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# Ultrasonic energy input influence on the production of sub-micron o/w emulsions containing whey protein and common stabilizers

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## ABSTRACT

Ultrasonication may be a cost-effective emulsion formation technique, but its impact on emulsion final structure and droplet size needs to be further investigated. Olive oil emulsions (20 wt%) were formulated (pH ~ 7) using whey protein (3 wt%), three kinds of hydrocolloids (0.1–0.5 wt%) and two different emulsification energy inputs (single- and two-stage, methods A and B, respectively). Formula and energy input effects on emulsion performance are discussed. Emulsions stability was evaluated over a 10-day storage period at 5 °C recording the turbidity profiles of the emulsions. Optical micrographs, droplet size and viscosity values were also obtained. A differential scanning calorimetric (DSC) multiple cool–heat cyclic method (40 to –40 °C) was performed to examine stability via crystallization phenomena of the dispersed phase.

Ultrasonication energy input duplication from 11 kJ to 25 kJ (method B) resulted in stable emulsions production (reduction of back scattering values, dBs ~ 1% after 10 days of storage) at 0.5 wt% concentration of any of the stabilizers used. At lower gum amount samples became unstable due to depletion flocculation phenomena, regardless of emulsification energy input used. High energy input during ultrasonic emulsification also resulted in sub-micron oil-droplets emulsions ( $D_{50} = 0.615 \mu\text{m}$  compared to  $D_{50} = 1.3 \mu\text{m}$  using method A) with narrower particle size distribution and in viscosity reduction.

DSC experiments revealed no presence of bulk oil formation, suggesting stability for XG 0.5 wt% emulsions prepared by both methods. Reduced enthalpy values found when method B was applied suggesting structural modifications produced by extensive ultrasonication. Change of ultrasonication conditions results in significant changes of oil droplet size and stability of the produced emulsions.

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## 1. Introduction

High intensity ultrasonication (HIUS) with a frequency range between 16 and 100 kHz, and 10 and 1000 W/cm<sup>2</sup> of power, is a technology that has various applications on the food industry. It can be used in processes such as cooking, freezing, drying, degassing, filtration and emulsification in order to assist or even replace conventional methods [1,2].

Ultrasound devices can generate very stable emulsions that require little surfactant [3,4], or even produce emulsions with small droplet sizes directly from separate oil and water phases [5]. Ultrasound generators are capable of producing emulsions in the sub-micron range or form translucent nano-emulsions with average droplet sizes up to 40 nm [6–8] and are more efficient in producing smaller droplets in comparison to rotor–stator systems with droplet size as small as 0.2  $\mu\text{m}$  [9]. However, a comparison of the average droplet diameter versus power consumption using different

emulsifying machines showed that the smallest droplet diameters were obtained when using the high pressure homogenizers [10]. On the other hand, micro-fluidization has been found to be more power efficient than ultrasound, but it is considered less practicable with respect to production cost as well as equipment contamination [3].

Considering that this technology is increasingly being up-scaled as its use is both time and power saving, thus making it a cost-effective emulsion formation technique [11–13] the interest in its improvement is high.

On the other hand, emulsifiers use in food industry is shifted towards food-grade ones, among them to proteins from different sources. Whey protein concentrates (WPCs) are widely used in food industry because they are considered high functional and nutritional ingredients. Their functionality is related to their protein content, mainly  $\beta$ -lactoglobulin ( $\beta$ -lg) and  $\alpha$ -lactalbumin ( $\alpha$ -la) [14].

When HIUS emulsification is used, whey proteins can result in emulsions of different particle size [15] or modify the viscosity of the continuous phase [16]. However, a better control of the process

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should be achieved as the effects of high-intensity ultrasound on the structural and functional properties of both food proteins and/or other hydrocolloids used as stabilizers in the mix need to be further studied.

Hydrocolloids commonly used in the production of emulsions when low fat food products such as light mayonnaise are produced can be xanthan, locust bean and guar gum [17,18]. Xanthan gum is a poly-anionic polysaccharide mainly considered a non-gelling agent that presents weak-gel shear-thinning properties and it is used in order to control the viscosity. It interacts with whey proteins and the nature of those interactions depends on proteins' iso-electric point (Ip). Thus, above Ip, as in this study, an electrostatic repulsion of protein–polysaccharide occurs because both components are negatively charged. Apart from xanthan, the presence of locust bean gum is reported to be detrimental to protein–protein or protein–polysaccharide interactions for the development of protein gels [19]. A range of textures from soft to rigid can be formed by changing gel condition formation based on whey protein–locust bean gum interactions [20,21] that may also find applications in emulsions/gel-like products. Furthermore, xanthan or locust bean gum successfully substitute starch in low fat emulsions [22]. On the other hand guar gum can change significantly the viscosity of emulsion containing whey proteins inducing their shear-thinning behavior [23] or can reduce coalescence of oil droplets in heated emulsions [24]. However, due to its lately high price locust bean gum is being routed as guar gum credible replacer [25].

Stability is related to both droplet size and viscosity change due to ultrasonication. The knowledge regarding the impact of HIUS application on both droplet size and stability using different polysaccharide/protein blends should increase.

The main objective of this study was the investigation of HIUS effect on WPC o/w model-emulsions production at pH 7, where proteins have better emulsifying properties, hence smaller droplets are formed compared to a pH near their isoelectric point ( $4 < \text{pH} < 6$ ) [26]. A formula containing reduced extra virgin olive oil content was selected for nutritional reasons and health benefits related to its composition in combination with the fact that no oxidation or hydrolysis effects are observed when ultrasounds (20 kHz, 120 s) are applied [27].

Additionally, in comparison to other edible oils such as corn and sunflower oil, olive oil presented the highest absence of off-flavoring after sonication which is related to the composition of the unsaturated fatty acids [28].

## 2. Materials and methods

Whey protein concentrate (WPC) Lacprodan 80 was kindly provided by Arla (Arla Foods Ingredients, Amba-Denmark). The composition of WPC powder in protein as stated by the manufacturer was protein  $78 \pm 2\%$ , fat maximum amount 8%, ash 2.74% and lactose  $7 \pm 2\%$ . Quantification of this whey protein by means of Reversed Phase-HPLC can be found in Panaras et al. [29]. Xanthan gum (XG), guar gum (GG) and locust bean gum (LBG) were obtained from Sigma (St. Louis, MO, USA). Extra virgin olive oil Altis (Elais Unilever, Athens, Greece) was purchased from a local store. Phosphate buffer solution powder ( $\text{pH } 7.0 \pm 0.2$ ) and sodium azide were purchased from Fluka (Fluka Chemie AG, Buchs, Switzerland).

