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Quantitation of Major Choline Fractions in Milk and Dietary Supplements Using a Phospholipase D Bioreactor Coupled to a Choline Amperometric Biosensor

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Current analytical methods lack the capacity of simultaneous determination of the content of free choline and phosphatidyl-bound choline, mainly phosphatidyl choline, in raw milk. Quantitative determination of total, free, and phosphatidyl-bound choline in milk and a dietary supplement is described using a phospholipase D packed bioreactor coupled to a choline oxidase-based amperometric biosensor. The response for choline and phosphatidyl choline was linear up to 0.5 mM and 1 mM, respectively, and the detection limits were 0.02 and 0.03 mM, respectively. The conversion efficiency of phosphatidyl choline to choline was 50% at 0.2 mL min^{-1}. The within days coefficient of variation for choline and phosphatidyl choline determination in milk samples was 2.8% and 3.2%, respectively. With the addition of an acid hydrolysis step, the method can quantify the concentrations of total, free, phosphatidyl-bound, and non-phosphatidyl-bound choline esters, thus permitting determination of major choline fractions in a complex matrix.

KEYWORDS: Biosensor; bioreactor; choline; dietary supplement; flow injection analysis; milk

INTRODUCTION

Choline plays at least three major metabolic functions in the body: (a) as a methyl donor, (b) as a precursor of the signaling lipids, platelet-activating factor, and sphingosylphosphoryl choline, and (c) as a precursor for acetylcholine, phosphatidyl choline, and sphingomyelin biosynthesis (*1*−*4*). Phosphatidyl choline and sphingomyelin are structural components of biological membranes and also serve as precursors for the intracellular messengers ceramide and diacylglycerol (*5*). Choline is widely distributed in foods (milk, egg yolk, liver, brain, soybeans, peanuts, etc.), mainly in the form of phosphatidyl choline and to a minor extent as sphingomyelin and free choline. The total choline content (free and bound) in milk samples ranges from ca. 1 mM in bovine (*6*, *7*) and human milk (*6*, *7*) to 5 mM in rat milk (*7*), while the relative distribution of the individual choline metabolites varies between species and with stage of lactation (*8*, *9*).

Measurement of choline in foods is difficult because in these matrixes it is present as free choline and/or as a moiety of other molecules. A number of methods have been developed for its determination. None of them is capable of a simultaneous determination of free and phosphatidyl-bound choline fractions in raw matrixes without a pretreatment step. The classical method determines spectrophotometrically the total choline content after multiple extraction and hydrolysis steps to release choline from the sample matrix followed by precipitation as a Reinecke salt (*10*, *11*). Other methods include microbiological assays (*12*), gas chromatography (*13*), capillary zone electrophoresis (*14*), HPLC (*15*), and choline oxidase and phospholipase D enzyme-based methods coupled with colorimetric (*16*), fluorescence (*17*), UV (*18*), or amperometric (*19*−*23*) detection.

The determination of choline-containing phospholipids, and in particular of phosphatidyl choline, the most common natural phospholipid occurring in all living cells with the exception of few bacteria and algae (*24*), can be performed by chromatographic methods (*25*−*27*) or by total phosphorus analysis (*28*) using molybdate−vanadate (*29*) or Bartlett's method (*30*). Alternatively, an enzymatic−spectrophotometric method has been introduced (*31*, *32*), based on two in-series reactions catalyzed by phospholipase D (PLD) and choline oxidase (ChO) followed by a colorimetric reaction involving enzymatically produced hydrogen peroxide, phenol, and 4-aminophenazone in the presence of a peroxidase. The method, however, could suffer from several drawbacks if the sample is not clear in aqueous medium. An improved enzymatic−spectrophotometric method was proposed by Campanella et al. (*33*), who used derivative spectrophotometry in order to eliminate the effect of any residual turbidity. Another approach is a bienzymatic gas diffusion amperometric biosensor operating in organic solvents (*34*) in which lecithin is more readily soluble. Major shortcomings of this device are the short lifetime (3 days when used in chloroform/hexane 50% v/v containing 1% methanol) and the incompatibility with flow injection systems.

Masoom et al. (*23*) have described a flow injection analysis (FIA) system including two in-series packed-bed enzymatic reactors (PBR) and an amperometric detector. This method was

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highly sensitive but suffered from interfering effects that made the sensor not suitable for complex matrixes such as milk. A way to overcome drawbacks of amperometric detection has been described by Lima et al. (*35*) Determination of total choline content in HCl-digested milk was accomplished by injection of HCl-digested samples into a flow system equipped with an enzymatic reactor containing choline oxidase immobilized on glass beads. This enzymatic reaction releases hydrogen peroxide which then reacts with a solution of iodide. The decrease in the concentration of iodide ion is quantified using an iodide ion selective tubular electrode based on a homogeneous crystalline membrane.

Interference problems associated with hydrogen peroxide-detecting amperometric biosensors can be successfully solved by the use of an anti-interferent barrier that is based on electrosynthesized nonconducting films with built-in permselectivity. Among these, overoxidized polypyrrole (PPy_{ox}) proved to be one of the most efficient anti-interferent barriers in amperometric biosensors that are based on electrochemical (*36*−*41*) or covalent immobilization of enzymes (*42*, *43*). We have recently reported a method for quantification of choline in milk, milk powder, and soy lecithin hydrolysates using an interference-free amperometric biosensor based on choline oxidase covalently immobilized onto an electropolymerized polypyrrole film (*44*).

