ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 1135–1148

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Determination of lecithin drug specialties and diet integrators; by means of first or second derivative enzymatic–spectrophotometric analysis

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Received 6 June 2001; received in revised form 27 December 2001; accepted 31 December 2001

Abstract

The possibility has been investigated of applying derivative analysis to a classical enzymatic–spectrophotometric method for lecithin determination for the purpose of developing an analytical direct method that does not require long pretreatment of the test sample even in the case of turbid samples. Several samples of drugs and food integrators containing lecithin were thus tested using both the standard and the derivative method and then comparing the results obtained. The RSD% values of measurements on real (food or drugs) samples were always <2.5. Using a first derivative spectrophotometric method, average recovery was always between 102 and 105%. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Lecithin; Analysis; Spectrophotometric; Derivative method

1. Introduction

Lecithin is the most common natural phospholipid. It is found in all living cells [1], except for a few bacteria and algae. It is an important constituent of biological membranes and is found in many animal tissues and organs. It is widespread also in the plant world and is abundant in legumes, cereals and seed embryos in general [2].

Vegetable lecithin is the main constituent of commercially available lecithins today and is derived mainly from soy beans. Lecithin's physico-chemical, biological and physiological properties make it useful in the pharmaceutical, food and cosmetics fields.

Moreover, owing to its high polyunsaturated fat content, lecithin is also used therapeutically as a dietetic support in cases of organic debilitation and altered fat metabolism. It also acts as a vehicle for essential fatty acids, such as linoleic and linolenic acids, which play an important role in the human body as growth factors, in the prevention of atherosclerosis and as precursors of prostglandins, substances that provide protection against hypertension, thrombosis and ulcers.

Owing to the difficulty of obtaining lecithin (phosphatidylcholine) of sufficient purity, the

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PII: S0731-7085(02)00155-3

lecithins available on the market contain variable percentages of other phosphatids. It is thus important to develop rapid and effective analytical methods for the purpose of quality control, clinical tests and to discover any commercial frauds.

The analysis of phospholipids can be performed by chromatographic methods [3–5], which are however laborious, time consuming, not easily automated. Furthermore separation–extraction [3,4] processes of the sample and derivatisation of the analyte are generally required. Alternatively, the phospholipid concentration can be obtained by total phosphorus analysis [6] using molybdate–vanadate [7] or Bartlett's method [8]; also these methods require prior sample treatment using perchloric acid or a perchloric acid–hydrogen peroxide mixture at about 180 °C. For this reason, in more recent years, a selective and direct enzymatic–spectrophotometric method has been introduced [9–12] based on two reactions in series catalysed 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 in the presence of peroxidase, performing the spectrophotometric measurement at $\lambda = 500$ nm. However, as explained in previous papers [13,14], this method may present several drawbacks if the sample is pigmented or is scarcely soluble in aqueous medium, such as to produce turbid or opalescent solutions. It was precisely to overcome these difficulties that the present authors previously proposed some methods of phospholipid determination based on the above two enzymatic reactions but using amperometric detection [13,14], obtaining results of indubitable interest. However, since the basic instrumentation needed to apply spectrophotometric methods is certainly much more widespread today in analytical laboratories than that needed for electrochemical methods, in the present research we went back to concerning ourselves with the spectrophotometric–enzymatic method.

The aim of the present research was to examine the possibility of applying derivative methods to the classical enzymatic–spectrophotometric analysis described above to determine lecithin in food or drug matrices in order to develop an analytical procedure alternative to the cited chromatographic or spectrophotometric methods [3–8] and that does not require any long or laborious pretreatment, even in the analysis of turbid or pigmented samples.

We focused in particular on the objective difficulty of performing a rapid, but sufficiently precise and accurate, assay of the phospholipids (lecithin) contained in real matrixes. In many cases the turbidity of the test solutions may mean that the spectrophotometric method can be applied only by significantly increasing the time of the analysis. In such cases, the samples must usually be subjected to a long series of extraction and/or separation pretreatments to reduce the causes of the turbidity if normal UV–Vis spectrophotometry is used. We also 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 pretreatment or in any case limiting it to rapid operations such as simple centrifugation.

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 ± 0.1) °C by means of a Colora ultrathermostat.

