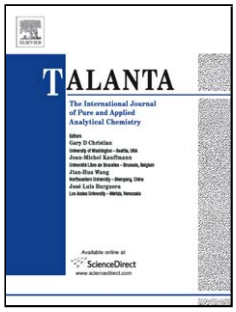

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PII: S0039-9140(08)00373-1
DOI: doi:10.1016/j.talanta.2008.05.005
Reference: TAL 9808

To appear in: *Talanta*

Received date: 24-1-2008
Revised date: 4-5-2008
Accepted date: 4-5-2008

Please cite this article as: L. Molina, G.A. Messina, P.W. Stege, E. Salinas, J. Raba, Immuno-column for on-line quantification of human serum IgG antibodies to Helicobacter pylori in human serum samples, *Talanta* (2007), doi:10.1016/j.talanta.2008.05.005

Immuno-column for on-line quantification of human serum IgG antibodies to Helicobacter pylori in human serum samples.

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Abstract

This study report an human serum IgG antibodies to *H. pylori* quantitation procedure based on the multiple use of an immobilized *H. pylori* antigen on an immuno-column incorporated into an a flow-injection (FI) analytical system. The immuno-adsorbent column was prepared by packing 3-Aminopropyl-modified controlled-pore glass (APCPG) covalently linking *H. pylori* antigens in a 3 cm of teflon tubing (0.5 i.d.). Antibodies in the serum sample are allowed to react immunologically with the immobilized *H. pylori* antigen, and the bound antibodies are quantified by Alkaline phosphatase (AP) enzyme-labeled second antibodies specific to human IgG. p-aminophenyl phosphate (pAPP) was converted to p-aminophenol (pAP) by AP and an electroactive product was quantified on glassy carbon electrode (GCE) modified with multiwall carbon nanotubes (MWCNT) (GCE-CNTs) at 0.30 V. The total assay time was 25 min. The calculated detection limits for amperometric detection and the ELISA procedure are 0.62 and 1.8 U mL^{-1}, respectively. Reproducibility assays were made using repetitive standards of *H. pylori* specific antibody and the intra and inter-assay coefficients of variation were below 5%. The immuno-affinity method showed higher sensitivity and lower time consumed, demonstrate its potential usefulness for early assessment of human serum immunoglobulin G (IgG) antibodies to *H. pylori*.

Keywords: *Helicobacter pylori*; alkaline phosphatase; p-aminophenyl phosphate; immuno-column; FIA

1. Introduction

The incidental discovery, in 1983, of a gastric bacterium led to a dramatic change in the field of gastroenterology [1]. *Helicobacter pylori* infects more than half the global population, causing peptic ulcer disease and chronic gastritis; it is also strongly associated with gastric malignancies. Indeed, it has been classified as a class I carcinogen [2]. The disease can be cured by eradication of *H. pylori* through the triple therapies based on a proton pump inhibitor with two antibiotics (clarithromycin and metronidazole or amoxicillin) [3]. In the absence of therapeutic intervention, infection by *H. pylori* lasts for the life of the host [4]. To prevent the indiscriminate use of multiple antibiotics, an accurate diagnosis for the presence of *H. pylori* infection becomes crucial.

H. pylori infection can be diagnosed by invasive techniques (endoscopy with biopsies for histology, culture and a rapid urease test) and non-invasive techniques (e.g. serology, the 13C-urea breath test and the stool antigen test) [5]. The availability of culture and PCR may be limited because they require highly experienced laboratory personnel. The EIA (enzyme immunoassay) and immunochromatographic method are more convenient, and can be interpreted either spectrophotometrically or visually and has good accuracy [6, 7]. Some noninvasive tests are based on serological procedures that detect immunoglobulin G (IgG) against *H. pylori* in human serum. Circulating anti-*H. pylori* IgG antibody has proved to be of considerable value in the diagnosis of active infection due to the reliable correlation between the presence of the antibody and gastric mucosal colonization [8,9].

Different immunological procedures have been described for the determination *H. pylori* in different samples. Immunochromatographic, microplate enzyme-immunoassay, electrochemical and piezoelectric biosensors [10-15]. Common serum IgG measurements are carried out using enzyme-linked immunosorbent assay (ELISA) [16]. ELISA for serum

IgG against *H. pylori* are based on measuring the quantity of IgG bound onto *H. pylori* antigens immobilized on the solid-phase. In all instances, the immobilized antigens are discarded after only one use.

An ideal diagnostic test should be easy to perform, fast, involve a few steps, and have high sensitivity and specificity. Conventional tests are time consuming and include several steps, which increase the likelihood of errors by the operator [17]. ELISA is amenable for automation, but at an increased cost that cannot usually be afforded by most laboratories in underdeveloped countries.

In general, enzyme immunoassays combine the specificity of the antigen–antibody reaction with the sensitivity and signal amplification of enzyme-catalyzed reaction. Alkaline phosphatase (AP) is widely employed for this purpose since its reactions are basically free of interferences, it is highly stable, and it has a high turnover, low cost, and broad substrate specificity [18].

Carbon nanotubes (CNTs) are a novel type of carbon material and can be considered as the result of folding graphite layers into carbon cylinders. The CNTs have generated great interest in future applications based on their field emission and electronic transport properties [19], their high mechanical strength and their chemical properties [20]. The research has been focused on their electrocatalytic behaviours toward the oxidation of biomolecules and their performance has been found to be much superior to those of other carbon electrodes in terms of reaction rate, reversibility and detection limit [21]. The uses of CNTs for preparation of immunosensors based on CNT-modified electrodes have been reported previously [22-26].

Heterogeneous enzyme immunoassays, coupled with flow injection (FI) system and electrochemical detection, represent a powerful analytical tool for the determination of low

levels of many analytes such as antibodies, hormones, drugs, tumor markers, and microorganism [27]. Electrochemical methods typically have the advantage of being highly sensitive, rapid, and inexpensive [28].

Here we report an human serum IgG antibodies to *H. pylori* quantification procedure based on the multiple use of an immobilized *H. pylori* antigen on a immuno-column incorporated into an a flow-injection (FI) analytical system. Antibodies in the serum sample are allowed to react immunologically with the immobilized *H. pylori* antigen, and the bound antibodies are quantified by AP enzyme-labeled second antibodies specific to human IgG. p-aminophenyl phosphate (pAPP) was converted to p-aminophenol (pAP) by AP and an electroactive product was quantified on GCE-CNTs at 0.30 V. The current obtained from the oxidation of the product of enzymatic reaction is proportional to the activity of the enzyme and, consequently, to the amount of antibodies bound to the immuno-column. This method allows for a rapid determination of anti-*H. pylori* IgG and minimizes the wastage of expensive antigens and other reagents and does not require highly skilled technicians or expensive and dedicated equipment.

