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RESEARCH ARTICLE

Solvent-free enzymatic hydrolysis of non-polar lipids in crude sunflower lecithin using phospholipase A_1 (Lecitase® Ultra)

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ABSTRACT

In this contribution, a new chromatographic method was applied to study the hydrolysis of non-polar lipids, i.e. triacylglicerols (TAG), diacylglycerols (DAG) and monoacylglycerols (MAG), when crude sunflower lecithin is treated with Lecitase® Ultra, an enzymatic preparation with phospholipase A_1 (PLA_1) activity. Results not only proved the enzyme lipase activity toward non-polar lipids in selected reaction conditions (aqueous system, T = 50 °C, pH = 5) but also suggested the occurrence of acyl-migration phenomenon observed by other authors in similar systems. Results showed that 1 h of reaction was enough to decrease the content of TAG in 54%, while DAG and MAG concentration increased from 0.4 to 3.5 and from 1.9 to 6.5 g/100 g of crude lecithin, respectively. Along the reaction, different contents of glycerides could be achieved, obtaining products with different composition which, in combination with the presence of phospholipids (PL) and/or lysophospholipids (LPL), could present specific emulsifying/stabilizing properties with a wide range of applications in food and pharmaceutical industry.

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Introduction

Crude vegetable lecithins, containing primarily phospholipids (PL) and oil, are obtained as a by-product in the degumming process of commercial oils. These crude lecithins are usually processed in order to remove the oil, obtaining a deoiled lecithin (or just "lecithin") which is composed mainly of phospholipids, with varied content of glycolipids, sphingolipids and carbohydrates depending on the source. Due to their surface-active properties, both crude and deoiled lecithin are used as emulsifiers in a vast range of foods, animal feed, and in pharmaceutical and technical applications (van Nieuwenhuyzen and Tomás 2008).

Although soybeans have been the primary source of vegetable lecithin for many years, nowadays, sunflower seeds (*Helianthus annuus* L.) are becoming a more attractive alternative. The expansion of genetically modified (GM) soybean crops is increasing rapidly and this raises concerns in consumers about genetically modified organisms (GMO) in foods, especially in Europe. These concerns are leading to a change in sourcing requirements for identity-preserved non-GM ingredients in the European food market, affecting lecithin production worldwide. As a result, considering that sourcing of non-GM soybean lecithin will become limited, it represents a market opportunity for high-quality identity-preserved sunflower lecithins (van Nieuwenhuyzen 2014).

Native and/or modified lecithins are used in many food industry processes mainly due to their versatile role as emulsifiers, viscosity regulators, anti-spattering and dispersing agents. These food additives are very efficient preventing destabilizing processes such as creaming, coalescence and sedimentation, thus increasing the shelf life of many products (Cabezas et al. 2012). Furthermore, modified lecithins, in particular enzymatically hydrolyzed lecithins, may present technological and commercial advantages over native lecithins, due to their enhanced oil in water (o/w) emulsifying properties and their improved capability to bind proteins and starch, which may aid to prevent undesired naturally occurring chemical and physical changes in various food products. Therefore, they appear as a very attractive additive in bakery and dairy industry as discussed in a previous work (Kasinos et al. 2014) as well as by other authors (Guo et al. 2005; van Nieuwenhuyzen and Tomás 2008; Cabezas et al. 2012).

Lipases, and a particular group: the phospholipases, are enzymes widely used for lipid modification with

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important applications in food industry. Phospholipase A_1 (PLA_1) (E.C. 3.1.1.32) constitutes a very diverse subgroup of phospholipases with 1,3-acyl hydrolase activity. PLA_1 displays broad substrate specificity and also shows some lipase activity, due to the similarity of PLA_1 sequence identity to that of lipases (Guo et al. 2005).

Lecitase® Ultra from Novozymes is an enzymatic preparation with PLA_1 activity, obtained from the fusion of lipase genes from *Thermomyces lanuginosa* and phospholipase genes from *Fusarium oxysporum*, expressing the stability of the lipase and the activity of the enzyme from *F. oxysporum*. In the last years, Lecitase® Ultra has started to be used for phospholipid hydrolysis in vegetable oil degumming; but some attention was also put on lecithin modification, intending to obtain products with higher amounts of lysophospholipids (LPL) in order to improve its emulsifying properties. Nevertheless, this enzymatic preparation has proved to have activity toward both PL and triacylglycerols (TAG), when used to hydrolyse soybean oil (Wang et al. 2010), or for the hydrolysis of ω-3 fatty acids from anchovy oil (Ranjan Moharana et al. 2016). In addition, some authors refer to the application of Lecitase® Ultra for partial hydrolysis of TAG to produce diacylglycerol-enriched oils (Wang et al. 2010; Liu et al. 2011). Nevertheless, although the study of the hydrolysis and/or esterification of TAG by Lecitase® Ultra and other lipases has extensively been performed using different oils, little attention was put in the hydrolysis of this species in crude lecithins.

When Lecitase® Ultra is used for PL modification in crude lecithins, non-polar lipids such as TAG could also be hydrolyzed giving 1(3),2-diacylglycerols (1,2-DAG) and 2-monoacylglycerols (2-MAG), due to its 1,3 lipase specificity. The emulsifying properties of DAG and MAG, and their use in industrial food processing are widely known. Furthermore, the synergistic actions of phospholipids and lysophospholipids with MAG and DAG have also been reported, and they are currently used together in several products such as margarine/shortenings and bakery goods (Szuhaj 2005). However, the optimal ratio between MAG–DAG and PL–LPL fractions can vary greatly depending on the application. Therefore, the possibility of a controlled *in-situ* production of MAG and DAG by enzymatic hydrolysis of TAG present in crude lecithins, could contribute to the development of products having additional properties to those containing only PL and LPL as surface-active compounds. Considering the fact that the raw material (crude lecithin) is a minimally processed by-product, this enzymatic modification could result in significant extra added value to the final product.

