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# Preparation of multiple Water-in-Oil-in-Water emulsions without any added oil-soluble surfactant

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## Abstract

We designed double emulsions without any added oil-soluble surfactant. Simple  $W_1/O$  emulsions stabilized solely by fat crystals were first prepared by dispersing the  $W_1$  aqueous phase in a surfactant-less fat phase at a temperature above its melting range, followed by cooling down to trigger bulk fat crystallization. The resulting  $W_1/O$  emulsions were arrested systems that were stable against gravitational and colloidal instabilities upon storage. The primary emulsions were in turn dispersed in a highly viscous external aqueous phase containing proteins to obtain  $W_1/O/W_2$  emulsions. We studied the shear and rheological conditions that determine the average oil globule size. The resulting materials had enhanced properties compared to conventional double emulsions including i) very slow passive delivery of the encapsulated species, ii) resistance to osmotic stress, iii) resistance to coalescence, and iv) thermo-responsiveness as the double globules could release the inner droplets' content upon warming.

**Keywords:** double emulsions, shear, fat crystals, thermo-responsiveness, osmotic stress.

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## 1 Introduction

Multiple water-in-oil-in-water emulsions ( $W_1/O/W_2$ ) are complex liquid dispersions of oil globules containing small aqueous droplets. These systems have a great potential in the food area for the entrapment and controlled release of aroma and flavor, protection of probiotics, taste masking and for lowering the fat content in foods by including aqueous droplets in the oil globules [1]. This type of double emulsion is of special relevance in the dairy sector [2].

Long-term kinetic stability is required for most of the practical applications. The stability issue in double emulsions involves both coalescence and diffusion phenomena, and has been extensively documented [3]. Coalescence may occur between the inner droplets, between oil globules and between the external phase and the inner droplets [4]. The oil globules are permeable to various hydrophilic species that can be transported without film rupturing [5]. Initially, the inner aqueous droplets comprise an encapsulated compound, and the external phase contains an osmotic regulator. Because of the concentration gradients, both solutes tend to be transferred from  $W_1$  to  $W_2$  and *vice versa* across the oil phase. The osmotic regulation process induces a continuous flow of water, varying the inner and outer volumes [5]. Water transport may have profound consequences for both the stability and properties of multiple emulsions. For instance, swelling of the internal droplets increase the globule volume fraction, which may produce significant changes in the rheological properties of the double emulsions [6].

The release of the encapsulated compound is induced by coalescence of the inner droplets on the globule surface and/or by diffusion/permeation across the oil phase [4]. The release kinetics has been shown to depend on various parameters: the type and concentration of surface-active species, the mean diameter and volume fractions of the inner droplets and of the oil globules [4], the oil type [7], and the osmotic pressure mismatch between  $W_1$  and  $W_2$  [6]. Among these parameters, the surfactant type plays a key role. Low-molecular weight surfactants generally do not allow longstanding

encapsulation [4]. In contrast, coalescence can be inhibited and diffusive transport can be slowed down using amphiphilic polymers and proteins [1,7].

One of the main constraints encountered in food related applications is the lack of food-grade emulsifiers able to efficiently stabilize W/O emulsions. The most widely used oil-soluble emulsifier is polyglycerol-polyricinoleate (PGPR) [8]. PGPR is highly effective for stabilizing W/O emulsions made with triglyceride oils [9]. Nevertheless, its use is regulated for many food products [10]. In addition, its presence induces an unpleasant off-taste when incorporated at the level required to ensure long-term stabilization of W/O emulsions (2-10 wt.%). Consequently, the use of PGPR represents one of the main technological drawbacks to the implementation of double emulsions in the food industry. Some recent efforts have been made to reduce the amount of PGPR in multiple emulsions, especially by incorporating hydrocolloids like casein [11], acacia gum [12], or whey proteins [13] in the inner droplets. These species can interact with PGPR at the oil/water interface and increase the yield of encapsulated compounds [1]. Lipophilic lecithin depleted of its major phosphatidylcholine (PC) component was found to favor the stabilization of W/O emulsions [14]. However, the droplets of W/O lecithin-stabilized emulsions formed large aggregates when edible lipids such as sunflower or olive oil were used as continuous phase.

The concept of using fat-based crystalline material for the solid (Pickering) stabilization of the internal W<sub>1</sub> water droplets of double emulsions has been described more recently. Saturated mono- and diglycerides are particularly suitable as these lipophilic surfactants form surface-active fat crystals that can stabilize emulsions by creating a mechanical barrier against droplet coalescence [15]. Fernandez-Martin *et al.* [16] used a mixture of crystallizable mono- and di-glycerides and PGPR to stabilize the W<sub>1</sub>/O emulsion. Frasc-Melnik *et al.* and Spyropoulos *et al.* [17,18] went a step further by completely suppressing PGPR. They formulated W<sub>1</sub>/O emulsions based on sunflower oil, stabilized with monoglyceride and triglyceride crystals added in the oil phase. These primary emulsions were then incorporated into an aqueous phase containing sodium caseinate to obtain W<sub>1</sub>/O/W<sub>2</sub> double

emulsions. After a storage period of several weeks, the emulsions retained their double structure, although partial coalescence between the globules was observed [17].

In this paper, our objective was to formulate food grade multiple emulsions without any added oil-soluble surface-active species (like PGPR or mono- or diglycerides), that are kinetically stable and that can be finely controlled in terms of globule size and internal droplets fraction. Owing to their considerable yield stress and viscoelastic properties over their melting domain, fats may potentially immobilize emulsified aqueous droplets and protect them against coalescence. This property was exploited to produce  $W_1/O$  emulsions. The primary emulsions were in turn dispersed in a highly viscous external aqueous phase containing sodium caseinate to obtain  $W_1/O/W_2$  emulsions. The average globule size was monitored by varying the shear rate under well-defined flowing conditions. The size variation was analyzed within the framework of Taylor's analysis of the elongation and breakup of droplets submitted to shear [19]. The encapsulation yield and the release kinetics from  $W_1$  to  $W_2$  under storage conditions at 4 °C and at 60 °C were probed by using sodium chloride initially dissolved in  $W_1$  as an ionic tracer. The obtained multiple emulsions were also examined for their stability against coalescence and for their ability to withstand osmotic stress conditions, in comparison to emulsions based on liquid oils containing PGPR.

## 2 Experimental Section

### 2.1 Materials

Most of the experiments were carried out with anhydrous milk fat (AMF) provided by Barry Callebaut (Belgium) and used without further purification. AMF is a mixture of triglycerides (>98 wt.%) with a range of melting temperatures from -40 °C to +40 °C. It is composed of approximately 6 % short chain fatty acids (less than 8 carbons), 20 % medium chains and 72 % long chains (more than 14 carbons), including 42 % saturated C16 and C18 chains. Unsaturated chains represented 27 % of the total fatty acids, oleic acid being the most abundant one with 22 % [20]. AMF also contains minor

components like sterols ( $\approx 0.3\text{wt.}\%$ ) as well as surface-active species ( $\approx 1\text{ wt.}\%$ ) like mono and diglycerides, free fatty acids and phospholipids. Other fats from various sources were also probed: cocoa butter from Barry Callebaut (Belgium), palm oil from Itega (France), coconut oil from ADM (France). Sunflower oil (Rustica, France) was purchased from a local supermarket. These fats were chosen because they all are partially crystallized at  $20\text{ }^{\circ}\text{C}$ , allowing the emulsification steps to be carried out at room temperature (see section 2.2). Sodium chloride (NaCl), D-(+)-glucose ( $\text{C}_6\text{H}_{12}\text{O}_6$ ), sodium dodecyl sulfate (SDS) ( $\text{C}_{12}\text{H}_{25}\text{O}_4\text{SNa}$ ), sodium azide ( $\text{NaN}_3$ ), Tween 80, and sodium caseinate (NaCAS) ( $M_w \approx 23\,300\text{ g.mol}^{-1}$ , containing 3 wt.% sodium), were purchased from Sigma-Aldrich (France). Polyglycerol polyricinoleate (PGPR) (esters of polyglycerol and polyricinoleate fatty acids,  $M_w \approx 1800\text{ g.mol}^{-1}$ ) was provided by Palsgraad (France), and Carrageenan Satiagel™ was from Cargill (France). The water used in the experiments was deionized with a resistivity close to  $15\text{ M}\Omega\text{.cm}$  at  $20\text{ }^{\circ}\text{C}$ .

