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Effectiveness of encapsulating biopolymers to produce sub-micron emulsions by high energy emulsification techniques

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Abstract

In this study, different emulsifying ingredients were used to produce sub-micron emulsions for encapsulation purposes. Maltodextrin combined with a surface-active biopolymer (modified starch, or whey protein concentrate), or a small molecule surfactant (Tween 20) were used as the continuous phase, while d-limonene was the dispersed phase. Results showed that biopolymers are not efficient ingredients to produce very small emulsion droplets compared with small molecule surfactants because of their slow adsorption kinetics. The main problem with surfactants also is instability of the resulted emulsions due to "depletion and bridging flocculation" caused by free biopolymers and competition between surfactant and surface-active biopolymers. In general, it was not possible to produce a fairly stable microfluidized emulsion with surfactants for encapsulation purposes.

Keywords: Re-coalescence; Tween 20; Emulsification; Microfluidizer; Droplet size distribution; Flocculation

1. Introduction

Emulsification is one of the important and critical steps in microencapsulation of food oils and flavours through spray drying and emulsion properties such as stability and droplet size[1] play a key role in optimizing the encapsulation efficiency during the process (Barbosa, Borsarelli, & Mercadante, 2005; Danviriyakul, McClements, Decker, Nawar, & Chinachoti, 2002; Liu, Furuta, Yoshii, & Linko, 2000, 2001; Risch & Reineccius, 1988). A stable emulsion with minimum droplet size can increase the retention of volatiles and shelf-life of encapsulated oil products through reduction of unencapsulated oil at the surface of powder particles (Ishido, Hakamata, Minemoto, Adachi, & Matsuno, 2002; Minemoto, Hakamata, Adachi, & Matsuno, 2002; Soottitantawat, Yoshii, Furuta, Ohkawara, & Linko, 2003, 2005). So, sub-micron emulsions can be of real benefit for encapsulation purposes.

The production and control of submicron emulsions with a narrow size distribution have been attracting considerable attention in recent years. Nano-(submicron) emulsions are kinetically stable systems that can be transparent (EDS <200 nm) or "milky" (EDS ≈ 500 nm) (Izquierdo et al., 2002; Tadros, Izquierdo, Esquena, & Solans, 2004), and because of their very small EDS and high kinetic stability, they have been applied in various industrial fields, for example, personal care and cosmetics, health care, pharmaceuticals, and agrochemicals (Forgiarini, Esquena, Gonzalez, & Solans, 2001; Schulz & Daniels, 2000; Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005; Sole, Maestro, Gonzalez, Solans, & Gutierrez, 2006; Sonneville-Aubrun, Simonnet, & L'Alloret, 2004). Production of nano-emulsions by "High-energy emulsification" methods like Microfluidization involves an application of very high

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[1] In rest of the discussion, instead of using different terms such as droplet diameter, droplet size, emulsion size, etc. which may become confusing, emulsion droplet size or simply EDS will be used.

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amounts of energy (e.g., high pressures) on a previously prepared coarse emulsion to produce very small emulsion droplets. Some workers believe Microfluidization is superior because, EDS distributions appeared to be narrower and smaller in Microfluidized emulsions than in the traditional emulsifying devices (Dalgleish, Tosh, & West, 1996; Pinnamaneni, Das, & Das, 2003; Robin, Blanchot, Vuillemard, & Paquin, 1992; Strawbridge, Ray, Hallett, Tosh, & Dalgleish, 1995). It is shown, however, that Microfluidization is unfavourable in specific circumstances such as higher pressures and longer emulsification times, as it leads to "over-processing", which is re-coalescence of emulsion droplets (Jafari, He, & Bhandari, 2006a, in press; Lobo & Svereika, 2003; Olson, White, & Richter, 2004).

Final EDS is the result of equilibrium between droplet break-up and re-coalescence. Between new droplet formation and its subsequent encounter with surrounding droplets, emulsifiers adsorb onto the created interface to prevent re-coalescence. If the timescale of emulsifier absorption is longer than the timescale of collision, the fresh interface will not be completely covered and will lead to re-coalescence, i.e., an EDS increase (Desrumaux & Marcand, 2002; Kolb, Viardot, Wagner, & Ulrich, 2001; Marie, Perrier-Cornet, & Gervais, 2002; Perrier-Cornet, Marie, & Gervais, 2005). Fast stabilization of new interfaces by sufficient emulsifier molecules is an efficient way to prevent re-coalescence (Brosel & Schubert, 1999; Floury, Desrumaux, Axelos, & Legrand, 2003; Karbstein & Schubert, 1995; Schulz & Daniels, 2000; Stang, Karbstein, & Schubert, 1994; Stang, Schuchmann, & Schubert, 2001). An effective emulsifier should adsorb rapidly at the fresh interface created during emulsification, reduce interfacial tension appreciably to facilitate droplet disruption, and prevent new droplets from flocculation by providing a protective layer around them.

There are many different emulsifiers available to incorporate into emulsions; some of them are solely emulsifier such as Spans and Tweens (Floury, Legrand, & Desrumaux, 2004; Marie et al., 2002) and some have both emulsifying and stabilising properties such as milk proteins and modified starches (Mohan & Narsimhan, 1997; Tesch, Gerhards, & Schubert, 2002). Slow emulsifiers, like biopolymers and high molecular weight surfactants can only be used effectively in emulsification systems with high residence times, such as colloid-mills, or multistage high pressure systems because, they get the chance to stabilize newly broken up droplets more than once. Small-molecule emulsifiers such as Tween 20 stabilize new interfaces in milliseconds, so that the droplets are unlikely to re-coalesce. When a mixture of emulsifiers is present, different molecules compete to adsorb at oil-water interface and lower the interfacial tension (Arboleya & Wilde, 2005; Dickinson, 2003; Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004; McClements, 2004). Since low molecular weight surfactants are much smaller in size than biopolymers, and because they can reduce interfacial tension more efficiently and quickly by adsorbing a large number of molecules within the same surface area, they are likely to dominate at the interface after equilibration, if both are present at high enough bulk concentrations (Kerstens, Murray, & Dickinson, 2006; Mackie, Gunning, Wilde, & Morris, 2000; Pugnaloni, Dickinson, Ettelaie, Mackie, & Wilde, 2004).