Recently, a new method to simultaneously quantify MAG, DAG and TAG by means of high temperature gas liquid chromatography and flame ionization detection (HTGLC–FID) was developed in our team (Pacheco et al. 2014), allowing identification and quantification of the three groups of glycerides in the same chromatographic run. This method resulted very efficient in the analysis of the composition of semisolid products obtained from enzymatic modification of vegetable oils and fats, achieving a comprehensive separation of the different groups of reaction products. Moreover, a detailed study of the production of structured lipids by acidolysis with an immobilized lipase has been successfully assessed using this analytical technique (Palla et al. 2012), and it has also allowed to evaluate the occurrence of the acyl migration phenomenon during the lipase-catalyzed interesterification of refined and fully hydrogenated soybean oil, and establish the effect of different reaction parameters (Pacheco et al. 2015).

Based on these facts, the aim of this contribution is to apply this novel and easy-to-implement HTGLC–FID method to study changes in glycerides composition during enzymatic modification of crude sunflower lecithin with Lecitase® Ultra, verifying the lipase activity of this enzymatic preparation toward TAG as well as DAG and MAG species, and evaluating the occurrence of acyl migration processes.

Materials and methods

Materials

Crude sunflower lecithin, obtained as by-product of sunflower oil water degumming process, was kindly provided by Oleaginosa Moreno Hnos S.A. (Argentina). Lecitase® Ultra, from Novozymes (Bagsvaerd, Denmark) with a claimed activity of 10,000 U/mL, was used as biocatalyst.

Purity of all standards used for chromatographic and phosphorus nuclear magnetic resonance spectrometric (^{31}P-NMR) determinations were greater than 96%. MAG, DAG and TAG standards were obtained from Sigma Chemical Co. (St. Louis, MO), while fatty acid methyl esters (FAME) standards were purchased from Supelco (FAME Mix 37, Bellefonte, PA). N-phosphonomethyl glycine (PMG) used as internal standard (IS) for PL and LPL determination was from Sigma-Aldrich (Steinheim, Germany). Pyridine was from J.T. Baker (Philipsburg, NJ), and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Fluka (Buchs, Switzerland), while deuterated water (D_2O, 99.8%, AtomD) was from Armar Chemicals

(Gottingen, Switzerland). All other reagents, gases and solvents were of analytical or chromatographic grade.

Substrate characterization

The general characterization of crude lecithin was carried out in triplicate according to the AOCS Official Methods (AOCS 2003). For that purpose, moisture (M%, AOCS Ja 2a-46), acid value (AV%, AOCS Ja 6-55), acetone insoluble matter (AIM%, AOCS Ja 4-46) and hexane insoluble matter (HIM%, AOCS Ja 3-87) were determined.

Phospholipid quantification was performed in duplicate by means of ^{31}P-NMR, following the procedure detailed elsewhere (Kasinos et al. 2014). Briefly, samples were prepared with distilled water and D_2O (1:1 ratio), containing 20 mg/mL of native crude lecithin, 10% (w/w) sodium deoxycholate (Sigma-Aldrich, Germany), 2 mM Na-EDTA (Acros Organics, Belgium) and 5 mM PMG as internal standard. ^{31}P-NMR spectra were performed on a Bruker Advance 500 spectrometer (Rheinstetten, Germany) operating at a ^{31}P frequency of 202.46 MHz, equipped with a BBI 5 mm probe. Measurements were performed at 40 °C. The following instrument settings were used: Inverse gate proton decoupling, 65,180 data points, 90° excitation pulse, number of scans of 256 with a relaxation delay time of 12.25 s and a decay acquisition time of 3.22 s.

Polar and non-polar fractions of crude lecithin were partitioned by solid phase extraction (SPE) using 500 mg bonded diol SPE cartridges (J.T. Baker Inc.), as described elsewhere (Carelli et al. 1997) and detailed in *TAG, DAG and MAG determination* section. Subsequently, fatty acid (FA) composition of both fractions was determined in quadruplicate according to IUPAC 2.301 and 2.302 methods (IUPAC 1992). This procedure allows determining the fatty acids bonded to the lipid molecules by alkali transmethylation followed by fatty acid esterification in acidic medium and subsequent hexane extraction. Fatty acid methyl esters profile was determined in quadruplicate by GLC–FID. Chromatographic conditions were the same as described in *FFA Determination* section.

Enzymatic hydrolysis

The reaction was performed in a batch, aqueous system at pH = 5 and T = 50 °C (as recommended by the manufacturer). The lecithin dispersion was prepared by adding 2% (w/w) of native crude lecithin in buffer solution (acetic acid/sodium acetate, 0.1 M). A water bath was used to control and maintain the temperature at 50 °C, and the mixture was stirred until homogeneous. Subsequently, 0.025% (v/v) of Lecitase® Ultra was added to the dispersion. This point was considered as the reaction time (t_r) equal to 0. The mixture remained in the water bath under continuous stirring (257 ± 5 rpm) at 50 °C, and aliquots were taken at 0.5, 1, 1.5, 3, 6, 16, 20 and 24 h and immediately incubated at 100 °C for 5 min to inactivate the enzyme.

TAG, DAG and MAG determination

Purification

Polar lipids present in the samples, such as PL and LPL, could interfere in the chromatographic determination and cause damages in the capillary column. For this reason, it was necessary to remove this fraction from the samples prior to determination. In a first place, freeze-drying was performed to remove the water (benchtop freeze-dryer, Rificor S.A., Argentina). In order to purify the non-polar fraction, SPE was performed according to Carelli et al. (1997) using 500-mg bonded diol SPE cartridges from J.T. Baker Inc. Samples were dissolved in chloroform (4–6 mg/mL) and injected in the conditioned cartridges. Non-polar lipids were released from the sorbent bed by passing 2 mL chloroform through. The elute was collected into a conical vial, evaporated to dryness under nitrogen and dissolved in 2 mL chloroform. Recovery of TAG, DAG and MAG in this purification step was assayed by thin layer chromatography (TLC), using a silica plate (20 cm × 20 cm, Kieserlburg 60, Merck), hexane:ethyl ether:acetic acid (80:20:2) as mobile phase, and a brief exposure to I_2 vapours for plate development.