### 2.1. Emulsion preparation

Whey protein phosphate buffer stock solution 20 wt% was prepared by agitation with a magnetic stirrer for 60 min. Solutions were left overnight at 5 °C to ensure complete hydration. A few drops of sodium azide 0.02 wt% were added to the whey solution

as an antibacterial agent to prevent from spoiling during storage. Xanthan gum solution 1 wt% was prepared by hot stirring in a water bath at 90 °C for 1.5 h. Locust bean gum solutions 1 wt% were prepared under heating and agitation at 60 °C for 60 min. Guar gum solutions 1 wt% were also prepared with agitation at ambient temperature for 60 min. Coarse emulsions of total 100 g (20 wt% olive oil, 3 wt% WPC) and gums (0.1–0.5 wt%) were prepared. The concentration used for WPC was based on preliminary experiments, in which emulsion stability was recorded as a function of WPC concentration. Gums concentrations were selected according to literature data. The critical micelle formation concentration of surfactants (CMC) can be used for finding out the appropriate surfactant concentration in order to avoid extensive agglomerates formation. However, accurate surface tension measurements should be carried out and droplet size in the system, meaning also oily volume, should remain the same. As droplet size changed with respect to process conditions and formula, CMC is considered to change significantly for taking it into account.

Emulsification was performed in two stages. First, the WPC stock solution and olive oil were mixed for 4 min at 6500 rpm using an Ultraturrax T25 device (IKA Werke, Staufen, Germany). Afterwards, appropriate weights of gum solutions were added and the mixing continued for another 4 min at the same speed. The final emulsions (100 mL) were prepared in a glass beaker (60 mm internal diameter) covered by ice to prevent the temperature rise above 50 °C using an ultrasound device (Sonopuls 3200, equipped with a 13 mm diameter VS 70T probe, Bandelin GmbH & Co, Berlin, Germany) operating at a frequency of 20 kHz and varying amplitudes and times (method A: single-stage and method B: two-stage). The probe was immersed 1 cm below the surface of the emulsion. The sonication device operates by controlling amplitude (100% amplitude corresponding to  $170 \mu\text{m}_{\text{ss}}$  for the specific probe used) or power (150 W maximum nominal power). It also has the ability to display or monitor the energy release (kJ) in the sample during sonication. In method A, 70% amplitude of sonication for 2 min was used, while in method B 70% amplitude for 3 min followed by 90% amplitude of sonication for 1 min (4 min in total).

In our case the ultrasonication time was limited to 4 min after preliminary experiments, considering also that the energy applied to the system and temperature rise were high. Furthermore, a total sonication time of 5 min at a frequency of 20–24 kHz was found to produce optimum results in a 15% o/w emulsion, when the dispersed phase was flaxseed oil [30]. Another approach is the reduction of amplitude (e.g. 20%) and significant increase in sonication time (e.g. 20 min), operating at 20 kHz, applied in protein solutions [31]. However, sonication time depends on equipment geometry and sample volume used [32,7] and comparisons are difficult to be made.

The pH of the final emulsions was adjusted to 7.0 with a few drops of HCl 1 M. Experimental data of energy release and temperature rise in the samples during emulsification are given in Table 1.

### 2.2. Particle size estimation

Oil droplet size measurements were performed on a Bruker Minispec NMR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with a Bruker ND 2176 probe at 20 °C. All samples were previously stored at 5 °C and measured about 24 h after preparation. Measurements were carried out in duplicate and results are demonstrated as the median diameter  $D_{50}$  and cumulative diameters  $D_{97.5}$  and  $D_{2.5}$ , representing the 50, 97.5 and 2.5% of droplets being smaller than each value, respectively. The method is described in detail elsewhere [33].

**Table 1**

Experimental parameters of ultrasonication according to sonication method applied.

Gum content (wt%)	Method A		Method B	
	Energy (kJ)	Temperature (°C)	Energy (kJ)	Temperature (°C)
0.1	11.84 (0.14)	45.10 (1.95)	np*	np*
0.25	11.68 (0.52)	43.05 (1.99)	25.66 (0.53)	31.42 (0.71)
0.5	11.52 (0.34)	45.80 (1.36)	25.77 (0.35)	33.05 (2.37)

In parenthesis standard deviation values.

\*Emulsions not further prepared with method B due to very high instability as seen in method A.

### 2.3. Apparent viscosity

Viscosity flow curves were obtained using a controlled stress SR-5 rheometer (Universal Stress Rheometer/Rheometrics Scientific, Inc., NJ) with plate–plate geometry and 0.5 mm gap. The diameter of the upper plate was 20 mm. The temperature was constant ( $25 \pm 0.2$  °C) by circulating water from a constant temperature circulator. Steady-state flow curves were obtained at shear rates between 0.01 and  $100 \text{ s}^{-1}$  approximately to avoid slippage in the case of final emulsions prepared with both sonication methods and up to  $1000 \text{ s}^{-1}$  in the case of 1 wt% gum solutions that had been subjected to various sonication treatments (70% amplitude for 1, 2 (method A) or 3 min and as in method B). Viscosity measurements were performed only in emulsions containing 0.5 wt% of various gums that were the most stable. In the case of gum solutions, a higher concentration of 1 wt% instead of 0.5 wt% was selected in order to better fit the precision of the rheometer geometry, since it is more accurate at relatively high viscosity values. The viscosity of 1 wt% gum solutions was calculated at a low shear rate ( $10 \text{ s}^{-1}$ ), mean values of at least two differently prepared formulations are reported and viscosity flow curves are demonstrated.

### 2.4. Light microscopy

Emulsion structure was observed in freshly prepared samples with a conventional optical microscope (Kruss Optronik, Germany) with a 10x magnification and several micrographs were obtained per sample from the center area of each slide. The emulsions were not subjected to dilution in order not to disturb their initial structure. Several photos were taken from random sample positions. The micrographs were recorded using a camera (SONY, Hyper HAD, CCD-Iris) connected to a computer.

### 2.5. Cold storage stability

Emulsion stability was evaluated with a vertical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France) during 10-day storage at 5 °C. This multiple light scattering device allows the optical characterization of any type of dispersion by using a mobile reading head composed of a transmitting NIR diode ( $\lambda = 850 \text{ nm}$ ) and two synchronous detectors analyzing the transmitted (T) and backscattered (BS) light with acquisitions every 40  $\mu\text{m}$ . Emulsion samples of about 6 ml were put in a glass tube and scanned from bottom to 80 mm tube height on a daily basis in order to obtain back scattering and transmission profiles that allow the calculation of instability due to creaming, sedimentation, coalescence as well as other phenomena. The procedure was replicated at least three times in order to obtain similar profiles. Emulsion instability due to clarification at the bottom of the cell is expressed by the serum index (SI)- elsewhere reported as creaming index- which is the height of the serum layer over the total height of the emulsion in the glass tube expressed in percentage. The height of serum and sample were calculated from peak thickness

and meniscus height accordingly, as depicted at the back scattering profiles plotted in reference mode.

$$\text{Serum index (SI), \%} = \left( \frac{\text{Height of the serum layer}}{\text{Total height of the emulsion}} \right) \times 100 \quad (1)$$

SI: serum index indicating emulsion phase separation (%).

Emulsion coalescence was evaluated from BS values at the middle of the sample and the variation of back scattering (dBS) with time during cold storage was calculated according Huck-Iriart et al. [34] to from Eq. (2).

$$\text{dBS} = \text{BS}_0 - \text{BS}_{10} \quad (2)$$

dBS: back scattering change of cold stored samples (%).  $\text{BS}_0$ , back scattering of freshly prepared emulsions (%).  $\text{BS}_{10}$ , back scattering of emulsions after 10 days of cold storage (%).