By the advent of modern emulsification systems and their potential application in encapsulation of food ingredients, understanding the mechanisms of emulsification and the behaviour of emulsion components along with the knowledge of factors affecting the emulsion properties during emulsification is essential. Also, there has been limited work to produce sub-micron emulsions with small molecule surfactants for encapsulation purposes. In fact, most of the published work in the emulsion territory is dealing with pure emulsions consisting water, oil and emulsifier. While in emulsification for subsequent encapsulation purposes, there is another constituent involved, so-called wall material or encapsulation matrix, which is mainly a biopolymer and has some direct and indirect influences on the emulsion properties. Therefore, the objectives of this work are to determine the optimum emulsification conditions and evaluate the influence of extreme emulsification conditions of Microfluidization on emulsion stability and droplet size by applying different surface-active biopolymers and surfactant.

2. Materials and methods

2.1. Materials

d-Limonene ($\rho = 840$ kg/m^3, $\eta = 8.8$ mPa s at 25 °C, RI = 1.487) was supplied by Quest International (NSW, Australia). Modified starch (Hi-Cap 100, waxy corn starch-modified, 5% moisture, solubility > 90%) and Maltodextrin (DE = 16–20, 5% moisture, bulk density = 600 kg/m^3) were purchased from National Starch and Chemical (Sydney, Australia), and Penford Limited (NSW, Australia), respectively. Whey protein concentrate (73% protein, 9% fat, 4% moisture, 5% lactose, 4% ash) was purchased from New Zealand Milk Products (ALACEN, Auckland, New Zealand). A non-ionic surfactant, i.e., Tween 20 (HLB = 16.7, $\eta = 350$ mPa s at 25 °C, RI = 1.468) was purchased from LabChem (NSW, Australia) and used as an added or a single emulsifying agent in some stages of this work. Analytical grade hydrochloric acid (HCL) and sodium azide (NaN_3) were purchased from Sigma Chemicals Company (Sydney, Australia). Distilled water was used for the preparation of all solutions. All general chemicals used in this study were of analytical grade.

2.2. Coarse emulsion preparation

All emulsions were produced in two stages, as described in our previous study (Jafari, He, & Bhandari, in press): (a) pre-emulsions were obtained with a rotor-stator system (L2R, Silverson Machines Ltd, UK). Silverson is a typical

colloid mill with a stator composed of a metal grating in which, 2 mm holes are drilled. (b) The coarse emulsions were then further emulsified using a microfluidizer. Sodium azide (0.02 wt%) was added to the emulsions as an antimicrobial agent.

2.3. Microfluidization

Previously prepared coarse emulsions were passed through an air-driven Microfluidizer (Model M-110 L, Microfluidics, USA), as described in our previous work (Jafari et al., in press). Pre-emulsion was fed to the system through a 200 mL glass reservoir. The device splits the pre-emulsion feed into two opposing channels in a stainless steel block (a ceramic interaction chamber); these channels narrow to approximately 75 μm in width, and the two jets of pre-emulsion are forced to collide head-on at high pressure, creating extreme shear. Through mechanical amplification of ×232, the typical pressure of the liquid jets flowing through the channels is about 120 MPa when the air pressure at the regulator is 530 kPa. The volume flow rate of the emulsions was measured and it was approximately 4×10^{-6} m^3/s at 60 MPa for one cycle. The experiments were duplicated.

2.4. Emulsion droplet size analysis

Size distribution of the oil droplets were determined by the laser light scattering method using Mastersizer 2000 (Malvern Instruments, Worcestershire, UK), as explained in our previous work (Jafari et al., in press). All measurements were done on two freshly prepared samples and results are reported as averages. The mean droplet diameter was expressed as the Sauter, or volume mean diameter (D32 and D43 respectively, sometimes written as d_{32} and d_{43}):

$$\mathrm{D32} = \sum n_i d_i^3 / \sum n_i d_i^2; \quad \text{Surface area moment (Sauter) mean diameter} \tag{1}$$

$$\mathrm{D43} = \sum n_i d_i^4 / \sum n_i d_i^3; \quad \text{Volume or mass moment (De Brouckere) mean diameter} \tag{2}$$

To determine the distribution width of droplet sizes, 'span' was used calculating from the following formula:

$$\mathrm{Span} = \frac{[\mathrm{d(v,90)} - \mathrm{d(v,10)}]}{\mathrm{d(v,50)}} \tag{3}$$

In this formula, d(v, 10), d(v, 50), and d(v, 90) are diameters at 10%, 50%, and 90% cumulative volume, respectively. In other words, [d(v, 90) – d(v, 10)] is the range of the data and d(v, 50) is the median diameter. The instrument also calculates the specific surface area (m^2/g) of the dispersed droplets that was reported in some of our results. However, these values should be treated cautiously since those calculated by Malvern Mastersizer are still controversial.

Table 1
Experimental parameters and their levels

No.	Parameters studied	Levels
1	Emulsifier type	Hi-Cap; WPC; Tween 20 Combination of Tween 20 and Hi-Cap
2	Emulsifier concentration	Hi-Cap in two levels: 10 and 40 wt%; Tween 20 in four levels: 0.5, 2.5, 5.0, and 10 wt%
3	Emulsion stability	Hi-Cap (10 wt%) emulsions produced by Microfluidizer versus Silverson Tween 20 (10 wt%) emulsions by Microfluidizer

2.5. Stability measurements

The stability of emulsions were evaluated by transferring about 25 g of the emulsions into a 50 mL plastic container (25 mm diameter and 40 mm height), tightly sealed with a plastic cap, and then stored for the required time (from 1 h up to 3 days) at room temperature (≈25 °C). The emulsion stability index (ESI) was defined as a relative ratio of the stored emulsion data (including D32, D43, and specific surface area) to the original emulsion data. It is expected that ESI will increase for droplet size data (i.e., ESI $\geqslant$ 1), while ESI should decrease for specific surface area (i.e., ESI $\leqslant$ 1) during storage, and both imply that the emulsion has been destabilized.

2.6. Experimental parameters and statistical analysis

The studied parameters and their levels are presented in Table 1. All the experiments were performed based on fully factorial designs and the results represent means of two replicates. General linear model of MINITAB (Version 14, 2004) was used to conduct an analysis of variance (ANOVA) to determine differences between treatments means. Treatments means were considered significantly different at $P \leqslant 0.05$ and the difference was considered very significant at $P < 0.01$. Some of the graphs were drawn by Excel (Microsoft Office 2003) and some by MINITAB 14.

