Emulsifying properties of hydrolized sunflower lecithins by phospholipases A_2 of different sources

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Abstract

Sunflower (*Helianthus annuus* L.) lecithins are obtained by gum purification from raw oil by a degumming process, which is a step of the refining process of raw vegetable oils. Food industry uses lecithins because of their multifunctional ingredients. Modification processes of the original phospholipid composition of native lecithin, such as enzymatic hydrolysis, are appropriate for certain applications. The aim of this work was to evaluate the emulsifying properties of different modified sunflower lecithins in oil-in-water (O/W) emulsions. In this study, modified sunflower lecithins, which were obtained by enzymatic hydrolysis with phospholipase A_2 from pancreatic porcine and microbial sources (hydrolyzed lecithin, SHLP and SHLM) were assessed using a deoiled lecithin (DSL) such as a control system. Modified lecithins were applied as emulsifying agent in O/W emulsions (30:70 wt/wt), ranging 0.1–2.0% (wt/wt). Stability of different emulsions was evaluated through the evolution of backscattering profiles (%BS), particle size distribution (in volume and surface), and mean particle diameters

(D [4,3], D [3,2]). Hydrolyzed lecithins presented the best emulsifying properties against the main destabilization processes (creaming and coalescence) for the emulsions studied in comparison with DSL. These modified lecithins represent a good alternative for the production of new bioactive agents. Furthermore, the use of a microbial phospholipase gives the possibility to obtain a spectrum of sunflower lecithins which functionality as bioactive agents could be applied to the development of foods with *kosher* and *halal* certification.

Keywords: sunflower lecithin, enzymatic hydrolysis, lysophospholipids, ^{31}P NMR, non-GMO product, *kosher* and *halal* certification

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1. Introduction

Lecithins are a mixture of acetone insoluble phospholipids, containing mainly phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), minor compounds such as phosphatidic acid (PA), and other minor substances such as carbohydrates and triglycerides (Schneider,1989). The production of sunflower oil, in Argentina, is of utmost importance from an economic point of view (Franco, 2008). In this country, sunflower lecithin might represent an alternative to soybean lecithin because it is considered a non-GMO product, which is in accordance with the preference of some consumers.

The introduction of changes in the original concentration of these phospholipids, by chemical or enzymatic modification of their structure can lead to obtain lecithins with different physicochemical and functional properties, with respect to native lecithin (van Nieuwenhuyzen & Tomás, 2008). The modification processes usually applied on native lecithins are the fractionation with ethanol (Sosada, 1993; Wu & Wang, 2004; Cabezas et al., 2009a, 2009b) and the enzymatic hydrolysis (Schmitt & Heirman, 2007; Cabezas et al., 2011a). Native and modified lecithins are used in a wide range of industrial applications: nutritional, pharmaceutical applications, food, cosmetics, etc. (Prosise, 1985; Wendel, 2000). In the food industry, lecithin represents a multifunctional additive in the manufacture of chocolate, bakery and instant products, margarines, and mayonnaise, due to the characteristics of its phospholipids (van Nieuwenhuyzen, 1981).

In particular, enzymatic hydrolyzed lecithin may present technological and commercial advantages over native lecithins: (1) enhanced O/W emulsifying property; (2) increased emulsion stability under acid conditions and in the coexistence with salts; (3) improved capability to bind proteins and starch; (4) excellent mold- or pan-releasing property. Consequently, the demand for lysolecithins was increasing in recent years (Hirai et al., 1998; Erickson, 2008).

The main application of lecithin at the food industry is associated with its rol as emulsifier agent for dispersions or emulsions (Hernández & Quezada, 2008). Emulsions are thermodynamically unstable systems from a physicochemical point of view. In virtue of that, it is important to characterize and know their behaviour against different destabilization processes (flocculation, coalescence, creaming, etc.) (McClements, 1999).

