical techniques for the determination of biotin can be classified in microbiological methods, bioassays, instrumental methods and binding assays [1,5]. The microbiological method is the most

common, based on the growth of microorganisms upon the presence of biotin in the culture media, since this vitamin is an essential nutrient for the microorganism under cultivation [6]. This assay is sensitive but holds low specificity. Moreover, it is tedious and time-consuming [7]. The biotin bioassay in animals, whose development was inhibited by artificial biotin deprivation, consists in fed them with known amounts of biotin. The weight gain is related with the logarithm of biotin amount [8]. This method is able to estimate the bioavailable biotin present in food samples [9]. The main instrumental methods include spectroscopy, colorimetry, chromatography and electrophoresis. These methods show high sensitivity, being simpler and faster than the microbiological method. The main advantage is the discrimination between biotin and biotin metabolites [10,11]. However, expensive and sophisticated equipment is needed which must be run by experienced personnel. The binding assays are based on the extremely specific and high affinity interaction between biotin and the glycoprotein avidin or its gly (association constant, $K_a = 10^{15} \text{ M}^{-1}$) leads to strong associations similar to the formation of a covalent bonding. This interaction is highly resistant to a wide range of chemical (detergents, protein denaturants), pH range variations and high temperatures [12,13]. These assays show higher precision and enhanced detection limits, in addition to their simplicity and ability to analyze a greater number of samples in a shorter period of time. Systems that distinguish between biotin and biotin metabolites based on bioaffinity reaction coupled to physicochemical methods such as colorimetry

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[14], fluorescence [15], chromatography [11,16], chromatography with electrochemical detection [17], quartz crystal microbalance [18] and electrochemical [19–24] were also developed. Finally, sophisticated analytical systems based on the reaction between biotin and anti-biotin antibody were recently developed [25–27].

Electrochemical sensors [28] and biosensors [29–31] have revolutionized the modern analysis because the simplicity and speed in the response by the direct transduction to electronic equipment [32]. In their development, the magneto graphite-epoxy composite electrodes (m-GEC) are widely employed as transducers [33–35]. They are cheap, easy to prepare, use, renew and modify. Moreover, these transducers are supports where the modified magnetic beads can be immobilized. The inclusion of these particles has improved the limits of detection, the signal-to-noise ratio, sensitivity and analysis time [36,37].

In this paper, we describe an electrochemical magneto biosensor for biotin detection in biotin fortified dietary supplement and infant formula commercial samples. The present strategy is based on a competition scheme where biotin and the biotin-HRP compete for the binding sites of streptavidin, which is immobilized on the magnetic beads surface. After molecular recognition, the beads are captured by a magneto electrode and put in the reaction cell along with the enzymatic substrates. Then, enzymatic products reduction currents are obtained, which are inversely related to biotin concentrations. The electrochemical response is measured using square wave voltammetry (SWV). The magnetic beads can easily be manipulated through the use of permanent magnets, and the matrix effect is also minimized due to improved washing and separation steps which allow the analysis to be made with a minimal pre-treatment step, without any pre-enrichment or purification step.

2. Materials and methods

2.1. Instrumentation

Cyclic voltammetry, square wave voltammetry and chronoamperometry were performed with a voltammetric analyzer Epsilon BAS, Bioanalytical Systems Inc (West Lafayette Indiana—USA) with a three electrode system based on magneto graphite-epoxy composite (m-GEC) as working electrodes [36–38]; platinum as auxiliary electrode and Ag/AgCl as reference electrode (Orion 92-02-00). The effective areas of electrodes were 0.43 cm² (RSD%=13%, $n=20$) by cyclic voltammetry and chronoamperometry with potassium ferricyanide.

2.2. Statistical analysis

Analysis of variance (ANOVA) was used to compare between the different samples and/or pretreatments. Parametric tests were preceded by tests for normal distribution per sample, and for homogeneity of variance between samples. A significance level of $P < 0.05$ was applied in all statistical tests.