## 2.2 Preparation of $W_1/O/W_2$ emulsions without oil-soluble surfactant

We first provide some basic concepts about the emulsification process. The conditions for droplet breakup can be described within the background developed by Taylor for simple fluids [19]. When an emulsion is submitted to a stress,  $\tau = \eta_c \dot{\gamma}$ , where  $\eta_c$  is the viscosity of the continuous phase at the applied shear rate  $\dot{\gamma}$ , the dispersed droplets undergo an internal flow. The equilibrium shape of the droplets is governed by two dimensionless parameters, the dispersed-to-continuous viscosity ratio,  $p = \eta_d/\eta_c$ , and the capillary number,  $Ca$ , defined as:

$$Ca = \frac{2\tau}{P_L} = \frac{D\eta_c\dot{\gamma}}{2\sigma} \quad (1)$$

where  $\sigma$  is the oil-aqueous phase interfacial tension and  $D$  is the droplet diameter. The capillary number is the ratio of viscous stress to Laplace pressure,  $P_L = 4\sigma/D$ . The viscous stress tends to elongate the droplet into a cylindrical shape, whereas Laplace pressure tends to maintain the spherical shape. The capillary number increases with the applied shear rate and, as a consequence, the droplet

becomes more elongated. Droplet break-up takes place when  $Ca$  exceeds a critical value,  $Ca_c$  [19], that depends on the type (simple shear, elongational, etc.) and history of the shear flow, as well as on the viscosity ratio,  $p$ . The average droplet diameter of the fragmented droplets can thus be written as:

$$D = \frac{2C_{ac}\sigma}{\eta_c\dot{\gamma}} \quad (2)$$

Eq. (2) indicates that a large viscosity of the continuous phase is required to fragment the droplets at relatively low shear rates. In our experiments, this condition was met by exploiting the rheological properties of the AMF oleogel made of fat crystals dispersed in liquid oil during the first emulsification step, and by dissolving a large amount of NaCAS in the  $W_2$  phase during the second emulsification step (see below).

The fabrication of our double emulsions involved the two following steps.

- **First step,  $W_1/O$  emulsion.** The  $W_1$  aqueous phase was prepared by dissolving NaCl at 0.5 mol.L<sup>-1</sup>. This solute was used as a tracer to measure the encapsulation yield, the release kinetics and also as a stabilizing agent to avoid coarsening phenomena [3]. Sodium azide was used as a bactericide agent and was dissolved in  $W_1$  at 0.02 wt.%. AMF was totally melted at  $T = 65^\circ\text{C}$  and the  $W_1$  phase, warmed at the same temperature, was progressively incorporated in the oil phase under manual stirring up to a fraction ranging from 10 to 60 wt.%. In total, 50 g were processed in a 100 mL-beaker. The system was then quenched under intense stirring by soaking the beaker in a large-volume cooling bath (500 mL). Two different quenching conditions were experienced, corresponding to two compositions of the cooling bath: one based on ice water at  $0^\circ\text{C}$  and the other based on a 10 wt.% NaCl brine solution at  $-5^\circ\text{C}$ . Figure S1 (supplementary material) shows the temperature variation of emulsions containing 30 wt.% of aqueous phase plunged into the two different cooling baths. The temperature decreases linearly with time and the cooling rates are  $-9.4^\circ\text{C.min}^{-1}$  in the water bath and  $-18.6^\circ\text{C.min}^{-1}$  in the brine solution. Once the emulsion temperature reached  $20^\circ\text{C}$ , the system was sheared using an Ultra-Turrax® T5 mixer (IKA, Germany) operating at 12000 rpm for 2 min to obtain the final primary water-in-oil ( $W_1/O$ ) emulsion.

- **Second step,  $W_1/O/W_2$  emulsion.** Right after the first step, a coarse multiple  $W_1/O/W_2$  emulsion was prepared by incorporating 30 g of the primary  $W_1/O$  emulsion into 70 g of the external aqueous phase,  $W_2$ , under manual stirring, at 20 °C. The external aqueous phase was composed of 12 wt.% NaCAS, 0.02 wt.% sodium azide and of 0.8 mol.L<sup>-1</sup> D-glucose to match the osmotic pressure of the inner aqueous phase,  $W_1$ , and consequently to avoid water transfer phenomena. The concentration of glucose was selected using tabulated values from the Handbook of Chemistry and Physics [21]. The osmotic contribution of NaCAS was neglected due to its high molecular mass and the reduced amount of sodium ions (3 wt.%).

Quasi-monodisperse multiple emulsions were finally obtained by shearing the pre-emulsions within a narrow gap in a Couette cell (TSR, France; concentric cylinders' configuration) at 20 °C. The inner cylinder of radius  $r = 20$  mm was rotated at a selected angular velocity,  $\Omega$ , which could reach up to 78.5 rad.s<sup>-1</sup>. The outer cylinder was immobile, and the gap between the stator and the rotor,  $e$ , was fixed at 200  $\mu$ m. For the maximum angular velocity, shear rates,  $\dot{\gamma} = r\Omega/e$ , as high as 7850 s<sup>-1</sup> could be attained in simple shear flow conditions. The final emulsions were stored at 4 °C.

## 2.3 Preparation of $W_1/O/W_2$ emulsions containing PGPR

A double emulsion using PGPR as the oil-soluble surfactant was fabricated for comparative studies. In a first step, a 1:1 (wt./wt.) mixture of the  $W_1$  aqueous phase (0.5 mol.L<sup>-1</sup> NaCl, 0.02 wt.% sodium azide) and of the oil phase (sunflower oil + 3 wt.% PGPR) was submitted to an intense stirring by means of an Ultra-Turrax® T5 mixer operating at 12000 rpm for 2 min, at 20 °C. Then, the primary  $W_1/O$  emulsion was manually dispersed at 30 wt.% in an aqueous phase containing 12 wt.% NaCAS, 0.02 wt.% sodium azide and 0.8 mol.L<sup>-1</sup> D-glucose, and sheared in the Couette's cell at 5250 s<sup>-1</sup>.

## 2.4 Emulsions characterization

The systems were observed using an optical microscope Olympus BX51 (Olympus, Germany) equipped with an oil immersion objective (X 100/1.3, Olympus, Germany) and a digital color camera



(Olympus U-CMAD3, Germany). When necessary, a cross-polarized configuration of the microscope was adopted to visualize AMF crystals.

The droplet size distribution of the primary  $W_1/O$  emulsion was measured using a Mastersizer 2000 Hydro SM from Malvern Instruments S.A (Malvern, UK). Measurements were performed directly after emulsification. Static light scattering data were transformed into size distribution using Mie theory (Kerker, 1969), by adopting a refractive index of 1.47 for the oil phase and of 1.34 for the  $W_1$  aqueous phase. The  $W_1/O$  emulsion (1 mL) was first diluted in sunflower oil (10 mL) containing PGPR at 4 wt.%, at room temperature. A small volume of the diluted  $W_1/O$  emulsion was then introduced under stirring in the dispersion unit containing sunflower oil. Because of the very high dilution factor, AMF crystals were fully dissolved and the light scattering signal was only due to  $W_1$  droplets stabilized by PGPR.

To measure the average size of the oil globules, 1 mL of  $W_1/O/W_2$  emulsion was diluted in 10 mL of a SDS solution at  $8 \times 10^{-3} \text{ mol.L}^{-1}$  corresponding to its critical micellar concentration (CMC). This surfactant has the ability to dissociate protein aggregates that bridge emulsion droplets [22]. A small volume of sample was then introduced under stirring in the dispersion unit containing a solution of Tween 80 (nonionic surfactant) at  $1.2 \times 10^{-5} \text{ mol.L}^{-1}$  (CMC) to avoid foaming and droplet deposition on the optics, and  $0.8 \text{ mol.L}^{-1}$  D-glucose to match the osmotic pressure of  $W_1$  droplets within the globules. In this case, since the globules were not optically homogeneous due to the presence of the inner water droplets and fat crystals, the size distributions were obtained using Fraunhofer's model [23]. This model is valid when the particle sizes are large when compared with the wavelength of the incident beam. The obtained results were systematically checked using optical microscopy.

The emulsions were characterized by their volume-averaged diameter  $D[4;3]$  and polydispersity index,  $U$ , defined as:

$$D = D[4,3] = \frac{\sum N_i D_i^4}{\sum N_i D_i^3} \text{ and } U = \frac{1}{\bar{D}} \frac{\sum N_i D_i^3 (\bar{D} - D_i)}{\sum N_i D_i^3} \quad (3)$$

where  $N_i$  is the total number of droplets with diameter  $D_i$ . The median diameter,  $\bar{D}$ , is the value for which the cumulative undersized volume fraction is equal to 50 %.