Enzymatic hydrolysis is carried out mainly by two groups of enzymes: phospholipases and lipases (Mustranta et al., 1995). Phospholipases A_2 catalyze the hydrolysis of the ester bond in the sn-2 position of glycerophospholipids, producing free fatty acids and the corresponding lysophospholipid. Advances in biotechnology and certain requirements of consumers (*kosher* or *halal* foods) have influenced the development of the production of microbial enzymes (bacteria, fungi, yeasts) which could be substitute of the traditionally obtained from porcine pancreas (Minchiotti, 2006; Cabezas et al., 2011b).

The aim of this work was analyze the emulsifying activity of sunflower lysolecithins obtained by phospholipases A_2 from diverse sources: bacterial (LysoMax PLA_2, Danisco) and porcine pancreas (Lecitase 10L, Novo Nordisk) in O/W systems. This study seeks to contribute to the oil industry with useful information for rescaling of the mentioned hydrolysis process, with the aim of increasing the aggregated value of sunflower lecithins.

2. Materials and Methods

2.1. Materials

Native sunflower lecithin was used as starting material, and was provided by a local oil industry (Vicentin S.A.I.C.). Enzymatic hydrolysis processes were carried out using a porcine pancreatic PLA_2 (Lecitase 10L, Novo Nordisk) and a microbial PLA_2 (*Streptomyces violaceoruber*, LysoMax PLA_2, Danisco). All solvents used were of analytical grade.

The sunflower lecithin used as starting material presented the following composition: 43.1% phospholipids (16.5% PI, 16.2% PC, 5.3% PE, and 5.1% minor phospholipids), 33.4% oil, and 23.5% of other compounds (glycolipids, complex carbohydrates).

All the solvents used were of analytical grade.

2.2. Enzymatic hydrolysis process

Enzymatic hydrolysis was carried out in a thermostated reactor at laboratory scale, using 27 g of native sunflower lecithin and 18 ml of 0.4 M $CaCl_2$. Initial pH was adjusted to 7 or 9 by adding 4 N NaOH solution. Then, the resulting mixture was set to the optimal temperature of each phospholipase, i.e. 60 ºC for porcine pancreatic PLA_2 and 50 ºC for microbial PLA_2, which were incorporated in a concentration of 2.0% ml lipase per 100 g lecithin. Next, continuous agitation (50 rpm) was applied during 5 h. Evolution of hydrolysis process was followed by measuring pH, using a pH meter for solid samples (840049 Puncture Tip, Saen S.R.L.). Products of enzymatic hydrolysis were subjected to a sudden decrease in temperature to stop the process of hydrolysis and then deoiled using acetone, according to AOCS Official Method Ja 4–46, procedures 1–5 (Cabezas et al., 2011a). After that, samples were stored at 0 °C. The hydrolysis process was carried out in duplicate.

Also, native sunflower lecithin was deoiled with acetone obtaining the deoiled sunflower lecithin (DSL). DSL was used as a sample control. Deoiling procedure was performed by duplicate.

2.3. Phospholipid composition

2.3.1. Sample Preparation

100 mg of each hydrolyzed sample were diluted in 1 ml of deuterated chloroform, 1 ml of methanol and 1 ml of Cs-EDTA (pH 8). The organic layer was separated after 15 min shaking, and analyzed by ^{31}P NMR (Cabezas et al., 2009a).

2.3.2. Quantitative ^{31}P NMR analysis

Quantitative ^{31}P NMR analysis was carried out in a Bruker Avance 600 MHz automatic spectrometer using triphenyl phosphate as internal standard (Spectral Service GmbH, Köln, Germany) (Diehl, 1997; 2001; 2008). Phospholipid content of samples

obtained under different conditions of enzymatic hydrolysis, was determined by this spectroscopic technique.

2.4. Oil-in-water (O/W) emulsions preparation

Commercial sunflower oil was used to prepare oil-in-water (O/W) emulsions with a formulation of 30:70 (wt/wt) according to Pan et al., 2004. Emulsions were prepared at room temperature in an Ultra-Turrax T25 homogenizer using S 25 N–10 G dispersing tool (7.5 mm rotor diameter) at 10,000 rpm for 1 min, with the addition of the different modified sunflower lecithins in a range of 0.1–2.0% (wt/wt). This process was carried out in triplicate for each case.

