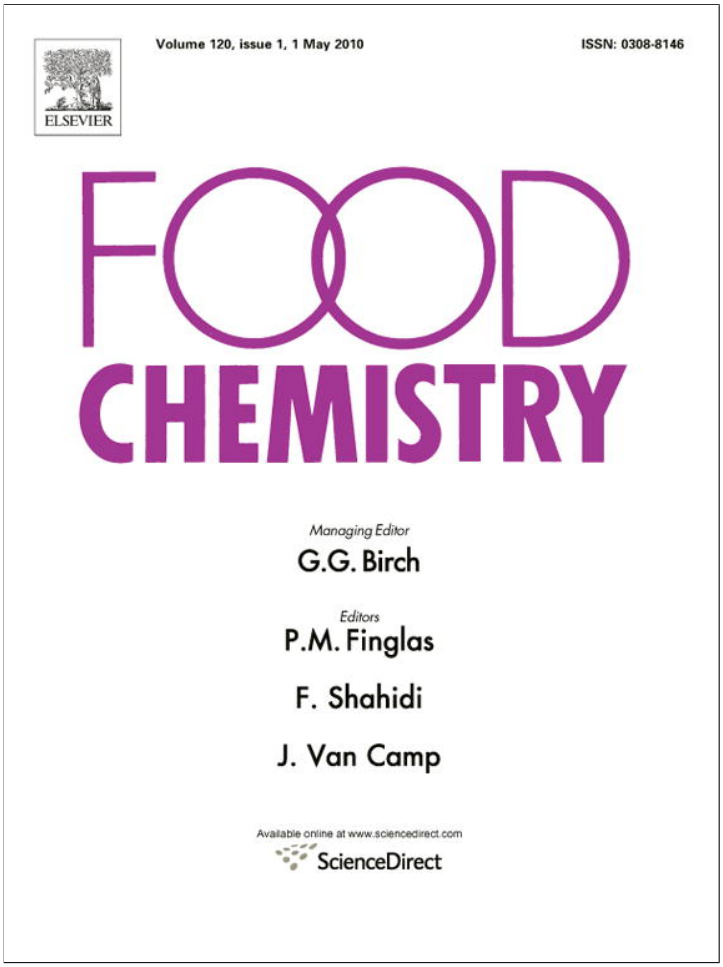

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Analytical Methods

Free-fatty acid profile obtained by enzymatic solvent-free hydrolysis of sunflower and soybean lecithins

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ABSTRACT

The free-fatty acid profile of sunflower and soybean lecithins generated by enzymatic hydrolysis was determined by capillary gas chromatography using the internal standard method. This procedure involves the previous lipid extraction, clean-up and fatty-acid methyl-esters preparation. Different conventional methods commonly employed to calculate the hydrolysis degree in both substrates were compared. The fatty-acid profile of sunflower and soybean phospholipids and its initial acidity composition were chromatographically determined. Results from recovery studies were satisfactory (98% and 108% for soybean and sunflower, respectively). The effect of temperature (50 and 60 °C) and pH conditions (pH 7 and pH 8) on lecithin-hydrolysis degree was evaluated, being temperature the most affecting parameter. The profile of the fatty acids liberated after hydrolysis depended on both the enzyme and the pH of reaction mixture. Finally, the chromatographic methodology employed in this paper may be useful to study the lecithin enzymatic modification in order to produce emulsifiers with specific characteristics.

1. Introduction

Argentina is an important producer of soy- and sunflower oil. During oil processing, the phospholipids present in the crude oil are removed in order to enhance physical oil stability. The gums produced after degumming process contain variable amounts of water, phospholipids, suspended matter and oil. Lecithin can be obtained by removing water, oil and other components like glycolipids. Lecithin exhibits emulsification properties in numerous applications in food and pharmaceutical industries, among others (van Nieuwenhuyzen, 1981). Nowadays, the local industry adds no processed gums to pellets and meals for animal feed in order to increase their nutritive value. However, modified lecithins have not been produced in Argentina yet with the purpose of using them like emulsifiers. Although the soybean is the most frequent and studied source of lecithin, phospholipids may be obtained from different crude vegetable oils like a by-product. Phospholipids have two fatty-acyl chains and a polar head group; the main phospholipids present in oilseed crops are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) (Padley, Gunstone, & Harwood, 1994). In consequence, their occurrence and distribution give the particular characteristics to lecithin (Cherry & Cramer, 1989). Sunflower seed can be considered a potential source of lecithin due to the distribution of the main phospholipid components, which appear to be similar to soybean lecithin (Holló, Perédi, Ruzics, Jeránek, & Erdélyi, 1993).

By changing the hydrophilic/lipophilic balance of phospholipids, it is possible to produce lecithin for different applications (Mustranta, Forssell, & Poutanen, 1995). Fatty-acid profiles of phospholipids depend on the lecithin source and may be modified by several enzymatic methods, such as hydrolysis, transesterification or alcoholysis, in order to enhance lecithin functional properties (Aura, Forssell, Mustranta, & Poutanen, 1995; Ghosh & Bhattacharyya, 1997; Hara, Nakashima, & Fukuda, 1997). Lipases and phospholipases are the biocatalysts that have been most often studied and in recent years employed to modify lecithin's native structure (Mustranta, Suortti, & Poutanen, 1994; Ghosh & Bhattacharyya, 1997; Kim, Garcia, & Graham Hill, 2007). Particularly, phospholipase A_2 (PLA_2) is specific for sn-2 position. The hydrolysis reaction can be placed either in organic solvent systems by W/O microemulsion using alcohols or in solvent-free systems (Mustranta et al., 1995; Aura, Forssell, Mustranta, Sourtti, & Poutanen, 1994; Yamazaki, Imai, & Suzuki, 2004). Modification reactions of phospholipids carried out without organic solvents are safer in order to produce food emulsifiers. Actually, biotechnology is an alternative for the classical chemical production of a lot of food additives. Chemical production usually involves the use of solvents that may be dangerous for human health and environmental

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safety. Enzymes are compatible with the green-chemistry concept; they are selective catalysts that generally can be used in a non-toxic solvent medium. Lysophospholipids obtained after phospholipid hydrolysis with phospholipases may exhibit interesting applications in food. This is due to their high hydrophilicity that enhances stability in sauces, mayonnaises, and margarines. In breadmaking, lysophosphatidylcholine improves product extensibility, volume and shelf-life (Holló et al., 1993; Knightly, 1989; van Nieuwenhuyzen, 1981).