#### 2.4.1 Viscosity measurements

The viscosity of  $W_1/O$  emulsions and of the external aqueous phase  $W_2$  were measured separately using an AR G2 controlled stress rheometer (TA Instruments, Delaware, USA). A cone-plate geometry of 60 mm diameter was adopted, with a cone angle equal to  $2^\circ$ , and a gap of 56  $\mu\text{m}$ . Samples were submitted to a ramped increase in shear stress ranging from 100 to 5000  $\text{s}^{-1}$  at 20  $^\circ\text{C}$ .

#### 2.4.2 Conductivity measurements

The ability of multiple emulsions to retain the solute (NaCl) encapsulated in the internal aqueous phase,  $W_1$ , right after the second emulsification step at 20  $^\circ\text{C}$  as well as under storage conditions at 4  $^\circ\text{C}$  and at 60  $^\circ\text{C}$ , was evaluated by measuring the amount of solute released in the external phase,  $W_2$ . For that purpose, conductivity measurements were performed at room temperature using a Consort C931 conductimeter (Consort bvba, Belgium). A small amount of the double-emulsions was collected and submitted to a 2-fold dilution using deionized water. The samples were then centrifuged at 1500 g (g being the earth gravity constant) for 5 min with a Rotanta 460 RF centrifuge (Hettich Lab technology, Germany) to separate the globules from the external aqueous phase. We checked that the applied centrifugation did not lead to coalescence phenomena that could produce further release of salt. For that purpose, the cream obtained by centrifugation was redispersed and observed under the microscope. Both the internal droplet size and the droplet concentration within the globules remained apparently unchanged. Conductivity measurements were performed on the supernatant phase devoid of globules, and salt concentration was deduced from a calibration curve obtained from solutions containing 6 wt.% NaCAS, 0.01 wt.% sodium azide and NaCl at different concentrations (see Figure S2, supplementary material).

## 2.5 Statistical analysis

All the above-mentioned analyses (light scattering, conductivity, viscosity) were performed in triplicate and values reported in the plots are expressed as means  $\pm$  mean deviations.

## 3 Results and Discussions

### 3.1 Primary W<sub>1</sub>/O emulsion

Primary W<sub>1</sub>/O emulsions containing 30 wt.% of internal aqueous droplets were prepared without any added oil-soluble surfactant in the oil phase. The emulsions were obtained by heating AMF at 65 °C, above its melting range, incorporating the aqueous phase and cooling the mixture to 20 °C under intense stirring to form an oleogel. This latter comprises fat crystals that stick together under the effect of van der Waals forces and intermolecular hydrogen bonds [24]. The equilibrium solid fat content of AMF at 20 °C is around 20 % [25]. At this temperature, the viscosity of the AMF oleogel was high enough to facilitate emulsification but low enough for easy handling.

Two quenching rates were implemented, as indicated in the experimental section (see also Figure S1, supplementary material). Figure 1 shows microscope images of the obtained emulsions and the corresponding size distributions of the water droplets. The emulsion resulting from the slowest quench (using ice water at 0 °C) had an average diameter,  $D = 3.74 \pm 0.15 \mu\text{m}$ , larger than that resulting for the fastest quench (using a cooling bath at -5 °C),  $D = 2.15 \pm 0.12 \mu\text{m}$ . The difference in the droplet average diameter emphasizes the impact of the cooling rate on the emulsification process. At high cooling rates, the nucleation rate increases leading to the formation of smaller crystals [26]. It is generally observed in emulsions stabilized by solid particles that the smaller the particles, the smaller the droplets, all else being equal [27].

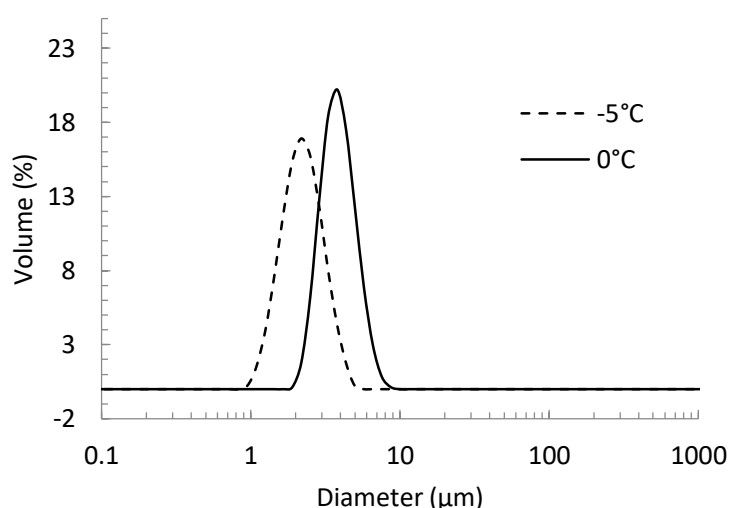


Figure 1. Size distributions and micrographs of primary  $W_1/O$  emulsions. The emulsions contained 30 wt.% of a  $0.5 \text{ mol.L}^{-1}$  NaCl aqueous phase dispersed in AMF and were processed in a cooling bath (a) at  $T = -5^\circ\text{C}$ , and (b) at  $T = 0^\circ\text{C}$ .

It has been demonstrated that small size crystals stabilize emulsions much more efficiently than large ones because of their higher surface coverage capacity [28]. It can also be argued that the concentration of crystals is generally much higher than required to cover the interface and crystals in excess form the oleogel network [24]. Emulsification thus takes place in the presence of a solid reservoir, which forms a network of aggregated crystals in which droplets are entrapped. It can be hypothesized that, once the agitation is stopped, the tiny partially uncovered droplets recombine until they reach a diameter comparable to the size of the mesh that confines them. Within this scheme, smaller crystals obtained at faster cooling rates would result in a finer mesh and consequently in smaller droplets, as observed experimentally.

This why, thereafter,  $W_1/O$  emulsions were formed using the fastest quench obtained with the brine solution at  $-5\text{ }^{\circ}\text{C}$ . The emulsions were kinetically stable when stored at  $20\text{ }^{\circ}\text{C}$  or at  $4\text{ }^{\circ}\text{C}$  since the size distributions did not vary over 4 weeks of storage. Instead, macroscopic separation into the two immiscible phases took place within a few hours when the primary  $W_1/O$  emulsions were warmed at  $50\text{ }^{\circ}\text{C}$ , a temperature such that the oil phase is fully molten. This observation reveals that fat crystals play a major role in the stabilization of the emulsions.

The  $W_1/O$  emulsions were observed under crossed-polarized light microscopy immediately after their fabrication. Figure S3 (supplementary material) reveals small bright spots reflecting the formation of tiny crystals (micron-sized or smaller). Maltese crosses whose size is comparable to that of the water droplets are also discernable. Such structures are generally observed in spherulite-like crystals or in droplets undergoing interfacial crystallization [29]. Fat crystals can be present in the continuous phase and at the oil/water interface. The stabilization of oil-continuous emulsions with fat crystals is thus often described as a combination of Pickering stabilization due to surface-active crystals adsorbed at the interface, and network stabilization due to physical trapping of droplets in the crystal network [30].

## 3.2 Multiple $W_1/O/W_2$ emulsions

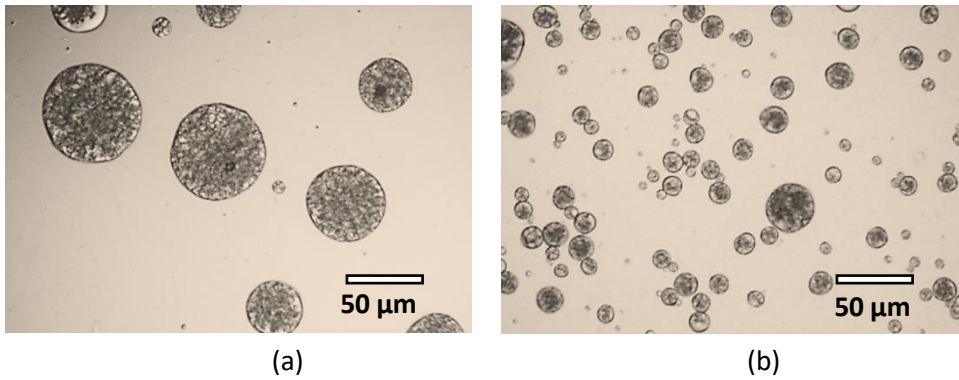
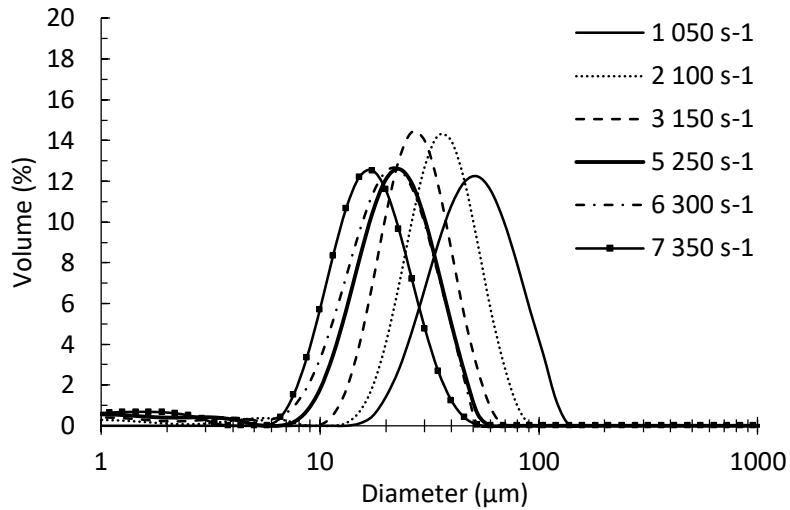
### 3.2.1 Influence of shear rate on globules size