### 2.6. Thermal analysis of XG emulsions

Thermographs of freshly made emulsions samples ( $15 \pm 1 \text{ mg}$  in a hermetically sealed aluminum pan) were obtained with a DSC Q100 (TA Instruments, DE, USA). The DSC was calibrated using mercury, distilled water and indium. An empty pan was used as reference. Samples were cooled from 40 to  $-40$  °C and heated to 40 °C, with a rate of 5 °C/min. The cooling/heating cycle was repeated two times within the experiment in order to investigate the influence of continuous and dispersed phase crystallization on the stability of emulsions. Enthalpy changes ( $\Delta H$ ), as well as onset ( $T_{\text{on}}$ ) and off-set ( $T_{\text{off}}$ ) temperatures of crystallization were determined from the measured peak areas in the DSC thermographs of the samples containing 0.5 wt% XG during the three heat-cool cycles with the TA Universal Analysis 2000, v.4.2E software.

### 2.7. Statistical analysis

Statistical analysis of the results was performed with Statgraphics Centurion XV (Statgraphics, Rockville, MD, USA) and *F*-test was applied in order to compare the mean values of selected properties (SI on day 10 of cold storage, dBS,  $D_{50}$ , apparent viscosity of gum solutions at  $10 \text{ s}^{-1}$ ,  $T_{\text{onset}}$ ,  $T_{\text{off}}$  and  $\Delta H$ ) at 95% level of confidence.

## 3. Results and discussion

### 3.1. Oil droplet size

During the emulsification process two different phenomena, droplet disruption and re-coalescence occur at the same time, the kinetics of each one individually can affect the final droplet size of emulsions [6,35]. As a consequence, concerning ultrasonic emulsification, an optimum energy input should be established to avoid over-processing regarding droplet size increase [30].

Particle size parameters of the emulsions prepared with both methods are shown in Table 2. Formulations prepared with low

**Table 2**

Particle size parameters of formulations containing 0.25 and 0.5 wt% stabilizers prepared with two ultrasonication methods.

	Method A			Method B		
	$D_{50}$ ( $\mu\text{m}$ )	$D_{97.5}$ ( $\mu\text{m}$ )	$D_{2.5}$ ( $\mu\text{m}$ )	$D_{50}$ ( $\mu\text{m}$ )	$D_{97.5}$ ( $\mu\text{m}$ )	$D_{2.5}$ ( $\mu\text{m}$ )
XG 0.25%	$1.107^f \pm 0.146$	$4.233 \pm 0.279$	$0.284 \pm 0.042$	$0.832^{cd} \pm 0.040$	$2.945 \pm 0.361$	$0.246 \pm 0.034$
XG 0.5%	$1.325^g \pm 0.096$	$2.641 \pm 0.161$	$0.709 \pm 0.127$	$0.786^{bc} \pm 0.79$	$2.527 \pm 0.188$	$0.274 \pm 0.048$
GG 0.25%	$1.093^{ef} \pm 0.061$	$3.572 \pm 0.421$	$0.385 \pm 0.028$	$0.843^{cd} \pm 0.089$	$2.245 \pm 0.035$	$0.372 \pm 0.102$
GG 0.5%	$1.330^g \pm 0.141$	$4.215 \pm 0.106$	$0.214 \pm 0.057$	$0.771^b \pm 0.069$	$1.871 \pm 0.143$	$0.561 \pm 0.035$
LBG 0.25%	$1.018^e \pm 0.139$	$3.389 \pm 0.242$	$0.341 \pm 0.042$	$0.876^d \pm 0.048$	$2.664 \pm 0.204$	$0.230 \pm 0.052$
LBG 0.5%	$1.077^e \pm 0.109$	$3.427 \pm 0.191$	$0.395 \pm 0.085$	$0.615^a \pm 0.049$	$2.225 \pm 0.120$	$0.383 \pm 0.038$

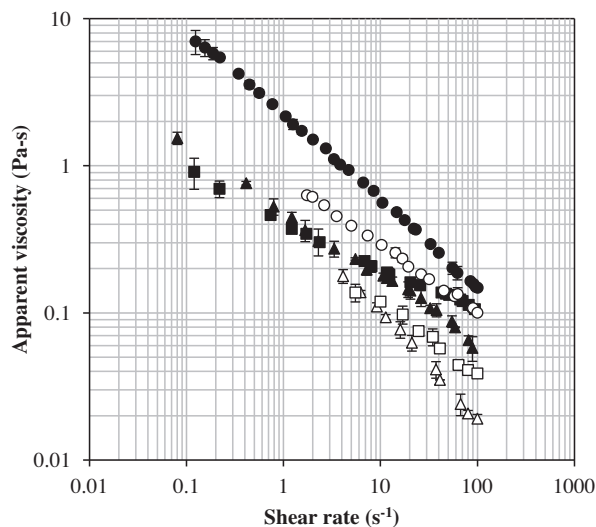
Results presented as average out of two measurements of two repeated formulations.

Mean values of the  $D_{50}$  followed by the same letters are not significantly different ( $P > 0.05$ ).

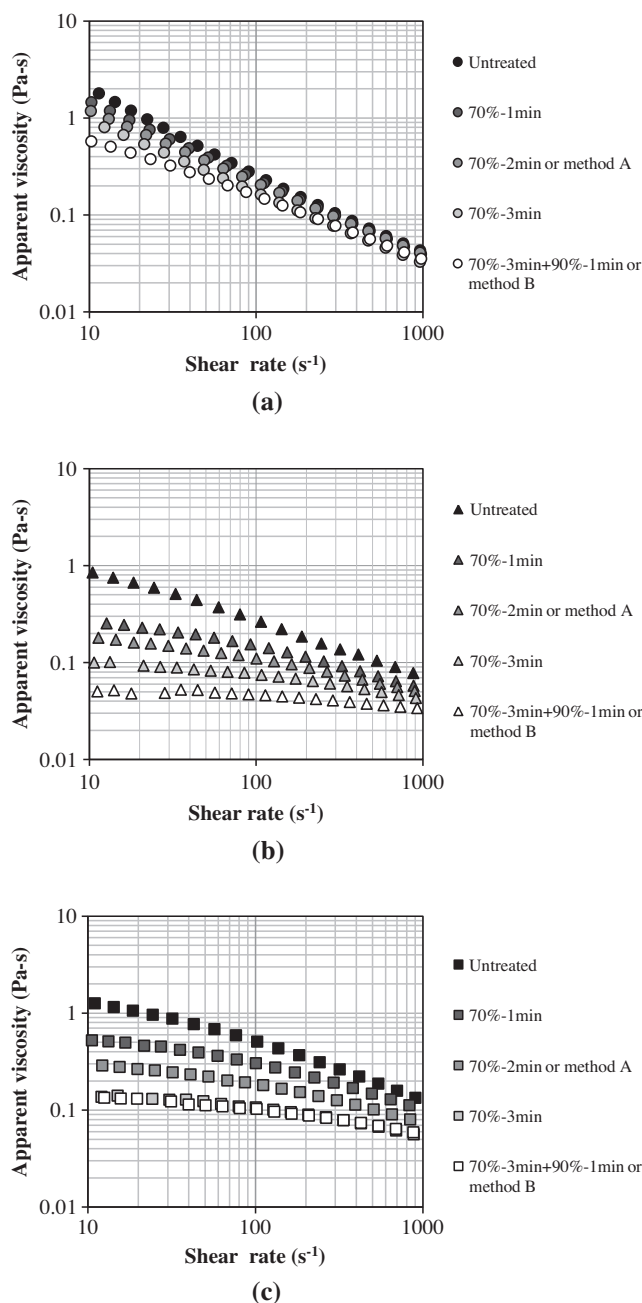
energy input (method A) exhibited an oil droplet median diameter  $D_{50}$  higher than  $1 \mu\text{m}$  in all cases ( $1.018$ – $1.325 \mu\text{m}$ ).