The phospholipid-hydrolysis reaction involves the appearance of free-fatty acids (FFAs), a fact that can be profited from in order to determine the lecithin-hydrolysis degree during the enzymatic reaction. Acid–base titration of FFAs is one of the analytical procedures most frequently used to determine the FFA content. This procedure is easy and fast, but the employment of a chemical indicator, like phenolphthalein, to determine the endpoint of the titration may be limited when the sample is coloured. A potentiometric titration can be utilised instead, but matrix interference obliges the use of organic solvents like methyl-isobutyl-ketone (IUPAC, 1992). Besides, automatic titration equipment is also recommended. Moreover, gas chromatographic techniques can also be employed to evaluate changes in fatty-acid composition during phospholipid modification (Holló et al., 1993). The study of the hydrolysis degree by itself results incomplete when considering the structure and functional properties of the products obtained. There is a lack of analytical procedures to evaluate directly the distribution of the FFAs generated in the hydrolysis. The experiences of lecithin modification under industrial conditions together with adequate techniques of analysis may be useful to evaluate the potential applications of these soybean and sunflower by-products in order to produce emulsifiers with implications on food or cosmetic industry.

The main aim of this work is to analyze the FFA profile generated after sunflower and soybean lecithin modification. The analytical procedure carried out involves the extraction, clean-up, fatty-acid methyl-esters (FAMEs) preparation and gas-chromatographic analysis. In addition, different analytical procedures to determine hydrolysis degree are compared.

2. Materials and methods

2.1. Substrates

Sunflower gum, provided by a local industry from water-degumming process, and commercial granulated soybean lecithin (FAS, Argentina) were used. The gums were stored at −20 °C until lecithin extraction. Sunflower lecithin was obtained from the gums by oil removal using three extractions with cold acetone at 0 °C (acetone/gum ratio of 1.5:2 v/w; shaken during 30 min). The insoluble matter (phospholipids) was collected and the solvent was removed under vacuum conditions in a rotary evaporator. Both lecithins were milled to a fine powder and used like substrate for the enzymatic-hydrolysis reaction.

2.2. Chemicals

Organic solvents were analytical grade, whereas *n*-hexane and 2-propanol for the determination of phospholipid distribution were HPLC grade from J.T. Baker Inc. (Phillipsburg, NJ). Silica gel 60, particle size 0.063–0.200 mm, 70–230 mesh (Merck, Darmstadt, Germany) was dried at 500 °C for 8 h, hydrated with 2% (w/w) of water, and stabilized for 12 h, prior to its use in column chromatography. Potassium hydroxide was used as titration reagent for hydrolysis-degree determinations, being their ethanolic solutions standardised every 30 days. Lauric acid 99% from Fluka (certified reference substance, Sigma–Aldrich, Steinheim) was employed as internal standard, while oleic acid 99% (certified reference substance, J.T. Baker, Phillipsburg, NJ, USA) was used for recovery studies. Others certified reference materials employed were Supelco FAME mix 37 and Supelco RM-3 mix (Bellefonte, PA, USA) for FAMEs identification for chromatographic analysis. Certified standard materials of L-α-PC, L-α-PE, L-α-PI from soybean and L-α-PA, sodium salt from egg yolk lecithin with purities greater than 98% were obtained from Sigma Chemical Co. (St. Louis, MO) for substrate characterisation.

2.3. Enzymes

Lecitase 10 L (Phospholipase A_2 (PLA_2), E.C.3.1.1.4) from porcine pancreas (10,580 U/mL) and Lecitase Ultra (Phospholipase A_1 (PLA_1), E.C.3.1.1.32) from *Aspergillus oryzae* (10,000 U/mL) were provided from Novozymes A/S, (Bagsvaerd, Denmark) and employed in a soluble form.

2.4. Substrate characterisation

Acetone-insoluble matter, hexane-insoluble matter, humidity and acid value of lecithin were determined using the AOCS official methods (Ja 4-46, Ja 3-87, Ja 2b-87, Ja 6-55), (AOCS, 2001). The phospholipid lecithin composition was determined by HPLC–UV using the AOCS Ja 7b-91 method (AOCS, 2001). Analyses were performed by triplicate. Lecithin fatty-acid composition was determined by gas chromatography of methyl esters (by duplicate) following AOCS Ce 2–66 method (AOCS, 2001).

2.5. Enzymatic-hydrolysis reaction

Lecithin was hydrolyzed at 50 and 60 °C in both Tris–HCl buffer pH 8 and phosphate buffer pH 7. Calcium chloride 0.2 M was added to assure a final concentration of 6 mM Ca^{+2} when Lecitase 10 L was used. The reaction system was magnetically stirred (400 rpm) in glass flasks employing a thermostated bath at 50 and 60 °C. The reaction mixture consisted on 20 mL of buffer, 400 mg of lecithin and 5 μL of enzyme. All the reactions were carried out at least by duplicate. Aliquots of 2 mL were withdrawn from the stirred reaction mixture at 30 min time reaction. These aliquots constitute the samples that were analyzed immediately by the following described methods.

2.6. Initial acidity and hydrolysis degree determination

In order to compare different methods for the initial acidity and hydrolysis degree determination, the following procedures (Methods I, II and III) were carried out.