3. Results and discussion

3.1. Influence of different surface-active biopolymers

Since our final goal was to prepare sub-micron emulsions for spray drying to produce encapsulated powders, we investigated the behaviour of two different biopolymers having emulsifying properties (Hi-Cap vs. WPC) during Microfluidization. The results (Table 2) showed that the biopolymer type had a very significant effect ($P < 0.01$) on EDS during Microfluidization. The effect of oil content

Table 2
The *P*-values obtained with ANOVA realised on the variables versus factors comparing emulsifying properties of WPC vs. Hi-Cap

Factor/response	D32 (nm)	D43 (nm)	Span	Surface area (m^2/g)
A: blocks (replications)	0.063	0.051	0.900	0.054
B: pressure (MPa)	0.001, VS	0.001, VS	0.017, S	0.001, VS
C: number of cycles	0.054	0.004, VS	0.008, VS	0.001, VS
D: oil content (%)	0.002, VS	0.001, VS	0.003, VS	0.001, VS
E: emulsifying agent (WPC vs. Hi-Cap)	0.001, VS	0.001, VS	0.001, VS	0.001, VS
B:C (interaction)	0.499	0.001, VS	0.001, VS	0.001, VS
B:D	0.012, S	0.898	0.149	0.043, S
B:E	0.001, VS	0.001, VS	0.001, VS	0.001, VS
D:E	0.001, VS	0.001, VS	0.001, VS	0.001, VS
B:D:E	0.857	0.316	0.756	0.018, S

Significance levels: VS = very significant ($P < 0.01$); S = Significant ($P < 0.05$).

and pressure was also very significant ($P < 0.01$). Another interesting result was the significant interaction between pressure and oil content ($P < 0.05$) regarding d_{32}, while for d_{43}, there was not such a significant interaction ($P > 0.05$). Other results are presented in Table 2.

When comparing emulsion size results of WPC with Hi-Cap (Fig. 1), it can be seen that although WPC emulsions had a much smaller d_{32} (e.g., 174 nm at 60 MPa for one cycle), their EDS in terms of d_{43} was higher than Hi-Cap emulsions. This was because of a wide and bimodal distribution of WPC emulsions (bigger spans). In fact, initial emulsions of WPC had two peaks: one peak around 300 nm and the other one around 4 μm that step by step, by increasing the Microfluidization pressure, this latter peak was shifting towards left (small size area) but still the size distribution was bimodal. ''Over-processing'' was also happening for WPC emulsions at higher energy densities. While, Hi-Cap emulsions had a narrower distribution (smaller span) with a single peak that was shifting to left

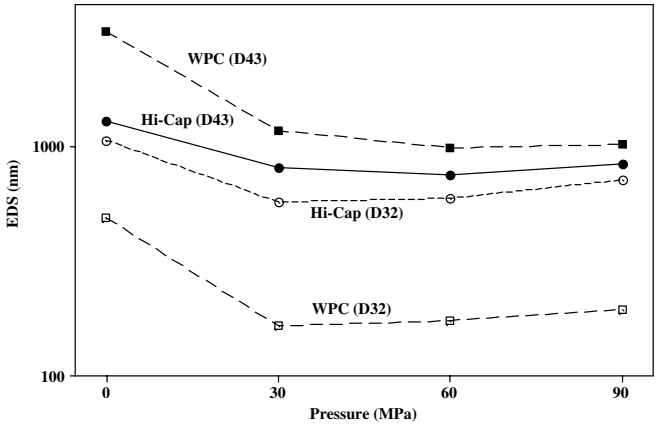

Fig. 1. Comparison of EDS at different Microfluidization pressures (one cycle). WPC: D32 (□) and D43 (■); Hi-Cap: D32 (○) and D43 (●). Emulsions had a continuous phase of 10 wt% Hi-Cap or WPC and 30 wt% Maltodextrin and a dispersed phase of d-limonene (ø = 0.1). Coarse emulsion was made by Silverson.

(smaller emulsion size) by increasing the pressure. This infers that Hi-Cap was behaving better during Microfluidization in terms of EDS and size distribution. It can be due to different emulsifier properties such as adsorption rate, molecular weight, conformational adjustment, excess surface concentration, etc.

Research has shown that adsorption of proteins includes a slow diffusion of the biopolymer from bulk phase to the subsurface region followed by adsorption of polymer segments at the oil-water interface (Arboleya & Wilde, 2005; Pugnaloni et al., 2004): Protein chains are normally highly folded in solution (in the form of aggregates) with hydrophobic amino acids tending to reside in the centre of molecule – away from water – while hydrophilic amino acids tend to be at the surface of molecule. During protein adsorption, it partially unfolds exposing its hydrophobic segments to the oil phase. It is shown that globular proteins such as WPC need more time to adjust their conformation compared with flexible coil proteins. These protein conformational changes are usually referred to as ''surface denaturation'' (Damodaran, 2005; McClements, 2004), and it can promote droplet flocculation (and then, re-coalescence) through increased hydrophobic attraction by new exposed segments and disulfide bond formation between proteins adsorbed onto different droplets. On the other hand, extensive protein–protein interactions at the interface may lead to the formation of an interfacial membrane, which may therefore provide better protection against droplet re-coalescence as it can be seen in our results with smaller d_{32} with WPC than with Hi-Cap. At high pressures, however, these interfacial membranes can be disrupted leading to re-coalescence of emulsion droplets and ''over-processing''.

A similar conformational adjustment has been proposed for methylcellulose derivatives (MC and HPMC), in which, these polymers also start to unfold after adsorption (Arboleya & Wilde, 2005). Although Hi-Cap would follow the same behaviour like other biopolymers, the extent of inter-polymer interactions and new exposed hydrophobic sites will be much smaller than proteins due to their lower surface active groups which are mainly those inserted chains of octenyl succinic acid. Regarding Octenyl-succinate (OSA) starches, which Hi-Cap is one of them, Tesch et al. (2002) proved that kinetics of their dynamic interfacial tension is similar to those of whey proteins and for both of them is rather slow comparing to Tweens. They suggested that whey proteins can be substituted by OSA starches (exactly comparable to our results) with a specific advantage at low pH values since, proteins stabilize emulsion droplets mainly by electrostatic repulsive forces, which strongly depend on pH: at pH values near the iso-electric point, there is a poor emulsification by whey proteins. While for OSA starches, steric hindrance is the main stabilizing mechanism independent of pH.

