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# Determination of Choline in Milk, Milk Powder, and Soy Lecithin Hydrolysates by Flow Injection Analysis and Amperometric Detection with a Choline Oxidase Based Biosensor

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A fast-response and interference-free amperometric biosensor based on choline oxidase immobilized onto an electropolymerized polypyrrole film for flow injection determination of choline in milk, milk powder, and soy lecithin hydrolysates is described. The sensor displayed an $I_{max}$ value of 1.9 ± 0.2 $\mu$A and an apparent Michaelis − Menten constant, $K'_M$, equal to 1.75 ± 0.07 mM. Detection limits of 0.12 $\mu$M could be obtained. Because even a slight deterioration of the anti-interference membrane can adversely affect measurement accuracy, a real time monitoring of the biosensor selectivity has been achieved by a dual Pt electrode flow-through cell where the enzyme modified electrode is coupled to an enzyme-free electrode in a parallel configuration. Finally, bracketing technique (alternate injections of sample and standards) allows a two-point calibration to be performed in real-time, correcting for any drift in sensor response.

**KEYWORDS: Choline; milk; milk powder; soy lecithin; amperometric biosensors; polypyrrole**

## INTRODUCTION

Choline is an important metabolic precursor for a number of biologically significant compounds such as lecithin, acetylcholine, and betaine, and it is involved in the synaptic transmission mechanism. Choline is an endogenous substance synthesized through a biochemical process based on methyl group transfers that require an adequate amount of serine, methionine, folic acid, vitamin $B_{12}$ and $B_6$ (*1*). Only recently it was suggested that a dietary source of choline may be required for adult humans, and the necessity of human daily intake classifies it as "vitamin-like" (*2*, *3*). Neonates need a dietary supply of choline, and for this reason, the American Academy of Pediatrics (*4*) has recommended that infant formulas contain at least 7 mg of choline/100 kcal (0.6 $\mu$mol/kcal).

Choline is widely present in foods, such as bovine and human milk, which also contain significant amount of its metabolites (see Table 3 in ref *5*), like phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomielin (*5*, *6*). The determination of choline is nowadays carried out by a number of techniques, such as the precipitation of choline as Reinecke salt in a colorimetric reaction (*7*) (the classical method) or by microbiological assays (*8*), gas chromatography (*9*) sometimes coupled with mass spectrometry (*10*), and HPLC (*11*, *12*).

Enzyme-based methods coupled with colorimetric(*13*), fluorescence (*14*), or electrochemical detection at enzyme modified electrodes (*15*−*19*) have been also described. Most of these methods require derivatization reactions, and/or the use of expensive instrumentation. Electrochemical biosensors based on immobilized enzymes offer a quick and simple measurement procedure, by low cost devices possessing high selectivity and sensitivity. The main drawbacks of such devices arise from the presence (unavoidable in complex matrixes) of interfering compounds causing a loss of selectivity and from gradual losses in enzyme activity deteriorating the biosensor sensitivity (*20*). Amperometric measurements of choline can be obtained by immobilized *choline oxidase* (ChO) biosensors monitoring the $O_2$ consumption or the $H_2O_2$ production during the enzymatic reaction. The preferred detection scheme is based on the measurement of $O_2$ consumption by a simple Clark type probe (*16*), although $H_2O_2$ detecting amperometric biosensors are intrinsically more sensitive and have a larger dynamic range (*15*−*19*). The main drawback of $H_2O_2$ detecting probes is represented by the high overpotential required to oxidize $H_2O_2$, which could deteriorate the biosensor selectivity. Several approaches (e.g., use of membrane, mediators, and differential measurements) have been described to cope with this problem. An $H_2O_2$-detecting biosensor based on ChO immobilized onto a carbon paste electrode containing *Horseradish Peroxidase* (HRP) and phenotiazine as electron mediator, has been described by Serradilla Razola et al. (*21*). $H_2O_2$ generated by ChO is reduced to $H_2O$ by HRP that is recycled to the native form by the mediator; the oxidized form of the mediator is finally reduced at the electrode polarized at 0.0 V, giving a cathodic current proportional to the choline content. A detection limit

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of 0.1 $\mu$M choline is found. Despite the low working potential, a loss of selectivity was observed because readily oxidizable species, such as catechol and phenol derivatives, interfere in the assay, being substrates of HRP; ascorbic acid was found also to interfere. The biosensor proved useful, however, for dynamic monitoring of choline released from living cells, provided a differential assay is performed.

