AAPS PharmSciTech **2003; 4 (4) Article 63 (http://www.aapspharmscitech.org).**

Quantification of Various Phosphatidylcholines in Liposomes by Enzymatic Assay

Submitted: July 14, 2003; Accepted: October 20, 2003

Holger Grohganz,[1] Vittorio Ziroli,[2] Ulrich Massing,[2] and Martin Brandl[1,2]

[1]Department of Pharmaceutics and Biopharmaceutics, Institute of Pharmacy, University of Tromsø, N-9037 Tromsø, Norway
[2]Tumor Biology Center, Department of Clinical Research, Breisacher Strasse 117, D-79106 Freiburg, Germany

ABSTRACT

The purpose of this research was to adapt a colorimetric, phospholipase D-based serum-phospholipid assay for the quantification of phosphatidylcholine (PC) in liposomes using a microtitre plate reader. PC from natural egg PC liposomes was quantified reliably. In contrast, poor sensitivity was found for liposomes composed of saturated PCs (dipalmitoyl-phosphatidylcholine [DPPC], hydrogenated egg PC). Triton X-100 was then added to the liposomes followed by heating above the phase transition temperature. This modified sample preparation resulted in recoveries of 102.6% ± 1.0%, 104.4% ± 7.6%, and 109.4% ± 3.2% for E80, E80-3/cholesterol, and DPPC liposomes, respectively. Absolute quantification of unknown PCs against a choline chloride standard is feasible, but relative measurements against the very same PC are recommended whenever possible. Validation experiments revealed an absolute quantification limit of 1.25 µg per assay, a good linearity in the range of 25 to 1000 µg/mL PC ($r^2 \geq 0.9990$) and a quite high accuracy (99.8%-101.4% of theory) and precision (relative standard deviation ≤ 3.2%) for all 3 PCs studied. The method is thus regarded as suitable for sensitive, rapid, and reliable routine quantification of PCs in liposomes.

KEYWORDS: quantification, phosphatidylcholine, liposome, microplate, enzymatic assay, colorimetric assay, phospholipase D

Corresponding Author: Martin Brandl, Department of Pharmaceutics and Biopharmaceutics, Institute of Pharmacy, University of Tromsø, N-9037 Tromsø, Norway;
Tel: +47 77646159; Fax: +47 77646151;
Email: martinb@farmasi.uit.no

INTRODUCTION

The level of phosphatidylcholine (PC) is a key variable of liposome-based drug formulations for sustained release and/or targeting. To date, routine quantification of PC mainly employs quantitative thin layer chromatography and various forms of wet digestion methods whereby colorimetric or turbidimetric analysis[1,2] are being used. A colorimetric analysis after extraction with organic solvents is also described in the literature.[3] All these methods are quite time consuming. For the determination of choline-containing phospholipids in physiological substrates such as serum, high density lipoproteins, amniotic fluid, and bile, various enzymatic assays have been described.[4-7] These assays are based on phospholipase D cleaving off the choline moiety, which is subsequently oxidized by choline oxidase forming stoichiometric amounts of hydrogen peroxide. The latter takes part in a peroxidase-catalyzed coupling of 4-aminoantipyrine with phenol, which is quantified colorimetrically. Test kits containing the required enzymes and substrates are now commercially available. Nie et al[8] described a microadaptation of this assay using a 96-well plate reader. Campanella tested a phospholipase D/choline oxidase-based biosensor for analysis of lecithin in dry food and pharmaceutical products upon dissolution in organic solvents.[9]

Here, we describe an adaptation of the enzymatic assay, which allows for quantification PCs in liposome dispersions in microtiter plate format. The objective of the research was to investigate the influence of reaction time and sample preparation with the purpose of establishing a micromethod with high reliability in the concentration range of 25 to 1000 µg/mL phospholipid. The method was furthermore to be validated for various lipids. A poor response in the case of saturated PC-containing liposomes and liposomes containing cholesterol was overcome by transferring the liposomes with tensides into mixed micelles.

