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Determination of choline containing phospholipids in serum, bile and amniotic fluids by the derivative enzymatic–spectrophotometric method

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Abstract

A comparison was performed of the analytical results obtained by applying a well-known enzymatic–spectrophotometric method for the determination of choline containing phospholipids in biological human fluids (sera, bile and amniotic fluids) following the standard procedure method, or else using the first or second derivative methods. In terms of result reproducibility the comparison was extended to include also a biosensor-based method developed in recent years by the present authors. Some advantages are associated with using the first derivative method in the case of serum samples containing blood traces, especially via the 'Gran's plot' method.

Keywords: Phospholipids; Analysis; Derivative method; Biological fluids

1. Introduction

Phospholipids are important constituents of biological cell membranes and are found in many animal tissues and organs. It is thus important to develop rapid and effective analytical methods for determining them in clinical tests.

The analysis of phospholipids can be performed by chromatographic methods [1–3], for which separation–extraction processes of the sample and derivatisation of the analyte are generally required, and so these methods are not easily automated. The phospholipid concentration can be also obtained by total phosphorus analysis using the molybdate–vanadate [4] or Bartlett's method [5]; also these methods require prior sample treatment using strong acidic and oxidant mixtures at about 180 °C. For this reason, in more recent years, a selective and direct enzymatic–spectrophotometric method was introduced [6–10], based on two in series reactions catalysed, respectively, by phospholipase D and choline oxidase enzymes, and on the subsequent reaction between hydrogen peroxide, obtained in the latter enzymatic reaction, with phenol and 4-aminophenazone (4-AAP) in the presence of peroxidase; the spectrophotometric measurement is performed at $\lambda = 500$ nm. However, as explained in a previous paper [11], this method may

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present some drawbacks if the sample is pigmented or turbid. It was precisely to overcome these difficulties that the present authors recently proposed a method of phospholipid determination [12] based on the above two enzymatic reactions, but using the first and/or second derivative in enzymatic–spectrophotometric analysis to directly determine the phospholipid (lecithin) content in several real matrices (food or drugs) [12] without the need for laborious sample pre-treatment or in any case limiting it to rapid operations such as simple centrifugation.

The aim of the present research was to examine the possibility of applying the new derivative method to determining choline-containing phospholipids directly in human serum, amniotic fluids and bile samples.

We investigated the possibility of using the first and/or second derivative in enzymatic–spectrophotometric analysis to directly determine lecithin content in several of these real solutions without the need for laborious sample pre-treatment or any separation method.

2. Experimental

2.1. Apparatus

The spectrophotometric measures were performed using a model 320 UV-Vis Perkin-Elmer double beam, double grating monochromator spectrophotometer. The output signal was digitised, put through a central processor unit (CPU) and then sent to the recording system and/or interface to the Perkin-Elmer model 3600 data station. Using dedicated software, this system allowed both the spectrum scanning parameters to be programmed and the spectrophotometric data to be stored on disk for further processing. The spectrophotometer was maintained at $(25 \pm 0.1)\,^{\circ}C$ by means of a Colora ultrathermostat.

The spectrophotometer was also able to convert the signal up to a fourth order derivative, with $\Delta\lambda$ ranging from 1 to 10. Quartz cuvettes with a 1.00 cm optical path length were used, together with a Julabo model UC 5B thermostat.

2.2. Reagents and materials

All the reagents and enzymes needed for the enzymatic–spectrophotometric method used were supplied by Poli (Milan) as a ‘sole reagent’ contained in the ‘Enzyfast Phospholipids-Trinder Method’ cod. 3220. The standard solution was supplied by the Poli company.

2.3. Samples analysed

The following biological samples were analysed:

- 3 samples of human serum belonging to male subjects,
- 3 samples of human serum belonging to female subjects,
- 1 sample of human bile,
- 1 sample of human bile containing traces of blood,
- 1 sample of amniotic fluid at 16th week of pregnancy,
- 1 sample of amniotic fluid at 17th week of pregnancy,
- 1 sample of amniotic fluid at 18th week of pregnancy.

The sera were examined without any preliminary treatment. It was necessary to perform prior dilution of the bile samples (about 10-fold), using the same buffer as that used in the enzymatic test. In the case of the amniotic fluid samples it was necessary to take samples about 50 times larger than those used in the standard method.