The obtained  $W_1/O$  emulsions were viscous pastes prone to harden over time. The freshly obtained emulsions were relatively fluid but, after a few hours at  $20\text{ }^{\circ}\text{C}$ , they exhibited considerable yield stress and firmness. As crystallization proceeds, solid bridges are formed between aggregated crystals, which further strengthens the 3D-fat network [31]. Polymorphic transitions leading to denser and stiffer crystals may also be at the origin of such rheological evolution [32].

The second emulsification step was always carried out less than 30 min after the fabrication of the  $W_1/O$  emulsion to avoid any significant polymorphic and structural evolution of the fat crystals. In this way, the viscosity of the primary emulsion remained low enough to process the material under

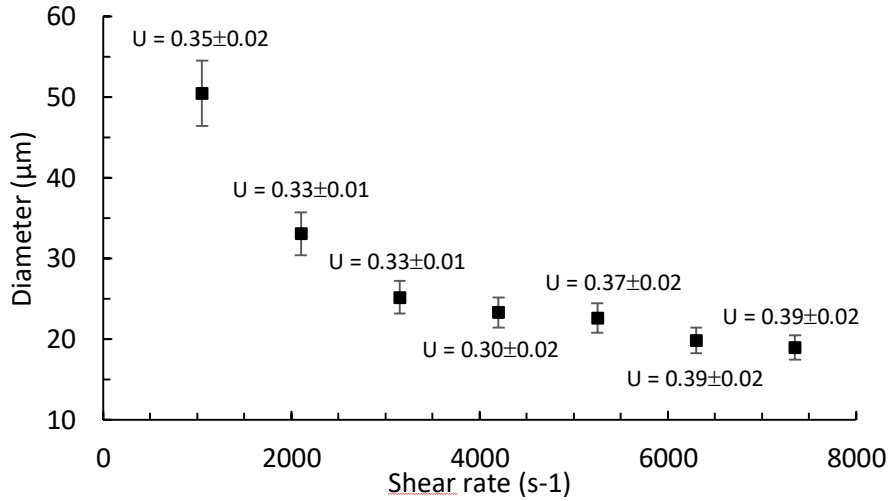
laminar flow conditions. Some preliminary trials, were carried out by submitting the mixture of the two phases ( $W_1/O$  emulsion and  $W_2$  aqueous phase) to vigorous agitation by means of an Ultra-Turrax® T5 mixer, operating at 12000 rpm for 2 min. Multiple emulsions were obtained but the turbulent mixing resulted in a significant release of the inner droplets. The obtained emulsions were polydisperse and a significant fraction of the globules did not contain inner aqueous droplets. From these preliminary experiments, we concluded that mild conditions in terms of agitation were required during the second emulsification step to preserve the multiple structure. We thus adopted an alternative method based on the application of a shear under laminar flow conditions in a highly viscous aqueous phase.

Multiple globules were obtained following the second step described in section 2.2. The applied shear in the Couette's cell was varied from 1050 to 7350  $s^{-1}$ . Figure 2 shows the evolution of the size distributions of the multiple globules obtained from a  $W_1/O$  emulsion initially containing 30 wt.% of aqueous droplets: as expected, increasing the applied shear rate resulted in a reduction of the globules size. In the same figure, we also report microscope images of the emulsions obtained at the lowest and highest shear rates (1050  $s^{-1}$  and 7350  $s^{-1}$ ). Large oil globules uniform in size are visible, and the smaller  $W_1$  water droplets are also distinguishable.



**Figure 2.** Evolution of the size distribution of double emulsions with the applied shear rate. Size distributions, micrographs of the emulsions obtained at the two limiting shear rates (a)  $1050 \text{ s}^{-1}$  (b)  $7350 \text{ s}^{-1}$ . The double emulsions were initially composed of 30 wt.% of  $W_1/O$  globules containing 30 wt.% of  $W_1$  droplets and were stabilized by NaCAS at 12 wt.% in the external aqueous phase,  $W_2$ . The emulsions were diluted with an isotonic D-glucose solution to facilitate the observation.

The average globule diameter,  $D$ , was plotted as a function of the shear rate,  $\dot{\gamma}$ , on Figure 3. The polydispersity index,  $U$ , is indicated nearby each experimental point. All emulsions exhibited narrow size distributions, as reflected by the relatively low value of  $U$  ( $< 0.4$ ). The applied fragmentation method not only successfully produces compartmented globules with a relatively narrow size distribution, but also allows tuning the globules diameter by varying the applied shear rate.



**Figure 3.** Evolution of the volume-average diameter and polydispersity of double emulsions with the applied shear rate. The double emulsions were initially composed of 30 wt.% of  $W_1/O$  globules containing 30 wt.% of  $W_1$  droplets and were stabilized by NaCAS at 12 wt.% in the external aqueous phase,  $W_2$ .

In simple emulsions, it has been observed that quasi-monodisperse droplet break-up occurs preferentially when the viscosity ratio,  $p$ , lies between 0.1 and 1 [33]. Goubault *et al.* [34] obtained monodisperse double emulsions comprising liquid oils within the same viscosity range. For  $Ca > Ca_c$ , the droplets exposed to shear are stretched into elongated threads that undergo a capillary instability and break into a chain of many droplets. If  $0.1 < p < 1$ , the radius of the elongated threads before rupturing is independent of the initial droplet size and this is the reason for the monodispersity of the final emulsion. The viscosities of  $W_1/O$  emulsions and of the  $W_2$  continuous phase were then measured to estimate parameter  $p$ . Each sample was submitted to a shear rate ramp from 100 to 5000  $s^{-1}$ , at 20 °C. Unfortunately, measurements were not reproducible for emulsions whose internal water fraction,  $\phi_i^0$ , was equal to or higher than 40-45 wt.%. Wall slipping occurred due to coalescence phenomena of the aqueous droplets on the solid surfaces of the rheometer, ultimately leading to the formation of an aqueous layer. This phenomenon was not observed for emulsions with lower droplet fractions, allowing a reliable determination of their viscosities. Also, it must be emphasized that the shear rates applied in the Couette's cell, was as high as 7350  $s^{-1}$ , beyond the accessible range of our rheometer ( $< 5000 s^{-1}$ ). **Figure S4** (supplementary material) shows the flow curves of the  $W_1/O$

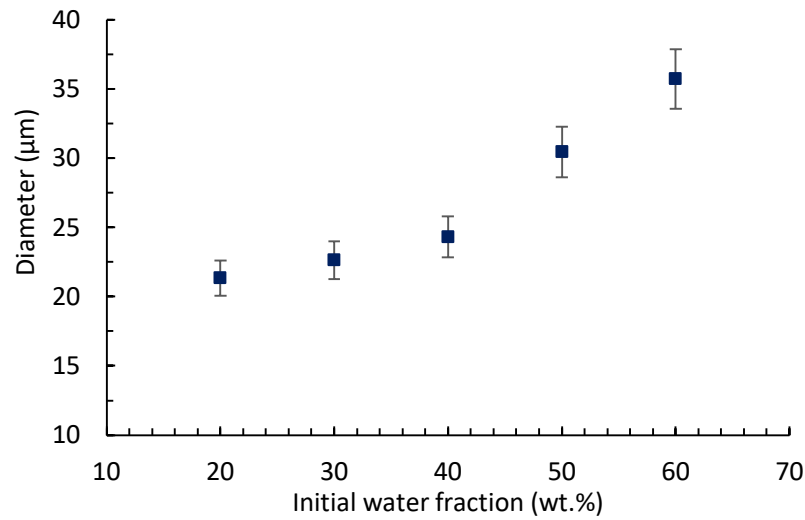


emulsions at two droplet fractions (10 and 30 wt.%) and of the external aqueous phase,  $W_2$ . All systems exhibit shear-thinning behavior. For the two emulsions under study,  $p$  lies between 0.3 and 1.2 for  $1000 \text{ s}^{-1} < \dot{\gamma} < 5000 \text{ s}^{-1}$ , suggesting that the right conditions are met for monodisperse fragmentation.

