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Polychlorinated Biphenyls (PCBs) Detection in Food Samples Using an Electrochemical Immunosensor

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In this work, a disposable electrochemical immunosensor, based on a competitive assay scheme, was applied to detect polychlorinated biphenyls (PCBs) in food. For this purpose, antibodies against PCBs were directly immobilized onto the carbon surface of a disposable screen-printed electrode. A competition between the PCBs present in the sample and a fixed concentration of an enzyme-labeled PCB was realized and evaluated by electrochemical detection. Alkaline phosphatase was used as the enzyme label, coupled with differential pulse voltammetry (DPV) as the electrochemical technique. The immunosensor was tested on aroclor mixture detection (1242 and 1248) and then on some typologies of food samples to evaluate the possible application for real sample analysis. Samples analyzed were from different matrixes, such as sheep milk, bovine adipose tissue, and bovine muscle. Results obtained were compared with the accredited results according to ISO 17025 methods for PCB detection (HRGC-LRMS) as a confirmatory analysis. Preliminary results show the possibility to use this device as a screening method in food sample analysis. The negligible matrix effect observed may lead to a simplified extraction procedure, and considerable time and consumable savings are the immediate benefits given by the proposed method.

KEYWORDS: Immunosensor; screen-printed electrodes; polychlorinated biphenyls; competitive immunoassay; differential pulse voltammetry

INTRODUCTION

Polychlorinated biphenyls (PCBs) are ubiquitous pollutants largely diffused in the environment. Their presence is mainly due to their properties according to low inflammability, chemical stability, and solubility in most organic solvents. Some applications resulted in a direct or indirect release of PCBs into the environment released due to inappropriate disposal practices, accidents, and leakages from industrial facilities. (*1*). Their residues have been identified in air, freshwater and marine sediments, fish and wildlife, human adipose tissue, serum, and milk (*2*, *3*).

The PCB contamination in meat (pork and chicken) often exceed the tolerance level set by the European Commission (200 ng, sum of seven indicator PCBs/g of fat, including PCB 28, 52, 101, 118, 138, 153, and 180) by a factor of 250 (*4*).

To develop routine controls and fast analysis times for PCBs in real samples is an important challenge. Current PCB measurement methods are based on complex laboratory-based instrumental techniques (GC/MS); these methods are generally time-consuming and expensive, and typically requiring sample preparation before the chromatographic separation (*5*). Immunosensor technology, on the contrary, offers a simple and alternative method for food applications (*6*−*8*), and this can be a useful instrument for the routine and specific measurement of compounds in decentralized locations.

The objective of this work has been to develop a simple protocol to detect PCBs in real samples, using the immunosensor technology coupled with electrochemical detection. In recent years, electrochemical immunosensors have been used to detect pesticides and pollutants (*9*−*13*), and some of them were based on disposable screen-printed electrodes as the electrochemical transducer. Screen-printed electrodes have been largely used for many applications in sensor and biosensor technology, and in development of immunosensors (*14*−*16*). These devices have already been used to assemble immunosensors to detect PCBs (*17*, *18*). The advantage of screen-printing technology is the mass production of electrodes at low cost, enabling their use as

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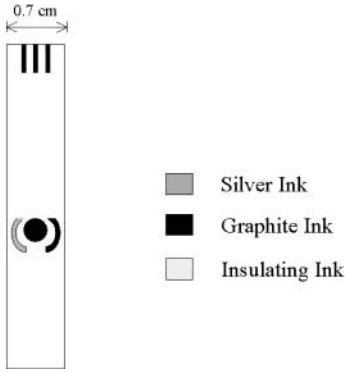

Figure 1. Scheme of a screen-printed electrode.

disposable sensors, and allowing analysis in complex matrixes (*19*). The small dimensions of the strip allow also the combination of these sensors with a portable measuring system.

The immunosensor reported in this paper is based on a competitive immunoassay scheme. The graphite surface of the screen-printed electrodes was modified by immobilization of antibodies specific against PCBs to obtain a selective surface for the realization of the immunological chain. A competition is then realized between a PCB congener alkaline phosphatase labeled and PCBs present in the tested solution. The extent of the affinity reaction is finally evaluated by measuring the enzymatic product α-naphthol produced during the revelation step, and the signal obtained is inversely related to the PCBs concentration in the sample. Applications to real samples analysis coming from different food matrixes have demonstrated the possible application as screening food-control method.