The aim of the present paper is the simultaneous determination of free choline and phosphatidyl-bound choline in untreated samples. For this purpose, the above-mentioned choline biosensor has been coupled with an immobilized PLD bioreactor in parallel configuration with a PLD-free reactor. Application to choline (free and phosphatidyl-bound forms) determination in untreated milk samples and to phosphatidyl choline determination in untreated dietary supplement samples is described. With the addition of an acid hydrolysis step, the total choline content can be also measured using the same device. The non-phosphatidyl-bound fraction can be estimated by subtracting the free and phosphatidyl-bound choline amounts from the total choline amount.

MATERIALS AND METHODS

Reagents. Phospholipase D (EC 3.1.4.4 from Streptomyces species, 4980 U mg^{-1}), choline oxidase (EC 1.1.3.17 from Alcaligenes Species, 14.6 U mg^{-1}), l-α-phosphatidyl choline (type XVI-E, from fresh egg yolk), choline chloride, bovine serum albumin (BSA, fraction V), and glutaraldehyde (GLU, grade II, 25% aqueous solution) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Choline chloride was kept desiccated under vacuum over P_2O_5. Aminopropyl-silylated controlled-pore glass (CPG, specific surface area of 189.25 m^2 g^{-1}, degree of substitution of 2.0−3.2 μmol m^{-2}, bead size of 100−200 μm, and pore size of 19.8 nm) was purchased from Shuller GmbH (Steinach, Germany). Pyrrole (Aldrich, Steinheim, Germany) was purified by vacuum distillation at 60 °C and stored under nitrogen atmosphere at −18 °C. Choline stock solutions (100 mM) were prepared in tridistilled water and stored in the dark at 4 °C. Phosphatidyl choline stock solutions (2 mM) were prepared in 50 mM ammonium acetate buffer, pH 7.0, containing 30 mM calcium chloride and 0.3% v/v Triton X-100 and were stored in the dark at 4 °C for a maximum of 1 week. More diluted choline and phosphatidyl choline solutions were prepared just before use. Real samples (milk and dietary supplement) were bought at a local store. All the other reagents were of analytical grade.

Apparatus. A PAR 273 (EG&G Princeton Applied Research, Princeton, NJ) potentiostat−galvanostat was used for the electrosynthesis of the PPy_{ox} films. The flow injection (FI) system (**Figure 1**) consisted of a Minipulse 3 peristaltic pump (Gilson, Villiers Le Bel, France) and a Rheodyne (Cotati, CA) model 7725 injection valve equipped with a 20 μL loop. For choline determination, the dual channel electrochemical detector (EG&G model 400, Princeton Applied Research, Princeton, NJ) contained a Pt dual electrode (3 mm diameter, 1 mm electrode gap) in parallel configuration, a Ag/AgCl reference electrode in 3 M NaCl, and four 0.05 in. thick gaskets (Bioanalytical System, Inc., West Lafayette, IN). The various parts were connected using 0.3 mm i.d. Teflon tubing. For data acquisition, the electrochemical detector was connected to a personal computer by an analog-to-digital converter (Metrabyte DAS 16, Keithley Inst., Inc., Cleveland, Ohio).

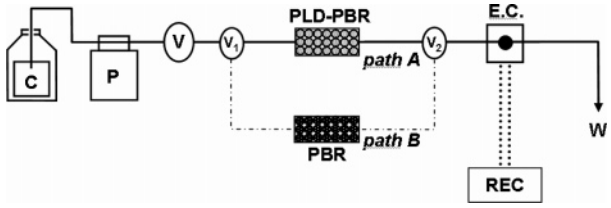

Figure 1. Schematic view of the experimental setup: C, carrier solution reservoir; P, peristaltic pump; V, six-port injection valve; V_1 and V_2, switching valves; PLD-PBR, phospholipase D packed-bed reactor; PBR, packed-bed (phospholipase D-free) reactor; EC, dual channel electrochemical detector with a dual electrode amperometric biosensor; REC, data acquisition system; W, waste.

Biosensor Preparation. Before surface modification, the Pt working electrode was cleaned with 65% (v/v) nitric acid, washed with tridistilled water, then polished with alumina (0.3 μm), washed extensively, and sonicated for 5 min in tridistilled water. Furthermore, the working electrode was electrochemically pretreated by cycling of the potential between −0.21 and + 1.19 V vs Ag/AgCl in 0.5 M sulfuric acid until the voltammogram reached a steady state. A polypyrrole (PPy) film was electrochemically grown on both Pt electrodes at +0.7 V vs Ag/AgCl in a deoxygenated 10 mM KCl solution containing 0.4 M pyrrole. The deposition charge was typically 300 mC cm^{-2} (estimated thickness around 0.6 μm). The PPy film was overoxidized overnight at +0.7 V vs Ag/AgCl in a 0.1 M PBS solution (pH 7.4) at room temperature.