3. Methods

3.1. Enzymatic–spectrophotometric determination

Phosphatidylcholine content was determined in human biological samples using the above cited spectrophotometric detection enzymatic method [6–10].

The method is based on the following enzymatic reactions:

$$\text{Phosphatidylcholine} + H_2O \xrightarrow{\text{Phospholipase D}} \text{Choline} + \text{Phosphatidic acid}$$

$$\text{Choline} + 2O_2H_2O \xrightarrow{\text{Choline oxidase}} \text{Betaine} + 2H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-AAP}^* \xrightarrow{\text{Peroxidase}} \text{Quinone chromogen}$$

*4-AAP = 4-aminophenazone

The standard method consists of adding 20.0 μl of sample to 2.0 ml of 'sole reagent'.

The reagent is obtained by mixing 36 ml of buffer with suitable quantities of freeze-dried enzymes before use, so that the final composition of the reagent is as follows: Pipes buffer (1,4-piperazine diethane-sulfonic acid) (75 mmol l^{-1}) at pH 7.9, containing phenol (7.0 mmol l^{-1}), 4-AAP (0.5 mmol l^{-1}) and 1.0 g l^{-1} of non ionic detergent, plus phospholipase D (2.5 U ml^{-1}), choline oxidase (10 U ml^{-1}) and peroxidase (2.5 U ml^{-1}).

Taking into consideration both the error due to sampling very small volumes and the need to obtain sufficient amounts of reagent to fill the cuvettes without difficulty, we preferred in each case to take 50.0 μl of sample and 3.0 ml of reagent.

The solution thus obtained was incubated at 37.0 °C for 15 min and, after cooling to room temperature, the absorbance at $\lambda = 500$ nm was measured and always read at most 1 h after the end of the thermostatting period prescribed for the method.

Throughout the investigation the absorption spectrum was always scanned between 360 and 700 nm. The scanning rate was set at 60 nm min^{-1} using a slit width of 2.0 nm. Derivative analysis was performed electronically using a $\Delta\lambda$ of 5 nm.

The absorbance reading was always performed against a distilled water blank. After the reading, the absorbance values of the solution containing only the sample in Pipes buffer ('sample blank') and the blank of the solution containing the 'sole reagent' in Pipes buffer, to which 50 μl of distilled water had been added ('reagents blank'), were always subtracted.

For the derivative spectra it was sufficient to subtract the 'reagents blank'.

Phosphatidylcholine solution supplied by the Poli company was used as standard both for the analysis of the biological fluids and for the preliminary calibration curves required for method optimisation, which were constructed by testing solutions of increasing standard phosphatidylcholine content.

For the 'standard' method, the readings were performed at a wavelength of 500 nm, that is at the absorption band maximum.

For the first derivative method the distance (expressed in arbitrary units) between the tangents to the maximum at 455 nm and the minimum at 557 nm were evaluated.

For the second derivative method a double reading was performed. In this case, a minimum actually appeared at 500 nm and two peaks at 412 and 600 nm, respectively. An evaluation was thus made of the distance (expressed in arbitrary units) between the tangent to the minimum and that to the maximum which occurs at 412 nm, or to that which occurred at 600 nm.

4. Results

Table 1 shows all the equations of calibration curves, their confidence interval and the correlation coefficients characterising the 'standard' and the derivative methods.

The main real samples analysed consisted above of a human serum samples drawn from both male and female subjects. In detail, samples taken from female subjects are marked as serum 1, serum 3 and serum 5, and those from male subjects as serum 2, serum 4 and serum 6, respectively.

Fig. 1 show the absorption spectrum obtained using the 'standard method' and first and second derivative

Table 1
Analytical data of analysed standard phosphatidylcholine samples

Method	Equation of calibration curve and confidence interval; $y = mx + b$; y = absorbance, x = mg l^{-1}; $y' = \Delta$l (a.u.), x = mg l^{-1}; $y'' = \Delta$l (a.u.), x = mg l^{-1}; $t = 2.78$; $1 - \alpha = 0.95$	Correlation coefficient
Standard method	$y = (0.014_5 \pm 0.000_4)x + (0.032_2 \pm 0.033_5)$	0.9997
First derivative method	$y' = (0.011_7 \pm 0.000_3)x + (0.021_2 \pm 0.025_3)$	0.9998
Second derivative method (reading at 412–500 nm)	$y'' = (0.011_4 \pm 0.001_9)x + (0.00_7 \pm 0.15_4)$	0.9925
Second derivative method (reading at 600–500 nm)	$y'' = (0.008_6 \pm 0.002_7)x + (0.015_6 \pm 0.215_5)$	0.9753