2.5. Optical characterization of emulsions

The backscattering of light was measured using a QuickScan Vertical Scan Analyzer (Coulter Corp., Miami, FL). The backscattering of monochromatic light (λ = 850 nm) of the emulsions was determined as a function of the height of the sample tube (ca. 65 mm) in order to quantify the rate of the different destabilization processes during the first 90 min. This methodology allowed to discriminate between particle migration (sedimentation, creaming) and particle size variation (flocculation, coalescence) processes (Pan et al., 2002). The basis of the vertical scan analyzer profiles has been exhaustively studied by Mengual (Mengual et al., 1999).

2.6. Particle size measurements

Particle size distribution, and De Brouckere (D[4,3]) and Sauter (D[3,2]) mean diameters of particles of the emulsions were determined with a particle size analyzer (Malvern Mastersizer 2000E, Malvern Instruments Ltd., Worcestershire,U.K.). Samples were diluted in the water bath of the dispersion system (Hydro 2000MU), which is a laser diffraction based particle size analyzer (Márquez & Wagner, 2010). This determination was carried out in triplicate for each case.

2.7. Statistical analysis

Data were evaluated by analysis of variance (ANOVA) using the software Systat® 12.0 (Systat, 2007). For this purpose, differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Compositional analysis of modified lecithins

Native sunflower lecithin used as starting material presented the following composition: 43.1% phospholipids (16.5% PI, 16.2% PC, 5.3% PE, and 5.1% minor phospholipids), 33.4% oil, and 23.5% of other compounds (glycolipids, complex carbohydrates).

The phospholipid composition of different modified sunflower lecithins obtained in this chapter is shown in Table 1. The sunflower hydrolyzed lecithins with a porcine pancreatic (SHLP) and microbial (SHLM) phospholipase A_2, at different levels of pH (7, 9), recorded a marked difference regarding the phospholipid composition in relation to the native sunflower lecithin (DSL). The hydrolyzed lecithins presented a high concentration of major lysophospholipids (> 64.9 mol LPL/ mol of total PL) compared to the native sunflower lecithin (≈ 1.5%), showing the efficiency of the enzymatic hydrolysis processes. In particular, the pancreatic PLA_2 (SHLP7, SHLP9) produced a higher hydrolysis degree of the main phospholipids in comparison with the microbial phospholipase (SHLM7, SHLM9).

PC presented a degree of hydrolysis in all performed conditions on the hydrolysis processes. These results can be correlated with the ones described by Penci, 2010. In that work, it was reported that phosphatidylcholine is the phospholipid with higher tendency to be hydrolyzed when using a porcine pancreatic PLA_2 with a very low amount of sunflower lecithins (1 mg). In this way, the residual PC and PI

concentration in the hydrolyzed lecithin was lower than the detection limit of the ^{31}P NMR equipment when the pancreatic porcine PLA_2 was used.

3.2. Optical characterization of O/W emulsions

Stability of the different O/W emulsions (30:70 wt/wt) was studied recording the backscattering (BS) profiles as a function of the cell length and time, by a vertical scan analyzer (QuickScan). For instance, Figure 1 shows two typical profiles obtained for emulsions with the addition of 0.1% of DSL and SHLP9.

The creaming destabilization process (i.e. migration of oil particles to the upper portion of the tube) is evidenced by a decrease of %BS values at the bottom of the tube. The QuickScan profiles corresponding to the zone I (10-20 mm) showed an increase of the emulsion stability against the creaming process, as a function of increasing concentration of different modified lecithins (Fig. 2). In particular, the hydrolyzed lecithins (SHLP and SHLM) generated a high stability in O/W emulsions than DSL, over the studied range of concentration. Moreover, O/W emulsions with 0.1-0.5% of DSL showed a sharp decrease of %BS in the Zone I.