A typical ChO biosensor was prepared as follows: A 300 μL volume of a PBS (pH 7.0, $I = 0.1$ M) solution containing 16 mg of BSA and 1 mg of ChO was carefully mixed with 30 μL of 2.5% (v/v) glutaraldehyde solution in PBS buffer. A 3 μL aliquot of the resulting solution was carefully deposited onto the surface of one of the Pt/PPy_{ox}-modified electrodes. The same procedure, except that ChO addition was omitted, was used for the modification of the other Pt/PPy_{ox} electrode. After air-drying at room temperature for at least 30 min, the sensors were thoroughly washed with PBS buffer to remove any weakly bound enzyme and to swell the membrane layers. When not in use, the electrodes were stored in a PBS solution, pH 7.4, at 4 °C in the dark.

Preparation of the Phospholipase D Packed-Bed Reactor (PLD-PBR). The PLD-PBR was prepared by packing 100 mg of aminopropyl-CPG in plastic columns (5.5 mm i.d., 9 mm length). The aminopropyl-CPG was activated by recirculating 10 mL of 2.5% (v/v) glutaraldehyde solution in PBS (pH 7.4, $I = 0.1$ M) for 2 h at a flow rate of 0.1 mL min^{-1} through the reactor (room temperature) and subsequently washing it with tridistilled water for 1 h. This procedure assured a 10-fold excess of glutaraldehyde with respect to the stoichiometric amount required by the amino groups present on the bead surface. The PLD solution (10 mL, containing ca. 500 U) was loaded onto the supporting material by circulating it for 5 h at a flow rate of 0.1 mL min^{-1}. The enzyme concentration was chosen on the basis of preliminary experiments, which showed that the conversion efficiency was not affected by changing the PLD amount in the 500−5000 units range. The enzyme solution was moved along the reactor by reversing the flow at time intervals of 30 min in order to increase the immobilization efficiency. The device was flushed with PBS buffer for 1 h and stored at 4 °C when not in use.

Sample Pretreatment. For the determination of free choline and phosphatidyl-bound choline, a 5 mL aliquot of raw milk was added with 15 μL of Triton X-100 (to give a final concentration of 0.3% v/v) and diluted 1:4 (v/v) with the carrier solution (50 mM ammonium acetate buffer (pH 7.0), 30 mM calcium chloride, and 0.3% (v/v) Triton X-100).

For quantification of phosphatidyl choline in a commercially available dietary supplement (35% (w/w) was the declared phosphatidyl choline concentration), a sample was finely ground in a mortar and then dissolved in the carrier solution so that the final phosphatidyl choline concentration was close to 0.1 mM. Before injection, samples were filtered through a 0.45 μm cellulose acetate membrane (Sartorius GmbH, Göttingen, Germany).

The determination of the total choline content was carried out on milk hydrolysates obtained following the Woolard and Indyk (*45*) procedure used in a collaborative study for the AOAC official method (*46*). Briefly, a 20 mL milk sample was mixed with 10 mL of 3 M HCl in a flat-bottomed flask with glass stopper. The flask was connected to a condenser, heated for 3 h in a covered water-bath (70 ± 2 °C), and agitated periodically. The acid digest was cooled and centrifuged for 15−20 min at 4000 rpm. A 10 mL aliquot of the supernatant was then transferred to a 20 mL volumetric flask, the pH was adjusted to 7.0−7.4 with a few drops of 50% (w/w) NaOH solution, and the solution was made to volume with the carrier solution. If not immediately analyzed, samples were stored frozen at −18 °C in the dark. Immediately before analysis, a further dilution with carrier solution (1:6 v/v, unless otherwise stated) was performed in order to bring choline concentration in the linear range of the biosensor.

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 carrier solution was a 50 mM ammonium acetate buffer (pH 7.0) containing 30 mM calcium chloride and 0.3% (v/v) Triton X-100. All the experiments were carried out at room temperature, and solutions were air-saturated. The PLD-PBR and the enzyme-free PBR were connected in a parallel configuration between the injection loop and the biosensor (see **Figure 1**). Unless otherwise stated, a flow rate of 0.2 mL min^{-1} was used.

Quantitative Determination of Total, Free, and Phosphatidyl-Bound Choline. Total and free choline concentrations could be estimated from a calibration curve obtained by injecting choline standards (0.01, 0.03, 0.05, 0.1, 0.3, 0.5 mM) each in triplicate. Samples (20 μL) were loop-injected through path B in **Figure 1**. We used for quantification a two-point calibration bracketing technique (*44*) because it corrects for temporal drift in biosensor sensitivity and avoids time-consuming calibration.

Phosphatidyl-bound choline concentration was estimated using the standard addition method. Phosphatidyl choline standard was added at 0.0, 0.25, 0.5, 0.75, and 1.0 mM (each concentration in duplicate) to a milk sample. Sample (20 μL) was loop-injected and directed through path A (**Figure 1**).

RESULTS AND DISCUSSION

The characterization and the optimization of the choline oxidase-based biosensor have been the object of a previous investigation (*22*). The bilayer configuration of the biosensor using the electrosynthesized PPy_{ox} film ensured anti-interference properties and a high enzyme loading of the biosensing membrane obtained by the co-cross-linking of the enzyme with glutaraldehyde and BSA. The best compromise between the amount of enzyme immobilized and fraction deactivated by cross-linking was achieved at 0.2% (v/v) glutaraldehyde and 50 mg mL^{-1} BSA. These values also provided good diffusional characteristics of the layer.