MATERIALS AND METHODS

Materials

The commercial phospholipid kit "Phospholipids B – enzymatic colorimetric method" was purchased from Wako Chemicals (Neuss, Germany). E80 (egg phospholipid with at least 80% PC and natural fatty acid composition) and E80-3 (saturated egg phospholipid with at least 80% PC) were a gift from Lipoid (Ludwigshafen, Germany). Dipalmitoyl-phosphatidylcholine (DPPC) was purchased from Genzyme Pharmaceuticals (Sygena Facility, Liestal, Switzerland). Cholesterol (Chol) was obtained from Croda Chemicals (Goole, UK) and recrystallized from methanol. Triton X-100 was obtained from Sigma-Aldrich Chemie (Steinheim, Germany); $CaCl_2 \times 6H_20$ AnalaR, from BDH Laboratory Supplies (Poole, England); and Tris-HCl pro analysi, from Merck KgaA (Darmstadt, Germany). High-performance liquid chromatography (HPLC)-grade chloroform and methanol were obtained from Merck KgaA. A Fluostar Galaxy titer plate reader by BMG Labtechnologies (Offenburg, Germany) together with Costar transparent 96-well plates (Corning, Corning, NY) was used for all measurements.

Methods

The coloring reagent solution was prepared by adding 45 mL of buffer solution (consisting of 50mM Tris buffer, 5 mg/dL calcium chloride, and 0.05% phenol) to the dry coloring reagent (phospholipase D 20 U, choline oxidase 90 U, peroxidase 240 U, 4-aminoantipyrin). Each plate well was filled with 50 µL of the phospholipid dispersion and 250 µL of coloring reagent. Plates were warmed to 37°C using the interior heating system of the titer plate reader and initially shaken for 5 minutes. The quantification was performed after various reaction times by measuring the absorption at λ = 492 nm after confirming that the final colored complex had a very broad absorption maximum between 480 and 510 nm (data not shown). All samples were prepared and measured in triplicate.

Liposome dispersions were produced by suspending appropriate amounts of phosphatidylcholine in 1.5 mL water or pH 8.0 buffer solution by shaking in a ball mill for 25 minutes at room temperature on addition of 5 glass beads (diameter ~1 mm). The resulting dispersion was transferred into a 50-mL volumetric flask and diluted with the corresponding medium to make a stock solution. In the case of cholesterol-containing liposomes, a homogeneous mixture was first achieved by dissolving phospholipid and cholesterol in a mixture of chloroform and methanol (2:1, vol/vol) and evaporating the solvent. The resulting dry powder was further processed as described above. The ratio of E80-3:cholesterol used for all experiments was 55:45 (mol/mol).

RESULTS AND DISCUSSION

Method Development

The investigation of liposome dispersions containing different phospholipids in aqueous dispersion led to different results. The analysis of liposome dispersions with increasing concentrations (up to 700 µg/mL) of unsaturated egg PC (E80) yielded a good correspondence between the absorbance and corresponding amounts of choline chloride (standard). In sharp contrast, a much lower, though linearly increasing absorbance was observed for saturated egg PC (in the form of E80-3/cholesterol liposomes) (**Figure 1**).

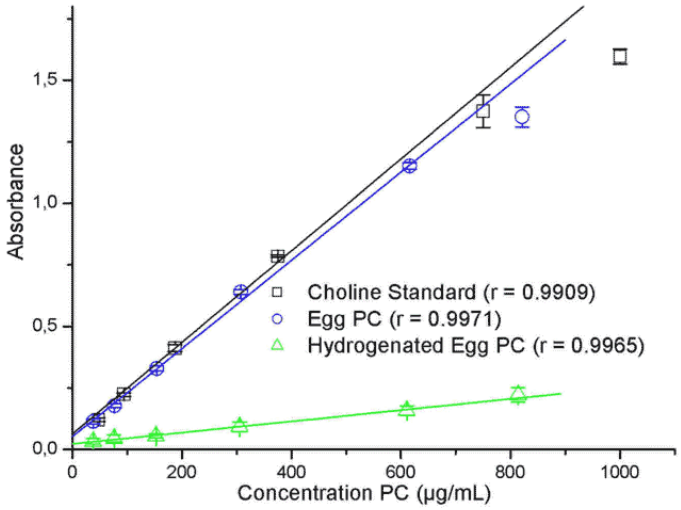

Figure 1. Absorbance obtained upon enzymatic reaction (15 minutes of incubation at 37°C) of choline chloride and 2 types of PC in water versus concentration of PC. Concentrations of E80 and E80-3 are standardized to PC content. Choline chloride concentration is given as corresponding PC concentration. All values are mean ± SD (n = 3).