Sample derivatization

Free hydroxyl groups of MAG and DAG must be silylated in order to increase the volatility and thermal stability of these compounds. The complete silylation reaction of partially hydrolysed glycerides is reached by means of adding MSTFA (as silylation reagent) and pyridine (as catalyst) to the purified non-polar fraction. The use of pyridine allows the reaction to proceed at room temperature within only 15 min, which represents a further advantage of the technique when unsaturated glycerides are being analyzed (Pacheco et al. 2014). In brief, 1 mL of the purified non-polar lipids sample was evaporated to dryness and the injection solution was prepared by adding 15 μL of piridine, 4.5 μL of tricaprin (CCC) solution (10.6 mg/mL in pyridine), 5.2 μL of tripalmitolein (PoPoPo) solution (5.0 mg/mL in pyridine) and 28 μL MSTFA. CCC was used as internal standard for MAG and DAG, while

PoPoPo was used for TAG quantification, since these compounds are not present in sunflower crude lecithin samples. The injection volume was 1 μL. Before injection, all solutions were mixed in an ultrasonic bath.

Chromatographic conditions

Glycerides quantification was performed by HTGLC in a 4890D series gas chromatograph (Agilent, Hewlett-Packard) equipped with a flame ionization detector. The injector was used in split mode. A metallic capillary column (MXT-65TG, 30 m × 0.25 mm × 0.1 μm film thickness; Restek, Bellefonte, PA) with a crosslinked 65% diphenyl 35% dimethyl polysiloxane stationary phase was used. Chromatographic conditions to simultaneously determine the three groups of glycerides (MAG, DAG and TAG) were as follows: split injector at 360 °C (60:1 split ratio); oven temperature programming: 40 °C (4 min), increment until 350 °C at 25 °C/min, and then to 354 °C at a rate of 0.2 °C/min, total run time 36.4 min; and constant FID temperature equal to 380 °C. Hydrogen was used as the carrier gas at a linear velocity of 41 cm/s. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, Rev. A.01.01). For quantification purposes, the internal standard method was used.

Quantification method

Calibration curves for MAG and DAG quantification were constructed using monopalmitin (MP) and dipalmitin (PP), respectively. Mass concentration ratios of these standard compounds to the internal standard (CCC) in each injection solution varied between 0.10 and 1.66. The concentration of CCC was constant for all points of the calibration curves and equal to 0.842 mg/mL.

Unfortunately, previous assays showed that relative response factors (RRF) for TAG with different degree of unsaturation degree were significantly different. Therefore, a single calibration curve for all TAG species could not be applied. Moreover, a maximum of 25 chromatographic peaks were obtained in the TAG range of the native and hydrolyzed crude lecithin samples but only ten commercial standards were available (PPP, POP, PSS, OPO, POL, SSS, SSO, SOO, OOO and LLL; where P, O, S and L are palmitic, oleic, stearic and linoleic acids, respectively). Therefore, the concept of relative retention time (*rrt*) which relates the retention time (*rt*) of the considered analyte to that of solvent and internal standard (PoPoPo), was used according to Eq. (1).

$$rrt = \frac{rt_{analyte} - rt_{solvent}}{rt_{IS} - rt_{solvent}} \quad (1)$$

This parameter, together with the FID response to each of the ten available TAG standards, allowed the construction of a calibration surface according to Eq. (2). Hence, the RRF of any TAG in the analyzed range – and consequently its concentration – could be obtained from Eq. (2), provided its *rrt* value and the parameters A, B and C.

$$\frac{1}{RRF} = (\mathrm{Arrt}^2 + \mathrm{Brrt} + \mathrm{C}) \quad (2)$$

Different injection solutions were prepared in order to obtain standard/PoPoPo mass ratios varying from 0.158 to 7.481. The PoPoPo concentration was 0.456 mg/mL for all solutions. Pyridine was added in order to obtain the desired final concentrations, while the added volume of MSTFA was 5 μL. Solutions were prepared in duplicate and injected in triplicate (injection volume, 1 μL).

It is important to mention that this method cannot distinguish between TAG positional isomers, although being capable of quantifying 1,2- and 1,3-DAG separately, as well as 1- and 2-MAG. Further details about the HTLGC technique as well as the quantification method can be found in Pacheco et al. (2014).

Free fatty acid determination

Although the analytical technique described in the previous section can be used in general to simultaneously quantify glycerides and free fatty acids (FFA), in this case the quantification of the latter was not completely accurate due to the high amount of FFA in the hydrolyzed samples. For this reason, FFA determination was carried out using a modification of the technique described in Penci et al. (2010) by means of a three stages procedure: extraction, purification and derivatization of the FFA, followed by GLC–FID analysis.

Liquid-liquid extraction (LLE)

FFA were extracted from 2 mL aliquots of reaction system in three steps: 10 mL isopropanol:heptane:sulfuric acid (4:1:0.1; v/v/v), 4 mL MilliQ water and 6 mL heptane, followed by 1 min centrifugation at 4500 rpm. The organic phase was collected, evaporated and redissolved in chloroform.