The spectrophotometer was also able to convert the signal up to a fourth order derivative, with $\Delta\lambda$ from 1 to 10. Quartz cuvettes with a 1.00 cm optical pathlength were used, together with an ALC CWS 4235 centrifuge and a Julabo model UC 5 B 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. As well as the standard supplied by the Poli company, another standard, obtained by dissolving egg yolk L-α-phosphatidylcholine (L-α-lecithin) supplied by the Sigma Company (Milan) cod.P-9671 in chloroform + *n*-hexane 1 + 1 (v + v) after careful weighing, was also used.

The following solvents were used: ethyl alcohol for RPE analysis, supplied by Carlo Erba (Milan); chloroform RS (stabilised with amylene) for HPLC, supplied by Merck (Germany); *n*-hexane for RPE analysis, supplied by Carlo Erba (Milan); methanol for RPE analysis, supplied by Carlo Erba (Milan).

2.3. Samples analysed

Lecithin content analysis was performed using diet integrators, drug specialties and fresh chicken eggs. In particular, indicating in parenthesis the number used to refer to the sample in the present paper, the following samples were purchased at the chemist's: granular diet integrator (no. 1), diet integrator in capsules (no. 2), two different drug specialties in capsules (no. 3 and no. 4); fresh chicken eggs were purchased at the local market.

The composition of the diet integrators and the drugs is shown in Table 1.

Table 1
Composition of drugs and food integrators, expressed as percent by weight, such as declared by the manufacturers

Sample no.	Components	% (w/w)
1 integrator (granules)[a]	Soya lecithin	≥97
	Phosphatidylcholine	*≥23*
	Linolenic acid	≥5
	Linoleic acid	≥58
	Moisture	≥1.5
2 integrator (capsules)[b]	Soya lecithin	32.9
	(*Phosphatidylcholine*)	*6.3*
3 drug (capsules)[b]	*Phosphatidylcholine*	*34.7*
	Mono and diglycerides (sunflower oil)	20.44
	Silicon dioxide	1.85
	Ethylvanillin	0.22
	p-Metoxyacetophenon	0.12
	Soya oil	41.77
	DL-α-Tocoferol	0.23
4 drug (capsules)[b]	*Phosphatidylcholine*	*36.7*
	Mono and diglycerides (sunflower oil)	21.62
	Silicon dioxide	1.95
	Ethylvanillin	0.20
	p-Metoxyacetophenon	0.11
	Soya oil	39.43

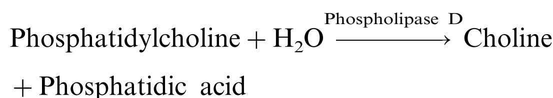
[a] Average composition in 100 g.
[b] Composition of each capsule.

3. Methods

3.1. Enzymatic–spectrophotometric determination

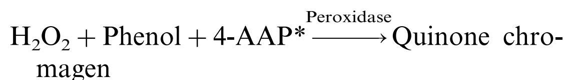
Phosphatidylcholine content was determined in real samples using a classical spectrophotometric detection enzymatic method [9–12].

The method is based on the following enzymatic reactions:



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

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{Choline oxidase}} \text{Betaine} + 2H_2O_2$$



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

*4-AAP, 4-aminophenazone.

The 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 using, so that the final composition of the reagent is as follows: Pipes buffer (1,4-piperazine diethane-sulphonic acid) (75 mmol l^{-1}) at pH 7.9, containing phenol (7 mmol l^{-1}), 4-AAP (0.5 mmol l^{-1}) and 1.0 g l^{-1} of non-ionic detergent. Lastly, 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 pink coloured 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, taking the reading immediately after thermostatting.

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.

For first derivative operation readings were taken between 455 and 557 nm, while for second derivative operation readings were made between 412 and 500 nm.

3.2. Pretreatment of test samples

The drug and diet integrator samples dissolved in the solvent proved to be most suitable for dissolving not only the phosphatidylcholine content but also the entire real matrix tested as quantitatively as possible. Its stability in solution was monitored for at least 1 day.

After carefully opening the capsules containing the drugs and the diet integrator with a scalpel, the content of each capsule was completely dissolved in a solvent mixture of chloroform + *n*-hexane 1 + 1 by volume. Only in the case of the granular diet integrator was it sufficient to use distilled water as solvent, taking care to subject the solution to strong stirring for about 10 min. A magnetic microstirrer supplied by Velp Scientifica (Italy), equipped with a magnetic anchor, was used. In this way a slightly opalescent solution was obtained which was subjected directly to the subsequent phases of the analysis. For the other samples, after the addition of the 'sole reagent' followed by thermostatting, centrifuging for about 15 min at 3500 rpm was instead necessary to eliminate the slight turbidity from the solution; the spectrophotometric reading was then performed immediately.