It appeared that the quantification of egg PC in liposomes could be performed using the method described. For saturated egg PC, however, the recovery was very low, possibly because of the following:

- slower reaction kinetics of phospholipase D with saturated PCs
- incomplete enzymatic reaction because of restricted accessibility of saturated PCs, when incorporated within (cholesterol-containing) liposomes

Hypothesis 1

According to the standard procedure recommended by the manufacturer, the samples had been incubated at 37°C for 15 minutes. This, however, might be too short in case of hydrogenated egg PC/cholesterol-liposomes. The experiment was thus repeated with an incubation time of 45 minutes. Furthermore, liposomes made of a second type of saturated PC (DPPC), but with no cholesterol present, were assayed. The results are given in **Figure 2**.

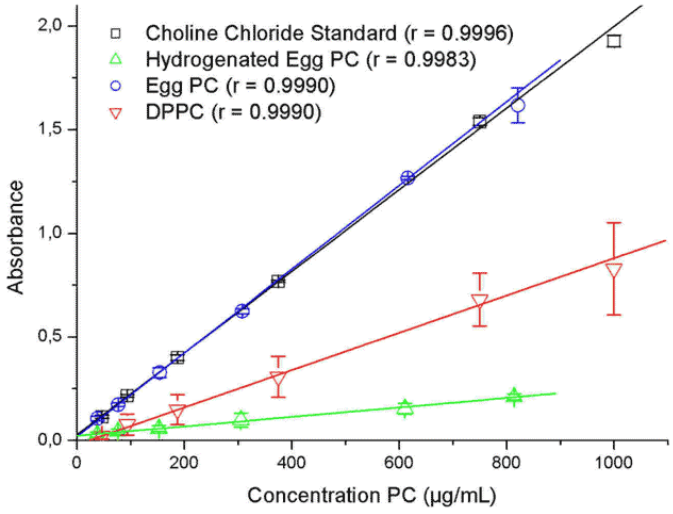

Figure 2. Absorbance obtained upon enzymatic reaction (45 minutes incubation at 37°C) of liposomes of 3 types of PC (egg PC, hydrogenated egg PC/cholesterol, and DPPC, respectively) as well as choline chloride standard in water versus concentration of PC. Concentrations pf E80 and E80-3 are standardized to their respective PC content. Choline chloride concentration given as corresponding PC concentration. All values are mean ± SD (n = 3).

Again, for liposomes containing egg PC a close to 100% recovery was seen, whereas for both types of saturated PC liposomes a linear, but lower response was obtained as compared with choline standard. As an increase in incubation time did not lead to a noticeable increase in the absorbance of hydrogenated PCs compared with the choline chloride standard, a too short incubation time may be ruled out as the main reason for the lower response.

Hypothesis 2

Next, attempts were made to find out if incorporation within liposomes restricts the accessibility of the PCs for phospholipase D. For vesicles made of egg yolk, a product-retardation of phospholipase D-catalyzed hydrolysis was described by Yamamoto.[10] Moreover, phospholipids with saturated fatty acid chains are known to form more rigid and stable membranes than natural phospholipids. The following techniques were used in order to potentially increase the accessibility of the choline moiety to enzymatic cleavage:

- transient warming of the liposome dispersion over the phase transition temperature
- transfer of the liposomes into mixed micelles by detergent and heat transfer

The influence of detergent and increased temperature on PC recovery was investigated using DPPC liposomes. Three samples were prepared by (1) just dispersing DPPC in buffer, (2) by dispersing DPPC in buffer and transient heating to 60°C, and (3) by heating the DPPC dispersion in the presence of 2.5% Triton X-100. Upon dilution to concentrations of 47 to 1000 µg/mL, addition of the color reagent, and incubation for 45 minutes reaction time absorbance was measured (**Figure 3**).

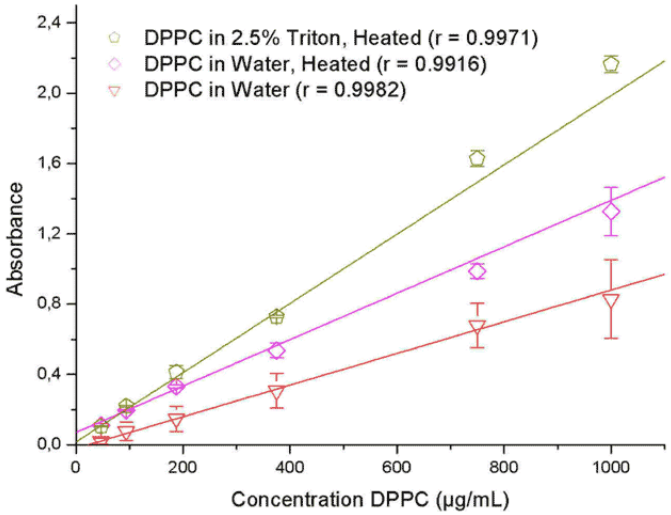

Figure 3. Absorbance obtained for enzymatic assay of DPPC liposomes treated by 3 different means (dispersion, dispersion + warming, and dispersion + addition of 2.5% Triton X-100 + warming) versus concentration of PC. Reaction time is 45 minutes. All values are mean ± SD (n = 3).