Purification (clean up)

A silica-gel column was prepared packing 1 g of pre-conditioned silica-gel (2% water content, particle size 0.063–0.200 mm, 70–230 mesh, Merck, Darmstadt, Germany) with chloroform in a glass column (15 mm inner diameter). Samples obtained in the LLE step were injected into the column and in a first stage glycerides were eluted using 15 mL of chloroform. This stage was necessary due to the high amount of oil and partially hydrolyzed glycerides (MAG and DAG) normally present in crude lecithins. Afterwards, 20 mL of chloroform:acetic acid (99:1 v/v) were used to elute the purified FFA fraction, and the samples were evaporated to dryness under nitrogen. Recovery studies for this purification step were performed by TLC using a silica plate (20 cm × 20 cm, Kieserlburg 60, Merck) and a solvent mixture of hexane:ethyl ether:acetic acid (80:20:2 v/v/v) as mobile phase.

Derivatization and chromatographic analysis

Purified fatty acids were converted to methyl-esters by acid esterification according to the IUPAC standard method 2.301 (IUPAC 1992). Prior to derivatization, lauric acid was added to the purified FFA extract, as internal standard for quantification. Subsequently, FAME were analyzed in an Agilent 4890D gas chromatograph (Agilent, Hewlett-Packard), equipped with a flame-ionization 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: hydrogen; split ratio: 1:100; injection volume: 1 μL. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, Rev. A.01.01). For this technique, response factors were determined in a previous work (Penci et al. 2010), being higher than 0.85 in all cases.

Statistical analysis

All experiments were performed in quadruplicate. The results were expressed as mean value ± standard deviation. The differences in mean values between samples were assessed using Student's *t*-test. For this purpose, differences were considered significant at $p < .05$.

Results and discussion

Substrate characterization

The general characterization of the crude lecithin is shown in Table 1. AIM% represents the content of phospholipids, glycolipids and carbohydrates, while oil and FFA correspond to the acetone soluble fraction. According to this characterization, oil phase (including FFA) represents approximately 32% (w/w) of the crude lecithin. This is in accordance to literature data (van Nieuwenhuyzen and Tomás 2008) and to the analysis by chromatographic methods, which gave a 30.1 ± 1.7% (w/w) of non-polar lipids including TAG, DAG, MAG and FFA in the native crude lecithin (non-modified lecithin). Similarly, PL and LPL content values are normal for crude sunflower lecithin, being phosphatidylcholine (PC, 39.76%) the major component of phosphoglyceride fraction, followed by phosphatidylinositol (PI, 27.86%), while phosphatidylethanolamine (PE, 15.91%), lysophosphatidylcholine (LPC, 8.04%) and others PL and LPL (8.43%) were in minor proportion. The rest of the parameters such as AV, HIM% and M% showed typical values for crude sunflower lecithin, indicating that the product used as raw material had not suffered changes during storage nor contamination along its processing (van Nieuwenhuyzen and Tomás 2008).

Total fatty acids bonded to lipid molecules in native crude sunflower lecithin, as well as its polar and non-polar fraction separately, were quali-quantitative determined by GC–FID. From the total fatty acid in the crude lecithin, 16.48% were saturated, with 12.38% palmitic acid (P, $C_{16:0}$) and 4.10% stearic acid (S, $C_{18:0}$), and 81.47% were unsaturated, with 16.80% oleic acid (O, $C_{18:1}$) and 64.67% linoleic acid (L, $C_{18:2}$). The remaining 2.05% included other FA, with less than 0.9% each. In addition, a significant difference was found in the fatty acid composition of the polar

Table 1. Characterization of crude sunflower lecithin used as substrate for hydrolysis experiments (Native lecithin).

Determination	Method	Value[a]
AIM (w/w%)	AOCS Ja 4-46	66.86 ± 0.66*
HIM (w/w%)	AOCS Ja 3-87	0.103 ± 0.015*
M (w/w%)	AOCS Ja 2a-46	0.451 ± 0.060*
AV (mg KOH/g lecithin)	AOCS Ja 6-55	22.94 ± 3.40*
PL + LPL (w/w%)	^{31}P-NMR	44.97 ± 0.92**
FFA (w/w%)	GLC-FID	1.42 ± 0.10***
TAG + DAG + MAG (w/w%)	HTGLC-FID	28.63 ± 1.60***

[a]Values shown as mean ± standard deviation.
*$n = 3$.
**$n = 2$.
***$n = 4$.
AIM: acetone insoluble matter; AV: acid value; DAG: diacylglicerols; FFA: free fatty acids; HIM: hexane insoluble matter; LPL: lysophospholipids; M: moisture; MAG: monoacylglicerols; PL: phospholipids; TAG: triacylglicerols; w/w%: g/100 g crude lecithin.

($C_{16:0}$ = 11.50%, $C_{18:0}$ = 3.52%, $C_{18:1}$ = 15.49%. $C_{18:2}$ = 67.52%, others = 1.97%) and non-polar ($C_{16:0}$ = 12.47%, $C_{18:0}$ = 4.24%, $C_{18:1}$ = 17.12%. $C_{18:2}$ = 64.12%, others = 2.05%) fractions, with a higher amount of saturated fatty acid in the latter. This difference has already been reported (Wang et al. 1997; Carelli et al. 2002) and it reflects the well known fact that in the biosynthesis of vegetable triacilglycerols, saturated fatty acids are almost exclusively esterified in *sn*-1 and *sn*-3 positions while unsaturated fatty acids are preferentially esterified in *sn*-2 position. Moreover, stereospecific analysis of TAG from sunflower seed oil identified linoleic acid as the major fatty acid in *sn*-2 position whereas oleic and palmitic acids were the major FA at the *sn*-3 position. Thus, as the *sn*-3 position of phospholipids is esterified with the phosphorylated group, it is reasonable to find a less proportion of palmitic and oleic acids in the polar fraction (Boukhchina et al. 2003).