MATERIALS AND METHODS

Materials. The samples were homogenized with an ultraturrax purchased from Ika-WERKE, (Germany), sonicated with Star sonic 90 purchased from Liarre (Italy), and centrifuged with a centrifuge 4237 R (ALC, Italy).

The sheep milk, bovine adipose, and bovine muscle extraction was performed by a Dionex ASE (Accelerated Solvent Extractor) extractor, with extraction cells (22−33 mL) (ASE Corp., Sunnyvale, CA). Purified glass columns 75 × 14 mm i.d. with interchangeable septums in PTFE were obtained from J. T. Baker (The Netherlands).

The capillary gas chromatograph was a HRGC series 8000 with electron-capture detector ECD 800 and autosampler AS 800 (Fisons, Rodano, Italy). The capillary column was a J&W DB-5MS, 30 m × 0.25 mm × 0.2 μm.

A quadrupolar mass spectrometer detector LRMS TRIO-1 (VG, Altrincham, England) combined with a capillary gas chromatograph HP-5890 series II (Palo Alto, CA) (Capillary column J&W DB-5 30 m × 0.25 mm × 0.2 μm), with split-splitless injector at fixed temperature was used for confirmatory analysis.

Electrodes used to build the immunosensor were printed in the Department of Chemistry of the University of Florence using a high performance, multipurpose precision screen printer DEK 245 (DEK, Weymouth, UK). The device appears like a planar strip containing three different electrodes. This three-electrode system is formed by a graphite working electrode, a silver-based reference electrode, and a graphite-based counter electrode (**Figure 1**).

All electrochemical measurements were performed using an Amel polarograph model 433/W (Amel, Milan). Differential pulse voltammetry (DPV) as the electrochemical technique was applied, using the following parameters: range potential 0/+700 mV, scan rate 70 mV/s, pulse amplitude 70 mV, pulse repetition 0.1 s, pulse width 50 ms.

Reagents. Inks for screen-printed electrode production were purchased from Acheson Italiana (Milan, Italy). The plastic substrate for printing was an Autostat HT5 polyester film purchased by Autotype Italia (Milan, Italy).

Table 1. List of the Samples Analyzed for Each of the Three Matrixes[a]

sample	denomination
blank in matrix	A
PCB 118 200 ppb	B
PCB 101 200 ppb	C
PCB 28 50 ppb	D
PCB 28 100 ppb	E
PCB 28 200 ppb	F
PCB 28 500 ppb	G
mixture of the seven congeners 200 ppb	H

[a] The ppb value is referred to the total PCB concentration.

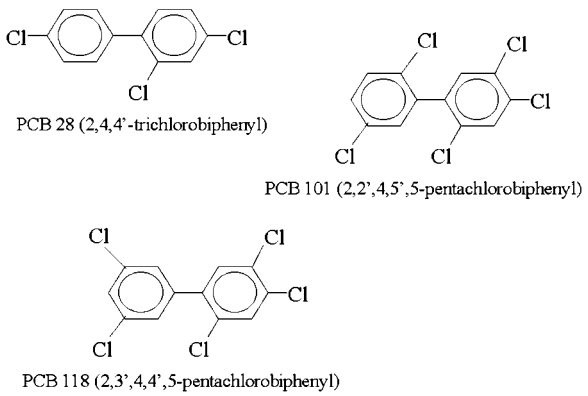

Figure 2. Structure of the three PCB congeners considered.

4,4′-Dichlorobiphenyl-AP conjugate (PCB 28-AP, tracer for the immunoassay) and purified IgG from sheep were kindly supplied by Prof. Fránek, Veterinary Research Institute, Brno, Czech Republic. These antibodies are developed against the 4,4′-dichlorobiphenyl congener BSA-conjugate (PCB 28-BSA conjugate), and as a function of their cross-reactivity, they are indicated as reactive toward aroclor 1242 and 1248 mixtures.

Purified rabbit-IgG was obtained from Sigma (Milan, Italy), which is a generic immunoglobulin from rabbit, used as a function of blocking agent for the sensor surface.

Standard solutions of aroclor 1242 and 1248 were purchased from Supelco, (Milan, Italy).

α-Naphthyl phosphate, diethanolammine (DEA), and polyoxyethylene-sorbitanmonolaurate (Tween 20) were purchased from Sigma (Milan, Italy).