A ceramic based multisite microelectrode array for rapid choline measurement in brain tissue has been described by Burmeister et al. (*22*). Choline oxidase was covalently immobilized on a Pt microelectrode (polarized at +0.7 V vs Ag/AgCl to amperometrically detect $H_2O_2$) covered by a Nafion layer acting as anti-interference barrier. A *choline oxidase* free adjacent microelectrode was used as control electrode. Measurements were performed by subtracting the control microelectrode normalized response from the choline detecting microelectrode normalized response. Detection limit was 0.4 $\mu$M choline, and good selectivity was observed over interferents such as ascorbic acid, uric acid, and DOPAC.

During the past decade, our group has demonstrated that some electrosynthesized polymers, such as poly(1,2-diaminobenzene) (*23*) and overoxidized polypyrrole (*24*) ($PPY_{ox}$), possessing "built-in" permselectivity allow a very efficient rejection of electroactive interferences and fouling species. The enzyme can be entrapped by the membrane during the electrosynthesis (electrochemical immobilization) or can be over-layered by other techniques such as covalent immobilization. Among covalent immobilization techniques, co-cross-linking of the enzyme with glutaraldehyde (GLU) and bovine serum albumin (BSA) is particularly attractive since a physically stable layer with high enzymatic activity can be obtained (*25*). Indeed the immobilization of *choline oxidase* and/or *acetylcolinesterase* onto a naked platinum electrode by co-cross-linking with BSA and GLU has been already described (*18*). The resulting biosensor was fully characterized and successfully used as amperometric detector for the simultaneous determination of choline and acetylcholine in brain tissue homogenates (*26*), by HPLC. A similar approach was used to construct a disposable biosensor for the rapid screening of the anticholinesterase activity in soil extracts (*27*). In both cases, an anti-interference layer was not required due to the presence of a chromatographic separation step or to the use of a solution containing only the enzyme substrate for the measurement of enzyme inhibition. Recently, *acetylcholinesterase* (or *butyrylcholinesterase*) has been co-immobilized (by BSA and GLU) with *choline oxidase* on a Pt electrode covered with some nonconducting electrosynthesized polymers; however, the potential of the electropolymerized films as anti-interferent barrier could not be appreciated, since the resulting probe was used for paraoxon determination (*28*), through inhibition measurement of the *acetylcholinesterase* activity, after incubation in a standard solution of paraoxon. In complex matrixes, like milk, milk powder, and soy lecithin, the use of this kind of enzymatic probe is possible, however, only if membranes with high rejection efficiency toward electroactive interference are incorporated into the biosensor.

In the present paper, *choline oxidase* has been immobilized, by co-cross-linking with BSA and GLU, onto an overoxidized poly(pyrrole) film electrosynthesized on a Pt electrode. The resulting biosensor couples the unmatched anti-interferent properties of an overoxidized polypyrrole ($PPY_{ox}$) layer with the high enzymatic loading achievable through enzyme immobilization by cross-linking technique. Application of such a biosensor to choline determination in milk, milk powder, and soy lecithin hydrolysates is demonstrated.