Method B was able to further decrease the average diameter in all formulations, thus producing finer droplets with a  $D_{50}$  between  $0.615$  and  $0.876 \mu\text{m}$ . Also, a drastic reduction of  $D_{97.5}$  was observed in all formulations containing the same type of stabilizer at the same concentration. As a result the  $D_{97.5}$  values ranged between  $2.641$ – $4.233 \mu\text{m}$  and  $1.871$ – $2.945 \mu\text{m}$  for method A and B accordingly. Thus, method B was more effective upon decreasing the size of bigger droplets resulting in narrower droplet size distributions.

This pronounced efficacy of method B was strongly related not only to increased duration (4 min), but also because a second stage at higher pressure amplitude (90%/1 min) was involved. An increase in the applied pressure amplitude led to intensive cavitation due to an increased number of bubbles generated by pronounced breaking of liquid threads. It is also known that an increase in amplitude and duration increase the energy release in the system that leads to subsequent temperature rise. These phenomena facilitate the dispersion of each phase into another by means of interfacial tension, viscosity and Laplace pressure decrease. Nevertheless, even though the temperature rise was greater in method A ( $\sim 43$ – $45^\circ\text{C}$ ) and viscosity decrease of emulsions and gum solutions lower (Figs. 1–3), still the total energy applied on the system as a consequence of limited duration and amplitude was not enough to create fine dispersions as in method B. Thereby smaller droplets were produced, while at lower amplitude a wider droplet distribution was observed. Same observations were noticed by Canselier et al. [36], McClements [37], Seekkuarachchi et al. [38], and Gaikwad and Pandit [39].

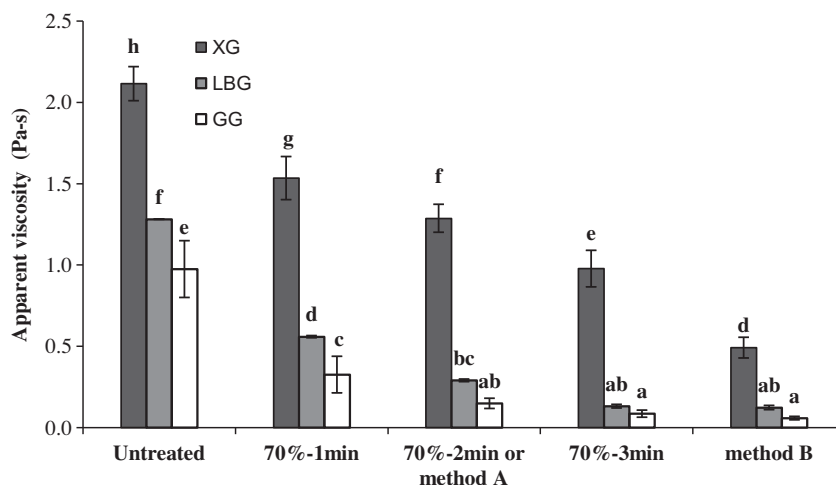


**Fig. 1.** Apparent viscosity of emulsions containing 0.5 wt% stabilizers prepared with method A; (●) XG, (▲) GG and (■) LBG and prepared with method B; (○) XG, (△) GG and (□) LBG.



**Fig. 2.** Apparent viscosity flow curves of 1 wt% gum solutions (a) XG, (b) GG and (c) LBG, as affected by different sonication treatments.





**Fig. 3.** Apparent viscosity values at  $10 \text{ s}^{-1}$  rate of 1 wt% gum solutions as affected by different sonication treatments. Mean values followed by the same letters are not significantly different ( $P > 0.05$ ).

It has been found that the addition of gum in the emulsion during homogenization enhanced the adsorption of the protein molecules onto the droplet surface due to increased protein/surface contact and led to reduced droplet size [40], but this is only merely in accordance with our findings. As shown in Table 2, the  $D_{50}$  diameters of emulsions prepared with method B decrease upon stabilizer concentration increase. The opposite phenomenon was observed in the case of method A, even though the differences of LBG emulsions were less evident ( $p > 0.05$ ). This controversy could be mainly attributed to the higher viscosities of the continuous phase during emulsification owned to decreased fragmentation of the hydrocolloids when the low energy input method was used. Tzoumaki et al. [41] have also observed an increase in the average droplet size of ultrasonically prepared WPI emulsions when chitin nanocrystals' concentration was increased above 0.5% attributed to high viscosity of the continuous phase. Concerning the stabilizer type, LBG was more efficient in decreasing the droplet size of emulsions prepared by both methods, while XG formulations contained bigger droplets especially those prepared at low energy input.

In general, sonication energy input increase was more efficient in reducing the droplet size than the stabilizers used, hence samples prepared with method B had lower droplet size and improved stability, regardless of composition. These findings are also in accordance to a recent study [42], in which it was found that the sonication power was significant in reducing the oil droplet size, whereas the continuous phase viscosity, surfactant characteristics and volume fraction had much lower influence on the final droplet size. Viscosity matters will be analyzed in more details in next section.

### 3.2. Apparent viscosity of 0.5 wt% emulsions and 1 wt% gum solutions

Viscosity as a function of shear rate of emulsions containing 0.5 wt% XG, GG and LBG prepared with both sonication methods is presented in Fig. 1. The flow curves of all formulations exhibited a typical shear-thinning behavior over the shear rate tested. XG samples presented higher viscosity values compared to emulsions containing galactomannans ( $\text{XG} \gg \text{LBG} \geq \text{GG}$ ).

Samples that underwent an elongated and more intensive sonication (method B) presented lower viscosity values, thus emulsification method affected the flow behavior of emulsions. Viscosity values followed the same order accordingly to the hydrocolloids used, regardless of the sonication energy input. Xanthan produced the thickest samples, i.e. higher viscosity values that were still

greater than the ones of samples containing LBG or GG after extended sonication.

Generally, the emulsion viscosity is influenced by the continuous phase viscosity, interfacial film viscosity and droplet size. Concerning droplet size and viscosity.