The tube zone between 40-45 mm (Zone II) is characterized by the accumulation of oil droplets after the creaming process (cream phase); Figure 3 shows the %BS values vs. time in Zone II. Emulsions formulated with hydrolyzed lecithins presented higher %BS values than those obtained using DSL, for all concentrations studied. The higher levels of %BS and the greater stability of these emulsions would be associated with the formation of dense cream phases with a lower proportion of continuous phase inside (Palazolo, 2006). However, emulsions with 0.1 -0.5% of DSL did not allow the formation of the cream phase. These results are related to the rapid decrease of %BS and the formation of an oil layer in the upper part of the tube (Fig. 1a) suggesting the occurrence of a cream phase destabilization by coalescence (Pan et al., 2002).

3.3. Particle size distribution

Particle size distribution in volume and surface of O/W emulsions obtained with different modified lecithins was measured just after emulsification (t=0); corresponding results can be seen in Figures 4 and 5, respectively. These distributions presented a bimodal or trimodal character depending on the concentration of aggregated lecithin, and the following particle size populations: (I) particle size < 4 μm; (II) particle size between 4 and 30 μm; (III) particle size > 30 μm. In this sense, only SHLP7 showed a trimodal character for all concentrations assayed. It should be noted that the SHLM7 presented a bimodal character for concentration in the range 0.1-0.5%, but with a high percentage of particles of the population II in comparison with DSL.

In order to complete the analysis of particle size distribution, Figure 6 depicts the evolution of De Brouckere (D [4,3]) and Sauter (D [3,2]) mean diameters as a function of the concentration of the different emulsifiers. Hydrolyzed lecithins generated values of D [4,3] and D [3,2] significantly lower than those corresponding to DSL. These results are correlated with the high stability of the O/W emulsions recorded when using hydrolyzed lecithins, considering the main destabilization processes determined by the corresponding QuickScan profiles (creaming or coalescence). It is worth to note that a high concentration of small particles produces a slow creaming process, according to the Stokes' law (McClements, 1999; Palazolo, 2006).

The hydrophilic-lipophilic balance value (HLB) is often used in connection with the performance of emulsifiers (McClements, 1999). The high concentration of hydrophilic phospholipids (lysophospholipids) presented in the hydrolyzed lecithins (SHLP and SHLM) increase this empirical value. In this sense, according to Carlsson (Carlsson, 2008), these modified lecithins with higher HLB values presented best properties as O/W emulsifying agents.

Also, the phase structure at the interface of the different phospholipids influences the emulsion formation and stability (van Nieuwenhuyzen & Tomás, 2008). LPC and LPE form hexagonal wide spread clusters. These structures have a great importance for the stabilisation of O/W emulsions. This behaviour is in relation to the low mean diameters and the high concentration of small particle populations recorded in emulsions using sunflower hydrolyzed lecithins (Figs. 4-6). However, PE gives reversed hexagonal phase, which are more difficult to arrange at the interface (van Nieuwenhuyzen, 1998). The presence of PE could explain the minor characteristics as emulsifying agent of DSL, and the high mean diameters when was using SHLM in contrast to when using SHLP (Fig. 6).

Taking into account the results presented in Figures 2 to 6, the addition of a concentration between 0.5 and 1.0% of hydrolyzed lecithin (SHLP, SHLM) is enough for covering all droplets surface. High concentrations of this modified lecithin do not show significant differences in the % BS values, nor in the mean particle sizes. On the other hand, concentration levels higher than 0.1% of hydrolyzed lecithins from different phospholipases at different initial pH levels showed similar characteristics in terms of their emulsifying activity. However, DSL presented an improved in the stability of O/W emulsions as a function of increasing concentration.

4. Conclusions

The best emulsifying properties exhibited by hydrolyzed sunflower lecithins (LHM7, LHP7, LHM9, LHP9) in comparison with the native lecithin could indicate that their application by the local oil industry could revalue this by-product of the refining process of crude sunflower oil.