The other question here can be what is the influence of high Microfluidization pressures on these biopolymers? There is a lot of controversy in the literature regarding this issue. The effects of hydrostatic high-pressure on proteins

are primarily related to the rupture of non-covalent interactions leading to disruption of the quaternary and tertiary structure of globular proteins, with relatively little influence on their secondary structure (Bouaouina, Desrumaux, Loisel, & Legrand, 2006). Dynamic high-pressure emulsification, where high pressures are experienced over very short times (e.g. about 10^{-4} s in the Microfluidizer), are different because force-induced phenomena of cavitation, shear, turbulence and temperature rise are involved simultaneously (Paquin, 1999). Desrumaux and Marcand (2002) by a differential scanning calorimetry (DSC) analysis showed that during ultra high-pressure emulsification, the conformation of whey proteins was changed (denaturation happened) that probably affected their emulsifying properties, but their molecular weight was not changed significantly (confirmed by electrophoresis). They found an optimum pressure of about 100 MPa in which, d_{32} and Span reached a minimum. By working on Methylcellulose as emulsifier, Floury et al. (2003) found that emulsification at high pressures (>150 MPa) could lead to degradation of long chain molecules and formation of polymers with significantly smaller molecular weight. They concluded overall, a pressure less than 150 MPa was optimum to produce real sub-micron emulsions without any "over-processing" in agreement with the results of Schulz and Daniels (2000) who found an optimum pressure of 90 MPa. Unfortunately in our experiments we could not increase the Microfluidization pressure to higher than 100 MPa because of the instrument limitations to confirm or reject these results. Generally, size distribution of WPC emulsions was becoming narrower by pressure up to 100 MPa but, there was some "over-processing".

On the other hand, some authors claimed high Microfluidizing pressures can facilitate interface adsorption of proteins by modifying their 3D-structures (a better unfolding) and resulting in smaller EDS. For example, Perrier-Cornet et al. (2005) proved that at pressures above 200 MPa, adsorption rate of whey proteins significantly increased (60%) corresponding to a very narrow EDS of sunflower oil. More recently, Bouaouina et al. (2006) showed dynamic high-pressure treatment did not affect the conformation of whey proteins but enhanced their stabilizing properties because of increased exposure of their hydrophobic sites. The efficiency of different biopolymers during dynamic high-pressure emulsification (e.g., Microfluidization) is a new area of research and more work needs to be done to fully understand their behaviour within these conditions.

3.2. Influence of the emulsifier concentration and adsorption rate

Replacing all the Maltodextrin with Hi-Cap through increase in proportions of Hi-Cap:Maltodextrin content (from 10:30 to 40:0%) helped to reduce EDS significantly ($P < 0.05$) in terms of d_{32} comparing with lower content of Hi-Cap (10%) as shown in Table 3. For example, emulsions produced at 40 MPa for one cycle had a d_{32} equal to 573 and 268 nm, for lower and higher emulsifier contents, respectively. This is possibly due to covering more interfacial area, higher rate of surface coverage and lower rate of droplet collisions because of the increase in emulsifier concentration and continuous phase viscosity. All these reasons will lead to a lower re-coalescence and consequently, smaller EDS.

Narsimhan and Goel (2001), Lobo and Svereika (2003), and Tcholakova, Denkov, and Danner (2004) have studied the effects of emulsifier type and concentration on EDS in a systematic way. They explained two regimes for emulsifier concentration: (a) emulsifier-rich regime in which, EDS does not depend on emulsifier concentration and is determined by interfacial tension and the energy density. (b) Emulsifier-poor regime in which, EDS strongly depends on the initial emulsifier concentration. By examining three different emulsifiers including WPC, SDS and Brij 58 (polyoxyethylene-20 cetyl ether), Tcholakova et al. (2004) found transition between the emulsifier-rich and poor regimes occurring at similar emulsifier concentrations, that was $C_S \approx 0.1$ wt%. Recently, Surh, Ward, and McClements (2006) found this transition occurs at about 0.9 wt% WPC. Determination of transient emulsifier concentration in extreme emulsification (e.g., Microfluidization) and with complex emulsions containing a high concentration of biopolymers could be different because of "over-processing" and re-coalescence.

Since the interface area is increasing dramatically by decreasing EDS (Fig. 2), particularly when the energy input is high such as Microfluidization, there should be enough emulsifier to adsorb at the fresh interface after successful disruption of big droplets and completely cover them. For example, surface area of the initial d-limonene droplets in Hi-Cap (10%) emulsions before Microfluidization was about 6 m^2/g that was then increased by 100% to approximately 12 m^2/g after emulsification at 20 MPa (one cycle). If emulsifier concentration is the limiting factor (emulsifier-poor regime), increase in energy density during Microfluidization will not lead to EDS reduction such as what happens in high pressures with emulsions containing 10% Hi-Cap (Table 3). Another reason for "over-processing" can be the slow adsorption rate of emulsifiers used in our study. Since residence time in the interaction chamber of Microfluidizer is in the range of milliseconds, the emulsifier should have a high adsorption rate (shorter than the residence time) to cover the fresh interface as quick as possible and protect them against re-coalescence before new droplets leave the emulsification zone (interaction chamber). Biopolymers such as Hi-Cap and WPC have a low adsorption rate mainly due to their high molecular weights and complex structures, and cannot quickly cover the newly formed droplets within the milliseconds, then, leading to EDS increase particularly, in situations with higher coalescence frequency (e.g., higher energy densities). So, the occurrence of "over-processing" in emulsions with lower contents of Hi-Cap (10%) at higher pressures and increased

Table 3
Microfluidized emulsions and role of the emulsifier

Pressure (MPa)	Emulsion data	Hi-Cap: Maltodextrin (10: 30%)	Hi-Cap: Maltodextrin (40: 0%)	Hi-Cap: Maltodextrin (10: 30%) + Tween 20 (10%)	Maltodextrin (40%) + Tween 20 (10%)
0	d_{32} (nm)	984[a]	958[a]	605[b]	260[c]
	d_{43} (nm)	1341[a]	5491[b]	1574[c]	474[d]
	Span	0.78	15.18	3.85	2.51
	Area (m^2/g)	5.97	19.40	11.50	27.85
20	d_{32} (nm)	575[a]	358[b]	252[c]	150[d]
	d_{43} (nm)	825[a]	3491[b]	455[c]	226[d]
	Span	1.25	13.12	1.98	1.80
	Area (m^2/g)	12.30	26.90	27.55	48.20
40	d_{32} (nm)	573[a]	268[b]	381[c]	154[d]
	d_{43} (nm)	761[a]	2675[b]	572[c]	227[d]
	Span	1.16	8.31	1.53	1.82
	Area (m^2/g)	12.05	43.55	18.20	46.90
60	d_{32} (nm)	569[a]	160[b]	381[c]	142[b]
	d_{43} (nm)	757[a]	2193[b]	505[c]	211[d]
	Span	1.17	3.78	1.44	1.83
	Area (m^2/g)	12.40	46.72	18.20	50.91
80	d_{32} (nm)	618[a]	148[b]	346[c]	146[b]
	d_{43} (nm)	776[a]	2135[b]	429[c]	215[d]
	Span	1.17	3.31	1.56	1.87
	Area (m^2/g)	11.25	48.10	19.95	50.02
100	d_{32} (nm)	632[a]	165[b]	380[c]	148[b]
	d_{43} (nm)	785[a]	1929[b]	477[c]	229[d]
	Span	1.18	4.98	1.31	1.94
	Area (m^2/g)	12.15	46.1	18.25	49.10

Pre-emulsions made by Silverson were Microfluidized at various pressures (one cycle). The dispersed phase was d-limonene ($\phi = 0.1$). Means within the same row, followed by different letters (a, b) are significantly different ($P \leqslant 0.05$).

number of Microfluidization cycles could be explained by the slow adsorption rate of emulsifier, higher droplet collisions (coalescence) and shorter residence time inside the interaction chamber at high energy densities, and deficiency in the emulsifier concentration to completely cover the new droplets.