Because heavy interference effects from electroactive components are expected in a very complex matrix such as milk, even a slight deterioration of the anti-interfering membrane (i.e., loss of selectivity) can adversely affect measurement accuracy. A real time monitoring of the biosensor selectivity has been achieved by a dual Pt electrode flow-through cell where the enzyme modified electrode is coupled to an enzyme-free electrode in parallel configuration; no differential measurement scheme was necessary, with the enzyme free electrode being used as a "control" for rejection efficiency. Furthermore, bracketing technique (alternate injections of sample and standards) allows a two-point calibration to be performed, correcting, in real-time, for any eventual drift in sensor response.

## EXPERIMENTAL SECTION

**Reagents.** Choline chloride, *choline oxidase* (EC 1.1.3.17 from Alcagenes spp. 14.6 U/mg solid), peroxidase (type I), bovine serum albumin (fraction V), glutaraldehyde (grade II, 25% aqueous solution) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without any further purification. Choline chloride was kept dried under vacuum over $P_2O_5$. All the other reagents were of analytical grade. Solvents were HPLC grade. The carrier stream was filtered through a 0.45-$\mu$m filter (HATF Millipore; France). Pyrrole (Sigma) was distilled under vacuum at 62 °C and kept under nitrogen atmosphere at −18 °C. Choline stock solution was prepared in tridistilled water and stored in the dark at 4 °C. Milk and soy lecithin samples were bought at a local store.

**Apparatus.** Flow injection experiments were performed using a peristaltic pump Gilson minipulse 3 (Gilson Medical Electronics, Villiers Le Bel, France), a Rheodyne (Cotati, CA) model '7725 injection valve equipped with a 20-$\mu$L loop, and an EG&G (Princeton, NJ) model 400 electrochemical detector. The latter included a thin-layer electrochemical cell with a Pt dual electrode (3 mm diameter, 1 mm electrode gap) in parallel configuration, and a Ag/AgCl, 3 M $Cl^-$ reference electrode. Four thin-layer flow cell gaskets (0.05 in. thick, Bioanalytical System Inc.: West Lafayette, IN) were used to define the flow channel. Signals were recorded by a Kipp and Zonen (Delft, Holland) model BD 112 flatbed recorder. Electropolymerization of pyrrole was carried out by an EG&G model 263 potentiostat-galvanostat. A long and narrow PTFE tubing (0.5 mm i.d.) was used to connect the sample injection valve to the electrochemical cell to ensure a Gaussian concentration profile.

**Sample Preparation.** Sample preparation was carried out following the procedure described by Woolard and Indyk (*29*) and used in a collaborative study for the AOAC Official method (*30*). Briefly, a 20-mL milk sample (or 5 g in case of solid samples such as milk powder or soy lecithin) was mixed in a flat bottomed flask with glass stopper with 10 mL (30 mL in case of solid samples) of 3 M HCl. The flask was connected to a condenser and heated for 3 h in a water bath at (70 ± 2) °C and occasionally shaken. A 10-mL of cooled hydrolysate was transferred to a 20-mL volumetric flask, and the pH was adjusted to 3.8−4.0 with a 50% w/w NaOH solution and made to volume with water. Hydrolysate aliquot were filtered through a 0.45-$\mu$m cellulose acetate membrane (Sartorius GmbH, Göttingen, Germany) before FIA, and if not immediately analyzed, frozen at −18 °C. When necessary, a further dilution with phosphate buffer solution ($I$ = 0.1, pH = 7.0) was performed to bring the sample choline concentration in the linearity range of the biosensor.

**Preparation of Modified Electrodes.** Before electrode modification, the Pt surface of the dual electrode flow cell was cleaned with hot $HNO_3$ followed by alumina (0.05-$\mu$m particles) polishing, extensive washing and sonication in tridistilled water. The polypyrrole (PPY) film was electrochemically grown (on both electrodes) at +0.7 V vs Ag/AgCl in a deoxygenated 10 mmol $l^{-1}$ KCl solution containing 0.4 mol $l^{-1}$ of pyrrole. The deposition charge was 300 mC/$cm^2$, because as already shown (*25*, *31*), this value gives the best anti-interferent properties. The PPY film (estimated thickness 0.6−1.0 $\mu$m) was then overoxidized overnight at +0.7 V vs Ag/AgCl in a 0.1 M phosphate buffer solution (PBS, pH = 7.0), washed and air-dried at room temperature.