TAG, DAG and MAG determination

The complete recovery of TAG, DAG and MAG in the purification step was confirmed by thin layer chromatography. Non-polar lipids were effectively purified, obtaining a fraction perfectly free of polar compounds for the HTGLC–FID determination. Figure 1 shows the MAG and DAG chromatographic profile in native and hydrolyzed lecithin (t_r = 1 h) for a typical run. In Figure 1(a,b), enlarged images of MAG and DAG zones are shown separately. The HTGLC technique was accurate to identify and quantify TAG, DAG, and MAG in the native sunflower lecithin and its hydrolysis products. Nevertheless, with this method no distinction could be done between 1- and 3-MAG nor between 1,2- and 2,3-DAG isomers.

Table 2 shows glycerides composition of native (t_r = 0) and hydrolyzed (t_r = 0.5–6 h) crude sunflower lecithin. All coefficient of variation values (CV%) were less than 10%, being 3–5% for MAG and DAG, and 5–10% for TAG. Results confirmed the lipase activity of Lecitase® Ultra, with a change in the composition of the three types of glycerides along the hydrolysis. The most important changes were observed within the first 3 h of reaction. In an initial stage (t_r = 0–30 min) MAG composition did not present significant changes, while DAG and TAG composition showed only a slight variation from their original composition in native lecithin. Afterwards, DAG and MAG composition in the modified crude lecithin increased to reach a maximum at t_r = 1 h, and then started to decrease, which indicates that the enzyme has also lipase activity toward this partially hydrolyzed glycerides.

Table 3 shows the composition of the different species of DAG in the native lecithin and in the hydrolyzed sample corresponding to the above mentioned maximum (t_r = 1 h). Something interesting to remark is the increase of some 1,3-DAG species, considering the specificity of the enzyme at the *sn*-1(3) position (Yang et al. 2006; Wang et al. 2010; Liu et al. 2012). For example, after 1 h of reaction 1,3-dipalmitin (1,3-PP) and 1,3-dilinolein (1,3-LL) concentrations changed from 0 to 32 and 194 mg/100 g lecithin, respectively, while 1,3-oleinlinolein (1,3-OL) composition increased in 1496 mg/100 g lecithin. This 1,3-DAG species could only have been formed by either the hydrolysis of a TAG, releasing one fatty acid from the *sn*-2 position, or by the conversion of a 1,2-DAG species via acyl migration of one FA in *sn*-2 position to *sn*-3 position. The 1,3 specificity of the enzyme has already been reported by other authors (Yang et al. 2006; Wang et al. 2010; Liu et al. 2012), therefore the hypothesis of the enzymatic cleavage on *sn*-2 position is dismissed in this discussion. Moreover, the acyl migration in glycerides and phospholipid species has been observed in similar systems (Wang et al. 2010; Jiang et al. 2014; Pacheco et al. 2015), supporting the second hypothesis.

Similarly, an increase of 1-MAG species was observed at short reaction times (t_r = 0–1 h). The composition of the different monoacylglycerol species is shown in Table 4. Putting aside the unlikely cleavage of the *sn*-2 acyl residue of 1,2-DAG, a 1-MAG species could be formed either by the conversion of the corresponding 2-MAG by acyl migration, or by hydrolysis of a 1,3-DAG. But considering the case of 1-monoolein (1-MO), for example, the total amount of 1,3-DAG containing at least one oleic acid (1,3-PO; 1,3-SO, 1,3-OO and 1,3-OL) in native lecithin (t_r = 0) was 1.47 mmol/100 g lecithin, and the 1-MO concentration increased in 6.19 mmol/100 g lecithin after 1 h of reaction. Therefore, the hydrolysis of 1,3-DAG cannot be the only source of 1-MAG species, reinforcing the hypothesis of acyl migration occurrence.

It is important to remark that the re-esterification of partially hydrolyzed glycerides is not taken into account in this study, due to the high amount of water present in the reaction system (more than 97 w/w%), which leads to a complete displacement of the equilibrium toward the hydrolysis.

Free fatty acids determination

The complete recovery of free fatty acids in the purification step was confirmed by thin layer chromatography. The initial amount of FFA in the crude lecithin