The emulsions at higher contents of Hi-Cap, therefore, could be expected to have smaller EDS as there are more emulsifier molecules and the chance of droplet stabilization is increasing, as confirmed by d_{32} results for higher Hi-Cap contents (40%). These emulsions, however, were bimodal in size distribution with higher d_{43} values than lower Hi-Cap concentrations (Table 3 and Fig. 3). Theoretically, d_{43} value is more sensitive to the presence of larger droplets than d_{32} value, and therefore, it could give a good indication of droplet re-coalescence. This might be related to lower adsorption rate of the Hi-Cap that would have a few consequences; some new droplets could not be covered within that very short time during droplet deformation and disruption in the interaction chamber of Microfluidizer or some of the fresh interface is covered incompletely that makes them very sensitive to coalesce. At the same time, rate (frequency) of droplet collisions would be very high; particularly at higher energy densities (pressures), because of more energy input, high volume flow rate and shorter residence times, that all lead to more re-coalescence and bigger EDS, creating a bimodal distribution.

Also, if sufficiently large stress is applied parallel to an interface that is already covered with emulsifier during pre-emulsion preparation, then some of the emulsifier molecules may be dragged along the interface, leaving some regions where there is an excess emulsifier and other regions where there is a depletion of emulsifier. The latter regions would be very sensitive to coalescence if droplets come close together. This process is only likely to be important if the adsorption rate of the emulsifier is relatively slow compared to duration of the applied stresses and collision frequency, such as Hi-Cap and this mechanism of instability has been approved for protein-stabilized emulsions (Damodaran, 2005; McClements, 2004). Another possible reason could be emulsifier tearing from the interface when there is a severe emulsification and this one has also been confirmed for proteins. The interesting result for higher Hi-Cap contents (40%) was that ''over-processing'' during Microfluidization was limited compared with lower concentrations of Hi-Cap (10%), since there was a gradual decrease in both d_{32} and d_{43} values and also, the dispersion index or Span (Table 3). This could be another support for reduction of re-coalescence at higher content of the emulsifier, as more new droplets will be stabilized and their

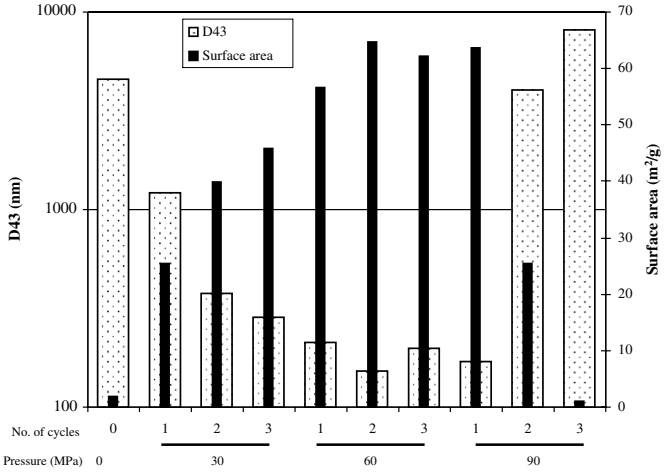

Fig. 2. Effect of Microfluidization pressures and number of cycles on specific surface area and d_{43} of the fish oil (10%) droplets in an emulsion consisting Hi-Cap (10%) and Maltodextrin (30%).

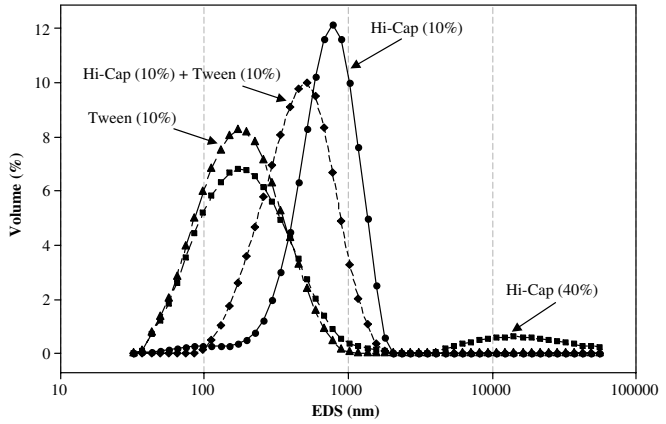

Fig. 3. Role of the emulsifier during Microfluidization (60 MPa, one cycle). The dispersed phase was d-limonene ($\varnothing = 0.1$), and the continuous phase is given in Table 3.

encounter will not necessarily result in coalescence, and thereby increase in EDS.

3.3. Influence of the combination of emulsifier and surfactant

There are two broad classes of emulsifying agents available to apply in food emulsions: small molecule surfactants (e.g., Tween series), and macromolecular emulsifiers, so-called biopolymers (e.g., Hi-Cap and WPC). In microencapsulation area, only the second group of emulsifiers (biopolymers) have been used extensively because of being able to form a "wall" around the "core" material which remains stable during spray drying and can protect the enclosed ingredient for a relatively long time. Limited application of surfactants in microencapsulation area has been reported and information in the literature is scant. In this part of our project, in order to investigate the influence of concentration and adsorption rate of surface-active agent on EDS during Microfluidization, we did some studies with a fast-adsorbing surfactant (Tween 20) either in combination with the existing emulsifier (Hi-Cap), or without it, to compare the results. Tween 20 (polyoxyethylene sorbitan monolaurate) is a water-soluble non-ionic surfactant with low molecular weight and a hydrophilic-lipophilic balance (HLB) of about 16.7 that is widely used in O/W emulsification (Kerstens et al., 2006; Uniqema, 2006). Hi-Cap, however, is an octenyl-succinate (OSA) starch, so-called modified starch, which is made by esterification of starch and anhydrous octenyl succinic acid under alkaline conditions to add hydrophobic side chains to the originally mere hydrophilic starch molecules (Tesch et al., 2002; Trubiano & Lacourse, 1988). These side groups anchor the molecule to the oil droplet surface, while the hydrophilic starch chains protrude into the aqueous phase and protect droplets against aggregation through steric repulsion.