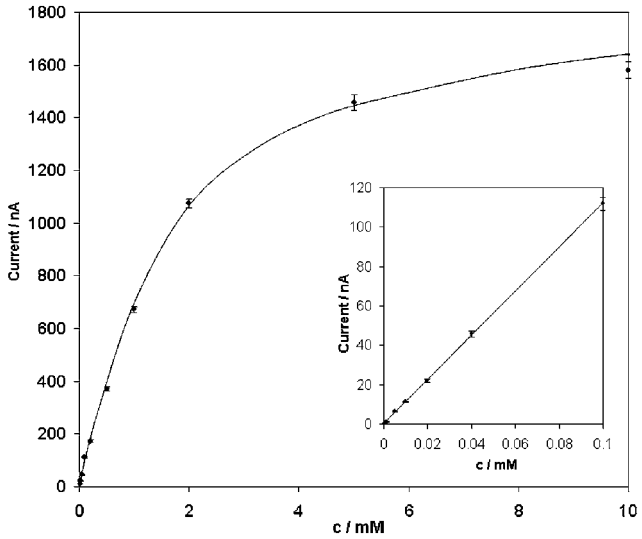


**Figure 1.** Calibration curve for choline obtained at a Pt/$PPY_{ox}$/BSA-GLU-ChO biosensor in a FIA system. Applied potential, +0.7 V vs Ag/AgCl; flow rate, 0.2 mL/min; injection volume, 20 $\mu$L; carrier, 0.1 M phosphate buffer (pH 7.0). Inset: calibration curve in the linear range of the sensor. Each datapoint is the mean of three independent measurements.

A 300-$\mu$L volume of a PBS solution containing 16 mg of BSA and 1 mg of ChO (corresponding to 7.3 U), was carefully mixed with 30 $\mu$L of 2.5% glutaraldehyde solution (25% glutaraldehyde solution diluted 1 + 9 with phosphate buffer). A 3-$\mu$L aliquot of resulting solution was pipetted on the surface of one of the Pt/$PPY_{ox}$ modified electrodes (avoiding air-bubble formation), carefully spread out to ensure complete surface coverage, and air-dried at room temperature. The same procedure (but omitting the ChO addition) was used for the modification of the other Pt/$PPY_{ox}$ electrode. After preparation, the sensors were soaked in a stirred PBS to remove any weakly bound or adsorbed enzyme and to swell the membrane layers; usually, about 15 min was necessary to obtain a stable and steady-state response to substrate addition. When not in use, sensors were stored in PBS at 4 °C in the dark.

**Flow Injection Analysis.** The $H_2O_2$ detection potential was +0.7 V vs Ag/AgCl and the electrochemical detector time constant was 0.1 s. The flow injection carrier stream was PBS ($I$ = 0.1, pH = 7.0). All experiments were performed at ambient temperature, and solutions were air saturated.

**Enzymatic−Colorimetric Method.** Enzymatic−colorimetric method was performed according to the method described by Woolard and Indyk (*29*). Briefly, the hydrolyzed sample, containing free choline, was added with choline oxidase, producing hydrogen peroxide that, in the presence of peroxidase, phenol, and 4-aminoantipyrine, gives a colored compound. Absorbance is measured at 505 nm and choline concentration calculated by interpolation from a standard calibration curve.

## RESULTS AND DISCUSSION

The immobilization procedure as well as the influence of various parameters (pH, GLU, BSA, ChO concentrations, enzyme-layer thickness and flow rate) have already been described in a previous paper (*18*).

A typical calibration curve is shown in **Figure 1** ; the fitting of data by the Michaelis−Menten equation gave an $I_{max}$ value of (1.9 ± 0.2) $\mu$A and a $k'_M$ equal to (1.75 ± 0.07) mM.