Five different egg yolks were selected and then homogenised. Lecithin extraction was performed on a weighed amount of homogenate. A fraction of yolk (4.0 g) was then weighed out and used for four consecutive extractions using 2 ml of solvent each time. The supernatant fractions obtained after each centrifuging operation (3900 rpm for 15 min) were collected and combined and the resulting solution made up to 50 ml with solvent. Measures performed on the solution from a subsequent similar extraction process revealed no further spectrophotometrically detectable presence of lecithin, showing that, after four repetitions, the extraction is practically quantitative. Indeed the lecithin concentration found in the extracts after the fourth repetition were found to be lower than the minimum spectrophotometrically detectable level.

In all cases in which the nominal phosphatidylcholine content of the samples was known, care was taken to develop solutions whose concentrations lay within the method's linearity range. As the weight of the capsule contents and the percentage of phosphatidylcholine (supplied by the manufacturers) were known, the solvent mixture volumes were selected in such a way that the final lecithin concentrations lay inside the method's linearity range (9.97–142.9 mg l^{-1}). The same was done for the granular sample, as also in this case the nominal value of the lecithin content was known.

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 the organic solvent used in the extraction had been added ('reagent blank'), were always subtracted.

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

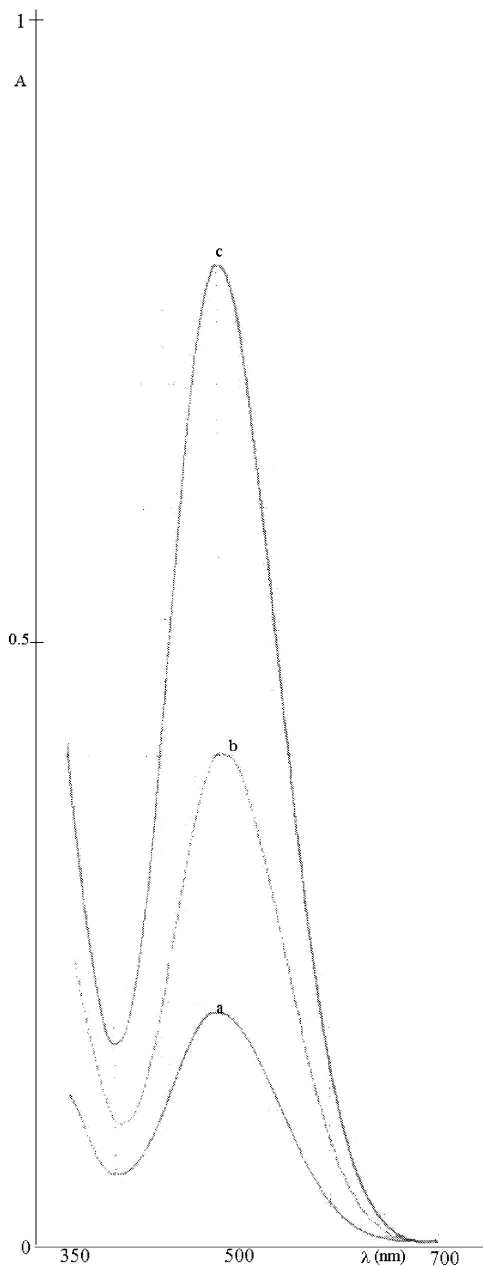


Fig. 1. Spectra obtained using 'standard' method for three different concentrations of standard lecithin solutions: (a) 9.967; (b) 24.793; (c) 49.180 mg l^{-1} of lecithin.

3.3. Preparation of standard lecithin solutions

Phosphatidylcholine supplied by the Poli company was used as standard in analysing the diet integrator granules in distilled water solution. For samples dissolved in the chloroform + *n*-hexane solvent mixture, standard solutions were prepared using the granular lecithin supplied by Sigma. The product was carefully weighed out on a Mettler model AE 240 electronic balance and then dissolved in the same chloroform + *n*-hexane solvent mixture, 1 + 1 by volume, then making up to volume in a verified volumetric flask. In this case, the final solutions containing the reagent and the standard solution, after thermostatting, were centrifuged before performing the spectrophotometric reading in order to completely eliminate the observed turbidity. The titre was then checked spectrophotometrically using the Poli standard.

The solutions obtained using Sigma lecithin were prepared daily. A significant alteration of the standard titre was already detectable 24 h after preparation, even though the solution was stored in a refrigerator in a sealed vessel in complete darkness.

