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Analytical determination of phosphatidylcholine: comparison of HPLC and enzymatic methods

A. Boix Montañés[a] & J. J. Permanyer Fábregas[ab]

[a]*Universidad de Barcelona, Facultad de Farmacia, Unidad de Nutrición y Bromatologia, Av. Juan XXIII s/n 08028 Barcelona, Spain*
[b]*Nutrexpa S.A., Central Analysis Laboratory, C/Lepanto 410-414, 08025 Barcelona, Spain*

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Two analytical techniques for the quantitation of phosphatidylcholine in food samples, high performance liquid chromatography (HPLC) and enzymatic analysis, have been tested in different cocoa powder and lecithin samples. The results given by the two techniques are highly correlated, although there are systematic differences between them. This is attributed to analytical interference due to the nature of the samples. © 1997 Elsevier Science Ltd

INTRODUCTION

Wettability and dispersability are important properties in the manufacture of instant cocoa products owing to the poor humectability of cocoa powder. Lecithin is used as an emulsifier to improve this characteristic behaviour.

Chemically, lecithin contains neutral lipids, polar lipids, and a great variety of minor components. Among the polar lipids, phospholipids are mainly responsible for its interesting industrial properties. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)are the most abundant phospholipids in an unmodified soy lecithin (Bernardini, 1981).

For the analytical quantitation of lecithin in a lecithinated cocoa, Association of Official Analytical Chemists' (AOAC) recommended method consists in a colorimetric determination of the amount of phosphorus present in the mineralization residue of a fat extract (AOAC, 1985). A blank assay of the non-lecithinated cocoa phosphorus content should be carried out. This means analyzing a sample of the cocoa before lecithination. This sample is seldom available, and so a constant substraction factor must be assumed in order to calculate the phosphorus in the sample that comes from the added lecithin.

As an alternative to this tedious and time-consuming method, the quantitation of phosphatidylcholine as a major phospholipid of the commercial lecithin used in this process could be a good alternative (Hurst & Martin, 1980).

Since there are different analytical possibilities for the determination of PC, different experimental comparisons of these methods can be found elsewhere in the literature: HPLC/TLC (Aitzetmüller & Handt, 1984), HPLC/NMR (Press *et al.*, 1981) and enzymatic determinations (Campanella *et al.*, 1986, 1990).

HPLC and enzymatic methods are two techniques commonly used in food analysis laboratories. Both techniques allow the quantitation of PC, and the aim of this work was to perform an experimental comparison of the determination of this analyte using an enzymatic and a normal phase HPLC method applied to commercial lecithin samples and lecithinated cocoa samples.

MATERIAL AND METHODS

Reagents and apparatus

Two series of samples were used, provided by several manufacturers: milled lecithinated cocoa, and commercial soybean lecithin. The alkalized 5% lecithinated cocoa samples were designated as LAC (lecithinated alkalized cocoa) and an alphanumeric code that identifies the different suppliers and their different samples.

In addition, various sorts of commercial soybean raw lecithin, with a nominal polar lipid content of 98% (L98), 62% (L62) and 30% (L30) were tested. These samples are similarly named with an alphanumeric code as in the cocoa samples.

For the enzymatic quantitation, a commercial enzymatic kit for the lecithin analysis in foodstuffs, based on

the method of Beutler and Henniger (1981), was used (Boehringer Mannheim, Germany, Cod.:529362). Final UV-absorbance determinations were carried out with a Hitachi UV/VIS spectrophotometer.

HPLC-UV determinations were carried out with a Perkin Elmer System, using silica stationary phases: Nucleosil 50–5 & 100–5 (Macherey-Nagel).

Standards of soybean phospholipids (L-α-PC and L-α-PE) were purchased from Sigma & Aldrich (Madrid). All chemicals were analytical grade: *n*-hexane, isopropylic alcohol (Scharlau, Barcelona), potassium chloride, *terc*-butanol, trichloroethane (Panreac, Barcelona) and deionized water.

