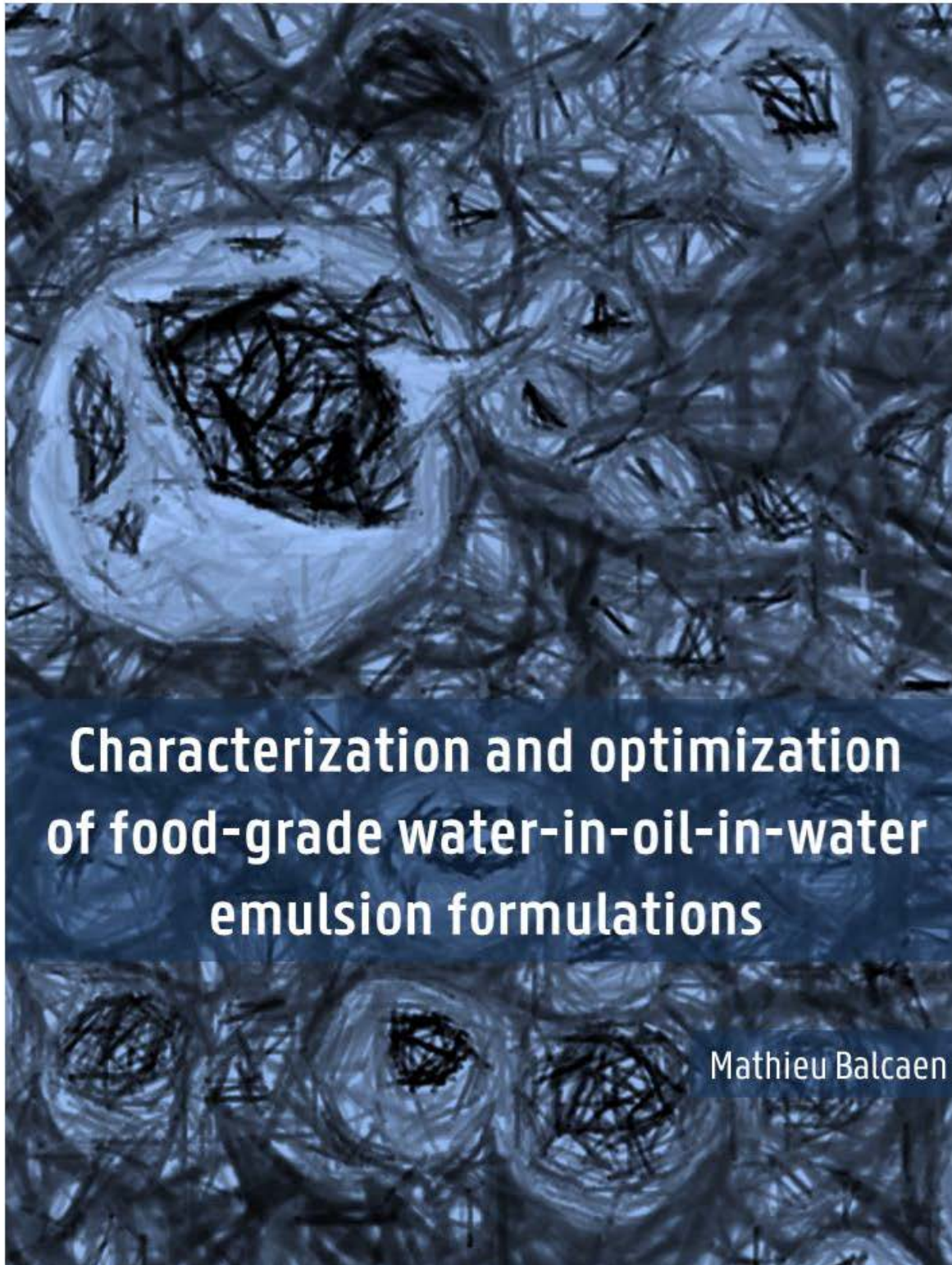




Characterization and optimization of food-grade water-in-oil-in-water emulsion formulations

Mathieu Balcaen

"My mom always said life was like a box of chocolates. You never know what you're gonna get."-
Forrest Gump

Promotor	Prof. Dr. Ir. Paul Van der Meeren Faculty of Bioscience Engineering Department of Green Chemistry and Technology Particle and Interfacial Technology Group Ghent University
Dean	Prof. Dr. Ir. Marc Van Meirvenne
Rector	Prof. Dr. Ir. Rik Van de Walle

Characterization and optimization of food-grade water-in-oil-in-water emulsion formulations

Mathieu Balcaen

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Dutch translation of the title:

Karakterisatie en optimalisatie van eetbare water-in-olie-in-water emulsie formulaties

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List of abbreviations

a	Radius
AMP	Ammonium phosphatide
C	Concentration
CLSM	Confocal Laser Scanning Microscopy
CPMG	Carr Purcell Meiboom Gill
Cryo-SEM	Cryogenic Scanning Electron Microscopy
D ₃₂	Surface weighted average diameter
D ₄₃	Volume weighted average diameter
D	Translational diffusion coefficient
D _s	Translational diffusion coefficient of water (free self-diffusion)
DLS	Dynamic light scattering
EV	Enclosed water volume fraction (based on the total sample volume)
EV _w	Enclosed water volume fraction (based on the total water phase)
g	Gravitational acceleration
G	Magnetic gradient strength (T/m)
H _{cream}	Height of the cream layer (mm)
H _{total}	Total sample height (mm)
HLB	Hydrophilic-lipophilic balance
Hoso	High oleic sunflower oil
HR-NMR	High Resolution Nuclear Magnetic Resonance
I	Echo intensity
I ₀	Echo intensity without magnetic gradient
K	Flow consistency index (Pa·s ⁿ)
k _b	Boltzmann constant ($1.381 \cdot 10^{-23}$ J/K)
LCT	Long Chain Triglyceride

LR-NMR	Low Resolution Nuclear Magnetic Resonance
M	Mass
MF	Microfluidizer
MCT	Medium Chain Triglyceride
n	Flow behavior index
N _A	Avogadro's constant ($6.022 \cdot 10^{23} \text{ mol}^{-1}$)
Na-Caseinate	Sodium caseinate
NMR	Nuclear Magnetic Resonance
O-170	Low-HLB sucrose ester; HLB $\approx$ 1, oleic acid $\approx$ 70% (di-, tri- and polyesters)
o/w	Oil-in-water
o/w/o	Oil-in-water-in-oil
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PFG	Pulsed Field Gradient
PGPR	Polyglycerol polyricinoleate
PGSTE	Pulsed Gradient Stimulated Echo
R	Decrease of the echo intensity due to relaxation effects
rpm	Rotations per minute
SD	Standard deviation
SLS	Static light scattering
T	Temperature (K)
<i>T</i>	Storage period (s)
T ₁	Longitudinal (spin-lattice) relaxation time
T ₂	Transverse (spin-spin) relaxation time

T_{2B}	Transverse (spin-spin) relaxation time of the bulk water phase
UT	Ultra-Turrax
TMACl	Tetramethylammonium Chloride
V	Volume
WPI	Whey Protein Isolate
w_1	First water phase
w_2	Second water phase
w/o	Water-in-oil
w/o/w	Water-in-oil-in-water
δ	Gradient pulse duration (s)
Δ	Diffusion delay (s)
Δ'	Diffusion delay corrected for the used pulse sequence and gradient shape (s)
γ	Gyromagnetic ratio ($2.675 \cdot 10^8 \text{ rad} \cdot \text{s}^{-1} \cdot \text{T}^{-1}$ for protons)
σ	Shape factor
σ_v	Standard deviation of the volume weighted droplet size distribution
π	Number pi (3.141592653...)
η	Viscosity (Pa.s)
ρ_1	Surface relaxivity related to the longitudinal (spin-lattice) relaxation time
ρ_2	Surface relaxivity related to the transverse (spin-spin) relaxation time

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Chapter 1: Introduction

1.1 General introduction on emulsions

An emulsion is a mixture of two or more liquids which are normally immiscible. Hereby, one immiscible phase is dispersed into the other immiscible phase which forms the continuous phase. Emulsions occur in a wide variety of industrial branches such as the food, cosmetic, pharmaceutical, chemical and refinery industries. Most often, they consist of a lipophilic and a hydrophilic phase, with one phase being dispersed as droplets in the other phase, stabilized using emulsifiers (Bernewitz et al., 2011). In food emulsions, these droplets usually have a size between 100 nm and 100 μm (McClements, 2015) and therefore, the mixture is macroscopically homogeneous while being inhomogeneous on the microscopic scale.

Because emulsions consist of two immiscible phases, they are generally thermodynamically unstable. However, due to the addition of emulsifiers, it is possible to obtain emulsions which are kinetically stable. This means that these emulsions are metastable systems, being stable for days, months or years (McClements, 2015).

1.1.1 Simple emulsions (w/o and o/w emulsions)

Conventional emulsions, occurring in food products, can be classified as either water-in-oil (w/o) or oil-in-water (o/w) emulsions (Figure 1.1). Hereby, the classification depends on which of both phases forms the continuous phase (McClements, 2015). A lot of daily consumed food products are in fact emulsions: examples of w/o emulsions are butter and margarine. Oil-in-water emulsions are even more prevalent: milk, a lot of dressing sauces, as well as mayonnaise, contain metastable oil droplets in a water continuous matrix.

1.1.2 Double emulsions (w/o/w and o/w/o emulsions)

Next to the more common w/o and o/w emulsions, more complex systems can be produced which are in fact emulsions of emulsions, called multiple emulsions. The most simple multiple emulsions are w/o/w and o/w/o type double emulsions (Figure 1.1) in which either a w/o or an o/w emulsion is dispersed into a water- or oil-continuous matrix, respectively (Muscholik and Dickinson, 2017). These complex emulsions were first described almost one hundred years ago. In fact, Seifriz (1925), next to double emulsions, also already described more complex multiphase systems such as the bimultiple and trimultiple emulsions.

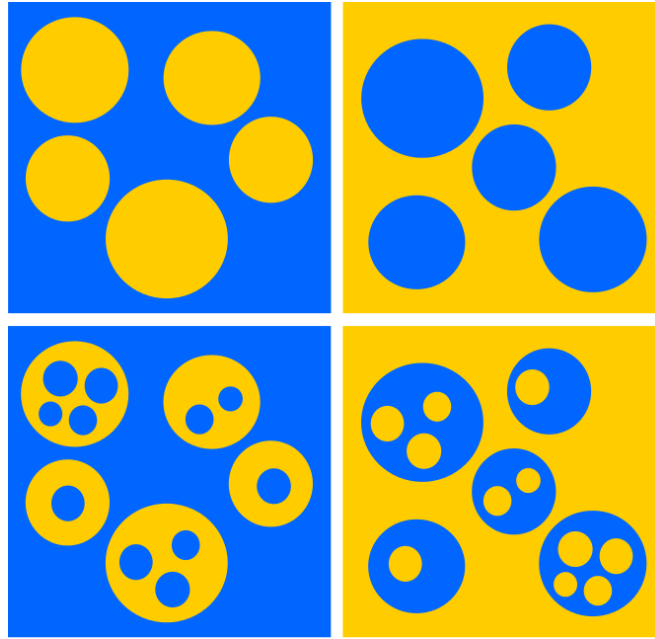


Figure 1.1: Schematic representation of the microstructure of simple o/w and w/o emulsions (top) and the corresponding w/o/w and o/w/o emulsions (bottom).

In this thesis, the research was focused on w/o/w type double emulsions, which are also the most commonly investigated double emulsion morphology (Muschiolik and Dickinson, 2017).

The encapsulation of water within an oily phase enables the application of w/o/w type double emulsions in the production of healthier foods by reducing the caloric content of the corresponding o/w type food products (Cofrades et al., 2013; Jiménez-Colmenero, 2013). Indeed, these emulsions are perceived in the same way as their oil-in-water counterparts (Oppermann et al., 2016). Moreover, the encapsulated water can contain water-soluble or water-dispersible ingredients which makes that w/o/w emulsions can be used for encapsulation purposes (Muschiolik and Dickinson, 2017).

1.2 Production of emulsions

1.2.1 w/o emulsions

Water-in-oil (w/o) emulsions generally consist of at least three ingredients: water, oil and a lipophilic emulsifier (Norn, 2014). However, in today's food industry w/o emulsions mainly occur as solid systems. Because of its solid state, a margarine (with 20% of water) can even be prepared without any emulsifier. The low water content can be emulsified by a simple mechanical treatment into the molten fat phase and kept emulsified because this treatment is maintained until the fat phase has crystallized again. Thus, the water droplets formed are

physically immobilized in a solid fat matrix. Therefore, there is no chance for coalescence to occur (Bueschelberger et al., 2014).

However, adding an emulsifier, which lowers the surface tension, enables the production of smaller water droplets. Moreover, adding a lipophilic emulsifier supports the formation of the right type of emulsion (w/o), which becomes increasingly important with increasing water content. Finally, when the solid w/o emulsions are melted, for example when butter is used for frying purposes, the stability of the emulsion is challenged again. Hereby, the used emulsifiers will be decisive in determining the destabilization kinetics of the melted w/o emulsion (Bueschelberger et al., 2014).

The production of w/o/w emulsions usually consists of a two-step process (part 1.2.2); in the second step, a liquid w/o emulsion is emulsified into the second water phase. This implies that a liquid w/o emulsion with a high stability is needed in order to produce a w/o/w emulsion. In most publications concerning liquid, edible w/o emulsions, the lipophilic (low-HLB) emulsifier polyglycerol polyricinoleate (PGPR) is used to stabilize the w/o interface. However, the use of PGPR should be avoided (or at least limited) because it can lead to off-tastes (Akhtar and Dickinson, 2001), there are legal restrictions and PGPR has a synthetic connotation.

As alternatives for PGPR, lecithin (Knoth et al., 2005a, 2005b; Ushikubo and Cunha, 2014), sorbitan esters (Márquez et al., 2007; Ushikubo and Cunha, 2014; Vaziri and Warburton, 1994), sucrose esters (Akoh, 1992; Akoh and Nwosu, 1992) and monoglycerides (Garti et al., 1998) have been proposed. Hereby, the monoglycerides are used to obtain Pickering stabilized emulsions. This means that the water droplets are stabilized using the adsorption of particles (in this case crystalized monoglycerides) at the w/o interface. For example, Frasc-Melnik (2011) was able to produce a stable w/o emulsion using fat crystal-stabilization by using only tri- and monoglycerides. This researcher even produced double w/o/w emulsions out of these primary emulsions. It was shown that using this composition, osmotic pressure gradients did not lead to the release of encapsulated solutes from the enclosed water phase. Finally, Ghosh and Rousseau (2009) prepared stable w/o emulsions with PGPR or Pickering stabilization by glycerol monostearate (GMS). These authors hereby noticed that GMS was only able to provide sufficient protection against droplet coalescence once it had crystalized. Emulsions which were prepared using GMS destabilized quickly when they were kept in a melted state.

Ushikubo and Cunha (2014) compared the emulsifying capacities of sorbitan mono-oleate, PGPR and standard soybean lecithin for two oil phases (soybean oil and n-hexadecane). These

authors concluded that PGPR produced the best water-in-soybean oil emulsions while the best water-in-hexadecane emulsions were obtained using sorbitan mono-oleate. For lecithin, a low interfacial tension at the water-soybean oil interface (1.2 mN/m) was measured at equilibrium but the initial interfacial tension was higher (9.2 mN/m) than in the other emulsifier systems. This indicates that, although lecithin can reduce the interfacial tension considerably, this reduction is slow. This observation indicates that lecithin cannot stabilize the formed droplets in a quick way. This research work shows that the performance of the lipophilic emulsifier strongly depends on the used oil phase, with PGPR being the best performer in vegetable oils containing long chain triglycerides.

Finally, it should be noted that some researchers focused on reducing the PGPR-content of w/o emulsions by combining this emulsifier with other lipophilic emulsifiers or by adding hydrophilic ingredients to the enclosed water droplets. Indeed, a number of publications have described a synergistic effect between hydrophilic and lipophilic emulsifiers, whereby the level of the latter could be reduced by increasing the former. For example, Su et al. (2006, 2008) showed that a lower PGPR-concentration could be used if sodium caseinate or acacia gum were incorporated into the water phase. The major advantage of the latter hydrophilic compound was that it provided stable emulsions over a wide range of pH conditions.

A lot of different production processes can be used in order to produce w/o emulsions. To prepare solid w/o emulsions such as butter or margarine, usually a scraped surface heat exchanger is used (Moonen and Bas, 2014). In order to produce liquid w/o emulsions, as with o/w emulsions, it is possible to use high-speed shearing (mainly rotor-stator devices), (ultra)sonication, high-pressure homogenization or membrane techniques as well as emulsification by microfluidics and other mild dispersion methods (Muschiolik and Dickinson, 2017). To produce fine, liquid w/o emulsions which are to be used in the subsequent production of w/o/w emulsions, usually a conventional rotor-stator type homogenizer is used to prepare a premix w/o emulsion. This premix is then usually subjected to high-pressure homogenization in order to accomplish a further substantial reduction in the mean droplet size of the water droplets.

1.2.2 w/o/w type double emulsions

As already mentioned in the previous part, the preparation of w/o/w type double emulsions usually consists of a two-step procedure (Figure 1.2) (Garti and Bisperink, 1998). A first water phase (w_1) is combined with an oil phase to produce a w_1/o -emulsion. Afterwards, this w_1/o -emulsion is used as internal phase to produce a double emulsion by combining the w_1/o -

emulsion with a second water phase (w_2). It should be emphasized that the composition of the enclosed and the external water phase in the double emulsion is not necessarily the same as of the water phases, w_1 and w_2 , which were used to produce the double emulsion. Indeed, water can exchange between both water phases during and after the production process.

In order to stabilize the external o/w interface, it is generally accepted that a hydrophilic (high-HLB) emulsifier or stabilizer has to be added to the second water phase. A wide range of these compounds has been added to the second water phase of w/o/w emulsions, such as small-molecular weight surfactants (polysorbates, lecithin), hydrocolloids (gum arabic, gelatin), and milk proteins (whey protein isolate, sodium caseinate) (Muschiolik and Dickinson, 2017). Hereby, the release of enclosed compounds is faster when low-molecular weight surfactants are used (Muschiolik et al., 2006). Finally, the o/w interface can also be stabilized through Pickering stabilization, using for example hydrophobically modified starch granules (Matos et al., 2013).

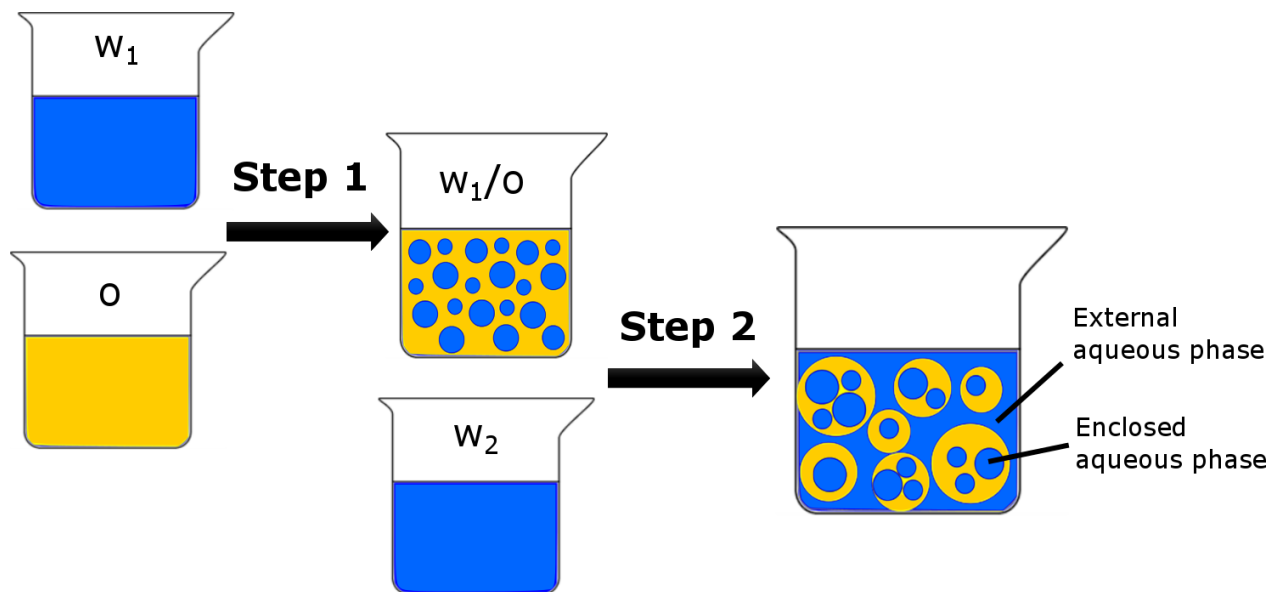


Figure 1.2: Schematic representation of the standard two step production process of w/o/w emulsions: a w/o emulsion is prepared in step 1, which is emulsified in a second aqueous phase in step 2.

Rotor-stator homogenizers are commonly used for the second emulsification step but under more moderate dispersing conditions as compared to during the production of the w/o emulsion. This lower shear in the second production step is needed to avoid destabilization of the previously formed primary w/o emulsion. Other emulsification devices that are routinely used during the second step of w/o/w production are sonicators (operating at moderate ultrasound intensity) and high pressure homogenizers (operating in a reduced pressure range) (Muschiolik and Dickinson, 2017).

Under laboratory-scale conditions, the use of some kind of a low-intensity emulsification method involving membranes or microchannels is becoming increasingly attractive to many investigators in order to ensure reliable and consistent fabrication of the double emulsion droplets while ensuring negligible disturbance of the smaller enclosed water droplets (Muschiolik and Dickinson, 2017).

Because double emulsions are prepared at a lower shear, the double emulsion droplets are usually quite large and the droplet size distribution is broad (Oppermann et al., 2015). Interestingly, the amount of water that is retained after the second emulsification step appears to be directly dependent on the final double emulsion droplet size rather than the used emulsification device (Schuch et al., 2014).

1.3 Stability of emulsions

As mentioned previously, emulsions are inherently unstable due to the high interfacial energy between the two immiscible phases. Because two interfaces (the w/o and o/w interface) are present, double emulsions are even more unstable.

Some common instability mechanisms, such as flocculation, coalescence, phase inversion and gravitational separation (creaming or sedimentation) are shown schematically in Figure 1.3. Hereby, it can be noted that these processes are connected: as an example, flocculation can lead to subsequent coalescence of droplets. Moreover, both flocculation and coalescence lead to an increase of the size of the dispersed bodies, which in turn increases the rate of gravitational separation. Whether creaming or sedimentation occurs is determined by the density of the dispersed and continuous phase (McClements, 2015).

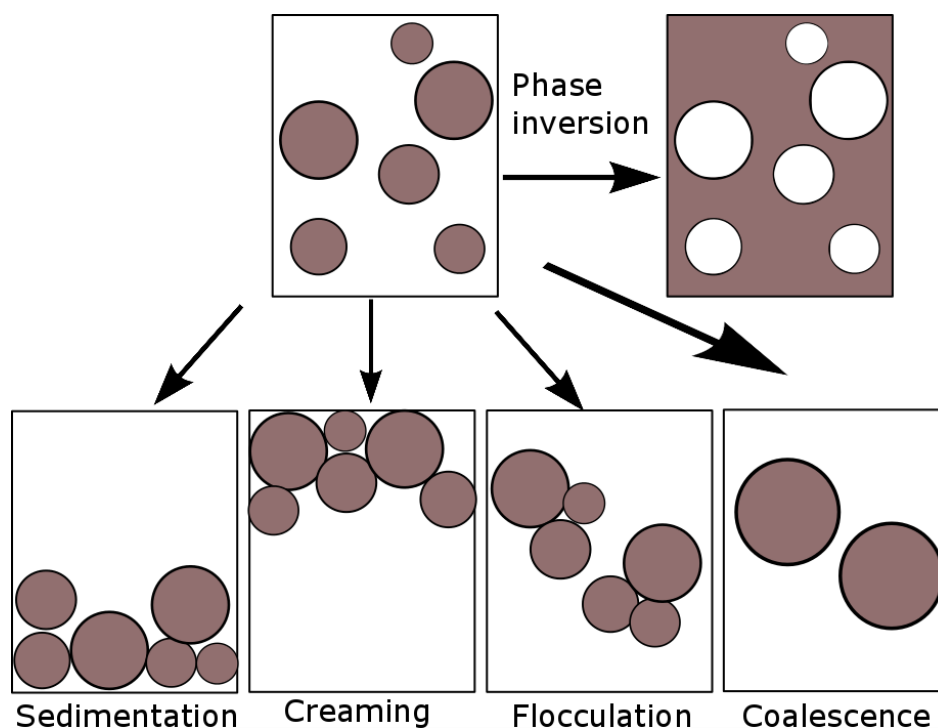


Figure 1.3: Schematic representation of some common physical destabilization mechanisms of emulsions.

Another instability mechanism which occurs in emulsions is the mass transport from smaller to bigger droplets (Figure 1.4). This is called Ostwald ripening and is caused by the higher solubility of smaller droplets as compared to bigger droplets (Kabalnov and Shchukin, 1992; Taylor, 1995). Molecules which are confined within droplets have an increased solubility in the continuous phase due to the high Laplace pressure inside droplets. This pressure is inversely related to their size ($P_{\text{Laplace}} = 2 \cdot \gamma / a$, in which γ is the interfacial tension and a is the droplet radius (Mezzenga et al., 2004)). In order to prevent Ostwald ripening, a compound can be added to the dispersed phase with a very low solubility in the continuous phase. For example, salts can be added to the dispersed water phase of w/o emulsions in order to prevent Ostwald ripening. Indeed, in that case, water transport from smaller to bigger droplets leads to a concentration gradient of the salt which leads to transport of water from the bigger droplets (containing a lower salt concentration) back to the smaller droplets which are characterized by a higher salt concentration (Mezzenga et al., 2004).

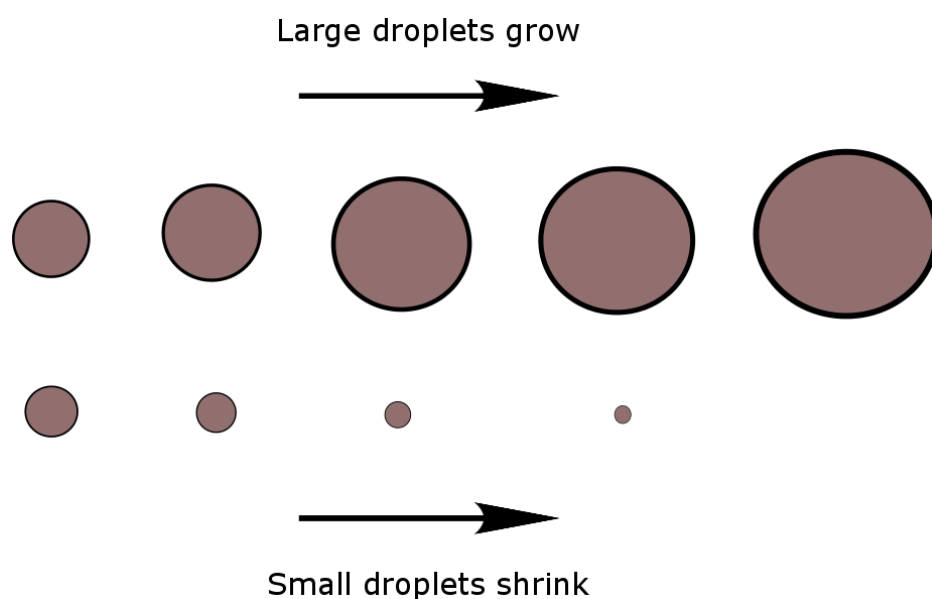


Figure 1.4: Schematic representation of Ostwald ripening which leads to the growth of large droplets at the expense of small ones.

In double emulsions, all the instability mechanisms which were mentioned above can take place. Because they consist of two emulsions, these effects can take place in one of the emulsions or in both at the same time. For example, coalescence can occur between enclosed droplets (internal coalescence of enclosed droplets), between the enclosed water droplets and the o/w interface (external coalescence of enclosed droplets) and finally also between the double emulsion droplets themselves.

Similar to Ostwald ripening in simple emulsions, water transport can occur from the small enclosed droplets, with a high Laplace pressure, to the external water phase with a low Laplace pressure. In fact, the latter water phase even has a slightly negative Laplace pressure due to the concave curvature of water at the o/w interface. Therefore, if no compound with a low oil solubility is added to the enclosed water phase of a w/o/w emulsion, water is transported from the enclosed to the external water phase. Because there is no force counteracting the Laplace pressure, the double emulsion droplets will eventually empty (Delample et al., 2014; Mezzenga et al., 2004).

If a compound with a low solubility in the oil phase is added to the enclosed water phase, water transport between the enclosed and the external water phase will occur until the Laplace pressure gradient equals the osmotic pressure gradient between both water phases. As long as both pressures are not balanced, water will be transported from one water phase to the other. Water influx will occur as long as the osmotic pressure gradient between the enclosed and

external water phase is higher than the Laplace pressure gradient between both phases while water efflux occurs if the opposite is the case. The osmotic balance between the enclosed and external water phase can therefore also be used to control the rheology and morphology, as well as the release of encapsulated ingredients by external coalescence of enclosed water droplets (Delamplé et al., 2014; Iqbal et al., 2013; Leal-Calderon et al., 2012; Mezzenga et al., 2004).

1.4 Characterization of emulsions

1.4.1 Droplet size distribution

The droplet size distribution of an emulsion has a big influence on its properties: it influences the gravitational, coalescence and microbiological stability upon storage as well as the rheology, appearance, texture, flavor and compound release rate (Bernewitz et al., 2011; Chantrapornchai et al., 1998; Pal, 1996; Vermeir et al., 2016). Moreover, the droplet size distribution provides insight into the efficiency of the used emulsifier and production process of the emulsion (Vermeir et al., 2016).

There are different approaches to determine a droplet size distribution. The most commonly applied techniques are static light scattering, dynamic light scattering and statistical image analysis of microscope pictures (Bernewitz et al., 2011). Hereby, a lot of these droplet size characterization techniques require dilution of the sample, which might have an influence on the droplet size distribution (Bernewitz et al., 2011; van Duynhoven et al., 2002). When w/o emulsions are characterized, the used dilution liquid often differs from the used oil phase as dilution in a viscous phase is difficult. For example, hexadecane (Choi et al., 2009; Yi et al., 2015), dodecane (Zhu et al., 2016) or dodecane containing 0.5 wt% sorbitan mono-oleate (Bonnet et al., 2009), have been used to dilute and subsequently analyze w/o emulsions.

The droplet size characterization of w/o/w emulsions is similar to the characterization of o/w emulsions. However, due to the presence of enclosed water droplets, more care has to be taken in the selection of the dilution medium, which is generally deionized water for o/w emulsion characterization. Indeed, osmotic gradients between enclosed and external water phase should be avoided in order to obtain an accurate estimation of the droplet size distribution of w/o/w emulsions.

In what follows, a brief overview of commonly used droplet size characterization techniques is provided. For some of the other, less frequently used, droplet size determination techniques, each with their own advantages and shortcomings, reference is made to Bernewitz et al. (2011) and van Duynhoven et al. (2002).

1.4.1.1. Light scattering

Scattering of light by droplets or particles frequently occurs in nature. For example, scattering of sunlight (which is white) by the molecules present in the atmosphere leads to the observed blue color of the sky. In fact, light of shorter wavelengths is scattered more by the molecules which form the atmosphere than light of a higher wavelength. The color with the shortest wavelength is therefore observed as the color of the sky (Simmons and Potter, 2000).

The scattering pattern of light of a certain wavelength by droplets depends on the droplet size as well as the properties of the dispersed and continuous phases. Hereby, one can consider the average scattered light intensity as a function of the angle (static light scattering) or the variation in scattered light intensity at a fixed angle (dynamic light scattering). In order to be able to derive the droplet size distribution, it is necessary that the light is scattered by only one droplet or particle which implies that these techniques generally require a high degree of dilution in order to prevent multiple scattering (Schatzel et al., 1990). In order to circumvent multiple scattering interference, new dynamic light scattering techniques were developed, such as photon cross correlation spectroscopy (Phillies, 1981; Schatzel et al., 1990) and diffusing wave spectroscopy (Weitz et al., 1993). Using these devices, more opaque systems, of which the concentration of droplets or particles is higher, can be analyzed.

1.4.1.1.1. Static light scattering (SLS)

Static light scattering is used to determine particle sizes between about 0.1 and 1000 μm and is therefore suitable for characterizing the droplets in most food emulsions (McClements, 2015). Static light scattering measurements are quite insensitive to the used refractive indices for larger droplets ($>25 \mu\text{m}$). However, the smaller the droplets get, the higher the sensitivity of the result towards the used refractive indices of the continuous and dispersed phase (Beekman et al., 2005). This technique is usually applied in the determination of the droplet size distribution of o/w or w/o/w emulsions whereas, w/o emulsions are often characterized using other techniques.

1.4.1.1.2. Dynamic light scattering (DLS)

As mentioned previously, dynamic light scattering techniques measure the fluctuation of the light intensity at a fixed scattering angle. Hereby, these slight variations in intensity with time are caused by the change in the relative positions of the particles due to their Brownian motion. Hence, the frequency of these fluctuations depends on the speed at which the particles move, and hence on their size (McClements, 2015). Because in DLS measurements, the Brownian

motion is followed, the droplets have to be sufficiently small (typically between 3 nm and 3 µm) in order to avoid a very long measurement time.

During these measurements, the translational diffusion coefficient (D) is measured and the size of the droplets is subsequently calculated using the Stokes–Einstein equation (Horne, 1995):

$$a = \frac{k_b T}{6\pi\eta D}$$

Where a is the radius, η is the viscosity of the continuous phase, k_b the Boltzmann constant and T the absolute temperature. In order to determine the droplet size distribution of a sample, next to the viscosity, also the refractive index of the continuous phase has to be known. When measuring the size distribution of w/o emulsions, the fact that the Brownian motion is followed is an additional reason to dilute the sample in a low-viscous organic solvent instead of the oil phase: in a viscous oil phase the water droplets diffuse much slower (Tscharnutter, 2006).

1.4.1.2. Microscopy

Imaging techniques are often applied. These are particularly useful in combination with statistical image processing. The used optical devices are usually light microscopes or confocal laser scanning microscopes (CLSM). Special attention has to be paid to the statistical parameters of the image interpretation: statistically meaningful sampling numbers for each and every size class have to be ensured. Usually, the samples have to be diluted. Several other effects, like droplet movement, artefacts or bubbles may have an effect on the obtained results.

Next to optical devices, electron microscopy techniques like transmission electron microscopy (TEM), scanning electron microscopy (SEM) and Cryo-SEM also provide direct insight into the emulsion's structure. However, the need for a more sophisticated, invasive sample preparation, makes the statistical interpretation even more difficult. When microscopy techniques are used to determine the droplet size distribution, the size regimes are limited by the respective resolution of the imaging device (Bernewitz et al., 2011).

1.4.1.3. ^1H NMR-based techniques

In contrast to most droplet size determination techniques, NMR-based techniques do not require dilution of the sample. Moreover, the size of individual droplets is determined, even if these droplets are flocculated. Light scattering techniques, on the other hand, measure the size of the flocs if droplets are present in this form (Van der Tuuk Opedal et al., 2009).

A frequently used NMR technique to determine the droplet size distribution is pulsed field gradient (PFG)-NMR diffusometry (Bernewitz et al., 2011; van Duynhoven et al., 2002). Using echo decay data, the diffusion of protons (^1H) can be followed and their diffusion coefficient can be determined. Murday and Cotts (1968) introduced a data analysis procedure whereby the droplet size distribution is estimated from the restricted diffusion coefficient of molecules in the dispersed phase, assuming the droplet boundaries to be impermeable (Vermeir et al., 2016).

Next to PFG-NMR diffusometry, T_1 - or T_2 -relaxometry can be used to characterize the droplet size (in fact the specific surface area) of emulsions (Amani et al., 2016; Godefroy et al., 2001; Peña and Hirasaki, 2003). Relaxometry has mainly been used in the characterization of oilfield emulsions (Amani et al., 2016; Godefroy et al., 2001; Peña and Hirasaki, 2003) and in the determination of the specific surface area of porous sedimentary rocks (Hurlimann et al., 1994; Korb et al., 2003). In this context, it can be mentioned that Peña and Hirasaki (2003) measured low values for the surface relaxivity, a parameter which links a change in specific surface area to a change in relaxation rate, for w/o emulsions with an oil phase free of paramagnetic impurities and stabilized using non-ionic surfactants such as edible w/o emulsions. As far as we know, the applicability of relaxometry for emulsions with a potentially low surface relaxivity, such as most edible emulsions, has not been fully ascertained.

Finally, it can be mentioned that both the aforementioned NMR techniques can be combined in order to obtain a droplet size distribution without prior assumptions of the shape of the droplet size distribution (Amani et al., 2016; Peña and Hirasaki, 2003). In contrast, unless regularization methods are applied, a lognormal distribution of the droplets is usually assumed when PFG-NMR diffusometry is used to determine the droplet size distribution (Vermeir et al., 2016).

Considering w/o/w emulsions, it is interesting to note that in principle, PFG-NMR diffusometry is capable of determining the droplet size of the water droplets inside the double emulsion droplets (Bernewitz et al., 2013). In contrast, light scattering techniques can only be used to characterize the w/o emulsions before production of the w/o/w emulsion. However, the production of a w/o/w emulsion might influence the droplet size distribution of the water droplets in the original w/o emulsion.

1.4.2 Enclosed water volume fraction of w/o/w emulsions

The determination of the enclosed water volume fraction of w/o/w type double emulsions may be achieved using an array of techniques, each with their own advantages and shortcomings. NMR-techniques are often applied, but these require expensive equipment, as well as long analysis times and a complicated data analysis (Bernewitz et al., 2013; Vermeir et al., 2014a). Another approach that is often used is the encapsulation of markers (Hattrem et al., 2014; Mun et al., 2011; O'Regan and Mulvihill, 2009). Next to the assumption that the marker behaves similarly as the enclosed water phase, also a difference in chemical potential between the enclosed and external water phases is created as well as an osmotic pressure difference, which has to be balanced. Moreover, the tracer has to be incorporated during double emulsion preparation, and hence these methods cannot be used for previously prepared emulsions.

1.5 Use of w/o/w emulsions for encapsulation purposes

As mentioned previously, w/o/w emulsions can be used to encapsulate water-soluble or water-dispersible ingredients. Hereby, the oil phase acts as a membrane which separates both aqueous phases. Double emulsions have already been used to encapsulate several kinds of compounds such as drugs in the pharmaceutical field (Okochi and Nakano, 2000; Schmidts et al., 2010), healthy ingredients in the functional foods field (Berendsen et al., 2015; Frank et al., 2012; Giroux et al., 2013; Jiménez-Colmenero, 2013) and pesticides in the agricultural field (ElShafei et al., 2010). Even the encapsulation of micro-organisms has been described (Rodríguez-Huezo et al., 2014). By dissolving lipophilic compounds in the oil phase, it is also possible to deliver lipophilic compounds using w/o/w emulsions. Therefore, using w/o/w emulsions, lipophilic (in the oil phase) and hydrophilic compounds (in the enclosed water phase) can be delivered using one system.

It should be noted that, already during production of the double emulsion, part of the enclosed water phase, and therefore also part of the enclosed hydrophilic compound, is released to the external water phase by external coalescence of the enclosed droplets. The amount which is released, is inversely proportional to the enclosed water volume fraction which is obtained after production. Concerning release of enclosed compounds after production of the double emulsion, several types of mechanisms on how the entrapped hydrophilic substance in a double emulsion is released have been proposed. However, there is still not a full consensus on these mechanisms and how to control them (Garti and Lutz, 2004). Pays et al. (2002) described two main possible routes of release. The first type is coalescence between the enclosed droplets

and the o/w interface (external coalescence of enclosed droplets), where the film rupturing is initiated by spontaneous formation of a small hole. The second type is known as compositional ripening and occurs without film rupturing. Instead, the entrapped chemical crosses the oil phase by diffusion.

Three mechanisms for diffusion of water and water soluble compounds through the oil phase have been described (Figure 1.5). These are reverse micellar transport, diffusion across very thin lamella and hydrated surfactant transport (Garti & Lutz, 2004).

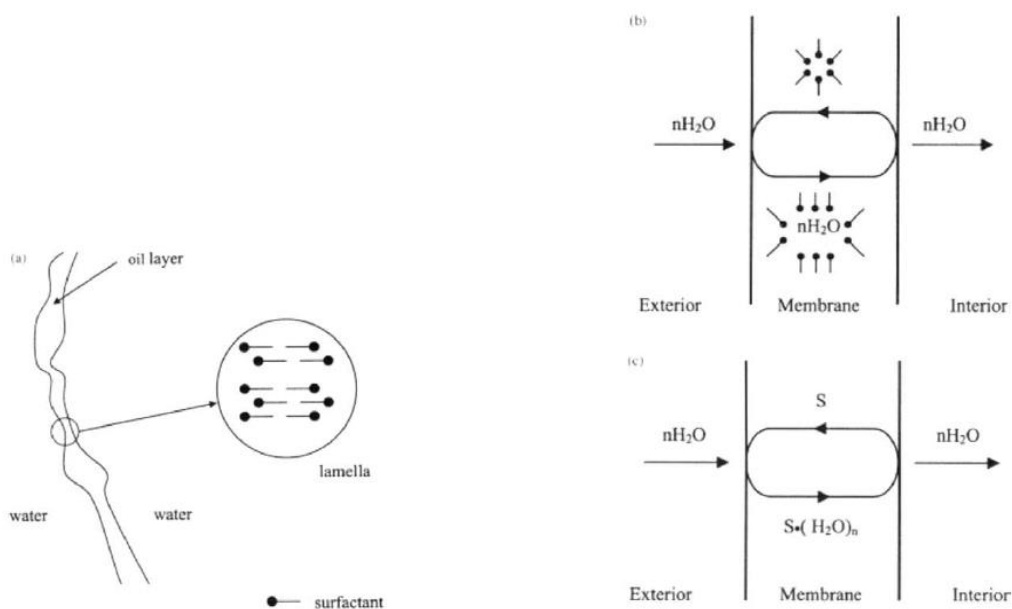


Figure 1.5: Diffusion mechanisms of water and water soluble compounds from the enclosed to the external water phase of w/o/w emulsions. a) transport through thin lamella of surfactant, b) transport via reverse micelles, c) transport via hydrated surfactants (Garti and Lutz, 2004).

Depending on the nature of the hydrophilic compound and the circumstances (such as temperature and pH), the hydrophilic compound is released from the enclosed to the external water phase at different rates (Magdassi and Garti, 1984). For example, amino acids with a more lipophilic character diffuse through the oil phase of w/o/w emulsions at a faster rate as compared to their more hydrophilic counterparts (Uribe Alvarez, 2017).

1.6 Thesis outline

Considering the number of publications concerning double emulsions (Figure 1.6), it is clear that the research interest towards these systems is still increasing. The extensive patent literature making claims for the viability of double emulsions in commercial food products, also points towards the high interest of food producers in these systems (Muschiolik and Dickinson, 2017).

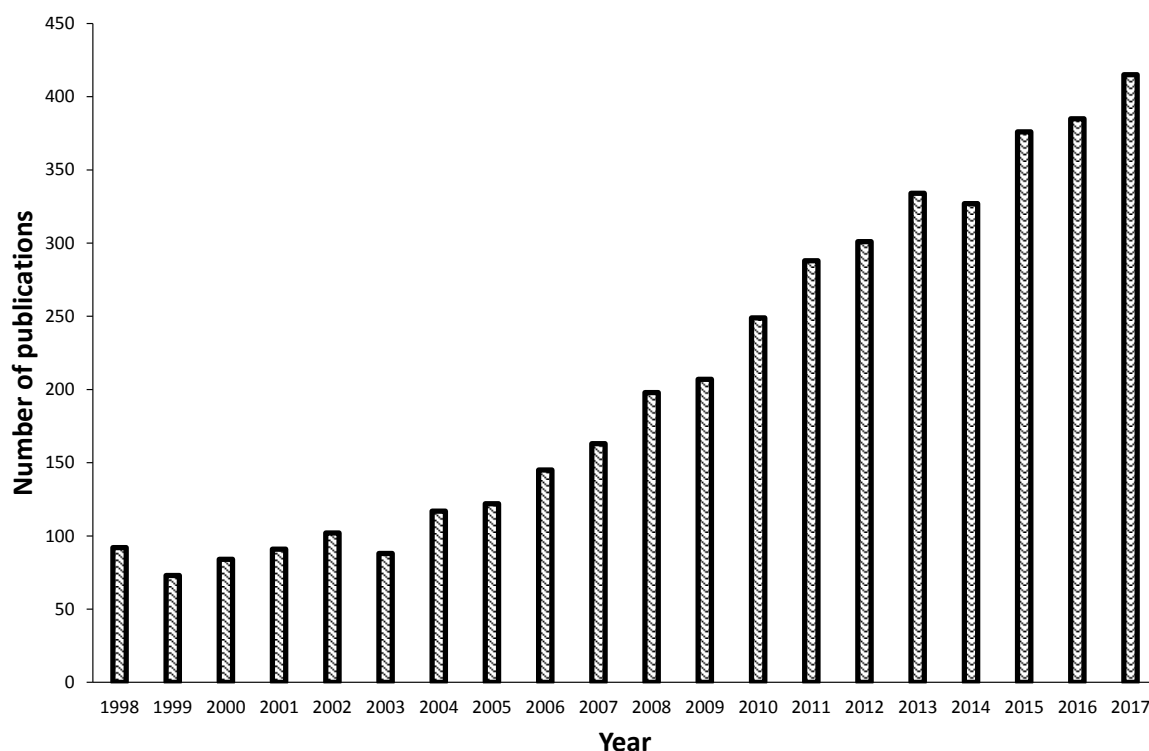


Figure 1.6: Number of publications with topic ‘double emulsions’ as a function of the publication year as found on web of science.

However, the actual industrial application of w/o/w emulsions, especially in the food industry, is very limited. In this context, Muschiolik and Dickinson (2017) define two outstanding issues which currently prevent the full realization of double emulsions in food products:

- 1) The lack of availability of large-scale production equipment to ensure efficient nondestructive emulsification during the second production step.
- 2) The limited range of food-grade ingredients available to successfully replace polyglycerol polyricinoleate (PGPR) as the primary emulsifier in w/o/w emulsions.

In our research work, we tried to contribute towards overcoming the second issue. In fact, this manuscript consists of two main parts (Figure 1.7). The first part focuses on the characterization

of w/o and w/o/w emulsions, which is a necessary prerequisite to be able to optimize their formulation. The second part of this manuscript is focused at optimizing the composition and production processes of w/o and w/o/w emulsions using the characterization techniques which were developed in the first part.

As already mentioned, w/o/w emulsions are usually prepared in a two-step process. In the second step, a liquid w/o emulsion with preferably submicron water droplets is mixed with the external water phase. However, such liquid w/o emulsions are not that widespread. Therefore, we examined whether existing droplet size characterization techniques could be used in order to characterize (submicron) liquid w/o emulsions (Chapter 2 and 3).

Because the use of PGPR as lipophilic emulsifier should be avoided or at least limited, some initial tests were performed to estimate the residual PGPR-concentration in the oil phase of w/o emulsions which were produced using different processing conditions (Chapter 4). Based on the measured residual PGPR-concentration, the formulation of the w/o and therefore also the w/o/w emulsions can be optimized.

Because one of the most important parameters of w/o/w emulsions is their enclosed water volume fraction, which defines the caloric content of the double emulsion droplets as well as how much of an enclosed hydrophilic compound is retained within the droplets, a simple technique was developed which enables the quick determination of this parameter (Chapter 5).

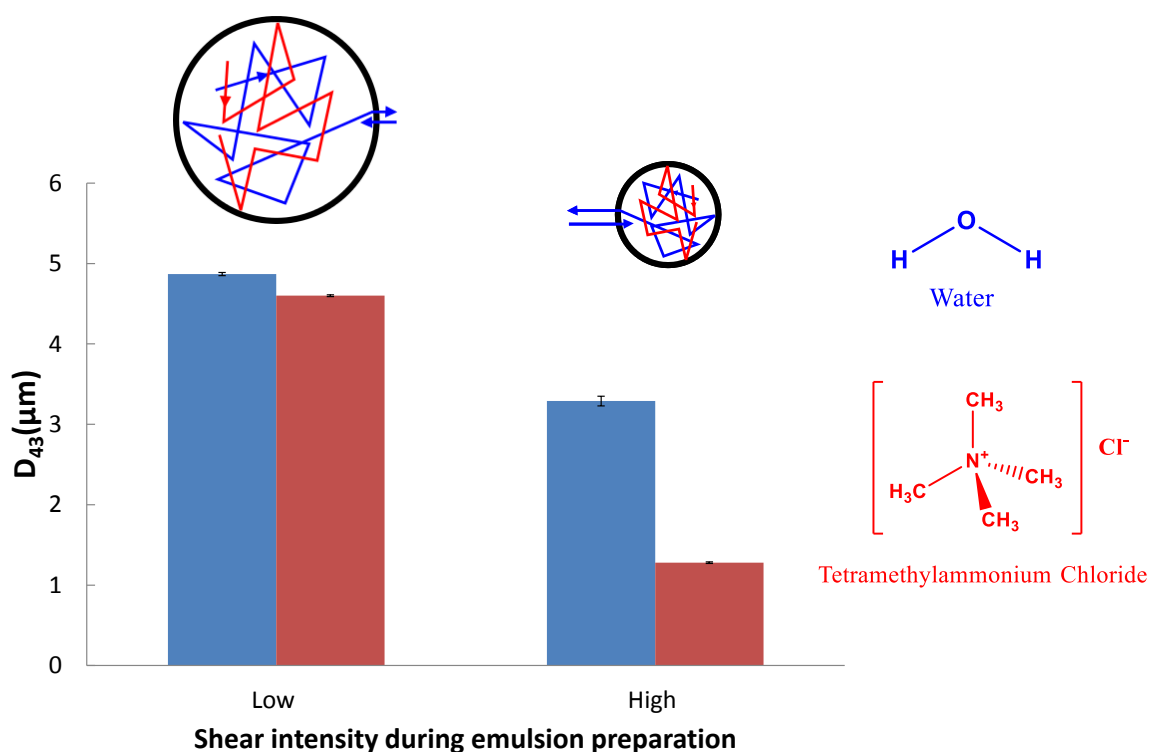
In the second part, which was aimed at improving the composition of the w/o/w emulsions, two pathways were followed. The first one was gelation of the enclosed water phase which might render the double emulsion less sensitive towards shear stresses (Chapter 6) while in the second one we examined alternative lipophilic emulsifiers (Chapter 7 and 8).

Finally, Chapter 9 shows that it is possible to produce double emulsions in one step. This might be interesting when these formulations are to be used in bulk food products. Indeed, the amount of steps in, and thus the complexity of, the production process is reduced while still water is encapsulated inside the oil-droplets. As such, this procedure could be a solution for the first issue raised by Muscholik and Dickinson (2017).

Schematic overview of this work	
Introduction (1)	
Part A: Characterization of w/o- and w/o/w-emulsions	
<u>w/o-emulsions:</u> - Determination of the droplet size distribution : a) ¹H-NMR (2) b) Dynamic light scattering (3) - PGPR-quantification (4)	<u>w/o/w-emulsions:</u> Enclosed water volume determination (5)
Part B: Production of w/o- and w/o/w-emulsions	
- Gelation of the (enclosed) water phase (6) - Alternative low-HLB emulsifiers: a) Ammonium phosphatide and low-HLB sucrose ester (7) b) Lecithin (8) - One-step production of w/o/w-emulsions (9)	
Conclusions (10)	

Figure 1.7: Schematic overview of this manuscript.

Chapter 2: Characterization of w/o emulsions using PFG-NMR diffusometry



- Liquid w/o emulsions were characterized using PFG-NMR diffusometry, dynamic light scattering, light microscopy and T_2 -relaxometry
- Due to the increase of solubility with decreasing droplet size, from a certain size downwards, PFG-NMR water diffusometry measured free diffusion between instead of hindered diffusion within droplets

Abstract

When the droplet size distribution of w/o emulsions is determined using PFG-NMR water diffusometry, water exchange through the oil phase can lead to erroneous (overestimated) results. However, it is generally accepted that measuring at lower temperature avoids this issue.

In this chapter, it was examined whether measuring at a lower temperature leads to satisfactory results when (sub)micron sized water droplets are present. It was shown that from a certain droplet size downwards, the increased water solubility resulted in water exchange at the w/o interface which led to the observation of free water diffusion through the oil phase. As a further consequence, low-resolution PFG-NMR based on water diffusion is incapable of accurately determining the droplet size distribution of these samples. On the other hand, the use of an oil insoluble marker in the water phase extended the application window of PFG-NMR diffusometry towards smaller droplet sizes. However, high-resolution equipment is required in the latter case.

Summarizing, our results revealed that low-resolution NMR diffusometry could only be used for supermicron w/o emulsions. Alternative techniques are needed when (sub)micron sized water droplets are present. In this respect, T_2 -relaxometry was shown to be capable of detecting differences in droplet size distribution between w/o emulsions, especially when the droplets were submicron in size.

Redrafted after: Balcaen, M., De Neve, L., Vermeir, L., Courtin, T., Dewettinck, K., Sinnaeve, D., Van der Meeren, P., 2018. Increasing water solubility with decreasing droplet size limits the use of water NMR diffusometry in submicron W/O-emulsion droplet size analysis. *J. Colloid Interface Sci.* 514, 364-375. <https://doi.org/10.1016/j.jcis.2017.12.049>

2.1 Introduction

Vermeir et al. (2016) showed that during PFG-NMR water diffusometry measurements on w/o emulsions, increasing temperatures led to increasing water diffusion through the oil phase. Due to the increased diffusion of water between droplets, a higher apparent droplet size was measured at higher temperatures. Therefore, in order to minimize the influence of water exchange between droplets, PFG-NMR measurements are usually conducted at a low temperature, typically 5 °C (Vermeir et al., 2016).

In this chapter, it was examined whether measuring at a low temperature indeed leads to satisfactory results, even in case (sub)micron sized water droplets are present in the w/o emulsion. Indeed, small droplets have a higher solubility than larger droplets or the bulk material, which is called the Kelvin effect (Davis et al., 1981). In addition, also an exploratory test was performed to determine whether a difference in droplet size in the produced edible w/o emulsions could be detected using T_2 -relaxometry.

2.2 Materials and Methods

2.2.1 Materials

The lipophilic emulsifier polyglycerol polyricinoleate (PGPR 4175) was kindly provided by Palsgaard A/S (Juelsminde, Denmark). Sunflower oil was acquired from a local supermarket while high oleic sunflower oil (Hoso; Iodine Value= 87; 82% C18:1) was acquired from Contined BV (Wageningen, The Netherlands).

The water phase contained 0.1M NaCl (VWR Chemicals: BDH Prolabo, Leuven, Belgium) in the emulsions analyzed using only low-resolution NMR while 0.1M tetramethylammonium chloride (TMACl, Sigma-Aldrich, Steinheim, Germany), in a mixture (50/50; m/m) of D₂O/H₂O, was used in the emulsions analyzed using both low- and high-resolution NMR. In the high-resolution tests, TMACl was used instead of NaCl because this salt is an oil-insoluble compound which can be detected using high resolution-NMR (Vermeir et al., 2016). Finally, all water phases also contained 0.02 wt% of the anti-microbial agent NaN₃ (Sigma-Aldrich, Steinheim, Germany).