$MgCl_2$ (1 M) and other reagents were analytical grade and purchased from Merck (Milan).

Purified water was obtained by PURELAB PRO 20, (USF). Solvents (petroleum ether, methanol, and isooctane) were all for organic trace analysis from Sigma (Italy). The PCB standards (PCBs 28, 52, 101, 118, 138, 153, and 180) were purchased from Dr. Ehrenstorfer GmbH, (Germany). Anhydrous sodium sulfate (from Sigma, Italy) was heated to 550 °C for 3−5 h. Basic alumina with Brockmann activity = 3 was from Merck (Germany).

Samples. All food samples were taken from different farms of the Abruzzo region and were provided by the Veterinary Institute of Teramo (IZS dell'Abruzzo e del Molise "G. Caporale").

They consist of samples extracted from different matrixes (sheep milk, fat from bovine adipose tissue, and muscle) spiked with seven congeners of polychlorinated biphenyls at different concentrations. These congeners are PCB 28, 52, 101, 118, 138, 153, and 180, indicated as markers from the Italian Legislation. The concentrations used are 50, 100, and 200 ppb of total PCBs.

For each of the three matrixes treated, eight compositions have been chosen (**Table 1**). It can be seen that only some congeners between the seven cited from the EU are considered; in **Figure 2** their structures are reported.

The blank (sample A) is used as reference, to normalize the response with respect to the matrix effect; the congener PCB 28 (present in the

samples D, E, and F) was chosen because it has been used as an immunogen to obtain the antibodies (*20*). PCB 101 and 118 (respectively in samples C and B) are included because these are present in a relevant percentage in aroclor 1242 and 1248 mixtures (*21*). Finally, to evaluate the possible effect of all seven congeners, a sample containing a mixture of them was analyzed. The concentrations analyzed are related to the limits set by the European Community; only sample G has a concentration of PCB 28 more than twice the maximum level permitted, and was used as a "highly polluted sample" control.

Extraction and Preparation of Samples. Samples were analyzed according to the standard procedure, matching the criteria proposed by European Directive (*22*). The absence of PCBs in all samples was checked using the same procedure reported below without spiking. The parameter optimization of the procedure used is reported in the literature (*23*, *24*).

Sample homogenization weight was 5 g. At this quantity, 5 g of anhydrous sodium sulfate was added. The mixture was extracted in an ASE cell with the following operative parameters: solvent petroleum ether, temperature 125 °C, pressure MPa 6.89, preheating time 5 min, heating time 6 min, static time 10 min, flush volume 60%, purge time 60 s. The extract was filtered on a Whatman 41 filter with sodium sulfate, to remove the remaining water phase.

The sample was dry evaporated under a cold gentle nitrogen stream.

A total of 50 mg of each sample was taken and spiked with 2.5, 5, and 10 ng to obtain the concentrations requested (50, 100, and 200 ppb) in PCB congeners. After 30 min, the sample was solubilized in 2 mL of petroleum ether.

All extracts (2 mL) were transferred in an alumina glass column, and the PCBs were eluted with 10 mL of petroleum ether. The eluted was dry evaporated under a cold gentle nitrogen stream and recovered with 1 mL of isooctane.

The alumina glass column was prepared as follows: 3 g of basic inactivated aluminum oxide was introduced into the glass column (75 × 14 mm i.d.), filled with petroleum ether after sedimentation, and then 0.5 g of anhydrous sodium sulfate was added.

Gas Chromatographic Analysis HRGC/ECD. Before using the HRGC/LRMS, a control with HRGC used as the detector, and electron capture detector (ECD) to check the presence of PCBs was made.

The gas chromatographic analysis was performed by an HRCG gas chromatograph (FISONS 8560) equipped with ECD. The injection was made using a split-splitless injector at 270 °C and splitting after 40 s. The PCBs were separated with a capillary column J&W DB-5MS, 30 m × 0.25 mm × 0.2 μm with helium as the carrier gas.

The following column temperature program was used: 98 °C isotherm 2 min, gradient 25 °C/min to 160 °C, isotherm 2 min, gradient 4 °C/min to 210 °C, isotherm 10 min, gradient 10 °C/min to 280 °C, isotherm 15 min.

Detector ECD, at 320 °C excited in constant current, washing with 40 mL/min of nitrogen.