2.3. Reagents and solutions

Magnetic beads of 1 μm diameter modified with streptavidin (Dynabeads M-280 Streptavidin, Cat. no. 602.10) were purchased from Invitrogen (Oslo, Norway). Biotin (Cat. no. B4501) and biotin-HRP (peroxidase-biotinamidocaproyl conjugate lyophilized powder, Cat. no. P9568) were obtained from Sigma. The streptavidin-activated magnetic beads, biotin and biotin-HRP stock solutions were prepared in citrate buffer (citrate 0.075 mol L⁻¹ and NaCl 0.75 mol L⁻¹, pH=7.0). Biotin stock was initially solubilized in NaOH 1 mol L⁻¹. All other reagents were of the highest available

grade, were supplied from Sigma or Merck. The o-phenilendiamine solution was prepared in ethanol:Milli-Q water (50:50). Working buffer solutions were phosphate 0.1 mol L⁻¹ and KCl 0.1 mol L⁻¹ at pH 6.0 and acetate/phosphate/borate 0.05 mol L⁻¹ and KCl 0.1 mol L⁻¹ (for buffering from pH 4.0 to 9.0).

2.4. Electrochemical magneto biosensor for biotin

The competitive assay for the biotin detection was performed according to Strategy 2 (see Fig. 1). The assay was performed in 2 mL Eppendorf tubes by adding small volumes of biotin standard or extracted samples to 0.35 mg mL⁻¹ of streptavidin-MB for achieving a final volume of 250 μL with citrate buffer. Then, an incubation stage was performed. After 3.5×10^{-8} mol L⁻¹ of biotin-HRP was added and incubated. Posteriori the magnetic separation and washing were done. The incubation stages and washing were carried out in shaking conditions at room temperature during 15 and 5 min, respectively. The magnetic separation was performed by m-GEC until the magnetic beads migrated to the electrode. The electrode was put in reaction cell along with the enzymatic cosubstrates and the electrochemical response was obtained by SWV in the range from 0 to -400 mV at 60 s. The reduction current intensity peak reported as current density ($\mu\text{A cm}^{-2}$) was sampled at -170 mV (RSD%=11%) vs. the Ag/AgCl reference electrode.

2.5. Calibration curve for biotin

The biotin concentration was analyzed by electrochemical magneto biosensor in the range from 1.4×10^{-8} to 3.57×10^{-7} mol L⁻¹. The biotin standard curve was plotted with the current densities ($\mu\text{A cm}^{-2}$) vs. the biotin concentration (mol L⁻¹). The four-parameter logistic equation was fitted according to Eq. (1):

$$y = (A - B) / [1 + 10 \exp((\log C - \log X) * D)] + B \quad (1)$$

where A and B are the maximal and minimal electrochemical response, respectively, C is the concentration producing 50% of the maximal response, X is the biotin concentration and D is the slope at the inflection point of the sigmoid curve.

The limit of detection (LOD) and quantification (LOQ) were calculated as the 90% and 80% of electrochemical signal of the standard curve, respectively. The repeatability and accuracy tests

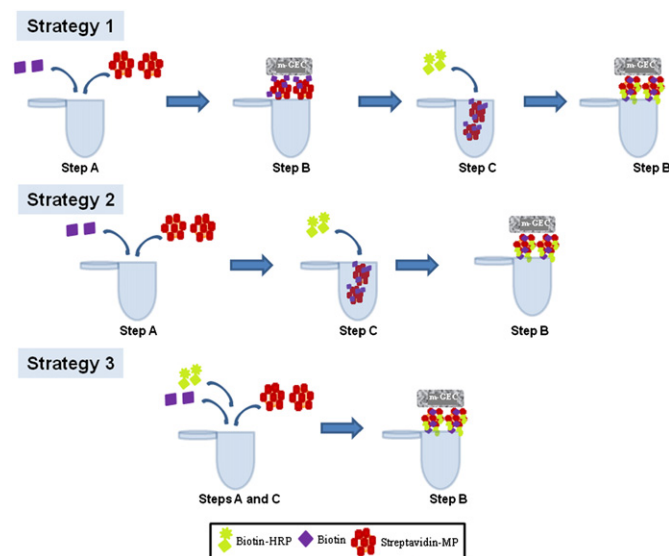


Fig. 1. Schematic representation of three competitive procedures such as Strategy 1, 2 and 3 for the determination of biotin. (A) incubation step of biotin with streptavidin-MB, (B) separation by m-GEC and washing steps, (C) biotin-HRP addition and incubation steps.

were determined to biotin standard concentration of $1.8 \times 10^{-7} \text{ mol L}^{-1}$ ($n=4$).