2.2.2 Preparation of the emulsions

2.2.2.1 Samples analyzed using low-resolution NMR

2.2.2.1.1 Samples analyzed using PFG-NMR diffusometry and T_2 -relaxometry under standard settings

5 wt% PGPR 4175 was added to Hosolux after which this phase was heated to 60 °C and stirred to obtain a homogeneous solution. The water phase was also heated to 60 °C after which the emulsions were prepared.

In a first production scheme, the 50/50 (m/m) emulsions were prepared using a rotor-stator homogenizer (Ultra-Turrax: type S25-10G, IKA®-Werke, Germany). Hereby, the water phase was added gradually during mixing. Three different mixing intensities were used namely 6500, 17500 and 24000 rpm; these emulsions are referred to as UT 6500-5wt, UT 17500-5wt and UT 24000-5wt, respectively.

In a second scheme, a premix was prepared using a bigger rotor stator homogenizer (Ultra-Turrax: type S50N-G45F, IKA®-Werke, Germany). Hereby, the water phase was added gradually during mixing at 5200 rpm. Afterwards, the emulsions were further processed using 1 pass through a Microfluidizer (type M110S, Microfluidics) at 140 , 420 or 840 bar during which the temperature was kept at 60 °C using a heating bath. The aforementioned emulsions are referred to as MF 140-5wt, MF 420-5wt and MF 840-5wt, respectively.

2.2.2.1.2 Samples analyzed using PFG-NMR diffusometry with variation of the parameters and T_2 -relaxometry at different temperatures

In order to determine the influence of the measurement temperature on the T_2 -relaxation time of the water phases in the w/o emulsions, two samples which contained 10 wt% PGPR 4175 in Hosolux were prepared at a different shear intensity. These samples were also analyzed using PFG-NMR diffusometry with varying diffusion delays and gradient pulse durations.

In a first production scheme, the 50/50 (m/m) emulsion was prepared using a rotor-stator homogenizer (Ultra-Turrax: type S25-10G, IKA®-Werke, Germany) at 6500 rpm; this emulsion is referred to as UT 6500-10wt.

In a second production scheme, a premix was prepared using the same rotor stator homogenizer (Ultra-Turrax: type S25-10G, IKA®-Werke, Germany). Hereby, the water phase was added gradually during mixing at 24000 rpm. Afterwards, the emulsion was further processed using 1 pass through a Microfluidizer (type M110S, Microfluidics) at 840 bar during

which the temperature was kept at 60 °C using a heating bath; this emulsion is referred to as MF 840-10wt.

2.2.2.2 Samples analyzed using both low- and high-resolution NMR

10 wt% PGPR 4175 was added to sunflower oil after which this phase was heated to 60 °C and stirred until a homogeneous solution was obtained. The water phase, containing 0.1M TMACl in a 50/50 (m/m) mixture of D₂O and H₂O, was also heated to 60 °C after which the emulsions were prepared, according to the production schemes described in section 2.2.2.1.2.

The emulsion obtained using the first production scheme is referred to as UT 6500-HR. The premix obtained in the second scheme is referred to as UT 24000-HR, whereas the emulsion obtained after one pass through the Microfluidizer at 840 bar is referred to as MF 840-HR.

2.2.3 Low-resolution NMR

2.2.3.1 PFG-NMR diffusometry

Low-resolution (LR) pulsed field gradient (PFG)-NMR diffusometry measurements were performed at 5 °C using a benchtop Maran Ultra spectrometer (Oxford Instruments, UK) operating at a frequency of 23.4 MHz. The samples of about 2.5 gram were filled in 18 mm outer diameter glass NMR-tubes (Oxford Instruments, UK). A monopolar rectangular gradient pulse was used. Thus, the standard Stejskal-Tanner equation was used in the determination of free diffusion coefficients (Sinnaeve, 2012). As described in the latter work, the shape factor σ equals 1 while Δ' equals $(\Delta - \delta/3)$ for this gradient pulse shape. For protons, the gyromagnetic ratio (γ) is equal to $2.675 \cdot 10^8 \text{ s}^{-1} \cdot \text{T}^{-1}$.

The free self-diffusion coefficient of the used water phase (D_s) was measured using the DSD script (Oxford Instruments, UK) and varying the gradient duration (δ) in ten steps between 0.05 and 2.75 ms while keeping the gradient strength (G) and the diffusion delay (Δ) constant at 0.14 T/m and 200 ms, respectively. The free self-diffusion coefficient of the water phase was measured in triplicate and amounted to $(1.34 \pm 0.04) \cdot 10^{-9} \text{ m}^2/\text{s}$ for the water phase (D₂O/H₂O; 50/50; m/m) containing 0.1M TMACl while a diffusion coefficient of $(1.29 \pm 0.01) \cdot 10^{-9} \text{ m}^2/\text{s}$ was measured for the water phase (H₂O) containing 0.1M NaCl.

Pulsed field gradient-NMR experiments on the w/o emulsions were performed using the stimulated echo pulse (STE) sequence, which was preceded by an inversion recovery sequence for suppression of the NMR-contribution from the oil phase in the emulsion. The inversion recovery experiment on the sunflower oil phase containing 10 wt% PGPR led to a τ_0 of 80 ms

while a τ_0 of 50 ms was measured for the high oleic sunflower oil containing 5 wt% PGPR. The measurements were performed by varying the gradient strength (G) between 0 and 3.17 T/m and, unless stated otherwise, using a diffusion delay (Δ) of 180 ms and a gradient pulse duration (δ) of 2.5 ms.

To determine the volume weighted average droplet size of the water droplet size distribution in the w/o emulsions, the Murday-Cotts model was fitted to the obtained echo decay curves using the diffusion coefficient as determined for the water phase. In order to estimate the experimental uncertainty, the standard error of the point estimate was calculated by taking the square root of the reciprocal of the Hessian (Zelterman, 2015).

2.2.3.2 T_2 -relaxometry

T_2 -relaxation (transversal relaxation) measurements were conducted using the Carr-Purcell-Meiboom-Gill (CPMG) sequence while keeping the receiver gain constant. Unless stated otherwise, the temperature was kept at 5 °C and T_2 -distributions were obtained by CONTIN analysis of the relaxation curve using the WinDXP 1.8.1.0 software (Oxford Instruments, UK).

As described by Godefroy et al. (2001), there is a relationship between the T_2 -relaxation time of the water phase and the droplet size given by equation 2.1:

$$\frac{1}{T_2} = \frac{1}{T_{2B}} + \frac{3 \cdot \rho_2}{a} \frac{1}{1 + \frac{\rho_2 \cdot a}{2 \cdot D}} \quad (\text{Equation 2.1})$$

In which ρ_2 is the surface relaxivity (m/s), a is the radius of the droplets (m), T_{2B} is the bulk T_2 -relaxation time (s) and D is the translational diffusion coefficient (m^2/s) of the liquid inside the droplets. Thus, what should in fact be considered is the difference between $1/T_2$ (s^{-1}) and $1/T_{2B}$ (s^{-1}):

$$\frac{1}{T_2} - \frac{1}{T_{2B}} = \frac{1}{T_{2,corr}} = \frac{3 \cdot \rho_2}{a} \frac{1}{1 + \frac{\rho_2 \cdot a}{2 \cdot D}} \quad (\text{Equation 2.2})$$

2.2.4 High-resolution PFG-NMR diffusometry

High-resolution (HR) pulsed field gradient (PFG)-NMR diffusometry measurements were performed using a Bruker Avance III spectrometer operating at a ^1H frequency of 500.13 MHz and equipped with a 5 mm DIFF30 high-gradient probe with a maximum gradient strength of 18 T/m. Measurements were performed at 5 °C and the temperature was controlled with a

Eurotherm 3000 VT digital controller. The samples were filled in glass NMR-tubes with a diameter of 5 mm (Euriso-top SAS, France).

PFG-NMR experiments were performed using a stimulated echo pulse sequence with bipolar sine bell shaped gradients and a δ and Δ of 2.4 ms and 180 ms, respectively. The free self-diffusion coefficients of water and TMACl in the water phase were measured upon varying G while keeping δ and Δ constant at 2.4 ms and 180 ms, respectively. Taking into account the shape factor (σ) of $2/\pi$ and a Δ' of $(\Delta - 5 \cdot \delta / 16 - \tau / 2)$ for the used gradient pulse (Sinnaeve, 2012), $D_{s, \text{water}}$ and $D_{s, \text{TMACl}}$ were measured to be $1.17 \cdot 10^{-9} \text{ m}^2/\text{s}$ and $5.60 \cdot 10^{-10} \text{ m}^2/\text{s}$, respectively.

To determine the volume weighted average droplet size of the water droplet size distribution in the w/o emulsions, the Murday-Cotts model was fitted to the obtained echo decay curves using the diffusion coefficient as determined for either the water- or the TMACl-molecules. In order to estimate the experimental uncertainty, the standard error of the point estimate was calculated by taking the square root of the reciprocal of the Hessian (Zelterman, 2015).

In order to determine the diffusion coefficient of PGPR in sunflower oil, a solution of 10 wt% PGPR in sunflower oil was prepared. The sample was filled in a glass NMR-tube with a diameter of 5 mm (Euriso-top SAS, France), equipped with a NI5CCI-B coaxial insert which contains D-aceton (Euriso-top SAS, France). PFG-NMR experiments were performed using a stimulated echo pulse sequence with bipolar sine bell shaped gradients and a δ and Δ of 2.4 ms and 180 ms, respectively. The peak corresponding to the proton next to polymerized OH-groups of ricinoleate was followed. This peak was found at 4.9 ppm when PGPR was dissolved in chloroform (Orfanakis et al., 2013). Due to the use of a different solvent (sunflower oil) in our experiment, the ^1H -peaks shifted to lower values and the peak of interest was found at 4.1 ppm.

2.2.5 Dynamic light scattering

Measurements were conducted using a Zetasizer 3000Hs (Malvern Instruments, UK) or a spectrometer PCS-100 SM (Malvern Instruments, UK) at a scattering angle of 150° and aperture of 50 μm . The latter spectrometer was equipped with a digital correlator K7032CN (Malvern Instruments, UK). Samples were diluted in 1-bromododecane (Alfa Aesar, viscosity at $25^\circ\text{C} = 3.37 \text{ mPa}\cdot\text{s}$) in order to prevent multiple scattering after which 3 subsequent measurements were conducted. Data analysis was performed using the monomodal option (cumulant analysis).

2.2.6 Light microscopy

The emulsions were diluted ten times in high oleic sunflower oil and observed under the microscope. The diluted emulsions were brought on a microscopy slide using a capillary after which a cover slip was gently applied. Image acquisition was conducted using an optical microscope (Olympus Cx40) with 100-fold objective coupled with a digital camera (AxioCam ERc 5S).

2.3 Results and Discussion

2.3.1 Samples examined using low-resolution NMR

2.3.1.1 Samples examined under standard settings

Six w/o emulsions were prepared with the same composition but with a varying shear intensity during production. Light microscopy, dynamic light scattering and two low-resolution NMR techniques, namely PFG-NMR diffusometry and T_2 -relaxometry (part 2.3.3), were used to determine the water droplet size distribution.

2.3.1.1.1 Light microscopy

As shown in Figure 2.1, a decreasing water droplet size was obtained with increasing shear intensity during production of the w/o emulsions. Light microscopy thus indicates that, as expected, the droplet size is reduced when higher shear intensities were used during production of the w/o emulsions.

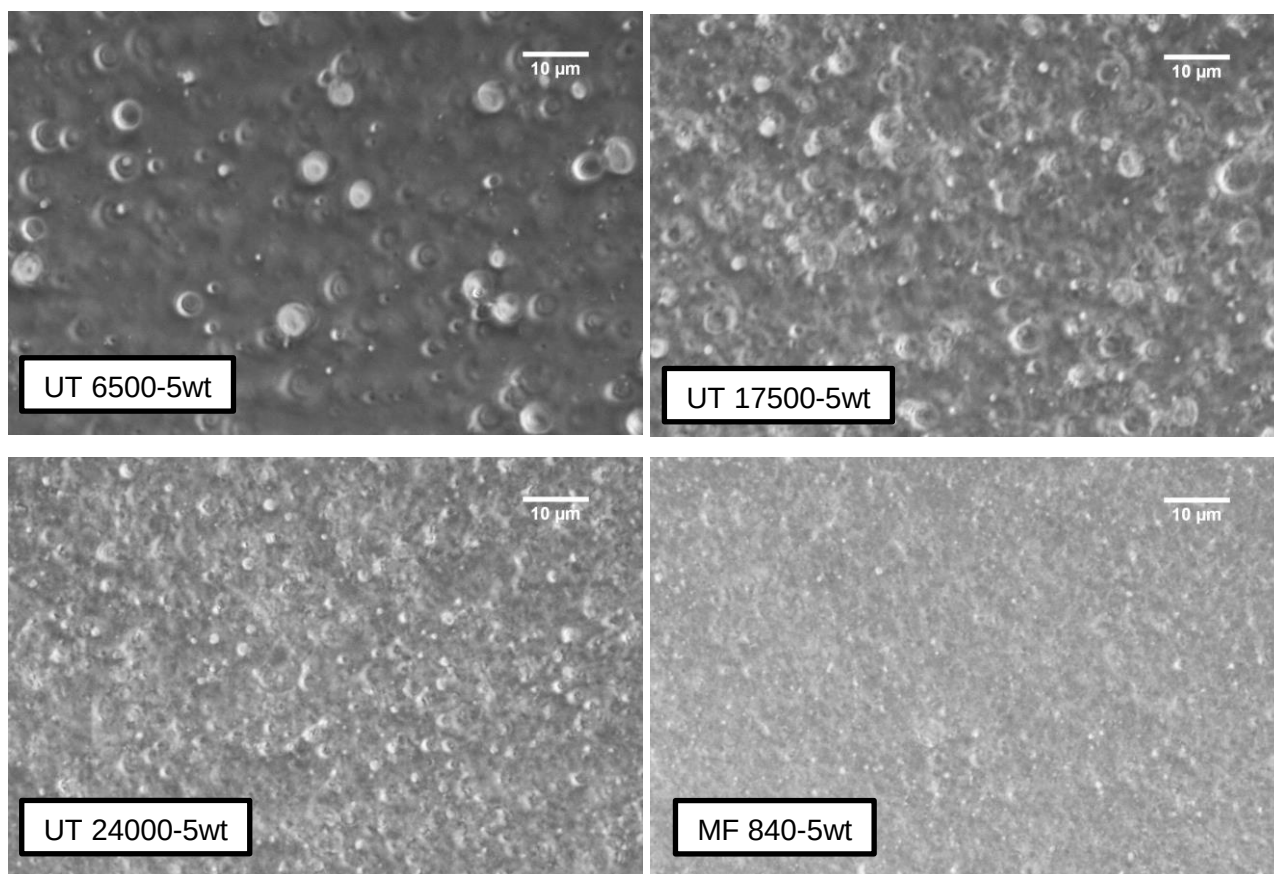


Figure 2.1: Pictures of the emulsions after 10x dilution in Hoso obtained from light microscopy using a 100x objective. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the used PGPR concentration in the oil phase.

2.3.1.1.2 Dynamic light scattering

Samples were diluted in 1-bromododecane because this solvent has a polarity which is similar to oil (dielectric constant of 4.1 and 3.1 at 20 °C for 1-bromododecane and glycerol trioleate, respectively (Haynes, 2015)) and a density which is similar to water (1037 kg/m³ at 25 °C (Cokelet et al., 1969)). Moreover, the viscosity (3.37 mPa.s at 25 °C) is smaller than the viscosity of the used oil phase (Boyson and Pashley, 2007; Cokelet et al., 1969). Due to the fact that the density difference between the continuous and dispersed phase is small, sedimentation is retarded during the measurement. Table 2.1 shows the obtained Z-average diameters and polydispersity indices of the emulsions as determined using cumulant analysis of the dynamic light scattering data.

These measurements clearly show that DLS after diluting the w/o emulsions in 1-bromododecane could also detect a difference in droplet size between the samples. In chapter 3, determination of the droplet size distribution of (sub)micron w/o emulsions using DLS is optimized.

Table 2.1: Z-average diameter and polydispersity index of the w/o emulsions; the average value and standard deviations were determined based on 3 repetitions after dilution in 1-bromododecane. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the used PGPR concentration in the oil phase.

Sample	Z-average diameter (nm)	Polydispersity index (-)
UT 6500-5wt	1072±30	0.399±0.284
UT 17500-5wt	849±17	0.535±0.022
UT 24000-5wt	586±8	0.444±0.038
MF 140-5wt	493±16	0.294±0.115
MF 420-5wt	355±3	0.312±0.053
MF 840-5wt	272±2	0.159±0.023

2.3.1.1.3 PFG-NMR diffusometry

Figure 2.2 shows the echo decay curves which were obtained for the w/o emulsions, prepared using different shear intensities. For the samples that were prepared using the lowest shear intensities (UT 6500-5wt and UT 17500-5wt), a decrease to about 17 and 42 % of the initial value was obtained, respectively. In contrast, a similar decay profile was obtained for the other 4 samples in which the signal decreased to about 60% of its initial value.

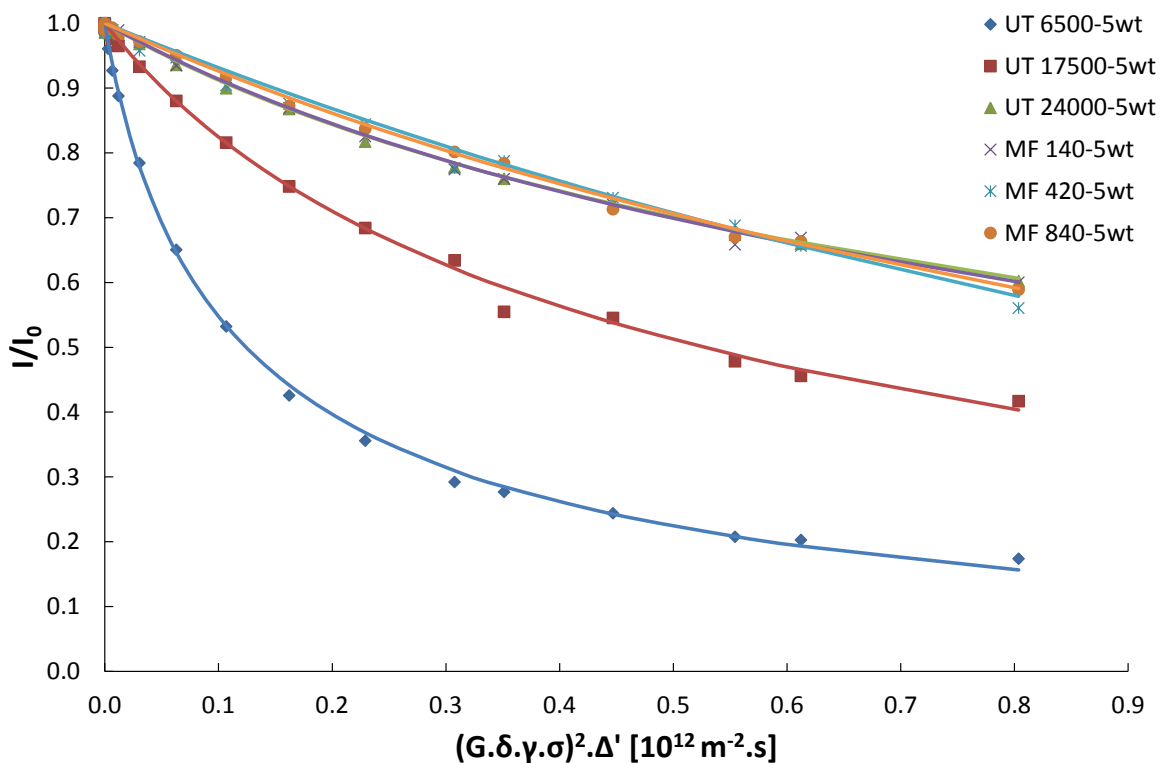


Figure 2.2: Echo decay curves of the w/o emulsions prepared using different shear intensities; the used gradient pulse duration was equal to 2.5 ms while the diffusion delay was set to 180 ms. Lines represent the fit of the Murday-Cotts model to the data. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the used PGPR concentration in the oil phase.

As the obtained echo decay was similar for the four samples which were prepared using the highest shear intensities, the obtained average droplet size and standard deviation of the droplet size distribution were similar when the Murday-Cotts model was fitted to these echo decay curves (Table 2.2).

Table 2.2: Volume weighted average diameter (D_{43}) and standard deviation of the volume weighted droplet size distribution (σ_v) of the w/o emulsions (together with their standard error) as obtained from fitting the Murday-Cotts model to the echo decay data. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the used PGPR concentration in the oil phase.

Sample	D_{43} (μm)	σ_v (μm)
UT 6500-5wt	6.36 $\pm$ 0.05	3.39 $\pm$ 0.10
UT 17500-5wt	3.82 $\pm$ 0.04	1.53 $\pm$ 0.16
UT 24000-5wt	2.95 $\pm$ 0.01	1.18 $\pm$ 0.08
MF 140-5wt	2.96 $\pm$ 0.03	1.11 $\pm$ 0.16
MF 420-5wt	3.01 $\pm$ 0.03	0.37 $\pm$ 0.27
MF 840-5wt	2.97 $\pm$ 0.02	0.71 $\pm$ 0.13

The results shown in Table 2.2 indicate that low-resolution PFG-NMR diffusometry, with the used parameter settings, did not detect a difference between the four samples that were produced under the highest shear intensities (UT 17500-5wt, and MF 140-5wt, MF 420-5wt and MF 840-5wt) whereas both light microscopy and DLS clearly pointed out a difference between these samples.

Hence, these results point to the fact that droplet size analysis by PFG-NMR water diffusometry has a lower boundary, whereby all smaller droplets give the same response, and hence the same apparent droplet size distribution.

2.3.1.2 Influence of the used diffusion delay and gradient pulse duration in low-resolution PFG-NMR diffusometry

The echo decay curves of the UT 6500-10wt (coarse emulsion; Figure 2.3) and MF 840-10wt (fine emulsion; Figure 2.5) were measured using LR-NMR with variation of the diffusion delay (Δ = 60/120/180/240 ms) and gradient pulse duration (δ = 2.5/8 ms).

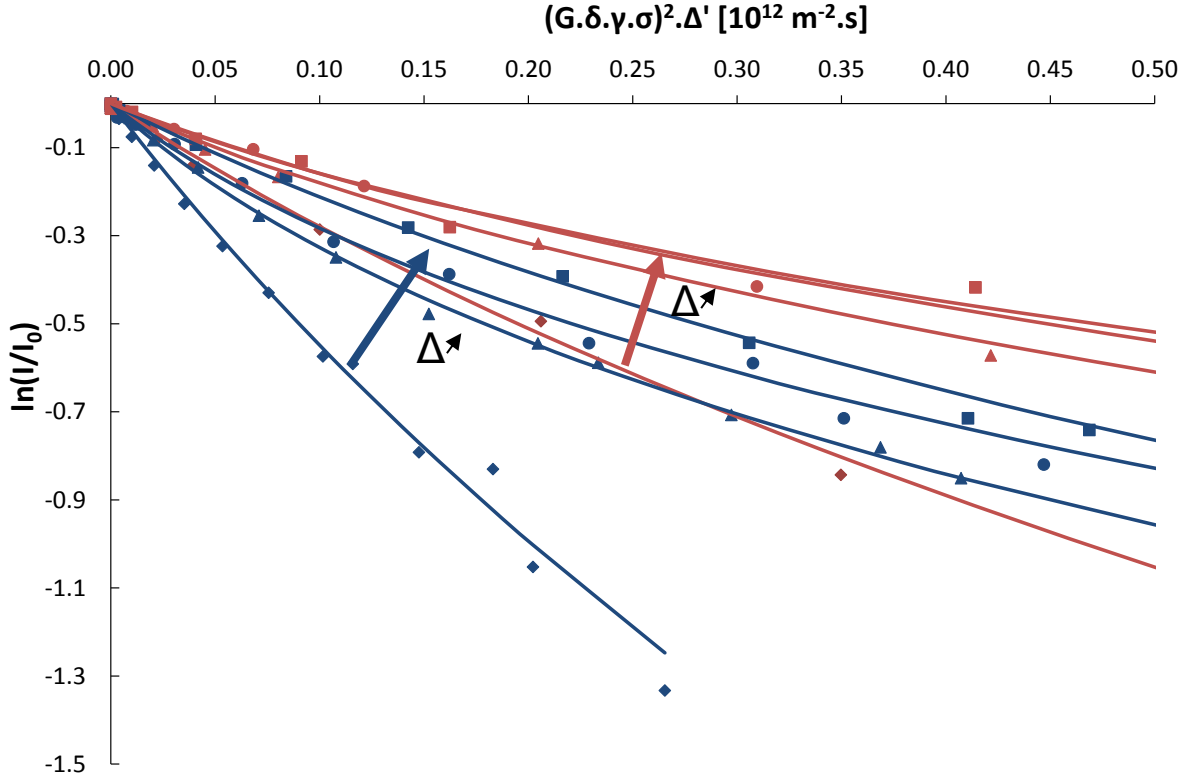


Figure 2.3: Echo decay curves of the w/o emulsion prepared at 6500 rpm with 10 wt% PGPR in the oil phase (UT 6500-10wt); the used gradient pulse duration was equal to 2.5 (blue) or 8 ms (red) while the diffusion delay (Δ) was set to 60 (diamonds), 120 (triangles), 180 (circles) or 240 ms (squares). Lines represent the fit of the Murday-Cotts model to the data.

As shown in Figure 2.3, the echo decay curves of the (coarse) UT 6500-10wt sample depended on the used diffusion delay and gradient pulse duration, which is expected for hindered diffusion. Fitting of the Murday-Cotts model led to a D_{43} of about 4 μm regardless of the used settings (Figure 2.4).

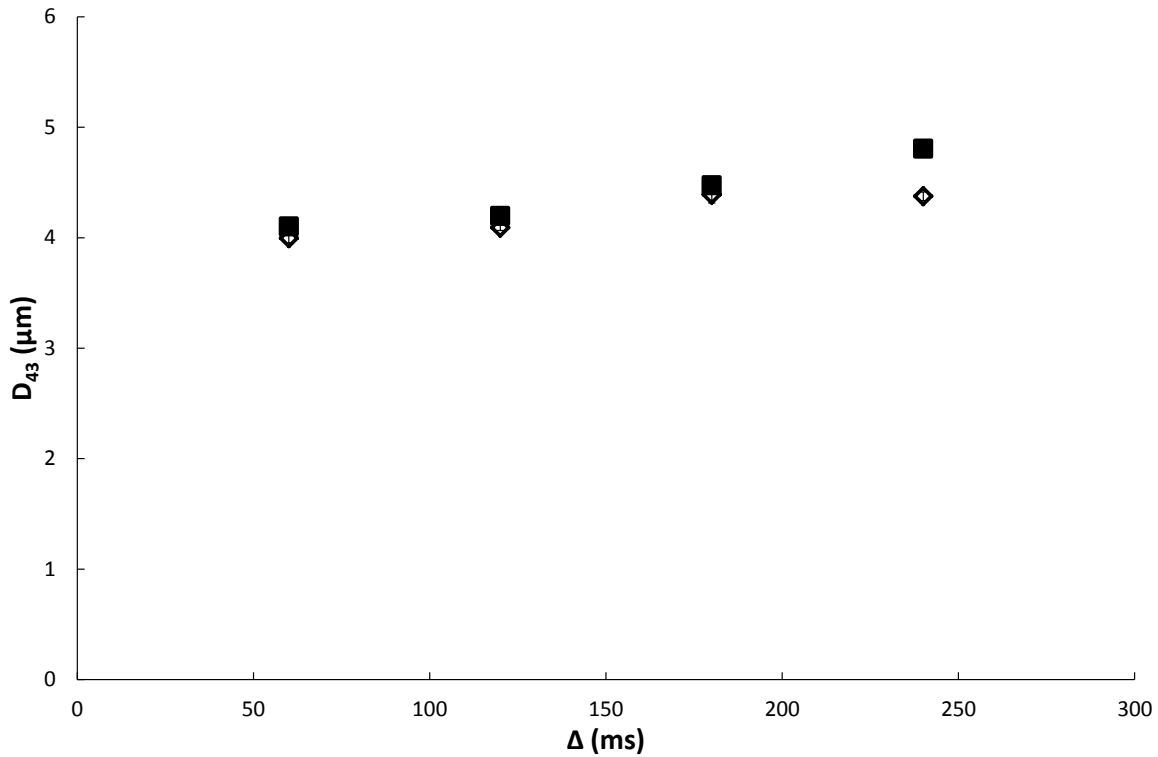


Figure 2.4: Volume weighted average diameter (D_{43}) of the w/o emulsion prepared at 6500 rpm with 10 wt% PGPR in the oil phase (UT 6500-10wt) as a function of the used diffusion delay (Δ), obtained from fitting the Murday-Cotts model to the echo decay curves which are shown in Figure 2.3. Diamonds represent the result for a gradient pulse duration (δ) of 2.5 ms while the squares represent the result for a gradient pulse duration of 8 ms. If no error bar is visible for a data point, the standard error is smaller than the used symbol.

The initial echo decay signal of the (fine) MF 840-10wt sample did not depend on the used diffusion delay and gradient pulse duration, which is typical for free diffusion (Figure 2.5). Also, from a certain gradient strength onwards, an increase of the slope can be seen when a diffusion delay of 60 ms was used. This indicates that eddy currents contributed to the signal; hence, diffusion delays smaller than or equal to 60 ms should be avoided.

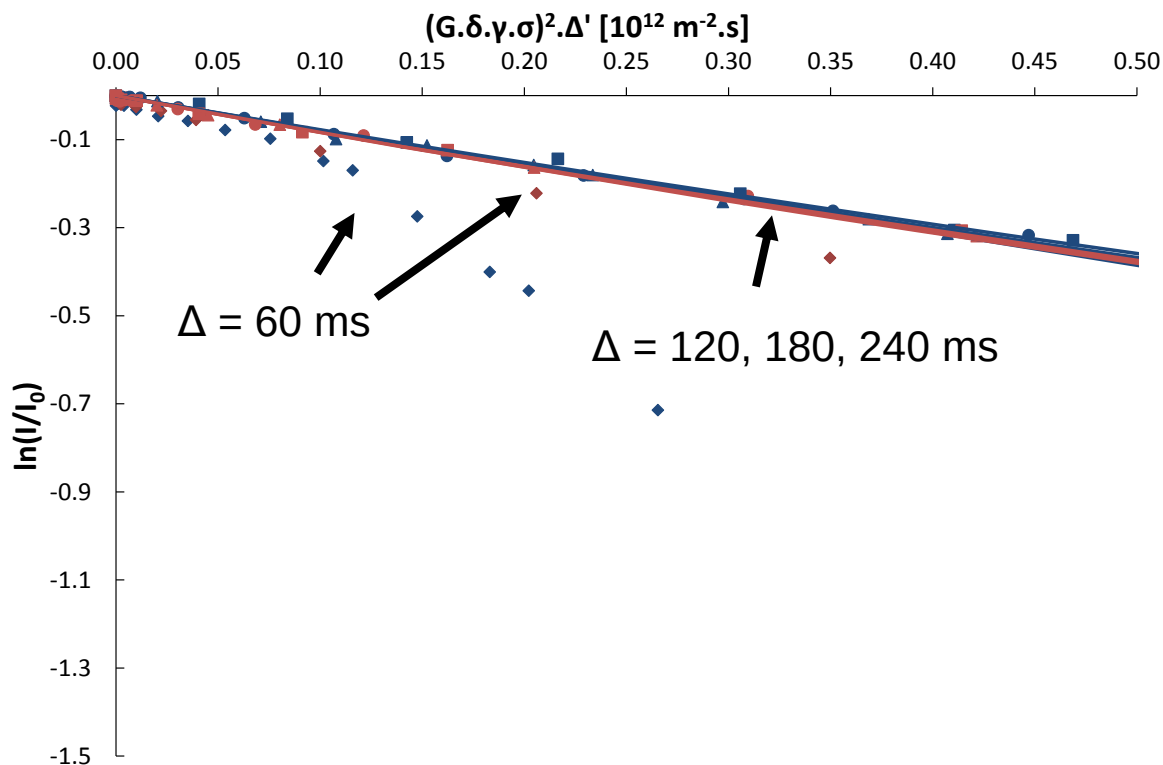


Figure 2.5: Echo decay curves of the w/o emulsion prepared using a Microfluidizer at 840 bar with 10 wt% PGPR in the oil phase (MF 840-10wt); the used gradient pulse duration (δ) was equal to 2.5 (blue) or 8 ms (red) while the diffusion delay (Δ) was set to 60 (diamonds), 120 (triangles), 180 (circles) or 240 ms (squares). Using a diffusion delay of 60 ms, from a certain gradient strength onwards, an increase of the slope is observed, attributable to eddy currents. The coinciding lines represent the fit of the Murday-Cotts model to the data obtained using diffusion delays of 120 ms or higher.

2.3.2 Comparison of low- and high-resolution PFG-NMR diffusometry

As described in part 2.3.1.1, low-resolution PFG-NMR diffusometry was not capable of detecting a difference between samples that did, however, contain droplets with a different droplet size distribution as indicated by light microscopy, dynamic light scattering as well as T_2 -relaxometry (part 2.3.3). Moreover, variation of the used diffusion delay and gradient pulse duration indicated that free instead of hindered diffusion occurred in the sample that was prepared at the highest shear intensity (part 2.3.1.2). Therefore, a set of new experiments was conducted in which low- and high-resolution PFG-NMR diffusometry were compared to confirm these observations and determine their cause.

Figure 2.6 shows that the echo decay curves of the samples, prepared using a higher shear intensity (and thus containing smaller droplets), coincided for small values of the ordinate. These small values may be either obtained by weak gradient strengths at long gradient pulse duration (i.e. 8 ms) or by stronger gradient strengths at short gradient pulse duration (i.e. 2.5 ms). As such, the indicated region corresponds to the whole x-axis interval that was considered during the LR-experiments shown in Figure 2.2. Hence, using the LR-NMR settings ($\delta=2.5$ ms), the two decay curves would coincide, as was the case with four of the six samples in Figure 2.2. However, using a δ of 8 ms, the decay was more pronounced and a difference between both samples could be distinguished, even with LR-NMR. As mentioned previously, independence of the echo decay curve from the used gradient pulse duration is a typical property of free diffusion, which implies free diffusion dominated the echo decay curve of fine emulsions for small values of the ordinate.

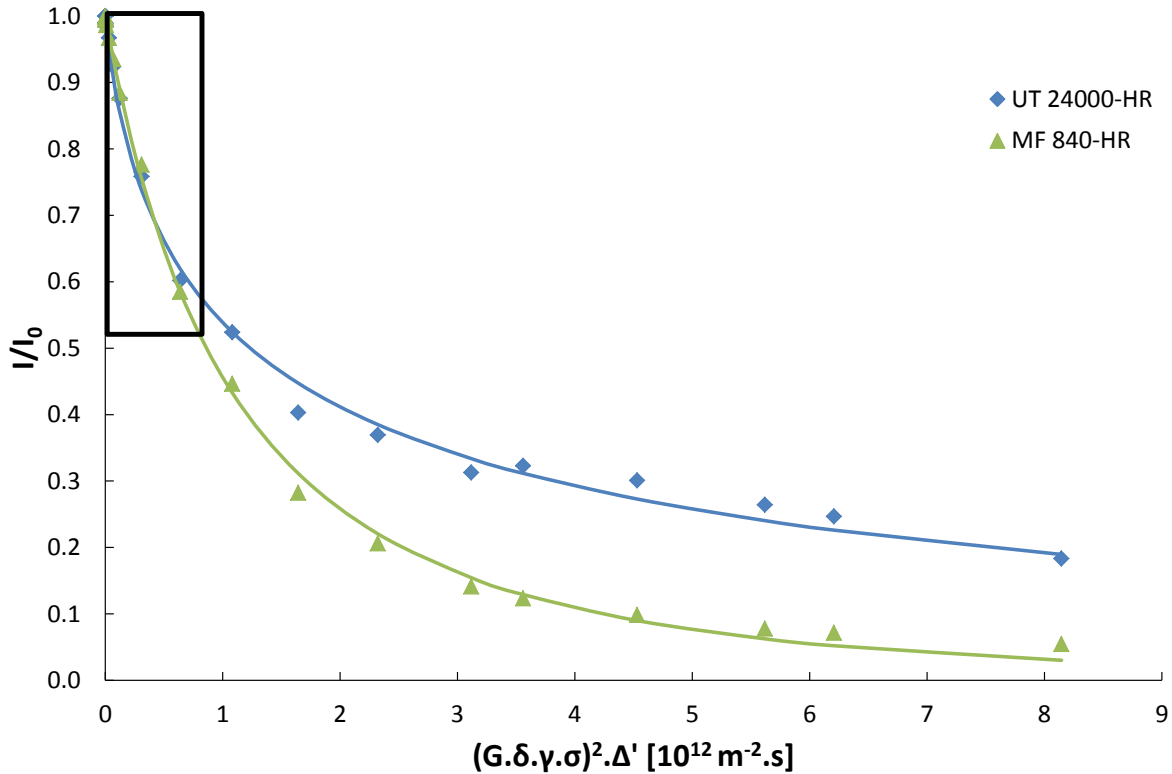


Figure 2.6: Echo decay curves of the water phases (LR-NMR) of the w/o emulsions prepared using different shear intensities. The used gradient pulse duration (δ) was equal to 8 ms while the diffusion delay (Δ) was set to 180 ms. The rectangle indicates the experimentally accessible window for LR-NMR measurements at $\delta=2.5$ ms. Lines represent the fit of the Murday-Cotts model to the data. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the code HR for the use of TMACI as salt (and 10 wt% PGPR in the oil phase).

As shown in Figure 2.6, a faster echo decay was recorded at bigger values of the ordinate for the sample which was prepared using the highest shear (MF 840-HR). This indicates that free diffusion contributed more to the signal of this sample as compared to the sample produced at lower shear (UT 24000-HR). In order to examine this effect, high-resolution PFG-NMR was conducted on the same samples, during which the diffusion behavior of water as well as an oil insoluble marker (TMACI) was followed separately (Figure 2.7).

As shown in Figure 2.7, the echo decay curves of the water phase, as determined using low-resolution NMR, and of the water molecules, as determined using high-resolution NMR, nearly coincided for the samples under consideration. This close resemblance proves that the inversion recovery sequence which was applied to remove the oil contribution from the LR-NMR data was effective and hence the LR-NMR data indeed reflected the behavior of water. Moreover, it shows that the echo decay curves which were obtained using different devices (high- and low-resolution NMR) were comparable.

Figure 2.7A shows that in the sample containing the biggest water droplets, TMACI and water have a similar decay profile, which indicates that their diffusion behavior is determined by the size of the restriction within which they can diffuse. The most interesting observation, however, is that for smaller droplets (Figure 2.7B and C), the echo decay curves of TMACI strongly deviate from the echo decay curves of the water molecules. This shows that, for these samples, the average molecular diffusion of the water molecules is not restricted to the same extent as the diffusion of the TMACI-molecules.

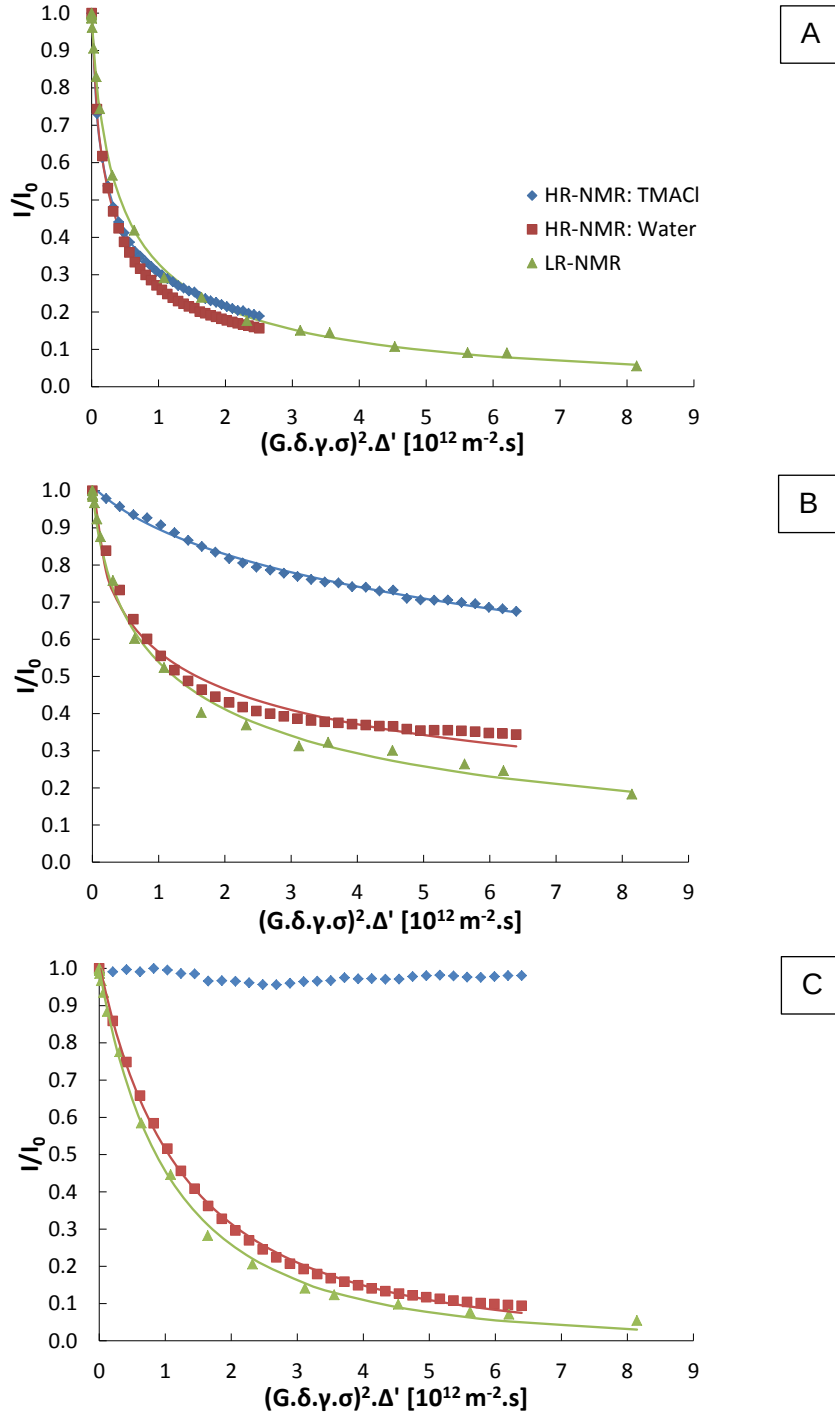


Figure 2.7: Echo decay curves of water and TMACI (HR-NMR) or of the water phase (LR-NMR) of the w/o emulsions prepared using different shear intensities. The used gradient pulse duration (δ) was equal to 8 ms while the diffusion delay (Δ) was set to 180 ms for the LR-NMR measurement. A) UT 6500-HR B) UT 24000-HR and C) MF 840-HR. Lines represent the fit of the Murday-Cotts model to the data. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the code HR for the use of TMACI as salt (and 10 wt% PGPR in the oil phase).

Table 2.3 shows the obtained volume weighted average diameter (D_{43}) and standard deviation of the volume weighted distribution (σ_v) for the different samples when the Murday-Cotts model was fitted to the obtained echo decay curves. It should be mentioned that for the MF 840-HR sample, fitting to the echo decay of TMACl did not yield satisfactory results as almost no decay of the echo signal was recorded (Figure 2.7C). However, from simulations of the decay profile as a function of droplet size (not shown), it follows that all droplets in this sample have to be smaller than about 0.5 μm in diameter.

Table 2.3: Volume weighted average diameter (D_{43}) and standard deviation of the volume weighted droplet size distribution (σ_v) of the w/o emulsions, each $\pm$ standard error, as obtained from fitting the Murday-Cotts model to the echo decay data of either water or TMACl in HR-NMR. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the code HR for the use of TMACl as salt (and 10 wt% PGPR in the oil phase).

	D_{43} (μm)		σ_v (μm)	
	Water diffusion	TMACl diffusion	Water diffusion	TMACl diffusion
UT 6500-HR	4.87 $\pm$ 0.02	4.60 $\pm$ 0.01	2.90 $\pm$ 0.03	3.65 $\pm$ 0.02
UT 24000-HR	3.29 $\pm$ 0.06	1.28 $\pm$ 0.01	2.70 $\pm$ 0.14	0.74 $\pm$ 0.07
MF 840-HR	2.94 $\pm$ 0.02	<0.50	0.67 $\pm$ 0.03	-

Table 2.3 thus shows that similar results were obtained when the diffusion of either TMACl or water was considered for the emulsion with the largest water droplet size. However, for the samples subjected to higher shear, a significant difference in resulting droplet size distribution parameters was observed based on either the water or TMACl echo decay curves. Indeed, the water decay and thus derived droplet size was hardly affected by further processing whereas the TMACl decay and derived size was. Moreover, an apparent lower limit of about 3 μm is observed based on water diffusometry. This is not in line with light microscopy, where droplets can hardly be observed in the samples processed at higher shear, indicating submicron droplets. On the other hand, the size information derived from the TMACl decay curve is much more in line with other observations. Indeed, dynamic light scattering measurements show a difference between all of the samples (Table 2.4). Hereby, it should be mentioned that the results of the UT 6500-HR sample are less reliable due to the high polydispersity.

Table 2.4: Z-average diameter and polydispersity index of the w/o emulsions, each $\pm$ standard deviation, determined after dilution in 1-bromododecane. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the code HR for the use of TMACI as salt (and 10 wt% PGPR in the oil phase).

Sample	Z-average diameter (nm)	Polydispersity index (-)
UT 6500-HR	630 $\pm$ 7	0.775 $\pm$ 0.083
UT 24000-HR	360 $\pm$ 6	0.424 $\pm$ 0.003
MF 840-HR	172 $\pm$ 2	0.147 $\pm$ 0.004

The observed difference in diffusion behavior between water and TMACI in small emulsion droplets might be explained by the higher solubility of water in the surrounding oil phase for smaller water droplets, called the Kelvin effect (Davis et al., 1981). Moreover, smaller droplets have a higher surface area, therefore a higher amount of collisions will occur between the water molecules inside the droplets and the droplet interface during the diffusion delay. Indeed, the amount of collisions is directly proportional to the specific surface area of the droplets. The Murday-Cotts model assumes that the droplet boundaries are impermeable which implies that all collisions between water and the interface should lead to reflection of the water molecules. However, part of these collisions might be non-reflecting. As a conclusion, it can be stated that the smaller the water droplets are, the higher the number of collisions between water and the interface will be and the higher the solubility of water in oil. The combination of both effects strongly increases the chance of the occurrence of a non-reflecting collision during the diffusion delay. When a non-reflecting collision occurs and water molecules leave the water droplet, they diffuse freely in the oil phase which leads to the observation of echo decay curves typical for free diffusion (i.e. independent from diffusion delay and gradient pulse duration).

From previous research, it is known that reduction of the diffusion delay might help in getting less contribution of the freely diffusing compound (Vermeir et al., 2016). However, similar (apparent) average droplet diameters were obtained at a diffusion delay of 60 ms. Hence, this value still seemed to be insufficient to solve the problem. Moreover, using this shorter diffusion delay, a contribution due to eddy currents became visible in the decay curves at higher gradient strengths (more than ± 1.5 T/m): at high ordinate values, the slope of the decay curves increased (Figure 2.5). In addition, to get to a decay of the same extent, the gradient pulse strength and/or duration will have to be increased. Finally, reducing the diffusion delay leads to a reduction in size of the biggest droplets that can be determined.

Trying to correct for free diffusion (fast decay in the echo decay curve) to get a droplet size estimate from the part with slow decay is also not feasible as the slow decay will be governed by the bigger droplets in the sample and hence will give rise to an overestimation. The reason is twofold: firstly, as will be described in chapter 6, the amplitude of the echo decay signal is linked to the relaxation time. As water inside bigger droplets has a higher relaxation time (Figure 2.9), this will lead to a larger contribution of these droplets to the echo decay curve. Moreover, as discussed previously, the number of collisions between the water molecules and the interface is lower in bigger droplets as compared to smaller droplets, which decreases the contribution of free diffusion to the echo decay curve of bigger droplets.

Diffusion of water in the emulsions containing small droplets thus seems to be a combination of free diffusion through the oil phase and restricted diffusion within the droplet. Free diffusion seems to be the dominating effect in the first 5 points of the echo decay curves of the MF 840-HR and UT 24000-HR samples, as indicated by the fact that both the diffusion delay and gradient pulse duration do not influence the obtained echo decay in this region. Based on the data in this region, a diffusion coefficient (D) can be determined using linear regression of the natural logarithm of the echo decay signal and the appropriate Stejskal-Tanner equation (Sinnaeve, 2012). This leads to a diffusion coefficient of $6.50 \cdot 10^{-13} \text{ m}^2/\text{s}$ and $6.14 \cdot 10^{-13} \text{ m}^2/\text{s}$, for the MF 840-HR and UT 24000-HR samples, respectively. If the same principle is used for the decay profiles of the 4 smallest w/o samples shown in Figure 2.2, a similar diffusion coefficient of $(6.75 \pm 0.01) \cdot 10^{-13} \text{ m}^2/\text{s}$ could be calculated.

Interestingly, when the diffusion coefficient of PGPR in a 10 wt% PGPR in sunflower oil solution was determined, it was found to be equal to $6.16 \cdot 10^{-13} \text{ m}^2/\text{s}$. Thus, as shown in Figure 2.8, the echo decay curves of PGPR in sunflower oil and water in either the MF 840-HR or UT 24000-HR sample coincided for low gradient pulse strengths. This shows that in fact the water molecules are moving at the speed of PGPR and thus hydrated PGPR molecules are leading to the observed free-diffusion component in the echo decay curve of water.

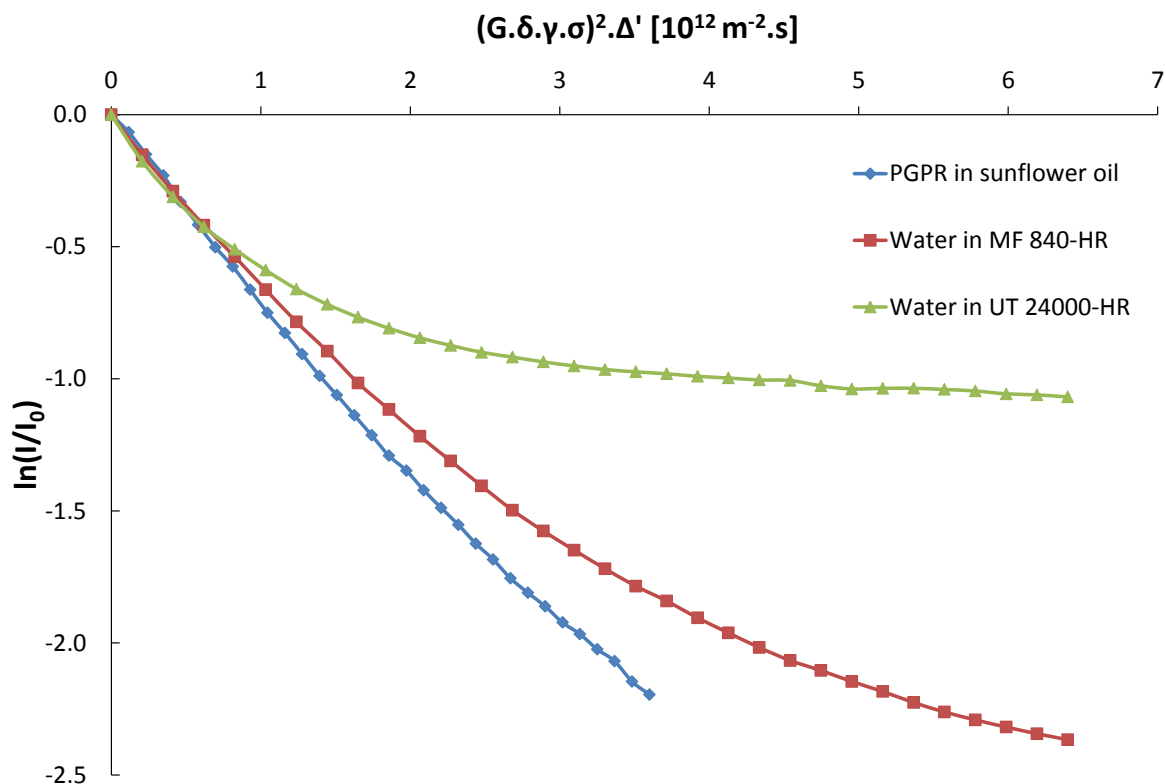


Figure 2.8: HR-NMR echo decay curves of 10 wt% PGPR in sunflower oil, water in the MF 840-HR sample and water in the UT 24000-HR sample. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the code HR for the use of TMACI as salt (and 10 wt% PGPR in the oil phase).

Considering the viscosity of the used oil phase at 5 °C (about 0.25 Pa.s), and using the Stokes-Einstein equation, the hydrodynamic diameter of the diffusing PGPR-species is calculated to be about 2.6 nm. This small diameter implies that the PGPR-diffusion speed is mainly governed by monomers of PGPR as this size corresponds better to the size of a PGPR molecule than of a micelle. Indeed, Orfanakis et al. (2013) measured a hydrodynamic diameter of PGPR monomers in toluene of about 8.8 nm. The fact that the measured hydrodynamic diameters do not completely correspond can be attributed to the different solvent system (toluene instead of sunflower oil) and measurement technique.

2.3.3 T_2 -relaxometry

The T_2 -distributions of the emulsions clearly separate the contribution of the oil protons (T_2 -relaxation time < 120 ms) from the contribution of the water protons (T_2 -relaxation time > 700 ms). Figure 2.9 shows the cumulative T_2 -relaxation time distributions for the water droplets in the w/o emulsions, which were prepared using different shear intensities. The shift of the T_2 -

relaxation time distribution towards lower values increased with increasing shear intensity during production, thus with decreasing droplet size. The fact that the sensitivity of the T_2 -relaxation time distribution towards the droplet size distribution strongly increased with decreasing droplet size is typically observed for emulsions with a low surface relaxivity. Indeed, the relaxation time does not vary linearly as a function of the droplet size but is only highly influenced by the droplet size from a certain size downwards, as can be observed from the limits of equation 2.2. Considering large values of the radius (a), the right-hand side of equation 2.2 approaches 0 and hence the T_2 -relaxation time of coarse emulsions approaches the T_2 -relaxation time of the bulk water phase. As the droplet radius becomes smaller, the right-hand side approaches $3 \cdot \rho_2/a$, which means that T_2 -relaxation time tends to zero for extremely small droplets and linearly increases with droplet radius with a slope of $1/(3 \cdot \rho_2)$.