The preliminary calibration curves required for method optimisation were constructed by analysing solutions of increasing phosphatidylcholine content obtained from the Poli standard.

4. Results

4.1. Development of standard and derivative methods

Fig. 1 shows the spectra recorded for increasing lecithin concentrations, respectively 9.967, 24.793 and 49.180 mg l^{-1}, using the 'standard' method. Fig. 2 shows the spectra obtained applying the first derivative method, while Fig. 3 shows the spectra referring to the second derivative method for the same lecithin concentrations.

The calibration curves were obtained using increasing volumes of lecithin standard supplied by the Poli company. The absorbance was always read at most 1 h after the end of the thermostatting period prescribed for the method.

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

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.

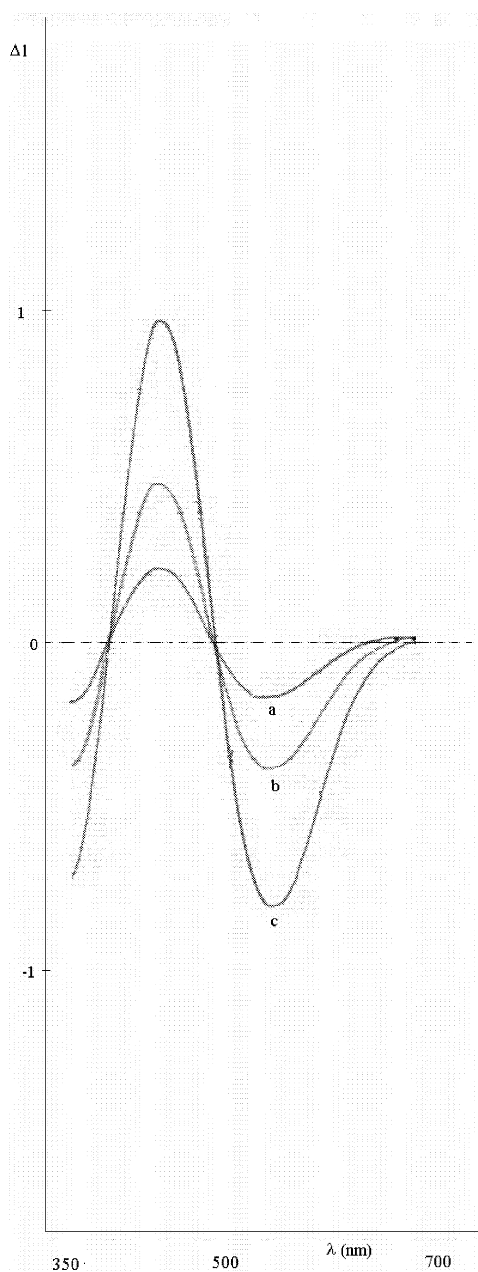


Fig. 2. Spectra obtained using first derivative method for three different concentrations of standard lecithin solutions: (a) 9.967; (b) 24.793; (c) 49.180 mg l^{-1} of lecithin.