2.6. Electrochemical magneto biosensor for biotin in food samples

The biotin quantifications were performed in the biotin-fortified dietary supplement and infant formula commercial samples, with biotin amount labels of 150 and 12.1 μg biotin in 100 g, respectively. They were obtained from local markets. The pretreatment of the samples were carried out according to an optimized procedure reported by Nelson et al. [39], where 2 g of sample were extracted with 5 mL of 1.5% formic acid solution during 5 min in agitation. Then, all samples were centrifuged for 15 min at 2000 rpm. 15 and 100 μL of supernatant of biotin-fortified dietary supplement and infant formula samples, respectively were analyzed by electrochemical magneto biosensor. The biotin concentration was ascertained from calibration curve and reported as μg biotin in 100 g sample.

3. Results and discussion

3.1. Electrochemical detection

The electrochemical detection is based in the HRP reaction with two substrates – being *o*-phenylenediamine and H_2O_2 used in this paper – by a ping-pong mechanism [36,40–42]. In the first stage, a peroxide molecule is bound to a free coordination site of iron (FeIII) in the HRP and is reduced to water in a rapid two-electron process, whereby the compound I is generated as the stable primary intermediary. The compound I is the oxyferryl species ($(\text{FeIV}=\text{O})\text{P}^*$) and is constituted by one oxygen atom from a molecule of peroxide, one electron comes from iron and other electron is withdrawn from the heme group to generate a porphyrin π cation radical. In the next stage the porphyrin π cation radical is reduced by one reduced *o*-phenylenediamine (OPD) molecule to compound II species ($(\text{FeIV}=\text{O})\text{P}$) which is subsequently reduced to the remaining native enzyme (FeIII) by other reduced OPD molecule. Consequently, two OPD molecules are oxidized to 2,3-diaminophenazine (DAP). Due to DAP is electroactive, OPD could be regenerated in the electrode surface by the reduction potential scan application in SWV.

The experimental conditions for seven cosubstrates were optimized and the best system was selected according to data reported by Kergaravat et al. [43]. On the one hand, a systematic optimization procedure of four factors involved in the enzymatic reaction was performed by using response surface methodology through a central composite design by Design-Expert 7.1.6. The evaluated factors were A=buffer pH (pH from 4.0 to 9.0, acetate/phosphate/borate 0.05 mol L^{-1} and KCl 0.1 mol L^{-1}), B=enzymatic concentration (9.5–38.2 nM), C=cosubstrates concentration ($0.5\text{--}10 \times 10^{-3} \text{ mol L}^{-1}$) and D= H_2O_2 concentration ($2\text{--}10 \times 10^{-3} \text{ mol L}^{-1}$). These factors were evaluated in very wide working ranges to determinate the kinetics of enzymatic reaction. In this stage an electrochemical response directly related with the enzymatic activity was searched due to the reaction must be independent of substrate concentrations and must fulfill a zero-order kinetics. In addition, it is well known that the hydrogen peroxide at high concentrations is a suicidal substrate therefore the cosubstrate: H_2O_2 ratio was also restricted to avoid the enzymatic inactivation. OPD was selected as HRP-cosubstrate due to it showed good characteristics in its role (high affinity and efficiency for the enzyme) in comparison with the other studied cosubstrates. On the other hand, the selection of the electrochemical technique between square wave voltammetry and amperometry were performed for each HRP-cosubstrate- H_2O_2

system. The HRP-*o*-phenylenediamine- H_2O_2 system presented the higher response by square wave voltammetry than other cosubstrates at reaction time of 60 s and the highest square wave voltammetry: amperometry current ratio with an approximate value of 7. Therefore, the optimal concentrations of enzymatic, OPD and H_2O_2 were 3.8×10^{-8} , 2.5×10^{-3} and $1.25 \times 10^{-3} \text{ mol L}^{-1}$, respectively and the phosphate solution at pH 6.0 was used as the buffer and supporting electrolyte.

3.2. Optimization of the electrochemical magneto biosensor

In order to optimize the stages that include affinity reaction, incubation, magnetic separation and washing, three different experimental procedures were evaluated (see Fig. 1) according to bibliography [34–36]. In all cases, the final concentrations of biotin and biotin-HRP were $3.5 \times 10^{-7} \text{ mol L}^{-1}$ and $3.5 \times 10^{-8} \text{ mol L}^{-1}$, respectively. The concentration of streptavidin-MB was kept in 0.35 mg mL^{-1} as recommended by the manufacturer. In the strategy 1, biotin was incubated with streptavidin-MB followed by magnetic separation and the washing. After, the biotin-HRP was added and incubated. Then, it was followed by magnetic separation and washing. Each magnetic separation and washing was performed to remove all non-specific adsorbed molecules. The strategy 2 was similar to strategy 1, but without magnetic separation and the washing, after incubating between biotin and streptavidin-MB. Finally, in the strategy 3 all the reagents were added together. After the incubation stage, magnetic separation and the washing were performed.