From Eq. (2), it is possible to derive an estimated value of the critical capillary number,  $Ca_c$ . This parameter characterizes the ability of liquid droplets to undergo fragmentation under well-defined flow conditions. For a given viscosity ratio,  $p$ , the higher  $Ca_c$ , the higher the shear stress to be applied in order to induce droplet break-up. By adopting the following values:  $D = 22.5 \text{ }\mu\text{m}$ ,  $\dot{\gamma} = 5250 \text{ s}^{-1}$ ,  $\sigma = 4 \text{ mN.m}^{-1}$  (measurement performed using the rising drop method ("Tracker" apparatus from Teclis Instruments (France) at  $45 \text{ }^\circ\text{C}$ ),  $\eta_c = 0.2 \text{ Pa.s}$  (at the applied shear rate  $\dot{\gamma}$ ), we obtain  $Ca_c \approx 3$ . For  $p = 1$ , Mabilite *et al.* [33] found  $Ca_c \approx 0.2$  for the fragmentation of simple emulsion droplets in conditions where the shear was applied suddenly (non quasi-static conditions), like in our experiments. In double emulsions, the hydrodynamic flow pattern within the oil globules is complex because of the presence of internal aqueous droplets and fat crystals. These latter exert a supplementary resistance to globule deformation and/or burst. This could explain the relatively large capillary number required to produce globule fragmentation in our case.

### 3.2.2 Influence of inner droplet fraction on globules size

The internal water fraction was varied from 20 to 60 wt.%. At large droplets fractions ( $\phi_i^0 > 50 \text{ wt.}\%$ ),  $W_1/O$  emulsions were prone to phase inversion. To prevent this instability, the  $W_1$  phase was always added progressively in AMF, within a time scale of 1 min. Only by incorporating the aqueous phase slowly could the proper consistency be achieved to facilitate further incorporation. The average droplet size was close to  $2\text{-}3 \text{ }\mu\text{m}$  in all cases, but the polydispersity tended to increase with  $\phi_i^0$  (see Figure S5, supplementary material). Once fabricated, the primary  $W_1/O$  emulsion was in turn emulsified at 30 wt.% in the external aqueous phase,  $W_2$ . The shear rate in the Couette's cell was fixed at  $5250 \text{ s}^{-1}$ .



359 **Figure 4.** Evolution of the average globule diameter with the initial inner droplet fraction. The double emulsions  
 360 were initially composed of 30 wt.% of  $W_1/O$  globules and were stabilized by NaCAS at 12 wt.% in the external  
 361 aqueous phase,  $W_2$ . The applied shear rate was equal to  $5250 \text{ s}^{-1}$ .

362

363 **Figure 4** presents the variation of the average globule size as a function of  $\phi_i^0$ . It can be observed  
 364 that the globule size increases with the droplet mass fraction. Our results about the impact of the  
 365 inner droplet fraction are in qualitative accordance with those obtained by Goubault *et al.* [34] in their  
 366 work concerning the fabrication of quasi-monodisperse  $W_1/O/W_2$  emulsions based on liquid oils. This  
 367 trend can be rationalized considering that the viscosity ratio,  $p$ , increases with the droplet fraction.  
 368 Indeed, in their seminal study about droplet fragmentation in simple emulsions, Mason & Bibette [35]  
 369 observed that the droplet diameter increased with  $p$ . In our case, at  $5000 \text{ s}^{-1}$ , from the data of **Figure**  
 370 **S4** (supplementary material), we deduce that  $p$  increases from 0.5 to 1.1 as the droplet fraction varies  
 371 from 10 to 30 wt.%.

### 372 3.2.3 Encapsulation yield

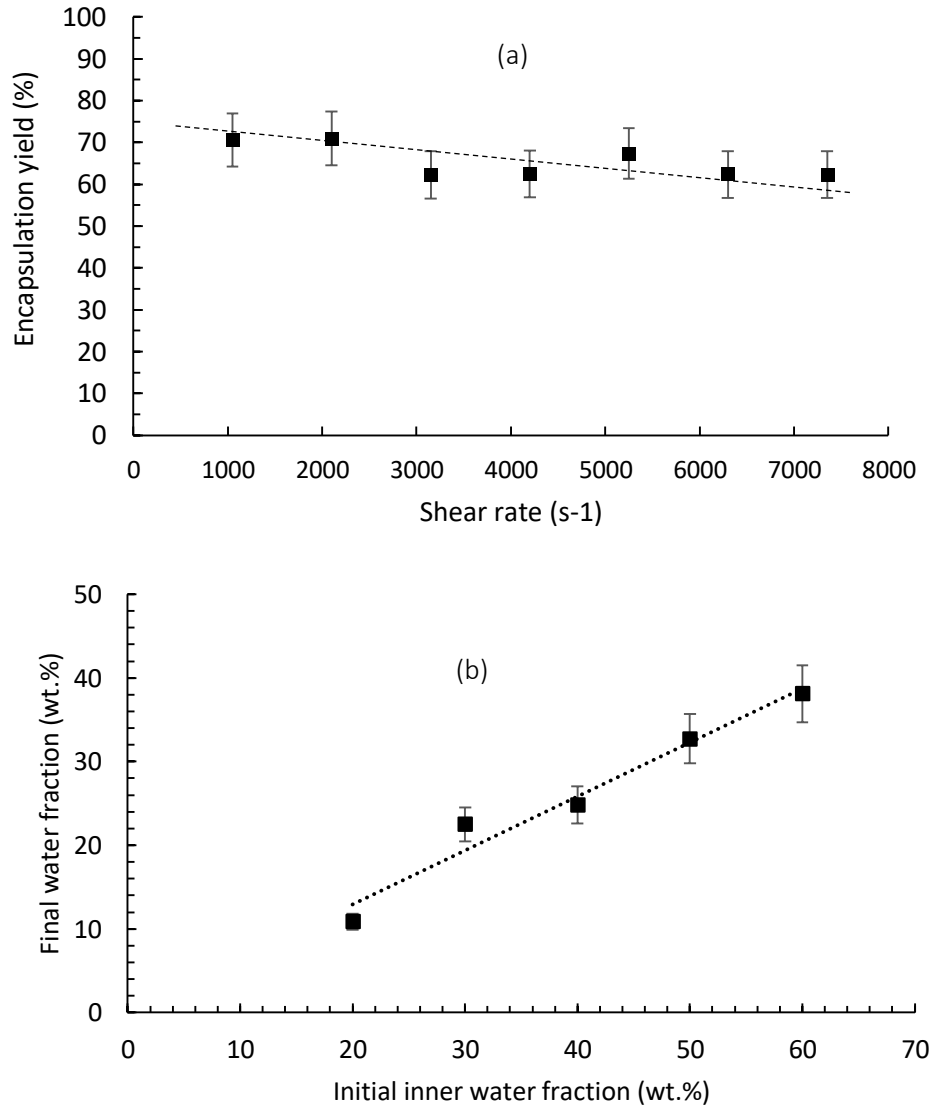
373 The encapsulation yield is defined as the fraction of NaCl that remains encapsulated in  $W_1$ . To  
 374 measure the encapsulation yield, a quantification of the salt (NaCl) released in the external  $W_2$  phase  
 375 was performed directly after emulsification in the Couette's cell at variable shear rates from 1050 to

7350 s<sup>-1</sup>. Conductivity measurements were carried out on the external aqueous phase of each emulsion following the method described in section 2.4.2. It will be demonstrated in section 3.3.2 that there was no salt diffusion across the oil phase for at least one month. The encapsulation yield, EY, was thus calculated assuming that the delivery was due to coalescence phenomena only and the volume of water released was considered in the calculation:

$$EY = 100 \times \left[ 1 - \frac{V_2^0 C_{2,NaCl}}{V_1^0 (C_{1,NaCl}^0 - C_{2,NaCl})} \right] \quad (4)$$

In Eq. (4),  $V_1^0$  and  $V_2^0$  are the volumes of  $W_1$  and  $W_2$  phases, respectively, initially introduced in the formulation,  $C_{2,NaCl}$  is the concentration of the salt released in  $W_2$ , deduced from conductivity, and  $C_{1,NaCl}^0$  (=0.5 mol.L<sup>-1</sup>) is the salt concentration in  $W_1$  droplets. The encapsulation yield can also be defined as the fraction of  $W_1$  aqueous droplets remaining in the globules right after the second emulsification step. The emulsions initially comprised 30 wt.% of globules, with  $\phi_i^0 = 30$  wt.%. Figure 5a shows that the encapsulation yield is only weakly varying (between 70% and 62%) with the applied shear rate.