An ammonium acetate buffer containing calcium chloride was used because (a) phosphate buffer, previously in the carrier stream, was replaced; this PLD is a calcium-dependent enzyme, which requires 30 mM Ca^{2+} to exhibit its maximum activity (*47*, *48*) and (b) a phosphate buffer could precipitate calcium. Biosensor response was not affected by the presence of 30 mM Ca^{2+} according to a one-way ANOVA test with a 95% confidence level. Triton X-100 was added at 0.3% (v/v) to the carrier solution to improve the solubility of phosphatidyl choline in the solution and to improve the interaction between lipid substrate and enzyme (*49*). Addition of Triton X-100 at 0.3% (v/v) decreased choline sensitivity for standard injections by ca. 85%; however, the remaining signal was sufficient to allow for quantification of choline.

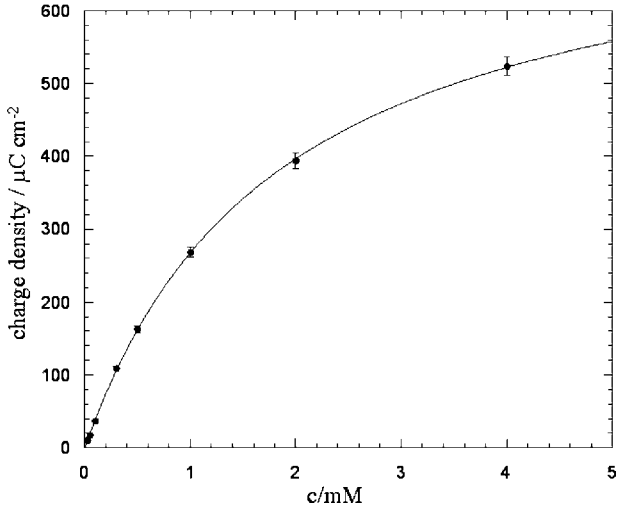

Figure 2. Calibration curve for choline obtained at a platinum/overoxidized-polypyrrole/BSA–glutaraldehyde–choline oxidase biosensor (path B in **Figure 1**). Applied potential: +0.7 V vs Ag/AgCl. Flow rate: 0.2 mL min^{-1}. Injection volume: 20 μL. Carrier solution: 50 mM ammonium acetate buffer solution (pH 7.0) containing 30 mM calcium chloride and 0.3% (v/v) Triton X-100. Each data point is the mean of three independent measurements; error bars represent standard deviations.

The dependence of the current response on the flow rate was investigated in the 0.1−0.5 mL min^{-1} range (data not shown). As expected, lower flow rates and, therefore, longer residence times led to higher production of hydrogen peroxide, i.e., to higher conversion efficiencies, but also to longer response times. A flow rate of 0.2 mL min^{-1} was selected as the best compromise between analysis time and the overall sensitivity of the device. Under the experimental conditions, a peak full width at half-height (a measure of the dispersion in the FIA system) of ca. 280 μL was obtained.

With the use of the ChO biosensor coupled with a PLD reactor, no pretreatment is required for quantification of free and phosphatidyl-bound choline. The type of packed-bed reactor (with or without PLD) had no significant effect on the peak full width at half-height and peak area as determined by a *t* test at the 95% confidence level.

A typical calibration curve, charge density (calculated from peak area) vs concentration, for choline is shown in **Figure 2**. An apparent Michaelis−Menten constant of 1.86 ± 0.04 mM, which is in agreement with previous reports (*22*, *44*), and a maximum value of charge density of 765 ± 7 μC cm^{-2} were obtained. The repeatability within days ranged between 2.0% and 3.4% ($n = 5$).

With the use of the PLD-PBR coupled with a ChO biosensor (path A in **Figure 1**), a linear response for choline and phosphatidyl choline standards was obtained in the range from 0.01 to 0.5 mM and from 0.02 to 1 mM, respectively. Unweighted linear regression analysis gave a slope of 10.9 ± 0.2 μC mM^{-1} for phosphatidyl choline and 23.3 ± 0.6 μC mM^{-1} for choline with intercept values not significantly different from zero according to a *t* test with a 95% confidence level and r^2 values greater than 0.998. Residue analysis (*F* test, 0.05 significance level) showed no lack of fit. The limit of detection, calculated at a signal-to-noise ratio of 3, for choline and phosphatidyl choline was 0.02 and 0.03 mM, respectively.

The conversion efficiency, calculated as the ratio of the slopes relevant to the linear range of the calibration curves for

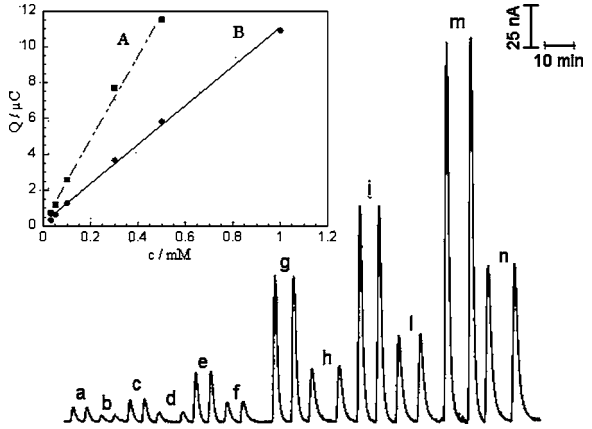

Figure 3. Flow injection peaks for alternate injections of choline standards (a, 0.03 mM; c, 0.05 mM; e, 0.1 mM; g, 0.3 mM; i, 0.5 mM; m, 1 mM) and phosphatidyl choline (b, 0.03 mM; d, 0.05 mM; f, 0.1 mM; h, 0.3 mM; l, 0.5 mM; n, 1 mM) using the phospholipase D packed-bed reactor (path A in **Figure 1**). Experimental conditions are the same as those in **Figure 2**. Inset: A, calibration graph for choline; B, calibration graph for phosphatidyl choline.