2. Materials and methods

2.1. Reagents and solutions

All reagents used were of analytical reagent grade. AP enzyme-labeled second antibodies specific to human γ-chain was purchased from Sigma Chemical (St. Louis, MO, USA). 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP) were purchased from Fluka Chemie (Steinheim, Switzerland). All other reagents employed were of analytical grade and used without further purifications. Aqueous solutions were prepared using purified water from a Milli-Q system.

The ELISA test kit for the quantitative determination of *H. pylori*-specific IgG class antibodies was purchased from EQUIPAR Diagnostici (Rome, Italy) and was used in accordance with the manufacturer´s instructions [29].

2.2. The flow-injection manifold

The immuno-column was prepared by packing varying lengths of teflon tubing with 3-Aminopropyl-modified controlled-pore glass (AP-CPG) bound *H. pylori* antigens. Both ends of the tubing were blocked with glass fiber. A pump (Wilson Minipuls 3 peristaltic pump, Gilson Electronics, Middleton, WI, USA) was used for pumping, introducing the sample. Figure 1 illustrates schematically the components of the single-line continuous-flow setup. The body of the amperometric detector was made of Teflon (Fig. 2). The GCE-CNTs are on the top of the amperometric detector micro flow cell. The potential applied to the working electrode was 0.30V versus Ag wire (pseudoreference electrode) and a Pt wire was used as counter electrode. At this potential, a catalytic current was well established. The pump tubing was Tygon (Fisher Accu Rated, 0.4 mm i.d., Fisher Scientific, Pittsburgh, PA, USA), and the remaining tubing used was Teflon (0.8 mm i.d. from Cole–Parmer, Chicago, IL, USA).

Amperometric detection was performed using the BAS LC-4C and the BAS 100 B (electrochemical analyzer Bioanalytical System, West Lafayette, IN) was used for cyclic voltammetric analysis.

The absorbancies were detected by Bio-Rad Benchmark microplate readers (Japan) and a Beckman DU 520 general UV/Vis spectrophotometer (Fullerton, CA, USA). The *H. pylori* antigens were sonicated by a Sonics Vibra Cell ultrasonic processor (Sonics & Materials, Newtown, CT, USA).

2.3 Preparation of the CNTs-modified GCE

Prior to the modification, the glassy carbon electrode surface was polished with 0.3 μm alumina slurries, rinsed thoroughly with purified water, sonicated 30 s into water and 30 s into acetone, and dried in air. One milligram of CNTs was dispersed with the aid of ultrasonic stirring for 45 min in methanol/water (50:50 v/v) in an aqueous 0.1% Nafion solution. A 20 μL aliquot of this dispersion was dropped on the GC electrode surface and then the solvent was evaporated under an infrared heat lamp [30].

2.4. *H. pylori*-specific IgG antibody immunoassay

A series of standards that covered the clinically relevant range (0–100 U $m1^{-1}$) was supplied with the ELISA test kit. A standard curve for the spectrophotometric procedure was produced by following the manufacturer´s protocol. Concentrations of *H. pylori*-specific IgG antibody were detected spectrophotometrically by measuring absorbance changes at 450 nm.

2.5. Preparation of the *H. pylori* antigens

The antigens was prepared from a sonicate *H. pylori* culture strain. The *H. pylori* were grown on blood agar plates at 37ºC for 3 days and then harvested, washed, and resuspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2). This preparation was subjected to sonication. The sonic amplitude level was set at 20% and the machine was operated using four cycles of 60 s regulated alternatively. The sonicated preparation was centrifuged at 1000g for 10 min, and the supernatant was stored in the 0.01 M PBS (pH 7.2), at -20ºC between uses.

2.6. H. pylori antigens *Immobilization.*

The immuno-column was prepared by packing varying lengths of teflon tubing with 3-Aminopropyl-modified controlled-pore glass (APCPG) and was allowed to react with an aqueous solution of 5% w/w glutaraldehyde at pH 10.00 (0.20 M carbonate) for 2 h at room temperature. After washing with purified water and 0.10 M phosphate buffer of pH 7.00, 0.5 mL of antigens preparation (100 μg mL^{-1} 0.01M PBS, pH 7.2) was coupled to the residual aldehyde groups overnight at 5 °C. The immobilized antigens preparation was finally washed with phosphate buffer (pH 7.00) and stored in the same buffer at 5° C between uses. The immobilized *H. pylori* antigens preparations were perfectly stable for at least one month.

2.7. Procedure for the immuno-column

This method was applied in the determination of IgG antibodies to *H. pylori* in 40 human serum samples. Prior to the analysis of each serum sample, the immuno-column was conditioned by flowing through 500 μL of desorption buffer (0.1 M glycine–HCl, pH 2) and then washed with 500 μL of 0.01 M PBS (pH 7.2). These solutions were pumped at a flow rate of 0.4 mL min^{-1}. The unspecific binding was blocked by flowing through 500 μL of 3% descremate milk in a 0.01 M PBS (pH 7.2). The serum samples were first diluted 50-fold with 0.01 M PBS (pH 7.2) and then 200 μL was injected into the PBS carrier stream at a flow rate of 0.15 mL min^{-1}. The immuno column was washed with 0.01 M PBS (pH 7.2) at a flow rate of 0.4 mL min^{-1}. 200 μL of anti-human IgG AP enzyme-labeled second antibodies (dilution of 1/ 2000 in 0.01 M PBS, pH 7.2) was then injected into the 0.01 M PBS (pH 7.2) carrier stream at a flow rate of 0.15 mL min^{-1}.

The immuno-column was then washed free of any traces of unbound enzyme conjugate with 0.01 M PBS (pH 7.2). DEA buffer (100mM diethanolamine, 50mM KCl, 1mM MgCl2, pH 9.6) was used to prepare the pAPP solution. 200 µL of substrate solution (2.7mM p-APP in a DEA buffer, pH 9.6) was injected into the carrier stream a flow rate of 0.15 mL min^{-1} and the enzymatic product (pAP) was measured on the surface of a GCE-CNTs coupled to a micro flow cell. For the next analysis, the immuno column was conditioned by flowing 500 µL of desorption buffer (0.1 M glycine–HCl, pH 2) and then washed with 500 µL of 0.01 M in PBS (pH 7.2) at a flow rate of 0.4 mL min^{-1}. A 10-port injector valve, with two different size loops, was used to inject the reagents.