Taylor equation describes their connection as follows:

$$r \sim \gamma / (\eta_c \times \gamma') \quad (3)$$

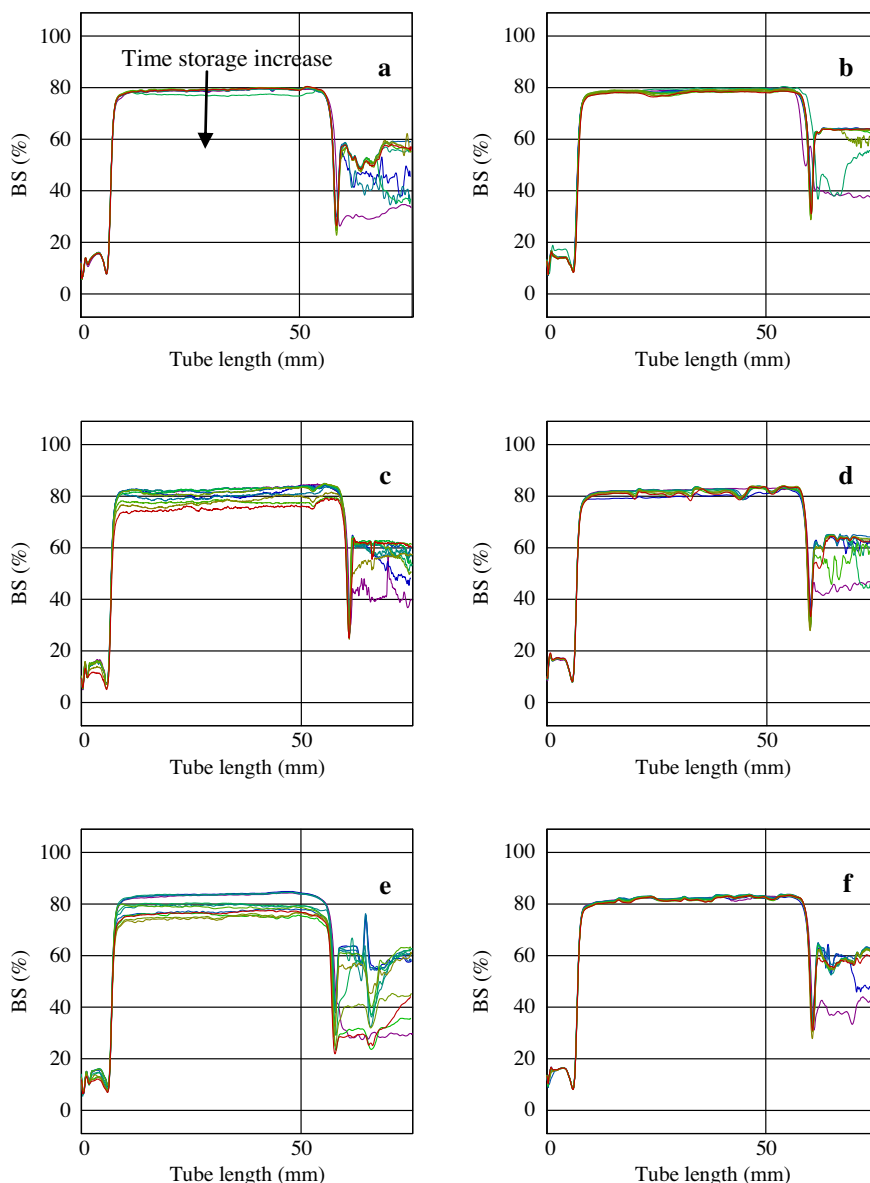
where,  $r$  is the droplet radius,  $\gamma$  is the interfacial tension,  $\eta_c$  is the continuous phase viscosity and  $\gamma'$  is the shear rate.

In their original form, the galactomannans used have different molecular weight and viscosities ( $\text{LBG} > \text{GG}$ ). Furthermore, they do not interact directly at the droplet interface, because they are neither charged macromolecules nor hydrophobic substances that could create interfacial films around oil droplets. On the other hand, xanthan gum, is a poly-anionic hetero-polysaccharide with gel-like properties. Its molecular weight and viscosity is very high, comparable to those of galactomannans used. Thus, Taylor equation may be not applicable in these type of emulsions, which are highly concentrated, so gums' viscosity after sonication is hardly related to droplet size.

A significant reduction of gum viscosity by increasing sonication energy input was also found in this study. Regarding viscosity reduction it is known that sonication results in pressure fluctuations, which propagate through the material resulting in the formation of microscopic bubbles that collapse within a few milliseconds (cavitation). As a result, extreme effects in the vicinity of these bubbles, where biopolymers exist, can occur. These include heating, high pressure and high shear rates [43–45] and can lead to cleavage of polysaccharides mainly due to glycosidic linkages breakage and structure changes (e.g. xanthan shift in ordered structure) [43].

Furthermore the chemical effects due to OH and H radicals are more prominent and influence the degradation of polymers in aqueous solutions at high frequency sonication (200–600 kHz) than at low frequency [46,47].

Although there are many reports on ultrasonic degradation of polysaccharides [48–51], these findings focus on rheological properties as affected by molecular weight reduction, thus information is given in terms of gum tailoring. Therefore, there are only limited data to our knowledge relating the effect of gum viscosity decrease on emulsion stability [52–54]. In Fig. 2 viscosity flow curves of 1 wt% gum solutions are demonstrated and Fig. 3 summarizes the effect of different sonication treatments on the apparent vis-



**Fig. 4.** Back scattering (BS), as a function of the tube length for samples stored at 5 °C (arrow denotes time) in emulsions containing 0.5 wt% gums, prepared with method A containing, (a) XG, (c) GG, (e) LBG and prepared with method B containing, (b) XG, (d) GG, (f) LBG.

cosity values at  $10\text{ s}^{-1}$  rate. XG solutions were characterized by higher viscosity values compared to GG and LBG ( $\text{XG} \gg \text{LBG} > \text{GG}$ ) in all cases of untreated or sonicated gum solutions, hence the viscosity of final emulsions is mainly affected by the final viscosity of the concurrently sonicated polysaccharide aqueous phase. As demonstrated by the flow curves in Fig. 2 galactomannan solutions (GG and LBG) presented a viscosity reduction when sonication time and intensity (method B) increased. Their viscosity was then reduced and the newtonian-like behavior was enhanced, especially after 3 min of sonication at 70% amplitude. It should also be noted that a more intensive treatment (method B) was not able to further decrease the viscosity of the galactomannan solutions, thus their viscosity reached a limiting value. On the contrary, XG solutions maintained their initial shear-thinning behavior and higher viscosity values were noticed even after 4 min of sonication (method B).

Our findings are in accordance with those of Tiwari et al. [43] who also showed that xanthan was more resilient against degradation and maintained its shear thinning behavior compared to guar by increasing ultrasonic intensity. This was shown by means of the consistency index  $k$ , which is a measure of viscosity (0.699 and

0.021 for XG and GG accordingly) and the flow behavior index  $n$  (0.594 and 0.932 for XG and GG accordingly), for which values near unity indicate a Newtonian behavior.

In total, when comparing untreated gum solutions with those that underwent the most intensive sonication (method B) there was a reduction of XG, LBG and GG viscosity by a factor of 4, 10 and 17 accordingly, thus GG was the most sensitive against ultrasonic depolymerization. Nevertheless, the viscosity decrease of the polymer, enhanced by temperature increase during sonication, improved the stability against coalescence of 0.5 wt% GG and LBG emulsions produced with method B (Fig. 4d and f) as a result of significant droplet size reduction. In the case of 0.5 wt% XG emulsions, coalescence was not affected by sonication method, assuming that the droplet size reduction in emulsions prepared with method B was able to counterbalance the polymer viscosity decrease.