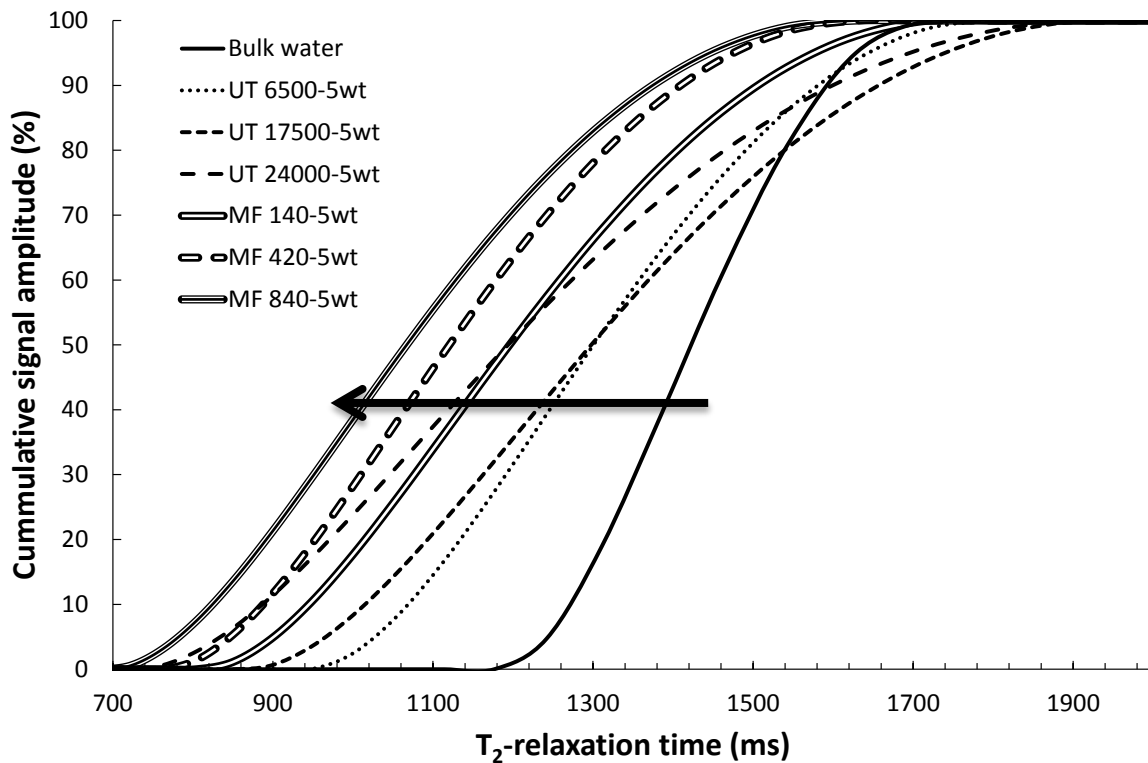


Figure 2.9: T_2 -relaxation time distributions of the water droplets in the w/o emulsion samples prepared using different shear intensities. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the used PGPR concentration in the oil phase.

Equation 2.2 indicates that, using the T_2 -relaxation time distribution of the samples, it is possible to determine the droplet size distribution. However, the surface relaxivity parameter (ρ_2) has to be known for the specific emulsion composition which requires independent sizing experiments. The use of PFG-NMR diffusometry in combination with T_2 -relaxometry in order to obtain the surface relaxivity parameter (ρ_2) has been described in literature (Amani et al., 2016; Peña and Hirasaki, 2003). However, our results indicate that for our samples, only from a certain size downwards, T_2 -relaxometry is capable of accurately determining a difference in droplet size distribution. In contrast, PFG-NMR is capable of determining a difference in droplet size only from a certain size upwards. The combination of both T_2 -relaxometry and PFG-NMR diffusometry in order to determine the surface relaxivity of the samples is therefore not feasible for these samples as the region of sensitivity towards the droplet size does not sufficiently overlap between both techniques. However, increasing the surface relaxivity might increase the droplet size range for which T_2 -relaxometry is sensitive, which might enable an overlapping droplet size range for T_2 -relaxometry and PFG-NMR diffusometry.

The addition of paramagnetic compounds to the oil phase might aid in increasing the surface relaxivity. Also, measuring at a higher temperature may increase the difference in T_2 -relaxation time between bulk and restricted water.

As shown in Figure 2.10, the difference in T_2 -relaxation time between the water droplets in either a UT- or MF-processed w/o emulsion and the bulk water phase indeed increased with increasing temperature. Hereby, the mode of the T_2 -distribution became less sensitive towards a temperature increase when the water droplets were smaller.

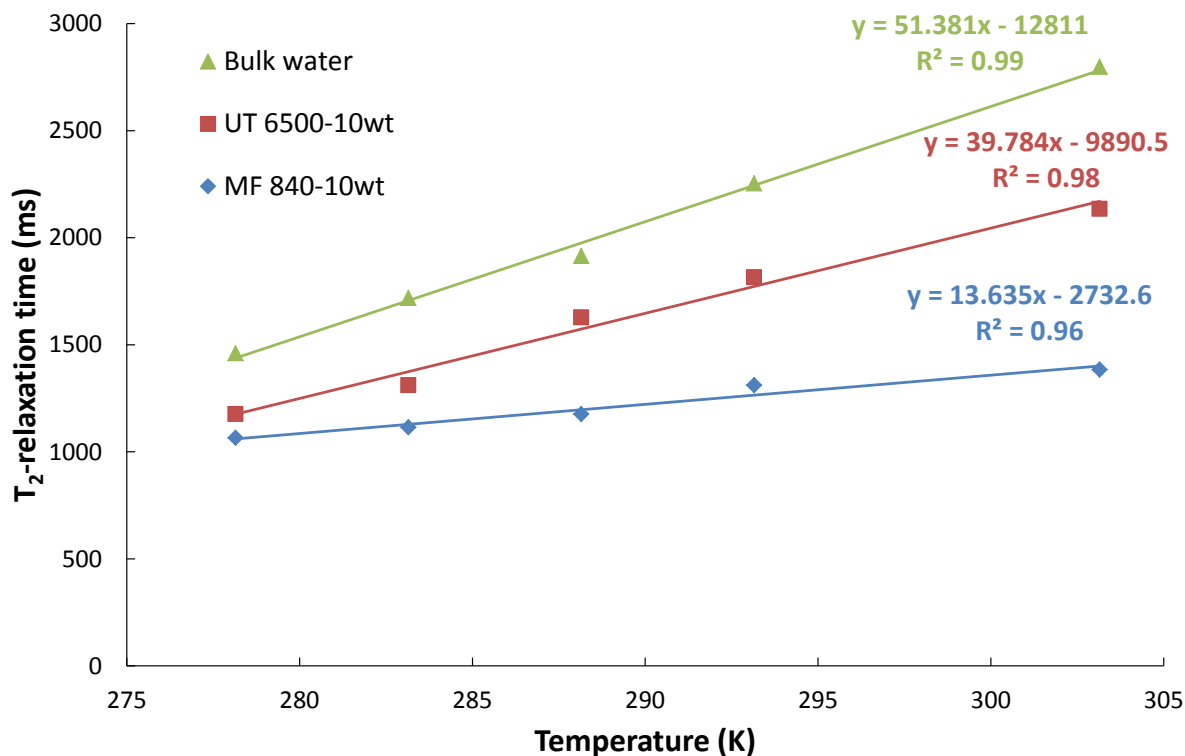


Figure 2.10: Influence of temperature on the mode of the T_2 -relaxation time distribution of the water phase and of the water droplets in the UT 6500-10wt and MF 840-10wt emulsions. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the used PGPR concentration in the oil phase.

However, the droplet size is linked to $1/T_{2,corr}$ and there is no strong effect of temperature on $1/T_{2,corr}$, which indicates that the surface relaxivity is not strongly temperature dependent (Figure 2.11). The average $1/T_{2,corr} \pm$ standard deviation in the temperature interval under consideration was 0.32 ± 0.04 and 0.13 ± 0.04 1/s for the MF 840-10wt and UT 6500-10wt samples, respectively.

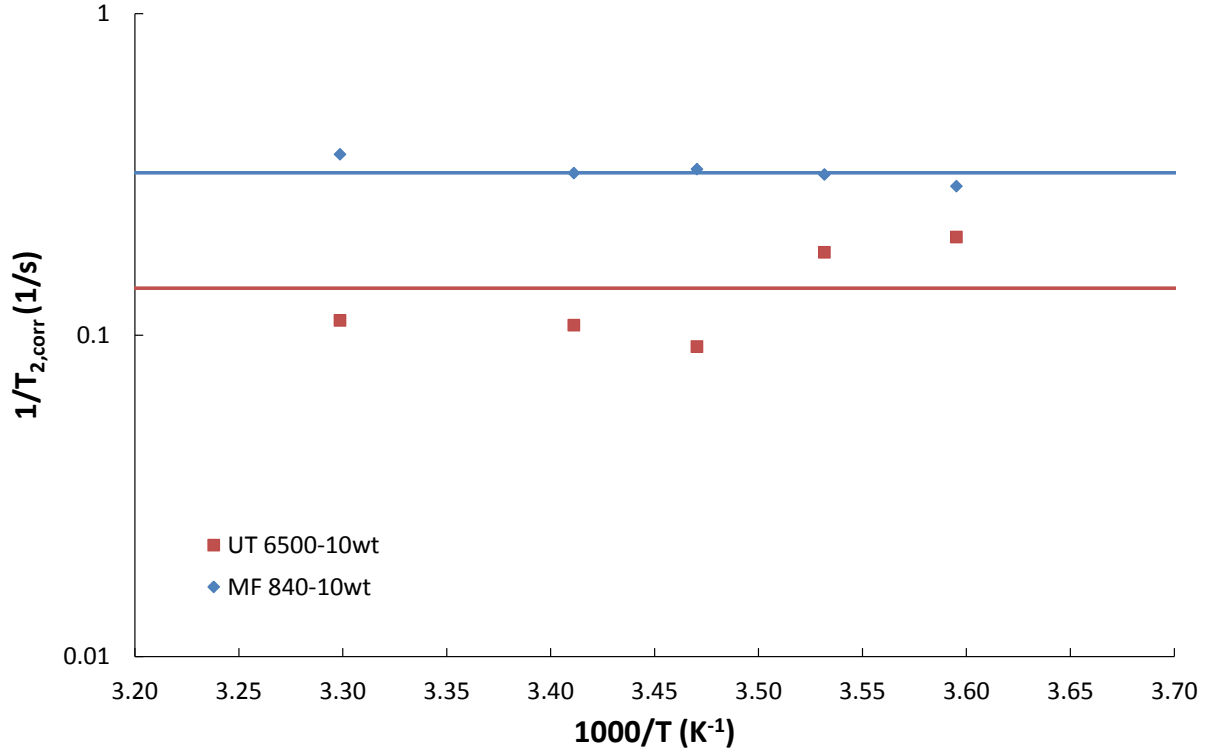


Figure 2.11: Influence of temperature on $1/T_{2,corr}$ of the water droplets in the UT 6500-10wt and MF 840-10wt emulsions. Codes represent the used emulsification device (UT = Ultra-Turrax, MF = Microfluidizer) followed by the stirring speed (UT) or the homogenization pressure (MF) and the used PGPR concentration in the oil phase.

From the aforementioned relaxometry measurements, it can be stated that conducting measurements at a higher temperature will increase the shift of the T_2 -relaxation time distribution towards lower values, relative to the T_{2B} -relaxation time, especially for small droplets. Therefore, the detection of droplet size distribution differences using T_2 -relaxometry should be conducted at a sufficiently high temperature. However, the droplet size itself is linked to $1/T_{2,corr}$ which is less sensitive towards changes in temperature.

It should be noted that, for the w/o emulsions which contain small water droplets, the ratio between their surface weighted average droplet sizes (D_{32}) can be calculated using the ratio between the $1/T_{2,corr}$ values. Indeed, these samples have the same composition and thus the same ρ_2 . Moreover, it is reasonable to use the surface limited scenario ($\frac{\rho_2 * a}{2 * D} \ll 1$) for droplet radii smaller than 3 μm , translational diffusion coefficients of the water molecules in the order of $10^{-9} \text{ m}^2/\text{s}$ and typical values for the surface relaxivity in the order of some $\mu\text{m}/\text{s}$. The surface

weighted average droplet size ratio between two samples (a and b) can therefore be calculated based on their average (corrected) T_2 -relaxation time using equation 2.3.

$$\frac{D_{32,a}}{D_{32,b}} = \frac{\frac{1}{T_{2,corr,b}}}{\frac{1}{T_{2,corr,a}}} = \frac{T_{2,corr,a}}{T_{2,corr,b}} \quad (\text{Equation 2.3})$$

Using equation 2.3, the surface weighted average droplet size (D_{32}), relative to the surface weighted average droplet size of the MF 140-5wt sample, can be calculated for the MF 420-5wt and MF 840-5wt samples. This gives a ratio of 0.77 and 0.63 for the MF 420-5wt and MF 840-5wt samples, respectively. This corresponds quite well to the ratio between the Z-average diameters of these samples relative to the Z-average diameter of the MF 140-5wt sample (Table 2.2), which equal 0.72 and 0.55, respectively.

2.4 Conclusions

In a previous study (Vermeir et al., 2016) the effect of the measurement temperature on the accuracy of droplet size determination of w/o emulsions, based on water NMR diffusometry, was determined. It was shown that when the temperature increased, also the measured droplet size increased when water diffusion was followed. In contrast, a constant droplet size distribution was determined when the diffusion of the ionic marker tetramethylammonium chloride was followed instead. This difference in diffusion behaviour was attributed to an increasing contribution of water diffusion through the oil phase when increasing the measurement temperature. Therefore, it was concluded that PFG-NMR water diffusometry should be conducted at a sufficiently low temperature. Because, like temperature, the water droplet size also influences the solubility of water (Kelvin effect (Davis et al., 1981)), we examined whether a decreasing droplet size affects the droplet size distribution as determined using water NMR diffusometry.

It was shown that from a certain droplet size downwards, water exchange at the w/o interface is substantial enough to lead to the observation of free diffusion of water through the oil phase using PFG-NMR diffusometry. Under the experimental conditions used in this manuscript, the lower limit was found at about 3 μm . However, this limit will probably depend on the composition of the w/o emulsion. This implies that the generally used low-resolution PFG-NMR technique (Bernewitz et al., 2011; van Duynhoven et al., 2002; Vermeir et al., 2016) is incapable of accurately determining the droplet size distribution of samples that contain (sub)micron sized water droplets. Therefore, care should be taken when small droplets (< 3 μm) are expected in a

sample because the echo decay might be substantially influenced by free diffusion through the oil phase whereby fitting to this echo decay leads to erroneous (i.e. overestimated) results.

High-resolution NMR revealed that water diffusion occurred at a speed similar to that of PGPR-monomers. This indicates that (hydrated) PGPR is not irreversibly adsorbed at the w/o interface but is exchanging and diffusing through the oil phase. Therefore, the concentration of PGPR in the oil phase will probably influence the contribution of free water diffusion through the oil phase.

Determination of the echo decay curve of the oil insoluble marker tetramethylammonium chloride in the water phase extends the application window of PFG-NMR diffusometry towards smaller droplet sizes. However, in order to obtain a sufficient echo decay for (sub)micron droplets, very high gradient pulse durations and/or strengths are required. Moreover, the marker has to be added before preparation of the w/o emulsion which renders the use of PFG-NMR in the determination of droplet sizes in existing w/o emulsions, containing small droplets, impossible.

Another NMR-technique which has been used in the determination of w/o emulsion droplet size distributions is relaxometry (Amani et al., 2016; Godefroy et al., 2001; Peña and Hirasaki, 2003). However, this technique has not been tested on edible w/o emulsions and Peña and Hirasaki (2003) noted that these emulsions usually have low surface relaxivities, which makes the relaxation time distribution of the dispersed phase less sensitive towards changes in specific surface area (and thus droplet size). We measured the T_2 -relaxation time distributions of edible w/o emulsions containing droplets with different sizes and were able to show that T_2 -relaxometry could detect a difference between these samples.

It was also shown that, in order to increase the influence of the water droplet size on the T_2 -relaxation time distribution, measurements should be conducted at a higher measurement temperature. Measuring at a higher temperature increases the shift of the T_2 -relaxation time distribution towards lower values, relative to the bulk T_2 -relaxation time. However, the droplet size is linked to $1/T_{2,corr}$ which is less sensitive to changes in temperature. The addition of paramagnetic compounds to the oil phase might increase the surface relaxivity parameter and therefore increase the sensitivity of the relaxation time distribution towards changes in the droplet size distribution.

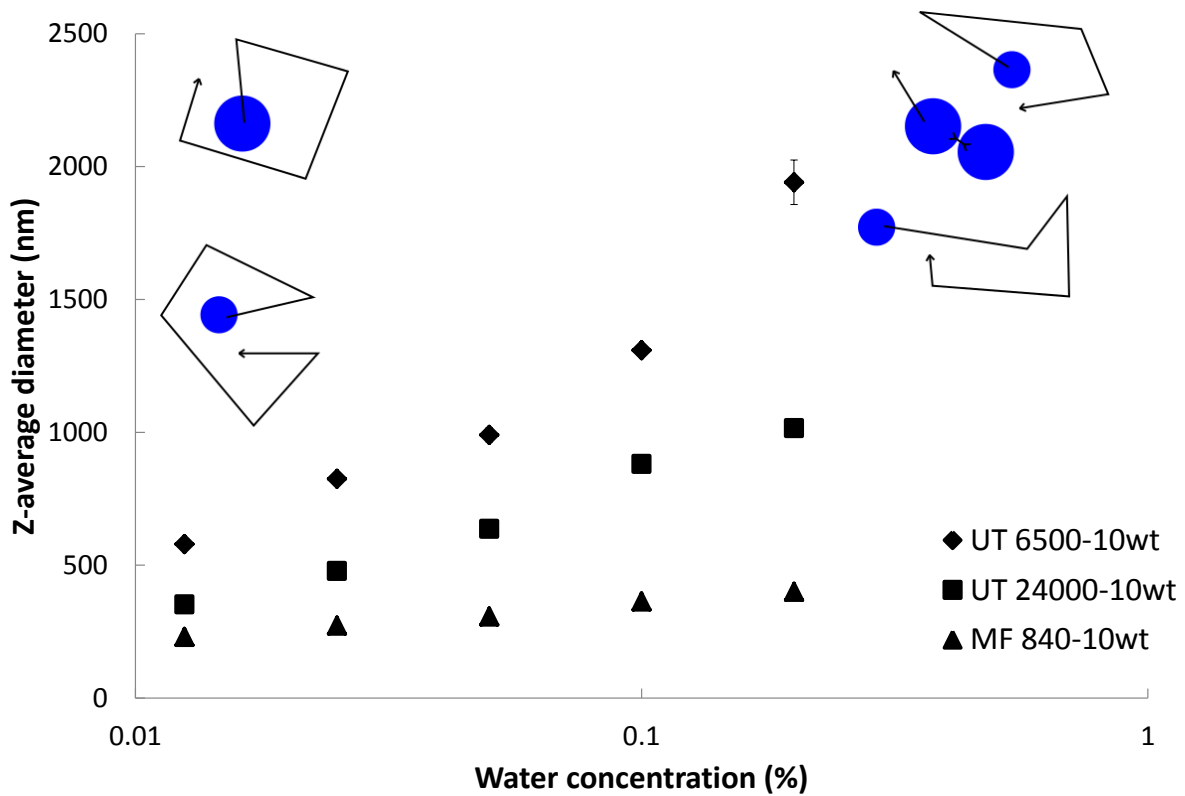
Finally, if T_2 -relaxometry is used to determine the droplet size distribution, an independent sizing technique is still necessary to determine the surface relaxivity parameter. A possible approach would be to determine the surface relaxivity for a w/o composition using an emulsion of which

the droplet size should be chosen in a region where both PFG-NMR and T_2 -relaxometry are sensitive towards droplet size changes. This region might also be increased by increasing the surface relaxivity using paramagnetic compounds. However, as shown in this chapter, the determination of the surface relaxivity can be omitted by dividing the corrected surface relaxation times of two samples by each other. This ratio equals the ratio of the surface weighted average droplet sizes of these samples. This approach can for example be used in quality control, during which the determination of the absolute droplet size is less important.

2.5 Acknowledgements

The 500 MHz NMR equipment is funded by the Hercules Foundation.

Chapter 3: Characterization of w/o emulsions using dynamic light scattering



- The method to measure (sub)micron sized w/o emulsions using dynamic light scattering was optimized
- Water saturation of the dispersion medium prevented dissolution of the water droplets
- The measured diameter was highly dependent on the droplet concentration

Abstract

As shown in the previous chapter, PFG-NMR water diffusometry was not capable of accurately determining the size of (sub)micron sized water droplets in liquid w/o emulsions. However, using dynamic light scattering (DLS), it was possible to detect a size difference between these samples. In order to optimize DLS to determine the water droplet size distribution of liquid w/o emulsions, different dilution liquids were tested. Also the effect of water solubility in the dilution medium, the effect of the measurement temperature and the effect of evaporation were examined.

Whereas DLS was indeed capable of detecting a droplet size difference between differently produced (sub)micron sized w/o emulsions, it was observed that the estimated droplet size was highly influenced by the specific measurement conditions. Firstly, interaction effects between water droplets gave rise to higher apparent diameters with increasing concentration. Therefore, the used concentration should be as low as possible. Moreover, the concentration which is used for the measurement should be mentioned. Secondly, due to the solubility of water in the dilution media, care should be taken when diluting the samples. Indeed, part of the sample can dissolve if the dilution medium is not (sufficiently) water saturated.

Summarizing, (sub)micron sized water droplets can be characterized using DLS. However, careful selection of the measurement and sample preparation conditions is necessary in order to obtain reliable data.

3.1 Introduction

Most droplet size analysis techniques require dilution of the sample, which might have an influence on the droplet size distribution (Bernewitz et al., 2011; van Duynhoven et al., 2002). Therefore, PFG-NMR diffusometry is frequently considered to be an ideal technique to measure the droplet size distribution since it does not require dilution of the sample. However, in the previous chapter, we showed that the diffusion behavior of water molecules cannot be used to determine the droplet size of liquid w/o emulsions in case (sub)micron sized water droplets are present. Indeed, the exchange of water between droplets leads to the measurement of free diffusion between droplets instead of hindered diffusion within droplets.

Chapter 2 also revealed that dynamic light scattering (DLS) could detect a size difference between w/o emulsions, which was not possible using water PFG-NMR water diffusometry. When w/o emulsions are characterized using light scattering, the used dilution medium often differs from the used oil phase as dilution in a viscous phase is difficult. For example, hexadecane (Choi et al., 2009; Yi et al., 2015), dodecane (Zhu et al., 2016) or dodecane containing 0.5 wt% sorbitan mono-oleate (Bonnet et al., 2009), have been used to dilute and subsequently analyze w/o emulsions. In this context, it should be mentioned that the used dilution medium has an influence on the interactions between water droplets. According to Leal-Calderon et al. (1997), changing the continuous phase can lead to non-flocculated w/o emulsions turning into flocculated ones or vice versa. This property can also be used as an advantage: as light scattering techniques will measure the size of the flocs if present (Van der Tuuk Opedal, 2009), diluting a flocculated sample into an appropriate dilution liquid might enable the determination of the size of the individual droplets. Finally, due to the fact that the Brownian motion of droplets is followed during a DLS-measurement, an additional advantage of diluting the w/o emulsions in a dilution medium with a lower viscosity is the fact that the diffusion speed of droplets is higher, which enables a lower measurement duration (Tscharnutter, 2006).

In this chapter, we first examined whether the limited solubility of water in the oil phase (Hilder, 1968) influences the measurement when the emulsion is diluted in the continuous oil phase. Therefore, we diluted the emulsion in an oil phase which had been saturated with water and compared the results with those obtained when the oil phase was not saturated with water.

Afterwards, the influence of the used dilution medium was checked: it was examined whether measuring in 1-bromododecane led to similar results as measuring in the continuous oil phase. As already mentioned in chapter 2, 1-bromododecane was selected as preferred dilution

medium because this solvent has a polarity which is similar to oil and a density which is similar to water. Moreover, the viscosity is smaller than the viscosity of the used oil phase (Boyson and Pashley, 2007; Cokelet et al., 1969). Due to the fact that the density difference between the continuous and dispersed phase is small, sedimentation is retarded during the measurement.

Finally, the optimal measurement conditions were used in the determination of the droplet size distribution of three w/o emulsions which were prepared using a varying shear intensity, thus having a different droplet size distribution. The results from DLS were hereby compared with qualitative information gained using cryogenic scanning electron microscopy (cryo-SEM).

3.2 Materials and Methods

3.2.1 Materials

The lipophilic emulsifier polyglycerol polyricinoleate (PGPR 4175) was kindly provided by Palsgaard A/S (Juelsminde, Denmark) and high oleic sunflower oil (Hoso; Iodine Value= 87; 82% C18:1) was acquired from Contined BV (Wageningen, The Netherlands). The water phase of the w/o emulsions contained 0.1M NaCl (VWR Chemicals: BDH Prolabo, Leuven, Belgium) and 0.02 wt% of the anti-microbial agent NaN_3 (Sigma-Aldrich, Steinheim, Germany).

As dilution media, high oleic sunflower oil (Contined BV, Wageningen, The Netherlands), 1-bromododecane (Alfa Aesar, Kandel, Germany) and dodecane (mixture of isomers, Acros Organics, Geel, Belgium) were used.

3.2.2 Preparation of the w/o emulsions

10 wt% PGPR 4175 was added to Hoso after which this phase was heated to 60 °C and stirred to obtain a homogeneous solution. The water phase was also heated to 60 °C after which the emulsions were prepared.

In a first production scheme, the 50/50 (m/m) emulsions were prepared using a rotor-stator homogenizer (Ultra-Turrax: type S25-10G, IKA®-Werke, Germany). Hereby, the water phase was added gradually during mixing. Two different mixing intensities were used namely 6500 and 24000 rpm. As in the previous chapter, these emulsions are referred to as UT 6500-10wt and UT 24000-10wt, respectively.

In a second production scheme, a premix was prepared using the same rotor stator homogenizer (Ultra-Turrax: type S25-10G, IKA®-Werke, Germany). Hereby, the water phase was added gradually during mixing at 24000 rpm. Afterwards, the emulsion was further processed using 1 pass through a Microfluidizer (type M110S, Microfluidics) at 840 bar during

which the temperature was kept at 60 °C using a heating bath. As in the previous chapter, this emulsion is referred to as MF 840-10wt.

3.2.3 Water saturation of dilution media

The dilution media were saturated with water by adding 20 % (v/v) of distilled water to 80 % (v/v) of the respective dilution media and end-over-end rotating of this mixture using a Stuart SB3 rotator (Cole-Palmer, Staffordshire, UK) for at least 4 hours at 20 °C and 2 rpm. Both phases were subsequently separated through centrifugation using a Sigma 3-16P device (Osterode am Harz, Germany) at 2000 g for 5 minutes. Most of the water was removed while a small volume was left to keep the dilution medium water saturated during storage at 20 °C.

To saturate the dilution medium at 30 °C, 20 % (v/v) of distilled water was shaken with 80 % (v/v) of the respective dilution media and placed in a water bath at 30 °C. The phases were shaken at least once an hour and kept in this state for at least 4 hours after which they were separated using centrifugation at 2000 g for 5 minutes; most of the water was removed after which the water saturated dilution media were stored at 30 °C.

3.2.4 Dynamic light scattering

Unless stated otherwise, the measurements were conducted at 20 °C with a Zetasizer 3000HS (Malvern Instruments, UK) device. An initial concentration of 0.2 % (m/v) of water in the dilution medium was prepared by diluting 24 mg of the w/o emulsions in 6 mL of the dilution medium after which subsequent dilutions were made by diluting 1-1 (v/v) with the same dilution medium. Unless stated otherwise, a cap was put on top of the cuvette. For every concentration, 3 subsequent measurements were conducted.

The average scattered intensity at 90° is expressed as kilocounts (kcounts) per second whereby the scattering intensity of the pure dilution medium is subtracted from the measured value. The correlogram was fitted using the monomodal option (cumulant analysis) whereby at least 25 kcounts per second were necessary in order to obtain repeatable measurements within an acceptable measurement time.

3.2.5 Characterization of the dilution media

In order to conduct a DLS-measurement, the viscosity and refractive index of the dilution medium is needed. These values were determined using a Brookfield LV-DV-II+ Pro viscometer with small sample adapter and spindle SC4-18 and a Officine Galileo 51510 refractometer,

respectively. Saturation of the dilution medium with water did not influence the measured parameters which are shown in Table 3.1.

Table 3.1: Viscosity and refractive index of the dilution media.

Dilution medium	Temperature (°C)	Viscosity (mPa.s)	Refractive index (-)
Hoso	20	82.58	1.469
1-Bromododecane	20	3.70	1.459
	30	3.02	1.455
Dodecane (mixture of isomers)	20	1.53	1.423

3.2.6 Cryo-SEM

Before analysis, the undiluted w/o emulsions were vitrified in liquid nitrogen and transferred to a PP3000T device (Quorum Technologies, East Sussex, UK) at -140 °C. The samples were sublimated for 45 min at -70 °C to remove the water from the water droplets. As the best images are obtained at horizontally cut surfaces, a top layer was removed from the sample using a knife before sublimation.

Before transferring the sample from the cryo-preparation to the SEM chamber, a thin layer of conductive material (Pt) was applied to the samples using argon gas. This sputter coating process prevents charging of the samples by the electron beam.

The droplets in the samples were visualized using a JSM-7100 F TTLS LV TFEG-SEM (JEOL Ltd, Tokyo, Japan) under high vacuum and a voltage of 3 keV.

3.2.7 Statistical analysis

The scattered light intensity (kcounts/s) as a function of water concentration (%) was analyzed using linear regression analysis which was performed using the Analysis ToolPak in Excel 2010.

3.3 Results and Discussion

In order to examine the effects of the measurement conditions on the DLS-measurements, the sample defined as UT 24000-10wt was used. Afterwards, the optimal measurement conditions were used to determine the droplet size of the samples which were prepared at different shear intensities.

3.3.1 Influence of water saturation of the dilution medium

Figure 3.1A shows the importance of water saturating high oleic sunflower oil when diluting the w/o emulsions. Indeed, considering the same degree of dilution, the scattered intensity was higher when the dilution medium was saturated with water.

If the dilution medium was saturated with water, measurements of the Z-average diameter were possible down to a water concentration of about 0.0125 %, whereas when the dilution medium was not saturated with water, the lower limit was found at about 0.05 %. The water solubility therefore seems to be slightly lower than the water solubility of 0.11 wt% in glycerol trioleate at 20 °C (Hilder, 1968). However, our water phase contained 0.1M NaCl while pure water was considered by Hilder (1968). Moreover, the oil phase which was not saturated with water was not dried prior to use and might have contained traces of water. Due to dissolution of the water droplets in the continuous phase, the measured droplet size at a given degree of dilution was lower in case the continuous phase was not saturated with water. However, even in case the dilution medium was saturated, there was still a limited solubility for water droplets which might be due to the small size of the droplets, which increases their solubility (Davis et al., 1981), or evaporation of water and subsequent dissolution of water droplets (see further).

Figure 3.1B shows that the water concentration has a big influence on the measured droplet size, which indicates the occurrence of interaction effects between the water droplets. Mettu et al. (2018) also recently measured and described the occurrence of an attractive interaction between PGPR-stabilized water droplets. When retracting one water droplet from the other, they measured an attractive minimum which they ascribed to short-range attractive interactions due to PGPR bridging between droplets when they are in close contact. Bridging between droplets can occur in case their surface is not fully covered by emulsifiers. Therefore, bridging is usually only observed in cases where a low emulsifier concentration was used. Unless desorption of PGPR occurred due to dilution of the w/o emulsions, the w/o emulsions which were prepared in this chapter can be expected to be fully covered by PGPR-molecules (Chapter 4). Another possible explanation of the observed attraction between water droplets is a stronger hydrophobic interaction between the polyricinoleate tails than between these tails and the continuous phase. In any case, the attractive energy only decreases the diffusion speed of the water droplets and does not lead to aggregation. As observed using light microscopy, water-in-sunflower oil emulsions stabilized using PGPR do not tend to aggregate (Figure 2.1). This implies that the thermal energy of the droplets is sufficient to overcome the attractive energy. The measurement of higher apparent diameters due to interaction effects between water droplets in water-in-oil

emulsions has also been observed by other researchers (Hamilton et al., 1990; Hou et al., 1988). In these works, micro-emulsions were considered and the interaction effects were less pronounced as compared to what we observed. However, both research works note that the magnitude of the interaction effects increases with increasing size of the droplets. This might again be linked to the higher thermal energy of smaller as compared to bigger droplets. Finally, it can be noted that Lim et al. (2013) observed a similar increase of the apparent diameter as ours with increasing concentration. These researchers were able to dilute their samples to a concentration at which the interaction effects were not influencing the diffusion anymore and a maximum concentration independent size of about 100 nm was obtained. In our measurements, further dilution was not feasible due to the limited difference in refractive index between continuous and dispersed phase, which led to a lower scattered intensity and a higher minimal concentration of droplets to perform a DLS-measurement. This means that we could not sufficiently dilute our emulsions to reach the region in which the measured droplet size became concentration independent. Figure 3.1B, however, shows that the influence of concentration was already lowered in the region below 0.025 wt%. The most accurate measurement of the droplet size distribution should therefore be found at the lowest possible concentration (i.e. 0.0125 wt% in this specific case).

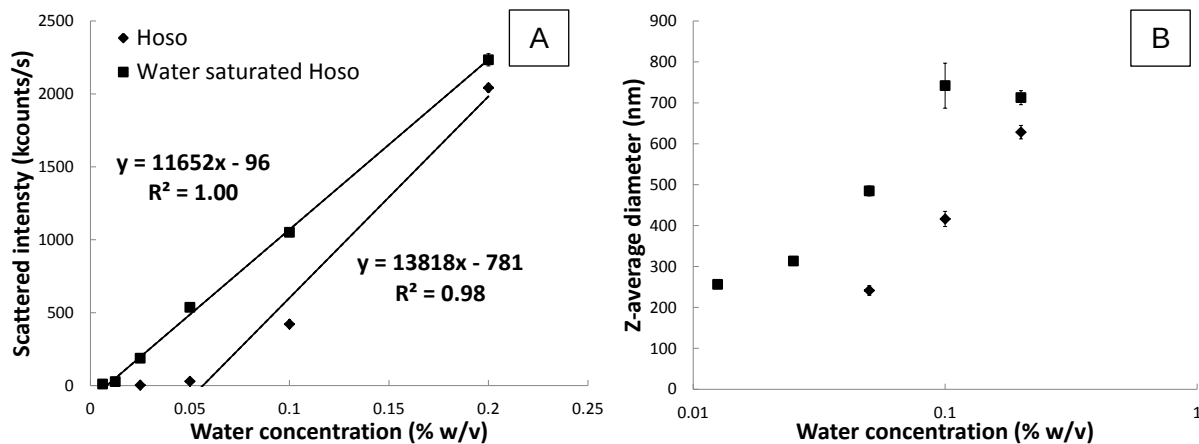


Figure 3.1: Scattered intensity (A) and estimated Z-average diameter (B) as a function of water concentration after dilution of the UT 24000-10wt emulsion in water saturated (squares) or non-saturated (diamonds) Hosos. If no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

By applying the principle which is used by Hou et al. (1988), it is possible to obtain a more accurate estimate of the Z-average diameter. These researchers determined the diffusion coefficient of water droplets without interaction effects (intrinsic diffusion coefficient) by measuring the diffusion coefficient of the droplets at different degrees of dilution and

extrapolating to infinite dilution. The diffusion coefficient is inversely proportional to the Z-average diameter (Lim et al., 2013). Therefore, extrapolation of the inverse of the Z-average diameter as a function of the water concentration to infinite dilution provides an estimate of the Z-average diameter without interaction effects. However, the linear part of the curve, at high dilution, has to be used in order to obtain the intercept at infinite dilution. Figure 3.2 shows that the curve only becomes linear at water concentrations below 0.05 %. This means that only 3 points could be considered in the regression analysis and very broad confidence intervals were obtained. Indeed, regression analysis revealed that with 95% certainty, the Z-average diameter (at infinite dilution) was between 176 and 308 nm. In order to obtain a more accurate estimate, more measurements should be conducted in the concentration range between 0.01 and 0.05 wt%. It should also be noted that the calculated Z-average diameter at infinite dilution (222 nm) was only slightly smaller than the Z-average diameter which was obtained when the DLS-measurement was conducted using a water concentration of 0.0125 % (256 nm). Therefore, measuring at 0.0125 % is a quick way to obtain a reliable estimate of the Z-average diameter.

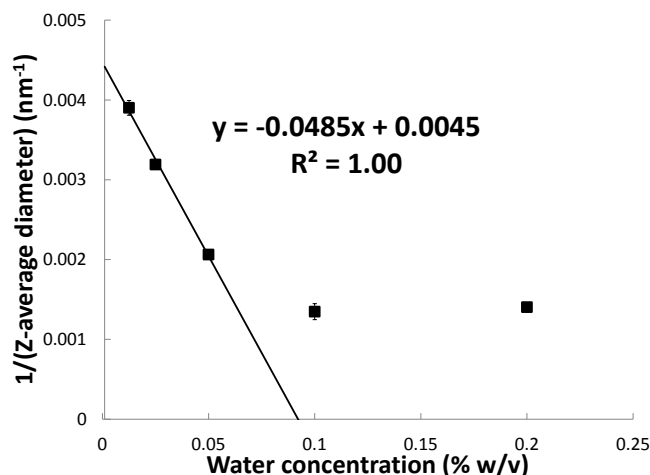


Figure 3.2: Inverse of the Z-average diameter as a function of water concentration after dilution of the UT 24000-10wt emulsion in water saturated Hosu. If no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

Because also in the linear regression analysis of the curve for scattered intensity as a function of water concentration for Hosu (Figure 3.1A), only 3 data points could be considered, broad confidence intervals were obtained for the slope and intercept. Both values included the slope and intercept of the curve obtained for water saturated Hosu. However, the statistical relevance of conclusions based on these very broad intervals is low. Looking at Figure 3.1A shows that there is mainly a shift in the curve; plotting the measured Z-average as a function of the scattered intensity indeed gives a good fit (Figure 3.3).

Considering the Z-average diameters as a function of scattered intensity (with scattered intensity below 1200 kcounts/s) for both dilution liquids a linear relation was found with determination coefficient of 0.99. The Z-average diameter levels off at higher scattered intensities which can probably mainly be attributed to multiple scattering. These results show that the dissolution of water does not really influence the measured droplet size distribution. Instead, an indirect effect is observed due to a difference in concentration of water droplets. Hereby, it should be mentioned that the presence of salt in the water phase probably avoids that the smallest droplets first dissolve, which would be expected from their higher curvature (Davis et al., 1981).

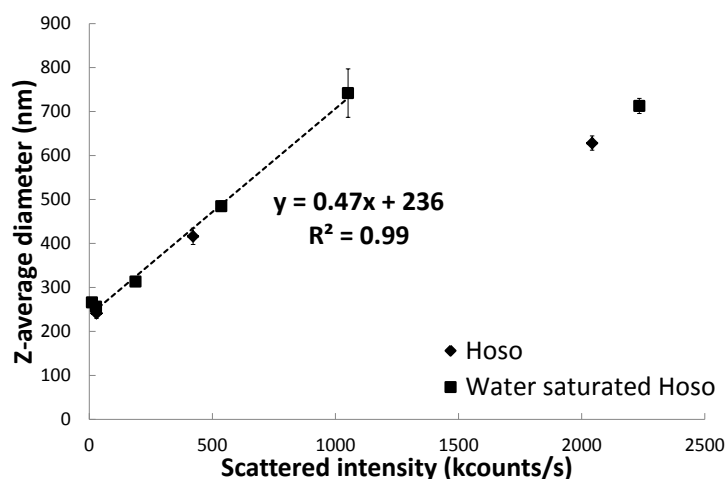


Figure 3.3: Estimated Z-average diameter of the UT 24000-10wt emulsion as a function of the scattered intensity for Hoso and water saturated Hoso as dilution liquids. Linear regression analysis was applied on the combined datasets of both dilution liquids with scattered intensities below 1200 kcounts/s. If no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

Figure 3.4A shows that water saturation was less crucial when 1-bromododecane was used as the dilution medium. This observation can be linked to a lower solubility of water in 1-bromododecane. However, Figure 3.4B clearly shows that similar interaction effects between droplets lead to a big influence of concentration on the measured Z-average diameter.

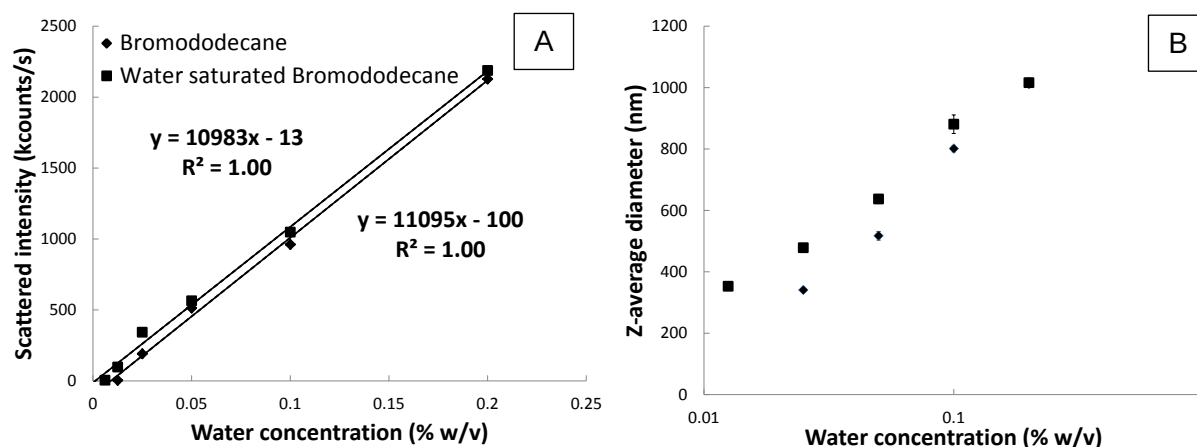


Figure 3.4: Scattered intensity (A) and estimated Z-average diameter (B) as a function of water concentration after dilution of the UT 24000-10wt emulsion in water saturated (squares) or non-saturated (diamonds) 1-Bromododecane. In case no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

3.3.2 Influence of water evaporation

Figure 3.5A shows a pronounced decrease of the measured scattered intensity as a function of time if the cuvette containing the sample with a concentration of 0.025 % in water saturated bromododecane was not closed. As this effect did not occur in a closed cuvette, evaporation of water and subsequent dissolution of water droplets was thought to be the reason of this observation. As shown in figure 3.5B, dissolution of water in the continuous phase also led to a decrease of the measured droplet size, in line with figure 3.4B. These measurements therefore show the importance of preventing the dissolution of water droplets in the continuous phase. Hereby, it is essential to keep the cuvette closed as well as to keep the headspace volume as small as possible in order to prevent evaporation of water from the dilution medium which induces further dissolution of the water droplets.

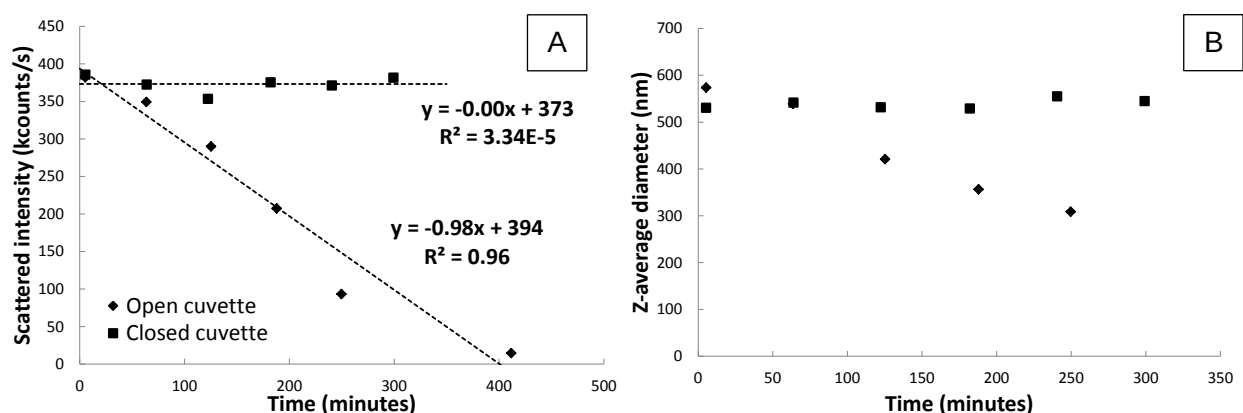


Figure 3.5: Scattered intensity (A) and estimated Z-average diameter (B) of 0.025 % (w/v) UT 24000-10wt in water saturated 1-Bromododecane as a function of time with an open (diamonds) or closed (squares) cuvette. If no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

3.3.3 Influence of the used dilution medium

Figure 3.6A clearly shows that the scattered intensity as a function of concentration for water saturated Hoso and water saturated bromododecane was similar. Indeed, the 95% confidence intervals of their slopes (10954-12350 and 10067-11900 (kcounts/s)/(% w/v) for water saturated Hoso and water saturated bromododecane, respectively) overlap. However, when water saturated dodecane was used as the dilution medium, the scattered intensity was much lower when the same concentration of droplets was considered. This effect can mainly be attributed to the smaller difference in refractive index between dodecane and water, which decreases the scattered light intensity. This effect, however, also led to the fact that the minimal concentration limit, at which reproducible DLS-measurements can be conducted, corresponding to a scattered light intensity of at least 25 kcounts/s, was found at about 0.025 % for dodecane while for bromododecane and Hoso, about 0.0125 % was sufficient.

Figure 3.6B shows that the measured droplet size as a function of concentration and therefore the interaction between water droplets was similar in bromododecane and Hoso. This implies that bromododecane can indeed be used instead of Hoso while maintaining a similar interaction between the dispersed water droplets. However, when dodecane was used as the dilution medium, the apparent diameter for a certain concentration and therefore the interaction between water droplets, stabilized with PGPR, largely increased despite of the lower scattered intensity as compared to Hoso or 1-bromododecane. As mentioned, this is an important issue; dilution can indeed lead to either flocculation or deflocculation depending on the used dilution liquid

(Leal-Calderon et al., 1997). This implies that the measured droplet diameter will be a function of the used emulsifier, concentration of droplets and dilution medium.

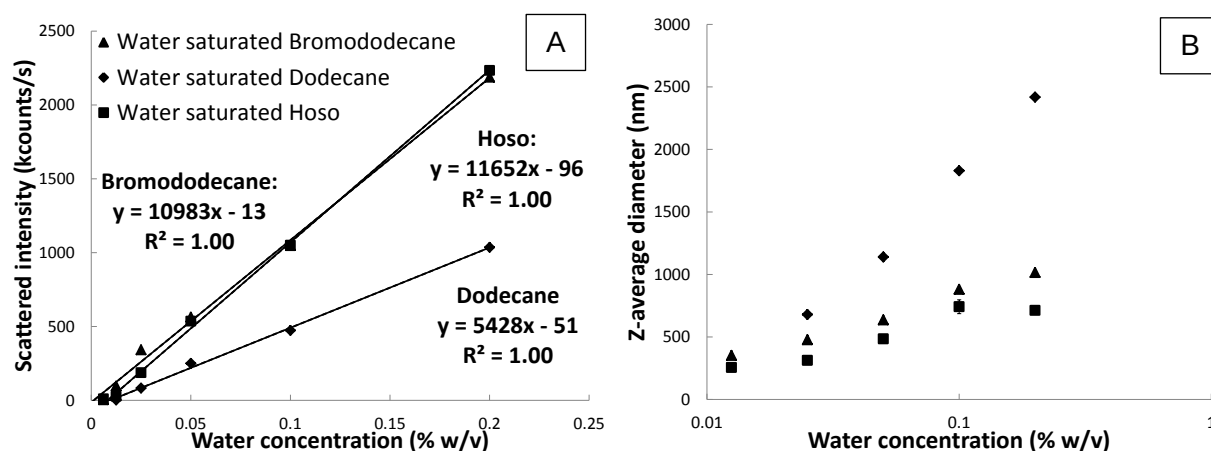


Figure 3.6 Scattered intensity (A) and estimated Z-average diameter (B) as a function of water concentration after dilution of the UT 24000-10wt emulsion in water saturated Dodecane (diamonds), Hoso (squares) or 1-Bromododecane (triangles). If no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

3.3.4 Influence of temperature

As shown in Figure 3.7A, the curve for scattered intensity versus concentration is similar at 20 and at 30 °C. However, at every concentration, the scattering intensity is slightly lower at a higher temperature. This can mainly be attributed to a slightly smaller difference in refractive index, although higher evaporation losses during preparation of the diluted sample when working at 30 °C can also not be ruled out.

Figure 3.7B shows that the interaction effects appear to be smaller at a higher temperature. The aforementioned observations therefore imply that measuring at a higher temperature reduces the interaction effects. Therefore, the necessary degree of dilution to eliminate these effects was reduced. However, the scattered intensity for a certain degree of dilution was lower when the temperature was increased, implying the need of a higher concentration in order to conduct a reproducible DLS experiment. Moreover, the risk of evaporative losses of water increases at higher temperature. It is therefore more convenient to measure at lower temperature.

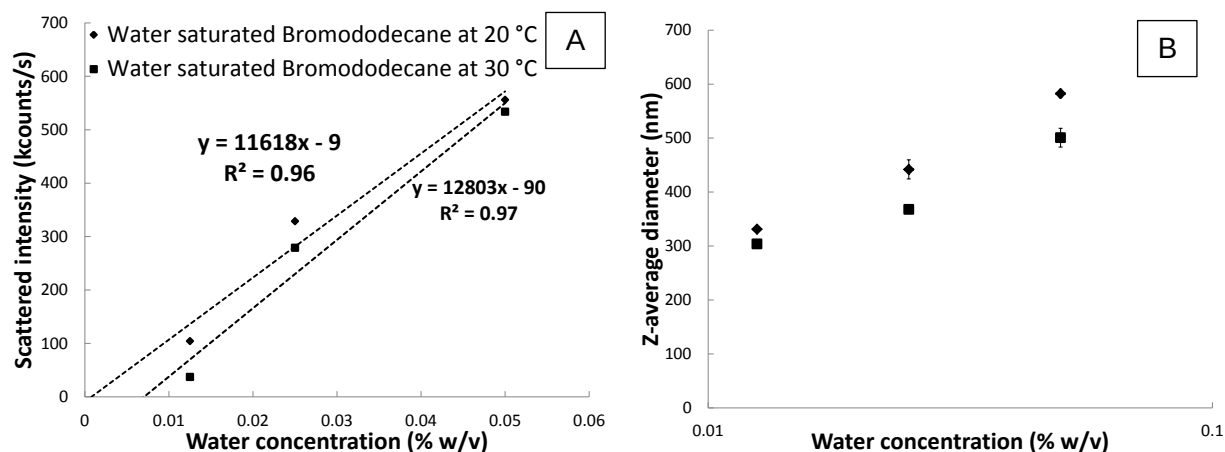


Figure 3.7: Scattered intensity (A) and estimated Z-average diameter (B) as a function of water concentration after dilution of the UT 24000-10wt emulsion in water saturated 1-Bromododecane at 20 (diamonds) or 30 °C (triangles). If no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

3.3.5 Measurement of droplet size of samples prepared at different shear rates

Three w/o emulsions were prepared with the same composition but different shear intensity during production. In the previous paragraphs, it was shown that measuring in water saturated 1-bromododecane at 20 °C and closing the cuvette leads to satisfactory results. Hereby, the concentration was varied using subsequent dilutions and the most reliable results were obtained at the highest possible dilution.

3.3.5.1 Dynamic light scattering

Figure 3.8A shows that the scattered intensity at a certain water concentration was higher when the shear intensity during preparation was higher and hence when smaller droplets were present in the sample. Whereas the Rayleigh theory (which holds for droplets much smaller as compared to the wavelength of the used light source) predicts an increased scattering for bigger droplets, the Mie theory (which holds for droplets with a similar size as the wavelength used) does not allow a general trend, but rather describes a complex relationship between scattered intensity and droplet size, which is frequently characterized by a maximum scattering intensity for droplets of some hundreds of nm in diameter. Considering the fact that the used DLS-device has a laser which emits light at 632.8 nm, the size of the droplets is in the range of the wavelength and their light scattering can be best predicted by the latter theory.

For the sample which contains the smallest droplets (MF 840-10wt), the increase of the scattered light intensity with increasing water concentration flattens. This can probably be attributed to multiple scattering of light by the droplets at these higher water concentrations.

Looking at figure 3.8B, it can be seen that for all samples, the measured Z-average diameter was again concentration dependent. Hereby, the interaction effects were smaller for smaller droplets, which corresponds to what was noted by Hamilton et al. (1990) and Hou et al. (1988).

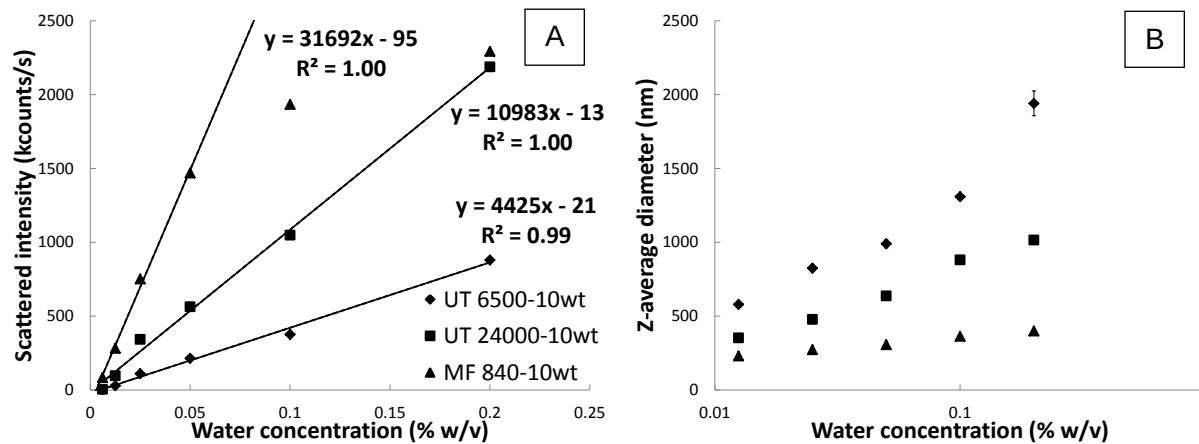


Figure 3.8: Scattered intensity (A) and estimated Z-average diameter (B) as a function of water concentration after dilution of the UT 6500-10wt (diamonds), UT 24000-10 wt (squares) and MF 840-10wt (triangles) emulsions in water saturated 1-Bromododecane. If no error bar is visible for a data point, the standard deviation is smaller than the used symbol.

In order to compare the DLS-results to the rather qualitative information which is obtained using cryo-SEM, the droplet size distribution should be considered instead of only an average value. Figure 3.9 shows the droplet size distributions which were obtained at a water concentration of 0.0125 %. This shows that the droplet size distribution mainly became more narrow with increasing shear intensity while the size of the smallest droplets remained more or less constant.