The use of a microbial phospholipase A_2 gives the possibility to obtain diverse sunflower lysolecithins, which functionality could be applied to the development of foods with *kosher* and *halal* certification.

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Table 1. Phospholipid (PL) composition of modified sunflower lecithins by ^{31}PNMR[a]

	DSL	SHLM9	SHLM7	SHLP9	SHLP7
PC	36.7	7.4	6.7	< 0.1	< 0.1
1-LPC	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
2-LPC	1.5	39.2	39.4	46.1	45.5
PI	35.3	11.2	12.3	< 0.1	< 0.1
LPI	< 0.1	18.6	15.5	26.5	27.9
PE	15.1	3.7	6.4	1.0	0.7
LPE	< 0.1	8.8	9.0	10.7	13.0
APE	1.8	< 0.1	< 0.1	< 0.1	< 0.1
PA	5.2	3.1	3.4	2.5	2.0
LPA	< 0.1	1.0	1.0	5.1	4.1
Other	4.5	7.1	6.4	8.0	6.7
PL / 100g lecithin	62.8	40.6	45.9	38.7	44.4

[a] Mean values are shown (n = 2). The coefficient of variation was lower than 5%

Figure 1. Backscattering (%BS) profiles of O/W emulsions (30:70 wt/wt) with the addition of: (a) DSL, 0.1%; (b) SHLP, 0.1%