A significant increase in absorbance was seen for the Triton-treated sample. A distinct, though smaller, increase was also seen for the sample that was just heated and recooled. This may indicate that both transient warming above the phase transition temperature and transfer of the liposomes into mixed micelles indeed improve the accessibility of phospholipase D to DPPC. It is obvious that the recovery of saturated PC from liposomes can be improved to levels close to theory by Triton-induced transfer into mixed micelles. This fits with the observation of Yamamoto, whereby phospholi-

Table 1. Limits of Detection and Quantification of PC From Liposomes Made of 3 Different Lipids*

Lipid	E80 Liposomes	E80-3/Chol Liposomes	DPPC Liposomes
Limit of detection	7.6 ± 2.4	4.6 ± 4.1	4.1 ± 3.4
Limit of quantification	23.1 ± 7.2	14.1 ± 12.6	12.4 ± 10.2

*Chol indicates cholesterol; DPPC, di-palmitoyl-phosphatidylcholine; and PC, phosphatidylcholine. Average ± SD of 6 measurements each (n = 3). All values are μg/mL.

pase D-catalyzed hydrolysis of egg yolk vesicles in the presence of a nonionic surfactant was found to be complete.

The following techniques were found appropriate for transformation of liposomes into micelles as judged by the disappearance of visual turbidity (ie, obtaining clear solutions):

- egg PC (E80) liposomes: addition of 2.5% Triton X-100 (without heating)
- hydrogenated egg PC (E80-3)/cholesterol liposomes: addition of 10% Triton X-100 and heating to 60°C for 10 to 15 minutes, recooling to room temperature
- DPPC liposomes: either heating to 60°C for 10 to 15 minutes with 2.5% Triton X-100 or overnight standing at room temperature with 10% Triton X-100

Final Method

For further experiments, the following final conditions of sample preparation and analysis were thus chosen: the phospholipid was dispersed in buffer solution. The mixture was redispersed in a ball mill at 30 Hertz for 25 minutes upon addition of 5 glass beads (diameter ~1 mm). Buffer solutions, pH 8.0, were supplemented with 2.5% (for E80 samples) or 10% (for E80-3/cholesterol and DPPC samples) Triton X-100 and 50mM Tris-HCl and 0.34mM $CaCl_2$, respectively. This dispersion was heated to 60°C, if necessary, then recooled and diluted to a concentration of 1000 μg/mL with the corresponding buffer. For the analysis, 50 μL of sample and 250 μL of coloring reagent were allowed to react for 45 minutes while being incubated in the titer plate reader at 37°C. UV absorbance was measured at λ = 492 nm against a blank consisting of 50 μL buffer containing Triton and 250 μL coloring reagent.

Validation

Limit of Detection and Limit of Quantification

The range of PC contents that can be quantified was investigated for each of the 3 PCs. The lower limits of detection and quantification were calculated according to the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines for the validation of analytical methods using the following formulas:

- Limit of detection = 3.3 x SD of blank / slope of calibration curve
- Limit of quantification = 10 x SD of blank / slope of calibration curve

For the determination of the limits, the averages of 6 measurements (ie, 6 separate plates) with 3 parallels on each plate were calculated. The lower limits of detection and quantification varied somewhat between the 6 separate measurements (**Table 1**).

Considering the means and SDs in **Table 1**, a common lower limit of quantification for all 3 phospholipids was set to 25 μg/mL. This corresponds to 1.25 μg per assay, a limit of quantification that is about an order of magnitude lower than what was reported earlier for both the enzymatic assay[8] and the phosphorus assays.[1,2] Kinetic experiments revealed that the enzymatic reaction does not in all cases reach the endpoint under the chosen conditions if PC in amounts of 1200 μg/mL and above are present (data not shown). The upper limit of quantification therefore was set to 1000 μg/mL.