Table 1. Effect of Flow Rates on Recovered Choline Concentrations from Phosphatidyl Choline Injections on a Phospholipase D Packed-Bed Reactor[a]

Flow rate (mL min^{-1})	max choline production (mM)	choline production rate (μmol h^{-1})
0.2	0.23 ± 0.01	2.8 ± 0.1
0.3	0.15 ± 0.02	2.7 ± 0.4
0.5	0.09 ± 0.01	2.7 ± 0.3

[a] Standard deviations are calculated directly from the data fitting. The number of samples for each flow rate is 5. The experimental conditions are the same as those in **Figure 4**.

phosphatidyl choline and choline, was equal to approximately 50% (**Figure 3**). This value was dependent on the flow rate used (**Table 1**) and the amount of active enzyme immobilized in the bioreactor. The latter quantity was calculated as follows: Steady-state current−time responses were measured at different phosphatidyl choline concentration (**Figure 4**). These data were fitted with the Michaelis−Menten equation (see inset in **Figure 4**) in order to obtain the i_{max} value (data not shown). This value is proportional to the maximal amount of phosphatidyl choline that can be converted into choline by the bioreactor in the presence of an excess of substrate. The correspondent choline concentration (column 2 in **Table 1**) was calculated by a choline calibration curve, obtained in the same experimental conditions. To obtain the choline production rate in the PLD bioreactor (column 3 in **Table 1**), the obtained values have been multiplied by the flow rate. The choline production rate was not affected by flow rate (0.2, 0.3, and 0.5 mL min^{-1}) as determined by a *t* test with a 95% confidence interval.

After 1 month of use, in the flow injection system, ca. 70% and 80% of the initial response was retained for choline and phosphatidyl choline, respectively. After 6 months of storage at 4 °C, 10% of the initial response was retained.

Determination of Total Choline Content in Milk. Total choline concentration of a milk sample was determined after hydrolysis of the sample (*44*) using the PLD-free PBR coupled with a ChO biosensor (path B in **Figure 1**). To correct for drifting of the biosensor response, the bracketing technique, which allows a two-point calibration, was used for quantification (see ref *44* for details). The choline concentration of the bovine

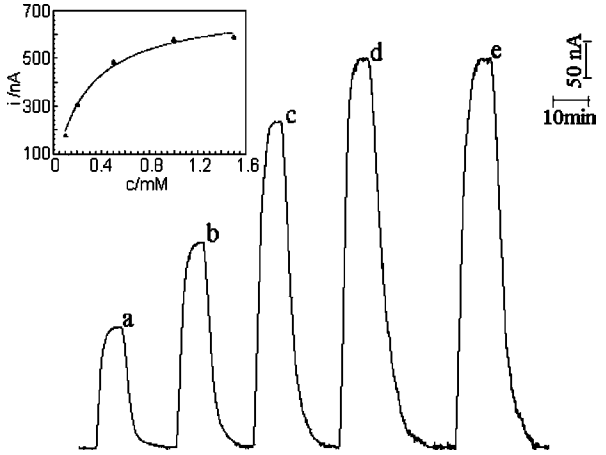

Figure 4. Current–time transients obtained by large volume (ca. 2 mL) injections of phosphatidyl choline standards at the following concentrations: a, 0.1 mM; b, 0.2 mM; c, 0.5 mM; d, 1.0 mM; e, 1.5 mM (path A in **Figure 1**). Inset: calibration curve. Applied potential: +0.7 V vs Ag/AgCl. Carrier: 50 mM ammonium acetate buffer solution (pH 7.0) containing 30 mM calcium chloride and 0.3% (v/v) Triton X-100. Flow rate: 0.2 mL min^{-1}.

milk sample, as determined by the bracketing technique (*44*), was 0.94 ± 0.04 mM ($n = 3$) which is similar to literature values (*6*, *7*).

Determination of Free Choline Content in Milk. Concentrations of free choline in milk were determined using the PLD-free PBR coupled with the ChO biosensor (path B in **Figure 1**). As can be seen from trace 1 in **Figure 5A**, the response of the ChO biosensor was not affected by adding to the milk sample increasing amounts of phosphatidyl choline standard. Furthermore, trace 2 demonstrates and validates the specificity of the biosensor response because it allows a real-time monitoring of the anti-interferent properties of the PPy_{ox} layer. The concentration of free choline in the milk sample, averaged from 10 injections, was 0.36 ± 0.03 mM (repeatibility: 2.8%), which is in good agreement with literature values (*6*, *7*). To our knoweledge, this is the first report on the determination of unesterified choline of an untreated milk sample using an amperometric biosensor.