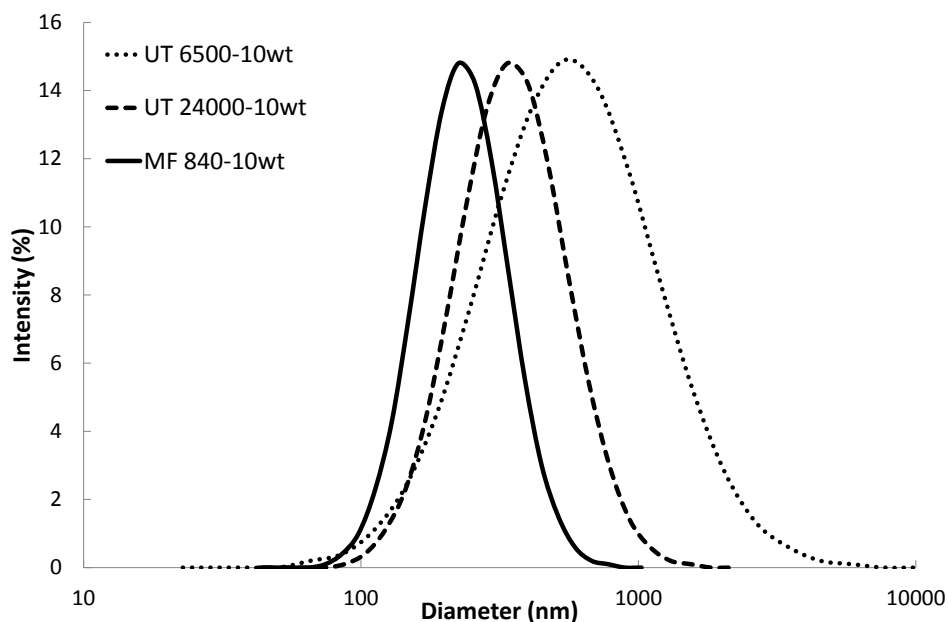


Figure 3.9: Intensity weighted distributions of the UT 6500-10wt (dotted line), UT 24000-10wt (dashed line) and MF 840-10wt (full line) emulsions, measured at a concentration of 0.0125 % (w/v) in water saturated 1-Bromododecane.

3.3.5.2 Cryo-SEM

Figure 3.10A and B show a lot of droplets with a diameter of about 1 μm in the sample prepared at the lowest shear (UT 6500-10wt) while the biggest droplets have a diameter of about 10 μm . In comparison, Figure 3.10C and D show that the biggest droplets found in the sample prepared at medium shear (UT 24000-10wt) were slightly bigger than 1 μm . Finally, Figure 3.10E and F clearly show that the biggest droplets found in the sample which was prepared at the highest shear (MF 840-10wt) had a diameter of about 1 μm with most droplets being submicron in size. The cryo-SEM images therefore agree with the distributions found using DLS with a low concentration of droplets (Figure 3.9).

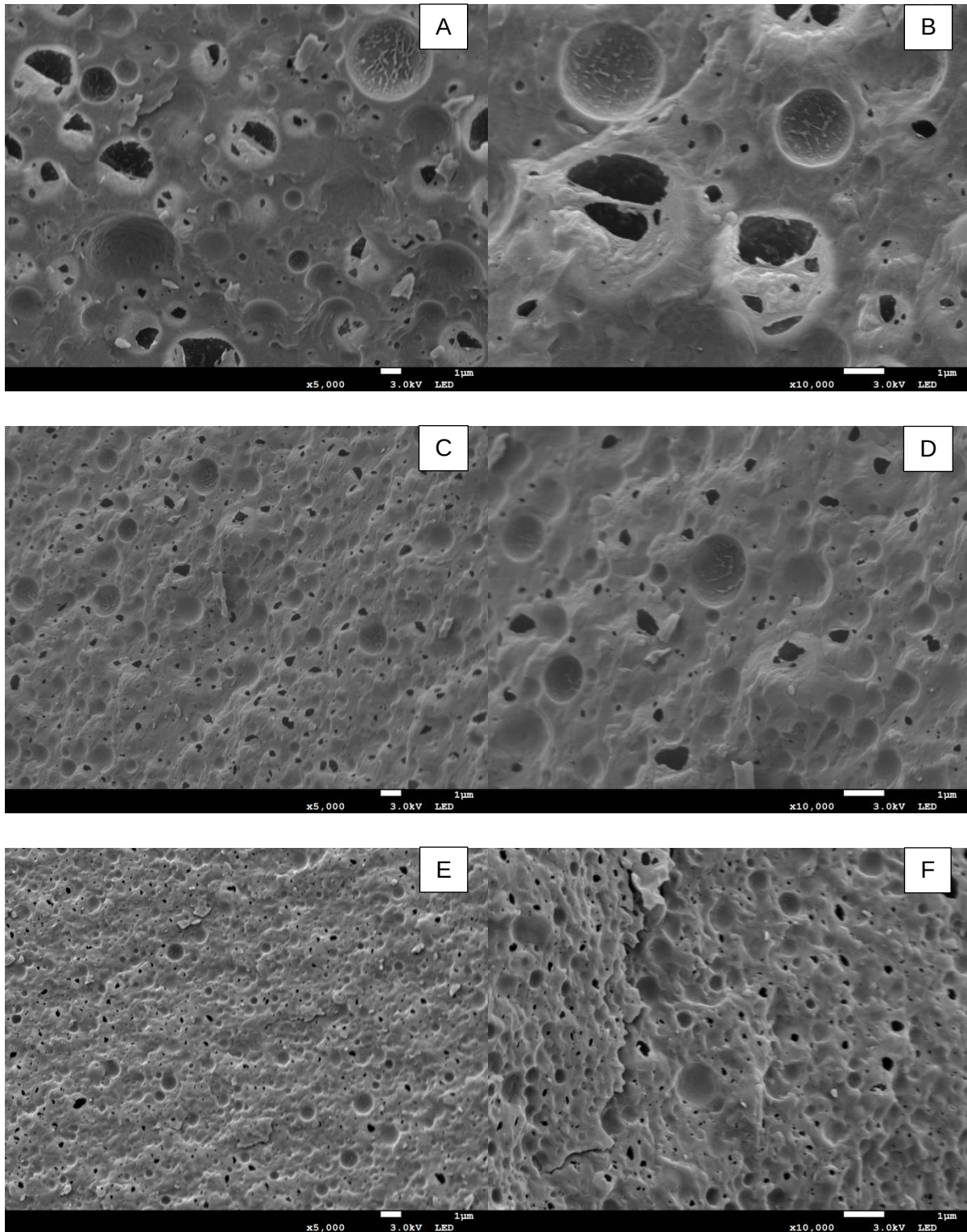


Figure 3.10: Cryo-SEM images of the UT 6500-10wt (A,B), the UT 24000-10wt (C,D) and the MF 840-10wt (E,F) w/o emulsions at 5000 (A,C,E) and 10000 times (B,D,F) magnification.

3.4 Conclusions

In this chapter, it was shown that the measurement of the droplet size distribution of (sub)micron sized liquid w/o emulsions is possible using dynamic light scattering. However, a lot of aspects have to be taken into account. Firstly, due to the fact that DLS-measurements require a high degree of dilution of the sample, the solubility of water in the dilution media has to be taken into account. Indeed, water saturation of the continuous phase largely prevents the dissolution of water droplets. Moreover, also evaporation of water from the dilution medium should be avoided as this shifts the equilibrium and leads to further dissolution of water droplets into the dilution medium. Hence, the headspace should always be as small as possible, vials should be closed and the temperature has to be chosen in a way that minimizes evaporation.

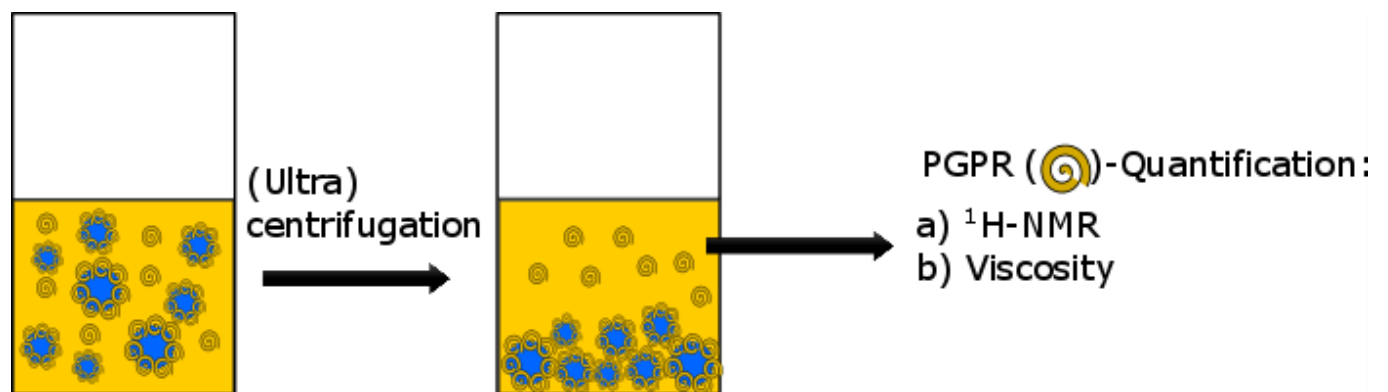
We also showed that the used dilution medium has a big influence on the measured droplet size. For every dilution medium, an increase of the measured diameter was recorded with increasing concentration. Hereby, the use of 1-bromododecane led to a similar behavior of the droplets as when they were dispersed in Hoso. However, the solubility of water in 1-bromododecane appeared to be lower than in Hoso, which reduces the need to saturate the dilution medium and also reduces the error when the dilution medium is not sufficiently saturated. In contrast to the aforementioned dilution media, dodecane increased the interaction between droplets, stabilized using PGPR, and thus the apparent diameter which was measured using DLS.

Finally, the droplet size distribution of three w/o emulsions was determined. Hereby, it was seen that there was less interaction between smaller droplets. Cryo-SEM confirmed that DLS-measurements of the samples after dilution to about 0.01 % (w/v) in water saturated bromododecane at 20 °C in a closed cuvette led to reliable results.

3.5 Acknowledgements

The Hercules foundation is acknowledged for its financial support in the acquisition of the scanning electron microscope JEOL JSM-7100F equipped with the cryo-transfer system Quorum PP3010T (grant no. AUGÉ-09-029) used in this research.

Chapter 4: Quantification of the PGPR-concentration in the oil phase



- The oil phase of w/o emulsions was separated using ultracentrifugation
- Both ^1H -NMR-measurements and viscosity measurements enabled the estimation of the residual PGPR-concentration

Abstract

The use of PGPR, the most commonly used low-HLB emulsifier in the preparation of liquid w/o emulsions, should be avoided or limited because this emulsifier can lead to off-tastes, there are legal restrictions and it has a synthetic connotation. In order to be able to minimize the required PGPR-concentration in w/o emulsions, it is necessary that the non-adsorbed (residual) PGPR-concentration can be quantified.

In this chapter, w/o emulsions were prepared at different shear intensities after which the oil and water phases were separated using ultracentrifugation. Afterwards, both ^1H -NMR and viscosity measurements were used in order to estimate the residual PGPR-concentration in the oil phase. Both techniques have their advantages and disadvantages but led to similar results. Therefore, in function of the experimental setting, one of these techniques can be used in order to estimate the residual PGPR-concentration in the oil phase which may help to further optimize the composition or processing conditions of the w/o emulsion.

4.1 Introduction

The most commonly used low-HLB emulsifier in the preparation of liquid w/o emulsions and subsequent w/o/w emulsions for potential food applications is polyglycerol polyricinoleate (PGPR). As the name already suggests, a PGPR-molecule contains parts of polymerized ricinoleate and parts of polymerized glycerol (Figure 4.1). The fatty acids in PGPR originate from castor oil, which mainly contains the fatty acid ricinoleate. The optimal degree of polymerization is equal to 5 monomers of ricinoleate per polyricinoleate oligomer. For the polyglycerol part, in order to get the best functionality, the amount of glycerol monomers per PGPR-molecule is maximized within EU-legislation. The latter defines the polyglycerol part of PGPR with a minimum of 75% di-, tri- and tetraglycerol and maximum 10% heptaglycerol or higher.

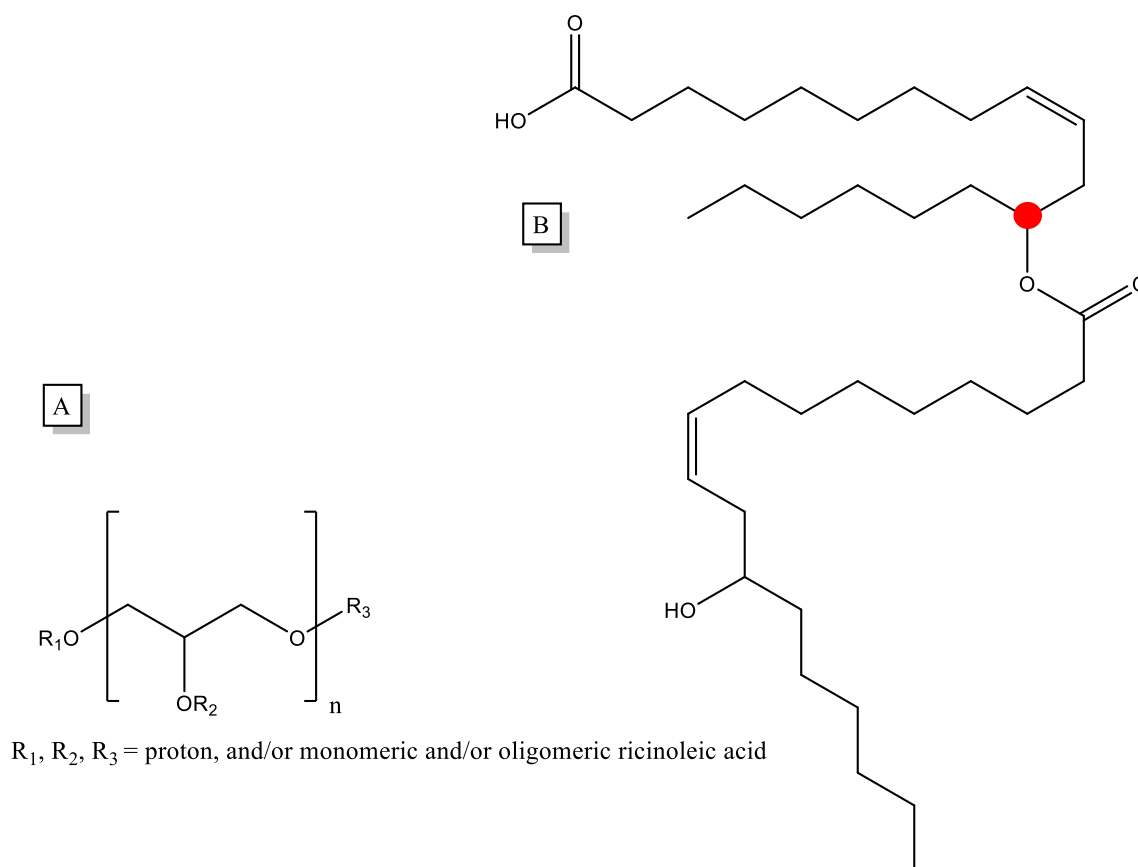


Figure 4.1: Chemical structure of PGPR (A) and of a ricinoleate dimer (B). The position of the carbon atom next to the polymerized OH-group of the ricinoleate dimer is indicated with a red dot.

However, as already mentioned in chapter 1, the use of PGPR should be avoided because it can lead to off-tastes (Akhtar and Dickinson, 2001), there are legal restrictions and PGPR has a synthetic connotation. In this context, it should be mentioned that the lack of an appropriate technique to determine the PGPR-concentration in food products, is one of the reasons why it is not allowed in a wider range of food products (Mortensen et al., 2017).

In this chapter, w/o emulsions were prepared using PGPR as emulsifier and varying the shear intensity during production. Subsequently, the water droplets were removed using ultracentrifugation and it was tested whether the residual PGPR-concentration could be estimated using ^1H -NMR and/or a much more simple viscosity measurement.

The height of the signal of the proton next to the polymerized OH-groups of polyricinoleate (Figure 4.1) was used to estimate the PGPR-concentration with ^1H -NMR. Therefore, this technique only considers the polyricinoleate part of PGPR. Mortensen et al. (2017) mentioned that PGPR cannot be measured directly in food products and that the methods employed to date all involve indirect determination of hydrolysis products by GC-FAME. The latter technique is therefore also linked to detection of ricinoleic acid or its esters in food. These types of methods cannot be used to quantify the PGPR-concentration exactly because the ricinoleate ratio in this food additive is not specified within the EU specifications for PGPR (E 476).

The fact that PGPR-molecules, due to their higher degree of polymerization, have a higher viscosity as compared to the triglycerides in vegetable oils, was used when the PGPR-concentration was estimated using viscosity measurements. Because PGPR consists of a mixture of different molecules, with different degrees of polymerization, it should be noted that this technique is also not providing an exact quantification of the PGPR-concentration because not every molecule in PGPR will adsorb at the w/o interface to the same extend. Moreover, the viscosity of the different molecules in PGPR will depend on their degree of polymerization. Therefore, both techniques which were employed in this chapter only give an estimation of the PGPR-concentration and not an exact value.

4.2 Materials and Methods

4.2.1 Materials

The emulsions described in part 2.2.2.1.1 were also used to conduct the experiments in this chapter. The same abbreviations were used to define the emulsions. In order of increasing shear during preparation UT 6500-5wt, UT 17500-5wt and UT 24000-5wt, as well MF 140-5wt, MF 420-5wt and MF 840-5wt, were used.

4.2.2 Sampling of the oil phases

The pure oil phases were obtained by centrifugation of the w/o emulsions for 12 hours at 20000 rpm (45000g) and 20 °C using a Beckmann L7-55 ultracentrifuge (Beckmann Coulter, USA) with 55.2 Ti rotor and 30 mL thickwall polyallomer tubes. If the obtained w/o emulsions were not completely separated (as was the case for the MF 420-5wt and MF 840-5wt samples), an additional centrifugation step was performed on the partly cleared oil phase using a Sigma 1-15P centrifuge at 14000 rpm (17968g) for at least 4 hours.

If centrifugation led to the formation of a water layer on the bottom of the tube, the percentage of water which coalesced, relative to the total amount of water which was used during preparation of the w/o emulsion, was determined. Hereby, the height of the water layer was measured using a ruler with nonius after which the percentage of water coalescence was calculated by taking into consideration that the tubes are cylindrical and filled up to a height of 35 mm. The diameter of the tubes is 13 mm and the bottom can be described by a halve sphere with radius 6.5 mm.

4.2.3 Use of ^1H -NMR to estimate the PGPR-concentration

The oil phases were diluted with CDCl_3 (55 mg sample was added to 450 μL CDCl_3) after which the ^1H -NMR spectrum was recorded using a Bruker Avance III 500 MHz spectrometer equipped with a BBI probe. The samples were measured at 25 °C. 1D ^1H -spectra were measured using 16 transients, an interscan delay of 1 second, an acquisition time of 4.37 seconds, and a spectral window of 15.00 ppm.

Finally, using the ^1H -NMR peak found at 4.90 ppm, which comes from the proton next to polymerized OH-groups of polyricinoleate, and which is therefore specific for PGPR (Orfanakis et al., 2013), the concentration of PGPR could be estimated. The position of the carbon-atom to which this proton is attached, is indicated with a red dot in Figure 4.1.

The ^1H -NMR spectra of the oil phases was dominated by the peaks of high oleic sunflower oil (Figure 4.2, top). However, as already mentioned, the peak at 4.90 ppm is specific for PGPR. As shown in the bottom figure of Figure 4.2, preparation of an emulsion indeed led to a decrease of the NMR-signal of PGPR. This shows that due to adsorption of PGPR at the w/o interface, the residual PGPR-concentration in the oil phase, and therefore its ^1H -NMR signal, decreased. The height of the signal relative to the height obtained for the oil phase containing 5 wt% PGPR was determined and multiplied by 5 to obtain an estimate for the concentration of PGPR in the oil phase (expressed in wt%).

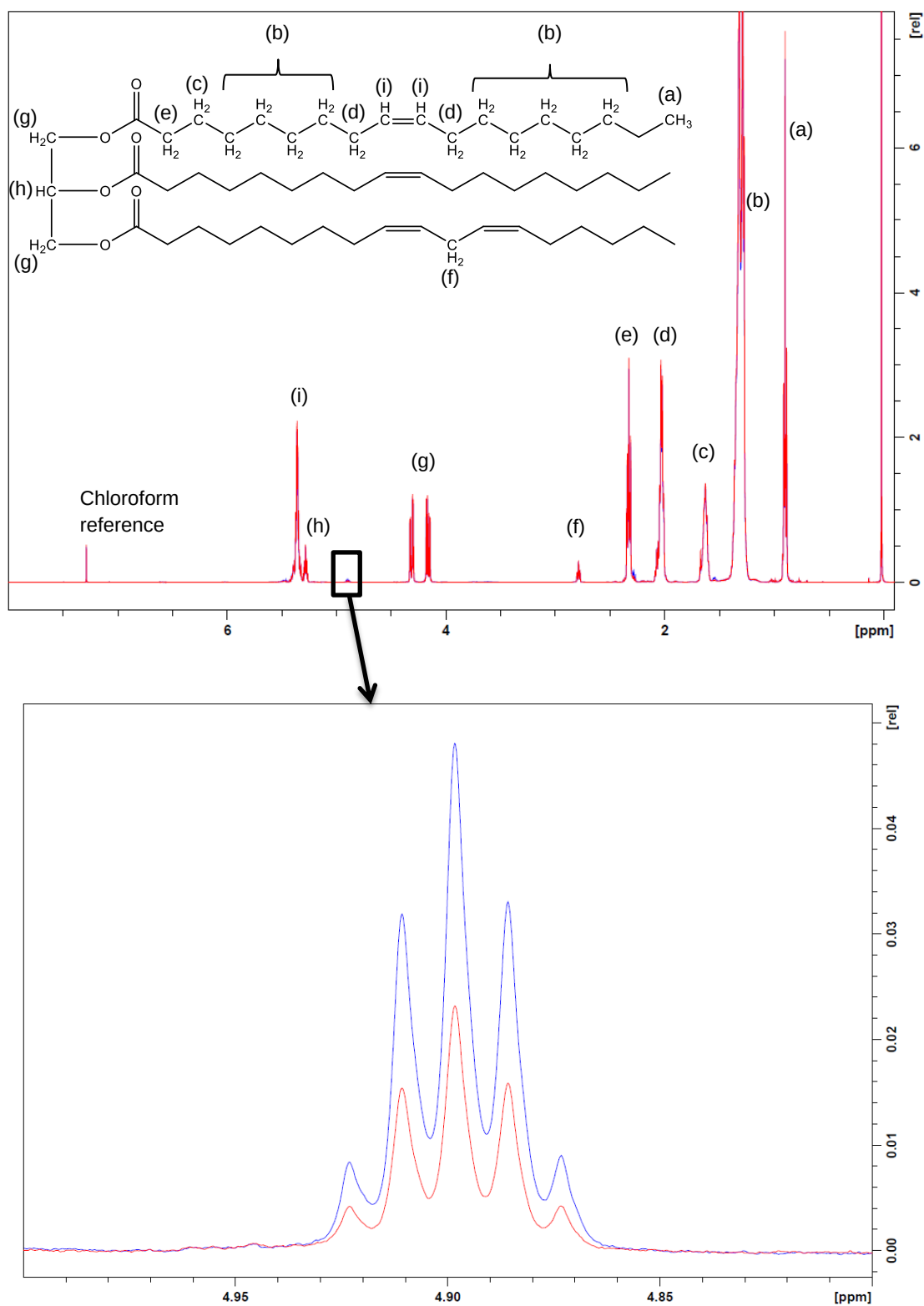


Figure 4.2: ^1H -NMR spectrum of 5 wt% PGPR 4175 in high oleic sunflower oil (blue) and the oil phase of emulsion UT 24000-5wt (red): complete spectrum (top) and detail of the signal specific for PGPR (bottom). The position of the ^1H -NMR signals of the protons present in a typical triglyceride in high oleic sunflower oil as well as its structure (with two oleic acid chains and one linoleic acid chain) are shown in the top figure.

4.2.4 Use of viscosity to estimate the PGPR-concentration

The viscosity of the oil phases was measured at 20 °C using a Brookfield LV-DV-II+ Pro viscometer (Brookfield, USA) with small sample adapter. Hereby, the shear stress was measured as a function of shear rate with three repetitions. Three shear rates were selected in order to obtain a torque between 10 and 100%, using a spindle SC4-18. The flow behavior of the oil phases was Newtonian.

The viscosity was calculated by considering the slope of the shear stress as a function of applied shear rate curve (with intercept forced through the origin) and considering the average of each of the measurements. In order to calculate the PGPR-concentration from the obtained viscosity, a standard curve (Figure 4.3) was determined by measuring the viscosity of high oleic sunflower oil containing varying concentrations of PGPR. Due to the high degree of polymerization, PGPR increased the viscosity of the oil phase (having a lower molecular weight).

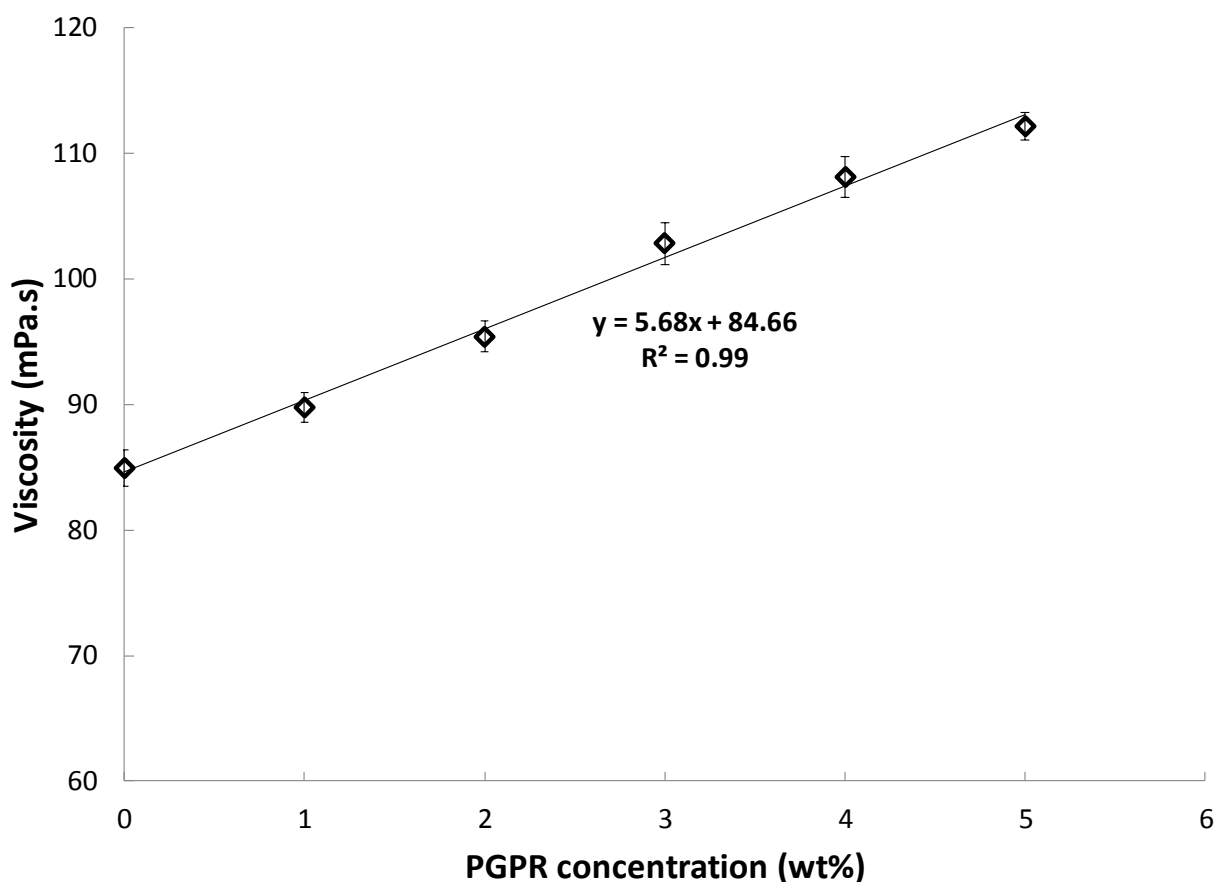


Figure 4.3: Viscosity of high oleic sunflower oil as a function of dissolved PGPR 4175 concentration at 20 °C.

4.3 Results and Discussion

Figure 4.4 shows that using both techniques, a decreasing residual PGPR-concentration was measured in the oil phase of the w/o emulsions with increasing shear intensity during their production. An increase of the shear intensity which was used to produce the w/o emulsions, decreased their droplet size. Indeed, smaller droplets have a higher specific surface area which needs to be stabilized by PGPR. Therefore, the amount of PGPR which remained in the oil phase decreased with increasing the shear intensity. Quantification of the residual PGPR-concentration in the oil phase therefore provided another indication that the droplet size distribution of the samples defined as UT 24000-5wt, MF 140-5wt, MF 420-5wt and MF 840-5wt was not the same, which was nonetheless indicated by water PFG-NMR diffusometry (Chapter 2).

It should be noted that the PGPR-concentration which was measured using ^1H -NMR was always slightly higher as compared to when the viscosity of the oil phase was used to estimate the residual PGPR-concentration. As already mentioned, the principles behind these measurement techniques are not the same, which might explain this difference. The result from ^1H -NMR is linked to the residual polyricinoleate concentration in the oil phase whereas the result from viscometry is linked to the viscosity and concentration of the remaining types of PGPR-molecules in the oil phase. It can be assumed that the use of ^1H -NMR leads to an overestimation of the residual PGPR-concentration due to the fact that it focuses on polymeric ricinoleate and therefore on the apolar part of any PGPR-molecule. The most apolar molecules will probably adsorb the least and therefore NMR might overestimate the residual PGPR-concentration. As viscosity is linked to the total PGPR-molecule the result might be closer to the truth.

Finally, these results also indicate that the used PGPR-concentration was not limiting: even in case of the highest shear (MF 840), there was still at least about half of the used PGPR which was not adsorbed at the w/o interface. In this context, it can be noted that in order to optimize the emulsion composition, 1 wt% PGPR was measured to be the CMC of PGPR in sunflower oil. Moreover, Nollet et al. (2018) mentioned that 1 wt% PGPR is also the minimal concentration which needs to remain in the oil phase in order to prepare stable w/o/w emulsions.

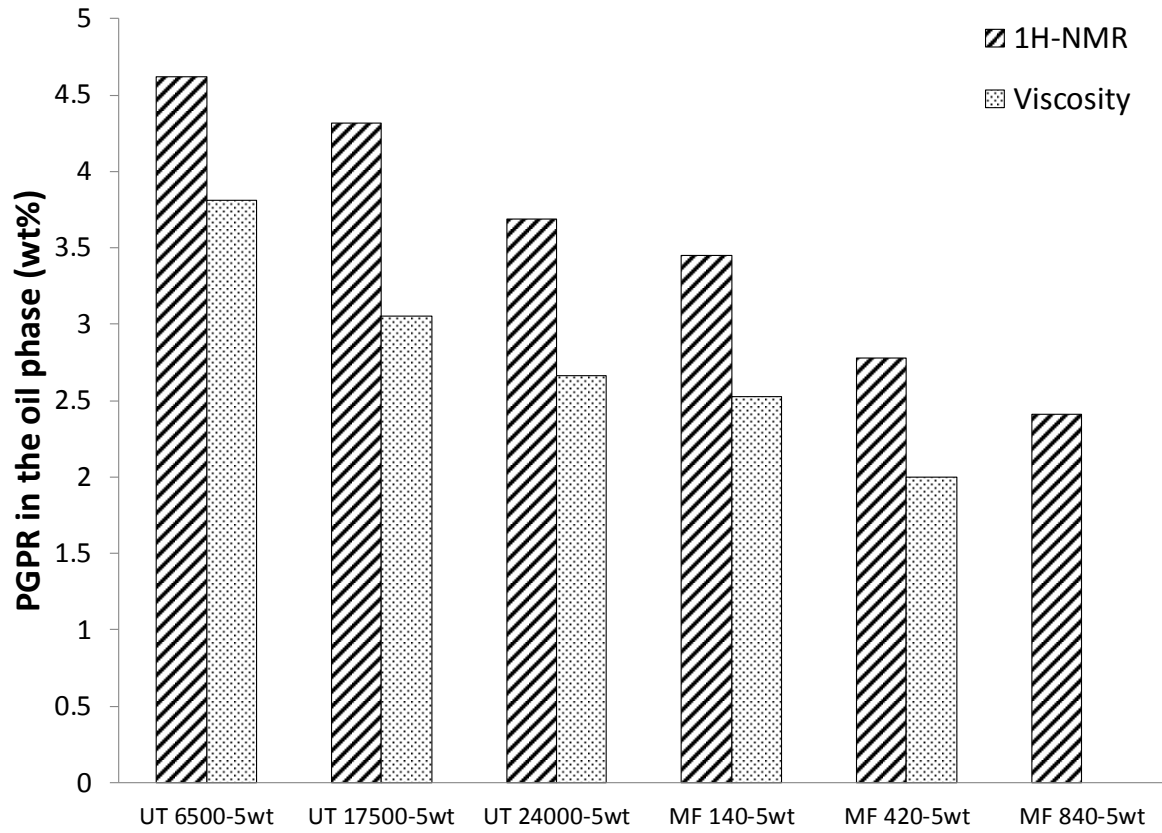


Figure 4.4: Residual concentration of PGPR in the oil phase of w/o emulsions which were prepared using varying shear intensities. The obtained oil volume for the MF 840-5wt sample was insufficient to measure the viscosity.

If the projected area per molecule of PGPR (pa_1) and the molecular mass (MM) are known, the surface weighted average diameter (D_{32}) of the water droplets can be estimated:

$$(C_0 - C) * x_{oil} = \left(\frac{6}{D_{32}} * \frac{x_{water}}{\rho_{water}} \right) / \left(\frac{pa_1 * N_A}{MM_{PGPR}} \right) \quad (\text{Equation 4.1})$$

In which C_0 is the concentration of PGPR used in the oil phase (kg/kg oil), C is the residual PGPR-concentration in the oil phase after emulsification (kg/kg oil), x_{oil} and x_{water} the mass fractions of water and oil in the emulsion (kg/kg emulsion), D_{32} the surface weighted average diameter (m), ρ_{water} the density of water (kg/m³), pa_1 the surface area per polar head group of PGPR (m²), MM_{PGPR} the molecular mass of PGPR (kg/mol) and N_A Avogadro's constant (mol⁻¹).

Nollet et al. (2018) determined the monolayer surface excess concentration of PGPR at the w/o interface of a system containing PGPR in sunflower oil and a water phase containing 0.1M NaCl. They calculated that 1.67 $\mu\text{mol}/\text{m}^2$ of PGPR molecules are necessary in order to form a complete monolayer. The surface area per polar head group was calculated to be equal to about 100 $\text{\AA}^2$. Considering a molecular weight of 3000 g/mol the D_{32} of the water droplets in the w/o emulsion could be estimated (Figure 4.5) when assuming that a complete monolayer was adsorbed at the w/o interface:

$$D_{32}(m) = \frac{6 \cdot 3 \text{ kg/mol}}{100 \cdot 10^{-20} \text{ m}^2 \cdot 6.02 \cdot 10^{23} \frac{1}{\text{mol}} \cdot (0.05 - C) \frac{\text{kg}}{\text{kg oil}} \cdot 1000 \frac{\text{kg}}{\text{m}^3}} \quad (\text{Equation 4.2})$$

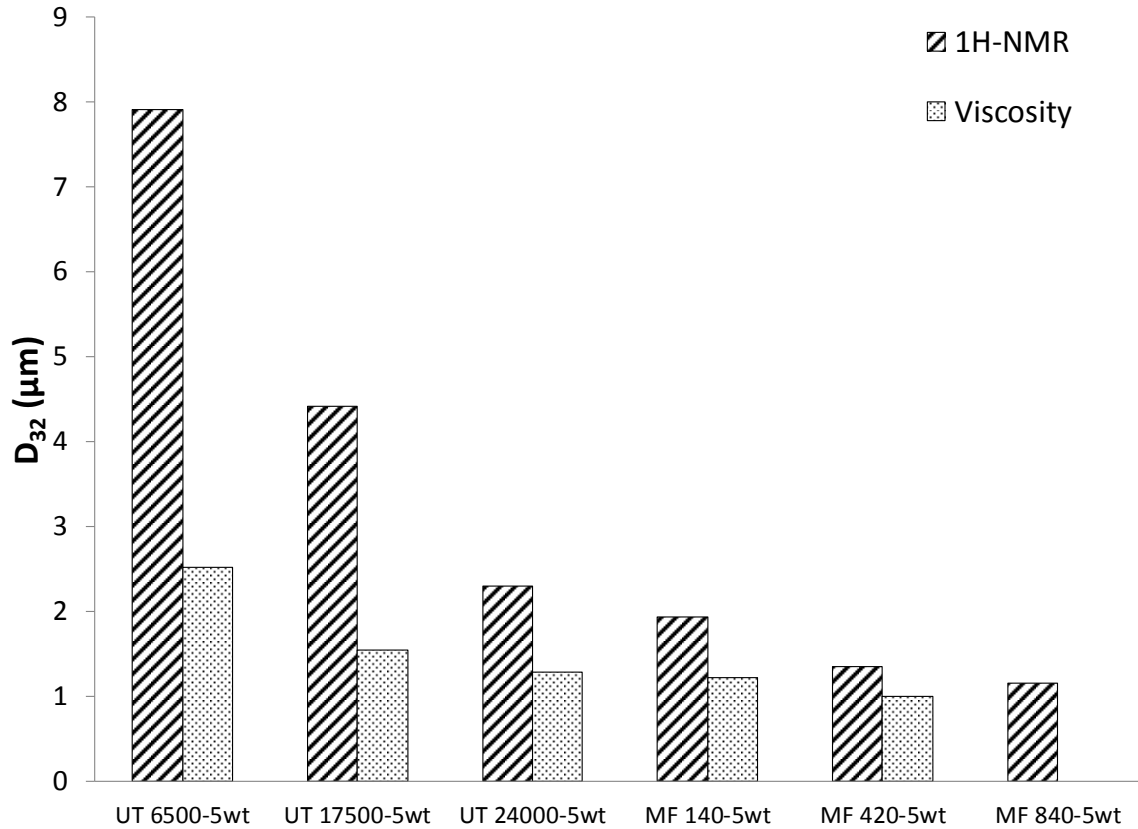


Figure 4.5: Estimated surface weighted average diameter from the residual PGPR-concentration in the oil phase as determined using either $^1\text{H-NMR}$ or viscosity. The obtained oil volume for the MF 840-5wt sample was insufficient to measure the viscosity.

Clearly the order of magnitude of estimated diameters corresponds to what was expected (in the μm -range). However, because the adsorbed amount of PGPR is in the denominator of equation 4.2, relatively small differences in measured residual PGPR-concentration lead to much bigger differences in calculated D_{32} .

As the T_2 -relaxation time also depended on the specific surface area, and thus on the surface weighted average diameter, these results can be compared. In part 2.3.3, the surface weighted droplet size (D_{32}), relative to the surface weighted droplet size of the MF 140-5wt sample was calculated using T_2 -relaxometry. This gave a ratio of 0.77 and 0.63 for the MF 420-5wt and MF 840-5wt samples, respectively. Doing the same using the data obtained from measuring the residual PGPR-concentration using ^1H -NMR, a ratio of 0.70 and 0.60 was obtained for the MF 420-5wt and MF 840-5wt samples, respectively. The results obtained by analyzing the residual PGPR-concentration in the oil phase and T_2 -relaxometry, which are both linked to the specific surface area of the w/o emulsion, indeed corresponded very well.

It should, however, be noted that the proposed technique relies on separating the water and the oil phase before analyzing the PGPR-content of the oil phase. Whereas coalescence did not occur when the samples described in this chapter (containing 5 wt% PGPR) were ultracentrifuged, this was the case in other experiments. When both the shear intensity and the PGPR-concentration were varied, it was observed that ultracentrifugation led to coalescence of the water droplets when the shear intensity was high and/or the PGPR-concentration was low (Figure 4.6). Moreover, in case 0.5 wt% Na-caseinate or WPI was added to the water phase, the sensitivity towards coalescence during ultracentrifugation was even higher.

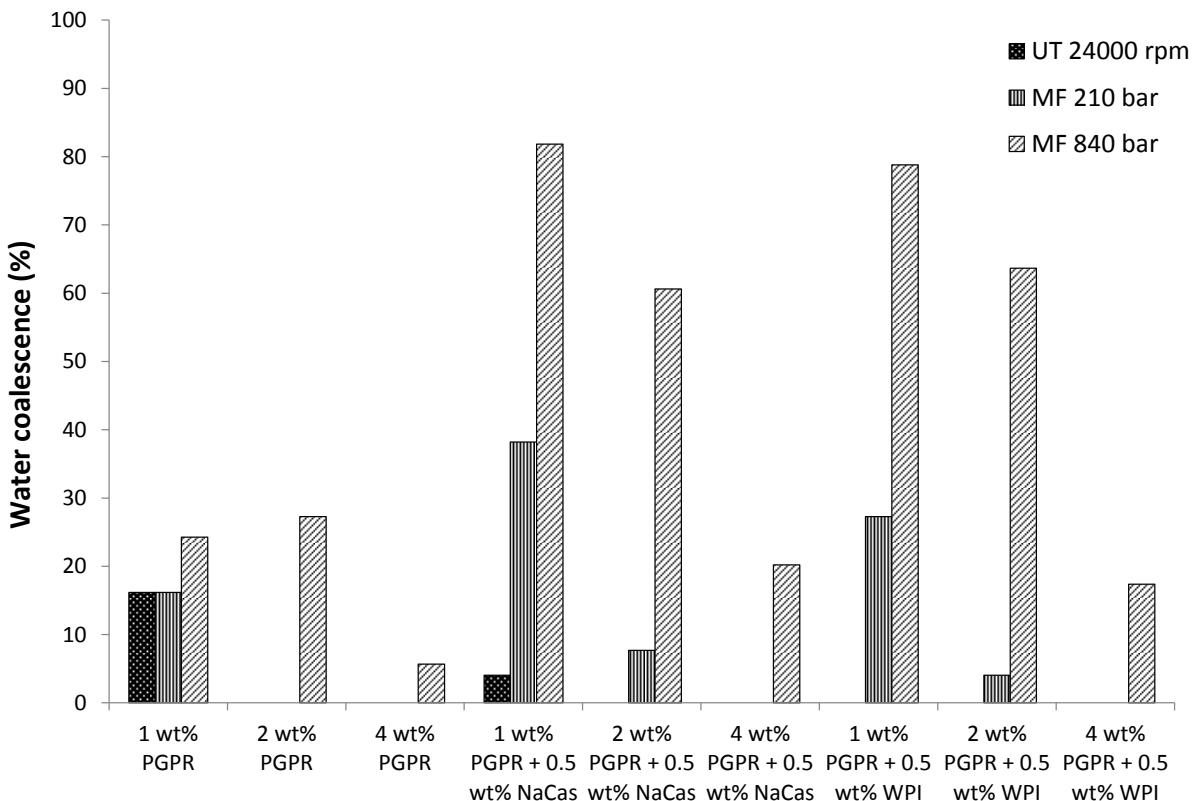


Figure 4.6: Water coalescence (%) that occurs when samples, which were prepared using different shear intensities and compositions, were ultracentrifuged at 225000g for 395 minutes.

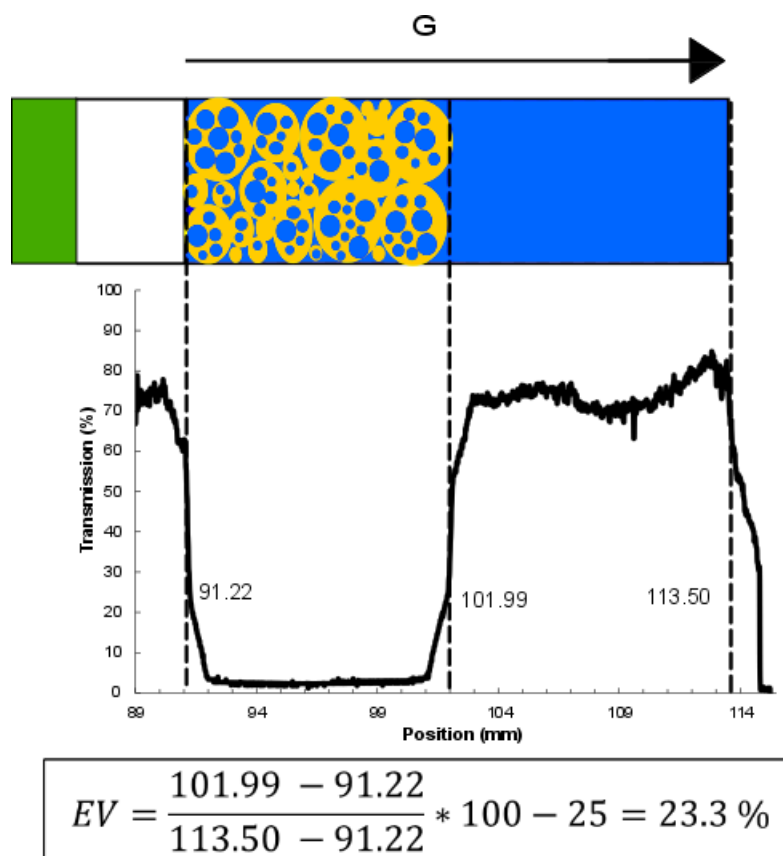
When water droplets coalesce during centrifugation, the measured PGPR-concentration in the oil phase might not correspond to the residual PGPR-concentration in the oil phase of the w/o emulsion. Indeed, the PGPR-molecules which were adsorbed at the surface of the coalescing droplets, might be released to the oil phase.

4.4 Conclusions

^1H -NMR and viscosity measurements are both efficient techniques to estimate the PGPR-concentration in an oil phase. The calculated PGPR-concentration is similar for both techniques whereby the estimate obtained using ^1H -NMR is systematically higher as compared to the one obtained using a viscosity measurement. However, it should be noted that both techniques are mainly (viscometry) or only (^1H -NMR) linked to the polyricinoleate part of the PGPR-molecule. Moreover, the water and oil phase have to be separated using centrifugation. If centrifugation influences the emulsion characteristics, for example through coalescence of part of the water droplets, this approach will lead to an underestimation of the adsorbed amount in the original emulsion (i.e. before centrifugation).

Viscosity measurements are a quick and relatively inexpensive way of estimating the residual PGPR-concentration in the oil phase and optimizing the composition or production process of the emulsion. However, the sample volume (about 10 mL with the used equipment) which is needed for viscosity measurements is higher than for ^1H -NMR measurements (less than 100 μL).

Chapter 5: Determination of the enclosed water volume fraction of w/o/w emulsions using analytical photocentrifugation



- The enclosed water volume fraction was determined through analytical photocentrifugation
- Results were similar between PFG-NMR and analytical photocentrifugation

Abstract

During the preparation of w/o/w type double emulsions, a part of the enclosed water phase is often released to the external water phase. Thus, it is important that the enclosed water volume fraction can be determined in a simple and straightforward manner after preparation. In this chapter, a method is developed to determine the enclosed water volume fraction of w/o/w type double emulsions using analytical photocentrifugation after which the results are compared to the enclosed water volume fraction as determined using pulsed field gradient Nuclear Magnetic Resonance (NMR). From the results, it can be concluded that analytical photocentrifugation is indeed a simple and straightforward method to determine the enclosed water volume fraction of double emulsions.

Redrafted after: Balcaen, M., Vermeir, L., Declerck, A., Van der Meeren, P., 2016. Simple and straightforward determination of the enclosed water volume fraction of W/O/W double emulsions by analytical photocentrifugation. *Part. Sci. Technol.* 34, 565-570.
<https://dx.doi.org/10.1080/02726351.2015.1090507>

5.1 Introduction

Akhtar and Dickinson (2001) proposed to determine the enclosed water volume fraction of a w/o/w double emulsion from the weight loss of the cream phase obtained after centrifugation upon drying. To our knowledge, this is the only publication in which centrifugation is used in the determination of the enclosed water volume fraction.

However, analytical photocentrifugation is expected to have a number of advantages as compared to the procedure described by Akhtar and Dickinson (2001). Analytical photocentrifugation is a technique in which separation processes can be monitored in situ by recording space- and time-resolved NIR light extinction profiles patented as the STEP-Technology (Detloff and Lerche, 2008). This optical detection system enables the continuous monitoring of the cream volume during centrifugation. Subtracting the oil content, the amount of water in the double emulsion droplets can be estimated without any additional experimental measurement. In a first part of this chapter, the parameters that enable an accurate estimation of the cream layer volume fraction were determined. In a second part, the enclosed water volume fraction as obtained by this newly proposed method was compared to the results derived from pulsed field gradient NMR (PFG-NMR).

5.2 Materials and Methods

5.2.1 Materials

The lipophilic emulsifier polyglycerol polyricinoleate (PGPR 4150; min. 75 % n-glycerols with $n=2$, 3 and 4; max. 10 % m-glycerols with $m \geq 7$) and the hydrophilic emulsifiers sodium caseinate (5.5 % moisture; 96 % protein on dry matter) and polysorbate 60 (Admul T60 K; max. 3% moisture; Kerry Bio-Science) were kindly provided by Palsgaard A/S (Denmark), Armor Protéines (Saint Brice en Cogles, France) and IMCD Benelux (Mechelen, Belgium), respectively. High oleic sunflower oil (Hoso; Iodine Value= 87; 82 % C18:1) was acquired from Contined B.V. (Wageningen, The Netherlands). The 0.1M KCl (VWR Chemicals: BDH Prolabo, Leuven, Belgium) solution used in both the w_1 - and w_2 -phase contained 0.02 wt% of the anti-microbial agent NaN_3 (Acros Organics, Geel, Belgium). All above mentioned chemicals were of analytical grade.

KCl was added to both aqueous phases in order to reduce Ostwald ripening effects due to the high Laplace pressure of the enclosed water droplets which in turn is related to their small size and thus high curvature (Mezzenga et al., 2004). The same concentration of salt (0.1M) was added to both water phases, due to which a slight exchange of water, because of the higher

Laplace pressure of enclosed droplets, can still be expected. However, the exchange will be very limited. Water is transported from the enclosed to the external water phase until the Laplace pressure difference equals the osmotic pressure difference:

$$\Delta\pi = \frac{2*\gamma}{a}$$

In which $\Delta\pi$ is the osmotic pressure difference (Pa), γ is the interfacial tension (N/m) and a is the internal water droplet radius (m).

Because the oil droplets are much larger than the water droplets, the curvature effect at the outer interface is neglected. The interfacial tension between sunflower oil containing PGPR above the CMC (>1 wt%) and a water phase containing 0.1M NaCl was measured to be 2 mN/m by Nollet et al. (2018). Assuming that the same interfacial tension is obtained in this system and the droplet has a radius of 750 nm after shrinking:

$$\Delta\pi = 5333 \text{ Pa} = i * C_{enclosed} * R * T - i * C_{external} * R * T$$

$$\text{So at } 25^\circ\text{C: } i * \Delta C = 2.15 \text{ mM}$$

$$\text{For KCl with } i = 2 \text{ this means that } \Delta C = 1.08 \text{ mM}$$

Therefore, the concentration of KCl in the enclosed water phase increases from 100 mM to 101.08 mM due to transport of water from the enclosed to the external water phase. This means that the enclosed water volume in this case changes with about 1%.

5.2.2 Emulsion preparation

5.2.2.1 w/o emulsion preparation

The first water phase (w_1) consisted of 0.1M KCl dissolved in milli-Q water containing 0.02 wt% NaN_3 . The oil phase consisted of high oleic sunflower oil containing 5 wt% PGPR. An Ultra-Turrax rotor-stator homogenizer (type S50N-G45F, IKA®-Werke, Germany) at 5200 rpm was used to prepare the w/o emulsion (50:50, v/v) at 60 °C.

5.2.2.2 w/o/w emulsion preparation

The second water phase (w_2) was mixed at room temperature with freshly prepared w/o emulsion in a 50:50 (v/v) ratio with an Ultra-Turrax rotor-stator homogenizer S25-10G (IKA®-Werke, Germany) at 24000 rpm for 1 minute. Hereby, the w_2 -phase only differs from the w_1 -phase in the presence of 2 wt% of either sodium caseinate or polysorbate 60. Afterwards, all

double emulsions were further mixed with a continuous Ultra-Turrax DK25 rotor-stator homogenizer containing a S25KV-25G-IL dispersing element (IKA®-Werke, Germany) at 24000 rpm for a fixed time.

A comparable emulsion without enclosed water was prepared by mixing 25 volume% of oil in the previously mixed water phases ($w_1 + w_2$, 75 volume%) with an Ultra-Turrax S25-10G (IKA®-Werke, Germany) at 24000 rpm for 1 minute and afterwards, during 2 minutes, with the above-described Ultra-Turrax DK25.

5.2.3 Determination of the double emulsion droplet size

The double emulsion droplet size was measured using a Mastersizer S (Malvern Instruments) equipped with a MS-17 automatic dilution unit and 300RF lens (size range of ± 0.05 -900 μm). The refractive indices were set to $1.5295 + 0.01i$ for the dispersed phase and 1.33 for the continuous phase (deionized water). The Mie theory was used to obtain the volume weighted average diameter (D_{43}). Measurements were performed by diluting the samples in deionized water until an obscuration between 10 and 30 % was obtained. The pumping and the stirring speed were both set to 30% of the maximal value and three measurements were performed on each sample.

5.2.4 Low-resolution NMR

NMR measurements were performed at 5 °C on a benchtop Maran Ultra spectrometer (Oxford Instruments, UK) operating at a frequency of 23.4 MHz. The samples were filled in 18 mm diameter glass NMR-tubes (Oxford Instruments, UK).

PFG-NMR experiments were performed using the stimulated echo pulse (STE) sequence, which was preceded by an inversion recovery experiment for suppression of the NMR-contribution from the fat phase in the emulsion. Measurements were performed varying the gradient strength (G) between 0 and 3.17 T/m while keeping the gradient duration (δ) constant (2.5 ms) and the diffusion delay (Δ) fixed at 200 ms.

5.2.5 Analytical photocentrifugation

Analytical photocentrifugation was performed at room temperature (using air cooling) with a LUMiFuge 116 instrument (LUM GmbH, Germany). Rectangular synthetic cells with 2.2 mm path length were used, filled with 0.35 mL sample. The light intensity was set to 100%. Enclosed water volume fractions were determined in duplicate after 10 hours of centrifugation at the maximum speed (3000 rpm; 1142g).