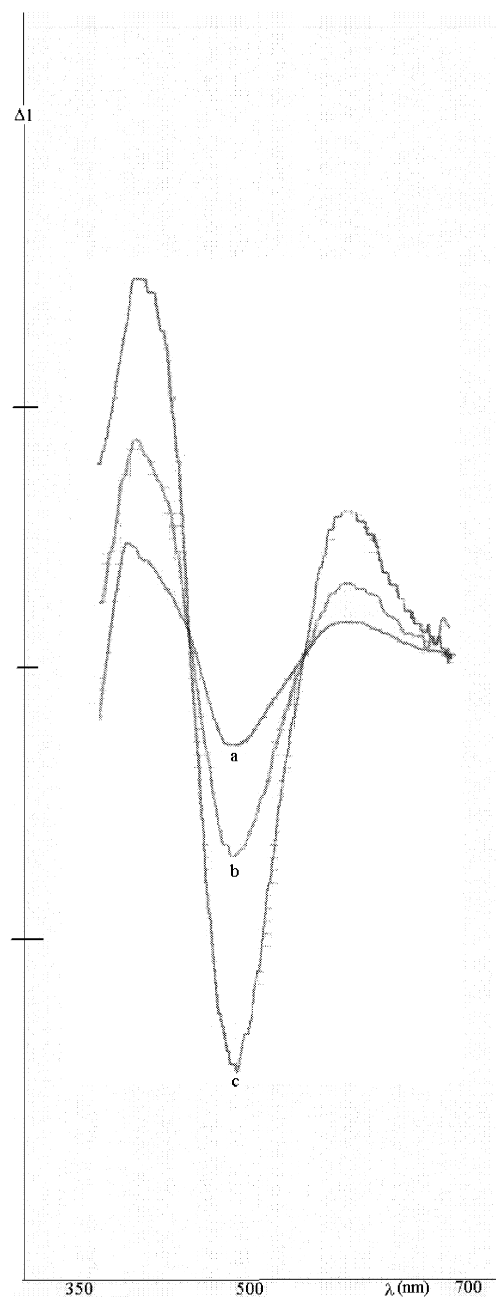


Fig. 3. Spectra obtained using second derivative method for three different concentrations of standard lecithin solutions: (a) 9.967; (b) 24.793; (c) 49.180 mg l^{-1} of lecithin.

For the second derivative method, a double reading was performed. In this case, a minimum actually appears 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 occurs at 600 nm.

Tables 2 and 3 list all the analytical data characterising the 'standard' and the derivative methods. The same tables also contain the equations referring to the calibration curves, the linear range, the analytical sensitivity, the minimum detection limit given at the minimum analyte concentration at which the recorded signal is three times the values of the standard deviation of the background noise and the precision of the measurements made in standard solutions, assessed as 'pooled' SD%.

4.2. Choice of solvent to dissolve capsule contents

One important phase of the investigation consisted in deciding on the best solvent in which to dissolve the drug products and food integrators analysed, and above all the phosphatidylcholine contained in them. It was actually found that, except for the diet integrator granules, the other samples in capsule form were completely insoluble in distilled water.

As the drug and foodstuff samples in capsule form presented very similar characteristics, the drug in capsule form no. 3 was chosen as matrix on which to carry out repeated solubilisation tests.

In all cases, the capsule was cut open and maintained for several minutes under stirring in 25 ml of the solvent tested.

The following solvents were tested: (1) EtOH; (2) $EtOH + H_2O$ 1 + 1 (v + v); (3) $EtOH + H_2O$ 1 + 2 (v + v); (4) $EtOH + H_2O$ 1 + 2 (v + v); (5) $CHCl_3$ with 5% EtOH; (6) $CHCl_3$ + hexane 1 + 1 (v + v). Fifty microlitres of each of these solutions were added to 3 ml of reagent solution and the method applied as described in Section 4.1. How-

Table 2
Parameters and analytical data for the used method

Method	Time of measurement[a] (min)	Time of analysis[b] (min)	Linear range (mg l^{-1})	Minimum detection limit (mg l^{-1})
'Standard'	10	30	9.97–142.9	1.63
First derivative	10	30	9.97–142.9	2.96
Second derivative (reading 412–500 nm)	10	30	9.97–142.9	6.98
Second derivative (reading 500–600 nm)	10	30	9.97–142.9	8.06

[a] Time necessary to record the spectrum of absorption, or first or second derivative spectrum, between 360 and 700 nm.
[b] In the case of sample's centrifugation it is also necessary to add about 15 min to the time of analysis.

Table 3
Analytical data for the used method

Method	Equation of calibration curve and confidence region $y = mx + b$; 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	'Pooled' SD%	Analytical sensitivity
'Standard'	$y = (0.0145 \pm 0.0004)x + (0.0322 \pm 0.0355)$	0.9997	2.71	0.545
First derivative	$y' = (0.0117 \pm 0.0003)x + (0.0212 \pm 0.0253)$	0.9998	3.18	0.466
Second derivative (reading 412–500 nm)	$y'' = (0.0114 \pm 0.0019)x + (0.007 \pm 0.154)$	0.9925	9.64	0.157
Second derivative (reading 500–600 nm)	$y'' = (0.0086 \pm 0.0027)x + (0.0156 \pm 0.2155)$	0.9753	20.75	0.071

Table 4
Results of the analysis using different solvents for the pharmaceutical sample no. 3

Solvent used	'Standard' method (mg)	First derivative method (mg)	Second derivative method (mg)
EtOH	180	160	167
EtOH + H_2O 1+1 (v+v)	191	171	174
EtOH + H_2O 1+2 (v+v)	220	193	227
EtOH + H_2O 1+2 (v+v)	104	100	98.2
$CHCl_3$ + 5% EtOH	105	100	114
$CHCl_3$ + hexane 1+1 (v+v)	324	313	327

Nominal value of phosphatidylcholine = 300 mg for capsule.

ever, in the cases of solutions 4, 5 and 6, after thermostatting, the solution was centrifuged for 15 min at 3500 rpm to reduce the effect of the visible turbidity. The subsequent spectrophotometric reading was performed on the supernatant.