2.6.1. Method I: titration

The increase of FFA concentration on the reaction mixture was determined by using AOCS Ca 5a-40 method (AOCS, 2001). Samples were mixed with 5 mL diethyl ether-ethyl alcohol 1:1 v/v and titrated with 0.1 N KOH ethanolic solution or its corresponding dilution using a microburette and phenolphthalein as indicator. Titration assays were performed at least by duplicate.

2.6.2. Method II: FFA extraction and titration

FFA extraction from the reaction mixture was made using the procedure proposed by Dole and Meinertz (1960). In brief, a 2 mL aliquot was manually shaken during 2 min with 10 mL of isopropyl-alcohol: heptane: sulphuric acid, (4:1:0.1, v/v/v). Afterwards, MilliQ water (4 mL) and heptane (6 mL) were added and the mixture centrifuged at 4500 rpm for 1 min. The upper phase, which

contained the FFA generated during reaction, was collected and analyzed by titration (by duplicate), as indicated in Method I.

2.6.3. Method III: chromatographic method

2.6.3.1. Clean-up. The FFAs were extracted from the sample, as indicated in Method II. A portion of the resulting extract was purified using silica-gel column chromatography. Approximately 1.5 g of hydrated silica gel (2% water content) was conditioned with chloroform in a glass column (15 mm inner diameter). Afterwards, the sample was transferred to the column and the FFAs were eluted with 20 mL chloroform/acetic acid (99:1, v/v). Some pigments were retained at the top of the column, while avoiding interferences during the chromatographic analysis. Phospholipids or lysophospholipids that might migrate to the organic phase during extraction can also be retained. After clean-up, the solvent was evaporated to dryness under nitrogen stream.

2.6.3.2. FAMEs preparation and chromatographic analysis. An internal standard (Lauric acid) was added to an aliquot of the purified FFA extract. FFAs were converted to methyl esters by acid-esterification according to IUPAC standard method 2.301 (IUPAC, 1992). FAMEs were analyzed with an Agilent 4890D gas chromatograph (Agilent Technologies, DE), equipped with a flame-ionisation detector, a split/splitless injector and a capillary column SP-2380 (30 m × 0.25 mm × 0.2 μm) (Supelco Inc., Bellefonte, PA). The operating conditions were as follows: injector and detector temperatures, 220 °C; oven temperature, 170 °C (10 min)–4 °C/min–210 °C (25 min); carrier gas, H_2; split ratio, 1:100; and injection volume, 1 μL. HP 3398A GC Chemstation Software (Hewlett Packard Company, Palo Alto, CA) was used for chromatographic analysis. Response factors were determined, being higher than 0.85 in all cases. FFA extraction and clean-up, FAME preparation, and GC analysis were performed by duplicate.

2.6.3.3. Recovery studies. Soybean and sunflower lecithin samples (100 mg) were enriched with oleic acid in order to evaluate the performance of the chromatographic method. The enriched samples (15 mg of oleic acid in 100 mg of lecithin) were extracted (Method II) and analyzed by chromatography (Method III). The assays were carried out by triplicate for both lecithins.

2.7. Calculation of hydrolysis degree (HD)

A general expression was used to calculate the HD. It is valid independently of the method employed to determine the FFA increment:

$$\mathrm{HD}(\%) = \frac{\{[\mathrm{FFA}] - [\frac{\mathrm{IA}}{M_O} \mathrm{L}_M]\} M_{\mathrm{PL}}}{\mathrm{L}_M \mathrm{AI}_M F} 100$$

Where FFA = total free-fatty acids determined after hydrolysis [mol], IA = initial free-fatty acid content [mg oleic acid/mg lecithin], M_O = oleic acid molecular mass [mg/mol], AI_M = acetone-insoluble matter [g/100 g of lecithin], L_M = lecithin mass [mg], M_{PL} = lecithin molecular mass [mg/mol], F = mol number of hydrolysed fatty acids per mol of phospholipid according to the enzyme attack position.

Gutiérrez-Ayesta, Carelli, and Ferreira (2007) employed similar expressions to study lipase catalytic ability to hydrolyse triglycerides and phospholipids. F is a factor that depends on the number of fatty acid (FA) moles liberated by phospholipid mol. If both the enzyme is specific to only one phospholipid position and acyl-migration does not take place, $F = 1$.

Due to the fact that lecithin is a mixture of phospholipids, the molecular mass can be estimated from its FA and its phospholipid composition (Carelli, Ceci, & Crapiste, 2002).

$$M_{\mathrm{PL}} = \left[\frac{W_{\mathrm{PC}}}{221.2 + 2M_{\mathrm{FA}}} + \frac{W_{\mathrm{PE}}}{179.1 + 2M_{\mathrm{FA}}} + \frac{W_{\mathrm{PI}}}{217.2 + 2M_{\mathrm{FA}}} + \frac{W_{\mathrm{PA}}}{136.0 + 2M_{\mathrm{FA}}}\right]^{-1}$$

With $M_{FA} = \Sigma[Wi/Mi]$, where Wi and Mi represent the mass fraction and the molecular weight of the ith FA, respectively. By means of this formula, the estimated average M_{PL} of soybean and sunflower lecithin were 738.43 and 747.13 g/mol, respectively.

2.8. Statistical analysis

The differences in mean values ($n = 3$) between IA and HD of soybean and sunflower lecithins from different methods as well as between mean percentages ($n = 4$) of liberated free-fatty acids after hydrolysis reactions were assessed with Tukey's test, being statistically different at a significance level of 5%. ANOVA analysis was applied to enzymatic hydrolysis data being HD the dependent variable. In all the cases an academic statistical package (InfoStat '2009', grupo infoStat, FCA, UNC, Córdoba, Argentina) was used.