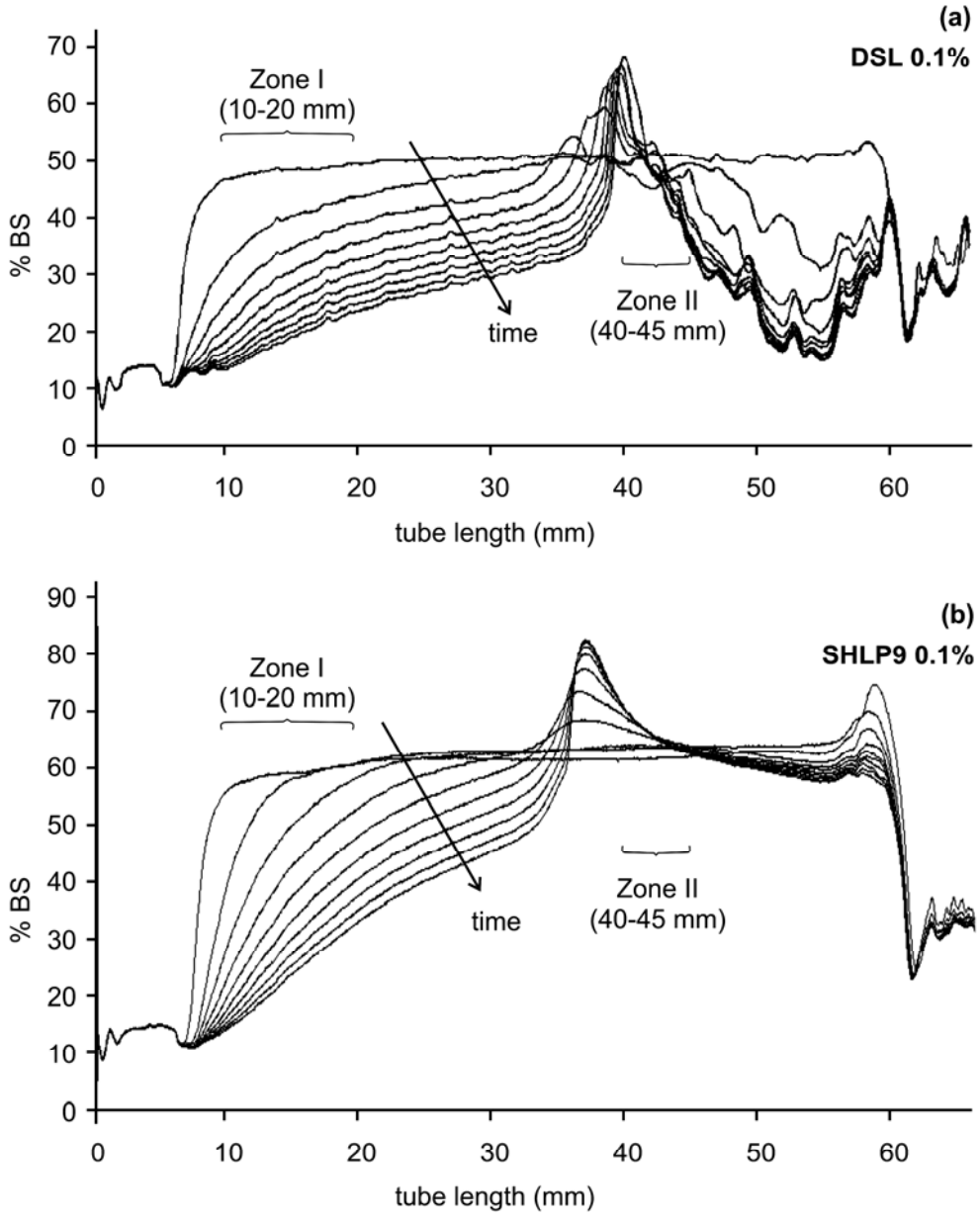

Figure 2. Backscattering (%BS) values of O/W emulsions (30:70 wt/wt) with the addition of different modified sunflower lecithins in Zone I (10-20 mm). Mean values (n = 3) ± sd

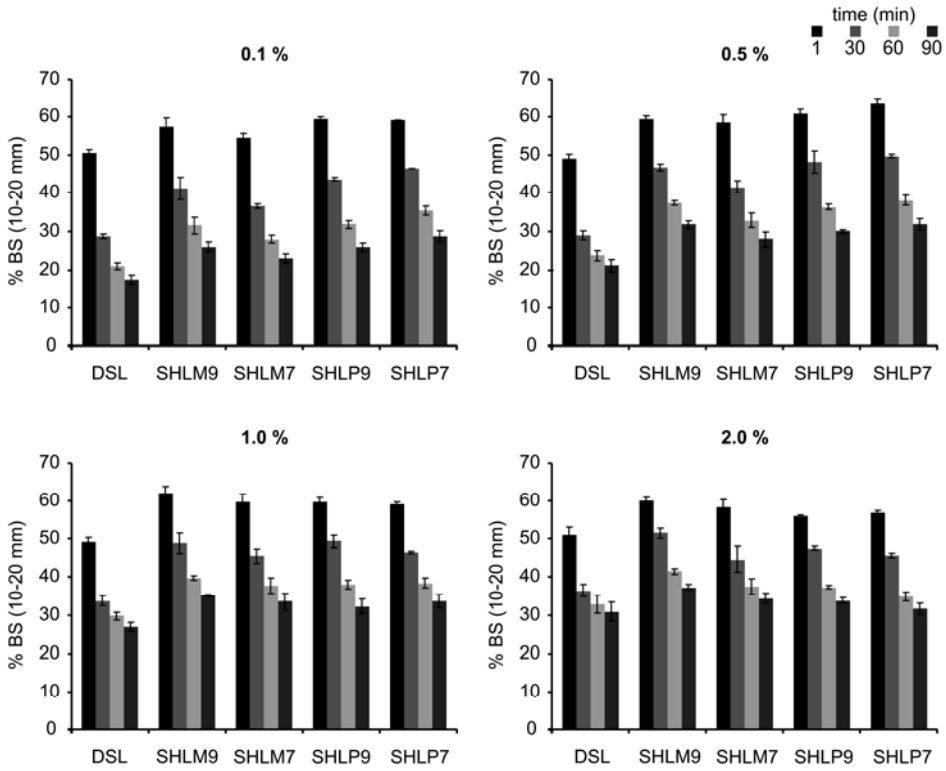

Figure 3. Backscattering (%BS) values of O/W emulsions (30:70 wt/wt) with the addition of different modified sunflower lecithins in Zone II (40-45 mm). Mean values (n = 3) ± sd