The limit of detection, defined as the concentration of choline that gives a signal-to-noise ratio equal to three, is of 0.12 $\mu$M; the repeatability ranged between 1.7 and 4.2% as shown in **Table 1**. Linearity was observed up to a concentration ca. 0.1 mM, that does not allow the determination of choline concentration in real samples without dilution.

**Table 1.** Repeatability of a Pt/$PPY_{Ox}$/Bsa-glu-ChO Biosensor in a FIA System at Different Choline Concentrations[a]

| choline concentration (mM) | repeatability (RSD%) $n$ = 5 |
|---|---|
| 0.001 | 4.2 |
| 0.1 | 3.0 |
| 1 | 1.7 |

[a] Experimental Conditions as in Figure 1.

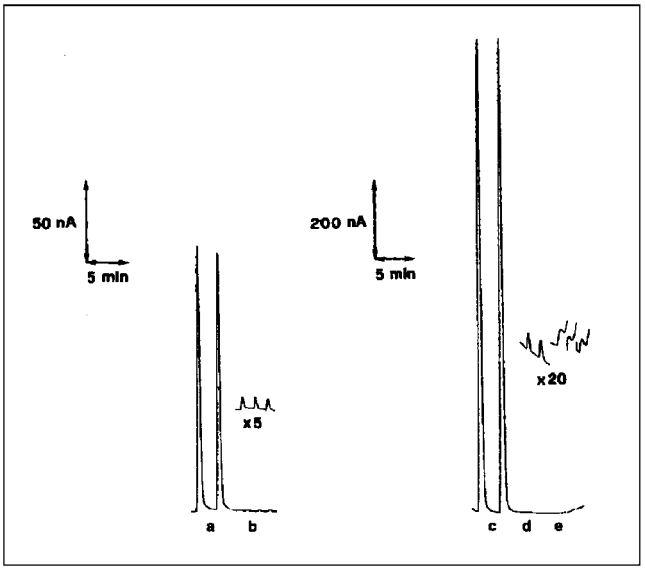


**Figure 2.** FIA peaks obtained by a Pt/$PPY_{ox}$/BSA-GLU-ChO biosensor for repeated injections of 0.5 mM choline (peaks **a**); 0.5 mM uric acid (peaks **b**, magnification factor ×5); 2.0 mM choline (peaks **c)**; 0.1 mM ascorbic acid (peaks **d**, magnification factor ×20) and 0.2 mM paracetamol (peaks **e**, magnification factor ×20). Flow rate: 0.2 mL/min; other conditions as in **Figure 1**.

Interestingly, the detection limit measured with a Pt/$PPY_{ox}$/BSA-GLU-ChO is ca. 10 times lower than the one obtained with the Pt/BSA-GLU-ChO biosensor (*18*). Considering that the enzyme loading, as well as the $I_{max}$ value, are the same in the two cases, this result could be ascribed to less marked Faradic, capacitive, and hydrodynamic noise components in the Pt/$PPY_{ox}$/BSA-GLU-ChO sensor.

The capability of the $PPY_{ox}$ membrane to efficiently reject interferents has been investigated, testing the most difficult to eliminate electroactive interferents (e.g., uric acid, ascorbic acid, and paracetamol) (see **Figure 2** ), even if some of them are not present in the analyzed matrix. This particular characteristic of the biosensor is to be ascribed to the peculiar permselective character of the $PPY_{ox}$ film (*18*, *32*), which is able to reject not only negatively charged ions such as ascorbate, but also neutral molecules (*33*) such as paracetamol. The choline bias, defined as the concentration of choline that gives the same current signal obtained by the injection of a given interferent at a specified concentration, is shown in **Table 2**; such low bias values are the prerequisite to the possibility of using the biosensor for measurements in very complex extracts without any further purification. In any case, the overall anti-interferent properties of the $PPY_{ox}$/GLU-BSA bi-layer membrane is definitely demonstrated by the absence of response observed at the enzyme-free "control electrode" upon injection of food hydrolysates (vide infra).