The electrochemical response of the enzymatic reaction was measured in each strategy by SWV and the reduction current density was inversely related with biotin concentration. A typical voltamogram is shown in the Fig. 2.

As is shown in Fig. 3, the strategy 3 presented the highest electrochemical signal ($6 \mu\text{A cm}^{-2}$, $n=5$), suggesting a pronounced competition between biotin and biotin-HRP. The electrochemical signals in the strategies 1 and 2 were 1.1 and $0.6 \mu\text{A cm}^{-2}$ ($n=5$), respectively. These results indicate that the first incubation of biotin alone with streptavidin-MB enhances the bound biotin amount to magnetic beads; subsequently biotin-HRP does not displace the bound biotin and saturates only bead free sites. The strategy 2, which retained more biotin in the beads, was selected for further studies due to the simplicity and rapidity due to this strategy avoids one step of magnetic separation and washing.

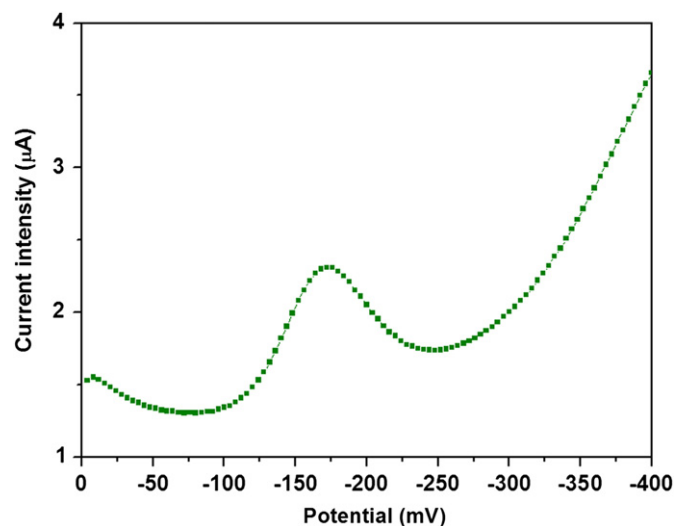


Fig. 2. Voltamogram obtained by SWV for the strategy 2 and 2.5×10^{-3} and $1.25 \times 10^{-3} \text{ mol L}^{-1}$ of OPD and H_2O_2 concentrations at buffer pH 6.0 as reaction medium and 60 s of reaction time.

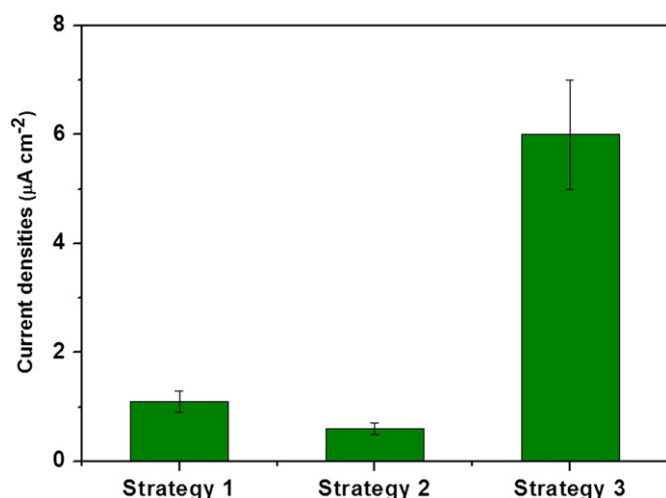


Fig. 3. The electrochemical response mean with deviation standard ($n=3$) for different strategies in the affinity reaction.