Determination of Phosphatidyl-Bound Choline in Milk. Concentrations of phosphatidyl-bound choline in milk were determined using the PLD-PBR coupled with the ChO biosensor (path A in **Figure 1**) The PLD-PBR coupled with the ChO biosensor measures choline from free and phosphatidyl-bound choline although choline from phospholipids lysophosphatidylcholine and sphingomyelin might be also released by PLD of Streptomices species (*32*). The obtained responses (see **Figure 5**), after subtraction for the contribution due to the free choline, provide a calibration curve by the "standard addition method". Data extrapolation gave the phospholipid-bound choline concentration (expressed as phosphatidyl choline) equal to 0.3 ± 0.1 mM (number of analyzed samples = 3); this value is in agreement with those reported in the literature, which show, however, a high variability (*6*, *7*).

The concentration of choline esters other than phosphatidyl-bound choline was roughly estimated (ca. 30% of the total choline, $n = 3$) by the difference between the choline concentration and the sum of the free choline and phosphatidyl choline concentration.

Determination of Free Choline and Phosphatidyl Choline in Dietary Supplements. The concentrations of free choline and phosphatidyl choline in dietary supplement samples were

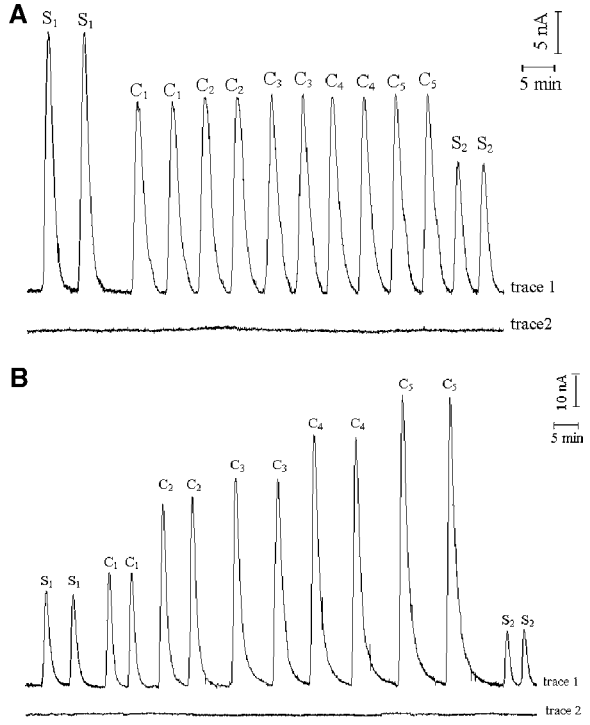

Figure 5. Flow injection peaks obtained using (**A**) the packed-bed (phospholipase D-free) reactor (path B in **Figure 1**) or (**B**) the PLD packed-bed reactor (path A in **Figure 1**). Trace 1: flow injection responses obtained by repetitive injection of 0.1 mM (S_1) and 0.05 mM (S_2) choline standards and milk samples that were diluted 1:4 (v/v) with carrier solution (described in the caption of **Figure 2**) and to which phosphatidyl choline standards were added at the following levels: 0 mM (C_1); 0.25 mM (C_2); 0.5 mM (C_3); 0.75 mM (C_4); 1.0 mM (C_5). Trace 2: response signal of a platinum/overoxidized-polypyrrole sensor during milk sample analysis. Other experimental conditions are the same as those in **Figure 2**.

measured by sequentially injecting the solution obtained as described in the Materials and Methods through the PLD-PBR and the PLD-free PBR. The concentration of phosphatidyl choline was 0.11 ± 0.02 mM or $38 \pm 6\%$ (w/w) ($n = 3$), which is in good agreement with the label information of 35% (w/w). The amount of free choline in the dietary supplement was below the detection limit.

Conclusions. In conclusion, the coupling of a PLD-PBR and a PLD-free PBR with a ChO-based amperometric biosensor allows the quantification of free choline and phosphatidyl-bound choline in raw milk and dietary supplements without any particular pretreatment in ca. 1 h. Acid hydrolysis of the milk samples allows additionally the estimation of the concentration of choline and non-phosphatidyl choline esters in milk so that a rough speciation scheme of the choline composition in milk can be estimated.