### 3.3. Light microscopy

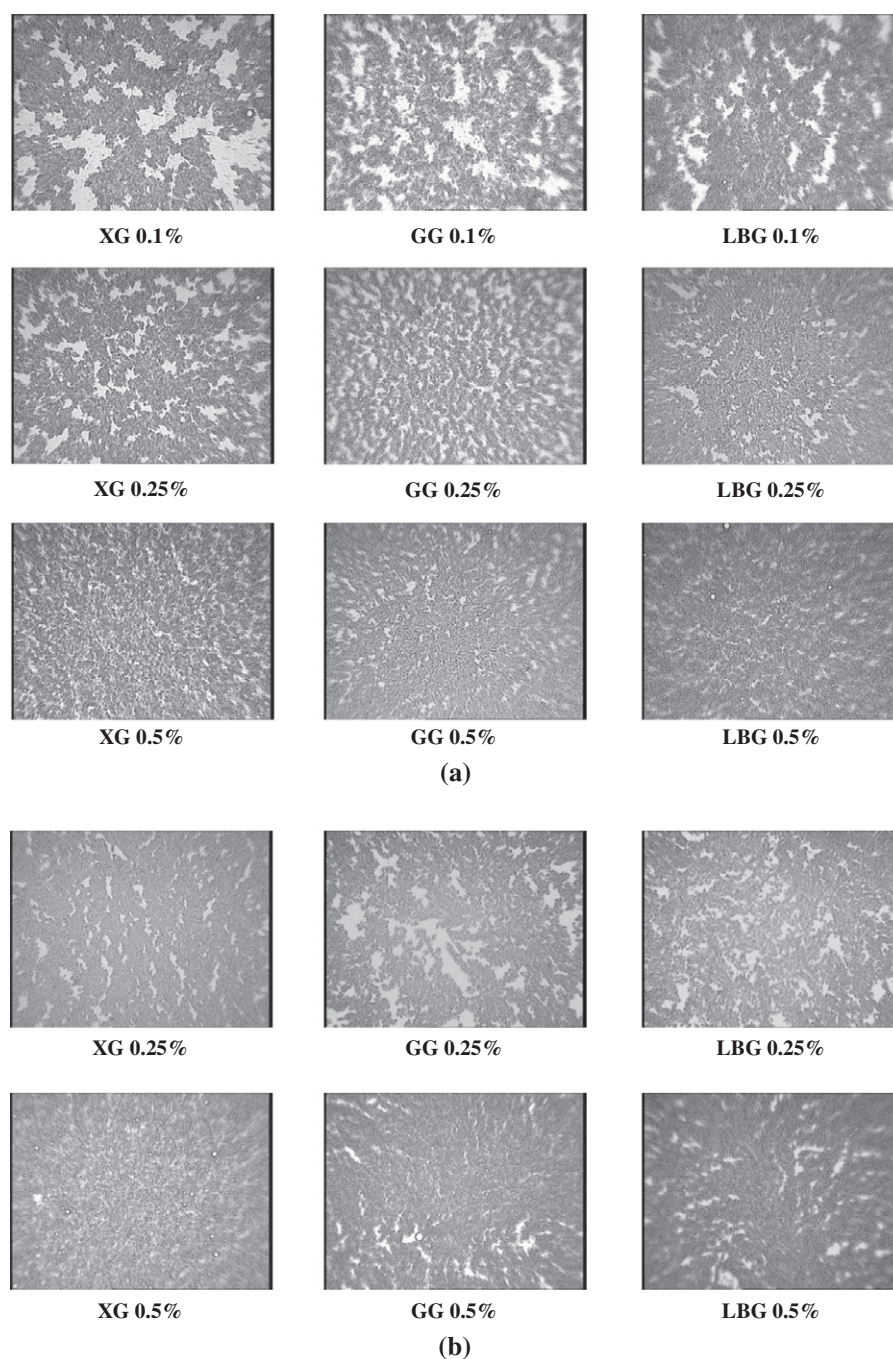
In Fig. 5 micrographs of all emulsions formulations prepared with both ultrasonic emulsification techniques are depicted. Emul-

sions containing 0.1 wt% gums had a loose structure, forming extended gaps in the aqueous phase. These gaps were formed due to depletion flocculation phenomenon, since reversible phase separation was observed upon gentle shaking. Thus, the weak flocks can easily be broken down to smaller sizes or even to single droplets [55]. The gaps formed seem to be more extended in the case of XG, which is typical due to its high molecular weight and repulsive interactions with proteins, in comparison to GG and LBG emulsions of the same concentration. This is in accordance to the high SI values found in samples containing 0.1 wt% XG, whereas samples containing GG and LBG exhibit better dispersion, resulting in lower SI values (Fig. 6a). Emulsions containing 0.25 wt% of polysaccharide prepared with both methods showed a lower degree of depletion flocculation than samples containing 0.1 wt% of stabilizers (meth-

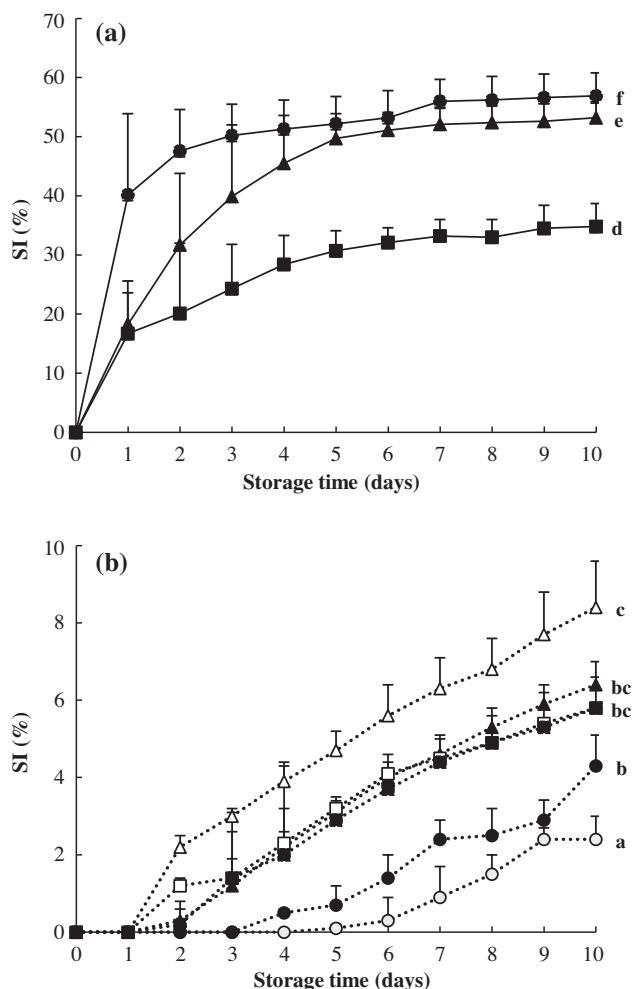
od A), thus exhibited lower values of SI. Furthermore, samples with 0.5 wt% had an even more homogenous structure and therefore stable emulsions were formed in terms of serum layer absence (Fig. 7).

### 3.4. Cold storage stability

Creaming, flocculation, coalescence and partial coalescence are examples of natural instability during storage. The presence of hydrocolloids in an emulsion and their concentration strongly influence emulsion stability. It is known that the addition of XG up to a critical concentration ( $\sim 0.2$  wt%) promotes extensive flocculation leading to creaming or coalescence, whereas at higher concentrations ( $\sim 0.5$  wt%) little or no flocculation occurs



**Fig. 5.** Micrographs of emulsions containing XG, GG and LBG prepared with two ultrasound techniques, using, (a) method A, (b) method B.