Recovery

The recovery of PC from E80, E80-3/cholesterol, and DPPC-liposomes upon transfer into mixed micelles was assessed by comparing the true PC-content (as indicated by the manufacturer) with the measured PC-content as evaluated against the chloride standard curve. The recovery of the various PCs was found as follows: E80 liposomes 102.6% ± 1.0% (n = 5), E80-3/cholesterol liposomes 104.4% ± 7.6% (n = 3), and DPPC liposomes 109.4% ± 3.2% (n = 6). Considering that both the natural phospholipids contained around 2.0% sphingomyelin, which also is a substrate of phospholipase D, the method is regarded as highly accurate for E80 and E80-3 liposomes. The recovery obtained for DPPC liposomes is considered satisfactory. In order to minimize experimental errors, however, a standard dispersion of the very same phosphatidylcholine should be used, rather than a choline chloride standard.

Table 2. Linearity of Calibration Curves for Egg PC, Hydrogenated Egg PC, and DPPC*

Lipid	Slope	r^2-Value
E80	1.767 ± 0.046	0.9999 ± 0.0001
E80-3	1.985 ± 0.065	0.9990 ± 0.0010
DPPC	2.357 ± 0.077	0.9999 ± 0.0001

*DPPC indicates di-palmitoyl-phosphatidylcholine; and PC, phosphatidyl-choline. Mean and SD values of 6 independent parallels. The difference in slope is due to the different PC content in the various phospholipids.

Table 3. Intra-Plate Variation*

Lipid	Accuracy at Level 200 µg/mL	Accuracy at Level 800 µg/mL	Precision at Level 200 µg/mL	Precision at Level 800 µg/mL
E80	98.9%-102.1%	98.4%-101.9%	2.2%-3.9%	0.7%-1.1%
E80-3	99.8%-102.4%	98.1%-101.3%	2.1%-3.1%	1.1%-1.8%
DPPC	99.4%-102.8%	96.0%-101.8%	0.7%-2.6%	1.7%-5.2%

*DPPC indicates di-palmitoyl-phosphatidylcholine. Range of averages (n = 6) of 6 plates is given. Accuracy is given as percentage of the true value. Precision is given as relative SD.

Table 4. Intermediate Precision (Day-to-Day Variation) and Accuracy for Various PCs*

Lipid	Accuracy at Level 200 µg/mL	Accuracy at Level 800 µg/mL	Precision at Level 200 µg/mL	Precision at Level 800 µg/mL
E80	100.3%	100.3%	3.2%	0.9%
E80-3	101.4%	99.9%	2.7%	1.5%
DPPC	100.7%	99.8%	1.6%	2.9%

*DPPC indicates di-palmitoyl-phosphatidylcholine; and PC, phosphatidylcholine. Accuracy is given as percentage of true value. Precision is given as relative SD of the mean (n = 6).

Linearity and Repeatability

For the determination of linearity (**Table 2**) as well as accuracy and precision (**Tables 3** and **4**), 6 separate plates were measured per lipid with each value of the standard curve (25-1000 µg/mL) prepared in triplicate. Each control sample concentration (200 and 800 µg/mL) was produced 6 times, pipetted 3 times into the titer plate, and measured. As recommended above, the same lipid was used for standard curve as for accuracy and precision measurements. Typical calibration curves for the 3 lipids are shown in **Figure 4**.

All lipids showed very high r^2- values (≥ 0.9990) demonstrating the method to be highly linear up to 1000 µg/mL lipid. The differences in slope between the different phospholipids are the result of different PC contents.

Both precision on the same plate (intra-assay precision, **Table 3**) and precision between plates (intermediate precisions, **Table 4**) reveal that the PC content of liposomes can be quantified accurately and reproducibly by the current method.

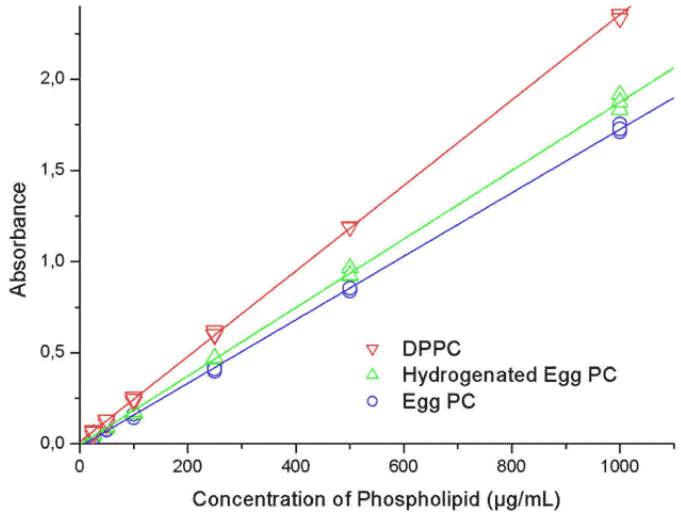

Figure 4. Calibration curves for egg PC, hydrogenated egg PC, and DPPC. Absorbance at λ = 492 nm over phospholipid concentration (n = 3).