The injection volume was 1 μL (for blank, standard solution, sample, sample + standard), using the same injection syringe, by auto sampler, 0.2 μL of an alkanes homologue series was injected (C10−C22), for retention index calculation.

The quantities of PCBs were determined by comparing their peaks areas with those of the corresponding standards.

The chromatographic peaks were analyzed with acquisition using a data system program.

Confirmatory HRGC-LRMS Analysis. The positive samples were confirmed using a high-resolution gas chromatograph and a low resolution mass spectrometer (HRGC-LRMS).

Confirmatory HRGC-LRMS analysis was performed on GC HP 5890 coupled with a low resolution mass spectrometer Trio 1.

The injection was made using a split-splitless injector at 270 °C and splitting after 30 s: according to the following temperature program: injection at 70 °C, isotherm for 2 min, linear increase from 70 to 160 °C at 20 °C/min isotherm for 1 min, linear increase from 160 to 240 °C at 3°/min, isotherm for 3 min, linear increase from 240 to 285 °C, isotherm 10 min.

The gas chromatography was performed with a J & W DB-5MS capillary column (30 m × 0.25 mm × 0.2 μm) with helium as carrier gas.

The mass spectrometer was operated in the selected ion monitoring (SIM) mode. The following masses were measured for each chlorination level of the analyzed PCBs: molecule mass (M) and M+2 for PCBs 28, 52 and M+2, M+4 for PCBs 101, 118, 138, 153, and 180. All peaks were identified by using the retention time and relative ion intensities. **Figure 3** shows one of the chromatograms obtained.

Sample Treatment for Immunosensor Analysis. Before the analysis, 1 mL of each sample was evaporated to eliminate the organic solvent (isooctane) and reconstituted with 500 μL of methanolic DEA buffer (DEA buffer containing methanol 2% v/v). During this procedure, the fat present in the organic solution of bovine adipose tissue and muscle samples precipitates, and this creates problems because of the major solubility of PCBs in the fat with respect to the aqueous solution. To reduce the fat interference, the reconstituted sample was centrifuged at 5000 rpm for 15 min; in these conditions, the fat formed a pellet that was easily washed with methanolic buffer and removed from the solution.

Finally, a small volume of labeled 4,4′-dichlorobiphenyl-AP was added, and then 10 μL of this solution was dropped onto the graphite surface and treated as in the standard analysis.

Immunosensor Analysis. The immunoassay scheme used is based on a direct competitive immunoassay procedure, as detailed in **Figure 4**.

The graphite working electrode was modified by the immobilization of antibodies against PCBs, and a competition was generated between the free antigen (the PCB molecule) and the same antigen labeled with an enzyme (scheme a)). An affinity complex Ag:Ab is formed during the incubation (scheme b), and the concentration of the analyte is determined by electrochemical measurement of an electroactive substrate (scheme c), produced by the reaction with the enzyme-label. Alkaline phosphatase (AP) was used as the label, and the activity of AP was electrochemically detected using differential pulse voltammetry (DPV) as the electrochemical technique. The response obtained is in inversely proportional to the concentration of the molecular target.

Screen-Printed Electrode Modification. The procedure used to modify the working-electrode surface has already been reported in previous work (*25*). The carbon-based working electrode was coated with 10 μL of carbonate buffer (CB) containing 10 μg/mL of rabbit-IgG as a precoating solution. After one night at +4°C, each electrode was rinsed with 300 μL of PBS-T buffer (phosphate saline buffer 0.1 M containing NaCl 0.1M and 0.05% p/v of Tween 20), dried carefully, and covered with 10 μL of CB solution containing 30 μg/mL of anti-aroclor antibody. The reaction was carried out for 2 h at room temperature, and then the electrode was rinsed again with 300 μL of PBS-T buffer. These modified electrodes can be used in the immunochemical test, or stored at +4°C in a dried chamber for several days without a decrease in the sensitivity.

Competition Reaction. The competition solution (in DEA buffer 0.1 M pH 9.6 added with $MgCl_2$ 1 mM, as the AP activator) was prepared by mixing the PCB standard solution (aroclor mixtures or real samples) with a small volume containing 4,4′-dichlorobiphenyl-AP conjugate at an optimized concentration. After the sample was stirred, 10 μL of this solution was deposited onto the graphite surface and incubated for 15 min, and then the electrode was washed and covered with 200 μL of enzymatic substrate solution (α-naphthyl phosphate 1 mg/mL in DEA buffer). After 5 min (the optimized time in which the enzymatic reaction takes place), the DPV measurement was started.