**Fig. 6.** Stability (serum layer formation) of emulsions during storage at 5 °C using (a) method A containing 0.1 wt% stabilizers (●) XG, (▲) GG and (■) LBG, solid lines (b) containing 0.25 wt% stabilizers prepared with method A; (●) XG, (▲) GG and (■) LBG and prepared with method B; (○) XG, (△) GG and (□) LBG, dotted lines. Mean values of SI at day 10 followed by the same letters are not significantly different ( $P > 0.05$ ).

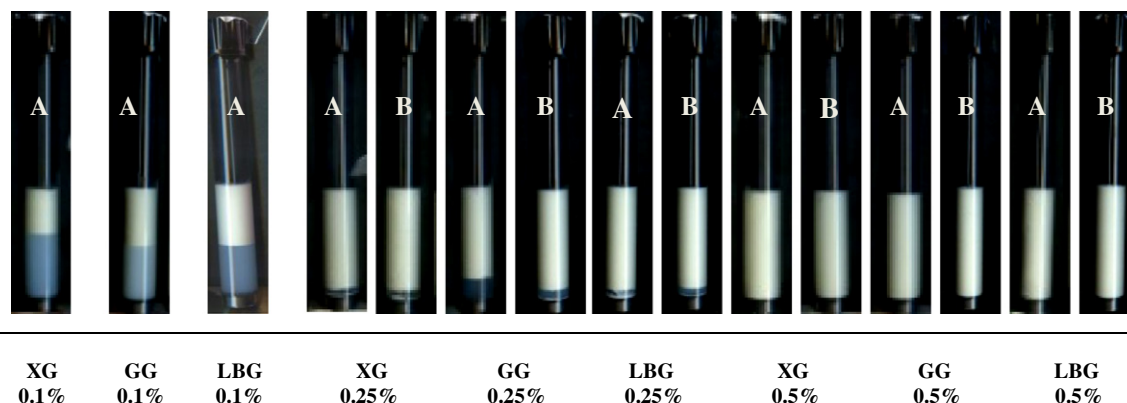
[40,56,57]. This is because even though the droplets are aggregated, their motion is limited, mainly because of the high viscosity of the continuous phase or the formation of a gel-network by the polysaccharides [58].

Fig. 6 demonstrates the serum index (SI) of emulsions prepared with both methods as affected by storage time, which represents the instability of the samples containing 0.1 and 0.25 wt% XG, GG and LBG. Formulations containing 0.1 wt% of XG presented the highest instability after 10 days of storage ( $SI = 59.6\%$ ), whereas LBG the lowest one ( $SI = 34.5\%$ ) (Fig. 6a). Due to their high instability emulsions at 0.1 wt% gum concentration were not subjected to the two-stage emulsification or further tested. Instability of the emulsions containing 0.25 wt% started later and proceeded at a much lower rate than those containing 0.1 wt% of polysaccharide. Emulsions containing 0.25 wt% of GG and LBG produced by method B presented generally similar SI values to those produced with the single-stage ( $p > 0.05$ ) (Fig. 6b). Even though the  $D_{50}$  was considerably reduced in these emulsions by higher energy input, a delay in creaming did not occur and destabilization was observed after 2 days of storage, while those prepared with method A started to cream on day 3 (Fig. 6b). Thus, creaming was not reduced by applying method B for GG and LBG emulsions, suggesting that the reduction of the droplet size could not counterbalance the aqueous phase viscosity reduction. On the contrary, there is a statistically significant difference for the emulsion of XG between the two methods with method B producing a more stable emulsion since the  $D_{50}$  was decreased at 0.832 nm and the viscosity of the XG was less affected by sonication treatment compared to that of the galactomannans (Fig. 3). As a general trend the SI of 0.25 wt% emulsions follows the order  $GG \geq LBG > XG$ . Similar findings about the influence of XG and LBG on emulsion stability have been reported [59,60]. Concluding, at low gum concentrations creaming depended on energy input and destabilization occurred despite the significant reduction of the droplet size in GG and LBG emulsions owned to significant viscosity reduction.

In Fig. 4 and Table 3, back scattering (BS) and variation values (dBS) for emulsions containing even higher gum concentration (0.5 wt%) are shown. These emulsions, regardless of the preparation method, remained stable during cold storage and even much longer (BS profiles obtained up to 30 days). This means that neither serum layer was observed nor BS peak near the zone of 50 mm, indicative for the creaming of droplets (Fig. 3). However, oil droplets coalesced as it can be seen by the decrease in BS values, since BS reduction is strongly affected by droplet size [61].

The reduction of oil droplet size can cause a decrease in the attractive forces acting between the droplets resulting in emulsions less susceptible to coalescence [62,63].

Formulations containing 0.5 wt% GG and LBG prepared with method A appear more susceptible to coalescence compared to XG. They exhibit greater differences of back scattering between preparation day ( $BS_0$ ) and the last day of cold storage ( $BS_{10}$ ) and



**Fig. 7.** Emulsion samples prepared using the two different emulsification methods (A and B) containing xanthan gum (XG), guar gum (GG) or locust bean gum (LBG). Serum separation on 10th day of cold storage in samples containing 0.1 and 0.25 wt% stabilizers can be seen.



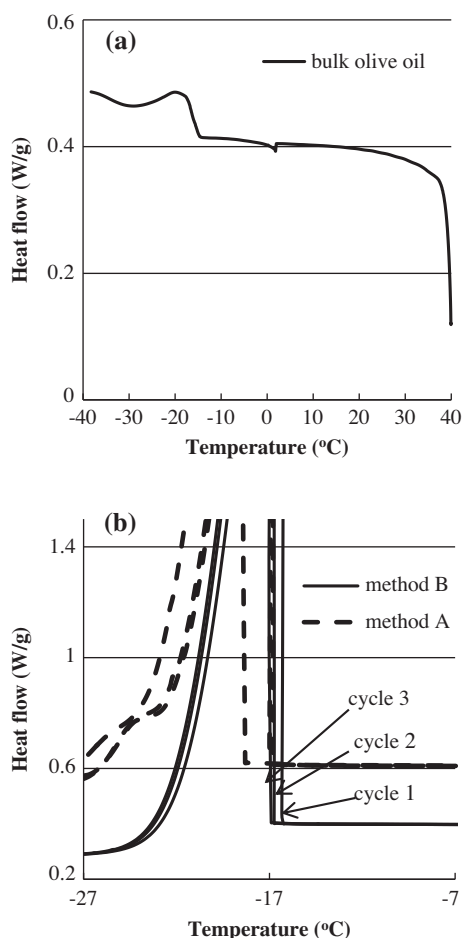
**Table 3**

Back scattering (%) and back scattering variation dBS (%) of emulsions containing 0.5 wt% stabilizers during cold storage. Storage time increase as indicates the arrow. Red lines correspond to 10 days cold storage.