It was found that by adding Tween 20 to the previous emulsions, it significantly ($P < 0.05$) affected EDS results. For example, d_{32} and d_{43} of emulsions made with Tween 20 (0.5%) after Microfluidization at 20 MPa for one cycle were about 265 and 563 nm, respectively. While in the same conditions without adding Tween 20, d_{32} and d_{43} were approximately 570 and 850 nm, respectively. This dramatic decrease in EDS could be possibly because of fast-adsorbing behaviour of Tween 20 that is crucial in high-energy emulsification since droplet deformation and disruption, emulsifier adsorption and droplet collisions, all take place in a very short time. It has been shown that low molecular weight surfactants such as Tween 20 contain a hydrophilic head group that arranges toward aqueous phase, and one or several hydrophobic tails that tend to go into the oil phase of the emulsions (Dickinson, 2003; Klinkesorn et al., 2004). They are very mobile and can rapidly cover the new oil-water interface during emulsification. On the other hand, high molecular weight emulsifiers such as Hi-Cap and WPC contain a mixture of hydrophobic and hydrophilic groups that makes them very slow at diffusing and adsorbing onto the fresh interface compared with small surfactants (Arboleya & Wilde, 2005; Pugnaloni et al., 2004). The other interesting result of adding Tween 20 (0.5% w/w) was its effect on the size distribution. At lower energy densities, there was a single peak log-normal distribution and by pressure rise, it became bimodal with two peaks appearing around 100 nm (representing d_{32}) and 800 nm (representing d_{43}), respectively. Although d_{32} decreased continuously by pressure from 265 nm at 20 MPa to 197 nm at 60 MPa, but neither the pressure nor the number of cycles had a significant effect on d_{32}. At the same time, d_{43} was minimal at the lowest energy densities (562 nm at 20 MPa for one cycle) and was increased gradually by pressure to about 715 nm at 60 MPa. This could be another evidence of "over-processing" because of increased coalescence frequency and probability at higher energy density.

Since our ultimate goal was to produce nano-emulsions with the lowest possible droplet size, three more levels of Tween 20 (2.5, 5.0, and 10.0% w/w) were used in three

pressures and three different cycles at each pressure in a fully randomised factorial design. Results for 10% Tween 20 and one cycle at each pressure are presented in Table 3. By increasing the surfactant concentration up to 2.5%, EDS was decreasing significantly ($P < 0.05$) as shown in Fig. 4 and emulsions at this stage had the smallest size with d_{32} and d_{43} equal to 124 and 183 nm at 60 MPa (one cycle), respectively. This could be explained by higher amounts of the emulsifier and surfactant present in the emulsion to cover the considerably high surface area of the fresh interface created during Microfluidization and also by higher adsorption kinetics of small molecule Tween 20. Emulsions had a fairly narrow distribution (span < 2.0) and "over-processing" was limited. Increasing the surfactant concentration beyond this point (2.5% w/w), however, was not advantageous since there was a significant increase ($P < 0.05$) in EDS, which is opposite to the normal trend. In other words, increasing the surfactant concentration not only did not help to reduce EDS, but also it caused a substantial EDS increase. For instance, when Tween concentration was further increased to 10%, d_{32} and d_{43} were increased to about 381 and 505 nm after 60 MPa Microfluidization (one cycle), respectively. One reason can possibly be the formation of free surfactant micelles in the emulsion bulk, which do not have any emulsifying properties because their hydrophobic chains or hydrophilic heads will be engaged inside these micelles (Becher, 2001; Bibette, Calderon, & Poulin, 1999). On the other hand, these surfactant micelles will trigger flocculation of emulsion droplets by depletion interactions (which will be discussed shortly) and thereby possible coalescence leading to an increase in EDS.

In general, although increasing the Tween 20 content to some extent can affect EDS results favourably but, final emulsions were very unstable since a slow phase separation was observed with these emulsions during a short time after Microfluidization; especially it was happening more quickly at higher Tween concentrations. This problem could arise from the influence of the combination of two

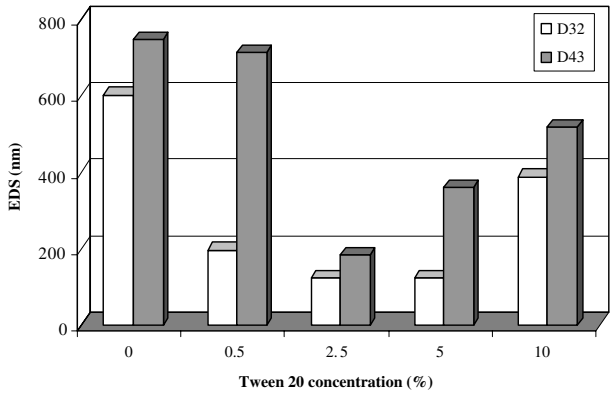

Fig. 4. Influence of surfactant (Tween 20) concentration on EDS during Microfluidization at 60 MPa for one cycle. Emulsion contained Hi-Cap (10%) and Maltodextrin (30%), with d-limonene (10%) as the dispersed phase.

emulsifiers (i.e., Hi-Cap and Tween 20) on emulsion properties and possible interactions and competitions between both existent emulsifiers. Since surfactants are much smaller in size than biopolymers, and because they can reduce the interfacial tension more efficiently and quickly, they are likely to dominate at the interface after equilibration, if both are present at high enough bulk concentrations (Kerstens et al., 2006), such as high levels of Tween 20 and Hi-Cap in our case. Even if biopolymer molecules do partially adsorb at the interface in the very initial stages of the emulsification process, soon after, however, an increasing number of small surfactant molecules adsorb at the interface and displace the biopolymer molecules, eventually remove all of the initial biopolymer film. For instance, Mackie et al. (2000) and Pugnaloni et al. (2004) by atomic force microscopy (AFM) confirmed that β-casein and β-lactoglobulin were totally displaced when Tween 20 was present in the O/W emulsions. Typically, in O/W emulsions, it has been reported that there is a complete displacement of β-lactoglobulin by non-ionic surfactants at higher surfactant/protein ratios (i.e., $\geqslant 15$) (Kerstens et al., 2006).