A standard curve for the immuno-column procedure was produced by following our protocol with a series of standards that covered the clinically relevant range (0–100 U mL^{-1}) supplied with the ELISA test kit. When not in use, the immuno-column was stored in in 0.01 M PBS (pH 7.2) containing sodium azide (0.01%) at 4 ºC. The stock solution of pAPP was prepared freshly before the experiment and stored under the exclusion of light for the duration of the experiment.

3. Results and Discussion

3.1. Electrochemical study of p-AP with the GCE-CNTs

The electrochemical behaviour of the hydrolysis products (pAP) of the enzyme substrates pAPP, was examined by cyclic voltammetry at GCE-CNTs. A cyclic voltammetric study of 5 mM of pAP in DEA buffer (pH 9.6), was performed by scanning the potential from -300 to 500 mV versus Ag /AgCl. CV showed well-defined anodic and corresponding cathodic peak, which corresponds to the transformation of pAP to *p*-benzoquinoneimine (QI) and vice versa within a quasireversible two-electron process (Fig.

3). A peak current ratio (I_{C1} / I_{A1}) of nearly unity, particularly during the recycling of potential, can be considered as criteria for the stability of QI produced at the surface of electrode under the experimental conditions.

3.2. Effect of Continuous-Flow Operation.

The immuno-column was prepared and incorporated into a FI manifold as illustrated in Fig. 1. To optimise the proposed method is necessary to have an understanding of the effect of the parameters governing the system. It has been shown that the theoretical framework developed for static ELISA system cannot be applied to describe the kinetics of antibody–antigen interactions occurring in a continuos flow immunoassay [31]. Several parameters differ significantly. The buffer flow reduces the limitations of diffusion as observed in static ELISA systems [32]. Furthermore, the surface density of immobilized antigens in the flow immunoassay is at least three orders of magnitude higher than in static ELISA systems, because the controlled pore glass increase the area for immobilization, about 3 orders of magnitude [33], and a high sensitivity can be attained by a rotating bioreactor and continuous-flow/stopped-flow/continuous-flow processing [34].

The proposed method manifolds follow the ELISA principles, but instead of using a microtiter plate, the reagents and washing buffers were pumped consecutively through the column containing immobilized antigen, coupled to amperometric detection for the determination of human antibody. AP enzyme-labeled second antibodies specific to human γ-chain was used as conjugate and the product of the enzymatic indicator reaction was measured at 0.30 V.

The implementation of continuous-flow permits: a) utilization of relatively low immunoreactants loading conditions, b) instantaneous operation under high initial rate

conditions, c) easy detection of accumulated products, and d) reduction of apparent Michaelis-Menten constant, K'_M. The main advantages of this system are its simplicity, and the easy with which it can be applied to the determination of specific IgG antibodies to *H. pylori* in serum samples.

The response obtained from the enzymatic reaction oxidation is proportional to the activity of the enzyme conjugated and consequently, to the amount of specific antibodies of serum samples bound to the immuno-column with *H. pylori* antigens immobilized.

3.3. Optimum conditions for the determination of the enzymatic products.

Various column lengths (1–5 cm) were prepared and incorporated into the FI manifold and the current noted when a 100 U mL^{-1} *H. pylori* specific antibodies control sera was injected and analyzed. The results as summarized in Fig. 4 show that immuno-columns longer than 3 cm would contain sufficient immobilized antigen to complex with antibodies to *H. pylori* of the range of patient sera investigated. An immuno-column of 3 cm long was adopted for further studies.

The sample size was studied in the range 50 to 500 μL and shows a maximum rate of response at 150 μL. For convenience a sample size of 200 μL was used to evaluate other parameters (Fig. 5)

The rate of enzymatic response under flow conditions was studied in the pH range 8–10 and show a maximum value of activity at pH 9.6 (Fig. 6). The pH value used was 9.6 in DEA buffer. The effect of varying pAPP concentration from 0.1 to 5 mM on the enzymatic response was evaluated. The optimum pAPP concentration found was 2.7 mM. That concentration was then used.

3.4. Quantitative test for the detection of H. pylori specific IgG antibody

Under the selected conditions described above, the absorbance of the enzymatic product is proportional to the concentration of *H. pylori* specific IgG antibody in the serum. A linear calibration curve for the detection of *H. pylori* specific IgG antibody in serum was produced over the range of 0-100 U mL^{-1}. The linear regression equation was i=0.0125 + 0.031 CHp, with the linear relation coefficient r = 0.998. The coefficient of variation (CV) for the determination of *H. pylori* specific antibody was 3.2 % (six replicates). The ELISA procedure was also carried out as described, absorbance changes were plotted against the corresponding *H. pylori* specific IgG antibody concentration and a calibration curve was constructed. The linear regression equation was A= 0.031 + 0.029 CHp, with the linear relation coefficient r = 0.996, the CV for the determination of 20 U mL^{1} *H. pylori* specific antibodies was 4.7 % (six replicates).

Taking the detection limit to be the concentration that gives a signal three times the standard deviation (SD) of the blank; for electrochemical detection and ELISA procedure was 0.62 and 1.8 U mL^{-1} respectively. This result shows that electrochemical detection was more sensitive than spectrophotometric method.

The sensitivity (S) is defined as the slope of the regression line signal vs. concentration, S for electrochemical detection and ELISA procedure was 0,031 μA/U mL^{-1} and 0.029 Abs/U mL^{-1} respectively.

The precision of the assay was checked with control serum at 20, 50 and 100 U mL^{-1} *H. pylori* specific antibody concentrations. The within-assay precision was tested with 5 measurements in the same run for each serum. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The results

obtained are presented in Table 1. The *H. pylori* assay showed good precision; the CV within-assay values were below 3.2 % and the between-assay values were below 5 %.

The accuracy was tested with dilution and recovery tests. A dilution test was performed with 100 U mL^{-1} *H. pylori* specific antibodies control sera with 0.01 M PBS, pH 7.2 (Fig. 7).

Reproducibility assays were made using a repetitive standard (n= 6) of 20 U mL^{-1} *H. pylori* specific antibody; the percentage standard error was less than 3.2 % (Table 2). When stored in PBS containing sodium azide (0.01%), the immunoreactor was found to retain 90% of its antibody binding property over a 1 month period.

The immuno-column was compared with a commercial spectrophotometric system for the quantification of *H. pylori* specific antibodies in 40 serum samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods (Fig. 8). These results suggest that the detectable concentration of *H. pylori* specific antibodies in this system has been already at the levels of clinical analysis, and the sensitivity has reached to the levels to meet the determination of *H. pylori* specific antibodies in serum even in light infected degree serum.

4. Conclusions

In this work, a immuno-column coupled with flow injection (FI) system for rapid sensitive and selective quantification of specific antibodies against *H. pylori* in human serum sample was developed.