Table 4 shows the results of the test performed using the solvents listed above. Taking into account that the nominal content of phosphatidylcholine for drug no. 3 (capsules) is 300 mg per capsule, on observing the results set out in Table 4, it is clear why the final choice of the solvent to use for dissolving the contents of the drugs in capsules fell on the mixture $CHCl_3$ + hexane 1 + 1 (v + v). Moreover, the choice of this mixture as a very good solvent both for phosphatidylcholine and drugs (as capsules) containing lecithin confirms previous observations in this regard [13], while it is clear that, using the other solvents considered, both the phosphatidylcholine and even more the content of the capsules are only partially soluble.

For the analysis of the food sample (egg yolk), for which it is not in any case possible to dissolve the entire matrix, a preliminary extraction of the lecithin was performed using the same solvent mixture in which the capsules were dissolved, with the sole addition of 4% methanol. This addition proved useful in the quantitative extraction of lecithin from this particular type of sample [13].

4.3. Analysis of foodstuff and drug samples

Two diet integrators containing phosphatidylcholine in the form of granules and capsules, a sample of chicken egg yolk and two drug specialties (capsules) were analysed.

The samples were pretreated and analysed as described in the preceding sections.

Table 5 illustrates the reproducibility of measurements performed on the two diet integrators and the two drugs obtained using the 'standard' or derivative methods. Table 6 shows the percentage relative differences between the experimental results obtained and the corresponding nominal values for lecithin, as stated by the manufactur-

Table 5
Reproducibility data obtained for analysis of drugs and dietetic integrators using enzymatic–spectrophotometric 'standard' method, or derivative methods

Sample no.	Method	Recovered value (%p/p)	RSD% ($n = 3$)
1 (granules)	'Standard'	24.23	1.65
	First derivative	23.94	0.96
	Second derivative	25.10	3.78
2 (capsules)	'Standard'	7.01	1.67
	First derivative	6.70	1.61
	Second derivative	7.20	2.67
3 (capsules)	'Standard'	37.50	0.47
	First derivative	36.20	0.66
	Second derivative	37.80	2.15
4 (capsules)	'Standard'	40.30	2.64
	First derivative	38.70	2.50
	Second derivative	42.70	4.30

Table 6
Comparison between nominal value (a) declared from manufacturers and (b), (c), (d) data recovered using spectrophotometric methods for drugs and diet integrators

Sample no.	Nominal value (%w/w) (a)	Value found using spectrophotometric standard method (%w/w) RSD% ≤2.6 (b)	Value found using spectrophotometric first derivative method (%w/w) RSD% ≤2.5 (c)	Value found using spectrophotometric second derivative method (%w/w) RSD% ≤4 (d)	$(b-a)/a$ (%)	$(c-a)/a$ (%)	$(d-a)/a$ (%)
1 (granules)[a]	≥23	24.2	23.9	25.1	+5.2	+3.9	+9.1
2 (capsules)[a]	6.3	7.0	6.7	7.2	+11.1	+6.3	+14.3
3 (capsules)[b]	35.0	37.5	36.2	37.8	+7.1	+3.4	+8.0
4 (capsules)[b]	36.7	40.3	38.7	42.7	+9.8	+5.4	+16.3

[a] Diet integrator.
[b] Drug.

Table 7
Recovery for pharmaceutical sample no. 4 (capsules)

Phosphatidylcholine found (mg l^{-1})	Phosphatidylcholine added (mg l^{-1})	Total phosphatidylcholine found (mg l^{-1})	Recovery (%)	Average recovery (%)
'Standard' method				
39.67	9.97	50.74	111	109
39.67	24.79	66.19	107	
39.67	49.18	93.28	109	
First derivative method				
38.03	9.97	48.70	107	105
38.03	24.79	63.56	103	
38.03	49.18	89.18	104	
Second derivative method[a]				
41.97	9.97	53.73	118	116
41.97	24.79	70.23	114	
41.97	49.18	99.51	117	
Second derivative method[b]				
41.97	9.97	53.73	118	116
41.97	24.79	69.98	113	
41.97	49.18	100.00	118	

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

Table 8
Reproducibility of analysis of chicken egg yolk sample using different spectrophotometric methods

Measurement no.	'Standard' method (mg of lecithin)	First derivative method (mg of lecithin)	Second derivative method (mg of lecithin)
1	210	204	215
2	206	202	204
3	207	200	211
Average	208	202	210
RSD%	1.00	0.99	2.65

ers in the case of those samples for which these values were available.