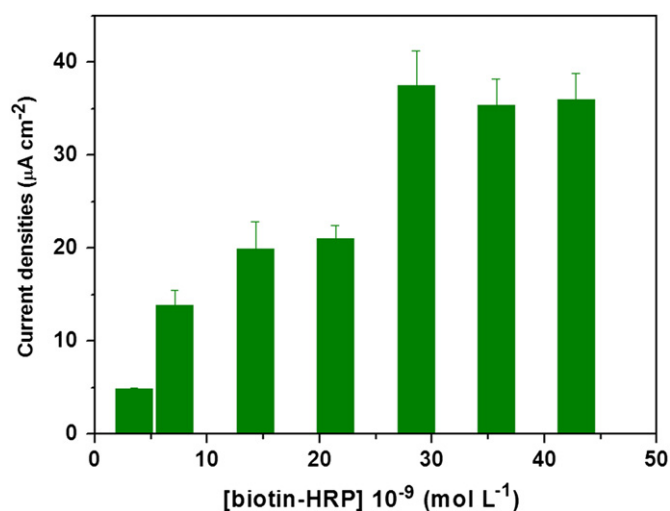


Fig. 4. The electrochemical response mean with deviation standard ($n=3$) for each biotin-HRP concentration. The experimental conditions were streptavidin-MB 0.35 mg mL⁻¹ and biotin-HRP from 3.7×10^{-9} to 4.5×10^{-8} mol L⁻¹.

On the other hand, the optimal concentration of biotin-HRP was studied by applying the strategy 2. In this case, the affinity reaction was evaluated by varying the biotin-HRP (in the range from 3.7×10^{-9} to 4.5×10^{-8} mol L⁻¹) with a fixed concentration of streptavidin-MB suggested by the manufacturer of 0.35 mg mL⁻¹. The current densities at different biotin-HRP concentrations were plotted. The results are displayed in Fig. 4 where it can be seen that the electrochemical signal reaches a plateau from 3.0 to 4.5×10^{-8} mol L⁻¹ of biotin-HRP. This result indicates the saturation of the binding sites of streptavidin-MB with the enzymatic conjugate biotin-HRP. Hence, the biotin-HRP concentration of 3.5×10^{-8} mol L⁻¹ was chosen for further assays.

3.3. Calibration curve for the biotin determination

The biotin standard curve of the current densities vs. biotin concentration by following the strategy 2 is shown in Fig. 5. The figures of merit for the chemical-measurement process were evaluated in order to characterize the biosensor performance [44]. Excellent results were obtained, such as an IC₅₀ was 1.34×10^{-7} mol L⁻¹ (RSD%=2%, $n=3$). The dynamic range estimated as 80% to 20% of electrochemical signal was ranged from

0.94 to 2.40×10^{-7} mol L⁻¹ of biotin concentration. The fitting of this method was evaluated by the analysis of eleven biotin calibration standards using the four-parameter logistic regression algorithm to fit the current densities vs. biotin concentration. “Goodness of fit” was confirmed with a correlation coefficient of 0.992. The LOD and LOQ values were obtained as 90% and 80% of maximal response, i.e. 8.4 and 9.4×10^{-8} mol L⁻¹ of biotin concentration. The Table 1 shows the relative standard deviation (%) and the recovery value (%) obtained by replicate analysis of a standard biotin concentration near to IC₅₀ (1.8×10^{-7} mol L⁻¹) ($n=4$). The known value of the biotin standard solution was compared with the experimentally obtained mean from the calibration curve by applying the *t*-Student test ($\alpha=0.05$) and this was successfully fulfilled. Both % RSD and % Recovery contributed to evaluate the repeatability and accuracy of a method. In this case, the obtained values are good, taking account of the level of analyzed concentration.

The obtained LOD by electrochemical magneto biosensor was compared with obtained values by several electrochemical methods used in the determination of biotin (see Table 2). Three methods based on the avidin-biotin assay with DPV detection [20–22] had slightly lower LOD than the obtained limit in this work, but an original application was only performed by one of them. In addition, the obtained limit was slightly higher than the reported limit for a method that uses a site-oriented anti-biotin antibody onto screen-printed graphite electrodes and SWV detection [26]. On the other hand, our limit was lower than limits reported for instrumental methods as HPLC [45], HPLC with electrochemical detection [17,46] and HPLC-MS/MS [11]. Moreover, this LOD was also similar to methods that use HPLC with derivatization [47] and HPLC combined with fluorescent detection [48–51].

3.4. Biotin in food samples: matrix effect, accuracy studies and interference effect

Real samples such as biotin-fortified commercial dietary supplement and infant formula were analyzed by the electrochemical magneto biosensor. The biotin amount in the samples were quantified being 131 (RSD%=19%, $n=3$) and 29 (RSD%=20%, $n=3$) μg 100 g⁻¹ of sample, respectively. The percentages of declared doses were 87% and 233% for biotin-fortified dietary supplement and infant formula, respectively. These values represent 26 and 5 μg in 100 kcal respectively, satisfying to regulation on biotin in food [4]. In addition this increase could be done for the manufacturer to prevent future losses of the vitamin in the process of fabrication and storage.