RESULTS AND DISCUSSION

Percentage of ASE Extraction Recovery. The percentage of recovery for the seven compounds was calculated. For this purpose, a blank sample (cleaned) of bovine adipose tissue was spiked with 25 ppb of each single congener. After the extraction procedure, gas-chromatographic analysis was performed and the final concentration was evaluated. The results obtained are reported in **Table 2**, and these demonstrate that this procedure can be used for PCB extraction from food matrixes.

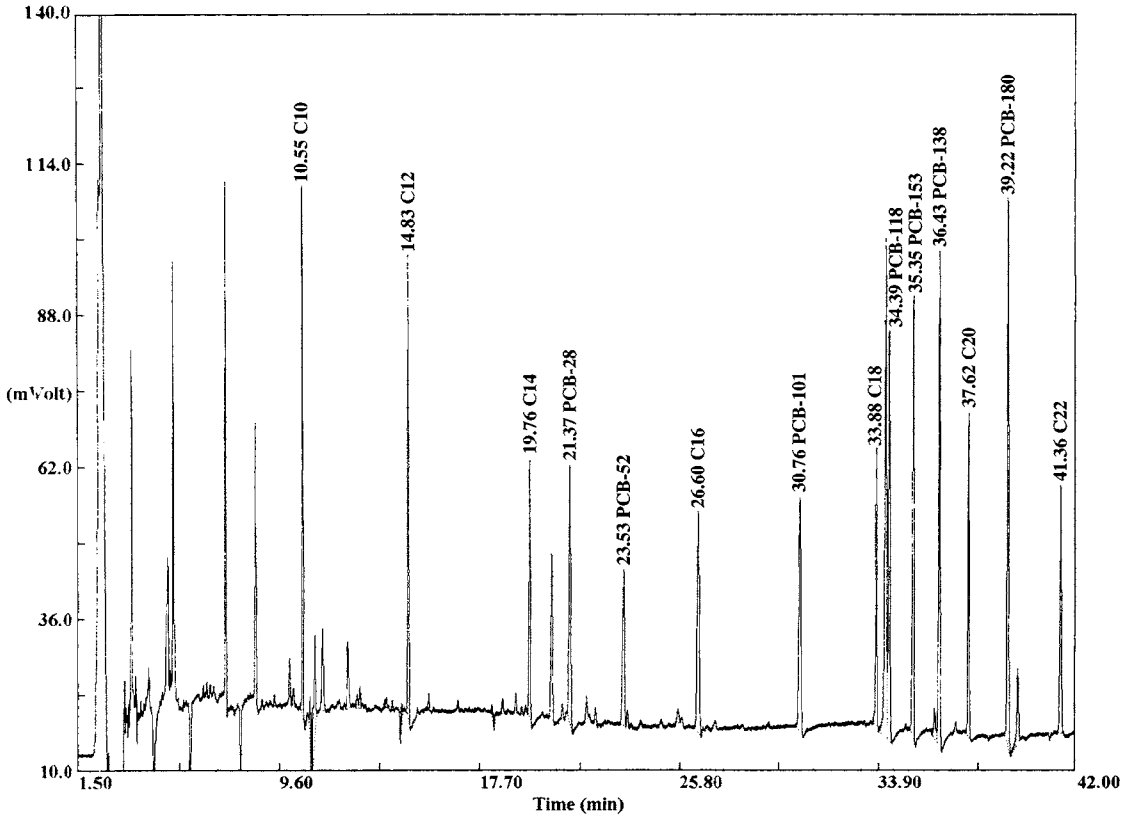

Figure 3. Standard PCB mixture reference (2 ppb) containing alkanes homologue series (from C10 to C22) for retention indexes calculation.

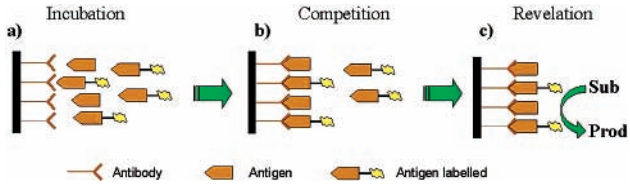

Figure 4. Scheme of the immunosensor reaction. The three steps are shown: incubation (a), competition (b), and electrochemical revelation (c).