The overall assay time (25 min) was shorter than the time reported for ELISA commercially test kits (160 min), this may be possible without reduce the selectivity. Also

minimizes the waste of expensive antigens and other reagents; shows physical and chemical stability, and accuracy.

In conclusion we took advantage of the simplicity of the ELISA system to develop a immuno-column that was capable of measuring the same levels of specific antibodies against *H. pylori* in human serum sample as detected by the conventional methods while having the advantages of speed and simplicity. Analytical results of clinical samples show the developed immunoassay has a promising alternative approach for detecting specific antibodies against *H. pylori* in human serum sample in the clinical diagnosis.

5. Acknowledgements

The authors wish to thank the financial support from the Universidad Nacional de San Luis and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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Figure 1. Block diagram of the continuous-flow system and detection arrangement. **P**: pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics, Inc. Middleton, WI). **C**: Carrier buffer line. **SI:** Sample injection. **W:** Waste line. **IC:** Immuno-column. **D**: BAS LC-4C (Bioanalytical System, West Lafayette, IN). **R**: Recorder.

Figure 2. Schematic representation of components in the amperometric flow cell. **A**: Assembled detector **B**: GCE-CNTs working electrode **C**: Top view of lower cell body **D**: Lower cell body. **a**: Electrical connection. **b:** O-ring. **c:** Auxiliary electrode. **d:** pseudoreference electrode.

Figure 3. Electrochemical study of p-AP with the GCE-CNTs. **a)** Cyclic voltammogram in aqueous solution of pAP 5 Mm in DEA buffer (pH 9.6). Scan rate: 100 mV s^{-1}.

Fig. 4. Relationship between immuno- column length and its antibody binding capacity.

Figure 5. Effect of sample size. Each value of i/μA based on five determinations.

Figure 6. Efect of pH on the rate of enzymatic response. Flow rate 150 μl min^{-1}. DEA buffer in the pH range 8–10 was used to prepare the 2.7mM of pAPP solution and 200 μL of each solution was injected in the system.

Figure 7. Dilution test results for 100 U mL^{-1} *H. pylori*-specific antibodies. Each value of i/μA is based on five determinations.

Figure 8. Correlation between proposed method and commercial photometric assays.

Table 1. Within-assay precision (five measurements in the same run for each control serum) and between-assay precision (five measurements for each control serum, repeated for three consecutive days).

[a] Control sera	Within-assay		Between-assay	
	Mean	**CV %**	**Mean**	**CV %**
20 U mL^{-1}	20.21	1.90	20.94	4.68
50 U mL^{-1}	50.20	3.14	49.46	3.75
100 U mL^{-1}	99.52	2.38	98.82	4.16

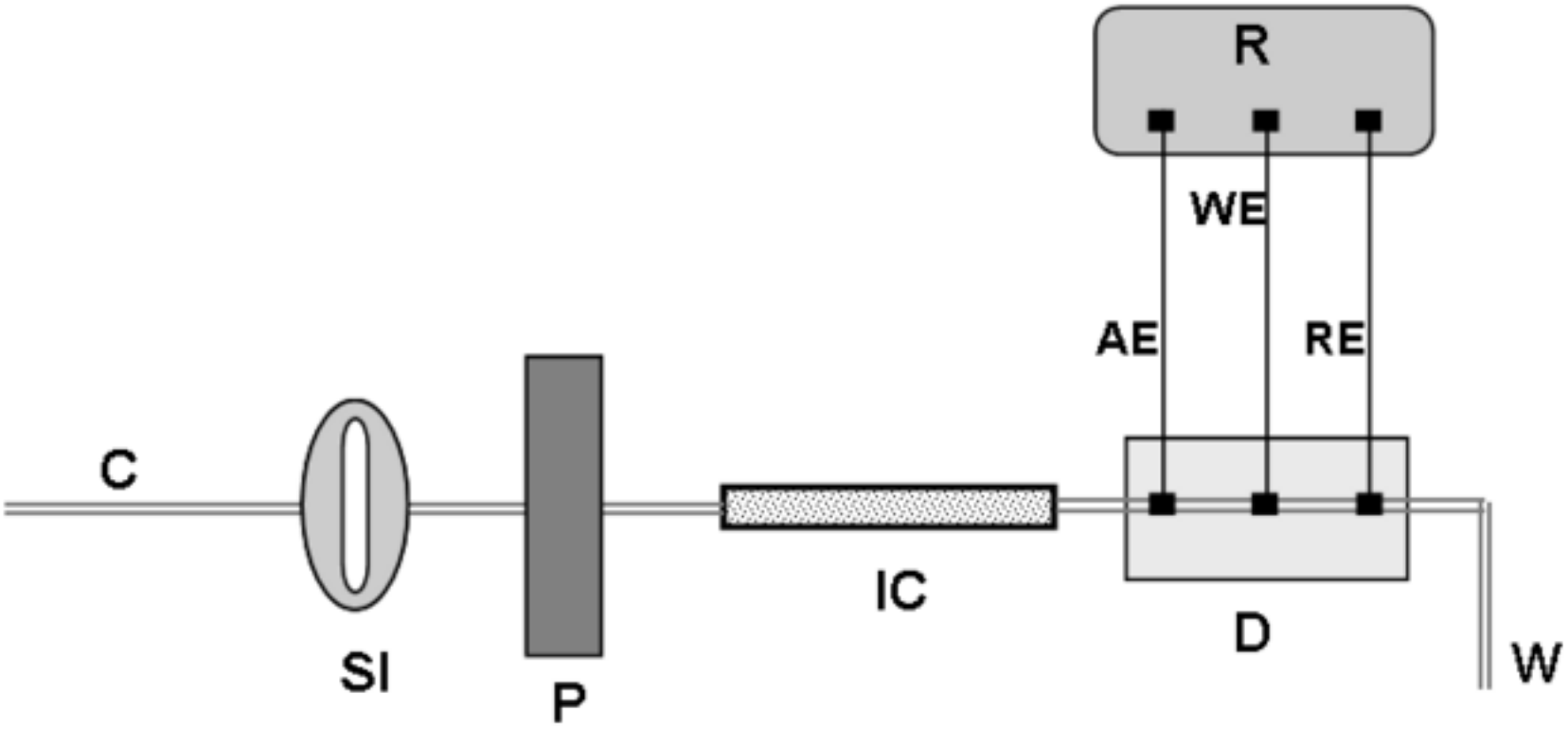
[a] U mL^{-1} *H. pylori* specific antibodies