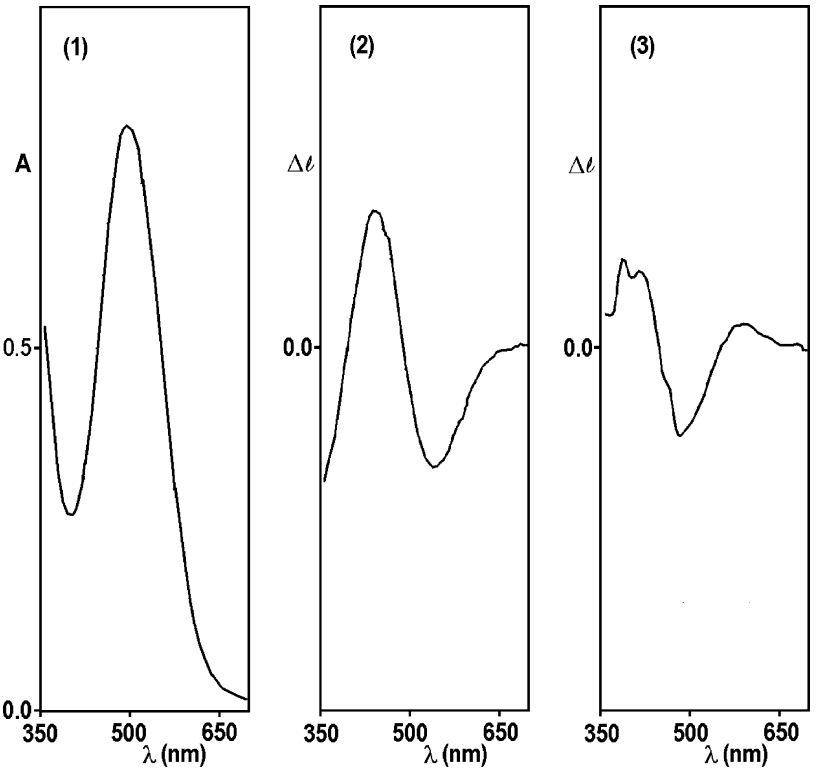

Fig. 1. (1) Absorbance spectrum of human serum sample. (2) First derivative spectrum of the same human serum sample. (3) Second derivative spectrum of the same human serum sample.

spectra of serum no. 1. These spectra are quite similar to those obtained for standard phosphatidylcholine [12].

Table 2 sets out the results of the analysis of these samples obtained either by the 'standard method' (i.e. reading off the absorbance at 500 nm), and by operating with the first or second derivative (thus determining the 'distance' between peaks and troughs falling on the wavelengths set out in Section 3.1). Table 3 shows the values obtained using the recovery tests for sera, obtained by 'standard addition' method (adding to the sample pure phosphatidylcholine at about the

Table 2
Results obtained in the analysis of human serum samples

Sample no.	Standard method (phosphatidylcholine in $g\,l^{-1}$) mean values ($n = 3$)	First derivative method (phosphatidylcholine in $g\,l^{-1}$) mean values ($n = 3$)	Second derivative method[a] (phosphatidylcholine in $g\,l^{-1}$) mean values ($n = 3$)	Second derivative method[b] (phosphatidylcholine in $g\,l^{-1}$) mean values ($n = 3$)
Serum no. 1	2.23 ± 0.10	2.23 ± 0.11	2.13 ± 0.11	2.01 ± 0.12
Serum no. 2	2.28 ± 0.05	2.23 ± 0.08	2.11 ± 0.09	2.06 ± 0.05
Serum no. 3	2.08 ± 0.07	2.06 ± 0.06	1.81 ± 0.06	2.16 ± 0.11
Serum no. 4	2.82 ± 0.01	2.83 ± 0.02	2.54 ± 0.07	2.58 ± 0.11
Serum no. 5	2.03 ± 0.10	1.96 ± 0.17	1.69 ± 0.07	1.45 ± 0.11
Serum no. 6	1.86 ± 0.09	1.82 ± 0.03	1.68 ± 0.11	1.52 ± 0.06

[a] Reading at 412–500 nm.
[b] Reading at 600–500 nm.