3. Results and discussion

3.1. Substrate characterisation

The results from soybean- and sunflower-lecithin characterisation are summarised on Table 1. FA composition for both lecithins accords with oil seed composition (Padley et al., 1994), being the main fatty acids concordant with the results obtained by other authors (Holló et al., 1993; Lantz, 1989). Soybean and sunflower lecithin present classical indexes in agreement with typical commercial specifications. Their phospholipid composition is within the ranges reported in the literature for soybean lecithin (PC: 12–46%, PE: 8–34%, PI: 1.7–21%, PA: 0.25–23%), and for sunflower phospholipid distribution (PC: 14–41%, PE: 17–24%, PI: 13–23%, PA: 3–22%) (Carelli et al., 2002; Cherry & Cramer, 1989; Holló et al., 1993). The sunflower gum, which is the raw material for sun-

Table 1
Soybean- and sunflower-lecithin characterisation.

Method	Commercial soybean	De-oiled sunflower
Acetone-insoluble matter[a] (%)	93.49 ± 0.36	90.93 ± 0.13
Hexane-insoluble matter[a] (%)	0.29 ± 0.01	0.89 ± 0.02
Moisture (Karl Fischer)[a] (%)	2.84 ± 0.15	3.18 ± 0.06
Acid value[a] (mg KOH/g)	25.7 ± 2.2	21.0 ± 2.3
Phospholipid distribution[a] (%)		
PC	39.0 ± 2.6	30.4 ± 0.3
PE	23.5 ± 0.2	20.2 ± 0.2
PI	11.2 ± 0.4	24.8 ± 0.8
PA	26.3 ± 2.1	24.6 ± 1.3
Fatty-acid profile[b] (%)		
C14:0	0.1	0.1
C16:0	19.9	18.9
C16:1	0.2	0.2
C18:0	4.5	4.9
C18:1	9.1	9.2
C18:2	57.4	64.2
C18:3	7.7	0.2
C20:0	0.2	0.3
C20:1	<0.1	0.1
C20:2	<0.1	-
C22:0	0.4	1.3
C22:1	0.2	0.2
C24:0	0.3	0.4
Unsaturated/saturated ratio	2.94	2.87

[a] Arithmetic means of triplicate determinations with their confidence interval at 95%.
[b] Arithmetic means of duplicate determinations.

flower lecithin, was also characterised showing the following phospholipid composition: PC = 42.8%, PE = 15.3%, PI = 18.4%, PA = 23.5%. De-oiling process with cold acetone resulted in a higher PE and PI content and in a loss of PC. The FFA profile obtained by chromatographic analysis of the initial free-fatty acid content (soybean: C16:0 = 25.2%, C18:0 = 12.6%, C18:1 = 8.3%, C18:2 = 47.1%, C18:3 = 5.6%, C22:0 = 0.7%, C24:0 = 0.6%; sunflower: C16:0 = 18.9%, C18:0 = 10.6%, C18:1 = 10.8%, C18:2 = 57.6%, C22:0 = 1.6%, C24:0 = 0.6%), was similar to the lecithin composition for soybean and sunflower, being linoleic and palmitic acid the most abundant.

3.2. Initial acidity and hydrolysis-degree determination – comparison of methods

In order to study the enzymatic hydrolysis of lecithin through the generation of FFAs, it is necessary to determine lecithin's initial acidity (IA, expressed mg oleic acid/mg lecithin), considering IA as the total FFA content present in substrates previous to the enzymatic modification. Hydrolytic damage or oil presence may be the source of FFAs. Three different methods were used to determine IA and HD: (I) direct titration of reaction medium, (II) FFA solvent extraction followed by titration, and (III) chromatographic method. For hydrolysis-degree (HD) determination, lecithins were hydrolysed with PLA_2 at pH 8 and $T = 50$ °C (enzyme load 5290 U/mL), which are described in Section 2. This condition was selected from a screening study so as to obtain low HD, thus allowing the best comparison between methods. The reactions were started with the enzyme incorporation. Blank assays (without enzyme) were carried out for all proposed methods (Methods I, II and III) without noticing FFA compositional changes in the reaction mixture. Table 2 compares the IA and HD values obtained in soybean and sunflower lecithin from the three above mentioned methods. In general, both substrates presented the same behaviour despite the method employed. The first method had an easy and fast implementation. However, the titration endpoint depends on the analyst's ability. Sunflower lecithin presented a coefficient of variation (CV) higher than the one for soybean lecithin, probably due to the difficulty in determining the titration endpoint as a result of the sample colour (dun-greenish). Method II involves the previous extraction of FFAs from the reaction mixture, and it requires more analytical time than Method I. In contrast, Method II has the advantage that the organic layer to be titrated is colourless. As result, this method showed lower CV values for both substrates. Method II should be evaluated more exhaustively for samples wherein lysophospholipids are the main component. Those compounds may be partially soluble on the organic layer or be present at the interface, causing interferences in the determination. The chromatographic method, called Method III, was evaluated through recovery studies. Satisfactory recovery percentages have been obtained using both lecithins: 98% (CV = 5%, $n = 3$) for soybean lecithin and 108% (CV = 1%, $n = 3$) for sunflower lecithin. The performance of silica gel clean-up was confirmed by TLC. No phospholipid band was observed after clean-up. Limit of detection (LOD, signal-to-noise ratio = 3) and limit of quantification (LOQ, signal-to-noise ratio = 10) were obtained for the internal standard being these values 0.2 and 0.7 μg/mL, respectively. Linearity was confirmed by C12:0 calibration curves (concentration range 0.18–85 μg/mL, $R^2 = 0.9976$). Method III presents the lowest CV for both substrates (Table 2); probably due to the clean-up procedure that removes interfering substances. The column chromatography lets pigments or phospholipids/lysophospholipids be retained. Otherwise, they can produce undesirable peaks on the chromatographic analyses or problems in the injection system. Method III involves the longest time of analysis. In addition, it is the most expensive since the majority of the materials must be chromatographic grade. In contrast, it presented the lowest CV and it is the only method that can give information about the FFA profile generated during hydrolysis. For these advantages, we decided to use this methodology in the following hydrolysis studies.