Sample preparation

Both analytical methods required similar but not identical sample preparation procedures. Commercial lecithin samples were treated by dissolution. In the case of the enzymatic analysis, 0.2 g lecithin was dissolved in 5 ml *terc*-butanol and diluted with deionized water to 25 ml (or 50 ml for L62 and L98 samples). For chromatographic analysis, samples were directly dissolved in chilled chloroform.

Phospholipids in cocoa samples were first extracted by organic solvents. In the main, two different binary solvent combinations have been described for carrying out a phospholipid extraction in an aqueous suspension: chloroform/methanol 2:1 (Folch *et al.*, 1951, 1957), and benzene/ethanol 1:1 (Schiefer & Beutler, 1985). In a preliminary study, we estimated that the benzene/ethanol extraction yielded larger amounts of individual PC than the corresponding chloroform/methanol extraction. This latter option was more suitable for a total phospholipids extraction.

According to these results, benzene/ethanol (BE) 1:1 extraction was adopted for the phosphatidylcholine extraction in cocoa samples.

Three g of a lecithinated cocoa powder were blended with 25 ml of BE, and afterwards with 40 ml of a 0.8% K Cl aqueous solution. After a vigorous shaking, two phases were separated by centrifugation (3500 rpm 10 min). The upper phase was aspirated, and the remaining aqueous suspension was re-extracted three times with 3×15 ml of organic solvent.

All the benzenic extracts were collected and evaporated to near-dryness in a vacuum rotary evaporator. For an enzymatic determination, the residue was redissolved in *terc*-butanol and water (1:9) until 25 ml. Otherwise, chromatographic determinations were carried out redissolving the residue in 25 ml trichloroethane.

Enzymatic method

The method allows the lecithin quantitation by means of a cascade reaction series that includes the hydrolysis of PC, three selective phosphorilations and the final oxidation of NADH. First, L-α-phosphatidylcholine is hydrolyzed by the enzyme phospholipase C to a diglyceride and phosphorylcholine, and the phosphorylcholine formed is hydrolyzed by alkaline phosphatase to choline. After heat-inactivation of the alkaline phosphatase, the formed choline is phosphorylated in the presence of adenosine-5′-triphosphate by the enzyme choline kinase. The resulting ADP formed in the preceding reaction is reconverted by phosphoenolpyruvate to ATP with the formation of pyruvate in the presence of pyruvate kinase. Finally, in the presence of L-lactate dehydrogenase, pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH). This last chain-reaction product (NAD^+) is formed in direct proportionality with the original phosphatidylcholine content, and can be quantified spectrophotometrically by a shift absorbance at 340 nm. A blank determination was carried out simultaneously with each determination series, substituting the sample solution with deionized water. Thus, the variation in absorbance can be expressed as dipalmitoylphosphatidylcholine (Boehringer Mannheim, 1989).

Intra-assay precision of the whole analysis was evaluated and expressed as the coefficient of variation (CV%), giving a 2.3, a 3.3 and a 7.2% for an L30-, an L62-type lecithins and an LAC sample, respectively.

Chromatographic method

The separation of phospholipids in molecular classes (i.e. PE, PI and PC) was carried out according to the different polar groups of each class, which requires the use of normal-phase HPLC.

As a mobile phase, a ternary solvent combination of *n*-hexane/isopropanol and an acetate buffer solution pH 4.2 (47:47:6) (Nasner & Kraus, 1981) was adopted. The injection volume was 25 μl. Because phospholipids suffer from specific chromophore groups, spectrophotometric UV detection for quantitation was carried out at 206 nm (Hax & Geurts van Kessel, 1977). A representative chromatogram of a lecithin sample with our analytical conditions is shown in Fig. 1. Major peaks identification was carried out injecting, in different runs, the corresponding trichloroethanolic solutions of phospholipid standards (PE and PC). The last peak was identified as PC, eluting isolately from the rest of phospholipids in a real sample.