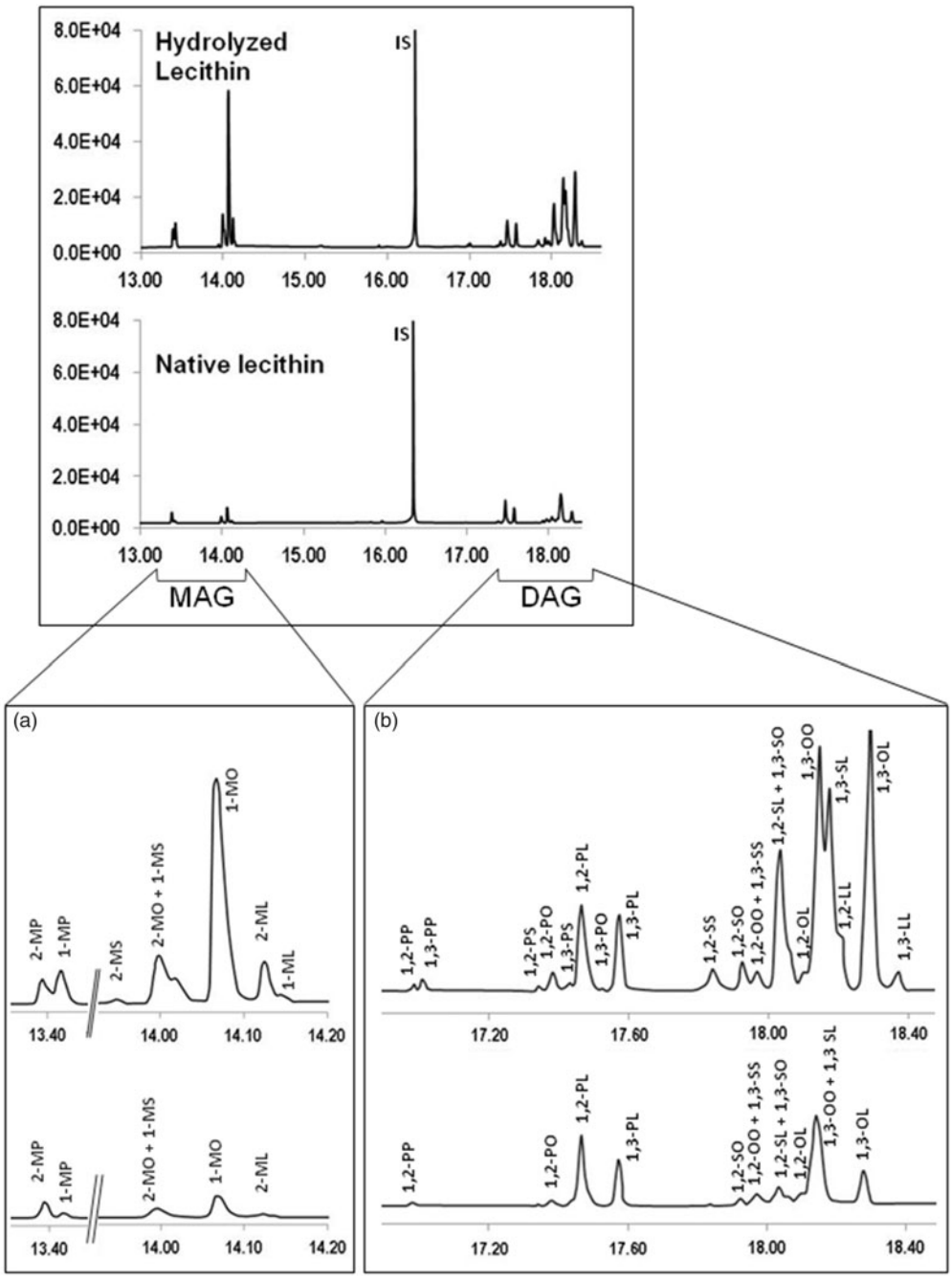

Figure 1. Chromatogram of native and hydrolyzed (reaction time: $t_r = 1$ h) crude sunflower lecithin for monoacylglycerols (MAG) and diacylglycerols (DAG). IS: internal standard (tricaprin, CCC); *a*: MAG zone; ML: monolinolein; MO: monoolein; MP: monopalmitin; MS: monostearin. No distinction is done between 1-MAG and 3-MAG isomers. *b*: DAG zone; L: linoleic acid; O: oleic acid; P: palmitic acid; S: stearic acid; no distinction is done between 1,2- and 2,3-DAG isomers. Chromatogram zones in *a* and *b* are not shown in the same scale.

and the fatty acids released during hydrolysis were determined for different reaction times from 0 to 24 h. These results are shown in Table 5 for the four main fatty acids in native and modified lecithin (palmitic, stearic, oleic and linoleic acid).

Results indicate that 90% of the total release of fatty acids has already occurred at $t_r = 6$ h. When considered individually, palmitic acid is released at a significantly higher rate: 70% of the total release is obtained within the first 60 min of reaction. Linoleic acid, even though it is the most abundant fatty acid in the raw material, is released at a much slower rate. These differences can also be appreciated in Figure 2, where only the amount of fatty acids released during

Table 2. Non-polar lipids composition of native (reaction time, $t_r = 0$) and hydrolyzed ($t_r = 0.5$–6 h) crude sunflower lecithin during solvent-free hydrolysis with Lecitase® Ultra (pH = 5, T = 50 °C).

Reaction time (h)	Monoacylglycerols (g/100 g lecithin)	Diacylglycerols (g/100 g lecithin)	Triacylglycerols (g/100 g lecithin)
0	0.41 ± 0.01^{a}	1.86 ± 0.09^{a}	26.36 ± 1.50^{e}
0.5	0.45 ± 0.02^{a}	2.79 ± 0.14^{b}	21.56 ± 1.64^{d}
1	3.47 ± 0.012^{d}	6.47 ± 0.13^{d}	12.10 ± 0.92^{c}
1.5	2.18 ± 0.10^{c}	3.11 ± 0.09^{c}	5.75 ± 0.53^{b}
3	0.82 ± 0.03^{b}	2.62 ± 0.10^{b}	2.2 ± 0.18^{a}
6	0.78 ± 0.04^{b}	2.36 ± 0.10^{b}	2.01 ± 0.19^{a}

Results shown as mean values ± standard deviation; $n = 4$.
Different letters in the same column indicate significant differences ($p < .05$). ("a" means the lowest value, while "e" means the highest value).

Table 3. Diacylglycerols composition of native and hydrolyzed ($t_r = 1$ h) crude sunflower lecithin during solvent-free hydrolysis with Lecitase® Ultra (pH = 5, T = 50 °C).

DAG	Native lecithin (mg/100 g lecithin)	Hydrolyzed lecithin ($t_r = 1$ h) (mg/100 g lecithin)
1,2-PP	3.72 ± 0.18^{a}	12.92 ± 0.29^{b}
1,3-PP	0.00 ± 0.00^{a}	32.36 ± 0.75^{b}
1,2-PS	1.86 ± 0.09^{a}	12.96 ± 0.23^{b}
1,2-PO	37.24 ± 1.70^{a}	64.71 ± 1.31^{b}
1,3-PS	9.31 ± 0.45^{a}	12.94 ± 0.26^{b}
1,2-PL	465.50 ± 21.93^{a}	517.68 ± 10.05^{b}
1,3-PO	0.00 ± 0.00^{a}	6.47 ± 0.18^{b}
1.3-PL	316.54 ± 15.01^{a}	452.97 ± 9.09^{b}
1,2-SS	1.86 ± 0.08^{a}	213.54 ± 4.31^{b}
1,2-SO	37.24 ± 1.76^{a}	129.42 ± 2.51^{b}
1,2-OO + 1,3-SS	55.86 ± 2.63^{a}	64.71 ± 1.31^{b}
1,2-SL + 1,3-SO	93.10 ± 4.38^{a}	1100.07 ± 22.03^{b}
1,2-OL	20.48 ± 0.96^{a}	32.36 ± 0.55^{b}
1,3-OO + 1,3-SL + 1,2-LL	633.08 ± 29.55^{a}	1941.30 ± 38.21^{b}
1,3-OL	186.20 ± 8.81^{a}	1682.46 ± 33.61^{b}
1,3-LL	0.00 ± 0.00^{a}	194.13 ± 3.78^{b}