Table 3
Data for recovery tests in human serum, by 'standard addition' method

Method	Recovery (%)	Average (%)
Standard method	Between 98 and 103	100
First derivative method	Between 97 and 104	100
Second derivative method[a]	Between 86 and 95	90
Second derivative method[b]	Between 90 and 106	98.5

[a] Reading at 412–500 nm.
[b] Reading at 600–500 nm.

same concentration of the sample, or two, or three more concentrated, respectively) and operating with 'standard method', or else with the first or second derivative method, respectively. No pre-treatment was necessary in the analysis of these samples.

Three samples of amniotic fluid were then analysed. In this case, as already mentioned in Section 2.3 above, it was necessary to analyse a much larger volume of sample than in the 'standard method' owing to the very low concentration of lecithin contained in these samples. Nevertheless, the absorption spectra and the first and second derivative spectra of the amniotic fluid samples do not differ significantly, except for absorbance values lower than those shown in Fig. 1.

Furthermore, the different volumes sampled and the consequent appreciable dilution of the solution to be analysed were naturally taken into account in the calculation in order to obtain the correct final concentration of lecithin contained in the sample. The results of the analyses performed on the amniotic fluids obtained using both the 'standard method' and operating in first and second derivative are shown in Table 4. Also the results of recovery tests using the 'standard addition' method in amniotic fluid are shown in this table.

Lastly, tests were performed also on human bile samples. In particular one human bile sample was used in the absence of any specific pathological alteration. In this case, as already mentioned in Section 2.3, it was necessary to dilute the original bile sample before testing. Then both the 'standard method' and the first and second derivative methods were applied as described in Section 3.1. The results of the tests carried out on this sample are shown in Table 5; also the results of the recovery tests obtained using the 'standard addition' method are shown in Table 5. In this case too the absorption spectrum of the diluted bile sample and the first and second derivative spectra do not differ significantly from those shown in Fig. 1.

Lastly, also a bile sample containing traces of blood was analysed. Also in this case prior dilution was performed, followed by application of the 'standard method' and the derivative methods. Fig. 2 shows the sample spectrum obtained using the 'standard method' and that of the relative 'blank'. The same figure also shows the spectra referring to this sample, obtained using the first and second derivative methods. The comparison of the spectrum obtained using the 'standard method' with that using the relative 'blank' clearly shows the interference (also on the 500 nm absorption peak) of the wide absorption band with a pronounced peak around 420 nm, partially overlapping the absorption band centered around 500 nm, which represents the analytical band of the 'standard method'. Also two small bands are observed in the 'blank' spectrum lying between 520 and 600 nm that further complicate the correct performance of the test. Indeed all this not

Table 4
Results obtained in the analysis of amniotic fluids and data for recovery tests in amniotic fluid samples, by 'standard addition'method

Sample (weeks of pregnancy)	'Standard method' (phosphatidylcholine in $mg\,l^{-1}$) mean values ($n = 3$)	First derivative method (phosphatidylcholine in $mg\,l^{-1}$) mean values ($n = 3$)	Second derivative method[a] (phosphatidylcholine in $mg\,l^{-1}$) mean values ($n = 3$)	Second derivative method[b] (phosphatidylcholine in $mg\,l^{-1}$) mean values ($n = 3$)
Sample 1 (16 weeks)	7.48 ± 0.98	6.71 ± 0.69	6.1 ± 1.7	4.4 ± 1.2
Sample 2 (17 weeks)	14.1 ± 1.2	14.03 ± 0.014	13.1 ± 1.0	12.59 ± 0.64
Sample 3 (18 weeks)	17.79 ± 0.25	16.86 ± 0.22	13.2 ± 1.9	12.3 ± 2.1
Mean recovery (%)	94	96	95	95

[a] Reading at 412–500 nm.
[b] Reading at 600–500 nm.