The matrix effect test was analyzed in the biotin-free powdered milk from three levels of standard biotin concentrations (Table 3). First, the absence of biotin was confirmed as a negative signal by electrochemical magneto biosensor according to the LOD (8.4×10^{-8} mol L⁻¹). Second, three different amounts of biotin were added to a biotin-free powdered milk sample for achieving concentrations of 27.8, 32.7 and 43.7 μg biotin in 100 g sample and three replicates per each biotin concentration were performed. After extraction of each sample, 50 μL of the supernatant was analyzed by the electrochemical magneto biosensor. The response was reported as μg biotin in 100 g sample. For the estimation of this effect, the known values of the added biotin concentrations were compared with the obtained values by applying the *t*-Student test ($\alpha=0.05$). In all cases the test was successfully fulfilled. The absence of matrix effect is related with the use of magnetic bead, as previously reported [34]. In this case, the magnetic beads could play a role in the preconcentration and separation of the biotin in the complex food sample. The recovery values for each level were ranged from 95% to 110% with an

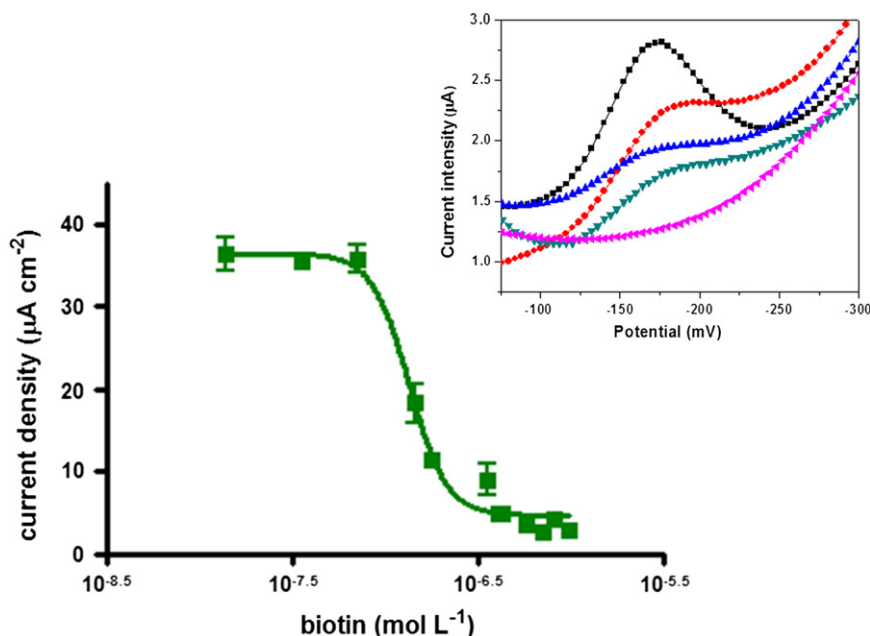


Fig. 5. Regression curve of biotin: current densities vs. biotin concentration. The experimental conditions were streptavidin-MB of 0.35 mg mL^{-1} , biotin-HRP of $3.5 \times 10^{-8} \text{ mol L}^{-1}$ and biotin from 1.4×10^{-8} to $3.57 \times 10^{-7} \text{ mol L}^{-1}$. The insert graph includes five typical voltammograms by SWV.

Table 1

Repeatability and accuracy studies performed with a biotin standard solution at a concentration of $1.8 \times 10^{-7} \text{ mol L}^{-1}$ by the electrochemical magneto biosensor following the strategy 2.

Standard biotin $10^{-7} \text{ (mol L}^{-1}\text{)}$	Obtained biotin $10^{-7} \text{ (mol L}^{-1}\text{)}$	%RSD $n=4$	%Recovery
1.8	2.2	13	107
	1.6		
	2.0		
	1.9		

Table 2

Electrochemical methods for biotin determination.