	Method A			Method B		
	BS <sub>0</sub>	BS <sub>10</sub>	dBS	BS <sub>0</sub>	BS <sub>10</sub>	dBS
XG	81.02 ± 1.22	79.19 ± 0.71	1.30 <sup>a</sup> ± 0.69	79.64 ± 0.49	78.58 ± 0.38	1.06 <sup>a</sup> ± 0.40
GG	82.40 ± 0.26	73.94 ± 0.91	8.65 <sup>b</sup> ± 0.86	82.64 ± 0.66	80.88 ± 0.95	1.3 <sup>a</sup> ± 0.04
LBG	83.01 ± 0.50	74.14 ± 0.28	8.99 <sup>b</sup> ± 1.10	83.35 ± 0.88	82.45 ± 0.96	0.90 <sup>a</sup> ± 0.22

Results presented as average out of three measurements.

Mean values of dBS in the same column followed by the same letters are not significantly different ( $P > 0.05$ ).



**Fig. 8.** DSC cooling thermographs of (a) bulk olive oil and (b) 0.5 wt% XG emulsions.

they are additionally characterized by lower viscosity values that can favor the mobility of the droplets. Emulsions prepared with method B were more stable in terms of coalescence. Only a slight reduction of BS values ( $\text{dBS} = 0.90\text{--}1.06$ ) (Table 3) was observed in all formulations despite the significant reduction of the continuous phase viscosity. Concluding, ultrasonication energy input increase (method B) resulted in stability improvement regardless of the composition when gum concentration in emulsions was 0.5 wt%.

### 3.5. Thermal analysis of 0.5 wt% XG emulsions

As it is shown in Fig. 4(a, b) emulsions containing 0.5 wt% XG were the most stable during cold storage, since only a small reduction in BS occurred and no serum layer formation was observed. In other words, coalescence of oil droplets was the only instability

phenomenon detected during cold storage. Nevertheless, the term coalescence involves two different instability mechanisms. The first one, also referred as “true coalescence”, is caused by extended contact of oil droplets that locally causes high Laplace pressures and breakage of the droplet emulsifier film layers with the subsequent creation of a bigger droplet. Secondly, the presence of solid particles in the dispersed phase can lead to “partial coalescence” that is encouraged when emulsions are subjected to temperature fluctuations [64]. Upon cooling or cold storage, crystalline regions associated with saturated triacylglycerols (TAGs) of the dispersed phase are formed. These fat crystals can penetrate into another droplet to cause collision, which can lead to “true” coalescence upon reheating of the emulsions [35,65,66].

This DSC cyclic heat–cool method was conducted in order to detect mainly the crystallization phenomena of the dispersed phase that could have a possible contribution on partial coalescence. A preliminary test using only olive oil was performed with a simple temperature ramp (Fig. 8a) within the same ranges ( $-40\text{--}40\text{ }^{\circ}\text{C}$ ). The cooling line shapes of olive oil (extra virgin) were quite similar to those reported by Chiavaro et al. [67], even though the major peak at around  $-38\text{ }^{\circ}\text{C}$  associated with the highly unsaturated TAGs was not clearly obtained within this temperature range (a much clearer thermograph was obtained when cooling at  $-50\text{ }^{\circ}\text{C}$ , data not shown). In our case another minor exothermic event with an onset temperature at  $-14.68\text{ }^{\circ}\text{C}$  was observed, that is attributed to the crystallization of more saturated TAG fractions of the extra virgin olive oil [68,69].

Fig. 8 demonstrates cooling shape lines of bulk olive oil (a) and samples of freshly prepared emulsions containing 0.5 wt% XG (b). The shape-line of method A has been slightly annotated for presentation reasons.

In Fig. 8(b) it can be seen that no crystallization of the dispersed phase occurred upon cooling for emulsions prepared with both methods (characteristic peaks were not observed), hence the olive oil remained well emulsified and no bulk oil derived by coalescence was detected during the procedure.

In Table 4 it can be seen that the emulsification method influenced the thermal properties of the emulsions. The aqueous phase crystallization of samples prepared with method A was characterized by more suppressed initiation temperatures ( $T_{\text{onset}}$ ) and increased enthalpy values. Another interesting aspect during the freeze–thaw cycles is that the crystallization enthalpies of the samples remained practically the same, assuming that the amount of freezable water was not affected by the process.

Still, the explanation of the influence of the ultrasonic treatment on the aqueous phase thermal properties remains complex, considering synchronous phenomena occurring such as xanthan gum depolymerization [70]. Ultrasonication is known to alter the functional properties of WPCs proteins in a less detrimental way. It has been shown that sonication treatment may enhance protein stability against aggregation [71], increase solubility and decrease the freezing point of WPC solutions [72]. Furthermore, the WPC, unlike the isolates (WPIs), contains important quantities of fat and lactose and therefore cannot be considered a pure substance, hence a

**Table 4**

Thermal properties of emulsions containing 0.5 wt% XG prepared with two ultrasonication methods.

	<i>T</i> max (°C)			$\Delta H$ (J/g)		
	Cycle 1	Cycle 2	Cycle 3	Cycle 1	Cycle 2	Cycle 3
<i>Method A</i>						
XG 0.25%	−16.93 <sup>ef</sup> ± 0.16	−17.10 <sup>f</sup> ± 1.93	−15.62 <sup>cde</sup> ± 0.57	252.9 <sup>b</sup> ± 35.78	253.95 <sup>b</sup> ± 37.41	253.95 <sup>b</sup> ± 36.56
XG 0.5%	−15.34 <sup>bcde</sup> ± 0.05	−17.00 <sup>ef</sup> ± 0.04	−16.53 <sup>def</sup> ± 0.09	294.40 <sup>b</sup> ± 0.71	291.30 <sup>b</sup> ± 0.28	292.10 <sup>b</sup> ± 0.10
<i>Method B</i>						
XG 0.25%	−12.63 <sup>ab</sup> ± 1.93	−14.23 <sup>abcd</sup> ± 1.19	−14.25 <sup>cde</sup> ± 2.31	240.00 <sup>a</sup> ± 0.57	240.65 <sup>a</sup> ± 0.07	240.45 <sup>a</sup> ± 0.07
XG 0.5%	−12.97 <sup>a</sup> ± 0.95	−13.07 <sup>a</sup> ± 0.44	−14.21 <sup>abc</sup> ± 0.58	237.80 <sup>a</sup> ± 1.71	237.97 <sup>a</sup> ± 1.62	237.77 <sup>a</sup> ± 2.03

Results presented as average out of two measurements.

Mean values followed by the same letters are not significantly different ( $P > 0.05$ ).

deeper knowledge on the overall crystallization phenomena encountered should be achieved.

#### 4. Conclusions

Olive oil micron-sized emulsions ( $D_{50} = 1.3 \mu\text{m}$ ) or sub-micron ( $D_{50} = 0.615 \mu\text{m}$ ) were formulated using whey protein, three kinds of hydrocolloids and two different emulsification energy inputs. Stable emulsions were only formed at 0.5 wt% stabilizer content. High energy input during ultrasonic emulsification resulted in emulsions of smaller droplet size and decreased viscosity. Nevertheless, these emulsions were more stable against coalescence even though the viscosity of the continuous phase was greatly affected by sonication. Galactomannans' solutions viscosity was greatly influenced by sonication treatment intensity comparing to XG ( $\text{XG} \gg \text{LBG} \geq \text{GG}$ ), influencing both creaming and coalescence.

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