We also measured the evolution of the final internal aqueous fraction, deduced from the encapsulation yield (see above), as a function of the initial one, at constant shear rate of 5250 s<sup>-1</sup> for a globule fraction equal to 30 wt.%. Figure 5b reveals that the final water fraction is roughly proportional to the initial one. The linearity enables a fine tuning of the final inner droplet content, as required in many practical applications, with an average encapsulation yield close to 65 % (derived from the slope of the straight line) for this shear rate.



**Figure 5.** (a) Evolution of the encapsulation yield with the applied shear rate. The double emulsions were initially composed of 30 wt.% of  $W_1/O$  globules containing 30 wt.% of  $W_1$  droplets and were stabilized by NaCAS at 12 wt.% in the external aqueous phase,  $W_2$ . The dotted line is a guide for the eyes. (b) Evolution of the final inner droplet fraction as a function of the initial one. The applied shear rate was equal to 5250 s<sup>-1</sup>.

### 3.3 Stability assessment

#### 3.3.1 Thermal responsiveness

The obtained double emulsions were thermally-sensitive. They could turn from  $W_1/O/W_2$  emulsions to simple  $W/O$  ones upon warming, within a short period of time. When heated above 40

°C, the oil phase was fully molten and this resulted in the fast release of the inner droplets, as revealed by conductivity measurements, using NaCl as a delivery probe. Full release was achieved after less than 10 min at 60 °C (See Figure S6, supplementary material). From this observation, we can also conclude that the stability of  $W_1$  droplets in the globules is ensured by fat crystals and that endogenous surface-active species in AMF are poor W/O emulsion stabilizers.

Due to their thermal sensitivity, double emulsions obviously cannot be pasteurized after fabrication in food applications. In this case, it is recommended to pasteurize  $W_1$  and  $W_2$  phases separately and to fabricate the double emulsions using sanitized blenders.

### 3.3.2 Encapsulation ability

An emulsion with 30 wt.% of globules,  $\phi_l^0 = 30$  wt.%, sheared at  $5250\text{ s}^{-1}$  was stored at 4 °C for almost 1 month to measure the percentage of salt released (=100-encapsulating yield) from the inner droplets to the external aqueous phase under quiescent storage conditions. The results are reported on Figure 6. The initial value ( $t=0$ ) corresponds to the delivery provoked by the emulsification process. Surprisingly, the delivery did not evolve over time, reflecting an outstanding retention ability of this type of double emulsion. Bonnet *et al.* [7] studied the release rate profiles of magnesium from multiple  $W_1/O/W_2$  emulsions based on liquid triglycerides including olein, a liquid fraction of AMF. PGPR and NaCAS were used as oil-soluble and water-soluble emulsifiers, respectively. The amount of magnesium released evolved regularly over time and was due to diffusion and/or permeation phenomena. In osmotically balanced double emulsions based on crystallizable oils, the presence of fat crystals has been found to reduce the release rate of encapsulated markers [31].

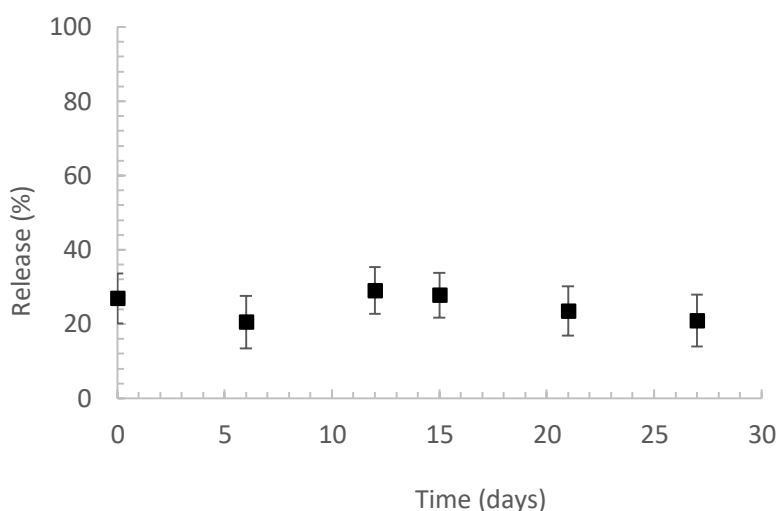


Figure 6. Evolution of the percentage of salt released during storage at 4 °C. The double emulsion was initially composed of 30 wt.% of  $W_1/O$  globules containing 30 wt.% of  $W_1$  droplets and was stabilized by NaCAS at 12 wt.% in the external aqueous phase,  $W_2$ . The applied shear rate was equal to  $5250 \text{ s}^{-1}$ .

In double emulsions, some inner droplets are in direct contact with the globule surface while others are not. Using a capillary technique (single droplet experiments), Wen & Papadopoulos [36] demonstrated that diffusion across thin liquid films is a relatively fast process. Assuming that the transport rate is mainly determined by diffusion/permeation through thin liquid films, droplet-droplet and droplet-globule contacts are obviously facilitating the release from the inner aqueous phase to the external one. Our results obtained with AMF suggest the presence of crystals around the droplets and on the globule surface, avoiding the formation of thin liquid films and thus preventing both coalescence and diffusion of NaCl ions towards the external aqueous phase. Also, the absence of oil-soluble surfactant is likely to impede any process involving reverse micellar transport [37].

### 3.3.3 Resistance to partial coalescence

It is well known that the incorporation of an oil-soluble surfactant in O/W emulsions based on crystallizable oils may induce partial coalescence. This instability is initiated in presence of fat crystals. Upon cooling, the spherical shape of the warm dispersed droplets which is controlled by surface tension evolves into a rough and rippled surface due to the formation of irregularly shaped/oriented

crystals. When fat crystals are formed nearby the interface, they can protrude into the continuous phase and pierce the film between adjacent droplets, forming an irreversible link. This phenomenon is termed as partial coalescence since the shape relaxation process is frustrated by the intrinsic rigidity of the partially solidified droplets [38]. In the case of emulsions stabilized with proteins, the strong interactions between them induce interfacial stiffness likely to impede partial coalescence. However, when a low molecular weight surfactant is added, its adsorption weakens the interactions between proteins and the gain in interfacial fluidity allows protruding crystals to pierce the interfacial films [38]. Partial coalescence is also encountered in double emulsions since an oil-soluble surfactant is usually added to stabilize  $W_1/O$  emulsions. For instance, Perez *et al.* [39] have studied partial coalescence in double  $W_1/O/W_2$  emulsions prepared with skimmed milk, PGPR as oil-soluble emulsifier and different crystallizable oils. They provided evidence that the presence of PGPR in the dispersed lipid phase promoted partial coalescence of the fat globules. This phenomenon was interpreted as being due to protein displacement by PGPR at the globule interface and/or by the increase of capture efficiency due to the modification of fat crystals by PGPR. Frasc-Melnik *et al.* [17] also observed partial coalescence between double emulsion globules containing fat crystals, which included saturated monoglycerides.

In our case, the high viscosity of  $W_2$  phase precluded creaming of the oil globules. Dilution was thus performed to concentrate the globules under the effect of creaming and to see whether they were prone to partial coalescence. The targeted composition was the following: 10 wt.% globules;  $\phi_l^0 = 30$  wt.%; 0.5 mol.L<sup>-1</sup> NaCl in  $W_1$ ; 0.02 wt.% sodium azide in  $W_1$  and  $W_2$ ; 4 wt.% NaCAS, and 0.8 mol.L<sup>-1</sup> D-glucose in  $W_2$ . The emulsions were first submitted to a 3-fold dilution under iso-osmotic conditions, using an aqueous solution containing only D-glucose at 0.8 mol.L<sup>-1</sup>. The stability at 4 °C was followed during 21 days by droplet sizing measurements and microscope observations. Because the viscosity of the continuous phase ( $W_2$ ) was considerably reduced, the globules formed a dense cream after a few hours of settling. However, the cream was readily redispersable upon manual shaking, with no apparent sign of partial coalescence. Such instability did not occur in our systems devoid of PGPR.

Figure 7 reveals that both the average globule size and the inner droplet fraction remained almost constant over the explored time interval.