Table 2
Initial acidity and hydrolysis degree of soybean- and sunflower-lecithins from different methods.

Lecithin	Method I		Method II		Method III	
	IA	CV (%)	IA	CV (%)	IA	CV (%)
Initial acidity determination						
Soybean	0.11^{B}	13.0	$0.09^{A,B}$	10.0	0.07^{A}	4.6
Sunflower	0.09^{B}	23.0	0.09^{B}	18.3	0.05^{A}	6.7
	HD (%)	CV (%)	HD (%)	CV (%)	HD (%)	CV (%)
PLA_2 hydrolysis reaction						
Soybean	11.84^{B}	4.3	9.20^{A}	1.8	9.02^{A}	5.3
Sunflower	17.09^{C}	17.1	14.52^{B}	12.8	9.55^{A}	4.7

IA, Initial acidity expressed in mg oleic acid/mg of lecithin; HD, hydrolysis degree; CV, coefficient of variation. All values are arithmetic means of triplicate determinations. Different capital letters in the same row are significantly different (Tukey's test, $p < 0.05$).

3.3. Hydrolysis studies

Hydrolysis studies with PLA_2 and PLA_1 were performed under the conditions indicated in Section 2. It is commonly accepted that water activity has a lot of influence on the lipase and phospholipase activity (Han & Rhee, 1998; Mustranta et al., 1994). In this study the hydrolysis experiences were carried out with a high content of water. So, it was assumed that the hydrolysis reaction was favoured. The re-esterification reaction was not taken into account because of the low ratio FFA/lecithin (FFA generated during hydrolysis). Consequently, this reaction is not considered to take place. This fact is in accordance with other authors (Svensson, Adlercreutz, & Mattiasson, 1992; Vikbjerg, Mu, & Xu, 2007). Since lecithin concentration was low (20 mg lecithin/mL reaction mixture), the water content was high during the reaction and HD was below 50% in all the experiences giving low concentration of FFAs. In this order, the factor $F = 1$ for both enzymes was assumed in the HD calculation.

Tables 3 and 4 summarise the hydrolysis results for soybean and sunflower lecithin that were obtained by employing the chromatographic method. The hydrolysis degree with its CV was evaluated for each substrate at 50 and 60 °C using pH 7 and pH 8. Vikbjerg et al. (2007) found that the highest content of glycero-

Table 3
Enzymatic hydrolysis and liberated fatty-acid (LFA) distribution using PLA_2.

Lecithin	pH 7				pH 8			
	50 °C		60 °C		50 °C		60 °C	
	HD (%)	CV (%)	HD (%)	CV (%)	HD (%)	CV (%)	HD (%)	CV (%)
Soybean	6.8	8.9	16.1	2.6	9.2	6.9	32.1	2.9
FA	LFA distribution (%)							
C18:1	8^{B}		6^{A}		10^{C}		8^{B}	
C18:2	84^{A}		87^{B}		83^{A}		$86^{A,B}$	
C18:3	8^{A}		7^{A}		7^{A}		6^{A}	
Sunflower	6.4	10.2	14.1	4.9	7.9	5.5	25.7	6.2
FA	LFA distribution (%)							
C18:1	8^{A}		11^{B}		7^{A}		10^{B}	
C18:2	92^{B}		89^{A}		93^{B}		$90^{A,B}$	

LFA, liberated fatty acids; HD, hydrolysis degree. All values are arithmetic means of quadruplicated determinations. Different capital letters in the same row are significantly different (Tukey's test, $p < 0.05$).

phosphorylcholine (GPC) was at 45 °C during PC acidolysis using PLA_2. GPC will be formed if the FA on the sn-1 position of a LPC (lysophosphatidylcholine) migrates to the sn-2 position. For PLA_2, experiences at this temperature were avoided. The highest HD was achieved under pH 8 and 60 °C for both enzymes. In this preliminary study, temperature factor showed more influence at pH 8 than at pH 7. When PLA_2 was used (Table 3) on a pH 7 buffer medium, an increment of the reaction temperature from 50 to 60 °C produced a duplication on the HD. However, at pH 8 the same increment triplicates HD values. By employing PLA_1 (Table 4), hydrolysis experiments achieved lower conversions than PLA_2. For PLA_1, at pH 7 fewer than 10% of the phospholipids were hydrolyzed independently of the reaction temperature. At 60 °C and pH 8 the HD duplicates the percentage reached at 50 °C. ANOVA analyses were carried out on HD% results. When PLA_2 is considered, significant differences ($F < F_{crit}$, $p < 0.05$) were found between substrates at 60 °C and both pH. As regard as PLA_1, only at pH 7 and 50 °C significant differences ($F < F_{crit}$, $p < 0.05$) between substrates are observed.

Table 4
Enzymatic hydrolysis and liberated fatty-acid (LFA) distribution using PLA_1.

Lecithin	pH 7				pH 8			
	50 °C		60 °C		50 °C		60 °C	
	HD (%)	CV (%)	HD (%)	CV (%)	HD (%)	CV (%)	HD (%)	CV (%)
Soybean	4.3	4.9	6.1	3.5	7.9	2.7	12.5	7.9
FA	LFA distribution (%)							
C16:0	61^{A}		76^{B}		66^{C}		74^{B}	
C18:0	6^{C}		2^{A}		5^{B}		2^{A}	
C18:1	26^{C}		$18^{A,B}$		21^{B}		17^{A}	
C18:2	4^{B}		1^{A}		4^{B}		5^{C}	
C20:0	3^{B}		3^{B}		4^{C}		2^{A}	
Sunflower	3.7	5.6	7.3	10.6	7.0	4.5	11.6	10.2
FA	LFA distribution (%)							
C16:0	$72^{A,B}$		69^{A}		74^{B}		75^{B}	
C18:0	4^{B}		4^{B}		7^{A}		3^{B}	
C18:1	22^{B}		23^{B}		15^{A}		$19^{A,B}$	
C18:2	–		2^{A}		1^{A}		–	
C22:0	1^{A}		1^{A}		2^{A}		1^{A}	
C24:0	1^{A}		1^{A}		1^{A}		2^{A}	

LFA, liberated fatty acids; HD, hydrolysis degree. All values are arithmetic means of quadruplicated determinations. Different capital letters in the same row are significantly different (Tukey's test, $p < 0.05$).