Table 7 shows the percentage recovery in tests performed on drug sample no. 4, obtained using the standard addition method. Tables 8 and 9, respectively show the reproducibility and recovery data (the latter obtained using the standard addition method) emerging from the tests performed on the chicken egg yolk sample.

As far as the analysis carried out on the latter sample is concerned, the experimental results reveal a lecithin content of about 5% by weight.

After dissolving the samples in capsule form in the solvent mixture chloroform + n-hexane $1+1$ (v + v), it was found advisable to immediately analyse the solution obtained by centrifugation. It was found that, in the relevant spectra obtained using the 'standard' method, in time a turbidity effect gradually occurred in the test solution.

For this reason the results, set out in the tables and referring to the 'standard' method, were obtained by taking into account the spectra recorded immediately after centrifugation.

5. Discussion

A comparison of the analytical data, summarised in Tables 2 and 3 and referring to the 'standard' and derivative methods, shows that, although there is no significant difference between the repeatability of measures obtained using the 'standard' method and those obtained by the first derivative method, the precision on the other hand decreases with increasing order of derivation. This was fairly predictable insofar as the signal/noise (S/N) ratio generally decreases with increasing order of derivation. Consequently, also the 'analytical sensitivity' decreases with increasing order of derivation. Also here, we observe that this decrease is instead very small on going from the 'standard' method to the first derivative method. Moreover, the minimum detectable limit tends to increase; above all on going from the first to the second order derivative, as the value of the background noise increases.

Indeed, measurement time, analysis time and the linear range practically do not change, while the value of the coefficient of correlation, which remains virtually the same for both the 'standard' and the first derivative methods, deteriorates perceptibly for the second derivative method.

In conclusion, the results obtained by applying either the 'standard' or first derivative methods to standard solutions in which no turbidity has yet developed and there is no interference due to particular absorption bands do not differ appreciably. In particular, the precision of the two methods is found to be comparable (approximately equal RSD%) (Table 3). Conversely, the application of the second derivative method does not present any particular advantage in the measurement of standard lecithin solutions. Indeed, because of the amplification of the background noise, the repeatability of the measurements and thus of the precision of the method deteriorates.

On the other hand, considerable advantages are gained by applying the first derivative method to samples in which turbidity develops. It must be recalled in this respect that other analytical techniques [3–8] generally demand the prior extraction of the lecithin from the real matrix containing it. The advantage of the measurement procedure proposed herein lies essentially in the fact that the matrix is dissolved directly together with the lecithin. The problem that may thus arise is due to the development of a certain degree of turbidity, which is in some cases non-negligible, during the development of heat. This turbidity is actually largely eliminated by simple centrifugation, which is performed after the enzymatic reaction and the development of heat. However, even the slight turbidity sometimes persisting even after this treatment can jeopardise the application of the 'standard' spectrophotometric method.

Table 9
Recovery tests for chicken egg yolk sample using different spectrophotometric methods

Phosphatidylcholine found in chicken egg yolk (mg l^{-1})	Phosphatidylcholine added (mg l^{-1})	Total phosphatidylcholine found (mg l^{-1})	Recovery (%)	Average recovery (%)
'Standard' method				
68.19	9.97	78.86	107	105
68.19	24.79	94.47	106	
68.19	49.18	118.84	103	
First derivative method				
66.23	9.97	76.70	105	102
66.23	24.79	91.76	103	
66.23	49.18	114.92	99	
Second derivative method				
68.85	9.97	79.52	107	106
68.85	24.79	94.88	105	
68.85	49.18	121.47	107	

Using the first derivative method, it is instead possible to completely eliminate also the effect of any residual turbidity and the first derivative method can thus be applied also to the direct analysis of complex lecithin-containing matrixes, such as the drugs and diet integrators in capsule form considered in this research, and in which this turbidity is often observed when the 'standard' enzymatic–spectrophotometric method is applied. The benefit obtained by first derivative operation was predictable and expected since, as is in any case extensively described in the literature regarding derivatived methods [15], first derivative operation allows absorbance variation to be determined as a function of the variation in wavelength. Furthermore, since absorbance due to turbidity alone remains constant in the visible spectrum (or at least varies very little) with varying wavelength, its contribution to the signal read off as a derivative is practically zero or at least negligible.