5.3 Results and Discussion

Analytical photocentrifugation enables the quantification of demixing by measuring the transmission as a function of sample height during centrifugation. Considering the fact that centrifugation leads to separation of the liquid w/o/w emulsion into a cream-layer, containing the w/o droplets and a serum-layer, containing most of the external water, the enclosed water volume fraction (EV(%)) can be approached by subtracting the oil volume fraction from the cream volume fraction:

$$EV (\%) = \frac{H_{cream}}{H_{total}} * 100 - volume\% (oil) \quad (\text{Equation 5.1})$$

With:

H_{cream} = Height of the cream layer (mm)

H_{total} = Height of the sample (mm)

$volume\% (oil)$ = Total volume fraction of oil in the double emulsion sample

As the sample cells have a constant thickness, the height of the cream phase is proportional to the volume of cream.

5.3.1 Determination of parameters

The profiles shown in Figure 5.1, which were obtained after 10 hours of centrifugation, clearly show a low transmission zone closer to the center of rotation (hence a cream layer) and a high transmission zone at larger distances from the center of rotation (hence a serum layer). In addition, Figure 5.1 reveals that, despite of the same oil content, a much thicker cream layer is obtained after centrifugation of the multiple emulsion, which is a direct indication of the presence of water inside the oil droplets. In order to be able to determine the cream volume fraction, the position of the bottom and the transmission value that indicates the interface between cream and serum, as well as between cream and air must be determined.

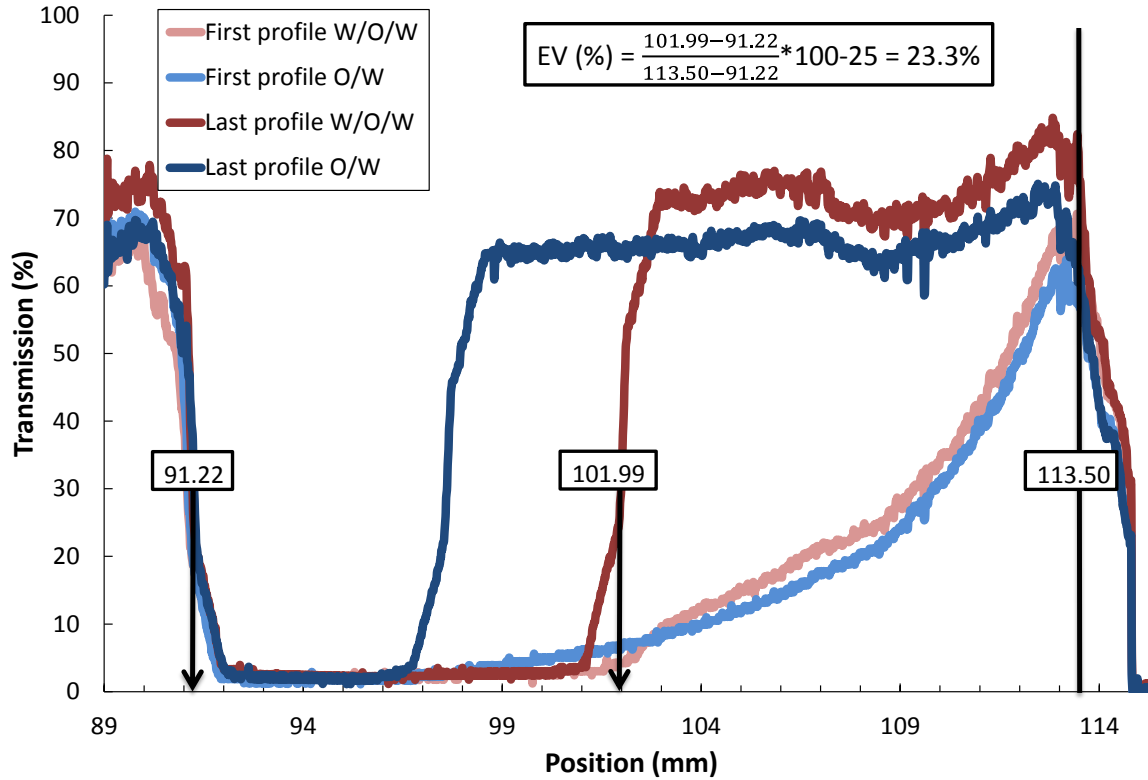


Figure 5.1: Transmission profiles of o/w 25:75 (v/v) and w/o/w 25:25:50 (v/v/v) emulsions, stabilized using sodium caseinate and mixed for 2 minutes, after 5 minutes and after 10 hours of centrifugation at 3000 rpm. The EV(%) is determined from the last profile of the w/o/w emulsion using equation 5.1.

5.3.1.1 Position of the bottom

According to the manufacturer, the bottom of the recipients is located at 113.50 mm from the center of rotation. To experimentally verify this value, eight sample cells were filled with different amounts of distilled water (ranging from 200 to 450 mg) of which the mass was carefully noted. At the interface between water and air, the transmission is reduced due to the meniscus effect. The radial position where the minimal transmission was observed, was taken to be the position of the interface. A plot of the radial position of the air water interface as a function of the mass of water yielded a straight line, corresponding to the equation: radial position (mm) = 113.38 – 0.055 x mass of water (mg). The intercept of the linear regression leads to the conclusion that with 95% certainty the bottom is located between 112.91 and 113.84 mm. In conclusion, the value specified by the manufacturer is corroborated and will be considered in all further calculations.

5.3.1.2 Front tracking of interfaces

As experimental profiles do not follow a perfect step-function, but have a less steep profile, the selection of the transmission value corresponding to the interface is important. Using the standard for synthetic cells, provided by the manufacturer, which has openings and closures of known size (Figure 5.2), the transmission value that has to be used for accurate front tracking can be determined.

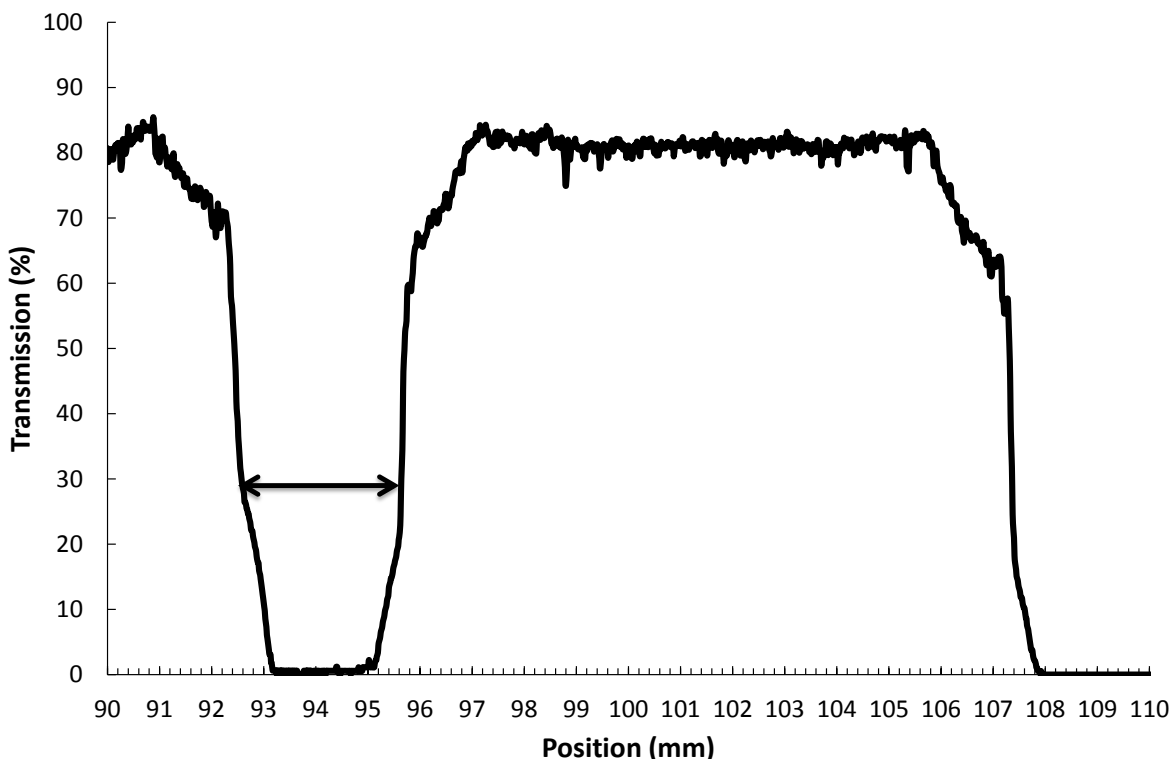


Figure 5.2: Transmission profile of the standard for synthetic cells; the arrow indicates a length of 3.0 mm.

As the solid part giving rise to the low transmission zone was measured to be 3.0 mm wide by a ruler with nonius, it follows that front tracking at 30% transmission is needed to obtain accurate data of the radial position. In fact, as long as the interfacial positions are determined using 30 to 40% transmission, the result will hardly be affected as the curve is steep in this region.

5.3.2 Droplet size of the double emulsions

As the droplet size has a big influence on both the creaming velocity and the compressibility of the emulsion droplets, this parameter was determined using static light scattering. The influence of mixing time on the volume weighted average droplet size (D_{43}) of the double emulsions stabilized using Na-caseinate is shown in Table 5.1.

Table 5.1: Volume weighted average diameter (D_{43}) $\pm$ standard deviation (SD) of the double emulsions prepared using sodium caseinate (based on three repetitions).

Mixing time (minutes)	1	2	3	4
$D_{43} \pm SD$ (μm)	22.24 $\pm$ 0.08	18.70 $\pm$ 0.09	16.04 $\pm$ 0.03	13.93 $\pm$ 0.01

The emulsion prepared using polysorbate 60 as the high-HLB emulsifier and 2 minutes of mixing time was characterized by a volume weighted average diameter of $13.52 \pm 0.03 \mu\text{m}$.

5.3.3 Importance of analytical photocentrifugation

A higher centrifugal speed does not only accelerate the cream phase separation, but also determines the equilibrium cream layer volume. Whereas a pseudo-equilibrium was reached after 30 minutes at 2000 rpm (508g), Figure 5.3 shows a cream layer compression upon further increasing the centrifugal speed to 2500 and 3000 rpm (793 and 1142g, respectively). This effect is thought to be due to a reduction in amount of interstitial water, which can be further enhanced by droplet shape deformation at higher centrifugal pressures. Hence, the highest possible centrifugal speed is desirable to ensure that the water in the cream layer is (mainly) due to enclosed water (within droplets) rather than to residual water present between droplets.

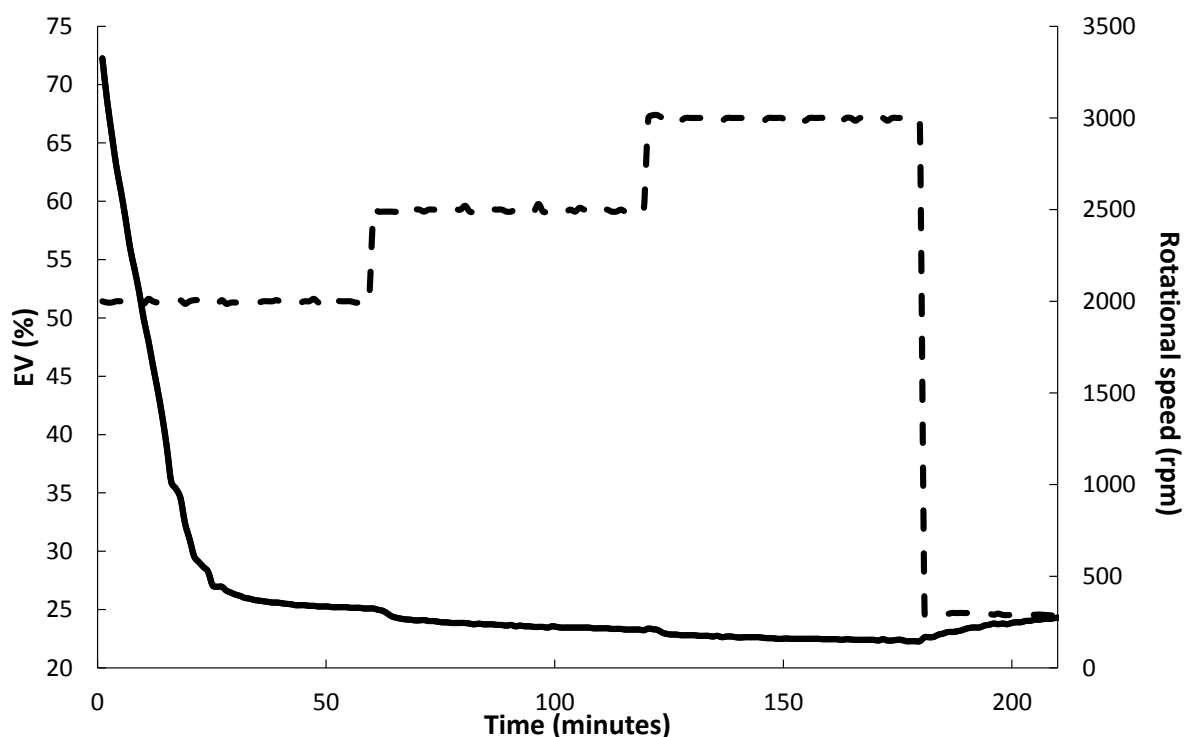


Figure 5.3: Rotational speed (dashed line) and enclosed water volume fraction (solid line) upon centrifugation of a sodium caseinate-stabilized w/o/w double emulsion (mixed for 2 minutes) as a function of centrifugation time.

Furthermore, a rebound effect, an increase in the apparent height of the cream-phase, was observed when the centrifugation speed was decreased from 3000 to 300 rpm (1142 to 11g; Figure 5.3 and Figure 5.4). This rebound observation highlights the importance of measuring the volume of the cream layer during, rather than after high speed centrifugation in order to reduce the interstitial water fraction. Furthermore, it indicates that the reduction of the cream phase height is due to the reduction of interstitial water rather than the loss of enclosed water.

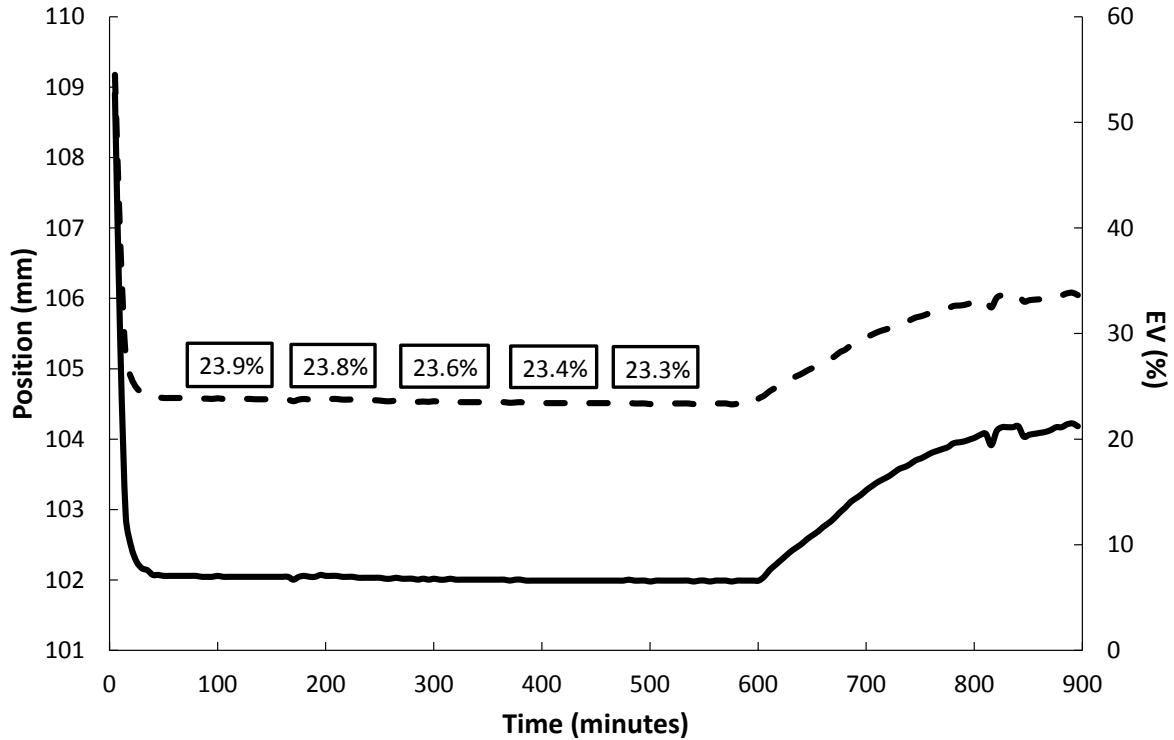


Figure 5.4: Enclosed water volume fraction (dashed line) and position of the interface between the cream layer and the serum phase (solid line) as a function of centrifugation time upon centrifugation of a sodium caseinate stabilized w/o/w double emulsion (mixed for 2 minutes) at 3000 rpm for 10 hours, followed by 300 rpm for 5 hours. The enclosed water volume fraction as determined after 100, 200, 300, 400 and 500 minutes high speed centrifugation is indicated.

In this chapter, the position of the interface between air and cream and between cream and serum is determined after 10 hours of centrifugation at 3000 rpm. However, Figure 5.4 clearly shows that most of the interstitial water is removed within 2 hours of high speed centrifugation: the apparent enclosed water volume fraction changed by less than 1% during the next 8 hours. Hence, the duration of high-speed centrifugation is not a critical parameter.

As interstitial water can hardly be completely eliminated, Akhtar and Dickinson (2001) proposed to introduce a correction based on the determination of the interstitial water volume of a simple o/w emulsion with comparable properties.

The light transmission through the cream layer of the o/w emulsion in Figure 5.1 was still close to 0% after centrifuging for 10 hours at 3000 rpm. As the transmission of a pure oil phase would be a lot higher, the low transmission of the cream layer indicates that coalescence did not occur and hence that there is still interstitial water present in the cream layer. The calculated enclosed

water volume fraction (%) for the polysorbate 60 stabilized o/w emulsion ($1.4 \pm 0.2\%$) was closer to zero than the value for the sodium caseinate stabilized o/w emulsion ($3.8 \pm 0.3\%$). This observation is in line with the well-known more pronounced interfacial tension reducing properties of small molecular weight emulsifiers (such as polysorbate 60) as compared to proteins. As the interfacial tension becomes lower, droplet deformation is facilitated, which indeed reduces the amount of interstitial water. Furthermore, sodium caseinate is charged and has a higher molecular weight than polysorbate 60, which might lead to a thicker, water-containing interfacial film.

When the water content of the cream phase is expressed relative to the cream layer volume (rather than to the overall volume), the interstitial water content of the cream layer was equal to $3.8/(25+3.8) = 13.2\%$ for the sodium caseinate stabilized o/w emulsion while a value of $1.4/(25+1.4) = 5.3\%$ was obtained for the polysorbate 60 stabilized o/w emulsion. Applying this correction, the enclosed water volume fraction for the data shown in Figure 5.1 is reduced from 23.3% to $48.3 \cdot (1 - 0.132) - 25 = 16.9\%$ whereby 48.3 is the volume% of cream corresponding to the first term in equation 5.1 whereas 25 is the volume% of oil which was used in the preparation.

5.3.4 Comparison of analytical photocentrifugation and PFG-NMR

Double emulsions stabilized using sodium caseinate as external emulsifier were mixed for one, two, three or four minutes using the Ultraturrax DK25. Vermeir et al. (2014b) found that increasing the mixing time not only leads to a decrease in droplet size, but also in enclosed water volume fraction as determined using T_2 -relaxometry. Using PFG-NMR on samples which were not centrifuged, the enclosed water volume fraction (%) was determined to be equal to 22.2, 20.3, 18.3 and 16.7% following one, two, three or four minutes of mixing, respectively. Also an emulsion stabilized using polysorbate 60 as external emulsifier was mixed for two minutes using the Ultraturrax DK25. Using PFG-NMR, the enclosed water volume fraction was determined to be equal to 20.0 %.

When the enclosed water volume fraction is calculated in duplicate from the volume fraction of cream obtained after high speed centrifugation diminished by the oil volume fraction, average values and standard deviations of 25.0 ± 0.4 , 23.3 ± 0.1 , $21.4 \pm 2.0.5$ and $20.3 \pm 0.4\%$ were obtained for one, two, three or four minutes of mixing, respectively (Figure 5.5).

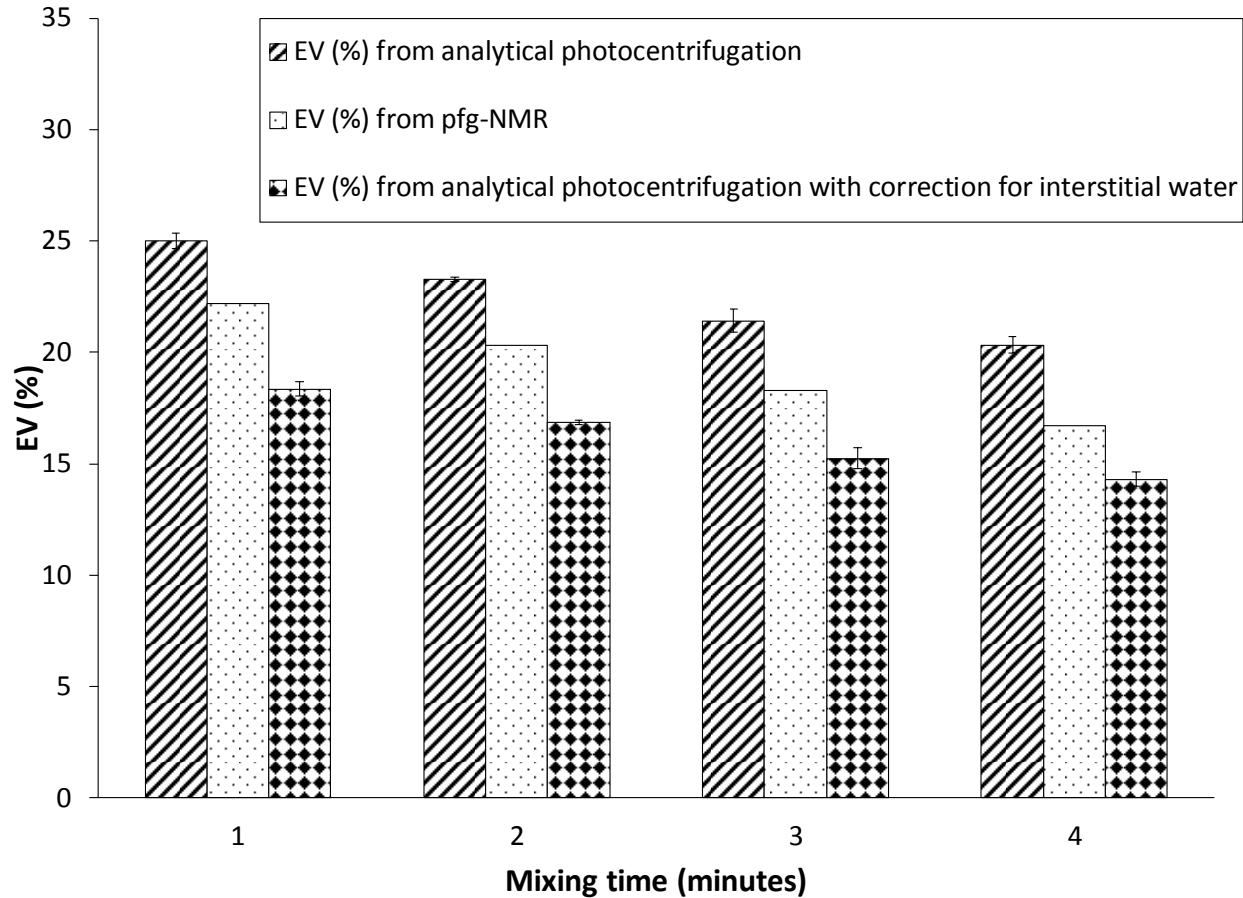


Figure 5.5: Enclosed water volume fraction of w/o/w (25/25/50; v/v/v) double emulsions as a function of the mixing time during the second homogenization step when stabilized using sodium caseinate, determined by analytical centrifugation and PFG-NMR. The error bars represent the standard deviation.

Whereas a similar (decreasing) trend is obvious, the estimated enclosed water volume fraction from analytical photocentrifugation was about 3% higher than from PFG-NMR in all cases. Whereas section 5.3.3 indicated that equation 5.1 may overestimate the enclosed water volume fraction due to interstitial water, Vermeir et al. (2014a) showed that PFG-NMR might underestimate the enclosed water volume fraction due to exchange of water between the enclosed and external water phase or between enclosed water droplets. This effect is however minimized in our experiments by measuring at a low temperature (5 °C). On the other hand, when the interstitial water is accounted for as proposed by Akhtar and Dickinson (2001), the calculated enclosed water volume fraction (%) was systematically lower than the value obtained using PFG-NMR. According to our opinion, this effect is most probably due to the higher compressibility of the bigger w/o droplets in the double emulsion than of the corresponding oil droplets in the o/w emulsion (Bibette et al., 2007); static light scattering indicated that the volume

weighted average diameter (D_{43}) was 18.7 and 8.7 μm for the droplets in the sodium caseinate stabilized w/o/w and o/w emulsion while the D_{43} was equal to 13.5 and 4.5 μm for the droplets in the polysorbate 60 stabilized w/o/w and o/w emulsion after 2 minutes of mixing. In fact, even if w/o/w and o/w emulsions of similar droplet size could be produced, it should be noted that the force acting on the droplets would be different because the density difference between dispersed phase and continuous phase is smaller in case of a double emulsion as compared to a simple o/w emulsion. Clearly, the use of parameters obtained using o/w emulsions to correct for interstitial water in the double emulsion cream layer is not a straightforward approach. As the proposed correction is not beneficial, we propose to use equation 5.1 without interstitial water correction as the resulting overestimation is limited provided that intensive centrifugation is performed and rebound is prohibited.

The enclosed water volume fraction of the polysorbate 60 stabilized emulsion, determined in duplicate using equation 5.1, was equal to 22.4% with a standard deviation of 0.3%. Similar to the experiment in which the effect of mixing was considered, this value is slightly higher as compared to PFG-NMR (20.0%), but the deviation is smaller for polysorbate 60 as compared to sodium caseinate. Applying the interstitial water correction, which has only a limited effect for polysorbate 60 stabilized emulsions as discussed previously, a value of $19.9 \pm 0.3\%$ was obtained, which is nearly identical to the value obtained using PFG-NMR.

It should be noted that in any case, the enclosed water volume fractions of double emulsions with the same composition can be easily compared using only the primary data (i.e. the volume fraction of cream) as the volume fraction of oil is a constant factor.

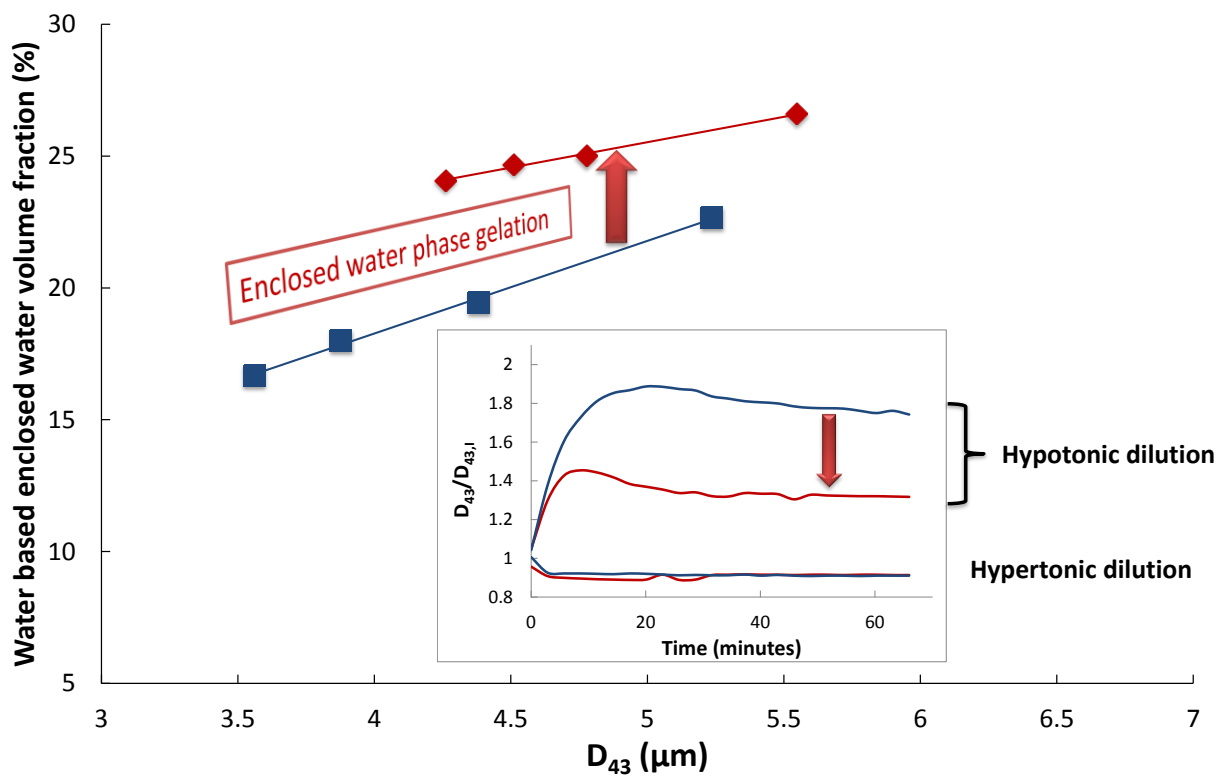
In this chapter, the determination of the enclosed water volume fraction of w/o/w type double emulsions was the main focus. However, a similar approach for o/w/o type emulsions might be possible, in which case the droplets sediment instead of cream in a centrifugal field.

5.4 Conclusions

Subtracting the oil content from the cream volume fraction, as determined by analytical photocentrifugation, provides a simple and straightforward tool for the determination of the enclosed water volume fraction of double w/o/w emulsions. As compared to PFG-NMR, analytical photocentrifugation was shown to overestimate the enclosed water volume fraction due to residual interstitial water in the cream layer. However, this effect was minimized by determining the cream volume during high speed centrifugation, especially for emulsions stabilized by small molecular weight emulsifiers. Comparing analytical photocentrifugation and

PFG-NMR, the former technique is less expensive, much simpler in data-analysis, more direct and can process several samples at the same time, which makes analytical photocentrifugation a highly attractive alternative for PFG-NMR.

Chapter 6: Double emulsions with a gelled enclosed water phase



- Enclosed water phase gelation was proven using low-resolution NMR T_2 -relaxometry
- W/o/w droplets with small diameter and high enclosed water content were produced
- Enclosed water phase gelation reduced shear-sensitivity of the double emulsion
- Enclosed water phase gelation influenced osmotic behavior of the double emulsion

Abstract

It has already been hypothesized and in some cases proven that gelation of the enclosed water droplets of w/o/w type double emulsions might allow the application of a higher shear in the second emulsification step while maintaining a high enclosed water content. In this contribution, thermal denaturation of a concentrated whey protein isolate solution, which is used as the enclosed water phase, was investigated. Low-resolution NMR T_2 -relaxometry measurements (using a CPMG-sequence) clearly showed that this thermal treatment effectively gelled the enclosed water phase. Using this approach, double emulsion droplets with a small average diameter (D_{43} of about 5 μm) and narrow droplet size distribution were produced while a high enclosed water content was maintained. Moreover, our results indicated that gelation could not prevent osmotic shrinking of the double emulsion droplets upon dilution in hypertonic solution. However, gelation of the enclosed water phase clearly reduced the degree of osmotic swelling of the double emulsion droplets upon hypotonic dilution. However, this effect is probably due to droplet-globule coalescence occurring at lower enclosed water volume increases in case the enclosed water phase is gelled.

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6.1 Introduction

It has already been hypothesized and in some cases proven that gelation of the enclosed water droplets might allow the application of a higher shear in the second emulsification step while maintaining a high enclosed water content (Iancu et al., 2009; Oppermann et al., 2015; Perez-Moral et al., 2014, p.; Surh et al., 2007). Hence, gelation of the enclosed water droplets might enable the production of double emulsion droplets with a sufficient amount of enclosed water as well as a small average diameter and narrow droplet size distribution. This implicates that double emulsions can be produced which are more stable, for example against flocculation and creaming (Ivanov et al., 1999; McClements, 2015).

Gelation can be achieved using a wide array of ingredients and preparation procedures. Whereas a reversible gel can be formed using starch (Iancu et al., 2009) or gelatin (Oppermann et al., 2015), irreversible gels can be formed using alginate in combination with Ca^{2+} -ions (Paques et al., 2013; Perez-Moral et al., 2014) or thermal denaturation of a whey protein isolate (WPI)-solution (Oppermann et al., 2015; Sağlam et al., 2011; Surh et al., 2007). In this work, the latter method has been preferred, since high protein foods are considered to be a potential candidate for body-weight control and treatment of obesity (Sağlam et al., 2011). Furthermore, several studies emphasized the importance of proteins in the diets of the elderly (Campbell et al., 2001; Evans, 2004; Kurpad and Vaz, 2000).

Gelation of the enclosed water phase of a w/o/w emulsion using thermal denaturation of a WPI-solution has already been described by other researchers. In the research reported by Surh et al. (2007), the water-in-oil emulsions under consideration were all equally stable in the second emulsification step, which may have been due to the high concentration of low-HLB emulsifier used. In contrast, Oppermann et al. (2015) observed a higher retention of enclosed water when a WPI-gel was used as the enclosed phase. However, the double emulsion droplets produced in the latter work were quite large (diameter of about 70 μm). Moreover, in both publications, a real proof of the enclosed water phase gelation was not provided. In this chapter, double emulsion droplets with a small average diameter and narrow droplet size distribution were produced. The enclosed water volume fraction of these double emulsion samples was determined using low resolution pulsed field gradient (PFG)-NMR diffusometry (Vermeir et al., 2014a), whereas low resolution NMR T_2 -relaxometry was used to prove that the enclosed water phase in the w/o/w or w/o emulsion was indeed gelled (Colsenet et al., 2005).

As the osmotic balance between the enclosed and external water phase is important to control the rheology and morphology as well as the release of encapsulated ingredients by external coalescence of enclosed water droplets (Delample et al., 2014; Iqbal et al., 2013; Leal-Calderon et al., 2012; Mezzenga et al., 2004), the osmotic behavior of the produced w/o/w emulsions was also examined. Whereas water can still migrate throughout the gel network, gelation might render the enclosed phase more resistive to expansion or contraction. As shown by Colsenet et al. (2005), the addition of whey proteins to a water phase decreases the self diffusion coefficient of the water molecules due to the combination of hydration and obstruction effects of the proteins. Hereby, the reduction of the water diffusion coefficient was bigger in case a gel was formed and the divergence between solution and gel increased with increasing protein concentration.

6.2 Materials and Methods

6.2.1 Materials

The lipophilic emulsifier polyglycerol polyricinoleate (PGPR 4150; min. 75 % n-glycerols with $n=2$, 3 and 4; max. 10 % m-glycerols with $m \geq 7$) and the hydrophilic emulsifier sodium caseinate (Na-caseinate; 5.5 % moisture; 96 % protein on dry matter) were kindly provided by Palsgaard A/S (Denmark) and Armor Protéines (Saint Brice en Cogles, France), respectively. Sunflower oil was acquired from the local supermarket, while whey protein isolate was acquired from Davisco (BiPRO, Davisco, Le Sueur Food Ingredient Company, USA). The 0.1M KCl (VWR Chemicals: BDH Prolabo, Leuven, Belgium) solution used in both the first and second water phase contained 0.02 wt% of the anti-microbial agent NaN_3 (Sigma-Aldrich, Steinheim, Germany). KCl was added to both aqueous phases in order to reduce Ostwald ripening effects due to the high Laplace pressure of the enclosed water droplets which in turn is related to their small size and thus high curvature (Mezzenga et al., 2004).

6.2.2 Emulsion preparation

6.2.2.1 Preparation of the first water phases (w_1)

The first water phase consisted of 0.1M KCl dissolved in deionized water containing 0.02 wt% NaN_3 and 5 or 10 wt% WPI. To induce gelation of these water phases, the solutions were placed in a water bath of 80 °C for one hour. At this temperature, the water phase is expected to turn into a gel due to thermal denaturation and aggregation of WPI (Puyol et al., 2001).

6.2.2.2 w/o emulsions preparation

The w/o emulsion containing 5 wt% WPI in the first water phase was prepared in duplicate; the double emulsions prepared using these primary emulsions are denoted with (1) and (2) hereafter. The oil phase consisted of sunflower oil containing 5 wt% PGPR. An Ultra-Turrax (type S50N-G45F, IKA®-Werke, Germany) at 5200 rpm was used to prepare the w/o emulsions (50:50, w/w) at 60 °C. The water phase was added gradually while stirring after which stirring was continued to obtain the final w/o emulsion. The obtained primary emulsion was split into two equal volumes: one sample was used as such in the preparation of a double emulsion while the other part was heated in a water bath at 80 °C for one hour to gel the water droplets.

6.2.2.3 w/o/w emulsion preparation

The second water phase (w_2) was mixed at room temperature with freshly prepared w/o emulsion in a 50:50 (w/w) ratio with an Ultra-Turrax S25KV-25G (IKA®-Werke, Germany) at 6500 rpm for 1 minute. This provided a macroscopically homogeneous but coarse double emulsion (premix). Hereby, the w_2 -phase differed from the w_1 -phase in the presence of 2 wt% of Na-caseinate and the absence of WPI. Afterwards, the premix was processed using a Microfluidizer (type M110S, Microfluidics) at 140 bar (driving air pressure of 1 bar) while avoiding creaming by swirling the emulsion. The emulsion was cooled during homogenization by a heat exchanger that was immersed in a water bath of 25 °C. After 1, 2, 3 and 4 passes, samples were taken.

6.2.3 Low-resolution NMR

NMR measurements were performed at 5 °C on a benchtop Maran Ultra spectrometer (Oxford Instruments, UK) operating at a frequency of 23.4 MHz. The samples of about 2.5 gram were filled in 18 mm outer diameter glass NMR-tubes (Oxford Instruments, UK) and their exact mass was carefully noted.

The free self-diffusion coefficients of the used water phases (D_s) were measured using the DSD script (Oxford Instruments, UK) varying the duration (δ) in ten steps between 0.05 and 2.75 ms while keeping the gradient strength (g) and the diffusion delay (Δ) constant at 0.14 T/m and 200 ms, respectively. A standard STE-sequence was used to obtain the data after which following relation can be used to obtain D_s :

$$I(\delta, \Delta, g) = I_0 * \exp(-D_s * q^2 * (\Delta - \frac{\delta}{3})) \quad (\text{Equation 6.1})$$

Whereby

$$q = \gamma * g * \delta \quad (\text{Equation 6.2})$$

$I(\delta, \Delta, g)$ are the echo intensities in the presence of gradient pulses of length δ while I_0 is the echo intensity in the absence of gradient pulses. γ is the gyromagnetic ratio ($2.675 \cdot 10^8 \text{ s}^{-1} \cdot \text{T}^{-1}$).

The free self-diffusion coefficient of the w_1 -phase with 5 wt% WPI was measured in duplicate and equaled $(1.217 \pm 0.007) \cdot 10^{-9} \text{ m}^2/\text{s}$ for the non-gelled water phase while this was $(1.205 \pm 0.028) \cdot 10^{-9} \text{ m}^2/\text{s}$ in case the water phase was gelled (average $\pm$ standard deviation). The diffusion coefficient of the w_1 -phase with 10 wt% WPI was measured to be $1.049 \cdot 10^{-9} \text{ m}^2/\text{s}$ for the non-gelled water phase while this was $9.918 \cdot 10^{-10} \text{ m}^2/\text{s}$ in case the water phase was gelled. Finally, the diffusion coefficient of the w_2 -phase was measured in duplicate and equaled $(1.180 \pm 0.009) \cdot 10^{-9} \text{ m}^2/\text{s}$ (average $\pm$ standard deviation).

Pulsed field gradient-NMR experiments on the double emulsion samples were performed using the stimulated echo pulse (STE) sequence, which was preceded by an inversion recovery experiment for suppression of the NMR-contribution from the oil phase in the emulsion. The inversion recovery experiment on the oil phase led to a τ_0 of 57.5 ms, which was used in all subsequent measurements. Measurements were performed varying the gradient strength (G) between 0 and 3.17 T/m while keeping the gradient duration (δ) constant (2.5 ms) and the diffusion delay (Δ) fixed at 180 ms.

As described in Vermeir et al., (2014a), the Murday Cotts model with free water contribution was fitted to the data taking into account the respective free self-diffusion coefficients of the water phases in order to determine the amount of encapsulated water. To have an estimate of the experimental uncertainty, the standard error of the point estimate was calculated by taking the square root of the reciprocal of the Hessian (Zelterman, 2015).

In order to use PFG-NMR to determine the enclosed water volume fraction, a correction is needed if the relaxation properties of the external water phase are different from the enclosed water phase. In Figure 6.1, the used radiofrequency sequence is shown as well as the used gradient pulses. During the τ_0 - and T -period, the signal decays due to longitudinal relaxation (next to the decay due to diffusion) while during both τ -periods, the signal decays due to transverse relaxation (next to diffusion). This means that if the enclosed and external water phases have a different T_1 - and T_2 -relaxation time, the signal intensity will be different for both phases not only through a difference in diffusion behavior but also due to relaxation effects.

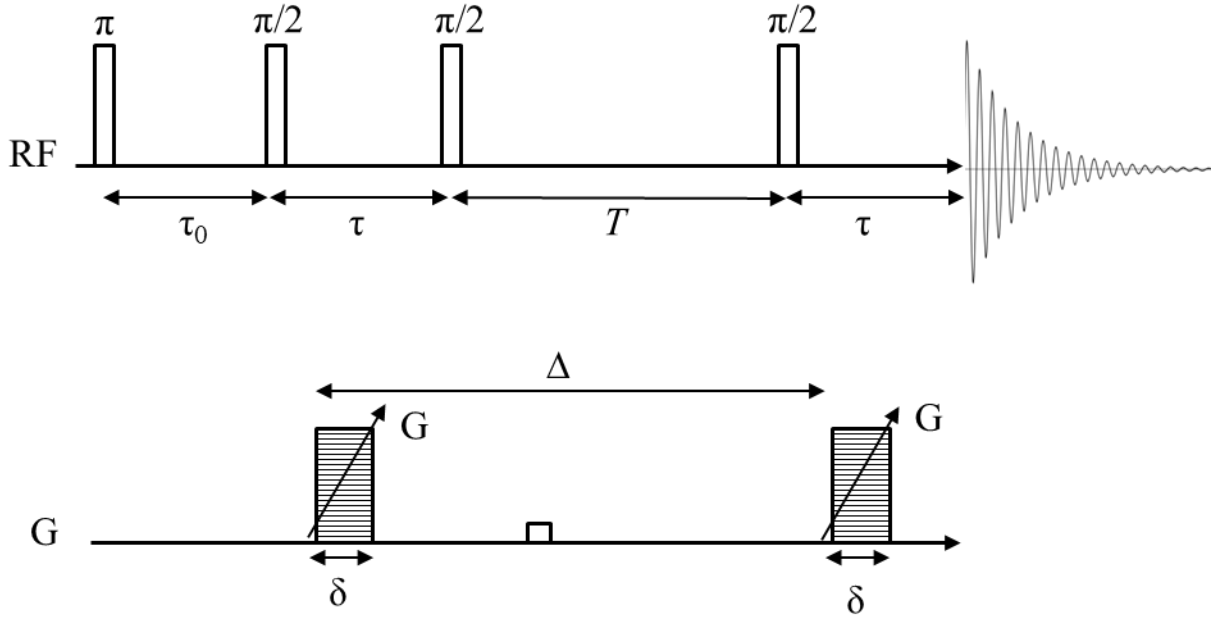


Figure 6.1: Schematic outline of the pulsed gradient stimulated echo (PGSTE) sequence.

According to Johnson Jr. (1999) the signal intensity of a water phase obtained after a STE-sequence equals:

$$I_{STE}(2\tau + T) = \left(\frac{M_0}{2}\right) * \exp\left(\frac{-2\tau}{T_2} - \frac{T}{T_1}\right) * \exp(-D * q^2 * \left(\Delta - \frac{\delta}{3}\right)) \quad (\text{Equation 6.3})$$

Herein, the first exponential factor is linked to relaxation effects while the second exponential factor is linked to diffusion.

Since in our experiments, an inversion recovery sequence is applied before the actual stimulated echo sequence, there is an additional decrease due to longitudinal relaxation during τ_0 . This leads to the following formula:

$$I_{IR+STE}(2\tau + T + \tau_0) = \left(\frac{M_0}{2}\right) * \exp\left(\frac{-2\tau}{T_2} - \frac{(T+\tau_0)}{T_1}\right) * \exp(-D * q^2 * \left(\Delta - \frac{\delta}{3}\right)) \quad (\text{Equation 6.4})$$

Wherein the reduction of the signal due to relaxation effects is equal to :

$$R_{IR+STE}(2\tau + T + \tau_0) = \exp\left(\frac{-2\tau}{T_2} - \frac{(T+\tau_0)}{T_1}\right) \quad (\text{Equation 6.5})$$

Therefore, in order to be able to correct for the difference in relaxation behavior of the used water phases, the T_2 - and T_1 -distributions of these phases were determined and the modes were

considered (Table 6.1). In our experiments τ_0 was equal to 57.5 ms and τ was fixed at 10 ms which implies T was equal to 170 ms.

Table 6.1: T_1 - and T_2 -relaxation times of the used water phases and the calculated signal decrease due to relaxation effects.

	T_1 (ms)	T_2 (ms)	R_{IR+STE}
W₂-phase	1311	897	0.82
5 wt% WPI-solution	1114	763	0.79
5 wt% WPI-gel	850	232	0.70
10 wt% WPI-solution	947	522	0.76
10 wt% WPI-gel	614	128	0.59

T_1 -relaxation (longitudinal relaxation) measurements were conducted using an inversion-recovery sequence which consisted of 100 data points from $\tau = 1000 \mu\text{s}$ to complete recovery of the longitudinal magnetization using sufficiently high relaxation delay values. Hereby, the τ -value is the time duration between the 180° and 90° radio frequency pulse. The T_1 -distributions were obtained using the WinDXP 1.8.1.0 software (Oxford Instruments, UK).

Finally, T_2 -relaxation (transversal relaxation) measurements were conducted using the Carr-Purcell-Meiboom-Gill (CPMG) sequence by keeping the receiver gain constant. Hereby, an exponentially decaying curve is obtained, which is characterized by a time constant T_2 . The T_2 -distributions were obtained by CONTIN analysis of the relaxation curve using the WinDXP 1.8.1.0 software (Oxford Instruments, UK).

6.2.4 Determination of the yield stress

To characterize the gel obtained using thermal denaturation of the WPI-solutions, the yield stress of the obtained gels was measured at room temperature using a YR-viscometer (Brookfield) at a run speed of 1 rpm, using a S-73 vane spindle and the secondary mark. Two measurements were performed.

6.2.5 Viscosity measurements

The viscosity of the w/o emulsions was measured using a rotational viscometer (Bohlin CSR-10, Malvern, UK) with plate-cone geometry whereby the diameter of the cone was 40 mm and the cone angle equal to 4° . The temperature was set to 20°C and the shear rate was varied between 1 and 50 s^{-1} using ten logarithmic steps. Every w/o emulsion was sampled five times

after which the viscosity of these samples was determined. The obtained shear stress to shear rate curve showed that these emulsions had a Newtonian flow behavior ($R^2 > 0.99$). From the slope of this curve, the viscosity could be determined taking into account all ten data points. Then, the average and standard deviation of the five viscosity measurements was calculated.

6.2.6 Determination of the droplet size distribution

The droplet size distributions of the double emulsion droplets were determined using a Malvern Mastersizer 3000 (Malvern Instruments) with Hydro MV accessory whereby the refractive indices were set to $1.53 + 0.01i$ for the dispersed phase and 1.33 for the continuous phase. The Mie theory was used to obtain the droplet size distribution as well as the volume weighted average diameters (D_{43}). Measurements were performed in 0.1M KCl solution to avoid osmotic gradients between the enclosed and external water phases. The stirring speed was set to 1500 rpm and sample was added until an obscuration between 2 and 6 % was reached.

Two independent measurements in this isotonic solution were performed, each of them consisting of three dependent measurements.

6.2.7 Light microscopy

The double emulsions were diluted ten times with 0.1M KCl solution and observed under the microscope to get an idea of the enclosed water droplet size as well as to get a visual proof of the fact that enclosed water was present in the obtained emulsions. The diluted double emulsion was brought on a microscopy slide using a capillary after which a cover slip was gently applied followed by a drop of immersion oil. Image acquisition was conducted using an optical microscope (Olympus Cx40) with 100-fold objective coupled with a digital camera (AxioCam ERc 5S).

6.2.8 Osmotic effects

The effect of a change in the osmotic pressure of the external water phase on the volume weighted average diameter (D_{43}) of the double emulsions was followed using an automated measurement sequence in the Mastersizer 3000. The double emulsions were diluted in hypo- (deionized water) and hypertonic (0.2M KCl) solution and 24 subsequent measurements were performed with a time-interval of 170 seconds in between two measurements (including 20 seconds of measurement). The applied settings were the same as for the isotonic droplet size distribution measurements (part 6.2.6).

Because often a fouling layer was formed on the glass walls of the flow-through cell during the measurement, 0.1 wt% polysorbate 60 was added to the water phases. The experimental results indicated that the addition of polysorbate 60 did not affect droplet size determination results. Moreover, a solution of 2 wt% Na-caseinate dissolved in deionized water was also used as dilution liquid, but the variability in the obtained measurements was bigger due to a higher background signal (data not shown).

6.3 Results and Discussion

6.3.1 Characterization of the enclosed water phases

For the gels prepared using 5 wt% WPI, the yield stress was measured to be equal to 998 ± 16 Pa. In contrast, for the gels prepared using 10 wt% WPI, the yield stress was out of range, even at a speed of 0.05 rpm. This shows that the yield stress of the gel prepared using 10 wt% WPI was at least 1600 Pa. These measurements show that using the proposed composition and heating procedure, gels were indeed obtained whereby the yield stress of the gel depended on the used WPI-concentration. However, to be able to prove gelation of the enclosed water phase of a w/o emulsion, another technique is needed.

As stated by Colsenet et al. (2005), the addition of whey proteins to the water phase leads to a shift of the T_2 -relaxation time of this phase towards smaller values. Considering the T_2 -relaxation time distributions of the used water phases (Figure 6.2), one can indeed see that the modal T_2 -relaxation time in Figure 6.2 shifted from 763 to 522 ms as the WPI concentration was increased from 5 to 10 wt%, whereas the modal T_2 -relaxation time of the W_2 -phase was equal to 897 ms. Furthermore, denaturation and subsequent aggregation by heating (and thus gel-formation) led to a more pronounced shift: upon heating, the T_2 -relaxation times of the 5 and 10 wt% WPI-solutions were reduced to 232 and 128 ms, respectively.

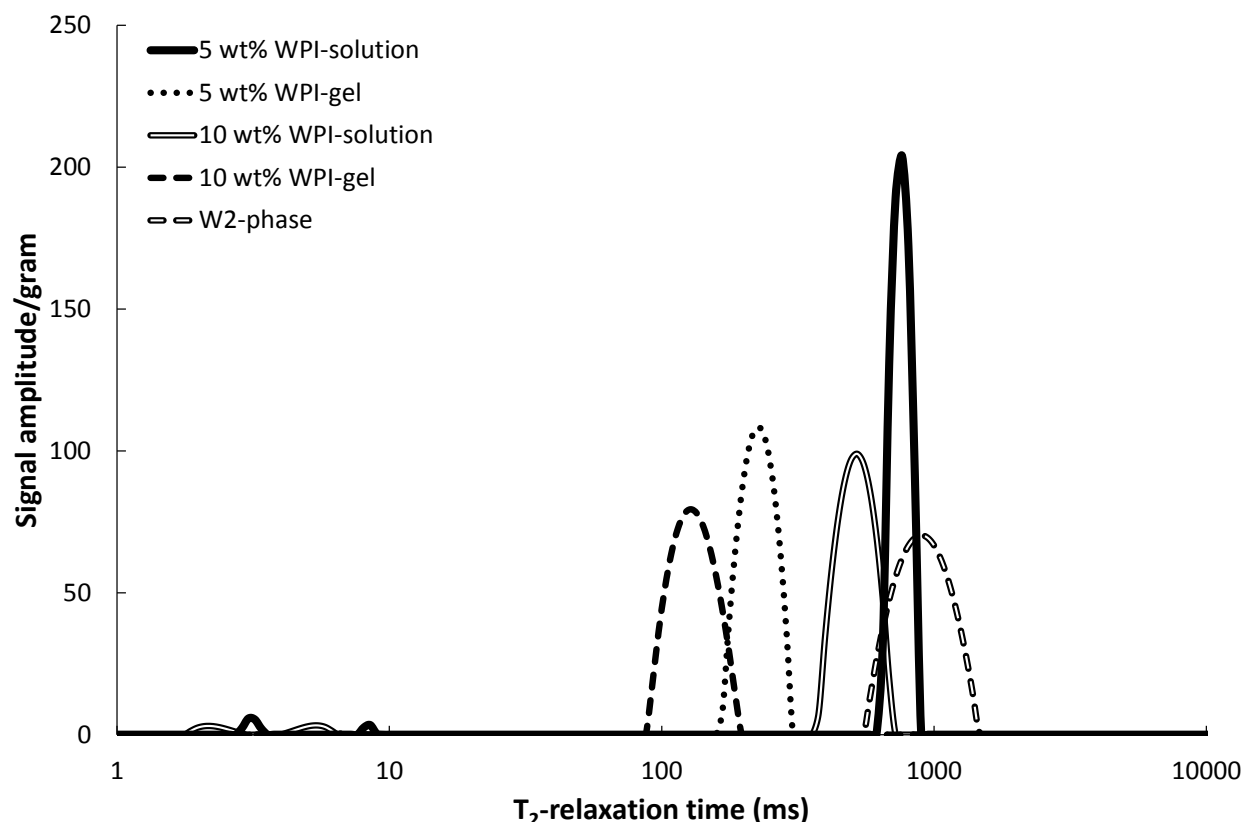


Figure 6.2: T_2 -distributions of the water phases used in the preparation of the w/o/w emulsions.

6.3.2 Proof of gelation of the enclosed water phase of w/o or w/o/w emulsions

Applying the CPMG-sequence to measure the T_2 -distribution of the w/o emulsions before and after heating thus makes it possible to check whether the water phase is indeed gelled. Subtracting the T_2 -distribution obtained for the unheated w/o emulsion from the T_2 -distribution of the same amount of the corresponding heated w/o emulsion, Figure 6.3 reveals that a peak disappeared at about 280 ms while a new peak appeared at about 120 ms upon heating the w/o emulsions containing 10 wt% WPI. These T_2 -relaxation times are slightly lower than those obtained for a 10 wt% WPI-solution or gel, respectively (Figure 6.2), which is due to the fact that the water in the w/o emulsion is contained within small droplets (Chapter 2). The difference in T_2 -relaxation time of the heated and unheated w/o emulsion clearly proves that the enclosed water phase of the w/o emulsions used in the preparation of the double emulsions was gelled by the applied heating step. A similar profile, albeit slightly shifted to higher T_2 -values, was obtained for the w/o emulsions containing 5 wt% WPI (data not shown). In fact, the T_2 -distributions of the w/o/w emulsions also enabled to check whether or not the enclosed water phase was gelled (data not shown).