Technique	Method description	LOD (mol L^{-1})	Original applications	Ref.
^a HPLC-SWV	Avidin-modified electrode	7.6×10^{-6}	Pharmaceutical drug	[17]
^b QCM sensor	Avidin immobilized on the crystal surface	7×10^{-8}	-	[18]
^c DPV	Competition between biotin and labeled biotin with daunomycin	3×10^{-8}	-	[20]
DPV	Competition between biotin and labeled biotin with daunomycin	2×10^{-9}	-	[21]
DPV	Competition between biotin and labeled biotin with Nile Blue A	1×10^{-9}	Vitamin tablet	[22]
^d A	Competition between biotin and biotinylated glucose oxidase	3×10^{-7}	-	[24]
A	Anti-biotin antibody immobilized in electrode and biotin-HRP	6×10^{-7}	-	[25]
SWV	Anti-biotin antibody immobilized in screen printed electrode using $\text{K}_4\text{Fe}(\text{CN})_6$ as electroactive substance	1.4×10^{-8}	-	[26]

^a SWV=Square wave voltammetry.

^b QCM=Quartz crystal microbalance.

^c DPV=Differential pulse polarography.

^d A=Amperometry.

acceptable RSD % value according AOAC [52], i.e. it was lower than 15% ($n=3$).

For the accuracy studies in food samples, biotin-fortified commercial dietary supplement and infant formula samples were spiked with three different concentrations of biotin by adding 76, 152 and $228 \mu\text{g}$ biotin to 100 g of dietary supplement and 7.4, 14.8 and $21.8 \mu\text{g}$ biotin to 100 g of the infant formula. After sample extraction, $10 \mu\text{L}$ of the supernatant of dietary supplement and $50 \mu\text{L}$ of infant formula were analyzed by the electrochemical magneto biosensor. The content of biotin was reported as μg biotin in 100 g sample. The recoveries were in the range from 80% to 120% with the R.S.D% less to 20%, $n=3$ (see Table 4).

These are excellent results, taking account of the simple-pretreatment of the samples and the matrix complexity.

The infant formula samples are generally derivatives of cow milk and are manufactured as breast-milk substitutes during the first months of life. The concentration of biotin in cow milk is approximately to $20 \mu\text{g L}^{-1}$, but it can lose by some pretreatments such as pasteurization or sterilization [53,54]. Despite the biotin is very stable and can be autoclaved without being affected, it can be easily oxidized [55,56]. del Campillo-Campbell et al. [57] have reported that biotin D-sulfoxide in cow milk could be the predominant metabolite in an aerobic environment. In addition studies of the biotin stability in artificial milk samples for infant have reported

that biotin may lose within 0–30% in a finished product, depending on the form of the product [58,59]. On the other hand, the biotin metabolism in human milk samples was deeply studied by Mock et al. [60,61]. Baby milk manufacturers recommend that at once reconstituted in water, milk should be drunk and consumed within 3 weeks of the opening of the container of powdered milk. Therefore, to estimate the effect of interferences generated as degradation products of biotin on magneto biosensor response, an experiment was designed. A biotin-free powdered milk sample was reconstituted and spiked with two levels of standard biotin concentrations (12 and 150 μg in 100 g). In addition two standard solutions of biotin were prepared at the same pH (6.0) and concentrations. All spiked samples and solutions were divided into aliquots and stored at 4 °C. Each aliquot was extracted and analyzed by magneto biosensor at three different days. The % recovery was calculated as the ratio between response value for each day and response value of the first day. Fig. 6A and B shows the % recoveries for samples and solutions at 12 and 150 μg biotin in 100 g, respectively vs. time. In all cases, the recoveries were decreasing in the course of time. However, the effect was more pronounced for the sample at 12 μg biotin in 100 g and more even for aqueous solution at the same concentration with significant differences among days according the ANOVA test ($P \leq 0.05$). After the first day of storage, for this level of biotin concentration, the recoveries were $97 \pm 7\%$ and $69 \pm 8\%$ for dairy matrix and aqueous solution, respectively. Based on these findings, we attributed the decrease of biosensor response to biotin degradation. Due to absence of matrix effect was demonstrated, the decrease less marked in milk matrix could be attributed to the protective effect of ascorbic acid whose concentration in this matrix should be approximately 2 mg in 100 mL [62,63]. Despite the fact that many scientific studies have reported that binding assays can not discriminate between biotin and its analogues, those studies were based on avidin and not on streptavidin [64,65]. Streptavidin has greater specificity for biotin than avidin and therefore its affinity reaction would have a bigger discriminatory power [66]. Although the developed system can not detect each degradation metabolite of biotin, available evidences in this work indicate that this system based on streptavidin-binding could estimate the biotin degradation. Nevertheless, these preliminary evidences should be confirmed in further studies by applying chromatographic separations coupled to binding assay where biotin and each metabolite should

be determinate against a standard curve of corresponding metabolite [67].