Surface activity properties of an emulsifier depend on the chemical structure. In general, phospholipids are a recognised emulsifier. But, by changing their original chemical composition, lecithin can be produced with novel applications (Ghosh & Bhattacharyya, 1997). If the FFAs generated during hydrolysis reaction are not removed or partially taken away, the composition of hydrolyzed phospholipids and the acidity content may affect the emulsifier's performance. Fujita and Suzuki (1990) investigated surface properties of mixtures of lysophospholipids, FFAs and monoglycerides (MGs) concluding that longitude and unsaturation grade of FA chains may affect their emulsifier performance. Taking into account these considerations, the distributions of the main fatty acids generated during hydrolysis are also shown in Tables 3 and 4. The results are in accordance with the FA stereometric analysis on these types of substances (Doig & Diks, 2003). It is well known that unsaturated FAs are mainly present at the sn-2 position of

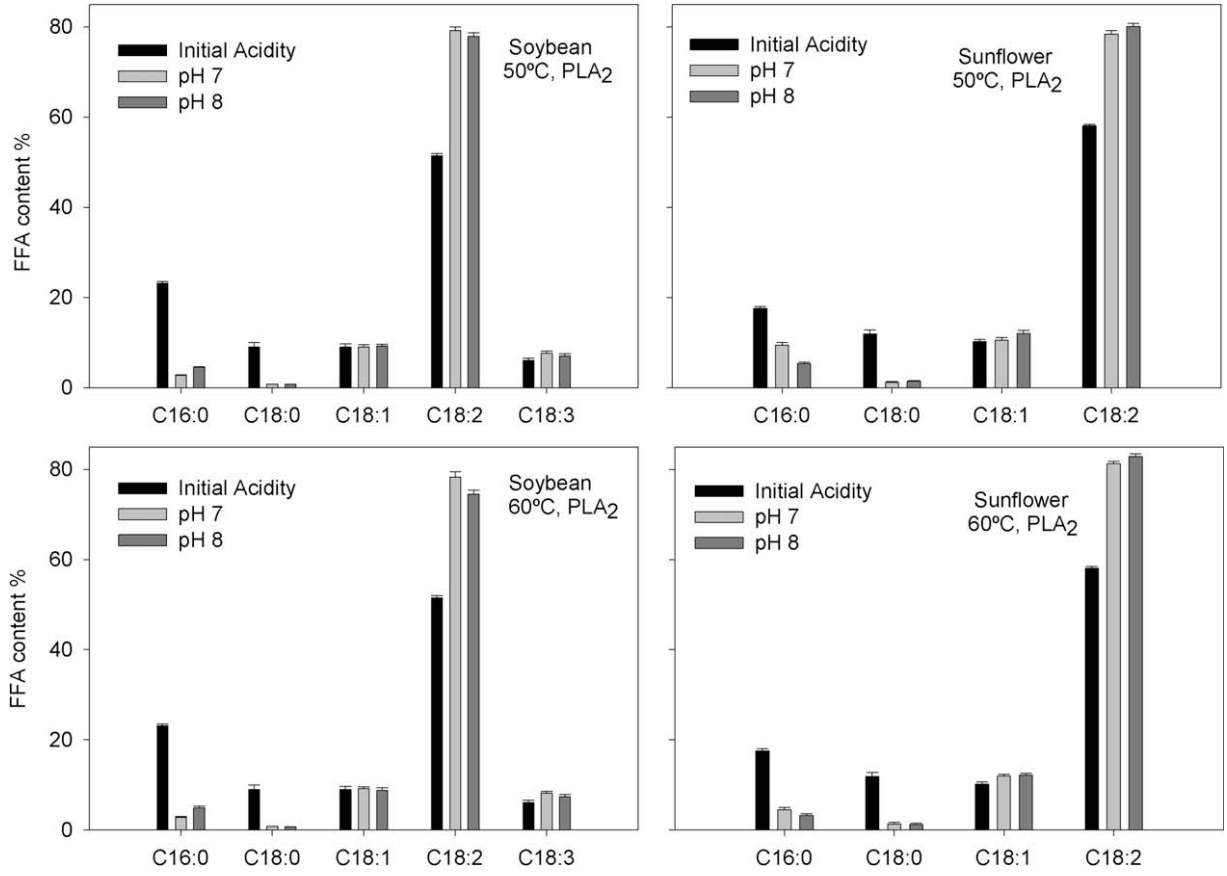

Fig. 1. Free-fatty acid profile obtained after hydrolysis using PLA_2.