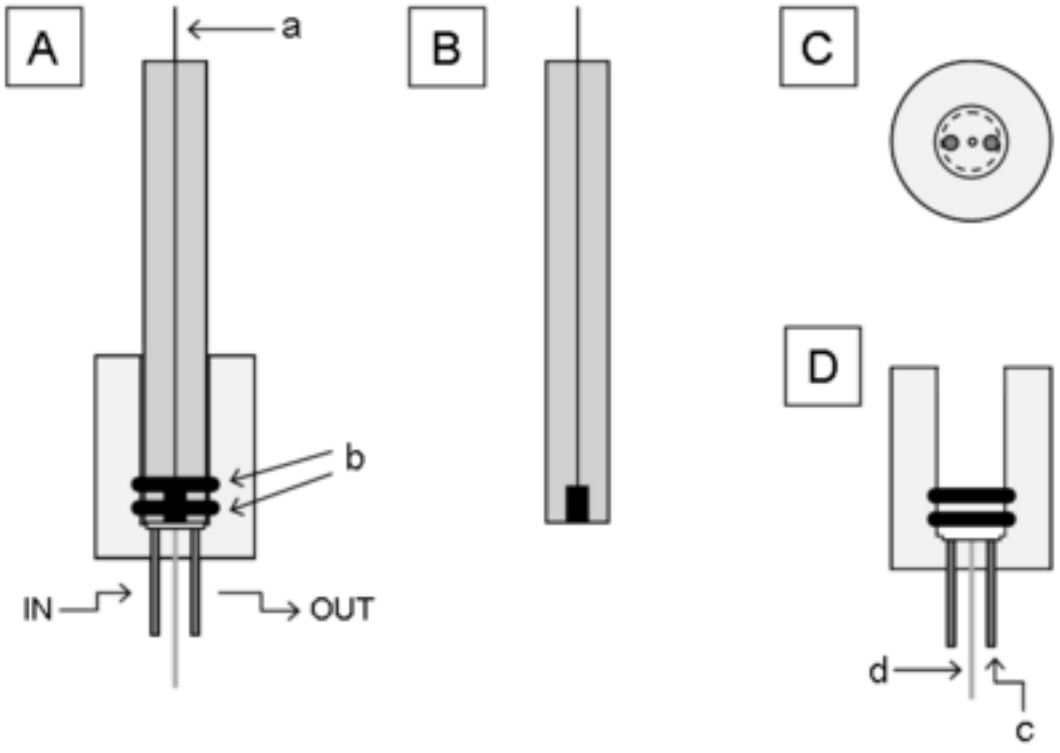
Table 2. Reproducibility assays using repetitive standards (n = 6) of 20 U mL^{-1} *H. pylori* specific antibody

Standards of 20 U mL^{-1} *H. pylori* **specific antibody**	**Proposed method (U mL^{-1})**	**ELIZA (U mL^{-1})**
1	20.20	19.31
2	19.87	19.53
3	20.54	20.29
4	20.09	20.89
5	19.72	19.05
6	19.94	19.39
[a] X ± SD	20.06 ± 0.288	19.74 ± 0.70

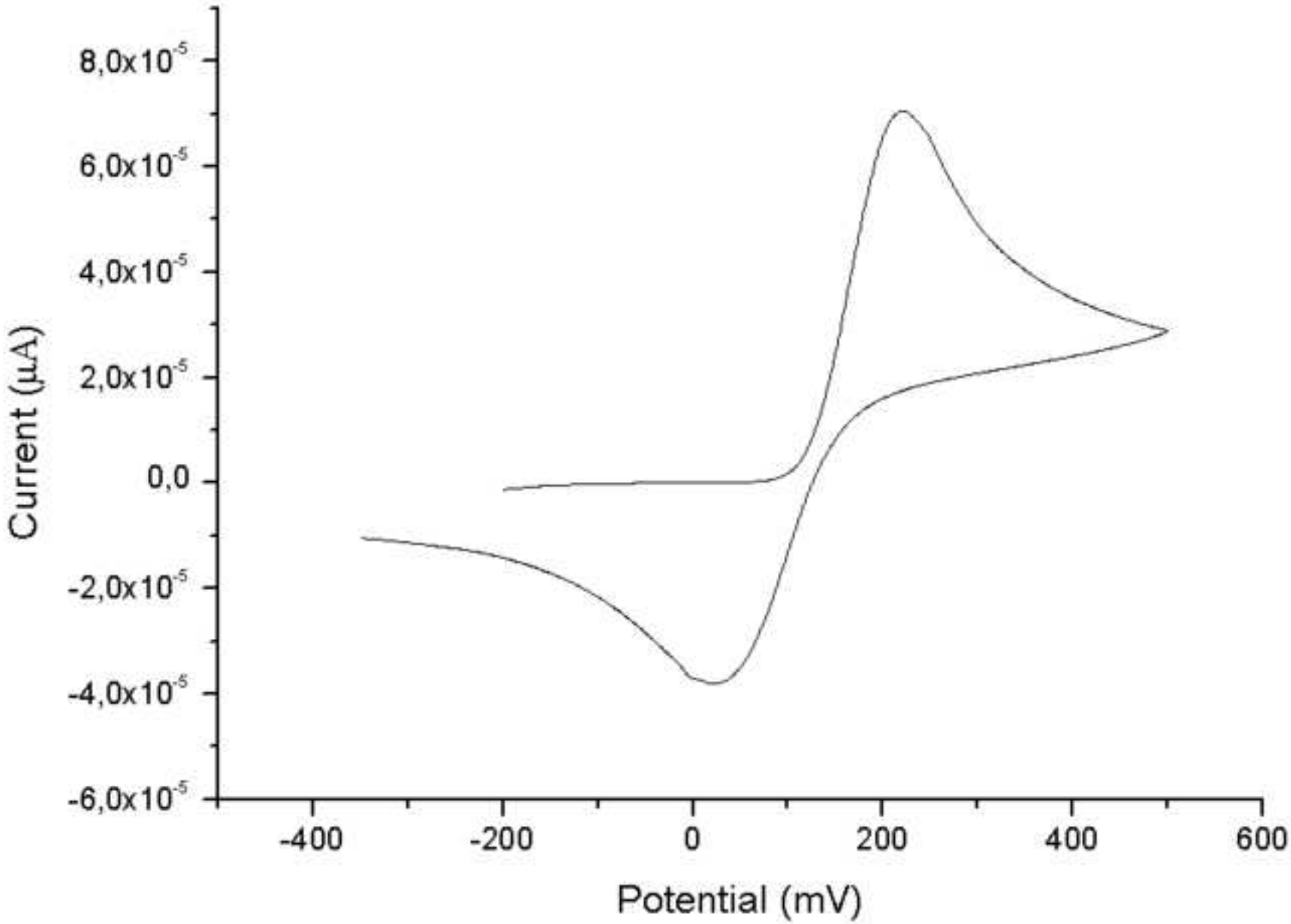
[a] *X* (U mL^{-1}), mean ± SD, standard deviation.