Validation study gave a precision of 3.7% at 6 μg PC and 3.8% at 30 μg PC. Calibration was achieved using an external standard of soybean phosphatidylcholine from 3 to 60 μg. The resulting peak areas of the external standard allowed the calculation of the calibration function, giving linear correlation coefficients of the regression equation of 0.9984 and 0.9978. Determination limits were calculated as three and ten times the blank variability (ACS, 1980; Knoll, 1985), using an appropriate t value, and gave the following limit values: 0.399 μg ml^{-1} (detection) and 0.599 μg ml^{-1} (quantitation).

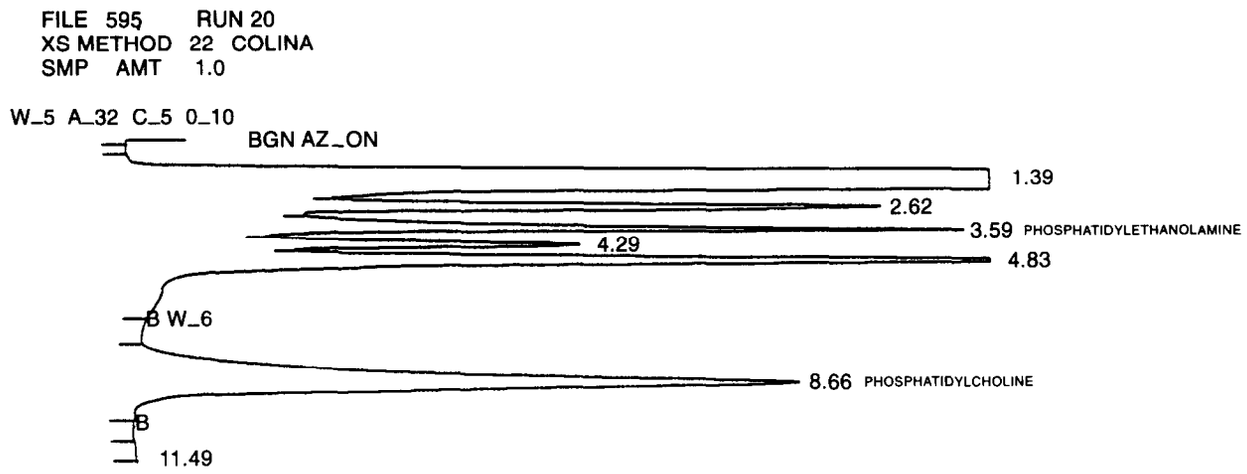

Fig. 1. Illustrative chromatogram of a commercial lecithin sample.

Comparison of PC results

Statistical comparison of both series of results was carried out applying the following tests:

- comparison of the medians of the paired data coming from both types of sample using Wilcoxon's non-parametric test.
- the non-parametric Spearman rank-correlation coefficient to express the degree of correlation, according to Snedecor and Cochran (1980).
- the Working and Hotelling confidence limits (95%) of the origin ordinate obtained with least squares regression to determine whether correlation passes through the origin. For this, the multiplier $\pm t_{0.05}$ of the 95% confidence limits is replaced by a multiplier $\pm (2F_{0.05})^{1/2}$. By this means it is possible to obtain a joint confidence band of the regression which simultaneously takes into account the errors on y and x. Thus, errors on x and y can be considered simultaneously, avoiding the need to assume the absence of errors in the x variable in a theoretical regression model (Snedecor & Cochran, 1980; Feinberg, 1988).

RESULTS AND DISCUSSION

The percentage values of PC in the samples are shown in Tables 1 and 2. PC results were compared, and the statistical data calculations are shown in Table 3.

The results of both types of sample can be significantly correlated because both Spearman rank-correlation coefficients are significantly different from zero.

Nevertheless, although the origin ordinates of the respective linear correlation straight lines cannot be significantly considered as different from zero, it can be clearly observed that all the chromatographic results are systematically lower than those of the enzymatic determination, particularly when cocoa samples are considered. This fact has also been noted by the statistically significant median-differences obtained with a Wilcoxon test.