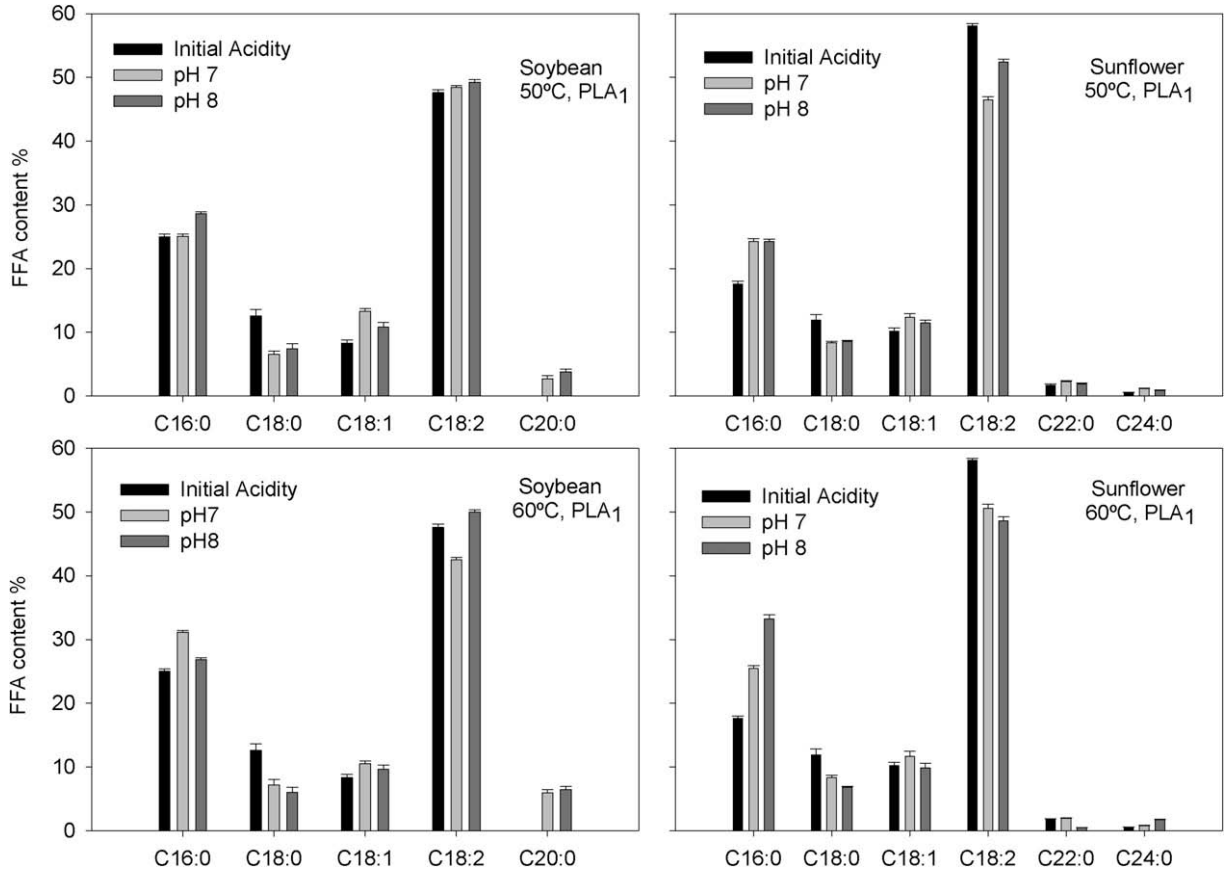

Fig. 2. Free-fatty acid profile obtained after hydrolysis using PLA_1.

phospholipids, triglycerides and other lipids. As expected, the fatty acids liberated using PLA_2 were the unsaturated ones in accordance with the sn-2 specificity of this enzyme. In these experiences, linoleic acid was the main FFA independently of the substrate and reaction conditions. Moreover, in presence of oleic acid, the occurrence of linolenic acid for soybean lecithin (Table 3) was noticeable.

Fig. 1 presents the FFA profile, where we considered the main shape was the one that had been obtained with PLA_2 during enzymatic hydrolysis. The increment on the unsaturated fatty acids is evident. In contrast, no augmentation in saturated FAs, like stearic or palmitic acid, was observed, thus showing that the acyl-migration of fatty acids from sn-1 to sn-2 has poor occurrence. This fact confirms the condition assumed for hydrolysis-degree calculation ($F = 1$). Peaks with retention time belonged to minor monounsaturated FAs such as C16:1 and C22:1 were absent initially, but they were observed in slight quantities during hydrolysis experiences. In general, for PLA_2, the pH or temperature parameters produce only slight changes on the percentages of C18:1 and C18:2. Moreover, no significant differences in the majority liberated fatty acids were detected with pH for both substrates (Table 3, Tukey's test, $p < 0.05$).

In contrast, different results were found employing PLA_1. This enzyme liberated saturated and unsaturated FAs with prevalence of palmitic and oleic acids accompanied with slight quantities of stearic acid (Table 4). Myristic acid was undetectable in the initial acidity, but in all cases traces were perceived in the reaction mixture after hydrolysis. Besides, other minor fatty acids were also detected in the reaction mixture after hydrolysis, which were the following ones: C18:2, C22:0 and C24:0 for sunflower lecithin and C18:2 and C20:0 for soybean substrate. Fig. 2 presents the FFA profile that was obtained with PLA_1. Significant differences (Table 4, Tukey's test $p < 0.05$) were found on majority liberated fatty acids with pH, for both substrates. This fact allows us to conclude that PLA_1 requires more control on the reaction conditions in order to produce modified emulsifiers with a specific FA composition. In all cases oleic acid was present in more concentration at pH 7 than at pH 8. Wang, Hammond, and Fehr (1997) have found that oleic acid was equally distributed between the sn-1 and the sn-2 positions on soybean phospholipid analysis, a fact that explains the oleic-acid liberation in the experiences with PLA_1.

4. Conclusion

In conclusion, three different methods for the evaluation of hydrolysis degree of lecithins using PLA_1 and PLA_2 were studied and compared, being the chromatographic method the most adequate for this purpose. Titration is a simple, fast method that is advised when the samples are colourless. A previous FFA extraction is useful for hydrolysis samples with strong colour. The chromatographic method provides information about the FFA profile and presents the lowest variation coefficient. This method could be helpful for other enzymatic reactions, such as acidolysis, interesterification, transesterification, etc. In this study, modified sunflower and soybean lecithins were obtained with different hydrolysis degree varying in accordance with reaction conditions. With respect to phospholipases, PLA_1 preserves the polyunsaturated fatty acids (PUFAs) in the modified phospholipid structure. In general, both lecithins showed similar hydrolysis performance in order to produce novel food additives. As a result, sunflower lecithin can be considered like a raw material for phospholipid emulsifiers.