Figure(s)

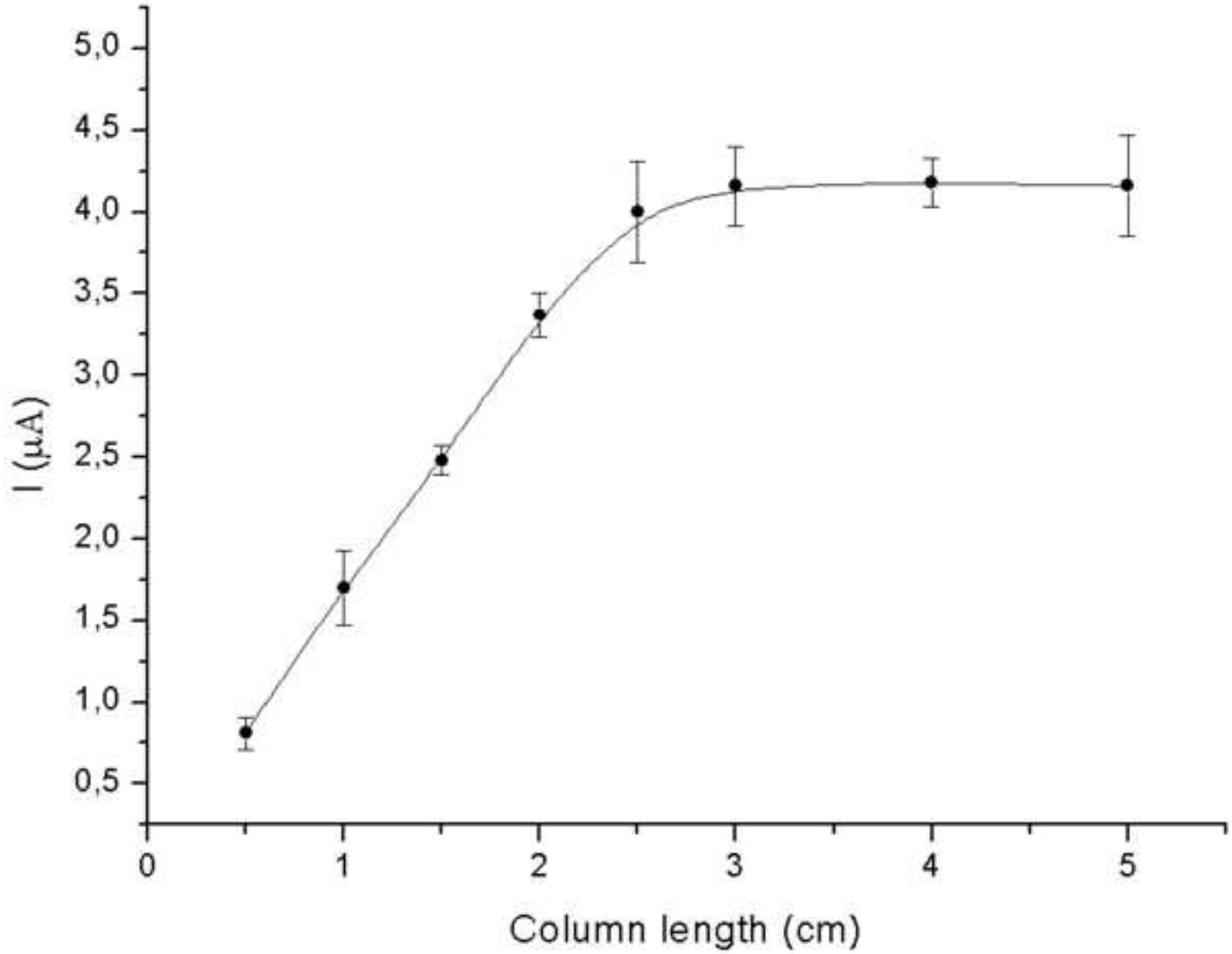




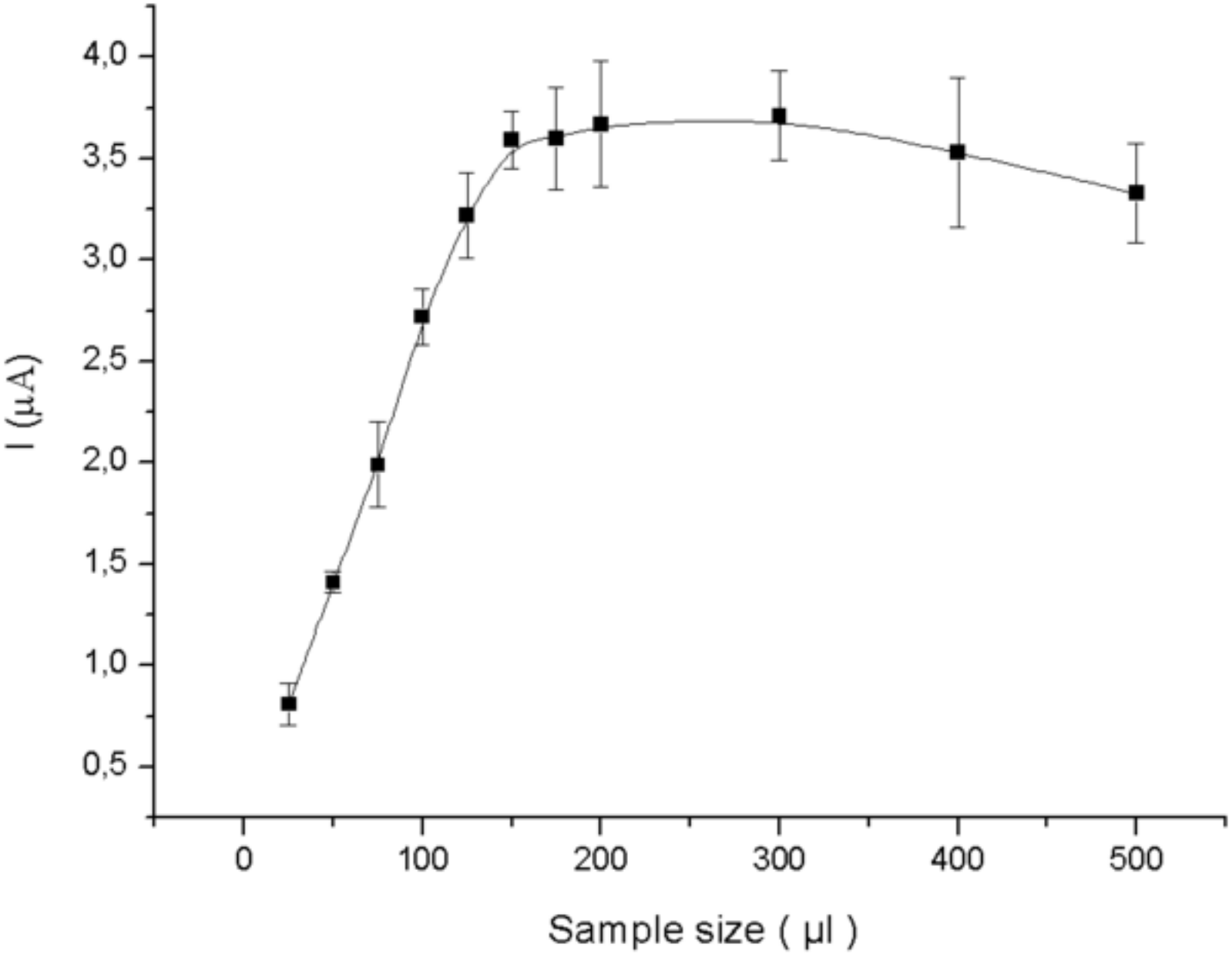
Figure(s)