Results shown as mean values ± standard deviation; $n = 4$.
Different letters in the same row indicate significant differences ($p < .05$). ("a" means the lowest value, while "b" means the highest value).
DAG: diacylglycerols; L: linoleic acid; O: oleic acid; P: palmitic acid; S: stearic acid; t_r: reaction time. No distinction is done between 1,2-DAG and 2,3-DAG.

Table 4. Monoacyglycerols (MAG) composition of native and hydrolyzed crude sunflower lecithin during solvent-free hydrolysis with Lecitase® Ultra (pH = 5, T = 50 °C).

MAG	Native lecithin (mg/100 g lecithin)	Hydrolyzed lecithin ($t_r = 1$ h) (mg/100 g lecithin)
2-MP	123.60 ± 4.32^{a}	173.40 ± 5.91^{b}
1-MP	20.60 ± 0.38^{a}	277.44 ± 10.58^{b}
2-MS	0.41 ± 0.10^{a}	3.47 ± 0.16^{a}
1-MS + 2-MO	68.80 ± 2.55^{a}	506.33 ± 17.20^{b}
1-MO	164.80 ± 5.09^{a}	2184.84 ± 74.20^{b}
2-ML	0.82 ± 0.8^{a}	312.12 ± 9.48^{b}
1-ML	32.96 ± 0.30^{b}	10.40 ± 0.34^{a}

Results shown as mean values ± standard deviation; $n = 4$.
Different letters in the same row indicate significant differences ($p < .05$). ("a" means the lowest value, while "b" means the highest value).
MAG: monoacylglycerols; MP: monopalmitin; MS: monostearin; MO: monoolein; ML: monolinolein; t_r: reaction time. No distinction is done between 1-MAG and 3-MAG.

the hydrolysis is shown (i.e. the total amount of FFA at each t_r minus the amount initially present in the crude lecithin).

These facts could suggest a preference of the enzyme for palmitic acid -a saturated and the shortest fatty acid in the raw material, preferentially esterified at *sn*-1(3) position-, which has also been observed by Wang et al. (2010) when Lecitase® Ultra was used for partial hydrolysis of soybean oil. These results could also back up the hypothesis of acyl migration occurrence, considering that in natural fats and oils saturated fatty acids are often located at *sn*-1(3) position, while unsaturated fatty acids often occupy *sn*-2 position (Wang et al. 2010). Wang et al. (1997) also observed a similar trend in soybean lecithin phospholipids: saturated fatty acids are primarily located in *sn*-1 position, oleic acid is distributed relatively equally between the *sn*-1 and *sn*-2 positions, and linoleic acid is more concentrated on *sn*-2 position. According to the 1,3 specificity of Lecitase® Ultra, palmitic acid,

Table 5. Main free fatty acid composition of native ($t_r = 0$) and hydrolyzed ($t_r = 1$–24 h) crude sunflower lecithin during solvent-free hydrolysis with Lecitase® Ultra (pH = 5, T = 50 °C).

	Fatty Acid (g/100 g crude lecithin)			
t_r (h)	P	S	O	L
0	0.26 ± 0.02^{a}	0.32 ± 0.02^{a}	0.64 ± 0.04^{a}	0.20 ± 0.02^{a}
1	7.70 ± 0.31^{b}	0.68 ± 0.04^{b}	2.69 ± 0.11^{b}	3.24 ± 0.12^{b}
2	8.97 ± 0.39^{c}	0.68 ± 0.03^{b}	$2.90 \pm 0.13^{b,c}$	5.44 ± 0.31^{c}
3	9.72 ± 0.32^{d}	0.69 ± 0.03^{b}	2.96 ± 0.09^{c}	9.52 ± 0.38^{d}
4	10.57 ± 0.41^{e}	0.75 ± 0.04^{b}	3.26 ± 0.12^{d}	14.61 ± 0.51^{e}
6	10.86 ± 0.39^{e}	0.74 ± 0.03^{b}	3.93 ± 0.09^{e}	17.68 ± 0.72^{f}
16	10.93 ± 0.40^{e}	0.93 ± 0.03^{c}	4.42 ± 0.11^{f}	20.64 ± 0.90^{g}
20	10.61 ± 0.46^{e}	0.93 ± 0.04^{c}	4.41 ± 0.13^{f}	21.20 ± 0.98^{g}
24	10.72 ± 0.41^{e}	0.90 ± 0.02^{c}	4.46 ± 0.10^{f}	20.79 ± 1.09^{g}

Results shown as mean values ± standard deviation; $n = 4$.
Different letters in the same column indicate significant differences ($p < .05$). ("a" means the lowest value, while "g" means the highest value).
Abbreviations: L: linoleic acid; O: oleic acid; P: palmitic acid; S: stearic acid; t_r: reaction time.