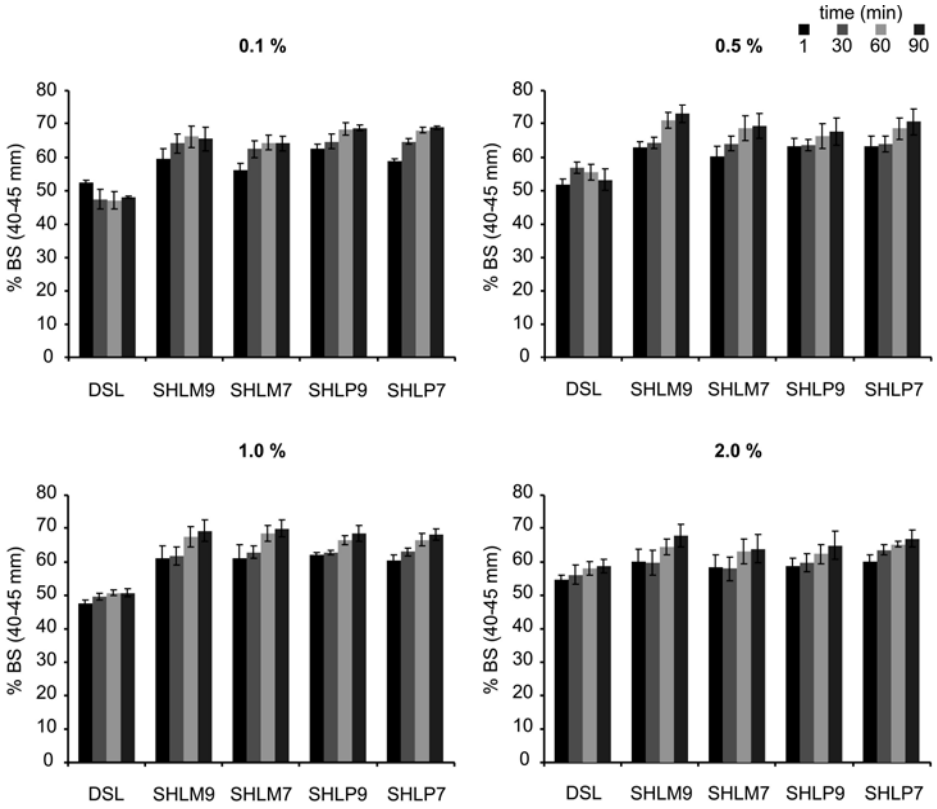

Figure 4. Volume particle size distribution for O/W emulsions with the addition of different modified sunflower lecithins. Mean values (n = 3)