8,0x10-5
6,0x10-5
4,0x10-5
2,0x10-5
0,0
-2,0x10-5
-4,0x10-5
-6,0x10-5
Current (µA)
-400
-200
0
200
400
600
Potential (mV)

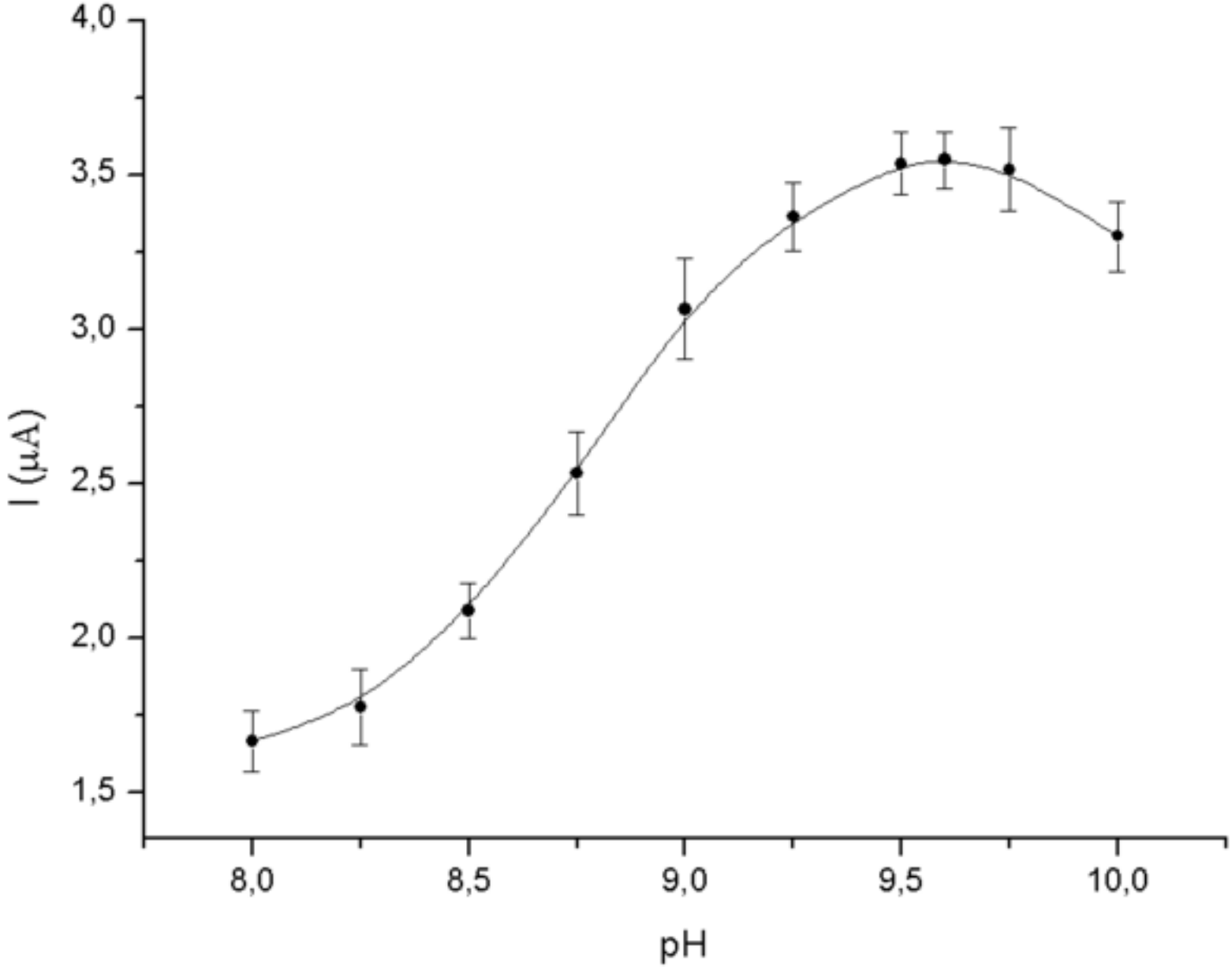
Figure(s)