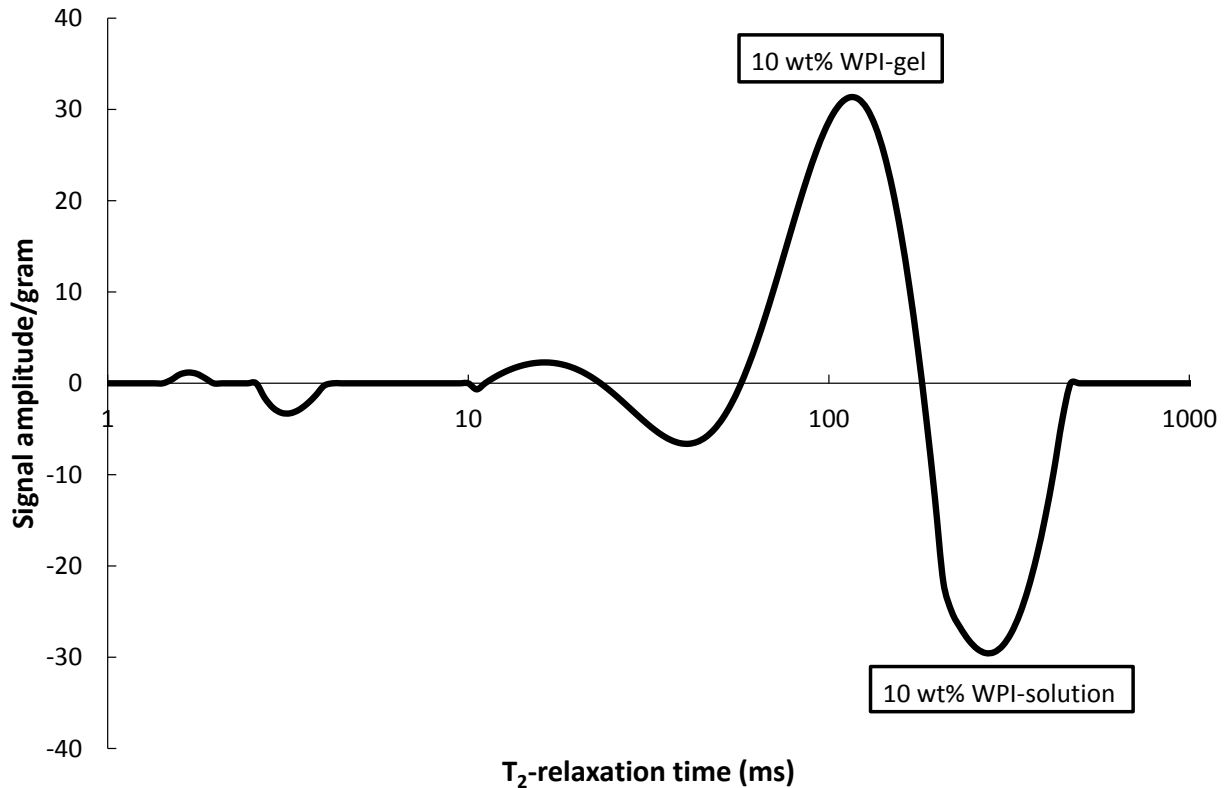


Figure 6.3: Difference between the obtained T_2 -distribution of a heated and unheated 10 wt% WPI-containing w/o emulsion (50/50; w/w).

6.3.3 Double emulsion droplet size

Plotting the volume weighted average diameter (D_{43}) as a function of the number of passes through the Microfluidizer clearly shows that as the amount of passes through the Microfluidizer was increased, the effect of an additional pass on the average diameter steadily diminished (Figure 6.4). Also the difference between 5 and 10 wt% WPI in the enclosed water phase did not seem to be a determining factor. The difference between the two samples containing 5 wt% WPI can probably mainly be attributed to small variations in the homogenization pressure as they were produced on different days while sample (1) containing 5 wt% WPI and the sample containing 10 wt% WPI were produced on the same day.

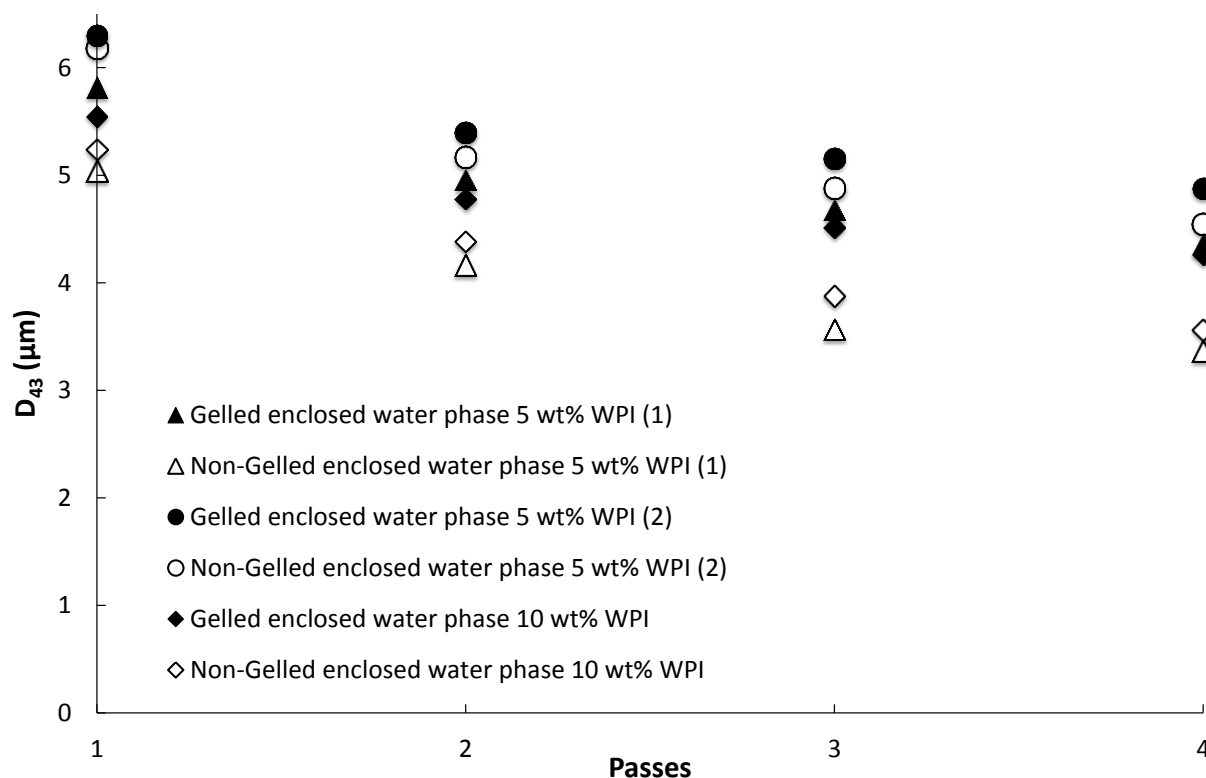


Figure 6.4: Influence of the number of passes through the microfluidizer on the volume weighted average diameter of w/o/w type (25/25/50; w/w/w) double emulsions containing either gelled or non-gelled enclosed water droplets as measured using static light scattering.

However, for every sample, gelation of the enclosed water phase appeared to lead to a bigger droplet size for the same processing conditions. As will be described in more detail in section 6.3.4, this observation can mainly be explained by the higher amount of enclosed water that was maintained when the enclosed water phase was gelled.

Figure 6.5 shows that very narrow, monomodal distributions were obtained for the emulsions with a gelled enclosed water phase. In contrast, for the emulsions with a non-gelled enclosed water phase, the distributions were somewhat skewed and broader. Increasing the number of passes led to a shift of the droplet size distributions towards smaller droplet sizes for both the gelled and non-gelled formulation.

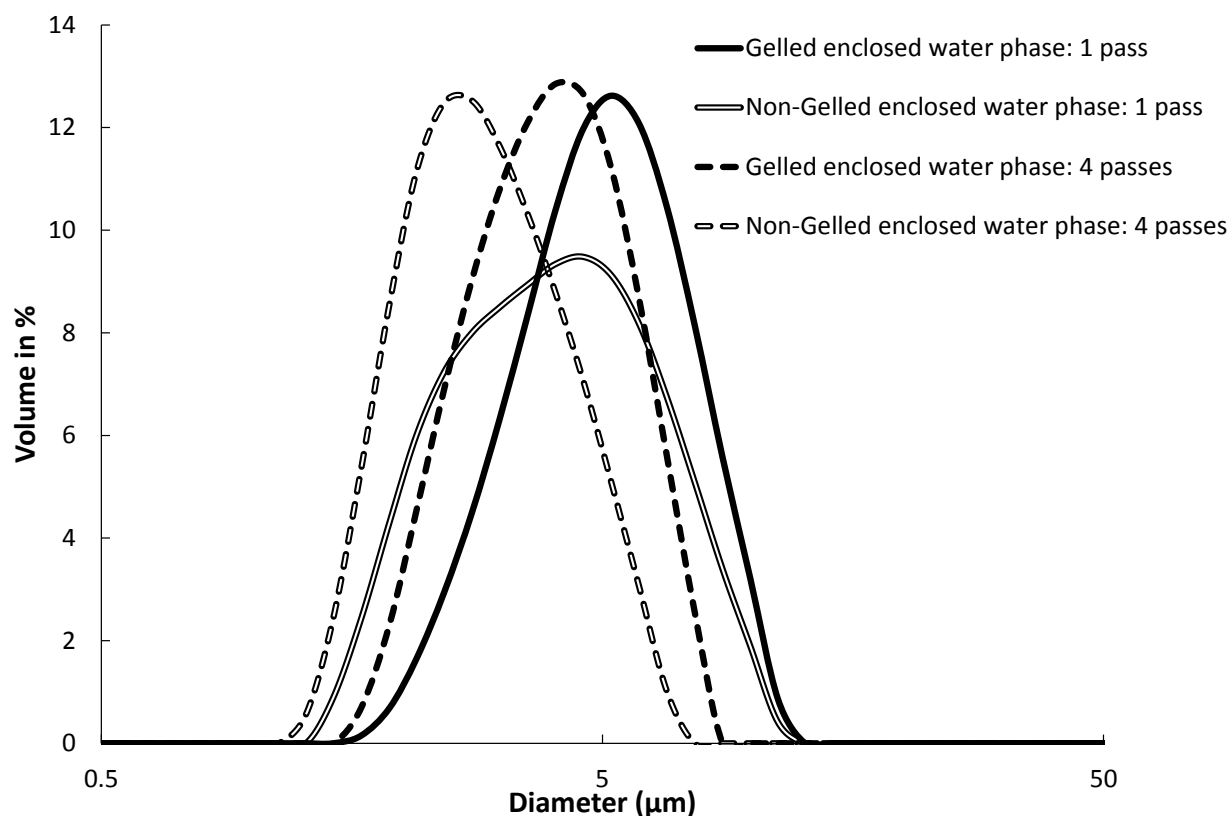


Figure 6.5: Differential droplet size distributions of w/o/w emulsions containing 5 wt% WPI (1) in gelled or non-gelled state in the enclosed water phase for 1 or 4 microfluidisation passes as measured using static light scattering.

6.3.4 Enclosed water volume fraction

Figure 6.6 clearly shows that enclosed water is present in both types of w/o/w emulsions regardless of enclosed water phase gelation. In addition, the physical appearance was very similar and no enclosed water droplets bigger than 1 μm in diameter seemed to be present in both types of double emulsion droplets.

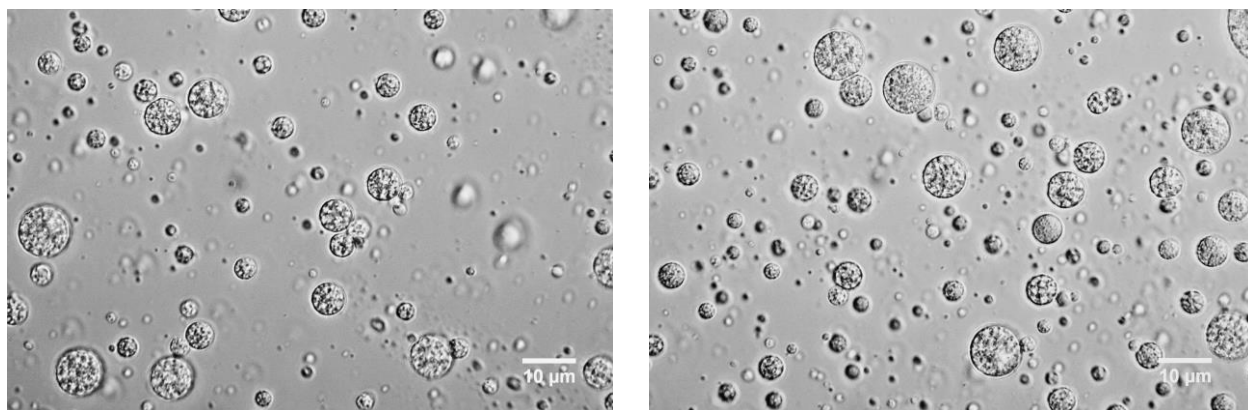


Figure 6.6: Pictures from light microscopy on w/o/w emulsions using a 100x objective containing either 5 wt% WPI in gelled (left) or non-gelled (right) state after 10x dilution in 0.1M KCl solution.

To quantify the enclosed water volume fraction in the double emulsions, PFG-NMR was used. In this chapter, in contrast to the other ones, the enclosed water volume fraction was defined as the fraction of water in the enclosed water droplets relative to the total amount of water present. This fraction is defined as the water based enclosed water volume fraction (EV_w). Hereby, one should keep in mind that the signal obtained using the PFG-NMR sequence is solely linked to the water molecules because proteins have T_1 - and T_2 -relaxation times which are much smaller.

Figure 6.7 clearly shows that gelation of the enclosed water phase containing 10 wt% WPI-solution led to a significant decrease of the obtained echo intensity: on average, the signal of the gelled w/o emulsion was reduced by 17 % as compared to the non-gelled w/o emulsion. As a further consequence, a correction, taking into account the reduction of the signal due to T_1 - and T_2 -relaxation during the measurement (R_{IR+STE}) will be necessary to compensate for this effect. The latter factor (R_{IR+STE}) can be calculated using equation 6.5.

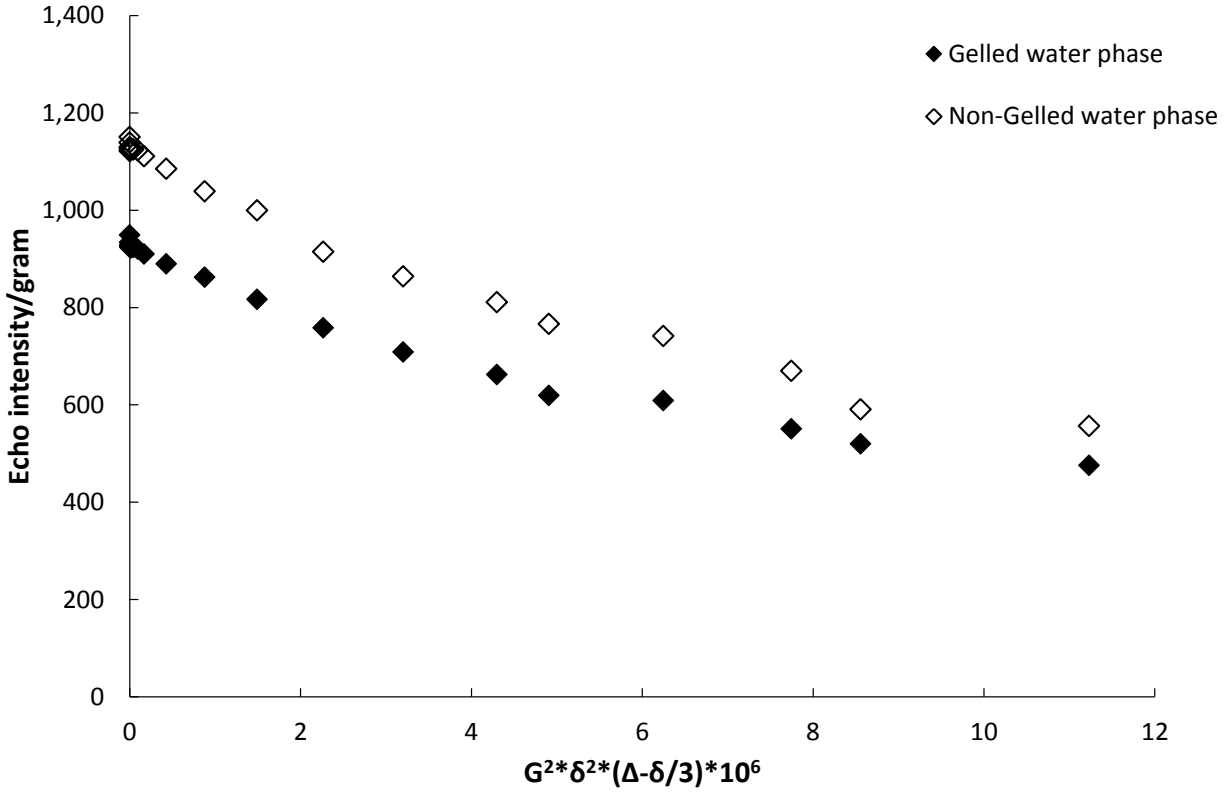


Figure 6.7: Echo decay signal intensity obtained for w/o emulsions containing 10 wt% gelled or non-gelled WPI-solution.

Dividing the R_{IR+STE} as obtained for the 10 wt% WPI-gel by the R_{IR+STE} of the 10 wt% WPI-solution indeed gives a reduction of the signal due to gelation to 78 % of the non-gelled solution, which is fairly close to the experimentally determined value of 83 %, as observed in Figure 6.7.

In order to determine the fraction of the water phase which is enclosed, the apparent enclosed water volume fraction ($EV_{w,a}$) as determined using the Murday Cotts model with free water contribution was corrected for the reduction of the signal due to relaxation effects:

$$EV_w(\%) = \frac{\frac{EV_{w,a}}{R_{IR+STE}(W_1)}}{\frac{1-EV_{w,a}}{R_{IR+STE}(W_2)} + \frac{EV_{w,a}}{R_{IR+STE}(W_1)}} * 100 \quad (\text{Equation 6.6})$$

As only a very gentle second emulsification step was used in the preparation of the premix samples and keeping in mind that only the water protons contribute to the signal, a water based enclosed water volume fraction of about 32.6 or 31.5 % is expected for the emulsions with 5 or 10 wt% WPI, respectively. However, the water based enclosed water volume fraction for the premix samples was calculated to be equal to 35.3 ± 1.2 , 33.6 ± 3.3 , 35.2 ± 1.2 and 33.8 ± 2.1 for the

Gelled 5 wt% WPI (1), (2) and the Non-Gelled 5 wt% WPI (1), (2) samples, respectively. For the emulsion containing 10 wt% WPI in gelled state a water based enclosed water volume fraction of $36.3 \pm 1.4\%$ was obtained while this was $35.6 \pm 1.5\%$ for the emulsion containing 10 wt% WPI in non-gelled state. The higher than expected enclosed water volume fraction is probably due to the fact that the osmotic pressure of the dissolved WPI is not negligible. In fact, the supplier mentions that the used WPI typically contains about 1.9 % of ash. Similarly, the osmotic pressure of the Na-caseinate solution will be larger than zero, but as less Na-caseinate is used than WPI, there is probably a (limited) net inward osmotic gradient. As water exchanges rapidly (as can be seen in part 6.3.5), the osmotic balance is quickly restored by the transport of external water to the enclosed water phase, which increases the enclosed water volume fraction. However, this does not have an influence on this experiment as we only want to compare gelled to non-gelled samples with the same concentration of proteins in the enclosed water phase.

For routine determination of the correction factor one might apply the STE-sequence without magnetic gradients on the samples. This gives the signal attenuation without contribution by diffusion. Keeping in mind the mass of the sample under consideration as well as its composition, a correction factor can be determined from the obtained intensity relative to the intensity obtained for the same amount of w_2 -phase. This approach is similar to what was shown in Figure 6.7 for a w/o emulsion containing either gelled or non-gelled WPI.

Because during the production of double emulsions, it is mostly desired to produce small droplets with a sufficient amount of enclosed water, a plot of both these parameters gives an indication of a desirable composition and processing condition. From Figure 6.8, it is clear that by gelation of the enclosed water phase, more water can be retained for similar droplet sizes. Hereby, the higher enclosed water volume fraction that is obtained in case the enclosed water droplets are gelled can not be attributed to a difference in viscosity of the primary emulsion with either a gelled or non-gelled water phase. Indeed, the viscosity of the w/o emulsion containing 5 wt% WPI in non-gelled state was measured to be 706 ± 50 mPa.s, while the viscosity was measured to be 646 ± 35 mPa.s in case the enclosed water droplets were gelled.

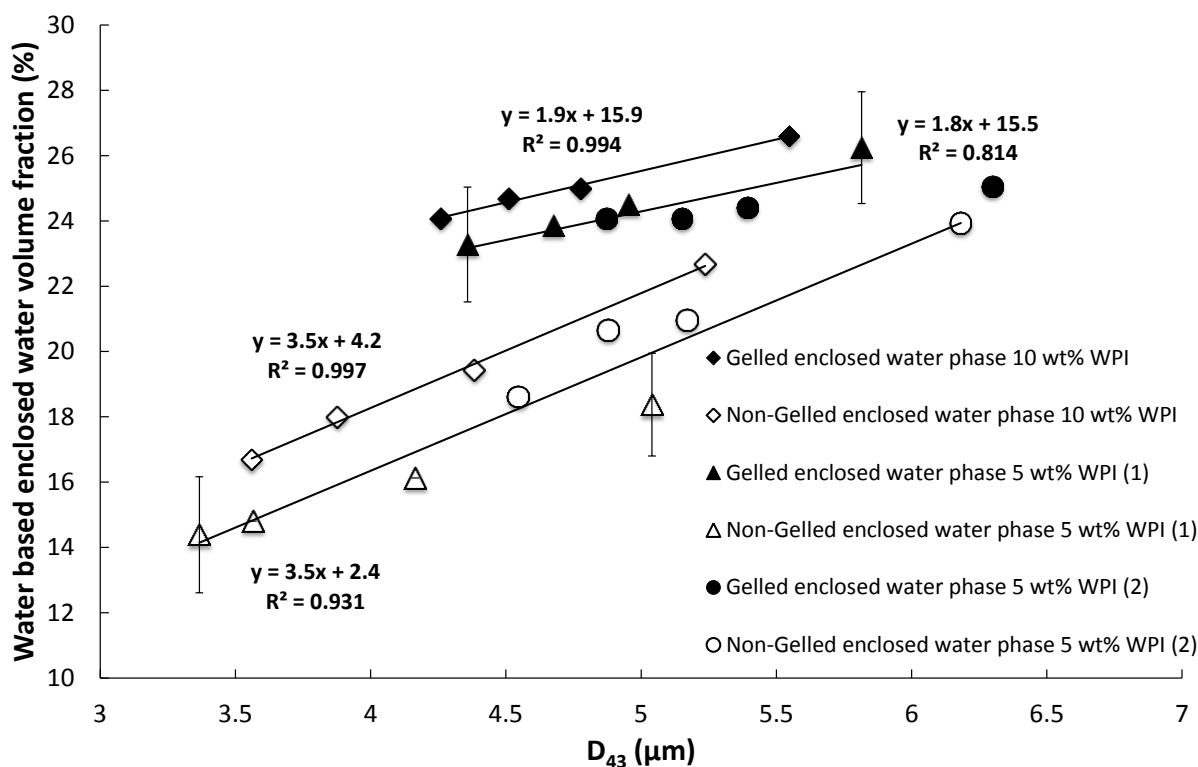


Figure 6.8: Water based enclosed water volume fraction as a function of volume weighted average diameter of w/o/w type double emulsions with gelled or non-gelled enclosed water phases which were prepared using a microfluidizer with one to four passes.

In Figure 6.8, the standard error of the point estimate of the water based enclosed water volume fraction is exemplarily shown for the double emulsions containing 5 wt% WPI (1) in gelled or non-gelled state and for one or four passes through the microfluidizer. In fact, in all measurements the standard error of the point estimate of the water based enclosed water volume fraction (%) varied between 1.2 and 3.3%. The 95 % confidence interval of the slope of both water based enclosed water volume fraction versus volume weighted diameter curves for the emulsions with a gelled enclosed water phase is equal to 1.8 ± 1.0 %/ μm and 1.9 ± 0.5 %/ μm for the emulsions with 5 or 10 wt% WPI, respectively. Hereby the point obtained for the highest diameter of the 5 wt% emulsion was not considered in the regression. In contrast, the 95 % confidence intervals of the slope of the enclosed water volume fraction versus D_{43} curves for the emulsions with a non-gelled enclosed water phase was equal to 3.5 ± 0.9 %/ μm and 3.5 ± 0.6 %/ μm for the emulsions with 5 or 10 wt% WPI, respectively. The slope of the emulsions with non-gelled enclosed water is thus significantly higher as compared to the emulsions in which the enclosed water phase is gelled. This clearly illustrates that emulsions with a gelled

enclosed water phase were more stable towards physical stresses during production, such as pumping in commercial production environments.

6.3.5 Stability towards osmotic gradients

To compare the influence of gelation of the enclosed water phase on the osmotic sensitivity, samples were selected with an enclosed water volume fraction and droplet size that was similar. For both 5 and 10 wt% WPI, the double emulsions passed once through the microfluidizer were selected. In case of the 5 wt% sample, sample (2) was selected.

As shown for the emulsion containing 10 wt% WPI-gel as enclosed water phase, the volume weighted average diameter did not change significantly upon dilution in isotonic medium containing 0.1 wt% polysorbate 60 during a 1 hour measurement sequence. This was the case for all samples under consideration. On the other hand, Figure 6.9 clearly indicates that when the double emulsions were dispersed in hypotonic medium, they started swelling very quickly. When the enclosed water phase was gelled, after about 10 minutes a maximum value was reached after which the average diameter started to decrease. In contrast, when the enclosed water phase was not gelled, a maximum value was reached after about 20 minutes. As the initial slopes of the gelled and non-gelled w/o/w emulsions were similar, the longer swelling emulsions were also swollen to a larger extent. The maximum in the curve is probably due to enclosed water droplets coalescing with the external water phase when more water has entered the enclosed droplets than the emulsifiers can stabilize.

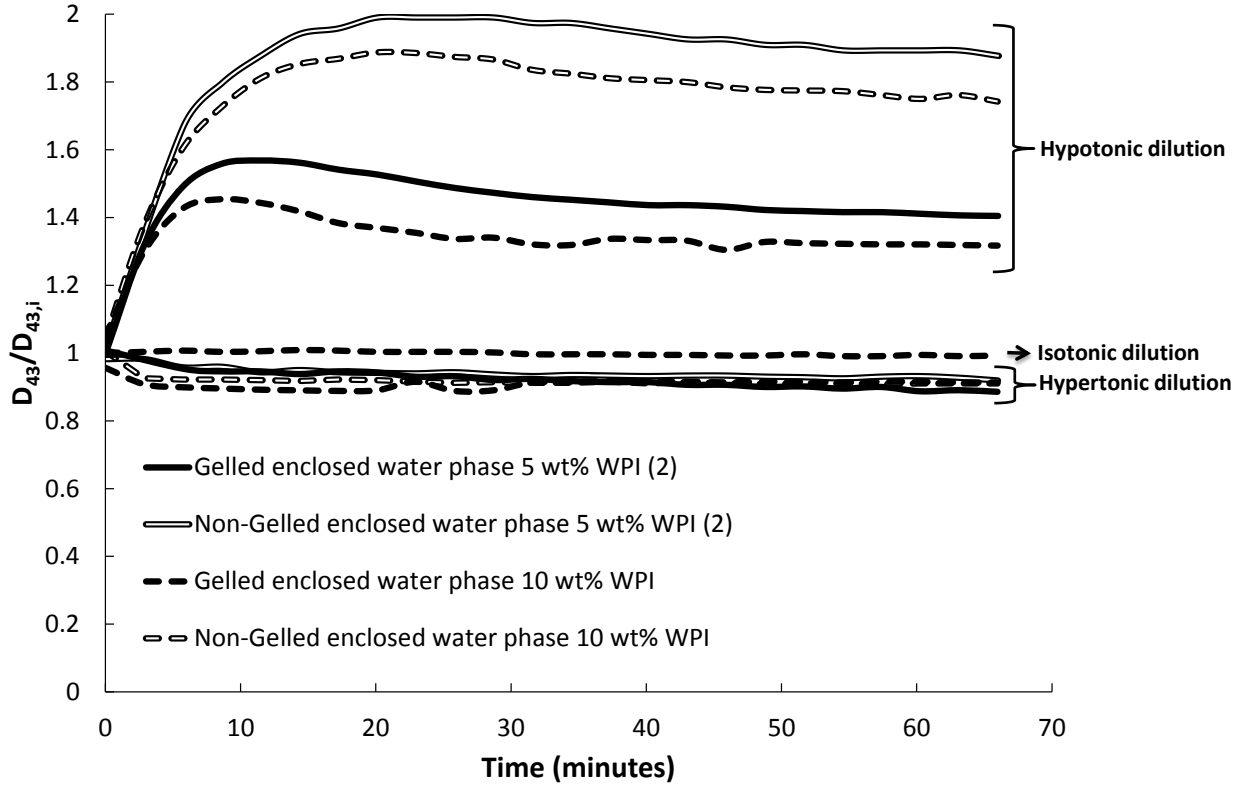


Figure 6.9: Evolution of the volume weighted average diameter of w/o/w type emulsion droplets with a gelled or non-gelled enclosed water phase upon hyper- and hypotonic dilution relative to the volume weighted average diameter as determined in isotonic solution.

In fact, the relative increase of the w/o interface can be estimated from the relative increase of the double emulsion droplet diameter ($D_{43}/D_{43,i}$) if the number of water droplets within each oil globule is assumed to be constant during osmotic swelling. Considering a density of about 0.92 g/mL for sunflower oil and 1 g/mL for the water phase, the volume ratio between water and oil in the primary emulsion is equal to about 48/52 (v/v). For the non-gelled w/o/w emulsion containing 5 % of WPI, the diameter nearly doubles, which means that the external surface area is increased by a factor of 4 and the droplet volume by a factor of 8. Assuming the retention of all enclosed water, the droplets contain 48 volume% of water, which implies that the volume of the entrapped water droplets is increased by a factor of about 15. Taking the third root, it follows that the enclosed droplet diameter is increased by a factor of about 2.47 and hence the w/o interfacial area by a factor of about 6.

Also the critical inner droplet volume fraction can be calculated which is the maximum volume fraction of water in the double emulsion droplet that is obtained during swelling. In aforementioned example the critical inner droplet volume fraction equals 93 %. As mentioned, all

these estimations are based on the maximal enclosed water volume fraction. The actual enclosed water volume fraction as obtained from the PFG-NMR-measurements can also be taken into account and gives rise to the relative w/o surface area increase and critical inner droplet volume fraction values which are mentioned in Table 6.2.

Table 6.2: Maximal relative increase of the w/o and o/w surface area and critical inner droplet volume fraction (%) as a function of the obtained maximal $D_{43}/D_{43,i}$.

Sample	Max. $D_{43}/D_{43,i}$	Relative increase o/w surface area	Relative increase w/o surface area	Critical inner droplet volume fraction (%)
Gelled 5 wt% WPI	1.57	2.46	3.92	85
Non-Gelled 5 wt% WPI	1.99	3.96	7.65	92
Gelled 10 wt% WPI	1.45	2.10	3.19	82
Non-Gelled 10 wt% WPI	1.88	3.53	6.13	91

In this respect, it should be kept in mind that PGPR might interact with the hydrophobic regions in whey proteins (Gülseren and Corredig, 2012) which could lead to a lower concentration of PGPR available for the stabilization of the extra interfacial area created during swelling. This hypothesis is sustained by the observation that the maximum degree of swelling is lower for 10 % WPI containing double emulsions, and is further reinforced by the observation that when no WPI is added to the enclosed water phase, swelling continues for more than one hour (data not shown). Due to denaturation, more hydrophobic regions of the proteins become exposed, which may lead to an even lower amount of available PGPR for stabilisation of the additional interfacial area produced upon swelling. This is in line with the experimentally observed lower maximum degree of swelling of double emulsions containing heat-treated WPI.

Moreover, it is likely that droplet-globule coalescence (i.e. coalescence of the inner droplets on the oil globule surface) is accelerated above a critical inner droplet volume fraction, when thin liquid films become highly compressed. Obviously, this critical volume fraction will depend on droplet deformability which itself depends on the interfacial tension and on the gelation state of the droplets. The interfacial tension as well as the viscoelastic properties of the interface may

change upon interaction between PGPR and either native or denatured WPI (Gülseren and Corredig, 2012) which may also explain the experimentally observed difference in the maximum degree of swelling. The critical inner droplet volume fractions that were obtained (Table 6.2) indicate that osmotic swelling leads to volume fractions which largely exceed the values for random or hexagonal close packing of monodisperse spheres (i.e. 64 and 74 %, respectively). Hence, droplets become largely compressed upon osmotic swelling.

When diluted in hypertonic medium, all droplets shrink. Shrinking is, however, more difficult to follow. When 0.2M KCl is used to dilute the double emulsion, the osmotic pressure difference between both water phases is much higher than the Laplace pressure difference and the equilibrium will be reached when $\pi_{\text{enclosed}} \approx \pi_{\text{external}}$. Even if all water remains enclosed and considering the fact that to obtain osmotic equilibrium between the enclosed and external water phase the enclosed water phase volume has to halve, the volume of the w/o droplet is reduced by only 25 %, and hence the double emulsion droplet diameter is reduced to about $\sqrt[3]{0.75} = 0.9$ of its initial value. The experimental results shown in Figure 6.9 indicate that this theoretical value is approached. Clearly, more sensitive techniques are needed to compare the behavior of double emulsions with a gelled and a non-gelled enclosed water phase when diluted in hypertonic media. It can nonetheless already be stated that gelation of the enclosed water can not protect the double emulsions against shrinking when stored in hypertonic medium.

According to Iancu et al. (2009), gelation might slow down the exchange of encapsulated materials. The influence of the gelation of the enclosed water phase using a WPI-solution on the exchange of encapsulated materials is thus an interesting subject for further research.

6.4 Conclusions

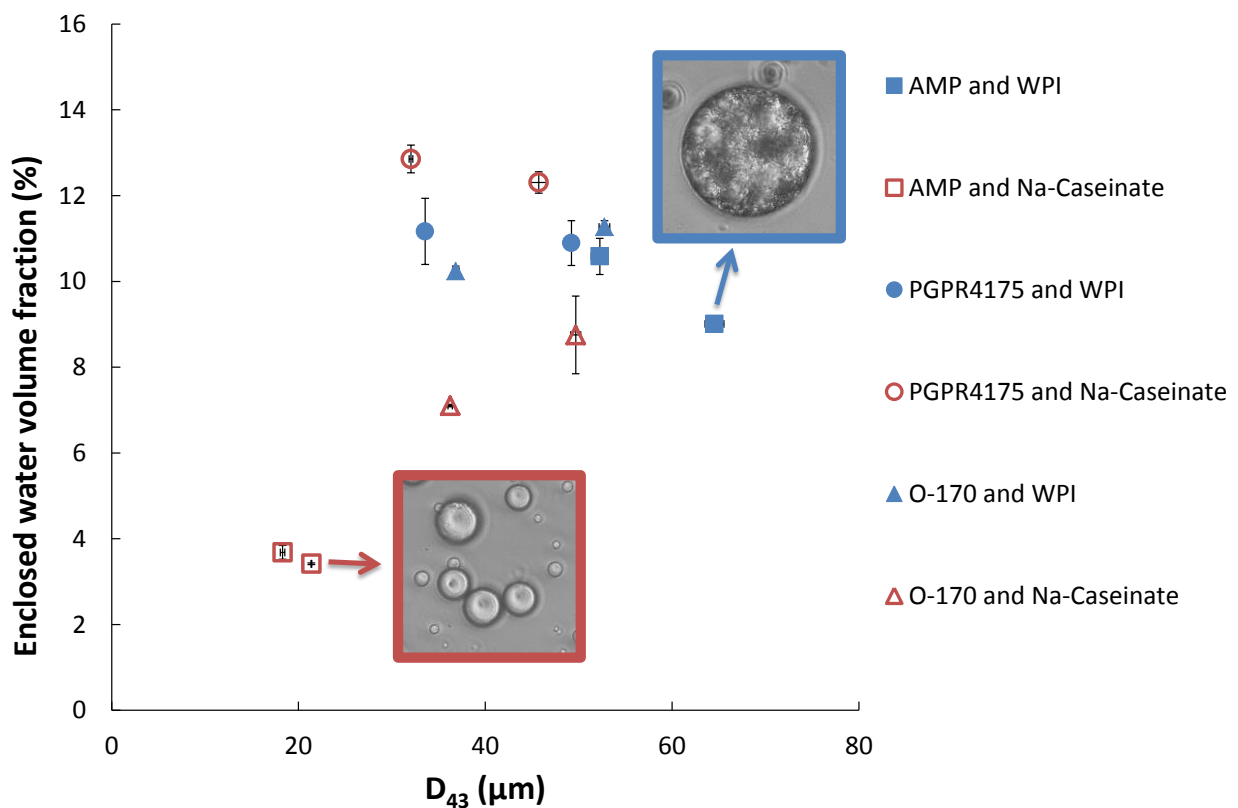
The first part of this chapter showed that low-resolution NMR T_2 -relaxometry using a CPMG-sequence allows to prove that the enclosed water droplets of w/o and w/o/w emulsions are gelled. Due to its effect on the T_1 - and T_2 -relaxation time of the water phase, gelation has an influence on the signal amplitude obtained using PFG-NMR. As a further consequence, a combined T_1 - and T_2 - correction is necessary in order to obtain an accurate estimation of the amount of encapsulated water by PFG-NMR when the composition of the external water phase is different from the enclosed water phase.

Upon gelation of the enclosed water phase, it was possible to produce double emulsions with a narrow droplet size distribution and a small average diameter (D_{43} of about 5 μm) while maintaining a higher enclosed water volume fraction as compared to samples with non-gelled

enclosed water. Moreover, when increasing the amount of passes through the microfluidizer, a faster decrease of the enclosed water volume fraction with decreasing droplet size is observed in case the enclosed water is not gelled. Hence, gelation helps to protect double emulsions against shear induced disruption of the enclosed water phase.

As far as osmotic effects are concerned, our results indicated that gelation could not prevent osmotic shrinking of the double emulsion droplets upon dilution in hypertonic solution. However, gelation of the enclosed water phase clearly reduced the maximum degree of osmotic swelling of the double emulsion droplets. However, this effect is probably due to droplet-globule coalescence occurring at lower enclosed water volume increases in case the enclosed water phase is gelled.

Chapter 7: Use of ammonium phosphatide and a low-HLB sucrose ester as alternatives for PGPR



- Stable w/o emulsions were prepared with alternatives for PGPR
- With WPI, all w/o/w emulsions produced had similar enclosed water volume fractions
- In the presence of Na-Caseinate, the enclosed water was released from AMP-stabilized w/o/w emulsions
- AMP- and O-170 stabilized w/o/w emulsions were more sensitive to osmotic imbalances than their PGPR-counterparts

Abstract

In most publications concerning edible w/o/w emulsions, the low-HLB emulsifier polyglycerol polyricinoleate (PGPR) is used to stabilize the w/o interface in combination with a high-HLB emulsifier which stabilizes the o/w interface. Therefore, PGPR was used as the reference low-HLB emulsifier and compared to two alternative low-HLB emulsifiers namely ammonium phosphatide (AMP) and low-HLB sucrose ester O-170. As high-HLB emulsifiers both random coil (sodium caseinate) and globular proteins (whey protein isolate) were used. Hereby, the use of WPI led to similar, high enclosed water volume fractions for all of the used low-HLB emulsifiers whereas the use of Na-caseinate led to almost no enclosed water in the emulsions containing ammonium phosphatide. Finally, the influence of osmotic pressure gradients on the release of an enclosed compound was examined. Therefore, the w/o/w emulsions were diluted in iso-, hypo- and hypertonic solutions after which the release of an enclosed marker compound was followed over time. Hereby, AMP- and O-170 stabilized w/o/w emulsions released the enclosed marker due to swelling under hypotonic dilution whereas hyper- and isotonic dilution never led to release of the enclosed marker, regardless of the used low-HLB emulsifier.

Redrafted after: Balcaen, M., Vermeir, L., Van der Meeren, P., 2017. Influence of protein type on Polyglycerol Polyricinoleate replacement in W/O/W (water-in-oil-in-water) double emulsions for food applications. *Colloids Surf. Physicochem. Eng. Asp.* 535, 105-113.

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7.1 Introduction

In this chapter, two alternative low-HLB emulsifiers were used to prepare w/o emulsions, namely ammonium phosphatide and the low-HLB sucrose ester O-170, which were compared to PGPR as the reference low-HLB emulsifier. To our knowledge, the use of these alternative low-HLB emulsifiers in w/o emulsions has been described only once until now.

Ammonium phosphatide was selected because this emulsifier is used as a PGPR-alternative in chocolate. Moreover, it has a similar structure to PC-depleted lecithin, of which the use in the preparation of w/o emulsions has already been described (Knoth et al., 2005a, 2005b, Scherze et al., 2006). Unfortunately, the PC-depleted lecithin which was used in these publications is no longer commercially available, whereas ammonium phosphatide is. In fact, ammonium phosphatides are phospholipids bearing ammonium counter ions. However, some higher molecular species such as double esters between diglycerides may also be formed during the production of ammonium phosphatides (GRAS notification for ammonium phosphatide, 2006).

Mazo Rivas et al., (2016) compared ammonium phosphatide and PGPR as emulsifiers in the production of w/o emulsions. They obtained stable w/o emulsions with each of these emulsifiers. However, the w/o emulsion prepared using PGPR contained droplets which were much smaller as compared to the emulsions prepared using ammonium phosphatide. Moreover, the emulsions prepared using ammonium phosphatide were flocculated and thus had a non-Newtonian flow behavior while the w/o emulsion prepared using PGPR was not flocculated and had a Newtonian flow behavior.

As a second alternative for PGPR, a low-HLB sucrose ester was selected because some types of sucrose esters (mainly high-HLB) are already used in food products. These emulsifiers suffer less from a chemical connotation and have no organoleptic issues. Akoh (1992) and Akoh and Nwosu (1992) already used a low-HLB sucrose ester (S-170), with mainly stearic acid in its lipophilic sidechains, as w/o emulsifier. These researchers obtained a w/o emulsion which did, however, phase separate within 48 hours of refrigerated storage. In contrast, the sucrose ester used in this chapter (O-170), contains mainly oleic acid in its lipophilic sidechains, which is more compatible with the fatty acid profile of the used oil phase.

As already mentioned, double emulsions usually contain a low-HLB emulsifier, used to stabilize the w/o interface, combined with a high-HLB emulsifier which stabilizes the o/w interface. PGPR has been combined with a lot of different high-HLB emulsifiers such as whey protein isolate (WPI) (Eisinaite et al., 2016; Mun et al., 2010; Schuch et al., 2015), complexes of WPI with

polysaccharides (Benichou et al., 2007; Berendsen et al., 2015; Lutz et al., 2009), polysorbates (Aditya et al., 2015) and Na-caseinate (Andrade and Corredig, 2016; Bonnet et al., 2009; Bou et al., 2014; Cofrades et al., 2014; Giroux et al., 2013; Vermeir et al., 2014b). In the production of the w/o/w emulsions in this chapter, the dairy proteins Na-caseinate and WPI, which both contain different types of proteins, were used as the high-HLB emulsifiers.

Caseinates are classified as random coil proteins while whey protein isolate contains globular proteins (Chandan, 2011). The used type of protein will have an influence on the o/w interface: random coil proteins tend to adsorb with the predominantly nonpolar segments protruding into the oil phase and the predominantly polar segments protruding into the aqueous phase while neutral regions lie flat against the interface. The interface formed by these molecules thus tends to be relatively open, thick and of low viscoelasticity. In contrast, globular proteins adsorb with the predominantly nonpolar regions on the surface of the protein facing the oil phase and the predominantly polar regions facing the aqueous phase. Therefore, these proteins tend to have a particular orientation at the interface because of which, globular proteins tend to form relatively thin, compact interfaces with a high viscoelasticity (McClements, 2015). Finally, it should also be mentioned that caseins, due to their strong amphiphilic nature and flexible structure, have a higher interfacial activity as compared to whey proteins (Britten and Giroux, 1993).

To determine which combinations of low- and high-HLB emulsifiers are interesting in the production of w/o/w emulsions, the enclosed water volume fraction of the double emulsions was determined after preparation. Moreover, also the storage stability of the enclosed water volume fraction was followed.

Finally, because encapsulation and controlled release of hydrophilic compounds is possible using w/o/w type double emulsions, the stability of the w/o/w emulsions towards different dilution liquids was tested. Hereby, the concentration of the water-soluble marker PTSA (1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt hydrate; Adachi et al., 2004; Mun et al., 2010; Tokgoz et al., 1996), which was added to the enclosed water phase, was followed in the external water phase after dilution of the double emulsions in different dilution liquids.

7.2 Materials and Methods

7.2.1 Materials

The lipophilic emulsifier sucrose ester O-170 (HLB approximately 1, approximately 70% oleic acid and 100% di-, tri- and polyester) was kindly provided by Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). The lipophilic emulsifiers polyglycerol polyricinoleate (PGPR 4175) and ammonium phosphatide (AMP 4448) were provided by Palsgaard A/S (Juelsminde, Denmark).

The hydrophilic emulsifier sodium caseinate (Na-caseinate; 5.5 % moisture; 96 % protein on dry matter) was kindly provided by Armor Protéines (Saint Brice en Cogles, France) while WPI was acquired from Davisco (BiPRO, Davisco, Le Sueur Food Ingredient Company, USA). High oleic sunflower oil (Hoso; Iodine Value= 87; 82% C18:1) was acquired from Contined BV (Wageningen, The Netherlands).

The 0.2M glucose (VWR Chemicals: BDH Prolabo, Leuven, Belgium) solution used in both the w_1 - and the w_2 -phase contained 0.02 wt% of the anti-microbial agent NaN_3 (Sigma-Aldrich, Steinheim, Germany). The w_1 -phase additionally contained 0.1 g/L of the fluorescent marker 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt hydrate (PTSA; 85% purity, Acros Organics, Geel, Belgium).

Glucose was added as an osmotic agent to reduce Ostwald ripening effects in the enclosed water droplets due to their high Laplace pressure which in turn is related to their small size and thus high curvature (Mezzenga et al., 2004). Glucose was used instead of a salt in order to avoid charge effects.

7.2.2 Emulsion preparation

7.2.2.1 w/o emulsion preparation

2.5 wt% of the lipophilic emulsifier was added to Hoso after which this oil phase was heated to 60 °C. To prepare the w/o emulsion, an Ultra-Turrax (type S50N-G45F, IKA®-Werke, Germany) at 5200 rpm was used to prepare the premix w/o emulsions (30/70, w/w) at 60 °C. The emulsifier concentration and ratio between water and oil were selected based on the works by Knoth et al. (2005a, 2005b) and Scherze et al. (2006). The water phase was added gradually during stirring after which stirring was continued to obtain the final premix. Afterwards, the obtained premix emulsion was processed using 1 pass through a Microfluidizer (type M110S, Microfluidics) operating at 840 bar while the temperature was maintained at 60 °C using a heating bath.

7.2.2.2 w/o/w emulsion preparation

In case PGPR was used as the low-HLB emulsifier, the w_2 -phase was mixed at room temperature with freshly prepared w/o emulsion in a 50/50 (w/w) ratio with an Ultra-Turrax S25-10G (IKA®-Werke, Germany) at 17500 or 24000 rpm for 3 minutes.

Because the other w/o emulsions were more viscous, a premix w/o/w emulsion was prepared. This premix was prepared by mixing for 1 minute at 6500 rpm using an Ultra-Turrax S25KV-25G-IL (IKA®-Werke, Germany) which is a rotor-stator homogenizer with a larger diameter. Afterwards, mixing was continued with an Ultra-Turrax S25-10G (IKA®-Werke, Germany) as described previously for the samples stabilized using PGPR.

The w_2 -phase only differs from the w_1 -phase in the presence of 2 wt% of either WPI or Na-caseinate and the absence of PTSA. All samples were stored at 5 °C after preparation.

7.2.3 Determination of the w/o/w droplet size distribution

The volume weighted average diameter (D_{43}) of the double emulsion droplets was determined using a Malvern Mastersizer 3000 (Malvern Instruments) with Hydro MV accessory. Hereby, the Mie theory was used and the refractive indices were set to $1.53+0.01i$ for the dispersed phase and 1.33 for the continuous phase. The stirring speed was set to 1500 rpm and sample was added until an obscuration between 10 and 20 % was reached. To avoid osmotic effects between the enclosed water phase of the double emulsion droplets and the external water phase, measurements were performed in a 0.2M glucose solution.

7.2.4 Light microscopy

Primary w/o emulsions were brought on a microscopy slide using a capillary after which a cover slip was gently applied followed by a drop of immersion oil.

The double w/o/w emulsions were diluted ten times with w_1 -phase and observed under the microscope to get a visual proof of the fact that enclosed water was present in the obtained emulsions. The diluted double emulsion was brought on a microscopy slide using a capillary after which a cover slip was gently applied.

Image acquisition was conducted using an optical microscope (Olympus Cx40) with 40-fold objective for the double- and a 100-fold objective for the primary emulsions coupled with a digital camera (AxioCam ERc 5S).

7.2.5 Analytical photocentrifugation

To determine the enclosed water volume fraction, analytical photocentrifugation was conducted at room temperature (using air cooling) with a LUMiFuge 116 instrument (LUM GmbH, Germany). Hereby, the enclosed water volume fraction is calculated by measuring the height of the cream layer during centrifugation and taking into account the amount of oil which was used, as described in Chapter 5. Rectangular synthetic cells with 2.2 mm path length were used, filled with about 0.35 mL sample. The light intensity was set to 100%.

The enclosed water volume fractions were determined in duplicate after the separation process was complete. Three hours of centrifugation at the maximum speed (3000 rpm; 1142g) sufficed.

Depending on the composition and the double emulsion droplet size, a small oil layer sometimes emerged on top of the cream layer during centrifugation. Moreover, big droplets sometimes stuck to the wall of the tube which made the border between the cream- and the serum layer less steep. To overcome the aforementioned problems, the 20% transmission value was used to determine the position of the top and bottom of the cream layer. Moreover, the top of the cream layer was determined after 5 minutes of centrifugation instead of at the end of centrifugation; the bottom was still determined after the separation process was complete.

Because mass ratios were used during the preparation of the double emulsion and analytical photocentrifugation is considering the volumes (Chapter 5), the mass ratio had to be converted to a volume ratio. Therefore, the temperature dependency of the density of the used oil phases as well as w_1 -phase were determined. Using the density at the temperature which is reached during centrifugation it is possible to calculate the volume ratios. Mostly, a temperature around 30 °C was reached at the end of centrifugation.

7.2.6 Density measurements

The density as a function of temperature was measured for Hoso and the w_1 -phase using a density meter (DMA 5000M, Anton Paar, Germany). The temperature was varied between 25 and 40 °C whereby density measurements were conducted every 3 °C. A linear relation between density and temperature was found for the oil phases ($R^2 > 0.99$) while a $R^2 > 0.99$ was found if a second order polynome was fitted to the density as a function of temperature for the water phase.

7.2.7 Determination of the sensitivity of the w/o/w emulsions towards dilution

PTSA is used as a fluorescent marker in the determination of the enclosed water volume fraction in double emulsions (Adachi et al., 2004; Mun et al., 2010; Tokgoz et al., 1996). The PTSA concentration was determined using a Cary Eclipse fluorescence spectrophotometer (Varian BV, The Netherlands) and an excitation wavelength of 374 nm while the emission was measured at 404 nm. The excitation and emission slits were set to 5 and 2.5 nm, respectively, and an averaging time of 1 second was used. Distilled water was used as the blank and to prepare the standard series.

To determine the sensitivity towards dilution in different solutions, samples of this double emulsion were taken after 2 days storage at 5 °C and diluted 1:1 (volume:volume) with the following liquids:

- 1) Isotonic solution (0.2M glucose and 0.02wt% NaN₃)
- 2) Isotonic solution containing Na-caseinate
(2 wt% Na-caseinate in 0.2M glucose and 0.02 wt% NaN₃)
- 3) Hypotonic solution (Deionized water)
- 4) Hypertonic solution (0.4M glucose)

After dilution in isotonic solution, samples were taken after 1, 30 and 60 minutes. For the other dilution tests, samples were taken 1, 5, 10, 15, 30 and 60 minutes after dilution. The samples were centrifuged immediately at 10000g for 1 minute to separate them into a cream- and a serum phase. Afterwards, the serum phase was sampled using a syringe and additionally clarified using a 0.45 µm nylon membrane filter (Diameter: 25mm; VWR International, USA).

The obtained serum phases were diluted twenty times using distilled water. The concentration of PTSA in this diluted water phase was calculated using a standard curve. Then, the percentage of PTSA which remains enclosed could be calculated. Hereby, it is assumed that PTSA is only released by external coalescence (between the w/o and the o/w interface). It should be noted that also during preparation of the double emulsion, part of the enclosed PTSA will be released due to shear-induced external coalescence of enclosed water droplets.

In the calculations, a correction is made for external coalescence of the enclosed water droplets, whereby possible osmotic effects, leading to dilution or concentration of PTSA in the external water phase, are neglected. Taking into account the aforementioned remarks, equation 7.1 was used to calculate the percentage of enclosed PTSA at every time point.

$$\text{Enclosed PTSA at time } t (\%) = \frac{M_{i,0} - M_{e,t}}{M_{i,0}} * 100 \quad (\text{Equation 7.1})$$

In which:

$$M_{i,0} = C_{i,0} * V_{i,0},$$

the total amount of PTSA which was used, $C_{i,0} = 0.1$ g/L, the theoretical PTSA concentration and $V_{i,0}$, the total volume of enclosed water used to prepare the emulsion. And

$$M_{e,t} = C_{e,t} * \frac{C_{i,0} * (V_{e,0} + V_{d,0})}{(C_{i,0} - C_{e,t})},$$

the amount of PTSA in the external water phase at time t , $C_{e,t}$, the concentration of PTSA measured in the external water phase at time t , $V_{e,0}$ and $V_{d,0}$, the volume of external water used to prepare the double emulsion and the volume used to dilute the w/o/w emulsion during the sensitivity test, respectively. These parameters are clarified in Figure 7.1.