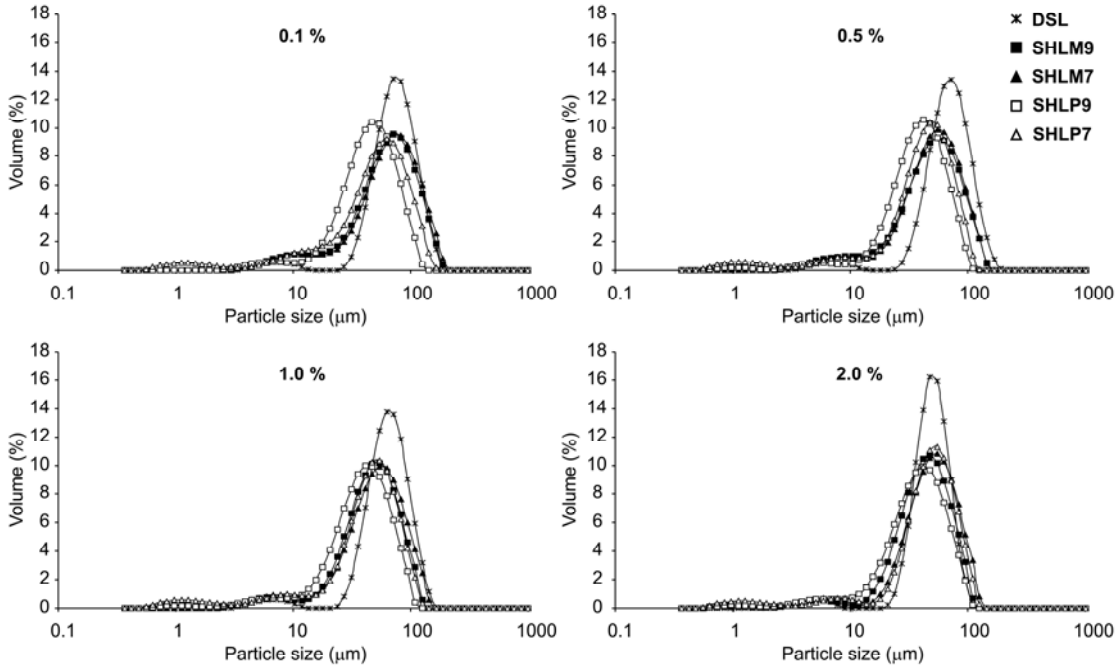

Figure 5. Surface particle size distribution for O/W emulsions with the addition of different modified sunflower lecithins. Mean values (n = 3)

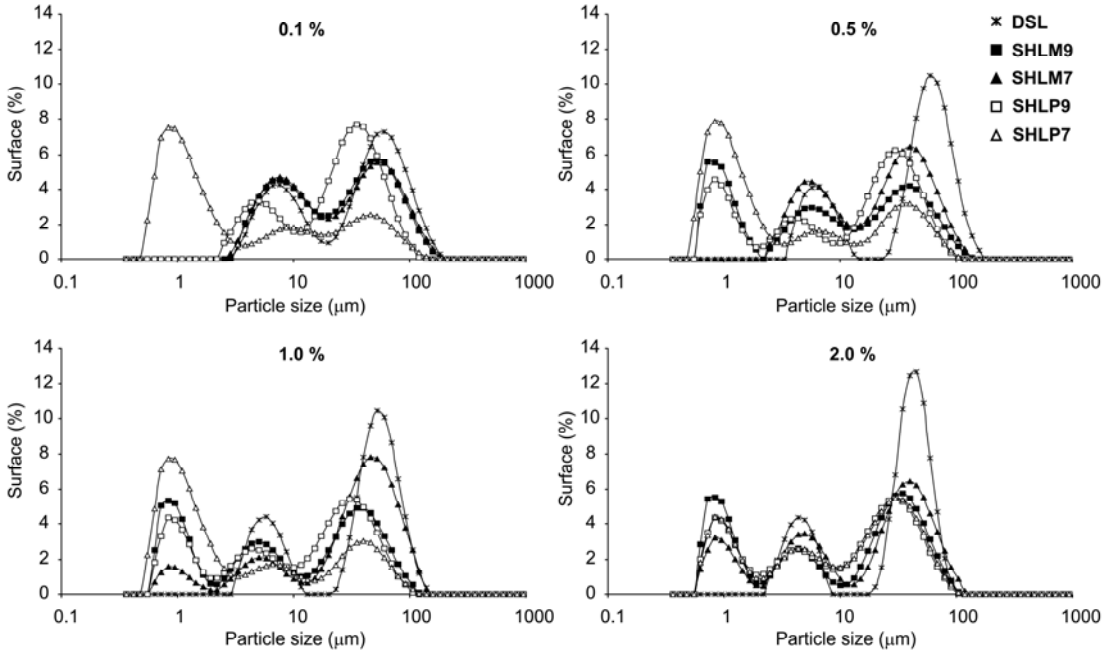

Figure 6. De Brouckere (D[4,3]) and Sauter (D[3,2]) mean diameters for O/W emulsions with the addition of different modified sunflower lecithins. Mean values (n = 3) ± sd