Table 5
Results obtained in the analysis of bile sample and data for recovery tests in bile sample, by 'standard addition method'

Method	Standard method ($g\,l^{-1}$ lecithin) mean value ($n = 3$)	First derivative method ($g\,l^{-1}$ lecithin) mean value ($n = 3$)	Second derivative method[a] ($g\,l^{-1}$ lecithin) mean value ($n = 3$)	Second derivative method[b] ($g\,l^{-1}$ lecithin) mean value ($n = 3$)
Results	40.6 ± 1.5	40.8 ± 1.2	41.4 ± 2.9	42.4 ± 3.7
Mean recovery (%)	101	102	104	107

[a] Reading at 412–500 nm.
[b] Reading at 600–500 nm.

only causes an appreciable interference at the wavelength at which the absorbance is read off using the 'standard method', but problems are caused also in the case of the derivative spectra both by the interference band peaking at about 420 nm which appreciably modifies also the trends of the first and second derivative spectra precisely at one of the read-off wavelengths (455 nm, in first derivative and 412 nm in second derivative, respectively), as well as by the two smaller bands between 520 and 600 nm, which tend instead to disturb the read-off at the 600 nm peak when the second derivative is used. For this reason, therefore, in the case of this sample type, in first derivative the readings were performed between 557 and 463 nm, while, for the second derivative spectrum, since readings can no longer be made at 412 nm, the analysis must be limited to the second portion of the spectrum (i.e. above 500 nm). Also in this case, however, readings

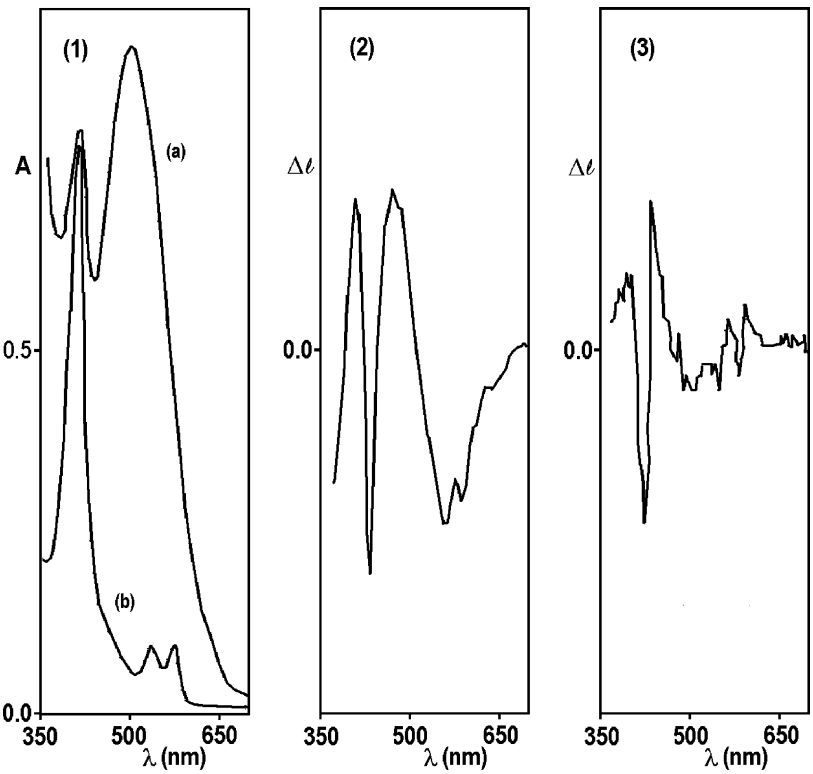

Fig. 2. (1) (a) Absorbance spectrum of human bile sample with blood traces obtained using 'standard method'. (b) Absorbance spectrum of relative 'sample blank'. (2) First derivative spectrum of the same human bile sample. (3) Second derivative spectrum of the same human bile sample.