Table 2. Recovery of Spiked PCB in a Sample of Bovine Adipose Tissue at a Concentration of 25 ppb

PCB congener	recovery (%)	SD (%)
28	99	10
52	93	10
101	102	5
118	101	9
138	102	5
153	104	7
180	95	7

In **Table 3** the precision data of PCB analysis by GC/ECD is reported, together with the linearity of the method in **Table 4**.

Standard Solution Results. Calibration curves for aroclor 1242 and 1248 mixtures were performed, and are reported in **Figure 5**. In **Figure 5B**, the raw signal obtained by DPV measurement for the aroclor 1248 is also shown. The different profiles obtained from the two calibration curves are related to the composition of the two aroclor mixtures. The signals are reported as B_x/B_o percentage, and they show the typical sigmoidal behavior characteristic of the competitive assays (dose−response calibration curve). The sensitivity of the system ranges between 0.01 and 100 ppm for both the aroclor mixtures,

Table 3. Precision data of PCB analysis by GC/ECD

substance	mean conc[a] (μg/kg fat)	standard deviation	coefficient of variation (%)
PCB-28	13.6	1.4	10.6
PCB-52	60.2	10.6	17.7
PCB-101	64.7	4.3	6.7
PCB-118	39.2	3.8	9.7
PCB-138	136.5	4.5	3.3
PCB-153	176.5	8.5	4.8
PCB-180	94.9	5.5	5.8

[a] All data were obtained by six replicates of a naturally contaminated fat sample.

Table 4. Linearity of Response of PCBs in GC/ECD Analysis

substance	standard solutions conc (ng/mL)[a]	correlation coefficient (r)
PCB-28	1–2–4–8	0.9996
PCB-52	1–2–4–8	0.9983
PCB-101	1–2–4–8	0.9984
PCB-118	1–2–4–8	0.9994
PCB-138	1–2–4–8	0.9998
PCB-153	1–2–4–8	0.9999
PCB-180	1–2–4–8	0.9961

[a] Standard solution used for the experiments was prepared in isooctane.

with a CV (calculated as an average for each assay) of 11%. The shape for the curve for aroclor 1248 is characteristic of the so-called "low-dose hook effect". This is occasionally encountered in competitive assays: at low antigen concentration, the binding can exceed the binding obtained at zero antigen concentration, and this could possibly be due to a positive cooperative binding of the antibody to the antigen (*26*).

Using these preoptimized conditions, the immunosensor was then applied to real sample analysis.