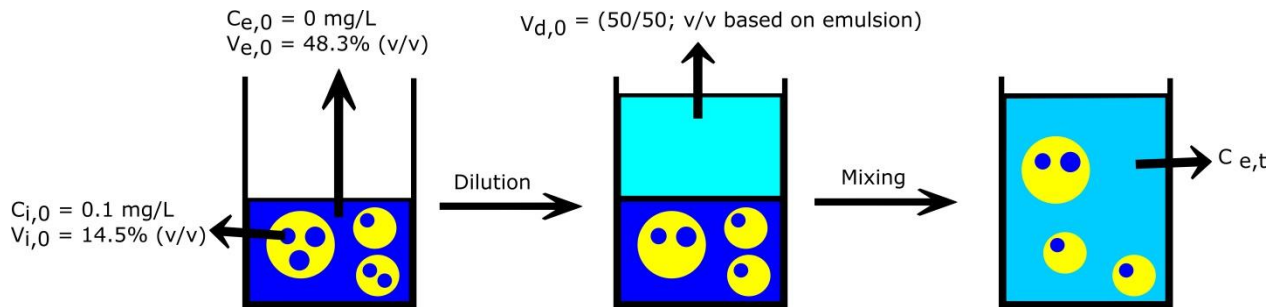


Figure 7.1: Scheme showing the concentrations and volumes considered in the determination of the enclosed PTSA fraction.

7.2.8 Statistical analysis

The evolution of the enclosed water volume fraction (%) as a function of storage time was analyzed using linear regression analysis which was performed using the Analysis ToolPak in Excel 2010.

7.3 Results and Discussion

7.3.1 Influence of the used low-HLB emulsifier on the w/o emulsions

With each of the tested low-HLB emulsifiers, stable w/o emulsions were prepared. These w/o emulsions, except from the one stabilized using PGPR, were flocculated. This increased their viscosity (visual observation) as compared to the emulsion stabilized with PGPR. The difference in aggregation behavior might be explained by the fact that the lipophilic side chain of PGPR is polymeric while the side chains of the alternative low-HLB emulsifiers are monomeric. Therefore, the presence of long chain triglycerides, present in sunflower oil, can lead to flocculation of the w/o emulsions stabilized using the latter emulsifiers (Leal-Calderon et al., 1997).

Light microscopy clearly confirmed that the emulsions prepared using ammonium phosphatide or O-170 were highly aggregated systems while when PGPR was used, very small, individual droplets can be observed (Figure 7.2).

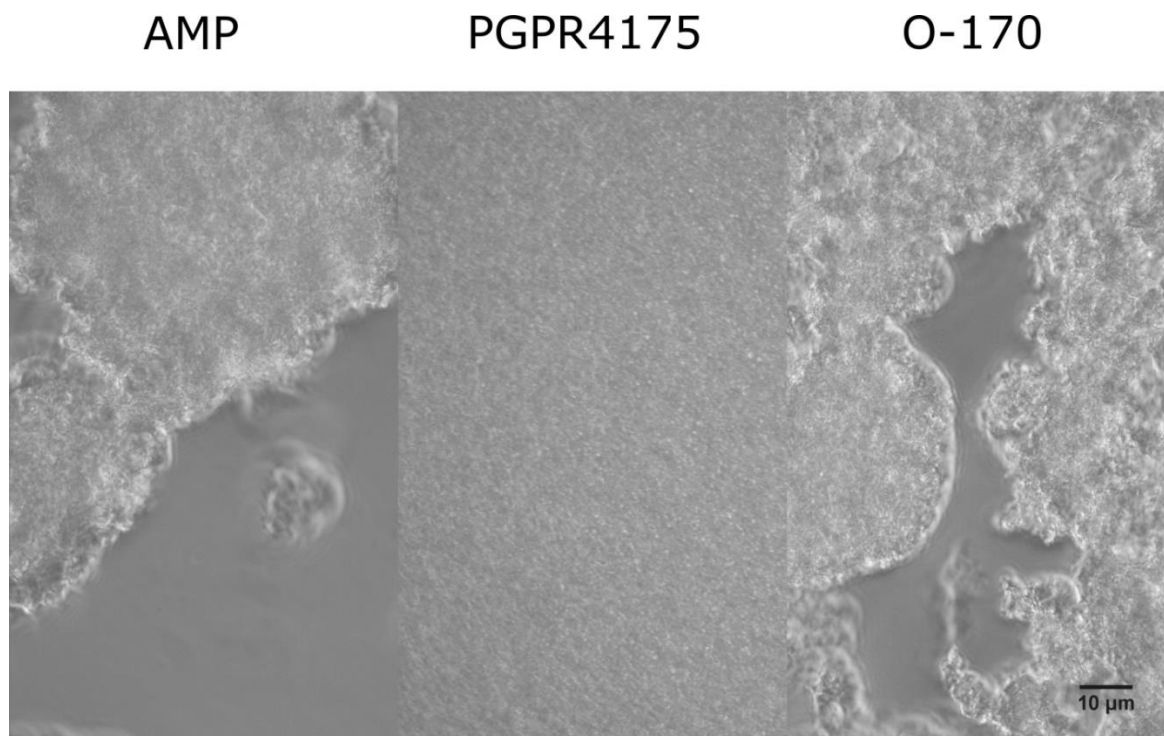


Figure 7.2: Pictures of the w/o emulsions stabilized using different low-HLB emulsifiers obtained from light microscopy using a 100x objective.

7.3.2 Influence of the used emulsifiers on the w/o/w emulsions

Using the aforementioned w/o emulsions, which were prepared using different low-HLB emulsifiers, w/o/w emulsions were prepared using two different hydrophilic emulsifiers (WPI and Na-caseinate). Hereby, two shear intensities were applied in order to vary the double emulsion droplet size.

7.3.2.1 Influence on the enclosed water volume fraction after preparation

Figure 7.3 shows the relationship between the enclosed water volume fraction (%) and the double emulsion droplet size for the double emulsions prepared using different compositions and shear intensities. Hereby, it should be taken into account that the maximum enclosed water volume fraction is equal to 14.5%.

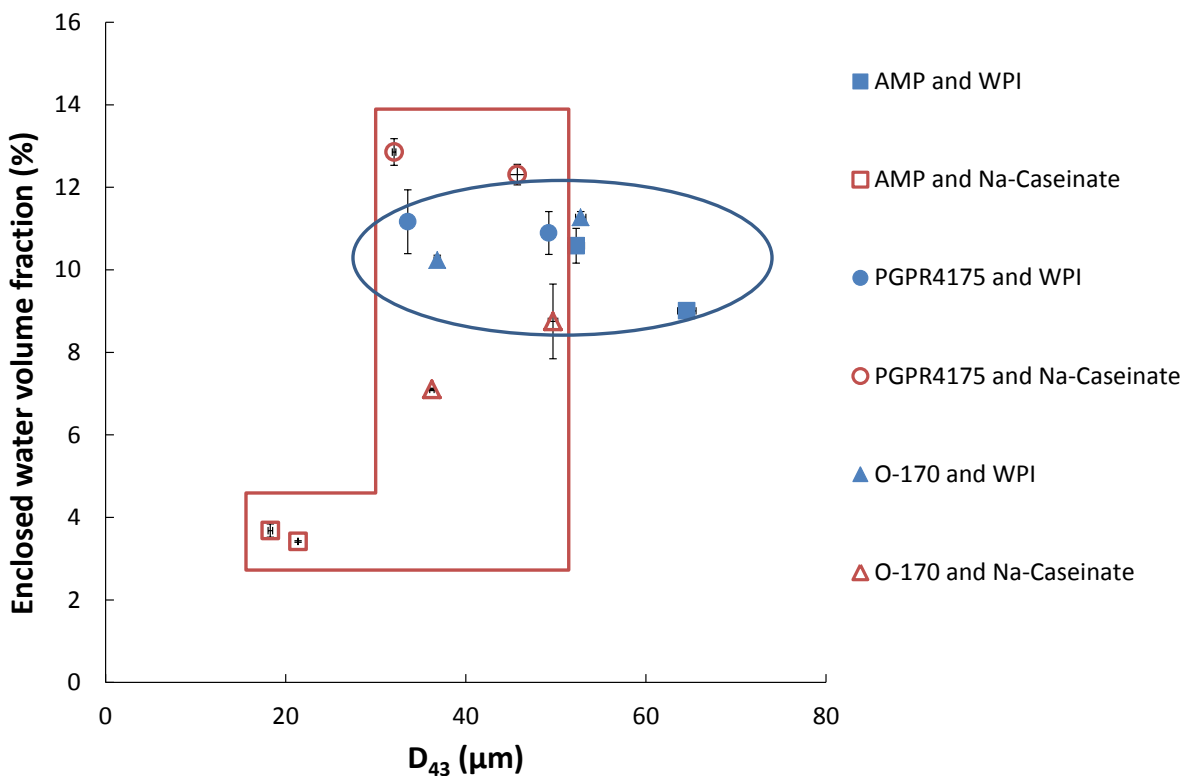


Figure 7.3: Enclosed water volume fraction after one day of storage at 5 °C with AMP (squares), PGPR (circles) or O-170 (triangles) as low-HLB emulsifier and WPI (closed symbols) or Na-caseinate (open symbols) as high-HLB emulsifier as a function of the volume weighted average diameter (D_{43}) of the double emulsion droplets.

Remarkably, as indicated with an ellipse in Figure 7.3, the enclosed water volume fraction which was obtained using WPI as high-HLB emulsifier was similar for all of the used low-HLB emulsifiers. In contrast, as indicated with a rectangle in Figure 7.3, when Na-caseinate was used as the high-HLB emulsifier, the enclosed water volume fraction was strongly dependent on the

used low-HLB emulsifier. When the enclosed water fraction was determined using pulsed field gradient NMR, the same conclusions could be drawn (data not shown).

Clearly, AMP is not capable of retaining a lot of water enclosed when this emulsifier is combined with Na-caseinate while the double emulsions prepared using O-170 and PGPR still enclosed most of the first water phase when combined with Na-caseinate. When PGPR was combined with Na-caseinate, it even led to a higher enclosed water volume fraction as compared to when WPI was used. The water content was qualitatively proven using light microscopy (Figure 7.4).

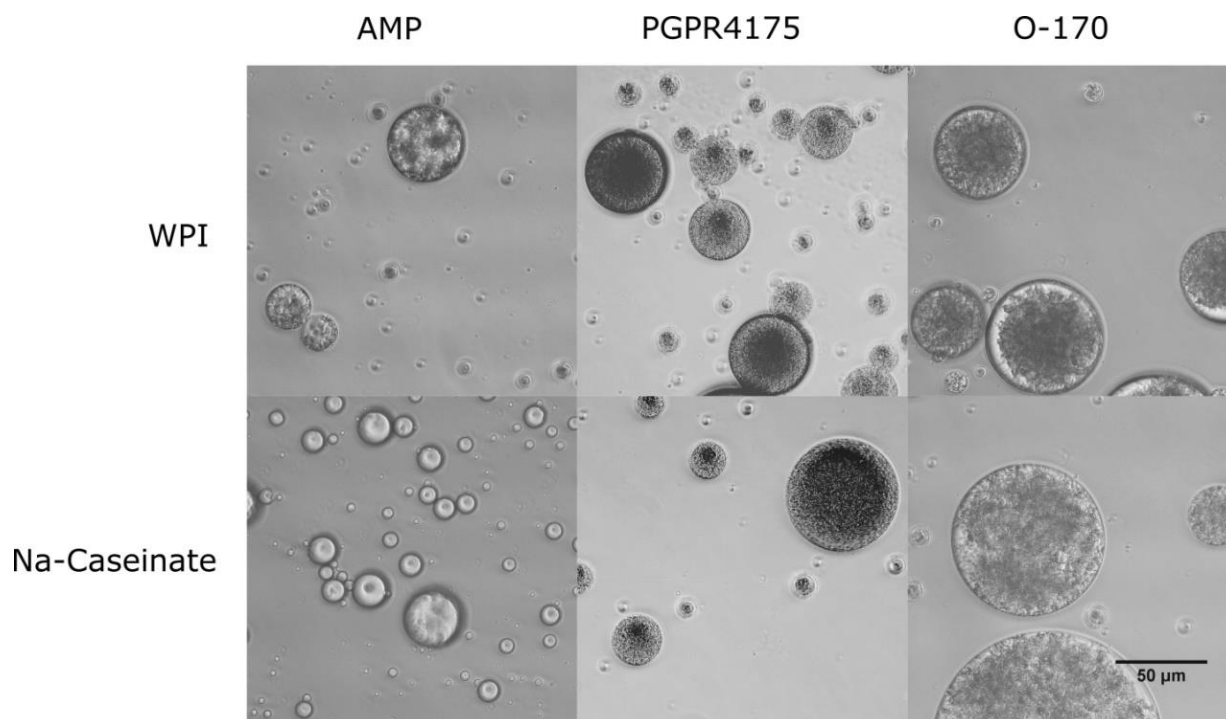


Figure 7.4: Pictures of the w/o/w emulsions prepared at 17500 rpm using AMP, PGPR or O-170 as low-HLB emulsifier and WPI or Na-caseinate as high-HLB emulsifier obtained from light microscopy using a 40x objective. The emulsions were diluted 10x in isotonic solution after one day of storage at 5 °C.

As shown in Figure 7.5, it is hypothesized that, in order to prevent external coalescence of the enclosed water droplets, a certain concentration of low-HLB emulsifier is necessary at the o/w interface. Indeed, low-HLB emulsifiers contain bulky lipophilic parts which are capable of repelling the enclosed water droplets, which are also mainly covered by the low-HLB emulsifier. In contrast, high-HLB emulsifiers mainly protrude into the water phase which implies these emulsifiers provide a smaller boundary towards external coalescence of the enclosed water droplets. The fact that a very low enclosed water volume fraction was obtained for the combination of AMP with Na-caseinate may therefore point towards Na-caseinate, being capable of occupying a critical part of the o/w interface area. Hereby the difference in result

between Na-caseinate and WPI may mainly be due to their difference in interfacial activity, with Na-caseinate having a higher interfacial activity than WPI (Britten and Giroux, 1993). An additional and complementary explanation might be the adsorption of a denser emulsifier layer (considering both the high- and low-HLB emulsifiers) at the o/w interface in case Na-caseinate is combined with O-170 or with PGPR as compared to when it is combined with AMP. In terms of repulsion, only steric repulsion will occur between O-170 or PGPR and the caseinate molecules. In contrast, AMP and Na-caseinate both bear a net negative charge at neutral pH and will therefore repel each other both sterically as well as electrostatically. The difference with WPI could be explained by the fact that caseinates are random coil proteins while WPI contains globular proteins. Therefore, as described in the introduction, the conformation of both types of proteins at the o/w interface is different. This hypothesis could be examined by changing the pH of the water phase: the stability of the double emulsion should depend on the pH in case the hypothesis is valid.

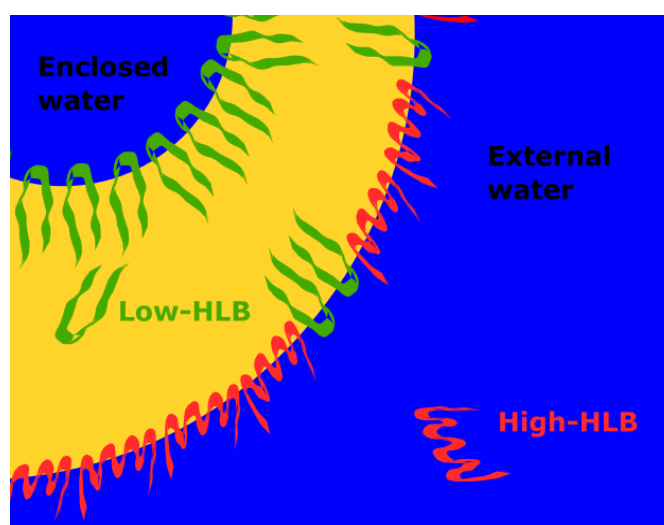


Figure 7.5: Scheme showing the hypothesized mechanism of stabilization of w/o/w emulsions against external coalescence: repulsion between bulky lipophilic groups of the low-HLB emulsifiers prevents release of enclosed water droplets.

The fact that the used low-HLB emulsifier will usually also adsorb at the o/w interface is not frequently emphasized. Indeed, previous research by other groups indicated that low-molecular weight emulsifiers are capable of at least partly replacing proteins at the o/w interface (De Feijter et al., 1987). Also, next to competition between both types of emulsifiers, different types of interactions, such as complexation, might occur between the low-molecular weight emulsifiers and proteins (Waninge et al., 2005).

The low-HLB emulsifiers which were used in this chapter, strongly vary in structure and molecular weight. AMP is an ionic emulsifier while O-170 and PGPR are both non-ionic emulsifiers. O-170 has a hydrophilic group consisting of sucrose (which consists of two circular moieties: glucose and fructose) while the hydrophilic group of PGPR consists of polyglycerol which is not circular. Finally, PGPR has a much higher molecular weight as compared to the other emulsifiers. It can therefore be expected that the competition and interaction between these low-HLB emulsifiers and proteins at the o/w interface will vary. For AMP, electrostatic interactions are possible while hydrogen-bridges and hydrophobic interactions are possible ways of interaction for all of the emulsifiers. Therefore, studying the interaction between low-HLB emulsifiers and different proteins at the o/w interface would be very interesting in order to determine new, promising compositions to prepare stable w/o/w emulsions. Also, the used concentration of both emulsifiers may be of utmost importance. Indeed, Cornec et al. (1996) examined the replacement of β -lactoglobulin (the main protein present in WPI) and β -casein (present in caseinate) by the low-HLB emulsifier Span 80. Hereby, at low Span 80 concentrations, β -casein was replaced to a higher extent than β -lactoglobulin, whereas the replacement of the proteins at higher Span 80 concentrations reached a plateau value whereby the degree of replacement was lower for β -casein.

Finally, the hypothesis shown in Figure 7.5 would imply that, in order to prevent external coalescence of enclosed water droplets, an o/w interface should be obtained which is largely stabilized by the low-HLB emulsifier. In the ideal case this low-HLB emulsifier would form a complex with a high-HLB emulsifier or stabilizer. In this way, both external coalescence of enclosed water droplets as well as coalescence between double emulsion droplets themselves could be avoided. This hypothesis has already been stated by Schuch et al., (2015). These researchers obtained the highest enclosed water volume fractions when PGPR was combined with carboxymethylcellulose, which had the lowest surface activity of the used high-HLB emulsifiers and stabilizers.

The stability of the double emulsions with a considerable enclosed water volume fraction was followed during storage. Hereby, the samples prepared at 17500 rpm (biggest droplets) were considered unless stated otherwise. Also, it was tested whether the addition of Na-caseinate might destabilize the double emulsion prepared using AMP and WPI.

7.3.2.2 Influence on the coalescence of double emulsion droplets

After 8 days of storage, the emulsion containing AMP and WPI, prepared at 17500 rpm, already had an oil layer on top indicating coalescence between the double emulsion droplets. The sample with the same composition but prepared at 24000 rpm (and therefore containing smaller droplets) did not have an oil layer at this time. However, after 30 days of storage, the same problem occurred for this sample. Moreover, also the sample prepared using O-170 and Na-caseinate had an oil-layer on top after 30 days of storage.

These effects could be due the presence of an emulsifier layer at the o/w interface with low stabilizing properties for this type of interface. In other words, an o/w interface with a high lipophilic emulsifier content. As already mentioned, this type of interface might be necessary in order to produce double emulsions with a high enclosed water volume fraction. However, if the o/w interface is not sufficiently stabilized, the double emulsion droplets can coalesce. The fact that with PGPR, this problem does not occur might be due to the bulky hydrophilic part of this (polymeric) lipophilic emulsifier.

Concluding, only the emulsions prepared using O-170 in combination with WPI and PGPR with WPI or Na-caseinate did not have an oil layer on top after 30 days of storage at 5 °C. It might be possible to reduce the occurrence of coalescence by producing smaller double emulsion droplets. However, this in turn might lead to an increase of the occurrence of external coalescence of enclosed water droplets. Indeed, smaller double emulsion droplets will have a higher external (o/w) surface area while the internal (w/o) surface area stays the same, which increases the chance of a collision between the w/o and o/w interfaces.

As already mentioned, another, additional option would be changing the o/w interface by changing the emulsifier composition, preferably creating an interface with emulsifier or stabilizer molecules which profoundly protrude both the oil and the water phase.

7.3.2.3 Influence on the storage stability: enclosed water volume fraction

Figure 7.6 shows the enclosed water volume fraction (%) of the double emulsion droplets as a function of storage time at 5 °C. As mentioned in the previous part, after 8 days of storage, the emulsion containing AMP and WPI, prepared at 17500 rpm, had an oil layer on top, therefore the sample prepared at 24000 rpm was followed instead.

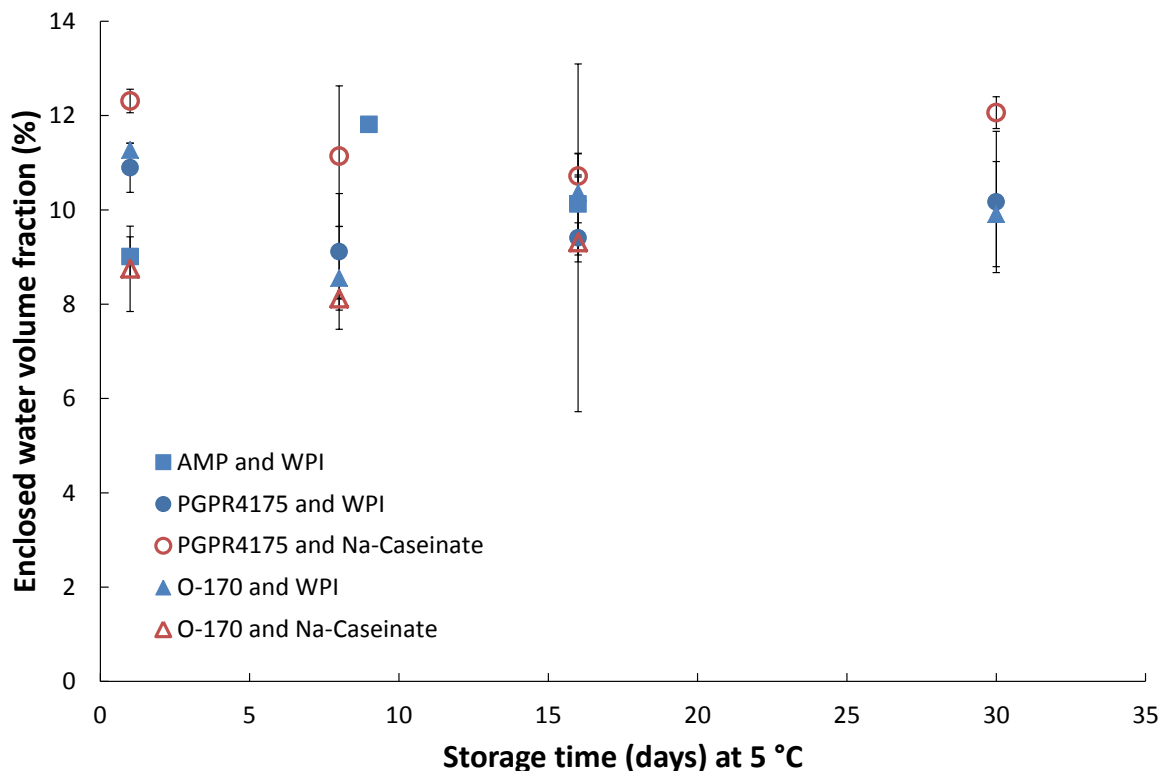


Figure 7.6: Enclosed water volume fraction as a function of storage time at 5 °C with AMP (squares), PGPR (circles) or O-170 (triangles) as low-HLB emulsifier and WPI (filled symbols) or Na-caseinate (open symbols) as high-HLB emulsifier. The AMP sample was prepared at 24000 rpm, the other samples at 17500 rpm.

The enclosed water volume fraction (%) appears to be quite constant for all the emulsions under consideration. Indeed, the slope of a linear regression of enclosed water volume fraction as a function of storage time was not significantly different from 0 at the 95% confidence level. These results indicate that the biggest problem for these big w/o/w emulsions is coalescence between the double emulsion droplets rather than external coalescence of enclosed water droplets.

Figure 7.7 indeed shows that the double emulsions still contained a lot of enclosed water, even after 30 days of storage at 5 °C.

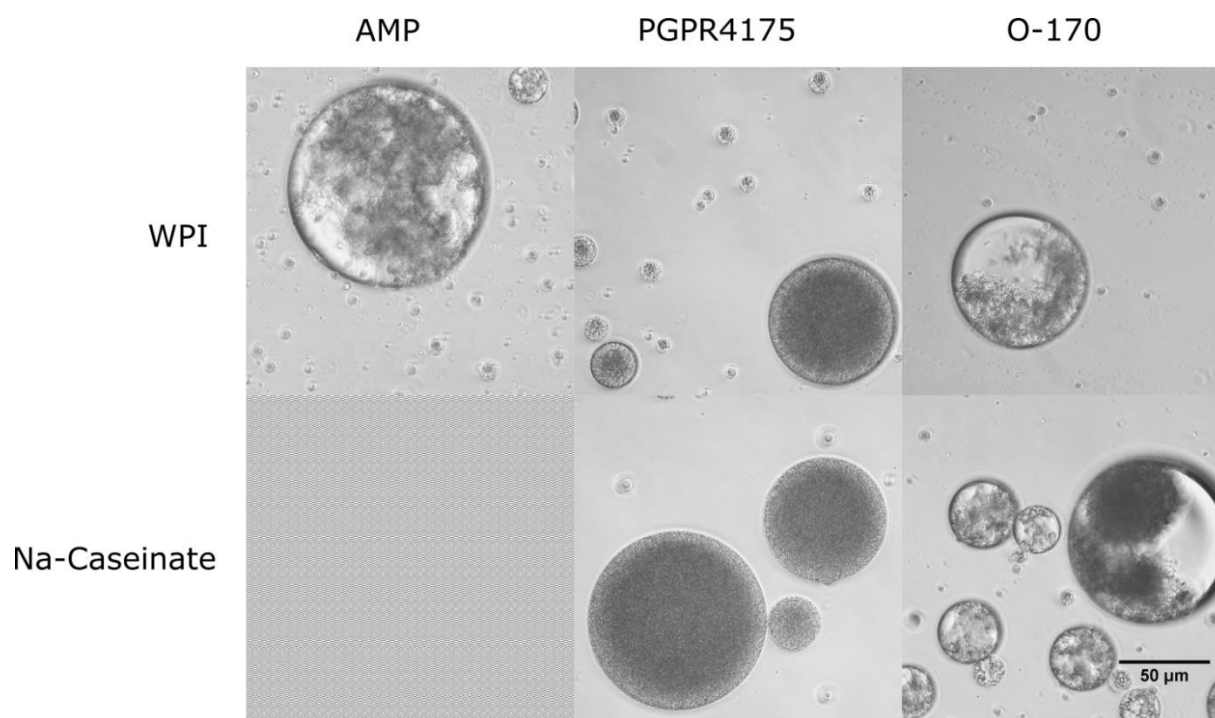


Figure 7.7: Pictures of the w/o/w emulsions prepared at 24000 rpm for AMP and 17500 rpm for PGPR or O-170 as low-HLB emulsifiers and WPI or Na-caseinate as high-HLB emulsifier obtained from light microscopy using a 40x objective. The emulsions were diluted 10x in isotonic solution after thirty days of storage at 5 °C.

7.3.2.4 Sensitivity of the w/o/w emulsions towards dilution

Because the enclosed water volume fraction was very low when ammonium phosphatide was used as low-HLB emulsifier and Na-caseinate as high-HLB emulsifier, it was tested whether a w/o/w emulsion stabilized by AMP and WPI was sensitive to the addition of Na-caseinate after production of this w/o/w emulsion. Therefore, this double emulsion was diluted in an isotonic aqueous phase containing 2 wt% Na-caseinate.

Figure 7.8 shows that the double emulsion prepared using AMP and WPI as emulsifiers was still sensitive towards the addition of Na-caseinate, even after preparation. Indeed, dilution with an isotonic solution containing Na-caseinate led to the release of the enclosed PTSA. This indicates that Na-caseinate is capable of adsorbing at the o/w interface and replacing WPI and/or ammonium phosphatide, even after preparation of the w/o/w emulsion using WPI. In this context, it can be mentioned that Dalgleish et al. (1991) proved that β -casein, found in caseinate, is indeed capable of replacing β -lactoglobulin, the main protein in WPI, at an o/w interface which was stabilized using β -lactoglobulin. However, no such research has been conducted on the interaction between ammonium phosphatide and proteins at the o/w interface.

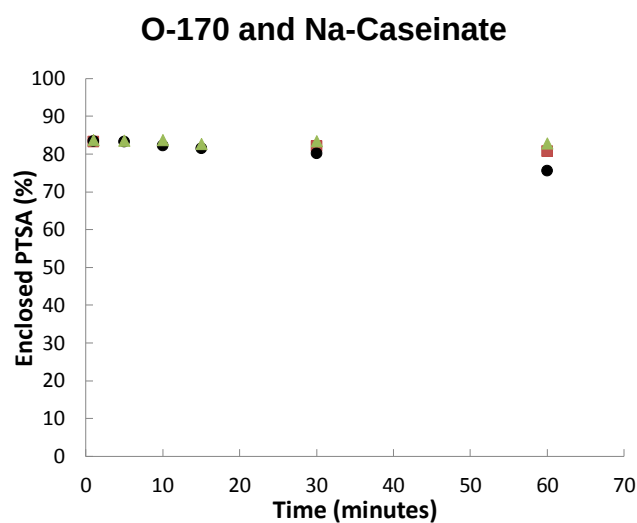
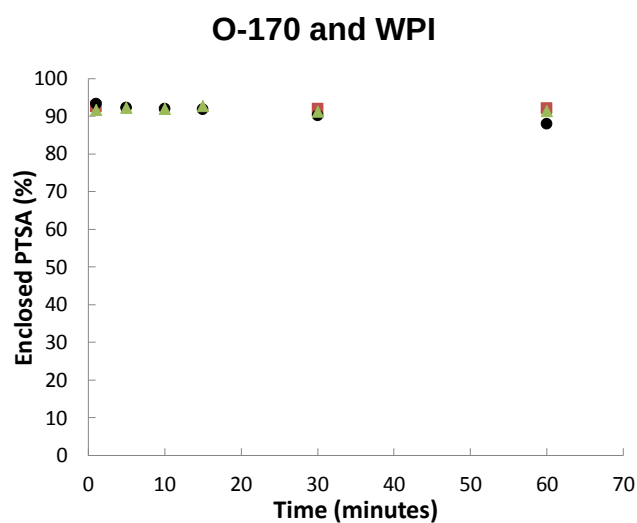
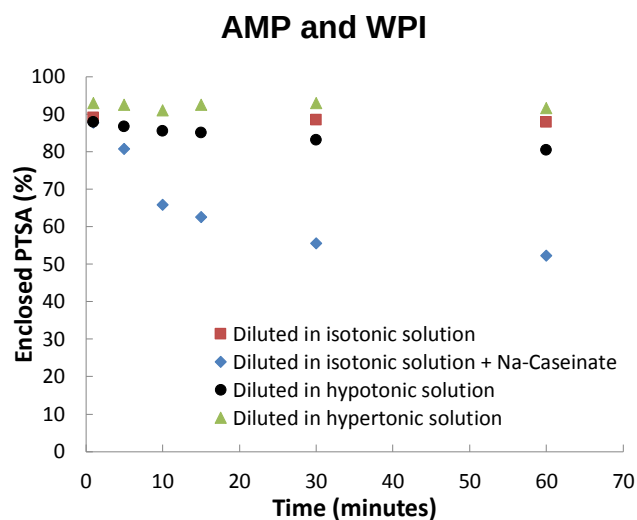


Figure 7.8: Percentage of PTSA which remains enclosed as a function of time at room temperature after 1:1 (volume:volume) dilution of the double emulsions in different aqueous solutions.

The release of PTSA due to an osmotic gradient between the enclosed and external water phase was also examined for all samples by dilution of the double emulsion with a hyper- (0.4M glucose) and a hypotonic (distilled water) aqueous phase with isotonic dilution (enclosed water phase without PTSA) as the reference. Hereby, it is expected that dilution in hypertonic medium leads to shrinking of the w/o and therefore w/o/w droplets while hypotonic dilution leads to swelling of the w/o and therefore w/o/w droplets (Chapter 6, Mezzenga et al., 2004). This change of droplet size might lead to external coalescence and therefore release of enclosed PTSA.

Both iso- and hypertonic dilution of each of the samples did not lead to release of the enclosed compound. Hypotonic dilution of the double emulsion prepared using ammonium phosphatide, however, led to the partial release of the encapsulated PTSA while release of PTSA after hypotonic dilution of double emulsions with O-170 as the low-HLB emulsifier was very low. Finally, when PGPR was used, there was no release of PTSA after hypotonic dilution.

None of the samples prepared using PGPR as low-HLB emulsifier released PTSA, regardless of the used dilution liquid. For the sample prepared using WPI as high-HLB emulsifier, for all tested combinations of dilution liquid and time the measured enclosed PTSA $\pm$ standard deviation was equal to $14.40 \pm 0.02\%$ with a minimum of 14.36%, whereas when Na-caseinate was the high-HLB emulsifier, a value of $14.31 \pm 0.03\%$ with a minimum of 14.21% was measured. These results therefore also indicate that PTSA did not diffuse through the oil phase as hardly any PTSA was detected in the external water phase of these samples, containing PGPR. Moreover these results indicate that no PTSA was released due to centrifugation at 10000g for 1 minute.

The effect of the dilution liquid on the release of an enclosed compound has practical implications. Indeed, when controlled release of an enclosed compound is required, it is interesting to note which dilution liquids lead to the release of enclosed components and which dilution liquids don't.

Based on the percentage of PTSA which remains enclosed after isotonic dilution, the enclosed water volume fraction can also be calculated. The average enclosed water volume fraction $\pm$ standard deviation of the three isotonic measurements equaled $12.8 \pm 0.1\%$, $13.4 \pm 0.0\%$, $11.9 \pm 0.2\%$, $14.4 \pm 0.0\%$, and $14.3 \pm 0.0\%$, for the sample stabilized using AMP with WPI, O-170 with WPI, O-170 with Na-caseinate, PGPR with WPI and PGPR with Na-caseinate, respectively. These confirm the enclosed water volume fractions which are shown in Figure 7.3: the highest values were obtained with PGPR, regardless of the used high-HLB emulsifier. The use of AMP

or O-170 in combination with WPI leads to similar and still high enclosed water volume fractions. Finally, using O-170 and Na-caseinate led to a lower enclosed water volume fraction while using AMP and Na-caseinate the enclosed water volume fraction was so low that no dilution tests were performed.

7.4 Conclusions

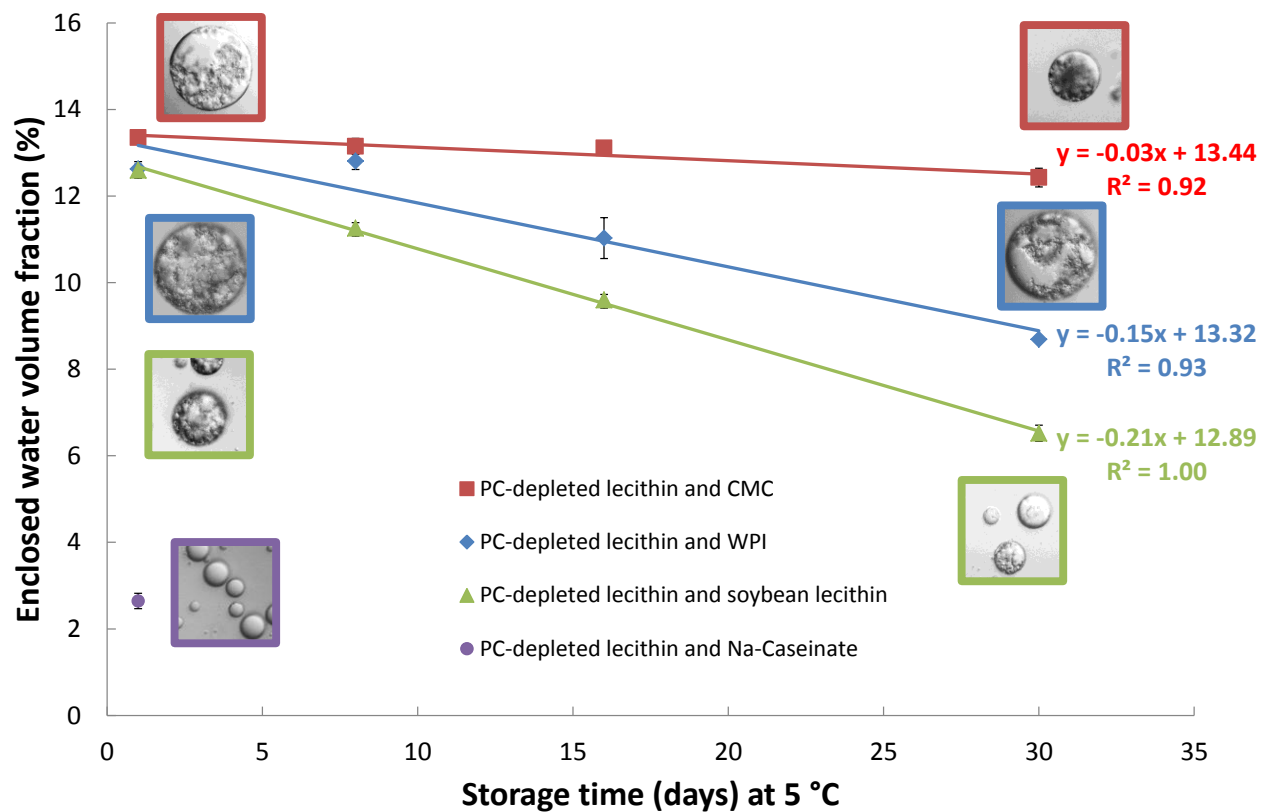
Replacement of PGPR in edible w/o and w/o/w emulsions is possible using ammonium phosphatide and low-HLB sucrose ester O-170. However, especially when AMP is used, the obtained w/o/w emulsions were less robust as compared to when PGPR is used.

When Na-caseinate was used as high-HLB emulsifier, the obtained enclosed water volume fractions strongly depended on the used low-HLB emulsifier. When AMP was used as low-HLB emulsifier, very low enclosed water volume fractions were obtained while using O-170 or PGPR, the enclosed water volume fractions were high. However, when WPI was used as high-HLB emulsifier, high enclosed water volume fractions were obtained regardless of the used low-HLB emulsifier.

Also the stability towards coalescence of the double emulsion droplets depended on the used low-HLB emulsifier. Hereby, emulsions prepared using O-170 in combination with WPI and PGPR with WPI or Na-caseinate did not have an oil layer on top after 30 days of storage at 5 °C whereas the emulsions prepared using AMP with WPI or O-170 with Na-caseinate did.

Finally, the double emulsions prepared using alternative low-HLB emulsifiers are more sensitive towards dilution, whereby the enclosed compounds are released. Indeed, swelling of the droplets in hypotonic solution could be compensated by PGPR whereas AMP and O-170 were not capable of fully compensating the effect, due to which release of the enclosed compound occurred. In contrast, shrinking of the double emulsion droplets in hypertonic solution never led to the release of the enclosed compound. Finally, addition of an isotonic solution, containing Na-caseinate to a w/o/w emulsion, prepared using AMP and WPI, also led to release of the enclosed compound. This implies that Na-caseinate is capable of at least partly replacing the adsorbed WPI and/or AMP.

Chapter 8: Use of lecithin as alternative for PGPR



- Using MCT-oil, it was possible to prepare w/o emulsions with each of the tested lecithin samples
- Using LCT-oil, only PC-depleted lecithin could be used to prepare a w/o emulsion
- The enclosed water volume fraction of double emulsions, prepared using PC-depleted lecithin and LCT-oil was highly dependent on the used high-HLB emulsifier

Abstract

In most publications concerning edible w/o/w emulsions, the low-HLB emulsifier polyglycerol polyricinoleate (PGPR) is used to stabilize the w/o interface in combination with a high-HLB emulsifier which stabilizes the o/w interface. Therefore, PGPR was used as the reference low-HLB emulsifier and compared to different lecithin samples.

As oil phase, either a medium chain triglyceride (MCT) oil or a long chain triglyceride (LCT) oil were used. When MCT-oil was used, each of the lecithin samples could be used to produce a stable w/o emulsion. In contrast, when LCT-oil was used, only with PC-depleted lecithin a stable w/o emulsion could be produced.

With each of the stable w/o emulsions, w/o/w emulsions were produced. Hereby, Na-caseinate, WPI, soybean lecithin and carboxymethylcellulose were used as high-HLB emulsifier or stabilizer. A big influence of the used high-HLB emulsifier on the resulting enclosed water volume fraction in the w/o/w emulsions was observed.

Finally, the influence of osmotic pressure gradients on the release of an enclosed compound was examined. Therefore, the w/o/w emulsions were diluted in iso-, hypo- and hypertonic solutions after which the release of an enclosed marker compound was followed over time. Hereby, the PC-depleted and WPI stabilized w/o/w emulsion released the enclosed marker due to swelling under hypotonic dilution whereas hyper- and isotonic dilution never led to release of the enclosed marker, regardless of the used low-HLB emulsifier.

8.1 Introduction

In the previous chapter we showed that it was possible to replace PGPR with either ammonium phosphatide or with a low-HLB sucrose ester: stable w/o emulsions were prepared with these low-HLB emulsifiers. Ammonium phosphatide was tested because its structure is similar to phosphatidylcholine-depleted (PC-depleted) lecithin, of which the use in the preparation of w/o emulsions has already been described (Knoth et al., 2005a, 2005b, 2007; Scherze et al., 2006). Indeed, lecithin, and especially PC-depleted lecithin, has been described as a possible alternative for PGPR in the production of w/o emulsions (Altuntas et al., 2017; Knoth et al., 2005a, 2005b, 2007; Mazo Rivas et al., 2016; Scherze et al., 2006). Because the PC-depleted lecithin used by these researchers was no longer commercially available when the research work shown in chapter 7 was executed, we used ammonium phosphatide instead. However, the production of a similar PC-depleted lecithin is currently being revived by Cargill (Schoeppe et al., 2016).

In this chapter, native, non-deoiled lecithin samples from different sources (sunflower and soybean oil) and thus with a different phospholipid composition, were used to produce w/o emulsions and compared to PC-depleted lecithin. In contrast to when PGPR is used as a low-HLB emulsifier, the used oil phase has a stronger influence on the resulting w/o emulsion when lecithin is used (Benichou et al., 2001; Knoth et al., 2005a; Lindenstruth and Müller, 2004; Weiss et al., 2005). Therefore, also two different types of oils were tested namely saturated medium chain triglycerides (MCT-oil) and a long chain triglyceride oil (LCT-oil; high oleic sunflower oil).

When a stable w/o emulsion was obtained, it was used in the preparation of different w/o/w emulsions. The production of w/o/w emulsions using lecithin as the low-HLB emulsifier is rarely described in literature (Akhtar and Dickinson, 2001; Altuntas et al., 2017; Luo et al., 2017; Piemi et al., 1998). Akhtar and Dickinson (2001) and Altuntas et al. (2017) used LCT-oil whereas the other researchers used MCT-oil as lipid phase in the emulsions.

Akhtar and Dickinson (2001) produced stable w/o emulsions using PC-depleted lecithin and pure rapeseed oil or rapeseed oil in combination with anhydrous milk fat. These w/o emulsions were used in the production of stable w/o/w emulsions with sodium caseinate (Na-caseinate) as the high-HLB emulsifier. These researchers also mention that the obtained w/o/w emulsions had a creamier mouthfeel as compared to when PGPR was used as the low-HLB emulsifier. Also, when anhydrous milk fat was added to the oil phase, the obtained enclosed water volume was slightly higher as compared to when pure rapeseed oil was used. In contrast, Altuntas et al.

(2017) were not able to produce stable w/o emulsions using soybean lecithin and hazelnut oil. Therefore, they combined lecithin with PGPR in order to produce w/o and w/o/w emulsions.

Piemi et al. (1998) and Luo et al. (2017) produced stable w/o and w/o/w emulsions using soybean lecithin and MCT-oil. Hereby, both research groups were not focussing on food applications. The former researchers tested the produced w/o/w emulsion as a transdermal delivery system while the latter researchers focussed on the different morphologies which were obtained when different emulsifier systems were used, one of which was a system stabilized using only lecithin.

In the previous chapter, we showed that when the w/o emulsions, stabilized using ammonium phosphatide, were used in the production of w/o/w emulsions, care had to be taken in the choice of the high-HLB emulsifier. Indeed, the enclosed water volume fraction was very sensitive towards the used high-HLB emulsifier. Both whey protein isolate (WPI) and Na-caseinate were tested as high-HLB emulsifiers. Hereby, the former consists of globular proteins while the latter consists of random coil proteins (McClements, 2015). In case Na-caseinate was used, very low enclosed water volumes were obtained, while using WPI, the enclosed water volume was very high. Similar results are expected when lecithin is used as low-HLB emulsifier. Therefore, we examined the influence of the used high-HLB emulsifier (either WPI or Na-caseinate) on the enclosed water volume fraction and storage stability of the produced double emulsions.

8.2 Materials and Methods

8.2.1 Materials

Non-deoiled soybean lecithin (Leciprime™; 13.3% PC, 10.2% PI, 9.6% PE, 5.4% PA and 61.9% AI) and sunflower lecithin (Topcithin™; 15.4% PC, 12.6% PI, 6.8% PE, 1.7% PA and 62.5% AI), as well as a phosphatylcholine-depleted soybean lecithin (PC-depleted lecithin; 5.3% PC, 15.4% PI, 14.2% PE, 3.9% PA and 67.4% AI) were kindly provided by Cargill (Vilvoorde, Belgium). The hydrophilic emulsifier sodium caseinate (Na-caseinate; 5.5 % moisture; 96 % protein on dry matter) was kindly provided by Armor Protéines (Saint Brice en Cogles, France), whey protein isolate (WPI) was acquired from Davisco (BiPRO, Davisco, Le Sueur Food Ingredient Company, USA), carboxymethylcellulose (CMC; Degree of substitution=0.65-0.90; Viscosity 2% solution=1500-3100 mPa.s) was acquired from Carl Roth (Karlsruhe, Germany) and powdered soybean lecithin (Emulpur™; 20.6% PC, 14.7% PI, 15.2% PE, 6.4% PA and 97.7% AI) was kindly provided by Cargill (Vilvoorde, Belgium). High oleic sunflower oil (Hoso; Iodine Value= 87; 82% C18:1) was acquired from Contined BV (Wageningen, The Netherlands) while MCT-oil

(Miglyol 812N) with approximately 58% C8:0 and 41% C10:0 was kindly provided by IMCD (Mechelen, Belgium). The 0.2M glucose (VWR Chemicals: BDH Prolabo, Leuven, Belgium) solution used in both the w_1 - and the w_2 -phase contained 0.02 wt% of the anti-microbial agent NaN_3 (Sigma-Aldrich, Steinheim, Germany). Glucose was added as an osmotic agent to reduce Ostwald ripening effects in the enclosed water droplets due to their high Laplace pressure which in turn is related to their small size and thus high curvature (Mezzenga et al. 2004). Glucose was used instead of a salt in order to avoid charge effects. To test the sensitivity of the double emulsions towards dilution in different kinds of aqueous solutions, an emulsion containing 0.1 g/L of the fluorescent marker 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt hydrate (PTSA; 85% purity, Acros Organics, Geel, Belgium) in the w_1 -phase was prepared.

8.2.2 Emulsion preparation

8.2.2.1 w/o emulsion preparation

2.5 wt% of lecithin was added to the oil phase after which this phase was heated to 60 °C. To prepare the w/o emulsion, a small Ultra-Turrax (type S25-10G, IKA®-Werke, Germany) at 24000 rpm or bigger Ultra-Turrax (type S50N-G45F, IKA®-Werke, Germany) at 5200 rpm was used to prepare the premix w/o emulsions (30/70, w/w) at 60 °C. The former rotor-stator homogenizer was used when the sample had a mass smaller than or equal to 100 grams while the latter was used in case the sample mass was bigger. The water phase was added gradually during stirring after which stirring was continued to obtain the final premix. Afterwards, the obtained premix emulsion was processed using 1 pass through a Microfluidizer (type M110S, Microfluidics) operating at 840 bar while the temperature was maintained at 60 °C using a heating bath.

8.2.2.2 w/o/w emulsion preparation

In case MCT-oil was used, the second water phase was mixed at room temperature with freshly prepared w/o emulsion in a 50/50 (w/w) ratio with an Ultra-Turrax S25-10G (IKA®-Werke, Germany) at 13500, 17500 or 24000 rpm for 3 minutes.

Because the w/o emulsions which were prepared using Hoso as the oil phase were very viscous, preparation of a premix was necessary. This premix was prepared by mixing for 1 minute at 6500 rpm using an Ultra-Turrax S25KV-25G-IL (IKA®-Werke, Germany) which is a rotor-stator homogenizer with a larger diameter. Afterwards, mixing could be continued with an Ultra-Turrax S25-10G (IKA®-Werke, Germany) as described previously.

The w_2 -phase only differs from the w_1 -phase in the presence of 2 wt% of either WPI, Na-caseinate, CMC or powdered soybean lecithin. All samples were stored at 5 °C after preparation.

8.2.3 Viscosity measurements

The viscosity of the w/o emulsions was measured using a LVDVII+pro rotational viscometer (Brookfield, USA) with small sample adapter. The viscosity was measured at 25 °C directly after preparation of the primary emulsions. Hereby the shear stress was measured as a function of shear rate. Five shear rates were selected in order to obtain a torque between 10 and 100%, using a spindle SC4-18 or SC4-34. The flow behavior of the oil phases was Newtonian while the flow behavior of the w/o emulsions, stabilized using lecithin, could be described using a power law function (Ostwald-de Waele power law). Three subsequent measurements were conducted. For the oil phases, the average viscosity of the three subsequent measurements was used. In contrast, for the w/o emulsions, the flow consistency index (K) decreased with each subsequent measurement. This indicates that due to shearing during the measurement, the w/o emulsion structure is partly disintegrated. Therefore, the consistency (K) and flow behavior index (n) obtained from the first measurement are used.

8.2.4 Determination of the w/o/w droplet size distribution

The volume weighted average diameters (D_{43}) of the double emulsion droplets were determined using a Malvern Mastersizer 3000 (Malvern Instruments) with Hydro MV accessory. Hereby the Mie theory was used and the refractive indices were set to $1.53+0.01i$ for the dispersed phase and 1.33 for the continuous phase. The stirring speed was set to 1500 rpm and sample was added until an obscuration between 10 and 20 % was reached. To avoid osmotic effects between the enclosed water phase of the double emulsion droplets and the external water phase, measurements were performed in 0.2M glucose solution.

8.2.5 Light microscopy

Primary w/o emulsions were brought on a microscopy slide using a capillary after which a cover slip was gently applied followed by a drop of immersion oil.

The double w/o/w emulsions were diluted ten times with the w_1 -phase and observed using a light microscope to get an idea of the enclosed water droplet size as well as to get a visual proof of the fact that enclosed water was present in the emulsions. The diluted double emulsion was brought on a microscopy slide using a capillary after which a cover slip was gently applied.

Image acquisition was conducted using an optical microscope (Olympus Cx40) with 40-fold objective for the double- and a 100-fold objective for the primary emulsions coupled with a digital camera (AxioCam ERc 5S).

8.2.6 Analytical photocentrifugation

To determine the enclosed water volume fraction, analytical photocentrifugation was conducted at room temperature (using air cooling) with a LUMiFuge 116 instrument (LUM GmbH, Germany). Hereby, the enclosed water volume fraction is calculated by measuring the height of the cream layer during centrifugation and taking into account the amount of oil which was used, as described in chapter 5. Rectangular synthetic cells with 2.2 mm path length were used, filled with about 0.35 mL sample. The light intensity was set to 100 %.

The enclosed water volume fractions were determined in duplicate after the separation process was complete. Three hours of centrifugation at the maximum speed (3000 rpm; 1142g) usually sufficed. Only in case powdered soybean lecithin was used as the high-HLB emulsifier, six hours of centrifugation at the latter speed were necessary to obtain a stable height of the cream layer. This increase in required centrifugation time could mainly be attributed to the fact that the superfluous soybean lecithin, which was also leading to a decreased transmission, was sedimenting during centrifugation.

When the double emulsion droplets were very big, a small oil layer emerged on top of the cream layer during centrifugation. Moreover, these big droplets sometimes stuck to the wall of the tube which made the border between cream- and serum layer less steep as compared to the emulsions prepared in chapter 5. To overcome the aforementioned problems, the 20% transmission value was used to determine the position of the top and bottom of the cream layer. Moreover, the top of the cream layer was determined after 5 minutes of centrifugation instead of at the end of centrifugation.

Because mass ratios were used during the preparation of the double emulsion and analytical photocentrifugation is considering the volumes (Chapter 5), the mass ratio needed to be converted to a volume ratio. Therefore, the temperature dependency of the density of the used oil phases as well as w_1 -phase were determined. Using the density at the temperature which is reached during centrifugation it is possible to calculate the volume ratios. Mostly a temperature around 30 °C was reached at the end of centrifugation.

8.2.7 Density measurement

The density as a function of temperature was measured for the MCT-oil, Hosol and the w_1 -phase using a density meter (DMA 5000M, Anton Paar, Germany). The temperature was varied between 25 and 40 °C whereby density measurements were conducted every 3 °C. A linear

relation between density and temperature was found for the oil phases ($R^2 > 0.99$) while a $R^2 > 0.99$ was found if a second order polynome was fitted to the density as a function of temperature for the water phase.

8.2.8 Determination of the sensitivity of the w/o/w emulsions towards dilution

A w/o emulsion whereby 0.1 g/L PTSA, which is a fluorescent marker, was added to the water phase and Hosol containing 2.5 wt% PC-depleted lecithin as the oil phase was prepared as described in part 8.2.2.1. Using this w/o emulsion, a w/o/w emulsion was prepared as described in part 8.2.2.2. Hereby, 2 wt% WPI was used as the high-HLB emulsifier and the double emulsion was prepared at 17500 rpm

As described in part 7.2.7, in order to determine the sensitivity towards dilution in different solutions, samples of this double emulsion were taken and diluted 1:1 (volume:volume) with following liquids:

- 1) Isotonic solution (0.2M glucose and 0.02 wt% NaN_3)
- 2) Isotonic solution containing WPI (2 wt% WPI in 0.2M glucose and 0.02 wt% NaN_3)
- 3) Isotonic solution containing Na-caseinate
(2 wt% Na-caseinate in 0.2M glucose and 0.02 wt% NaN_3)
- 4) Hypotonic solution (Deionized water)
- 5) Hypertonic solution (0.4M glucose)

The collection and analysis of samples was performed as described in part 7.2.7.

8.2.9 Statistical analysis

The evolution of the enclosed water volume fraction (%) as a function of storage time was analyzed using linear regression analysis which was performed using the Analysis ToolPak in Excel 2010.

8.3 Results

Because most results can be explained using the same basic principles, these principles are elaborated in a separate discussion (part 8.4).

8.3.1 Influence of the used oil phase

8.3.1.1 Influence of the used oil phase on the w/o emulsions

8.3.1.1.1 Influence on stability and characteristics

Using medium chain triglycerides as the oil phase, w/o emulsions could be prepared using each of the lecithin samples. In contrast, using high oleic sunflower oil as the oil phase, a stable w/o emulsion could only be obtained with PC-depleted lecithin. When either sunflower or soybean lecithin were used, phase separation between the water and the oil phase occurred within less than one hour. This indicates the importance of lecithin with a reduced PC-content when lecithin-stabilized w/o emulsions are to be prepared in long chain unsaturated oil phases.