Table 6
Results obtained in the analysis of bile sample with blood traces and data obtained for recovery tests in bile sample with blood traces, by 'standard addition' method

Method	Standard method (direct) method ($g\,l^{-1}$ lecithin) mean value ($n = 3$)	Standard method, ('Gran's plot method) ($g\,l^{-1}$ lecithin) mean value ($n = 3$)	First derivative method[a], ('Gran's plot method) ($g\,l^{-1}$ lecithin) mean value ($n = 3$)	Second derivative method[b], (Gran's plot method) ($g\,l^{-1}$ lecithin) mean value ($n = 3$)
Results	27.8 ± 1.8	22.5 ± 1.8	20.6 ± 1.4	33.3 ± 2.9
Mean recovery (%)	128	104	100	92

[a] Reading at 557–463 nm.
[b] Reading at 500–592 nm.

had to be made at 592 nm, rather than at 600 nm, so as to keep the observed interference to a minimum.

Above all, Table 6 shows the results obtained using the normal 'standard method' (which in this case is referred to also as the (direct) 'standard method') for the bile sample containing traces of blood. Indeed, in view of the strong likelihood of interference, as mentioned above, it was decided to apply the 'Gran's plot' method [13] to this sample, i.e. a method which gave good analytical performances when applied to real matrices [14,15]. Table 6 shows also the results obtained using this method, by operating together with both the 'standard method', and with first and second derivatives. Fig. 3 shows the plots obtained using the 'Gran's plot' method together with an indication of sample dilution and of the correlation and extrapolation carried out to obtain the required concentration value.

Lastly, Table 6 also shows the results obtained using the 'standard addition' method based on the results of biliary phospholipid concentration, as shown in the same table.

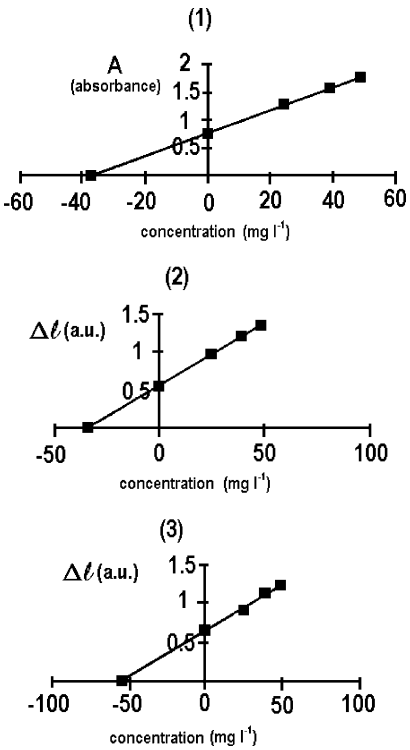

Fig. 3. Graphical applications of 'Gran's plot' method. (1) 'Standard method', sample diluted 61.00 times before the analysis, concentration obtained $36.8\,mg\,l^{-1}$, $r = 0.9994$. (2) First derivative method, sample diluted 61.00 times before the analysis, concentration obtained $33.8\,mg\,l^{-1}$, $r = 0.9999$. (3) First derivative method, sample diluted 61.00 times before the analysis, concentration obtained $54.6\,mg\,l^{-1}$, $r = 0.9979$.

5. Discussion

In a previous paper [12] a detailed discussion of the results obtained by applying either the 'standard' method or first derivative methods to standard solutions in which no turbidity had yet developed and there was no chromatic interference due to particular absorption bands led to the conclusion that, when using standard solutions, the results obtained by the 'standard method' and those obtained with the first derivative did not differ appreciably. In particular, the precision of the two methods was found to be comparable (R.S.D.% approximately equal) (Table 7). Conversely, the application of the second derivative method did not present any particular advantage in the measurement of standard lecithin solutions. Indeed, because of the amplification of the background

Table 7
Comparison of precision data and recovery tests, by 'standard addition method', in the determination of choline-containing phospholipids in different human fluids, found by the biosensor method and by the enzymatic–spectrophotometric method (standard method, or first derivative method, or second derivative method)

Analysed medium	Biosensor Method mean value ($n \geq 3$)		Enzymatic–spectrophotometric (standard method) mean value ($n = 3$)		Enzymatic–spectrophotometric (first derivative method) mean value ($n = 3$)		Enzymatic–spectrophotometric (second derivative method[a]) mean value ($n = 3$)	
	R.S.D. %	Recovery (%)	R.S.D. %	Recovery (%)	R.S.D. %	Recovery (%)	R.S.D. %	Recovery (%)
Standard solutions	2.7		2.7		1.7		4.5	
Human sera	3.3	97–100	3.4	94–101	2.4	96–102	4.5	88–95
Human bile	2.0	104–106	3.7	99–102	2.5	101–103	7.1	102–107
Amniotic fluids	3.2	95–103	7.7	≈94	3.9	≈96	17.2	≈95

[a] Reading at 412–500 nm.

noise, the repeatability of the measurements and thus of the precision of the method deteriorated.