The use of biosensors in continuous monitoring might be hampered by changes in enzyme activity (causing a drift in

**Table 2.** Choline Bias Produced by Typical Interferents at the Given Concentration in FIA Experiments (Bias Is Defined As Choline Concentration Overestimation Caused by Injection of a Given Interferent at the Specified Concentration)

| interferent | bias/$\mu$M |
|---|---|
| ascorbic acid (100 $\mu$M) | 4.6 |
| uric acid (500 $\mu$M) | 3.4 |
| paracetamol (200 $\mu$M) | 3.4 |

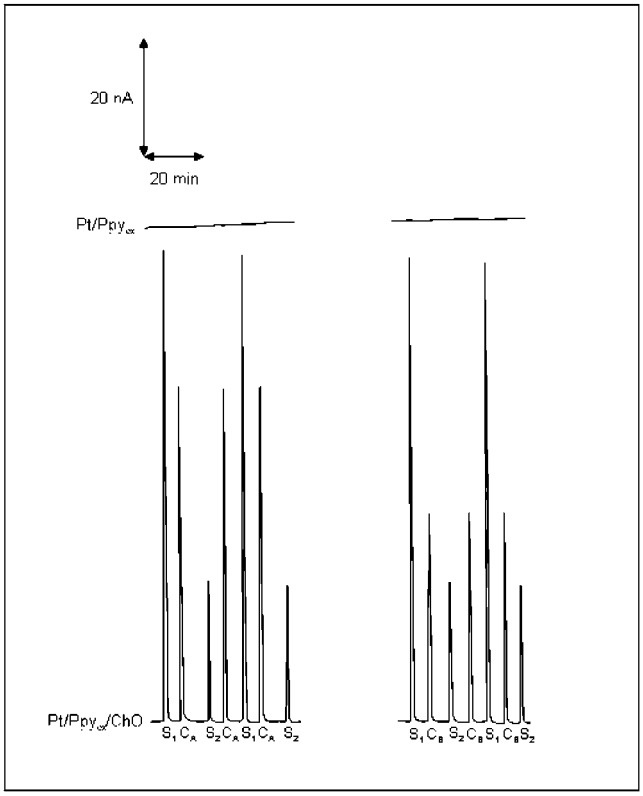


**Figure 3.** Bottom: FIA responses obtained at a Pt/PPY$_{ox}$/BSA-GLU-ChO biosensor by sequential injections of a 0.1 mM choline standard (**S$_1$**), a 0.03 mM choline standard (**S$_2$**), soy lecithin hydrolysate (**C$_A$**), and milk powder hydrolysate (**C$_B$**). Overall dilution factor: 1:18 for milk and 1:500 for soy lecithin samples. Top: response signal of a Pt/PPY$_{ox}$/BSA-GLU (enzyme free) sensor. Other conditions as in **Figure 1**.

sensor response) and unexpected loss of selectivity (i.e., deterioration of anti-interferent properties of the membrane layer). The above-mentioned problems have been faced as described below. First of all, sample measurements have been performed between two injections of standard solutions, with a choline concentration higher and lower than the one expected in the sample. This bracketing technique allows a two-point calibration to be performed in real time, thus avoiding errors coming from changes in enzyme activity. At the same time, the biosensor selectivity can be continuously checked using a Pt/PPY$_{ox}$/BSA-GLU sensor in parallel configuration, as described in the Experimental Section. The enzyme-free modified electrode allowed a real-time monitoring of the anti-interferent properties of the PPY$_{ox}$/BSA-GLU bi-layer, ensuring that the biosensor response to sample injections is due only to choline.

**Figure 3** shows typical "bracketed" sample injections; the signal recorded at the enzyme-free sensor is also shown. It is worth noting that no interference was detected at the "control electrode".