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References

AOCS, (2001). In: D. E. Firestone (Ed.), *Official methods and recommended practices of AOCS* (4th ed.). Illinois: The American Oil Chemists Society, AOCS Press.

Aura, A. M., Forssell, P., Mustranta, A., Sourtti, T., & Poutanen, K. (1994). Enzymatic hydrolysis of oat and soya lecithin: Effects on functional properties. *Journal of the American Oil Chemists Society, 71*(8), 887–891.

Aura, A. M., Forssell, P., Mustranta, A., & Poutanen, K. (1995). Transesterification of soy lecithin by lipase and phospholipase. *Journal of the American Oil Chemists Society, 72*(11), 1375–1379.

Carelli, A. A., Ceci, L. N., & Crapiste, G. H. (2002). Phosphorus-to-phospholipid conversion factors for crude and degummed sunflower oils. *Journal of the American Oil Chemists Society, 79*(12), 1177–1180.

Cherry, J. P., & Cramer, W. H. (1989). Plant sources of lecithin. In B. Szujah (Ed.), *Lecithins: sources, manufacture & uses* (pp. 16–31). Illinois: American Oil Chemists Society.

Doig, S. D., & Diks, R. M. M. (2003). Toolbox for exchanging constituent fatty acids in lecithins. *European Journal of Lipid Science and Technology, 105*, 359–367.

Dole, V. P., & Meinertz, H. (1960). Microdetermination of long-chain fatty acids in plasma and tissues. *The Journal of Biological Chemistry, 235*(9), 2595–2599.

Fujita, S., & Suzuki, K. (1990). Surface activity of the lipid products hydrolyzed with lipase and phospholipase A-2. *Journal of the American Oil Chemists Society, 67*(12), 1008–1014.

Ghosh, M., & Bhattacharyya, D. K. (1997). Enzymatic alcoholysis reaction of soy phospholipids. *Journal of the American Oil Chemists Society, 74*(5), 597–599.

Gutiérrez-Ayesta, C., Carelli, A. A., & Ferreira, M. L. (2007). Relation between lipase structures and their catalytic ability to hydrolyze triglycerides and phospholipids. *Enzyme and Microbial Technology, 41*, 35–43.

Han, J. J., & Rhee, S. J. (1998). Effect on salt hydrate pairs for water activity control on lipase-catalyzed synthesis of lysophospholipids in a solvent-free system. *Enzyme and Microbial Technology, 22*, 158–164.

Hara, F., Nakashima, T., & Fukuda, H. (1997). Comparative study of commercially available lipases in hydrolysis reactions of phosphatidylcholine. *Journal of the American Oil Chemists Society, 74*(9), 1129–1132.

Holló, J., Perédi, J., Ruzics, A., Jeránek, M., & Erdélyi, A. (1993). Sunflower lecithin and possibilities for utilization. *Journal of the American Oil Chemists Society, 70*(10), 997–1001.

IUPAC, (1992). In: C. Paquot, A. Hautgenne (Eds.), *Standard methods for the analysis of oils, fats and derivatives* (7th ed.). Oxford: International Union of Pure and Applied Chemistry. Blackwell Scientific Publications Inc.

Kim, I. H., Garcia, H. S., & Graham Hill, C. Jr., (2007). Phospholipase A1- catalyzed synthesis of phospholipids enriched in n-3 polyunsaturated fatty acid residues. *Enzyme and Microbial Technology, 40*, 1130–1135.

Knightly, W. H. (1989). Lecithin in baking applications. In B. Szujah (Ed.), *Lecithins: sources, manufacture & uses* (pp. 174–195). Illinois: American Oil Chemists Society.

Lantz, R. A. (1989). Industrial methods of analysis. In B. Szujah (Ed.), *Lecithins: sources, manufacture & uses* (pp. 163–173). Illinois: American Oil Chemists Society.

Mustranta, A., Suortti, T., & Poutanen, K. (1994). Transesterification of phospholipids in different reaction conditions. *Journal of the American Oil Chemists Society, 71*(12), 1415–1419.

Mustranta, A., Forssell, P., & Poutanen, K. (1995). Comparison of lipases and phospholipases in the hydrolysis of phospholipids. *Process Biochemistry, 30*(5), 393–401.

Padley, F. B., Gunstone, F. D., & Harwood, J. L. (1994). Occurrence and characteristics of oils and fats. In F. D. Gunstone, J. L. Harwood, & F. D. Padley (Eds.), *The lipid handbook* (pp. 47–223). London: Champan & Hall.

Svensson, I., Adlercreutz, P., & Mattiasson, B. (1992). Lipase-catalized transesterification of phosphatidylcholine at controlled water activity. *Journal of the American Oil Chemists Society, 69*(10), 986–991.

van Nieuwenhuyzen, W. (1981). The industrial uses of special lecithins: a review. *Journal of the American Chemists Society, 58*(10), 886–888.

Vikbjerg, A. F., Mu, H., & Xu, X. (2007). Synthesis of structured phospholipids by immobilized phospholipase A_2 catalyzed acidolysis. *Journal of Biotechnology, 128*, 545–554.

Wang, T., Hammond, E. G., & Fehr, W. R. (1997). Phospholipid fatty acid composition and stereospecific distribution of soybean with a wide range of fatty acid composition. *Journal of the American Oil Chemists Society, 74*(12), 1587–1594.

Yamazaki, K., Imai, M., & Suzuki, I. (2004). Soybean lecithin using hog pancreas phospholipase A_2 influenced by the hydrophobic character W/O microemulsions systems. *Biochemical Engineering Journal, 19*, 171–174.