Surfactant micelles and unadsorbed biopolymers located in the aqueous phase can induce destabilization of emulsions by mechanisms of "depletion flocculation" (Dickinson, 2003; McClements, 2004). This arises when droplets approach within a distance less than the mean diameter of the free biopolymer molecule. The exclusion of the biopolymer from the intervening gap is related to an inter-droplet attractive force, because of the solvent tendency to flow out from the gap under the influence of the local osmotic pressure gradient. At very low concentrations of free biopolymer, the entropy loss linked to droplet aggregation prevails over the depletion effect and the emulsion remains stable. Maybe this can be a possible explanation for better stability of our emulsions made with very low levels of Tween 20 (0.5%), since displacement of Hi-Cap molecules from the interface was limited in this situation. Beyond a critical biopolymer concentration, however, reversible depletion flocculation occurs, resulting in enhanced sedimentation and phase separation (Dickinson, 2003), such as what happened in our emulsions at higher levels of Tween 20 (>0.5%).

Another possible reason can be the formation of some networks between surfactant and biopolymer. These surfactant-biopolymer interactions can occur through a variety of different mechanisms, with the two most important being electrostatic and hydrophobic interactions (McClements, 2005). The binding of surfactant to the biopolymer can lead to large change in stability of the emulsions as in our case; many droplets were flocculated and caused a phase separation. In fact, when two droplets coated with Tween 20 are in close proximity, Hi-Cap molecules make contact with the surfaces of these droplets, creating a bridge between the two droplets. Formation of multiple contacts of this type will promote flocculation and enhance the rate of creaming. This phenomenon is commonly

known as ''bridging flocculation'' and is more likely when the used biopolymer is a weak and slow emulsifier (Damodaran, 2005), such as Hi-Cap. By examination of the resulted emulsions with optical microscopy and also through laser scattering droplet size analysis (data not presented), we found that there was a huge flocculation of droplets without any apparent coalescence as by a simple shaking, a fairly stable emulsion reformed and its EDS was still comparable to initial results.

The important point is that possibly, the process of Microfluidization makes the ''depletion flocculation'' and ''bridging flocculation'' worse, since phase separation is happening very fast when Tween 20 and Hi-Cap are present together. The reason for this could be the very short residence time of the emulsions in the interaction chamber of the device. In these circumstances, molecules of surfactant have more chance to adsorb at the interface than Hi-Cap molecules because of their smaller size, more effective interfacial packing, and the fast droplet disruption occurring during emulsification. Hence, there would be a high concentration of the free Hi-Cap molecules in the form of aggregates at the continuous phase. At the same time, droplet collision frequencies are very high due to intensive energy input and substantial energy densities. It is no wonder why the resulted emulsions break down very quick after Microfluidization. By making some emulsions with sodium caseinate and Tween 20, the same behaviour was observed that suggests combination of protein and surfactant also leads to displacement of biopolymer and considerable flocculation as happened with H-Cap.

3.4. Influence of the surfactant

Finally at this stage, we used Tween 20 (10%) alone without the existence of surface-active biopolymers (Hi-Cap) to eliminate any competition between these two emulsifiers. The result (Table 3) was as expected because the produced emulsions had to some extent, the smallest EDS (d_{32} and d_{43} were 142 and 211 nm, respectively, after Microfluidization at 60 MPa for one cycle) with a fairly narrow distribution (Span = 1.8). More importantly, they were more stable this time, since no phase separation happened after Microfluidization. Our final results showed that these emulsions still are not as stable as emulsions made with Hi-Cap, since EDS data for the Microfluidized emulsions a few hour after production and also results for the reconstituted emulsions prepared from encapsulated powders (data not shown) revealed a significant increase ($P < 0.05$) in EDS. For example, original emulsions after Microfluidization at 60 MPa (one cycle) had a d_{32} and d_{43} of about 0.14 and 0.21 respectively, while the results for the same emulsions after 1 h storage were 200 and 360 nm, and for the reconstituted spray dried powders were approximately 0.75 and 3.83 μm. This can be related to the lower stability of Tween emulsions compared with Hi-Cap ones and the influence of spray drying process including atomization on the emulsion stability.

In fact, biopolymers such as Hi-Cap have both emulsifying and stabilizing properties and in spite of their lower adsorption rates, emulsions made by these emulsifiers are more stable due to better protection of droplets (sometimes with multi-layers of biopolymers and formation of repulsive forces on droplets) and reducing the coalescence frequency by increasing the continuous phase viscosity. While, small surfactants such as Tween 20 have just emulsifying features and their main advantage is fast adsorbing rate. Another possible reason can be the influence of existed biopolymer (maltodextrin). In a study by Klinkesorn et al. (2004), they found corn oil O/W emulsions made with maltodextrin and Tween 80 are unstable because of ''depletion flocculation'' by the non-adsorbed maltodextrin. According to their results, at 15 wt% maltodextrin (critical flocculation concentration), a rapid creaming and complete phase separation occurred. When the maltodextrin concentration increased further to 25 or 35 wt%, creaming rate was decreased appreciably, and they attributed this to the increase in continuous phase viscosity which slows down the movement of droplets. This instability caused by free maltodextrin can be limited in our case, as maltodextrin concentration in the emulsions was high (40%) and we did the spray drying quickly after preparing the emulsions (within 1 h). Also, an EDS analysis by laser scattering immediately before spray drying process, verified the initial EDS results.

3.5. Stability of the emulsions

Stability of the emulsions was evaluated by analysing their EDS data over a period of time. As Hi-Cap stabilized emulsions were very stable after Microfluidization, their stability was evaluated within 3 days after their production. Regarding Tween emulsions, their stability was analysed within 3 h after their formation. Emulsion stability index (ESI) was defined as the ratio of newly measured data (d_{32}, d_{43}, and specific surface area) to the original data. An increase of ESI for droplet size data (ESI > 1.0) and a decrease of ESI for specific surface area (ESI < 1.0) of the droplets show that the emulsion has been destabilized (Fig. 5).