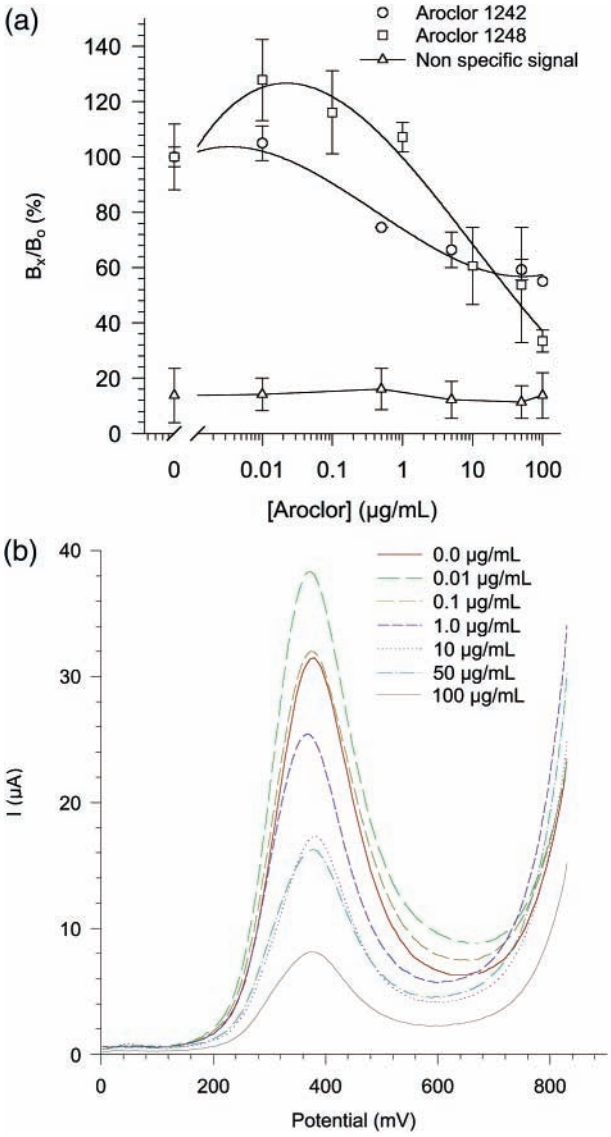

Figure 5. (A) Calibration curves for aroclor 1242 (○) and 1248 (□) mixtures obtained using the immunosensor. Also the nonspecific signal is reported (△). (B) Electrochemical signal obtained by DPV measurements related to the aroclor 1248 mixture calibration curve.

Real Sample Analysis. The procedure used for the standard solution analysis was tested on both negative and PCB spiked food samples and compared to gas-mass results. Different matrixes were chosen (sheep milk, fat from bovine adipose tissue, and muscle), and the PCB standard used to spike the food matrixes were PCB 28, 52, 101, 118, 138, 153, and 180 and a mix of them, using three different concentrations, 50, 100, and 200 ppb. This last value (200 ppb) was considered the admissible limit concentration by the Italian Legislation (*27*) matching the criteria proposed by the European Directive (*28*). We also tested a control sample (sample G) reconstituted with 250 μL of methanolic buffer to have a PCB 28 concentration of 500 ppb.

Preliminary experiments were performed in blanks extracted to evaluate the effect of the matrix on the electrochemical response, reported in **Figure 6**. From this figure, it is possible to evaluate that the averages of the signals obtained are very similar to the current normally obtained with the blank in buffer. This result demonstrates that there is a minimum effect of the medium, and that there is no fouling effect on the electrode surface during the measurement. The largest standard deviations

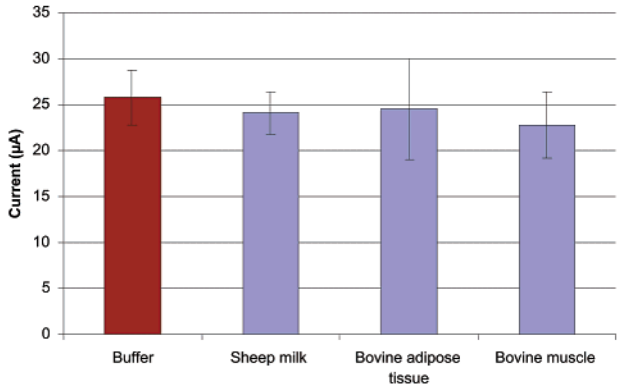

Figure 6. Comparison of the signals obtained with different blank matrixes.

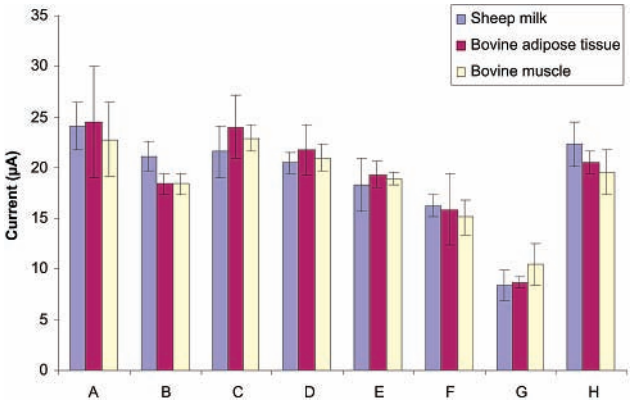

Figure 7. Comparison of samples; effect of different matrixes on PCB detection. Results are reported as absolute signal (current).

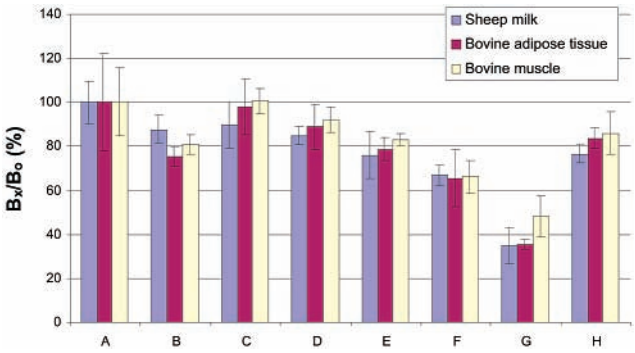

Figure 8. Comparison of samples; effect of different matrixes on PCB detection. Results are reported as B_x/B_0.

obtained for the analysis in the bovine adipose tissue and muscle is possibly due to the high presence of fat with respect to the sheep milk. This is eliminated from the solution, as it tends to adsorb PCBs, increasing the irreproducibility of the extraction and of the measure.