Robustness

In order to evaluate how deviations from the chosen reaction time influence the result, the absorbance was determined at 30, 45, and 60 minutes of reaction time for all phospholipids and concentrations. A Student *t* test was performed (after performing an F test to see if a *t* test was applicable) in order to check whether the response at 45 minutes reaction time differed statistically from other reaction times (30 and 60 minutes). No relevant differences between different reaction times were found for all PCs at contents of 25, 50, 100, and 250 µg/mL (ie, at these concentration levels the reaction was completed after 30 minutes and the color stayed stable for up to 60 minutes). For the highest concentration, 1000 µg/mL, the results are slightly different. The numbers of statistically significantly different parallels as compared with the total number of valid parallels are given in **Table 5**.

Table 5. Statistically Significantly Different Means Compared With the Total Number of Valid Parallels at Distinct Reaction Times and a Concentration of 1000 µg/mL for 3 PCs*

Type of PC	Comparison Between 30 and 45 Minutes	Comparison Between 45 and 60 Minutes
E80	3 out of 6	1 out of 4
E80-3/Chol	6 out of 6	3 out of 6
DPPC	5 out of 5	0 out of 5

*Chol indicates cholesterol; DPPC, di-palmitoyl-phosphatidylcholine; and PC, phosphatidylcholine.

For the highest concentration level studied here (1000 µg/mL), absorbances after an incubation time of 45 minutes mostly were significantly higher than after 30 minutes, whereas after 60 minutes, most parallels did not differ significantly from the 45-minute values. At this concentration level, a reaction time of less than 45 minutes may negatively affect the result and must be avoided. This holds true especially for E80-3/Cholesterol-liposomes.

CONCLUSION

The quantification of PCs incorporated in liposomes by a phospholipase D–based microenzymatic assay has been found incomplete in case of saturated PCs. This effect has been overcome by detergent-induced transfer of the liposomes into mixed micelles, or alternatively by dissolution by organic solvents. A modification of the assay is presented, suitable for quantification of both natural hydrogenated and synthetic PCs in liposomes. Absolute quantification of unknown PCs against choline standard appears feasible, but relative measurements against the very same PC are recommended whenever possible. During validation, good accuracy and precision of the PC test in the range of 25 to 1000 µg/mL for all 3 liposome formulations under investigation were demonstrated.

REFERENCES

1. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem*. 1959;234:466-468.

2. Eibl H, Lands WE. A new, sensitive determination of phosphate. *Anal Biochem*. 1969;30:51-57.

3. Stewart JC. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal Biochem*. 1980;104:10-14.

4. Takayama M, Itoh S, Nagasaki T, Tanimizu I. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin Chim Acta*. 1977;79:93-98.

5. Gurantz D, Laker MF, Hofmann AF. Enzymic measurement of choline-containing phospholipids in bile. *J Lipid Res*. 1981;22:373-376.

6. McGowan MW, Artiss JD, Zak B. A procedure for the determination of high-density lipoprotein choline-containing phospholipids. *J Clin Chem Clin Biochem*. 1982;20:807-812.

7. Bradley CA, Salhany KE, Entman SS, Aleshire SL, Parl FF. Automated enzymatic measurement of lecithin, sphingomyelin, and phosphatidylglycerol in amniotic fluid. *Clin Chem*. 1987;33:81-86.

8. Nie Y, He JL, Hsia SL. A micro enzymic method for determination of choline-containing phospholipids in serum and high density lipoproteins. *Lipids*. 1993;28(10):949-951.

9. Campanella L, Pacifici F, Sammartino MP, Tomassetti M. Analysis of lecithin in pharmaceutical products and diet integrators using a new biosensor operating directly in non aqueous solvent. *J Pharm Biomed Anal*. 1998;18:597-604.

10. Yamamoto I, Nishii M, Tokuoka E, Handa T, Miyajima K. Product-retardation and -activation of catalytic hydrolysis by phospholipase D in small unilamellar vesicles of egg yolk phosphatidylcholine. *Colloid Polym Sci*. 1997;275:627-633.