Our results confirmed that emulsions made with Tween 20 were generally less stable than their Hi-Cap counterparts, since their EDS increased significantly ($P < 0.05$) after a few hours storage, and phase separation was slowly happening. This could be mainly because of ''depletion flocculation'' of the free maltodextrin molecules and Tween 20 micelles and then, coalescence of the closely enough flocculated droplets through their interfacial film rupture and formation of bigger droplets to enhance their thermodynamic stability. In fact, coalescence of emulsions stabilized by small molecule surfactants is largely governed by their ability to keep droplets apart, rather than the resistance of the droplet membrane to rupture. Non-ionic surfactants such as Tween 20, do this by having polymeric hydrophilic head group that provide a large steric overlap and

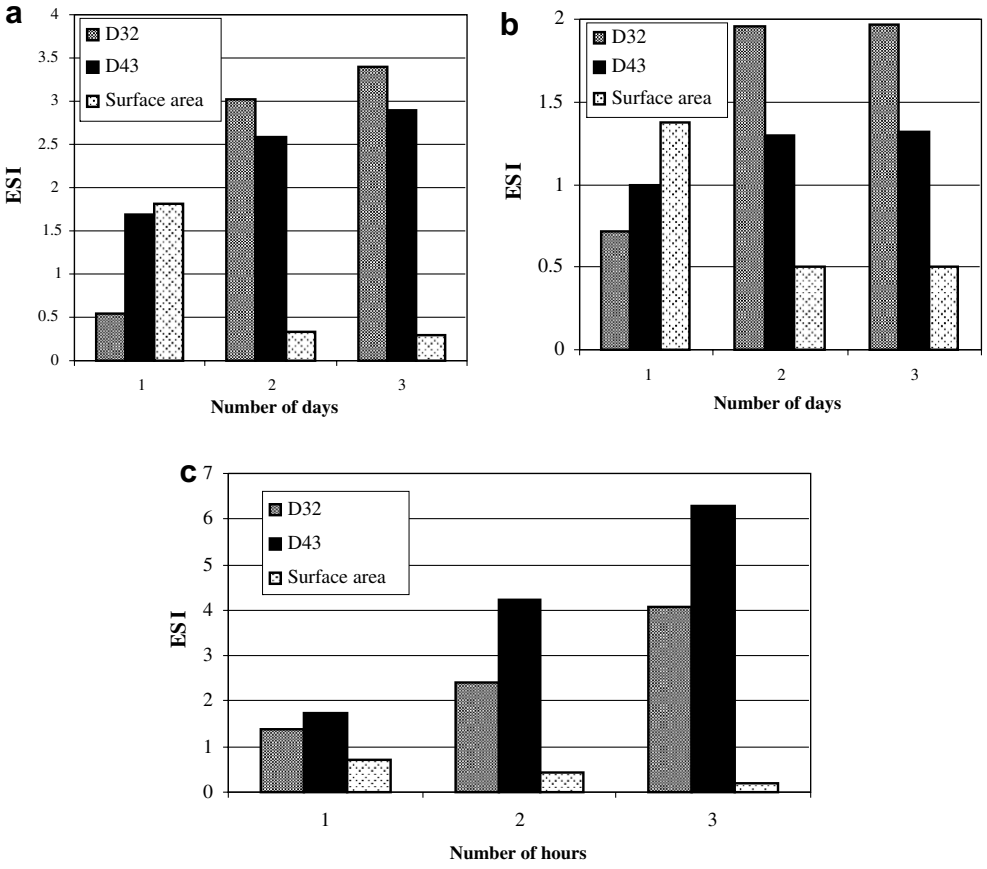

Fig. 5. Stability of Hi-Cap (10%) emulsions made by Microfluidizer at 60 MPa for one cycle (a), or Silverson at the highest speed for 10 min (b) and Tween 20 (10%) emulsions produced with Microfluidizer at 60 MPa for one cycle (c). Dispersed phase was d-limonene (ø = 0.1).

hydration repulsion (McClements, 2005). Considering Hi-Cap emulsions, they were generally more stable, as it can be seen in Fig. 5, mainly because of strong repulsive forces between droplets (due to a combination of electrostatic and steric interactions) provided by Hi-Cap, and formation of membranes which are highly resistant to rupture, and so coalescence. Also, emulsions prepared using OSA starches are more stable compared to fast adsorbing surfactants because of a higher continuous phase viscosity which helps to reduce the re-coalescence of new droplets, in agreement with the results of Tesch et al. (2002).

When comparing stability of emulsions produced by Microfluidizer or Silverson, it was found that they follow the same trend with approximately same EDS results after 3 days storage (Fig. 5a and b): a gradual increase of ESI for droplet size and a sharp decrease of ESI for surface area of droplets that is reasonable. The interesting result was reduction of d_{32} of these emulsions 24 h after their production, as ESI calculated for d_{32} decreased from 1.0 to 0.55 (Microfluidized emulsion) and/or 0.71 (emulsions with Silverson). In fact, emulsions at this stage become bimodal with a bigger span; big droplets become bigger and their number increased while, the number of medium droplets decreased. This can be explained by "Ostwald ripening" that is the instability process by which, larger droplets grow at the expense of smaller ones due to higher solubility of smaller droplets and molecular diffusion through the continuous phase (Capek, 2004). In other words, large droplets become bigger and small droplets become smaller, which is exactly what we observed in our case. This process is different from coalescence since no film rupture is happening between flocculated droplets (Bibette et al., 1999; Damodaran, 2005). It has been shown that Ostwald ripening is higher for O/W emulsions containing oils which are slightly water soluble such as flavour oils (Buffo & Reineccius, 2001) and so, d-limonene that is slightly polar oil. Another reason can be coalescence of droplets during storage since the coalescence rate for bigger droplets is much higher than small droplets, so medium and big droplets become bigger while, small droplets still are not coalesced and their number is increasing. During the following days, however, ESI in general was increased for both EDS data because of more Ostwald ripening and higher coalescence rate.

3.6. Conclusion and further remarks

We cannot expect to decrease emulsion droplet size as long as higher energies are supplied to break down bigger droplets into smaller ones, and as long as there are emulsifier molecules in the emulsion. When emulsions are

prepared for encapsulation purposes by modern emulsification systems, as specific surface area increases dramatically by reducing EDS, there should be enough emulsifier molecules to adsorb onto the fresh interface and stabilize and protect them against re-coalescence. Our results showed that even by increasing the biopolymer content to the maximum, EDS cannot be reduced appreciably because of extreme emulsification conditions during Microfluidization that is creation of very high energy density at a very short time on a small volume of the emulsion. In fact, emulsification is very fast in these systems and such emulsifiers should be used that have higher adsorption rate (smaller molecule size) and their structure and capabilities will not deteriorate during high-energy emulsification. Although the application of biopolymers is inevitable in microencapsulation due to their film and wall matrix forming properties for covering the active ingredients and produce encapsulated powders. Modified-starches such as Hi-Cap have the advantage of being independent from pH and ionic strength of the emulsion compared with proteins which loose their emulsifying abilities in different emulsion environment conditions.

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