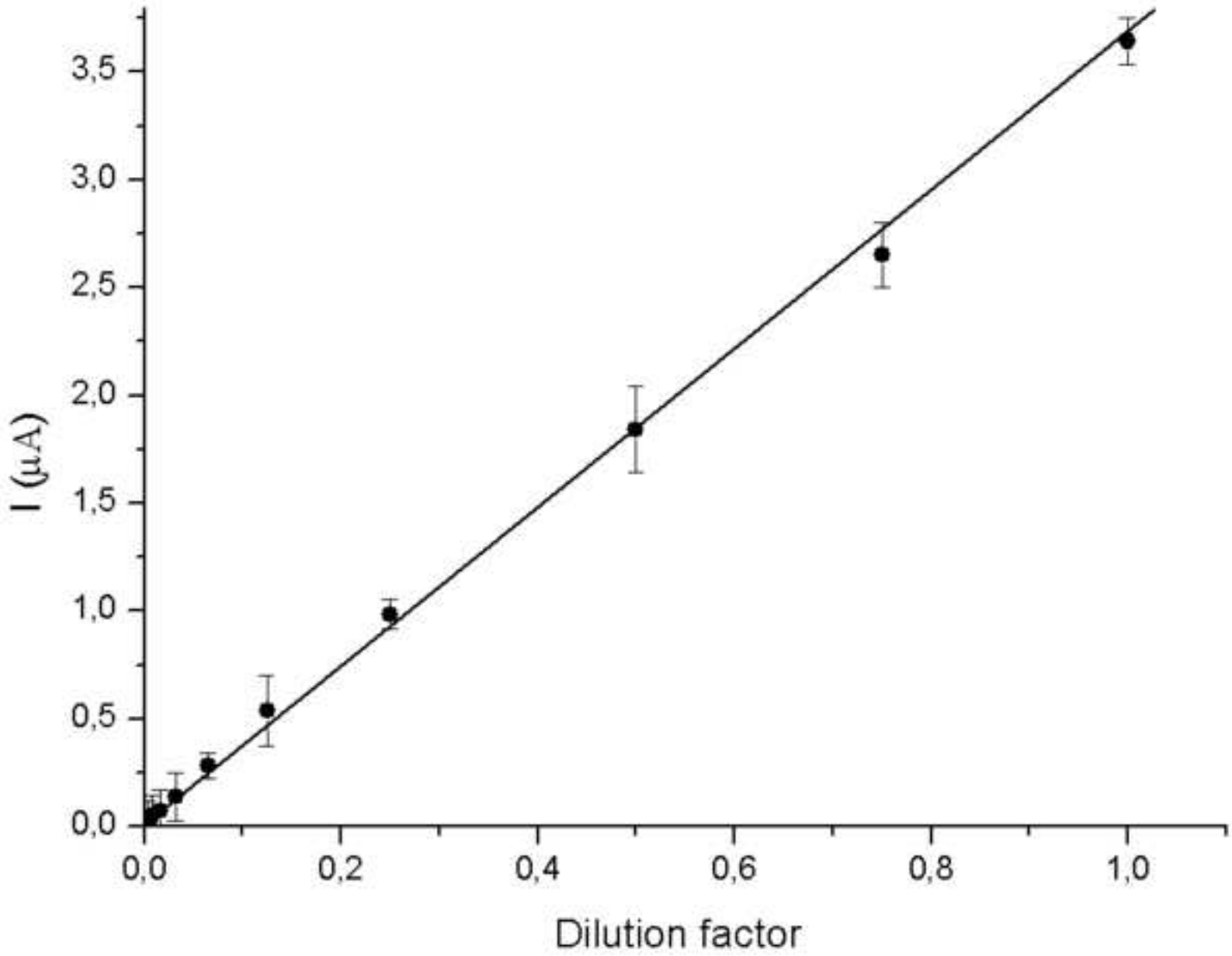
Figure(s)



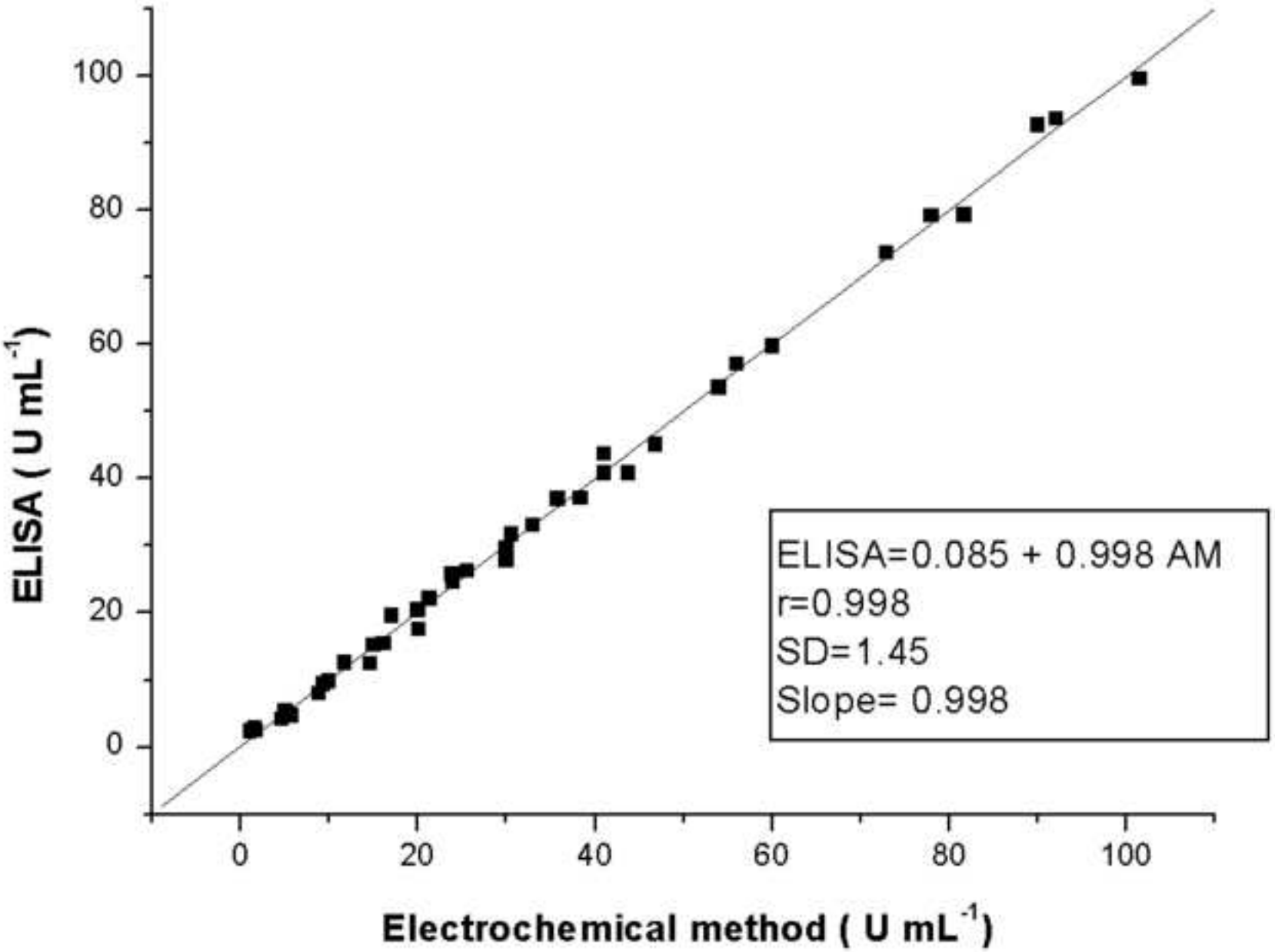
Figure(s)



Figure(s)



Figure(s)


ELISA (U mL^{-1})
Electrochemical method (U mL^{-1})
0
20
40
60
80
100
ELISA=0.085 + 0.998 AM
r=0.998
SD=1.45
Slope= 0.998