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LITERATURE CITED

(1) Zeisel, S. H.; Blusztajn, J. K. Choline and human nutrition. *Annu. Rev. Nutr.* **1994**, *14*, 269−296.
(2) Zaloga, G. P.; Bortenschlager, M. D. Vitamins. In *Nutrition in Critical Care*; Zaloga, G. P., Ed.; Mosby-Year Boo: St. Louis, MO, 1994; pp. 217−242.
(3) Zeisel, S. H. Biological consequences of choline deficiency. In *Choline Metabolism and Brain Function*; Wurtman, R., Wurtman, J., Eds.; Raven Press: New York, 1990; pp 75−99.
(4) Zeisel, S. H. Diet and brain function: available information and misinformation. In *Pediatric Nutrition: Theory and Practice*; Grand, R. J., Sutphen, J. L., Dietz, W. H., Eds.; Butterworths: Boston, MA, 1987; pp 801−808.
(5) Downes, C. P.; Currie, R. A. Lipid signaling. *Curr. Biol.* **1998**, *8*, R865−R867.
(6) Zeisel, S. H.; Char, D.; Sheard, N. F. Coline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. *J. Nutr.* **1986**, *116*, 50−58.
(7) Holmes-McNary, M. Q.; Cheng, W. L.; Mar, M. H.; Fussell, S.; Zeisel, S. H. Choline and choline esters in human and rat milk and in infant formulas. *Am. J. Clin. Nutr.* **1996**, *64*, 572−576.
(8) Blanc, B. Biochemical aspects of human milk−comparison with bovine milk. *World Rev. Nutr. Diet.* **1981**, *36*, 1−89.
(9) George, D. E.; Lebenthal, E. Human breast milk in comparison to cow's milk. In *Textbook of Gastrienterology and Nutrition in Infancy*; Lebenthal, E., Ed.; Raven Press: New York, 1981 pp 295−320.
(10) Casson, C. B.; Griffin, F. J. The determination of egg in certain foods by enzymatic hydrolysis of the phospholipides. *Analyst* **1959**, *84*, 281−286.
(11) Martinez, S. E. V. Simultaneous determination of choline and betaine in some fish materials. *Analyst* **1983**, *108*, 1114−1119.
(12) Lied, E.; Braekkan, O. R. Determination of total choline in biological materials. *Int. J. Vitam. Nutr. Res.* **1975**, *45*, 438−447.
(13) Saucerman, J. R.; Winstead, C. E.; Jones, T. M. Quantitative gas chromatographic headspace determination of choline in adult and infant formula products. *J. AOAC* **1984**, *67*, 982−985.
(14) Carter, N.; Trenerry, V. C. The determination of choline in vitamin preparations, infant formula and selected foods by capillary zone electrophoresis with indirect ultraviolet detection. *Electrophoresis* **1996**, *17*, 1622−1626.
(15) Ikarashi, Y.; Maruyama, Y. Liquid chromatography with electrochemical detection for quantitation of bound choline liberated by phospholipase D hydrolysis from phospholipids containing choline in rat plasma. *J. Chromatogr.* **1993**, *616*, 323−326.
(16) Takayama, M.; Itoh, S.; Nagasaki, T.; Tanimuzu, I. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta* **1977**, *79*, 93−98.
(17) Fleming, J. K.; Gadsden, R. H. Assessment of phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin in human serum. *Clin. Biochem.* **1987**, *20*, 249−256.
(18) Boehringer Mannheim GmbH Biochemica. UV-method for the determination of L-lecithin in foodstuffs and other materials. In *Methods of Enzymatic Food Analysis*; Mannheim, Germany, 1984.
(19) Campanella, L.; Tomassetti, M.; Bruni, M. R.; Mascini, M.; Palleschi, G. Lecithin determination in foods and drugs by an amperometric enzymatic sensor. *Food Addit. Contam.* **1986**, *3*, 277−288.
(20) Karube, I.; Hara, K.; Satoh, I.; Suzuki, S. Amperometric determination of phosphatidylcholine in serum with use of immobilized phospholipase D and choline oxidase. *Anal. Chim. Acta* **1979**, *106*, 243−250.
(21) Panfili, G.; Manzi, P.; Compagnone, D.; Scarmiglia, L.; Palleschi, G. Rapid assay of choline in foods using microwave hydrolysis and a choline biosensor. *J. Agric. Food Chem.* **2000**, *48*, 3403−3407.
(22) Guerrieri, A.; De Benedetto, G.; Palmisano, F.; Zambonin, P. G. Amperometric sensor for choline and acetylcholine based on a platinum electrode modified by a co-cross-linked bienzymatic system. *Analyst* **1995**, *120*, 2731−2736.
(23) Masoom, M.; Roberti, R.; Binaglia, L. Determination of phosphatidylcholine in a flow injection system using immobilized enzyme reactors. *Anal. Biochem.* **1990**, *187*, 240−245.