The results of the derivative enzymatic–spectrophotometric analyses performed on biological samples considered in this work showed that serum samples in particular can be analysed without any pre-treatment. In the case of amniotic fluids, on the other hand, it was often necessary to operate on a sample volume that is greater than the one envisaged for the method owing to the very low phospholipid concentrations usually found in this type of sample. In the case of bile, on the other hand, a preliminary dilution of the sample was required using the same buffer as for the enzymatic test, indeed a rapid preliminary examination showed that even when only very small volumes of bile were sampled their concentration in phosphatidylcholine was such as to produce absorbance values so high that they exceeded the upper limit of the method's linear range.

However, above and beyond these methodological considerations, the results of the tests carried out on these biological samples basically show that, also for this type of sample, at least when they come from individuals free of any specific disease, the conclusions that may be drawn are similar to all intents and purposes as regards repeatability and reproducibility to those described above when using standard choline solutions containing phospholipids. In practice, the standard method and the first derivative method are equivalent, while the precision of the second derivative method is certainly not as good as that of the first two.

The advantages of the (first) derivative method were apparent above all in the case of the analysis of bile samples containing traces of blood. In the latter case, the aim was to minimize chromatic interference by slightly varying the wavelength at which the first and second derivative peaks and troughs were read. It was also deemed advisable to apply the 'Gran's plot' method. The results show that also in these cases the first derivative method may still be usefully applied if the peak reading wavelength is shifted slightly. In fact, when this was done, the recovery tests (see Table 6) confirmed the reliability of the first derivative method compared with the 'standard method', both when using the 'direct method' and the 'Gran's plot' method.

Lastly, the present research allowed several preliminary analytical comparisons to be made between the standard or derivative spectrophotometric methods described and an (enzymatic–amperometric) biosensor method developed by us in recent years [16,17] and applied to the same kind of biological fluids as those investigated in the present work. A comparison of this kind, based on precision and 'recovery' data, is outlined in Table 7. It can be seen how the best precision results obtained when operating on standards, sera, bile or amniotic fluids, are obtained using the biosensor method, or else using the spectrophotometric method in first derivative, and how these data, for both methods, are fully comparable (Table 7). On the other hand, the precision data are good and comparable for the 'standard' spectrophotometric method when operating on standard solutions or on sera, but much less so when operating on bile and above all on amniotic fluids. On the other hand, the precision data obtained when operating in second derivative are

certainly worse for the reasons outlined above and recoveries (Table 7) obtained using the first derivative spectrophotometric method apparently tend to be slightly better than those obtained using the biosensor method. Lastly, the LOD [18] of the spectrophotometric 'standard method' is 1.6 mg l^{-1}, that one of the 'first derivative method' 2.9 mg l^{-1} and that one of the 'second derivative method' 6.9 mg l^{-1}, while the LOC [18] of all the three methods ranges between 5 and 10 mg l^{-1}.

6. Conclusions

It may be concluded that the normal values of phospholipids in the serum of adult individuals expressed as mg l^{-1} of lecithin are generally in the range of 1250–3000 mg l^{-1}. However, the minimum and maximum values reported in the literature vary considerably (from 1040 to 4280 mg l^{-1}). This is essentially because the phosphatidylcholine concentration in the serum tends to increase with age and to vary with diet. Furthermore, there is no significant difference between the normal values for females and for males, except during pregnancy, when phosphatidyl concentration in the serum tends to rise in women [19].

All this is in full agreement with the results we obtained for the sera tested, which are shown in Table 2. This also seems to confirm how not only the precision of the first derivative spectrophotometric method but also its accuracy are quite satisfactory and that the derivative method may thus be considered a valid alternative also to direct, non spectrophotometric, methods including, for example biosensor methods [16,17], which are known to be generally unaffected by chromatic interference. However, these interferences may be offset, as we show in the present work, also by using the first derivative spectrophotometric method and making suitable slight modifications to the working wavelengths.

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