4. Conclusions

The determination of biotin in two food samples was successfully achieved by the electrochemical magneto biosensor. In order to do that, the biotin-HRP competed with free biotin in the sample for the binding sites of streptavidin on the magnetic microbeads according to the strategy 2. Then, the electrochemical response was detected by SWV and was related with the biotin concentration in the samples. The developed method with a LOD of $20 \mu\text{g L}^{-1}$ (9 μg of biotin in 100 g sample) has a simple and fast procedure since derivatization processes were not necessary. Therefore, this method was carried out with a simple sample

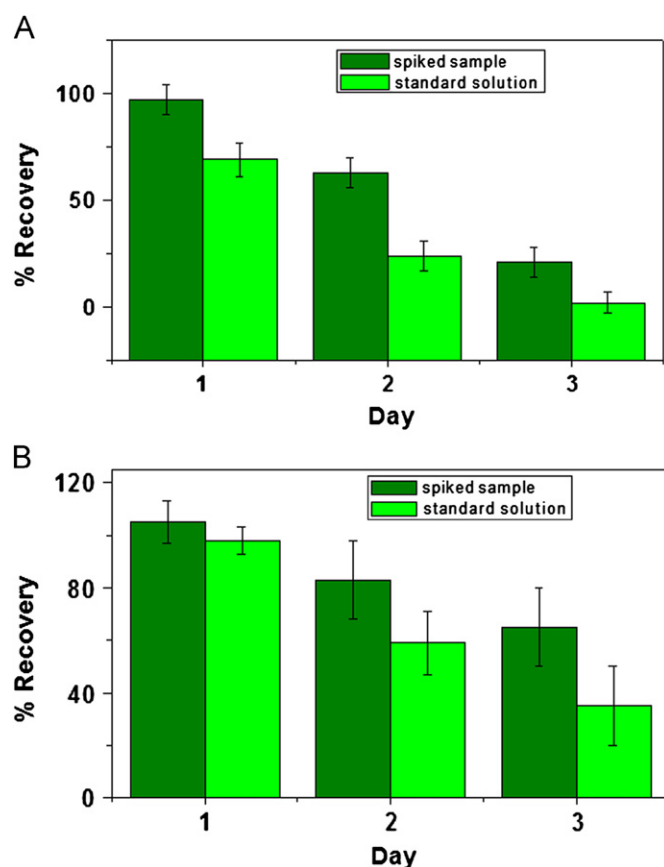


Fig. 6. %Recoveries for spiked samples and solutions vs. time. (A) spiked sample and solution at 12 μg biotin in 100 g, (B) spiked sample and solution at 150 μg biotin in 100 g, ^a Each column represents the mean of at least five measurements $\pm$ standard deviation.

Table 3
Matrix effect study in biotin-free power milk.

Added biotin ($\mu\text{g } 100 \text{ g}^{-1}$)	Obtained biotin ^a ($\mu\text{g } 100 \text{ g}^{-1}$)	% Recovery	RSD % <i>n</i> = 3
27.8	30	108	4
32.7	31	95	14
43.7	46	105	10

^a Each value represents the mean of at least three measurements $\pm$ standard deviation.

Table 4
Recovery values for accuracy studies in food samples based on the electrochemical magneto biosensor.

Samples	Labeled biotin ($\mu\text{g } 100 \text{ g}^{-1}$)	Obtained biotin ^a ($\mu\text{g } 100 \text{ g}^{-1}$)	Recovery assay			
			Added biotin ($\mu\text{g } 100 \text{ g}^{-1}$)	Obtained biotin ($\mu\text{g } 100 \text{ g}^{-1}$)	% Recovery	RSD% <i>n</i> = 3
Dietary supplement	150	131	25	76	220	117
			152	76	277	96
			228	76	370	105
			7	7	35	86
Infant formula	12	29	6	15	47	120
			15	15	50	95
			22	22	50	95

^a Each value represents the mean of at least three measurements $\pm$ standard deviation.

pretreatment based in an only extraction in buffer solution. The total analysis time was of 40 min *per* 20 assays.

In the future, the perspective of our work will be based on the use of screen-printed magneto electrodes. In the validation stage, the method that will include these electrodes should be coupled to chromatographic separations to specifically quantify biotin and its metabolites in complex matrix.

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