Results for the polluted samples for the three different matrixes analyzed are reported in **Figures 7** and **8**. In **Figure 7**, the currents obtained for each sample analyzed (from A to H) are plotted, grouped by considering the three matrixes of provenience. To analyze these results, the current measured for each sample was then normalized with respect to the corresponding blank matrix (sample A), considered as 100% of the response. In this way, the B_x/B_0 plot of **Figure 8** was obtained, in which all the signal percentages are compared. The error bars for each of the histograms are the standard deviation calculated on four repetition for each measurement. Results are also shown in **Table 5**.

Table 5. Results Obtained from the Immunosensor Analysis of Samples Taken from Different Organic Matrixes[a]

	sheep milk		bovine adipose tissue		bovine muscle	
	average %	SD (%)	average %	SD (%)	average %	SD (%)
A	100	9.7	100.0	22.2	100.0	15.8
B	87.7	6.2	75.1	4.3	80.8	4.6
C	89.5	10.4	98.0	12.7	100.4	5.8
D	84.9	4.2	88.8	10.1	92.1	5.6
E	75.9	10.8	78.8	5.2	83.0	2.6
F	66.7	4.6	64.8	14.5	66.2	7.7
G	34.8	8.2	35.5	2.4	48.2	9.2
H	92.5	9.2	83.7	4.6	85.8	9.6

[a] Signals are reported as a percentage calculated with respect to the corresponding blank in matrix.

Figure 8 shows that a decrease of the signal with respect to the blank was obtained for the samples, which is independent from the matrix analyzed.

The major percentage decrease was obtained for samples D E, F, and G, which contain PCB 28; in this case the signals are also correlated with the increase of the concentration of the congener. This behavior is reasonable, because PCB 28 was used as the immunogen in the antibody production, and thus the system presents the highest sensitivity against this compound than for the others. In particular for sample G, reconstituted with a half volume of buffer with respect to the other samples, a signal that is about the 35% of the blank with a good reproducibility was obtained, demonstrating that by changing the volume of the buffer during the reconstitution step, it is possible to obtain a more concentrated sample for an easier detection. Sample H (which contains all seven cited congeners) also gave a decrease of the signal (about 14% with respect to the blank), but not as intensive as for the previous three samples. The same result was obtained for sample B (containing 200 ppb of PCB 118).

Different behavior was observed for sample C (containing 200 ppb of PCB 101); in this case, a current decrease was observed only for the extract from sheep milk, whereas for the other two matrixes, an increase of the current was obtained. This result could be due to the high solubility of PCB 101 in the fat, and its low recovery in the methanolic buffer used for the measurements.

CONCLUSIONS

The ability of this low-cost and disposable electrochemical immunosensor to detect polychlorinated biphenyls in a complex matrix, such as food, was demonstrated. The fully optimized assay presented a dynamic range of 0.01−50 ppm in standard solutions, with an IC_{50} (as average from the calibration curves of aroclor 1242 and 1248 mixtures) of 5.2 ppm. These preliminary results demonstrate that the analysis in food matrixes can be performed by the immunosensor without further pretreatment steps with acceptable precision and reproducibility, in comparison to bioassay data with gas chromatographic responses. The signal decrease observed at 200 ppb for a mixed PCB polluted sample also demonstrates the ability of this system to work under the tolerance level set by the European Commission.

The minimum matrix-effect observed indicates that the response of the immunosensor is weakly influenced by the type of real sample; in addition, the normalization of the signal as B_x/B_0 with respect to the corresponding blank further minimizes the difference between the matrixes. This large applicability coupled with the high sensitivity may lead to the use of this system as a preliminary indicator before using the HRGC/MS analysis in the food laboratory.

ABBREVIATIONS USED

PCBs, polychlorinated biphenyls; DPV, differential pulse voltammetry; AP, alkaline phosphatase; Ag, antigen; Ab, antibody; CB carbonate buffer; DEA, diethanolamine.

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