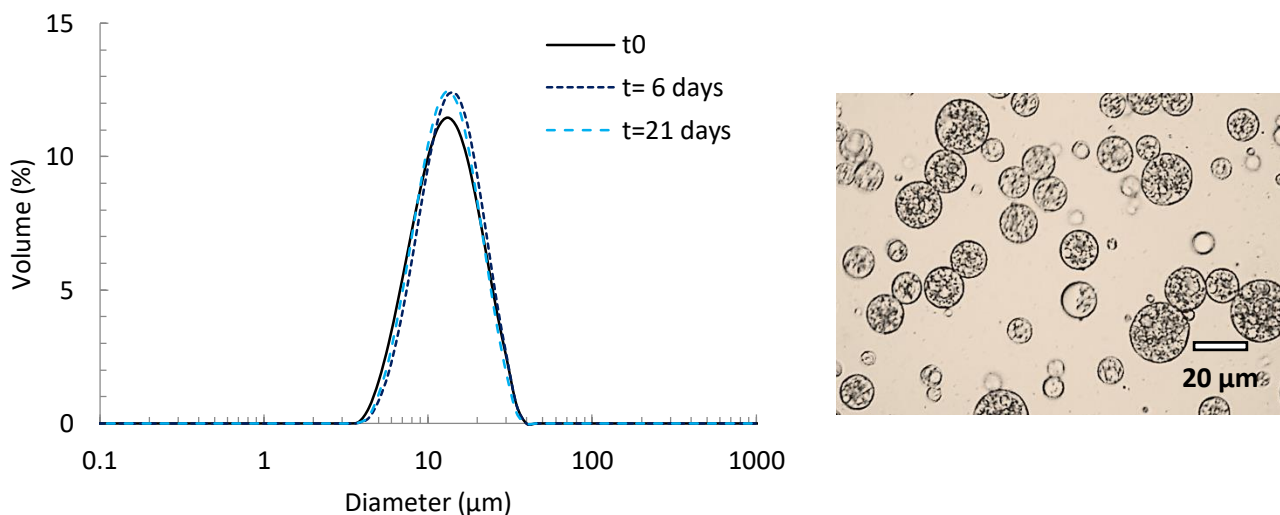


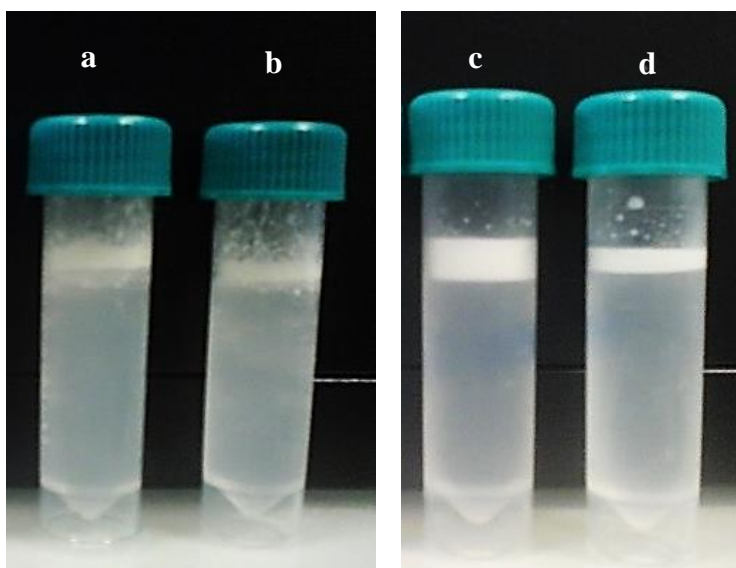
Figure 7. Evolution of the size distribution of a double emulsion upon prolonged storage ( $t_0$ , 6 days, 21 days). The double emulsion was initially composed of 30 wt.% of  $W_1/O$  globules containing 30 wt.% of  $W_1$  droplets and was stabilized by NaCAS at 12 wt.% in the external aqueous phase,  $W_2$ . The emulsion was submitted to a 3-fold dilution in an aqueous phase containing  $0.8 \text{ mol.L}^{-1}$  D-Glucose and was stored at  $4^\circ\text{C}$ . The micrograph was taken after 21 days of a storage.

### 3.4 Resistance to an osmotic shock - Swelling process

We compared the resistance to osmotic stress of two double emulsions, one based on AMF without added oil-soluble surfactant (emulsion 1), and the other one based on liquid oil (sunflower) and PGPR (emulsion 2). Initially, both emulsions had similar structural features. The droplet fraction in the primary  $W_1/O$  emulsion was  $\phi_i^0 = 50 \text{ wt.}\%$  for both systems. The average diameters of the aqueous droplets were close to  $2\text{--}3 \text{ }\mu\text{m}$  and the size of the oil globules was around  $25\text{--}30 \text{ }\mu\text{m}$ . Immediately after fabrication, the emulsions were submitted to a 10-fold dilution (by wt.) with either pure water or an iso-osmotic solution. Due to the large osmotic pressure mismatch, an osmotic swelling phenomenon was expected to occur for the emulsions diluted with pure water, consisting in the transfer of water from the external  $W_2$  phase into the inner  $W_1$  droplets through the oil phase [6].



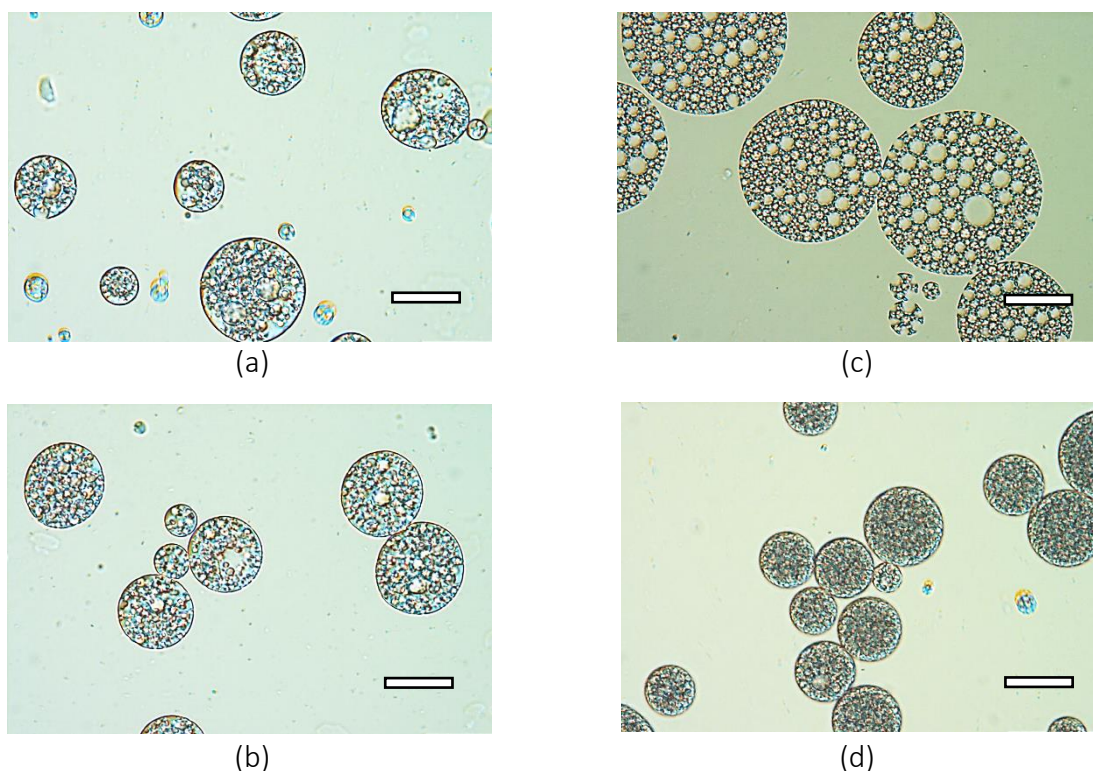
The macroscopic aspect of the double emulsions after 2 hours of settling at 4 °C is shown in **Figure 8**. The globules form a cream layer sitting at the top of the tubes. A thin cream layer is formed for emulsion 1, irrespective of the diluting osmotic conditions (Tubes a,b), reflecting the absence of water transfer. The height of the cream layer in emulsion 2 is much larger for the system diluted with pure water (Tube c), due to the swelling phenomenon. Under iso-osmotic conditions, the thickness of the cream layer (Tube d) is comparable to that of emulsion 1. The diluted emulsions were observed under the microscope after 2h of storage. **Figure 9** reveals the absence of swelling for emulsion 1 whatever the conditions (**Figure 9a,b**), and for emulsion 2 diluted under iso-osmotic conditions (**Figure 9d**). Conversely, the globules of emulsion 2 diluted with pure water (**Figure 9c**) are noticeably larger than the initial ones and contain large tiny packed inner droplets, evidencing water transfer.