(24) Kirk, R. E.; Othmer, D. F. *Encyclopedia of Chemical Technology*; John Wiley and Sons: New York, 1981; Vol. 14.
(25) Krull, U. J.; Thompson, M.; Arya, A. Chromatographic analysis of phospholipids and cholesterol oxidation. *Talanta* **1984**, *31*, 489−495.
(26) Cantafora, A.; Di Biase, A.; Alvaro, D.; Angelico, M.; Marin, M.; Attili, A. F. High-performance liquid chromatographic analysis of molecular species of phosphatidylcholine—development of quantitative assay and its application to human bile. *Clin. Chim. Acta* **1983**, *134*, 281−295.
(27) Beare-Rogers, J. L.; Bonekamp-Nasner, A.; Dieffenbacher, A. Determination of the phospholipid profile of lecithins by high-performance liquid chromatography. Results of a collaborative study and the standardized method. *Pure Appl. Chem.* **1992**, *64*, 447−454.
(28) Tomassetti, M.; Campanella, L.; Salvi, A. M.; D'Ascenzo, G.; Curini, R. J. Total phosphorus determination in human bile: comparison between two spectrophotometric methods. *Pharm. Biomed. Anal.* **1984**, *2*, 417−424.
(29) Murison, J.; Festi, D.; Ross, P. E.; Bouchier, I. A. D. The estimation of phospholipids in bile. *Clin. Chim. Acta* **1976**, *68*, 159−166.
(30) Bartlett, G. R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **1959**, *234*, 466−468.
(31) Annoni, G.; Dioguardi, M. L.; Tripodi, A.; Zuin, M. Enzymatic assay of choline-containing phospholipids in bile. *Clin. Chem.* **1980**, *26*, 669−670.
(32) Anaokar, S.; Garry, P.; Standeferm, J. C. Enzymatic assay for lecithin in amniotic fluid. *Clin. Chem.* **1979**, *25*, 103−107.
(33) Campanella, L.; Magrì, A. L.; Sorbo, A.; Tomassetti, M. Determination of lecithin drug specialties and diet integrators; by means of first or second derivative enzymatic-spectrophotometric analysis. *J. Pharm. Biomed. Anal.* **2002**, *29*, 1135−1148.
(34) Campanella, L.; Pacifici, F.; Sammartino, M. P.; Tomassetti, M. A new organic phase bienzymatic electrode for lecithin analysis in food products. *Bioelectrochem. Bioenerg.* **1998**, *47*, 25−38.
(35) Lima, J. L. F. C.; Delerue-Matos, C.; Vaz, M. C. V. F. Enzymatic determination of choline in milk using a FIA system with potentiometric detection. *Analyst* **2000**, *125*, 1281−1284.
(36) Centonze, D.; Guerrieri, A.; Malitesta, C.; Palmisano, F.; Zambonin, P. G. Interference-free glucose sensor based on glucose oxidase immobilized in an overoxidized nonconducting polypyrrole film. *Fresenius' J. Anal. Chem.* **1992**, *342*, 729−733.
(37) Centonze, D.; Zambonin, C. G.; Palmisano, F. Determination of glucose in nonalcoholic beverages by a biosensor coupled with microdialysis fiber samplers. *J. AOAC Int.* **1997**, *80*, 829−833.
(38) Palmisano, F.; Centonze, D.; Guerrieri, A.; Zambonin, P. G. An interference-free biosensor based on glucose oxidase electrochemically immobilized in a nonconducting poly(pyrrole) film for continuous subcutaneous monitoring of glucose through microdialysis sampling. *Biosens. Bioelectron.* **1993**, *8*, 393−399.
(39) Palmisano, F.; De Santis, A.; Tantillo, G.; Volpicella, T.; Zambonin, P. G. Microbial detection by a glucose biosensor coupled to a microdialysis fiber. *Analyst* **1997**, *122*, 1125−1128.
(40) Palmisano, F.; Guerrieri, A.; Quinto, M.; Zambonin, P. G. Electrosynthesized bilayer polymeric membrane for effective elimination of electroactive interferents in amperometric biosensors. *Anal. Chem.* **1995**, *67*, 1005−1009.
(41) Palmisano, F.; Centonze, D.; Quinto, M.; Zambonin, P. G. A microdialysis fiber-based sampler for flow-injection analysis: determination of L-lactate in biofluids by an electrochemically synthesized bilayer membrane-based biosensor. *Biosens. Bioelectron.* **1996**, *11*, 419−425.
(42) Guerrieri, A.; De Benedetto, G. E.; Palmisano, F.; Zambonin, P. G. Electrosynthesized nonconducting polymers as permselective membranes in amperometric enzyme electrodes: a glucose biosensor based on a co-cross-linked glucose oxidase/overoxidized polypyrrole bilayer. *Biosens. Bioelectron.* **1998**, *13*, 103−112.
(43) Palmisano, F.; Rizzi, R.; Centonze, D.; Zambonin, P. G. Simultaneous monitoring of glucose and lactate by an interference and cross-talk free dual electrode amperometric biosensor based on electropolymerized thin films. *Biosens. Bioelectron.* **2000**, *15*, 531−539.
(44) Pati, S.; Quinto, M.; Palmisano, F.; Zambonin, P. G. Determination of choline in milk, milk powder, and soy lecithin hydrolysates by flow injection analysis and amperometric detection with a choline oxidase based biosensor. *J. Agric. Food Chem.* **2004**, *52*, 4638−4642.
(45) Woollard, D. C.; Indyk, H. E. The routine, enzymatic estimation of total choline in milk and infant formulas. *J. Micronutr. Anal.* **1990**, *7*, 1−14.
(46) Woollard, D. C.; Indyk, H. E. Determination of choline in milk and infant formulas by enzymatic analysis: collaborative study. *J. AOAC* **2000**, *83*, 131−138.
(47) Coulon-Morelec, M. J.; Faure, M. Methods of phosphatidic acid preparation I. Extraction and purification of phosphatidic acid from from wheat germ. *Bull. Soc. Chim. Biol. (in French)* **1967**, *49*, 273−278.
(48) Faure, M.; Troestler, J. Methods of phosphatidic acid preparation II. Phosphatidic acid production from enzymatic degradation of lecithin by phospholipase D. *Bull. Soc. Chim. Biol. (in French)* **1967**, *49*, 279−290.
(49) Gurantz, D.; Laker, M. F.; Hofmann, A. F. Enzymatic measurement of choline containing phospholipids in bile. *J. Lipid Res.* **1981**, *22*, 373−376.

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