In these cases, therefore, the agreement between the values obtained and nominal values increases significantly when the first derivative method is applied, compared to the results when the 'standard' method is applied. This emerges clearly from the test results shown in Table 6. Indeed the relative percent difference between observed and nominal values, computed for samples containing phosphatidylcholine in capsule form, is 3–6% on average for the first derivative method versus 5–11% for the 'standard' method (Table 6). Also in this case, however, the strong background noise accompanying the second derivative spectra prevents any useful application. This is confirmed by the results shown in Table 6, which seem to discourage any application of the second derivative method also in these cases.

Lastly, also the data on the recoveries obtained using the standard addition method shown in Table 7 provide further confirmation. Conceivably the 'standard' method could still ensure good results if the 'suitably turbid blanks' were subtracted from the observed absorbance. Even with the strong reduction in transmittance that this would entail, this operation would not solve the problem entirely. Indeed, as already mentioned, the turbid samples obtained simply by dissolving the matrix require a rapid spectrophotometric reading of the supernatant. Although the supernatant is separated rapidly from the precipitate, with the passing of time new turbidity effects are actually observed in the solution. This prevents correct spectrophotometric measures being made using the 'standard' method even after the subtraction of specific 'blanks'. In this case, it is actually practically impossible to correctly reproduce a 'blank', the absorbance of which gradually increases in time.

As we have seen, the problem may instead be solved in these cases using the first derivative method, which practically eliminates this drawback completely, ensuring greater accuracy without the need to perform the readings very rapidly.

Analysis of the chicken egg yolk revealed a lecithin content of about 5% (Tables 8 and 10), which is in line with the (often extremely variable) literature data. Moreover, from the recovery tests (Table 9) performed using the standard addition method, also in this case better recoveries are obtained using the first derivative method than with the 'standard' method.

Lastly, lecithin content determinations were carried out in our laboratory using a bienzymatic amperometric biosensor [13,14]. It was thus possible to compare lecithin values determined using the biosensor and described in previous papers with those obtained using the standard or derivative methods for the drug and foodstuff samples tested in the present investigation. A comparison of the results obtained is shown in Table 10.

It is immediately apparent that, by using first derivative spectrophotometry, an appreciable improvement is obtained in precision versus the amperometric detection method. Agreement with nominal values is unequivocally better in at least two cases out of four; while in two others it is slightly worse.

6. Conclusions

In conclusion, this investigation confirms that the enzymatic–spectrophotometric method for phospholipid determination described herein is highly specific and precise, but also sufficiently

Table 10
Comparison of results obtained using both enzymatic–spectrophotometric and enzymatic–amperometric methods for the analysis of several pharmaceutical forms or diet integrators

Sample no.	Nominal value (%w/w) (a)	Value obtained by biosensor (%w/w) RSD% $\leq$6.1 (b)	Value obtained by spectrophotometric 'standard' method (%w/w) RSD% $\leq$2.6 (c)	Value obtained by spectrophotometric first derivative method (%w/w) RSD% $\leq$2.5 (d)	Value obtained by spectrophotometric second derivative method (%w/w) RSD% $\leq$4 (e)	$(b-a)/a$ (%)	$(c-a)/a$ (%)	$(d-a)/a$ (%)	$(e-a)/a$ (%)
1 integrator (capsules)	$\geq$23	23.8	24.2	23.9	25.1	+3.5	+5.2	+3.9	+9.1
2 integrator (capsules)	6.3	6.6	7.0	6.7	7.2	+4.8	+11.1	+6.3	+14.3
3 drug (capsules)	35.0	31.0	37.5	36.2	37.8	−11.4	+7.1	+3.4	+8.0
4 drug (capsules)	36.7	32.1	40.3	38.7	42.7	−12.5	+9.8	+5.4	+16.3
Egg	$\approx$7[a]	5.2	5.2	5.1	5.4	–	–	–	–

[a] Literature data.

robust and rugged. In practice, operating conditions are particularly soft and small variations in the working parameters have comparatively little effect on the results obtained. Above all, it shows the occasions on which it is truly advantageous to use derivative methods, namely, in the case of real samples, whenever the solutions used have a turbidity that is difficult to eliminate. Lastly, it was also observed experimentally that, in these cases, the best results are obtained using the first derivative, practically without making any changes in the executive procedure followed by the method, as already described in the literature [9,10].

Acknowledgements

This work was financially supported by National Research Council (CNR) of Italy, Targeted Project 'MADESS'.

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