An obvious limitation in the chromatographic quantitation is the fact that the external standard calculation must be based on results obtained with calibration standards with approximately the same degree of unsaturation of the samples, since the response of the detector depends greatly on that. For this reason, we

Table 1. Percent values (w/w) of phosphatidylcholine in lecithinated cocoa

Sample	% $PC_{Enzymatic}$	% PC_{HPLC}
LAC A1	0.51	0.46
LAC A2	0.23	0.11
LAC B1	0.44	0.31
LAC B2	0.50	0.39
LAC B3	0.34	0.29
LAC C1	0.37	0.20
LAC D1	0.68	0.53
LAC E1	0.43	0.35
LAC F1	0.61	0.53
LAC F2	0.40	0.39

Table 2. Percent values (w/w) of phosphatidylcholine in lecithin

Sample	% $PC_{Enzymatic}$	% PC_{HPLC}
L98A1	24.49	22.00
L62A1	14.17	12.83
L62A3	13.07	12.74
L62A4	11.79	9.82
L62A5	13.04	11.40
L62A6	13.82	14.12
L62B1	13.71	11.76
L62B2	14.87	14.85
L62C1	14.32	12.34
L62D1	15.33	13.67
L30A1	7.69	7.44
L30A2	7.57	6.91
L30A3	6.55	5.17

Table 3. Statistical comparison between both pair data series

	Lecithin results	Cocoa results
Medians (HPLC/ Enzymatic)	12.34/13.71	0.37/0.43
Sum of all signed ranks	−85.0	−55.0
p value (2-tailed)	0.0012	0.0020
ρ (Spearman)	0.9231 ($p < 0.01$)	0.9394 ($p < 0.01$)
Origin ordinate	0.59628	0.13015
Working's 95% C.I.	−1.59050 to 2.78306	−0.01285 to 0.27314

consider as an adequate approximation the use of a soy phospholipid standard for the external calibration, due to the fact that there is no standard reference material.

Besides that, the enzymatic quantitation must be done by using a conversion factor, from absorbance differences of the final solution to a percentage of dipalmitoylphosphatidylcholine in the samples. Both circumstances allow us to attribute a theoretically higher analytical selectivity, and therefore a better final accuracy, for the chromatographic method.

The non-equivalency of the results of the phosphatidylcholine quantitation by different enzymatic methods has occasionally been evidenced, alluding to differences in the analytical selectivity (Campanella *et al.*, 1990). This lack of an adequate selectivity can be attributed to the particular enzymatic chain-reactions or to the last product quantitation.

In fact, this enzymatic determination includes, among others, the use of phospholipase C (E.C. 3.1.4.3) and choline kinase (E.C. 2.7.1.32), which constitute one of the most selective enzyme combinations that can be used. Additionally, although the 340 nm wavelength is not absolutely selective, the measurement of absorbance differences (and not the absolute value) induced by the last enzymatic reaction contributes to increasing the selectivity of the method for choline phospholipids.

Notwithstanding, phospholipase C can have a little sphingomyelinase activity (Bradley *et al.*, 1987), which cannot be higher than 0.05% (Schiefer & Beutler, 1985). Otherwise, the choline kinase substrate specificity does not extend to diethyl- and dimethylamine ethanol, with a cross-reactivity of 50% (Boehringer Mannheim, 1989) where these substrates were present in the matrix.

All these facts could lead to systematically higher results than the true phosphatidylcholine content, expressed as dipalmitoylphosphatidylcholine, depending on the different compositions of the sample matrices.

All the preceeding considerations indicate that, in our case, there appears to be an obvious significant correlation between the two analytical determinations. But, on the other hand, there is also a clear discordance in the accuracy of those analytical methods that depend on the sample matrix components and that could be attributed to a lower analytical selectivity of the specific enzymatic determination of phosphatidylcholine. These facts lead to the conclusion that the results of a phosphatidylcholine determination in lecithin, or in lecithinated cocoa, can be affected by the analytical technique that has been employed, owing to differences in analytical specificity, and can be more evident with cocoa samples, where the occurrence of a clear matrix effect has been observed.

We have then assumed the chromatographic determination as a reference one, while the enzymatic determination seems to be a faster determination, but subjected to limitations when interanalytical comparisons must be done.

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