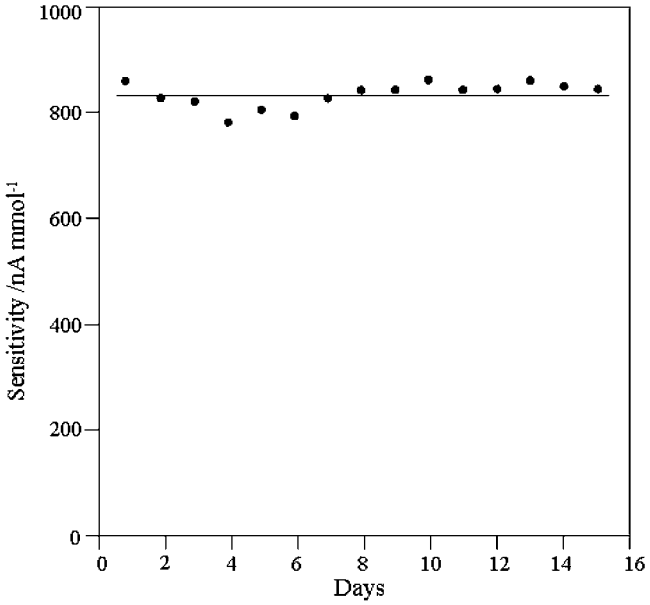


**Figure 4.** Sensitivity of a Pt/(PPy)$_{ox}$/BSA-GLU-ChO biosensor as a function of time. Sensitivity was measured daily after 15 consecutive injections of milk hydrolysate. Experimental conditions as in **Figure 1**.

**Table 3.** Choline Concentration and Relevant RSD for a Number of Real Sample Hydrolysates[a]

| extract sample | choline concentration/mM or *mg/100 g of sample | RSD % ($n = 3$) |
|---|---|---|
| full cream milk | 0.823 | 0.5 |
| partially skimmed milk | 0.79 | 4.2 |
| skimmed milk | 0.752 | 0.7 |
| ewe milk | 0.903 | 0.5 |
| goat milk | 0.441 | 1.0 |
| milk powder | 63.3* | 1.0 |
| soy lecithin | 1588* | 0.9 |

[a] The RSD % value has been calculated on three replicates.

The stability of the sensor was evaluated (see **Figure 4** ) over a 15-day period (biosensor stored at 4 °C when not in use). Each point in **Figure 4** represents the sensitivity (measured daily as the slope of the calibration curves obtained in the linear range of the biosensor) after 15 consecutive injections of milk hydrolysate. As it can be seen, in the explored period of time, the sensor sensitivity remained essentially unchanged: the relative standard deviation was 2.9% ($n = 15$). Furthermore, the rejection efficiency of the bilayer polymeric membrane also remained unchanged.

After one month of intensive use in the flow injection system, ca. 70% of the initial sensitivity was still retained. As far as the shelf life is concerned, 10% of the original response was still displayed after a 6-month storage (+4 °C) period.

**Table 3** shows the choline concentration for a number of real samples, with the RSD % value calculated on three replicates. The results obtained for each sample were not significantly different from those obtained by a classical enzymatic−colorimetric method (*29*), according to a proper *t*-test at the 95% confidence level.

**Conclusions.** A Pt/PPY$_{ox}$/BSA-GLU-ChO amperometric biosensor for choline determination in milk, milk powder, and lecithin hydrolysates is described for the first time. A real time monitoring of the biosensor selectivity has been achieved by a dual Pt electrode flow-through cell where the enzyme modified electrode is coupled to an enzyme-free electrode in parallel

configuration. Bracketing technique (alternate injections of sample and standards) allows a two-point calibration to be performed in real-time, correcting for any drift in sensor response.

On-line coupling of this biosensor to a phospholipase enzymatic reactor for choline determination in untreated milk sample is currently under investigation.

## ACKNOWLEDGMENT

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