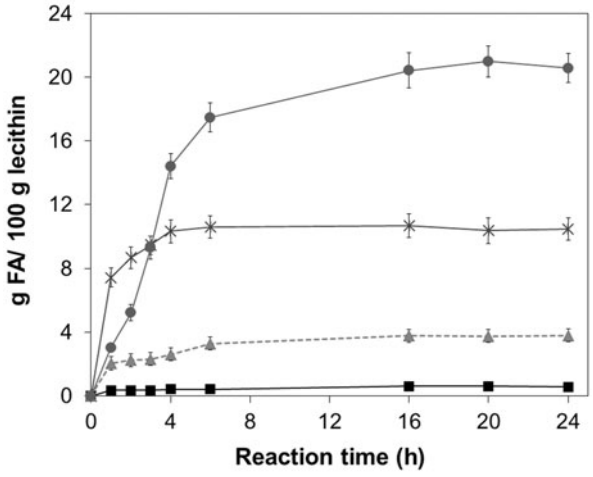

Figure 2. Evolution of fatty acid release during enzymatic hydrolysis of crude sunflower lecithin, treated with Lecitase® Ultra. Reaction conditions: pH = 5; T = 50 °C; reaction time: t_r = 1–24 h. L: linoleic acid ($C_{18:2}$, -×-); O: oleic acid ($C_{18:1}$, -■-); P: palmitic acid ($C_{16:0}$, -▲-); S: stearic acid ($C_{18:0}$, -●-).

primarily located in *sn*-1(3) position, is released preferentially in the first part of the reaction; afterwards, oleic and -specially- linoleic acid, normally found in *sn*-2 position, migrate to *sn*-1(3) becoming available for enzymatic cleavage, resulting in a higher release of this two unsaturated fatty acids.

In addition, in their studies Wang et al. (2010) postulated two routes for the hydrolysis of TAG in the partial hydrolysis of soybean oil using Lecitase® Ultra, as shown schematically in Figure 3. The results reported in the present work suggest that TAG hydrolysis in crude sunflower lecithin follows the same two routes when using this enzymatic preparation. In an initial stage of the reaction, the hydrolysis of TAG seems to follow route 1, due to the *sn*-1,3-specificity of Lecitase® Ultra, releasing mostly saturated fatty

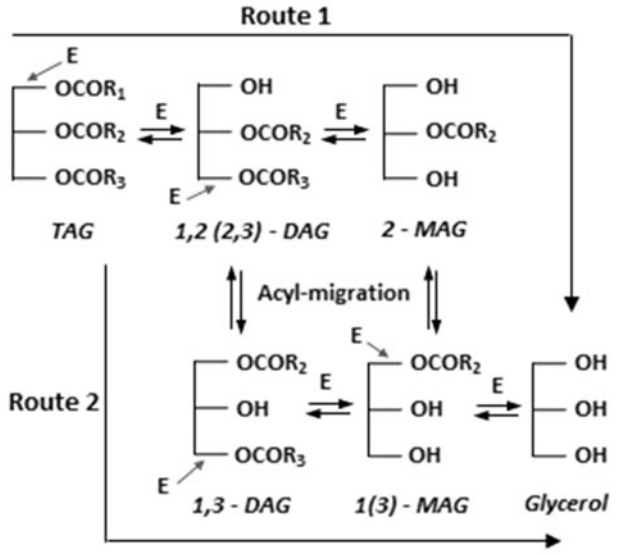

Figure 3. Reaction routes postulated for glycerides solvent-free enzymatic hydrolysis in crude sunflower lecithin, catalyzed by Lecitase® Ultra (pH = 5, T = 50 °C). DAG: diacylglycerols; E: enzyme; MAG: monoacylglycerols; R_1, R_2, R_3: acyl residues; TAG: triacylglicerols.

acids from the *sn*-1,3-position. In a second stage, the partial hydrolysis reaction simultaneously follows route 2, due to the conversion of 1,2-DAG into 1,3-DAG via acyl migration, increasing the release of unsaturated fatty acids originally located at the *sn*-2-position. In the final stage of the reaction, route 2 appears to be the primary route followed, since 1,3-DAG are preferentially hydrolyzed by the enzyme over 1,2-DAG, as already mentioned.

It is important to remark that FA are also released from PL and LPL by the enzyme (and not only from the non-polar lipids). The acyl migration phenomenon was also observed in this polar fraction, and although this has been partially reported in a previous contribution (Goñi et al. 2015), it will be further discussed in future contributions.

Although some authors affirmed that acyl migration cannot be totally avoided in this kind of systems, it has been suggested that this phenomenon can be controlled, adjusting some reaction parameters (Yang et al. 2005). This means that further analysis must be performed in order to maximize or adjust the composition of the species of interest, keeping in mind that the optimal conditions will depend on the required composition of the final product. For instance, if acyl migration in MAG could be controlled, a higher concentration of these surface-active species could be achieved.

Taking into account that the purpose of enzymatic modification of crude sunflower lecithin is to improve the emulsifying/stabilizing properties of this oil's processing by-product, the hydrolysis of TAG and the

subsequent increase of DAG and MAG in the raw material would be very interesting, adding other specific characteristics to the final product. This indeed opens a challenging field of study, in order to correlate the composition of the hydrolysis mixtures produced under different reaction conditions (e.g. time, temperature and pH) with their potential applications as surface-active additives.

Conclusions

The analytical techniques were successfully applied for the study of non-polar lipids hydrolysis during enzymatic modification of crude sunflower lecithin, and Lecitase® Ultra activity toward TAG, DAG and MAG species was demonstrated. The acyl migration phenomenon suggested by the observed results could lead to further studies in order to confirm its occurrence, and to better understand this process and the variables that have influence on it.

Finally, using Lecitase® Ultra to enzymatically modify crude sunflower lecithin, varied products with different concentrations of MAG, DAG as well as PL and LPL could be achieved, with a wide range of applications in food and pharmaceutical industry.

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Disclosure statement

The authors declare that they have no conflict of interest.

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