**Figure 8.** Comparative behavior of various emulsions submitted to a strong dilution. (a) Emulsion based on AMF, without lipophilic surfactant, diluted with pure water; (b) Emulsion based on AMF, without lipophilic surfactant, diluted with an iso-osmotic solution; (c) Emulsion based on sunflower oil and PGPR, diluted with pure water; (d) Emulsion based on sunflower oil and PGPR, diluted with an iso-osmotic solution. A 10-fold dilution was performed in each case and the resulting emulsions were observed after 2h-storage at 4 °C.

It has been demonstrated that osmotic equilibration occurs at a relatively fast rate, within a few hours, in systems containing liquid triglycerides [6]. From the images of **Figure 8** and **Figure 9**, it is obvious that the swelling process is fast for emulsions containing liquid oil and PGPR (**Figure 9c**) and is

almost undiscernible in presence of fat crystals (Figure 9a). The same observation has been made by Nadin *et al.* [30] in fat crystal-stabilized water-in-oil emulsions designed as a controlled release matrix for the delivery of salt towards an external aqueous phase. It was demonstrated that PGPR-based emulsions were sensitive to the presence of a salt concentration gradient, whereas fat crystal-stabilized emulsions showed little response. A different result was obtained by Frasch-Melnik *et al.* [17]: when an osmotic pressure gradient was applied to their double emulsions stabilized by fat crystals, the internal droplets tended to swell. We would like to stress that the amount of saturated monoglyceride and tripalmitin introduced in sunflower oil (c.a. 5 wt.%) was much lower than the AMF solid fat content in our experiments, which was around 50 % at 4 °C [25]. In our case, it can again be argued that fat crystals around  $W_1$  droplets form a thick and continuous solid layer hindering the formation of thin liquid films for the diffusive transport of water. This property makes double emulsions based on crystallized oil remarkably resistant to osmotic shocks. For emulsion 1, swelling was not visible even after 7 days of storage at 4 °C.

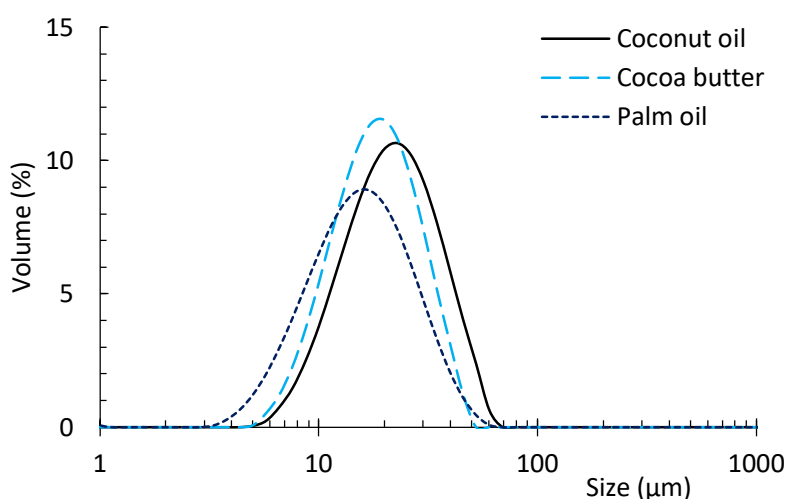


**Figure 9.** Micrographs of multiple emulsions submitted to dilution. (a) Emulsion based on AMF, without oil-soluble surfactant, diluted with pure water; (b) Emulsion based on AMF, without oil-soluble surfactant, diluted with an

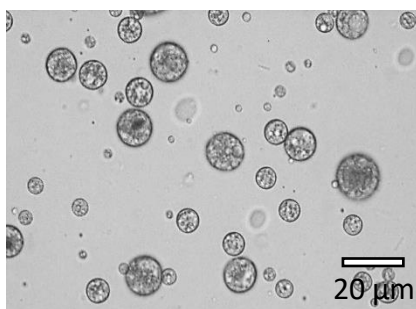
iso-osmotic solution; (c) Emulsion based on sunflower oil and PGPR, diluted with pure water; (d) Emulsion based on sunflower oil and PGPR, diluted with an iso-osmotic solution. A 10-fold dilution was performed in each case and the resulting emulsions were observed after 2h-storage at 4 °C. The scale bar corresponds to 20  $\mu\text{m}$ .

### 3.5 Probing the generality of the approach

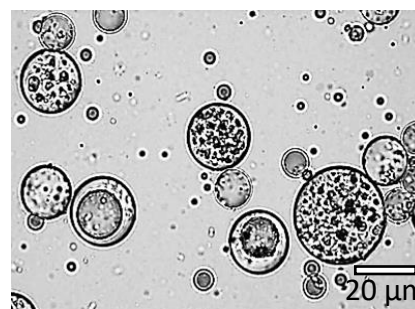
The generality of our approach was proved by replacing AMF by fats from various vegetal sources: cocoa, palm and coconut. In all cases the emulsions had the following initial composition: 30 wt.% globules;  $\phi_i^0 = 30 \text{ wt.}\%$ ; 0.5  $\text{mol.L}^{-1}$  NaCl in  $W_1$ ; 12 wt.% NaCAS and 0.8  $\text{mol.L}^{-1}$  D-glucose in  $W_2$ . They were obtained following the protocol described in the experimental section, and the applied shear rate during the second emulsification step was  $5250 \text{ s}^{-1}$ . Figure 10 shows microscopic images of the double emulsions right after fabrication. We also reported the corresponding size distributions measured by static light-scattering. The size distributions were relatively narrow in all cases, especially for the double emulsions based on cocoa butter (Figure 10a). Although the encapsulation yields were not measured, it can be stated from the images that a significant fraction of the inner droplets remained encapsulated in the globules.



(a)



(b)



(c)

*Figure 10. Generalization of the concept with other crystallizable oils. The oil phase used was (a) cocoa butter; (b) palm oil; (c) coconut oil. Multiple emulsions were initially composed of 30 wt.% of W<sub>1</sub>/O globules containing 30 wt.% of W<sub>1</sub> droplets and was stabilized by NaCAS at 12 wt.% in the external aqueous phase, W<sub>2</sub>. Emulsions were diluted with an isotonic D-glucose solution to facilitate the observation.*

## 4 Summary and Conclusions

Aside the stability issue, there is strong interest in formulating double emulsions from sustainable and label-friendly ingredients, and in reducing the number of additives to meet consumers' requirements. In this paper, we designed food grade double emulsions of the W<sub>1</sub>/O/W<sub>2</sub> type, without adding any oil-soluble surfactant. In the primary W<sub>1</sub>/O emulsion, the aqueous droplets were stabilized by endogenous triglyceride crystals forming an oleogel. So far, double emulsions described in the literature contained either PGPR or fat-based exogenous crystalline material like triglycerides and surface-active partial glycerides.

The method used was based on two successive emulsification steps. A W<sub>1</sub>/O emulsion stabilized only by triglyceride crystals was first produced. To this end, a W<sub>1</sub> aqueous phase was mixed with an oil phase without added surfactant at a temperature above its melting range. The system was cooled down to 20 °C under stirring to trigger bulk fat crystallization. The primary W<sub>1</sub>/O emulsion was then dispersed in a highly viscous external aqueous phase containing sodium caseinate to produce a W<sub>1</sub>/O/W<sub>2</sub> emulsion. The process was rather versatile and could be reproduced with fats from several sources including milk, palm, cocoa and coconut.

The obtained multiple emulsions have a widespread application potential including the encapsulation of hydrophilic drugs and their controlled delivery, the implementation of taste masking strategies, the preparation of low-fat products without compromising taste, etc. The average globule size was finely tuned through the application of a controlled shear and the size distributions were quite narrow. Monodispersity is a valuable property since it enables fabrication of materials with reproducible and well-defined properties. In addition, the obtained materials based on AMF exhibited

598 outstanding properties: they withstood osmotic shocks and they were not subject to partial  
599 coalescence under storage conditions despite the presence of fat crystals.

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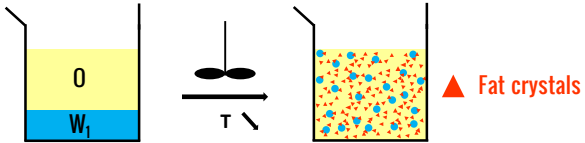
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First step:  $W_1/O$  emulsion



Second step:  $W_1/O/W_2$  emulsion