Figure 8.1A shows that spherical water droplets were obtained when MCT-oil was used. However, the water droplets appeared to be more aggregated as compared to the ones produced by Knoth et al. (2005a). This can probably be explained by a difference in the water droplet size distribution: the interaction between water droplets increases with increasing droplet size and therefore, also their tendency to aggregate increases. To produce the w/o emulsion, Knoth et al. (2005a) used a special laboratory-scale pressure homogenizer with a ball valve at 80 bar whereas we used a Microfluidizer at 840 bar. Scherze et al. (2006) already showed that the used emulsification device influenced the droplet size of w/o emulsions to a higher extent when PC-depleted lecithin was used as compared to when PGPR was used as the low-HLB emulsifier. When lecithin was used, smaller water droplets were obtained with the pressure homogenizer with ball valve at 80 bar as compared to with a high pressure homogenizer at 1000 bar. In fact, of all the tested emulsification devices, the smallest droplet size was obtained with the former device. This also indicates that the w/o emulsion which was prepared in this chapter probably contains bigger water droplets as compared to the emulsion prepared by Knoth et al. (2005a).

As shown in Figure 8.1B, the emulsion prepared using Hoso was highly aggregated. Some spherical cavities were visible. However, it appears that water is mainly held in an aggregated structure which consists not only of spherical particles. Knoth et al. (2005a) described these water bodies as lumps.

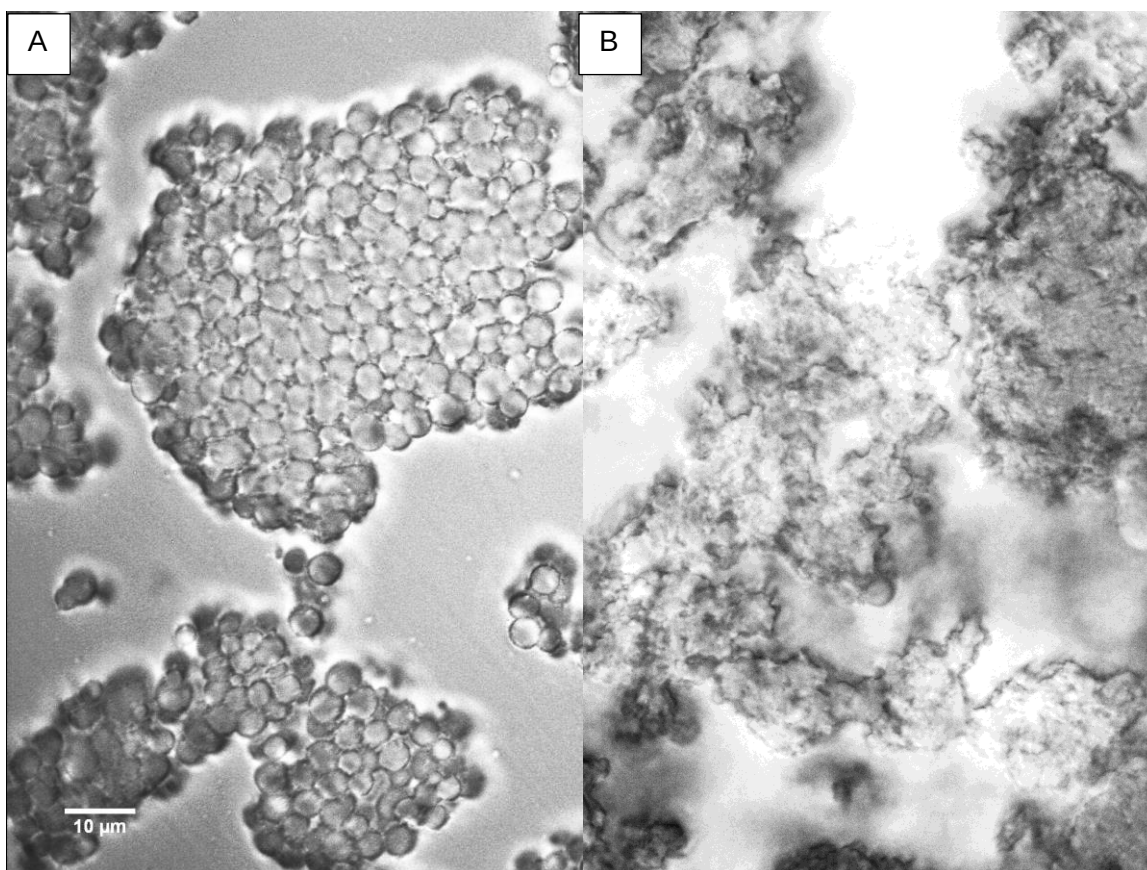


Figure 8.1: Pictures of the w/o emulsions stabilized using PC-depleted lecithin and MCT-oil (A) or Hosolipid (B) as the oil phase obtained from light microscopy using a 100x objective.

8.3.1.1.2 Influence on the viscosity

In Table 8.1, the flow consistency (K) and flow behavior indices (n) of the w/o emulsions are given. Clearly all w/o emulsions showed a shear thinning behavior ($n < 1$). Moreover, the consistency of the w/o emulsion with Hosolipid as the continuous phase was a lot higher as compared to when MCT-oil was used.

Table 8.1: Flow consistency (K) and flow behavior index (n) of the w/o emulsions.

Oil Phase	Emulsifier	K (Pa.s ⁿ)	n
Hosolipid	PC-depleted Lecithin	7.72	0.28
MCT-oil	PC-depleted Lecithin	1.17	0.31
	Sunflower Lecithin	1.79	0.28
	Soybean Lecithin	1.33	0.42

The viscosity of the oil phases used in the preparation of the w/o emulsions was determined in triplicate: the viscosity $\pm$ standard deviation was 70.01 ± 0.43 mPa.s for 2.5 wt% PC-depleted lecithin in Hosono while 2.5 wt% of PC-depleted, soybean or sunflower lecithin in MCT-oil gave a viscosity $\pm$ standard deviation of 26.02 ± 0.43 , 26.03 ± 0.34 and 26.11 ± 0.50 mPa.s, respectively.

Comparing the consistency of the w/o emulsions (corresponding to the shear stress at a shear rate of 1 s^{-1}) to the viscosity of the oil phases (also corresponding to the shear stress at a shear rate of 1 s^{-1}) which were used, shows that the increase in viscosity was a lot higher when Hosono was used as compared to when MCT-oil was used. Indeed, an increase with a factor of about 110 was noted for Hosono while an increase between about 45 and 70 times was obtained for MCT-oil.

This observation indicates that aggregation was stronger when Hosono was used as the oil phase of the w/o emulsions as compared to when MCT-oil was used, which corresponds with the visual observation in Figure 8.1 and with the results of Knoth et al. (2005a). Considering the consistencies shown in Table 8.1, it might be assumed that –when using MCT-oil– aggregation increases in the order PC-depleted lecithin < soybean lecithin < sunflower lecithin. The difference in aggregation behavior of the water droplets, stabilized using different lecithin samples, might again be linked to a difference in the water droplet size distributions. Hereby, the tendency to aggregate is smaller for smaller droplets and therefore, it can be assumed that the smallest water droplets were produced using PC-depleted lecithin.

8.3.1.2 Influence of the used oil phase on the w/o/w emulsions

Two w/o/w emulsions were prepared using PC-depleted lecithin and WPI as the low- and high-HLB emulsifier, respectively. Hereby, the used oil phase was either Hosono or MCT-oil.

8.3.1.2.1 Influence on the double emulsion droplet size

As shown in Figure 8.2A, independent of the homogenization speed, the double emulsion droplet size was higher in case Hosono was used as the oil phase. This can be related to the much higher viscosity of the w/o emulsions prepared using Hosono as compared to MCT-oil (part 8.3.1.1.2).

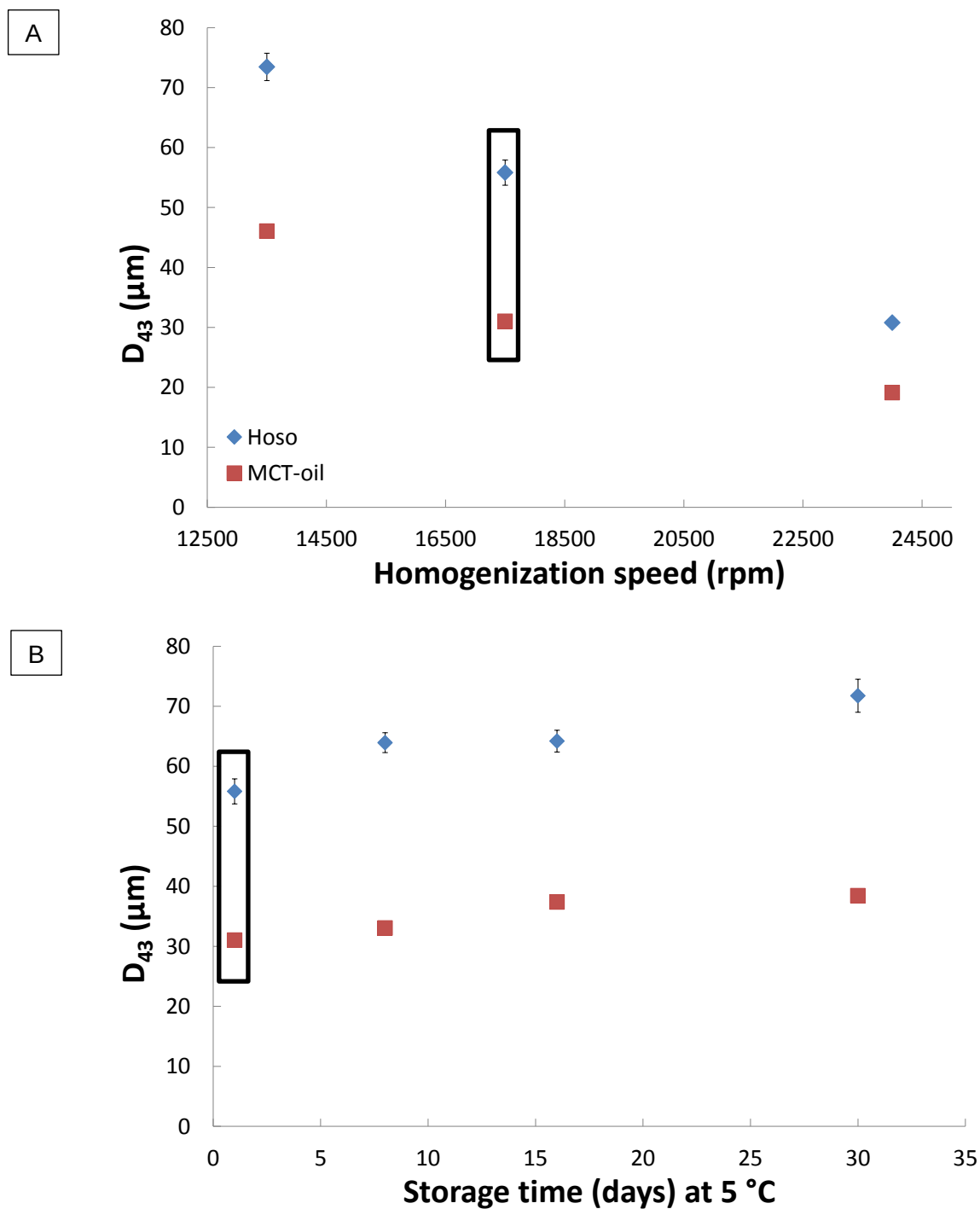


Figure 8.2: A) Volume weighted average diameter (D_{43}) after one day of storage at 5 °C of the double emulsion droplets with Hosol or MCT-oil as the oil phase, PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, as a function of homogenization speed of the Ultra-Turrax S25-10G. B) Volume weighted average diameter (D_{43}) of the double emulsion droplets with Hosol or MCT-oil as the oil phase, PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, prepared at 17500 rpm as a function of storage time at 5 °C.

The double emulsion droplet size of the emulsions prepared at 17500 rpm was followed as a function of the storage time: Figure 8.2B shows that there was a slight increase of the volume weighted average droplet size during storage independent of the used oil phase. Hereby, during a 30 day storage period at 5 °C, the diameter increased with about 24% in case MCT-oil was used while an increase with about 29% was measured when Hoso was used as the oil phase.

8.3.1.2.2 Influence on the enclosed water volume fraction

Figure 8.3A shows that using either of the two oil phases, a high enclosed water volume fraction was obtained. Indeed, the enclosed water volume fraction can maximally be equal to about 14.5% for Hoso and 14.6% for MCT-oil (due to their difference in density). Hereby, the enclosed water volume fraction decreased with a decreasing double emulsion droplet size. For similar droplet sizes, the enclosed water volume fraction was slightly lower in case Hoso was used as the oil phase. The presence of enclosed water in both types of double emulsions is shown in Figure 8.4.

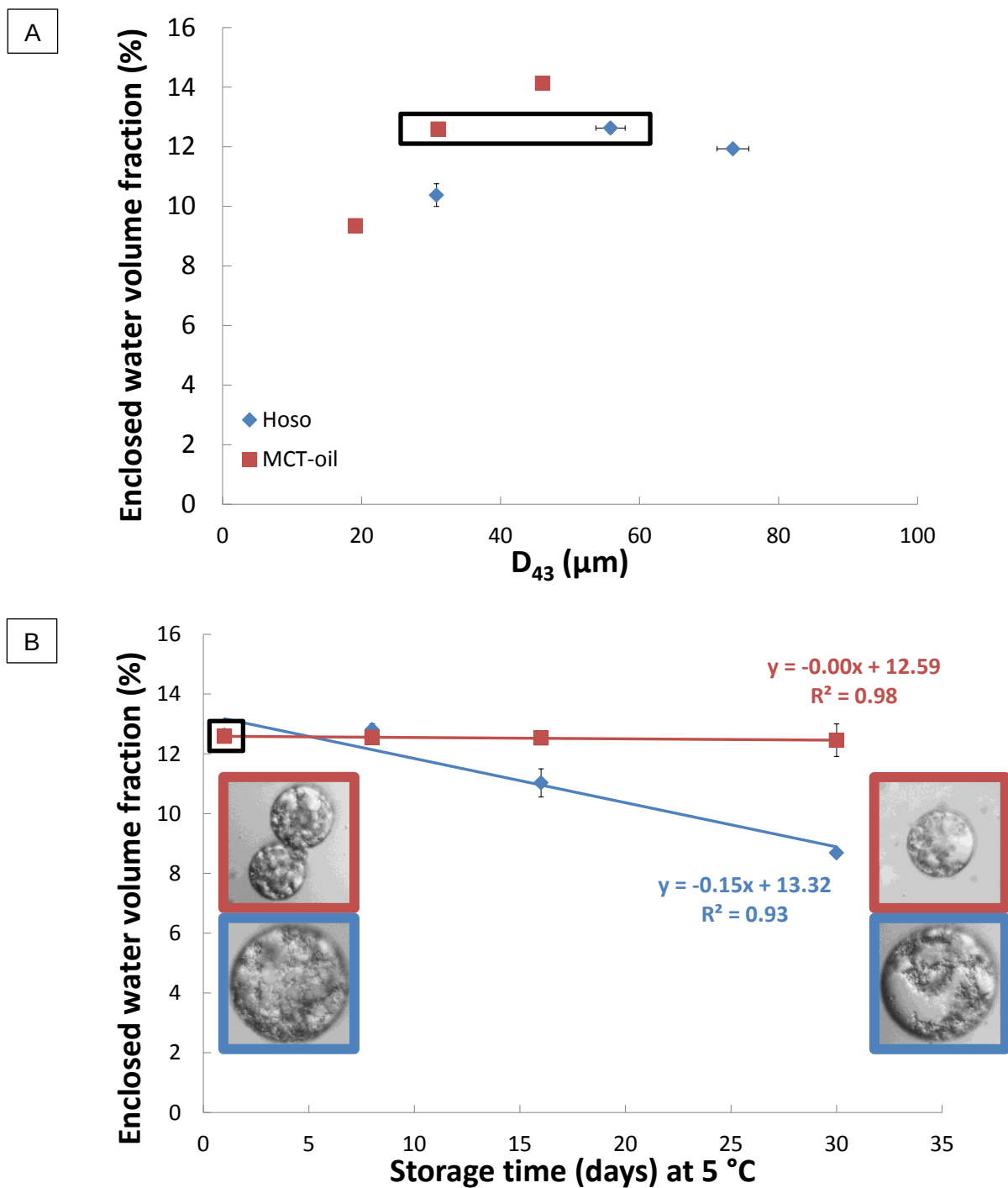


Figure 8.3: A) Enclosed water volume fraction after one day of storage at 5 °C with Hosoi or MCT-oil as the oil phase, PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier as a function of the volume weighted average diameter (D_{43}) of the double emulsion droplets. B) Enclosed water volume fraction of the double emulsion droplets, with Hosoi or MCT-oil as the oil phase, PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, prepared at 17500 rpm as a function of storage time at 5 °C. The insets show a picture of these emulsions obtained using a light microscope with 40x objective after 1 and 30 days of storage at 5 °C. The oil phase is MCT-oil (red) or Hosoi (blue).

The storage stability of the double emulsion prepared using MCT-oil, in terms of enclosed water volume fraction, was a lot higher as compared to when Hoso was used (Figure 8.3B). When a linear regression analysis is conducted on the enclosed water volume fraction (%) as a function of storage time, a decrease $\pm$ standard error of 0.00 ± 0.00 %/day was calculated for MCT-oil while a decrease of 0.15 ± 0.03 %/day was calculated for Hoso.

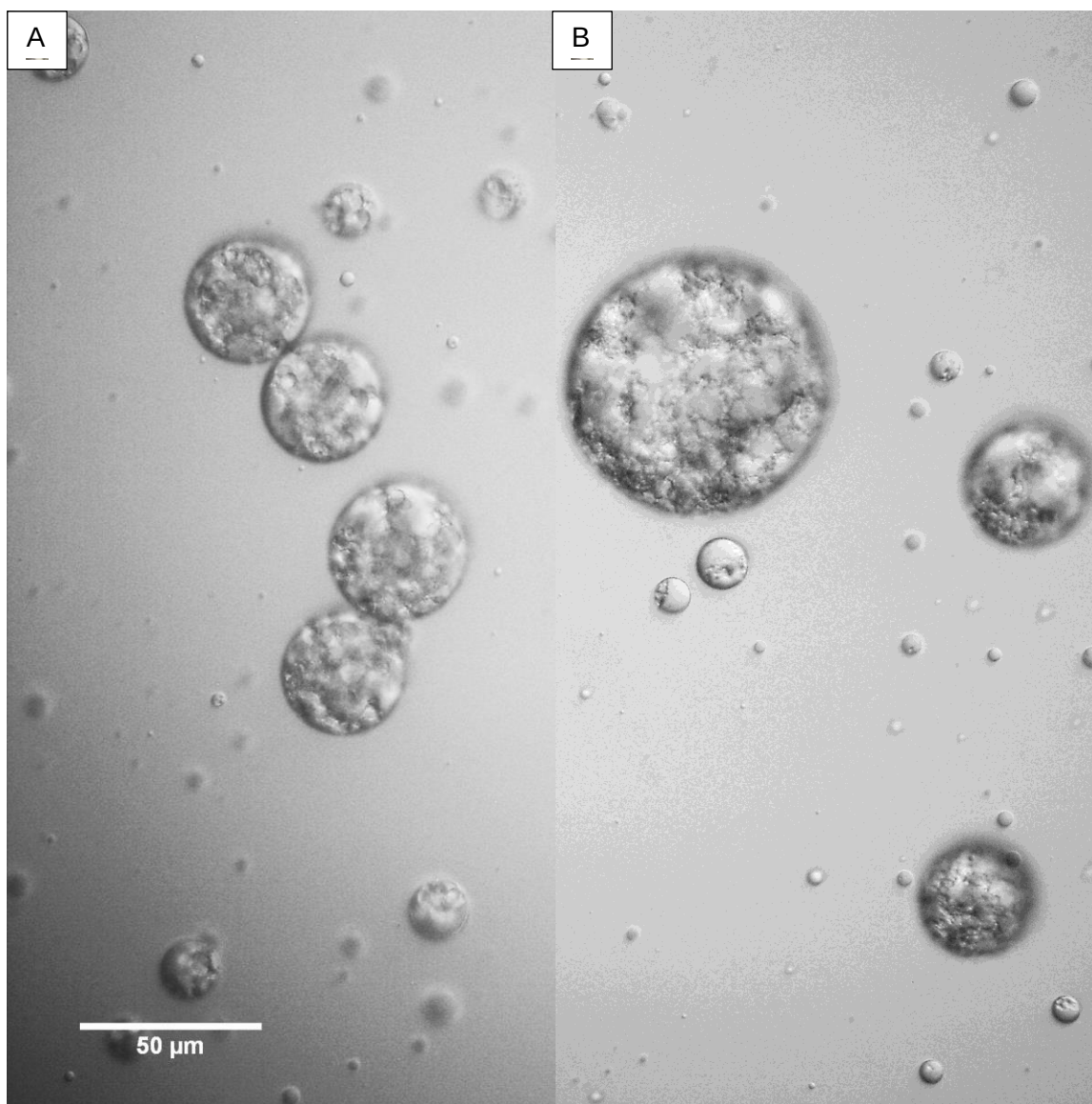


Figure 8.4: Pictures of the w/o/w emulsions prepared at 17500 rpm and stabilized using PC-depleted lecithin and WPI as the low- and high-HLB emulsifiers, respectively. Pictures were obtained using light microscopy and a 40x objective. The oil phase was MCT-oil (A) or Hoso (B) and the emulsions were diluted 10x in isotonic solution after one day of storage at 5 °C.

8.3.2 Influence of the lecithin composition

As already mentioned, using either of the lecithin samples (sunflower, soybean or PC-depleted lecithin), it was possible to produce w/o emulsions as long as the used oil phase was MCT-oil. Therefore, w/o/w emulsions were prepared which only differed in the used lecithin to stabilize the w/o emulsion using MCT-oil as the oil phase.

8.3.2.1 Influence on the double emulsion droplet size

Figure 8.5A shows that the composition of the lecithin, which was used to stabilize the primary w/o emulsion, had no strong influence on the double emulsion droplet size directly after preparation. The double emulsion droplet size seems to be mainly linked to the viscosity of the w/o emulsions, which was shown to be similar (Table 8.1). In terms of droplet size stability, the biggest difference was observed between sunflower and PC-depleted lecithin (Figure 8.5B). After 30 days of storage at 5 °C, the droplet size decreased with about 13% in case sunflower lecithin was used whereas, when PC-depleted lecithin was used, the droplet size increased with about 24%. Finally, when soybean lecithin was used, the measured droplet size increased with only 3% after 30 days of storage.

A decrease of the double emulsion droplet size can be explained by the loss of enclosed water from the double emulsion droplets. The loss of enclosed water as a function of storage time when sunflower lecithin was used, was indeed confirmed by the measurements of enclosed water volume fraction as a function of storage time (Figure 8.6B).

An increase in double emulsion droplet size can be caused by an increase of the enclosed water volume fraction (due to transport of water from the external to the enclosed water phase) or by coalescence and/or flocculation of double emulsion droplets. Because no significant increase in the enclosed water volume fraction was measured for the sample produced using PC-depleted lecithin (Figure 8.6B), it can be assumed that part of the double emulsion droplets coalesced and/or flocculated. The fact that the double emulsion droplet size of the emulsions containing PC-depleted lecithin increased might be linked to the reduced phosphatidylcholine-content of this lecithin: phosphatidylcholine has good o/w stabilizing properties whereas phosphatidylethanolamine (which is enriched in PC-depleted lecithin) is rather suited for the stabilization of w/o interfaces (part 8.4).

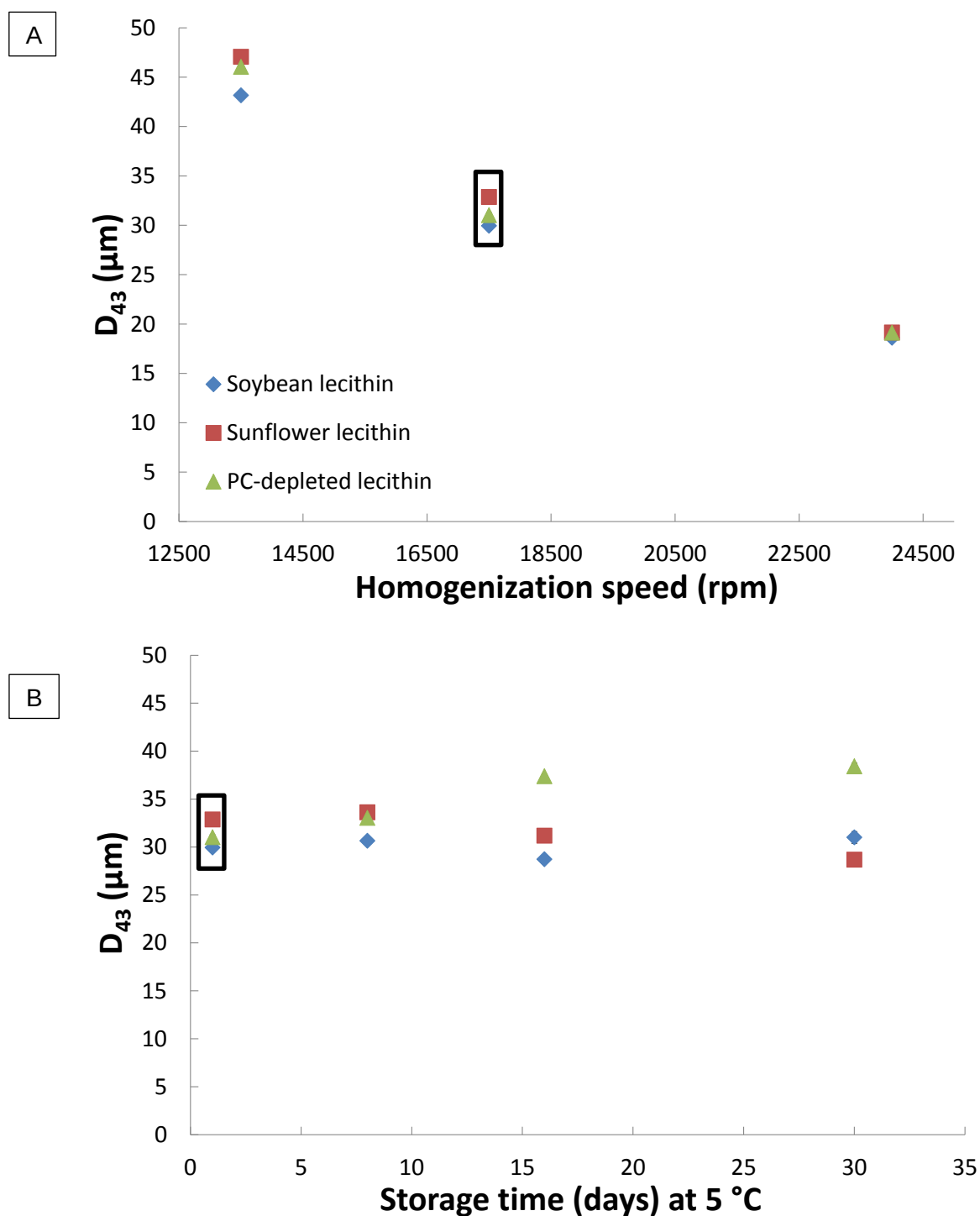


Figure 8.5: A) Volume weighted average diameter (D_{43}) after one day of storage at 5 °C of the double emulsion droplets with MCT-oil as the oil phase, sunflower, soybean or PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, as a function of homogenization speed of the Ultra-Turrax S25-10G. B) Volume weighted average diameter (D_{43}) of the double emulsion droplets with MCT-oil as the oil phase, sunflower, soybean or PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, prepared at 17500 rpm as a function of storage time at 5 °C.

8.3.2.2 Influence on the enclosed water volume fraction

The lecithin composition did not have a strong influence on the enclosed water volume fraction versus double emulsion droplet size relation after preparation (Figure 8.6B). Increasing the shear during preparation (decreasing the double emulsion droplet size) led to a slight decrease of the enclosed water volume fraction. The double emulsion droplets which were produced using the highest shear (with a volume weighted average droplet size of about 19 μm) still retained between 64 and 80% of the first water phase.

However, there was a big influence of the lecithin composition on the storage stability of the enclosed water volume fraction (Figure 8.6B). Indeed, when PC-depleted lecithin was used, the enclosed water volume fraction did not decrease during 30 days of storage at 5 °C (change of 0.00 ± 0.00 %/day). In contrast, a decrease with 0.17 ± 0.02 %/day and 0.37 ± 0.05 %/day was calculated for soybean and sunflower lecithin, respectively. Therefore, using PC-depleted lecithin, the highest storage stability could be obtained while using sunflower lecithin the storage stability was very low. Finally, using soybean lecithin, an intermediate stability was obtained. As seen on the inset picture of the w/o/w emulsion prepared using sunflower lecithin, these emulsions were almost completely devoid of enclosed water after storage for 30 days at 5 °C (Figure 8.6B, red).

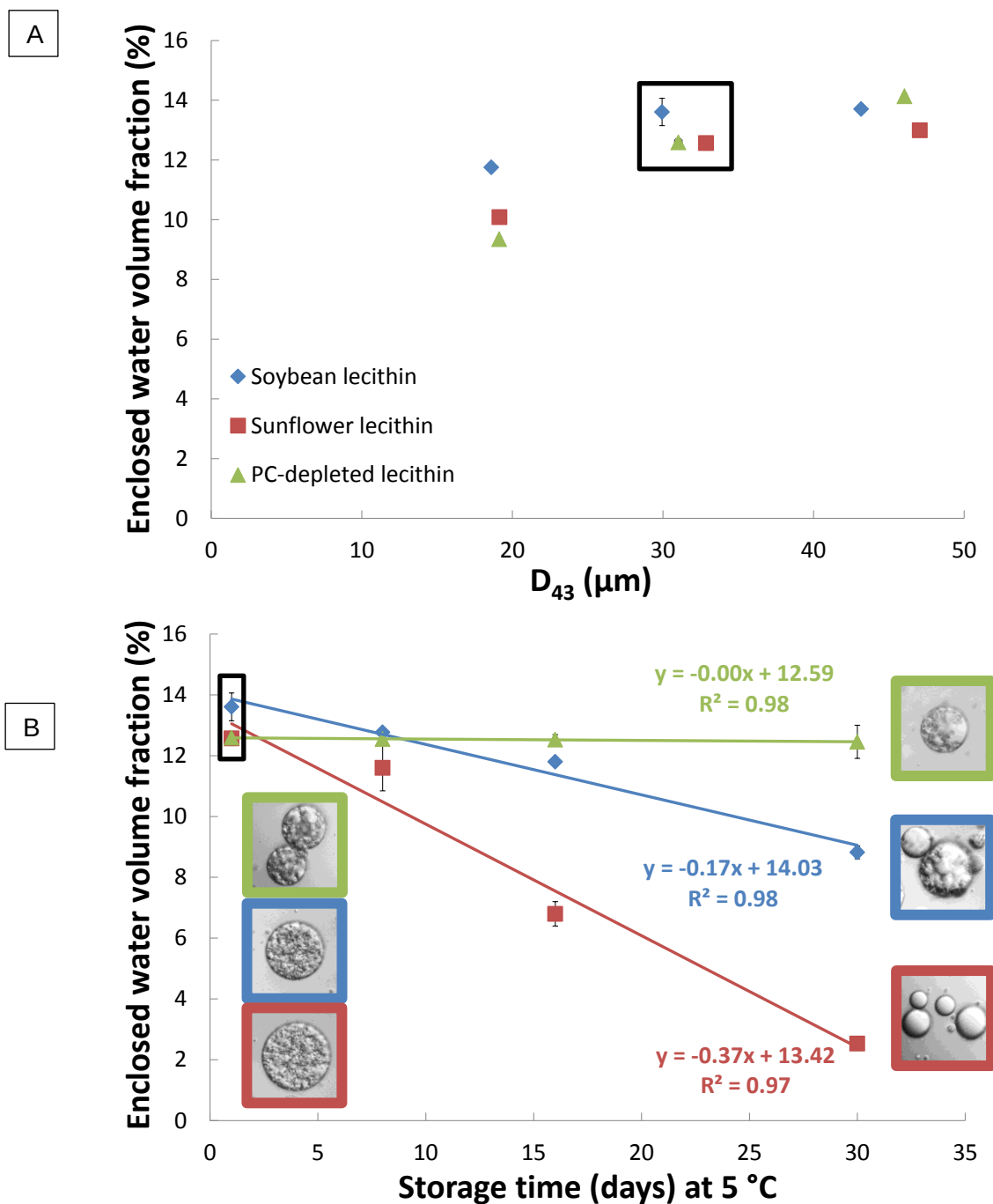


Figure 8.6: A) Enclosed water volume fraction after one day of storage at 5 °C with MCT-oil as the oil phase, sunflower, soybean or PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, as a function of the volume weighted average diameter (D_{43}) of the double emulsion droplets. B) Enclosed water volume fraction of the double emulsion droplets with MCT-oil as the oil phase, sunflower, soybean or PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, prepared at 17500 rpm as a function of storage time at 5 °C. The insets show a picture of these emulsions obtained using a light microscope with 40x objective after 1 and 30 days of storage at 5 °C. The used lecithin is sunflower (red), soybean (blue) or PC-depleted lecithin (green).

8.3.3 Influence of the used high-HLB emulsifier on the w/o/w emulsions

Double emulsions were prepared using long chain unsaturated triglyceride oil (Hoso) as the oil phase and PC-depleted lecithin as the low-HLB emulsifier, the high HLB-emulsifier was varied.

8.3.3.1 Influence on the double emulsion droplet size

It was assumed that there could be lecithin, which was used to stabilize the w/o interface, left to stabilize the o/w interface of a double emulsion. This was indeed observed: w/o/w emulsions could be prepared without adding an additional high-HLB emulsifier to the external water phase (results not shown). However, when no additional high-HLB emulsifier is added to the external water phase, the obtained double emulsion droplets are very big.

Figure 8.7A shows that when whey protein isolate was used, the biggest droplets were obtained, which can be related to the lower surface activity of this protein as compared to lecithin and Na-caseinate. However, the surface activity of CMC is very small (Schuch et al., 2015), which shows that lecithin, which is not consumed in the stabilization of the w/o interface, can indeed help to stabilize the o/w interface. Hereby, the increased viscosity due to the addition of CMC to the external water phase has a beneficial effect during preparation as it reduces the viscosity ratio between dispersed and continuous phase and therefore the droplet size (Leal-Calderon et al., 2012).

When Na-caseinate was used as high-HLB emulsifier, the double emulsion droplets were a lot smaller. Moreover, when having a look at the enclosed water volume fraction (Figure 8.8A), it appears that there is almost no water present in these double emulsions. Two additional w/o/w emulsions were prepared using 2 wt% Na-caseinate as the high-HLB emulsifier but at a lower shear intensity. Even these emulsions did not contain a considerable amount of enclosed water (biggest D_{43} in Figure 8.8A).

Considering the storage stability of the double emulsion droplet size (Figure 8.7B) it can be mentioned that during 30 days of storage at 5 °C, the droplet size increased with 14 and 26% in case CMC and WPI were used, respectively. In contrast, when powdered soybean lecithin was used, the droplet size decreased with 13%.

An increase in double emulsion droplet size can be caused by an increase of the enclosed water volume fraction or by coalescence and/or flocculation of double emulsion droplets. Because for none of the tested compositions, the enclosed water volume fraction increased during storage (Figure 8.8B), the increase in droplet size can only be explained by coalescence and/or

flocculation of the double emulsion droplets. As already mentioned, PC-depleted lecithin is rather suited for the stabilization of w/o interfaces. The o/w interface of the double emulsions, which can be assumed to be at least partly covered by the phospholipids, might therefore not be sufficiently stabilized against coalescence and/or flocculation.

Remarkably, although the highest increase in double emulsion droplet size was measured when WPI was used as high-HLB emulsifier, the release of enclosed water was also higher for this sample as compared to when CMC was used (Figure 8.8B). This implies that, although the double emulsion droplet size decreased due to (partial) release of the enclosed water phase, an overall increase of this parameter was measured. The increase due to coalescence and/or aggregation of the double emulsion droplets therefore contributed more to the measured droplet size than the decrease due to release of enclosed water. It should however be kept in mind that the double emulsion, stabilized using WPI had a much bigger initial droplet size as compared to the other samples. Because the attractive interactions between droplets increase with increasing droplet size (Hamilton et al., 1990; Hou et al., 1988), the bigger droplet size of this emulsion might be part of the reason of the highest droplet size increase.

Finally, when powdered soybean lecithin was used as high-HLB emulsifier, a decrease of the droplet size during storage was measured which can mainly be linked to the loss of enclosed water (Figure 8.8B).

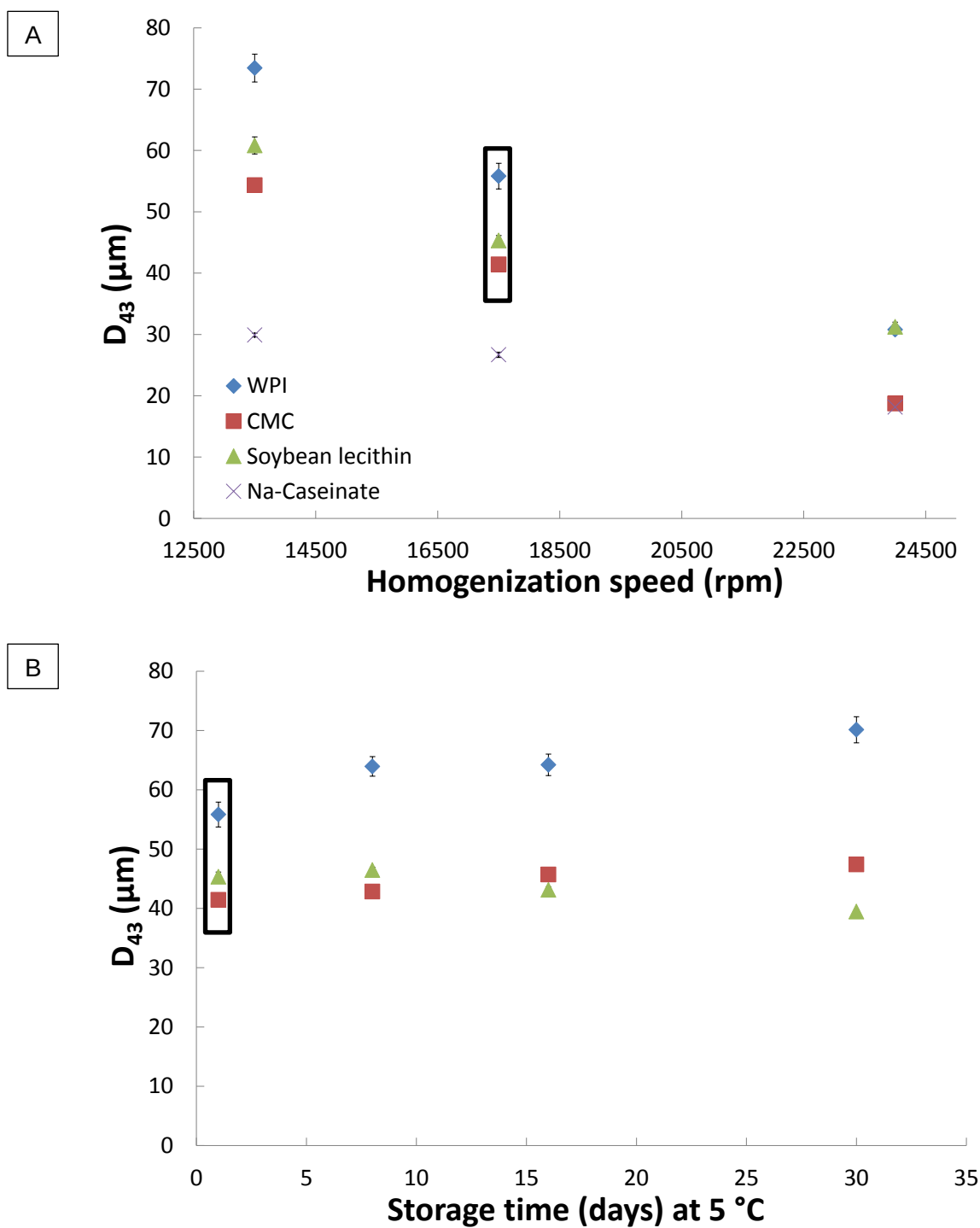


Figure 8.7: A) Volume weighted average diameter (D_{43}) after one day of storage at 5 °C of the double emulsion droplets with Hosu as the oil phase, PC-depleted lecithin as the low-HLB emulsifier, and varying the high-HLB emulsifier as a function of homogenization speed of the Ultra-Turrax S25-10G. **B)** Volume weighted average diameter (D_{43}) of the double emulsion droplets with Hosu as the oil phase, PC-depleted lecithin as the low-HLB emulsifier, and varying the high-HLB emulsifier prepared at 17500 rpm as a function of storage time at 5 °C.

8.3.3.2 Influence on the enclosed water volume fraction

As shown in Figure 8.8A, the used high-HLB emulsifier did not have a strong influence on the enclosed water volume fraction versus double emulsion droplet size relation after preparation unless the used high-HLB emulsifier was Na-caseinate. In the latter case, almost independent of the droplet size, the enclosed water volume fraction was very low. For the other high-HLB emulsifiers, the use of higher shear during preparation (decreasing the double emulsion droplet size) led to a slight decrease of the enclosed water volume fraction. The double emulsion droplets which were produced using the highest shear, with a volume weighted average droplet size between 19 and 31 μm , still retained between 70 and 86% of the first water phase, respectively.

However, following the enclosed water volume fraction as a function of storage time at 5 °C (Figure 8.8B) reveals that CMC greatly enhanced the storage stability of the w/o/w emulsions. Indeed, the enclosed water volume fraction decreased with 0.15 ± 0.03 and 0.21 ± 0.00 %/day when WPI and soybean lecithin were used, respectively. In contrast, a very slow loss of enclosed water volume during storage of 0.03 ± 0.01 %/day was noted in case CMC was added to the external water phase.

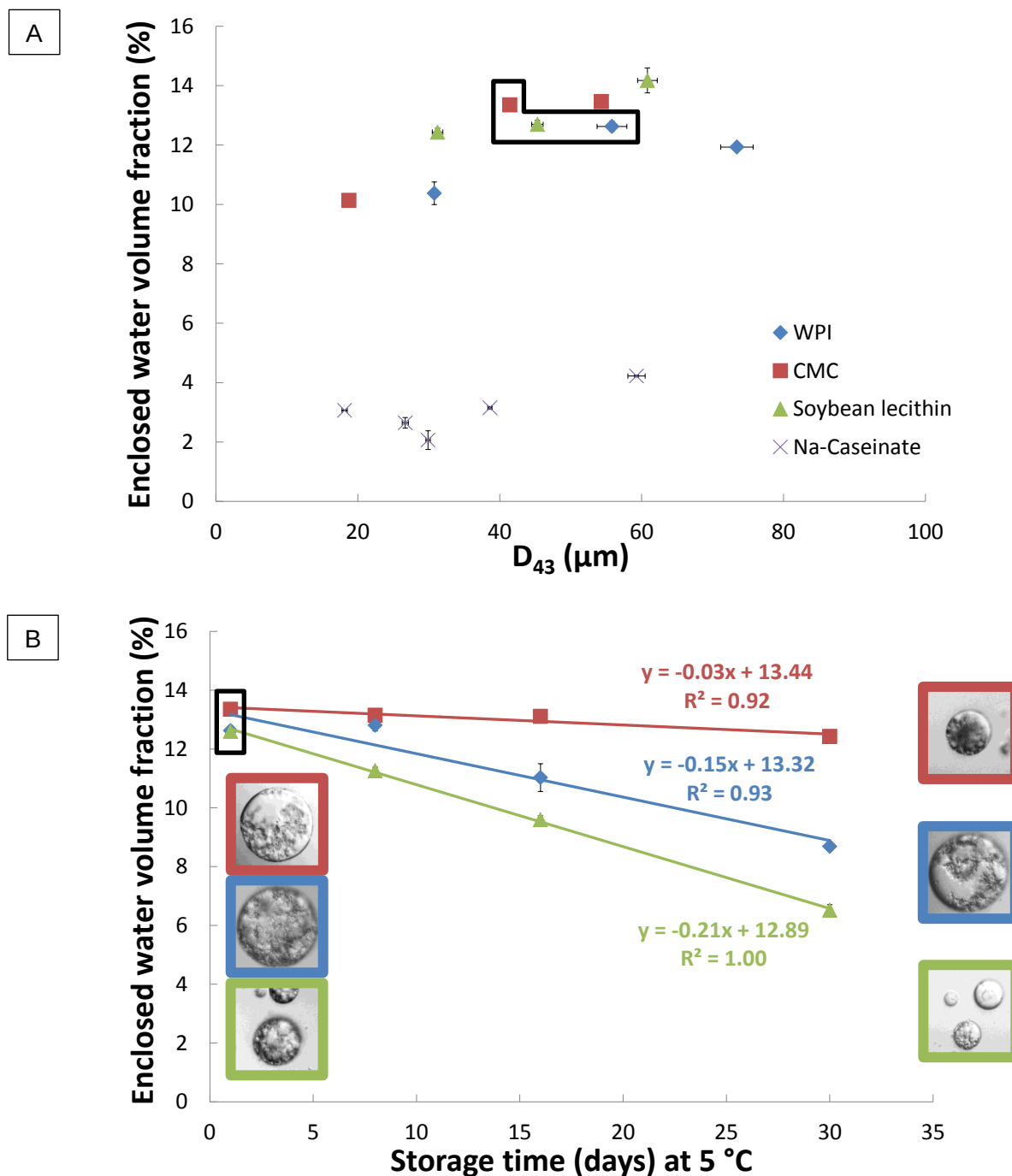


Figure 8.8: A) Enclosed water volume fraction after one day of storage at 5 °C of the double emulsion droplets with Hosolip as the oil phase, PC-depleted lecithin as the low-HLB emulsifier, and varying the high-HLB emulsifier as a function of the volume weighted average diameter (D_{43}) of the double emulsion droplets. B) Enclosed water volume fraction of the double emulsion droplets with Hosolip as the oil phase, PC-depleted lecithin as the low-HLB emulsifier, and varying the high-HLB emulsifier prepared at 17500 rpm as a function of storage time at 5 °C. The insets show a picture of these emulsions obtained using a light microscope with 40x objective after 1 and 30 days of storage at 5 °C. The used high-HLB emulsifier is CMC (red), WPI (blue) or soybean lecithin (green).

8.3.4 Sensitivity of the w/o/w emulsions towards dilution

Because the enclosed water volume fraction was very low when Na-caseinate was used as high-HLB emulsifier, it was tested whether the w/o/w emulsions, prepared using PC-depleted lecithin as low-HLB emulsifier and WPI as high-HLB emulsifier, were sensitive to Na-caseinate when this compound was added after production of the w/o/w emulsion. Therefore, the double emulsions were diluted in an isotonic aqueous phase containing Na-caseinate. In order to be able to compare the results of the aforementioned dilution, the double emulsion was diluted in two other isotonic solutions namely the w_1 - and w_2 -phase (containing 2 wt% WPI).

The release of PTSA due to osmotic gradients between enclosed and external water was also tested by dilution of the double emulsion with a hyper- (0.4M glucose) and a hypotonic (distilled water) aqueous phase. Hereby it is expected that dilution in hypertonic medium leads to shrinking of the w/o and therefore w/o/w droplets while hypotonic dilution leads to swelling of the w/o and therefore w/o/w droplets (Chapter 6; Mezzenga et al., 2004). The change of droplet size might lead to external coalescence of enclosed water droplets and therefore release of enclosed PTSA.

Figure 8.9 clearly shows that the addition of a solution containing 2 wt% Na-caseinate in isotonic medium led to the release of the enclosed compound. This effect was, however, absent if the double emulsions were diluted in an isotonic water phase or in an isotonic water phase containing WPI. This result suggests that Na-caseinate was capable of replacing WPI and/or lecithin at the o/w interface, which subsequently led to the release of enclosed water phase. It can be noted that the release of water due to the addition of Na-caseinate was slightly higher than in case ammonium phosphatide was used as low-HLB emulsifier (Chapter 7). This can probably mainly be attributed to the fact that the concentration of surface active agents was higher in case ammonium phosphatide was used. Whereas the same apparent emulsifier concentration was used (2.5 wt% in the oil), the PC-depleted lecithin also contains oil whereas ammonium phosphatide does not.

It is also interesting to note that about 82% of the PTSA, which was added to the w_1 -phase before preparation, remained enclosed in the double emulsions after preparation. This implies that about 18% of the enclosed water was lost due to external coalescence of enclosed water droplets during preparation of the double emulsion. The enclosed water volume fraction of the double emulsion can be calculated from this result and was equal to $0.82 \cdot 14.5 = 11.9\%$, which

corresponds fairly well with what was determined for a similar w/o/w emulsion using analytical photocentrifugation (12.6%).

Looking at the influence of osmotic gradients, hypotonic dilution led to the release of enclosed water due to swelling breakdown of the double emulsion droplets whereas dilution in hypertonic medium did not lead to external coalescence of the enclosed water droplets. Hereby, in contrast to when ammonium phosphatide was used (Chapter 7), the release due to hypotonic dilution was higher than due to the addition of Na-caseinate. Again this can probably mainly be attributed to the fact that the concentration of lipophilic, surface active compounds present in the oil phase was lower as compared to ammonium phosphatide in chapter 7.

As already mentioned in the previous chapter, these observations have practical implications. Indeed, when controlled release of enclosed compounds is required, it is interesting to note which dilution liquids lead to the release of enclosed components and which dilution liquids don't. From the results shown in Figure 8.9, it is clear that w/o/w emulsions in which lecithin was used as low-HLB emulsifier were quite sensitive towards dilution.

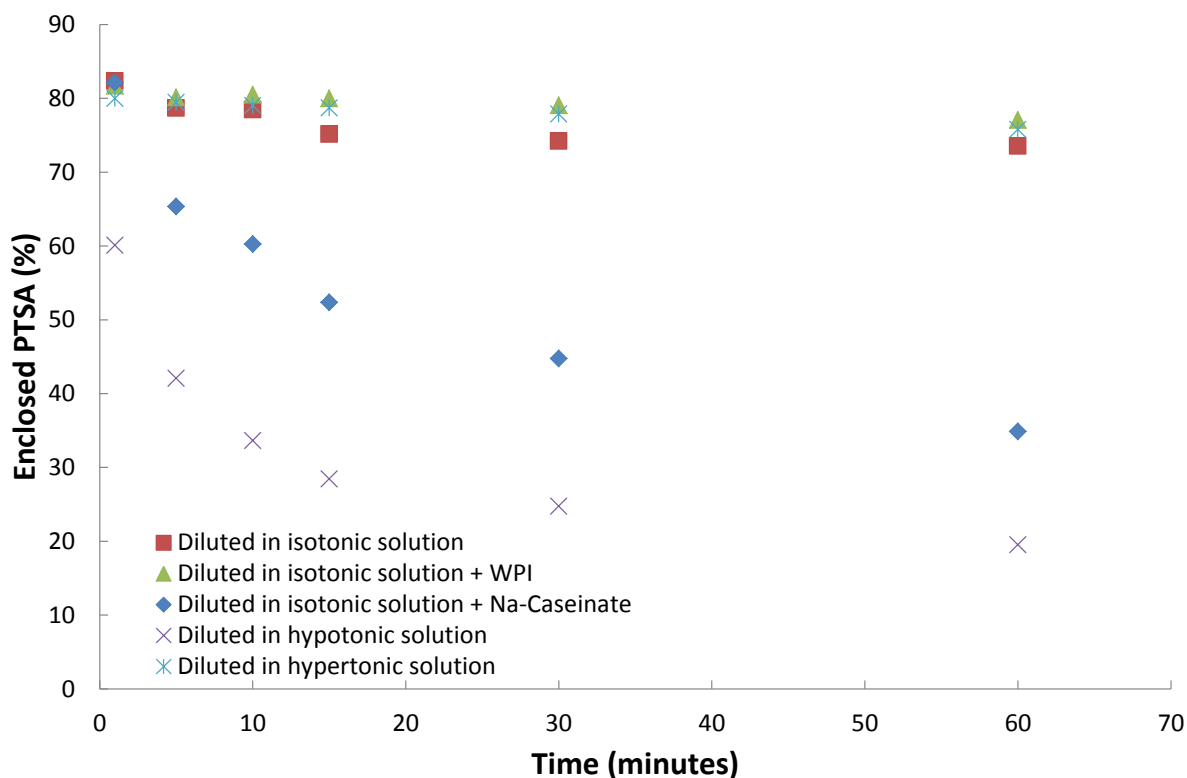


Figure 8.9: Percentage of PTSA which remains enclosed as a function of time at room temperature after 1:1 (volume:volume) dilution of the double emulsion in different aqueous solutions.

8.4 Discussion

Two mechanisms can help to explain the majority of the results. Firstly, as already mentioned by Kabalnov et al. (1996), the spontaneous curvature of lecithin can change depending on the continuous oil phase, the phospholipid composition and the temperature (Table 8.2). Hereby, a negative curvature favors the formation of w/o emulsions while a positive curvature favors the formation of o/w emulsions.

Table 8.2: Factors affecting phospholipid monolayer spontaneous curvature (Kabalnov et al., 1996).

Factors decreasing spontaneous curvature	Factors increasing spontaneous curvature
<i>Phospholipid composition</i>	
Unsaturated acyl chain	Saturated acyl chain
Phosphatidylethanolamines	Phosphatidylcholines
<i>Temperature</i>	
Increased temperature	Decreased temperature
<i>Oil phase composition</i>	
Hydrocarbons	Fluorocarbons
Short-chain oil	Long-chain oil

An additional explanation might be a difference in the hydration behavior of the different phospholipids as a function of the used oil phase. Indeed, degumming of vegetable oils relies on the fact that hydrated PC is insoluble in the oil phase. Moreover, this hydrated PC is capable of entraining PE (phosphatidylethanolamine), PI (phosphatidylinositol) and PA (phosphatidic acid) (Bockisch, 1998). Kanamoto et al. (1981) showed that PE is only removed on degumming of soybean oil if also PC is present with which PE can form mixed micelles. Therefore, it might be assumed that using PC-depleted lecithin, a w/o emulsion can be produced in Hosono because the removal of PE (and possibly other phospholipids) due to hydration and subsequent removal as mixed micelles with PC is lower.

Using Table 8.2, also most of the stability differences which were observed for the produced w/o/w emulsions can be explained. Firstly, the storage stability of the enclosed water volume fraction was higher with MCT-oil as compared to with Hosono as the oil phase. This can be linked to the fact that MCT-oil is a medium chain oil which can penetrate the lipophilic bush of the emulsifier more easily than the long acyl chains in Hosono. This in turn leads to a lower

spontaneous curvature of the phospholipids in the emulsion with MCT-oil as compared to the emulsion with Hoso. The formation of holes which enable contact between the water phases, requires a high positive curvature (Figure 8.10) as already mentioned by Kabalnov et al. (1996) for coalescence between w/o droplets and Pays et al. (2001) for external coalescence in w/o/w emulsions.

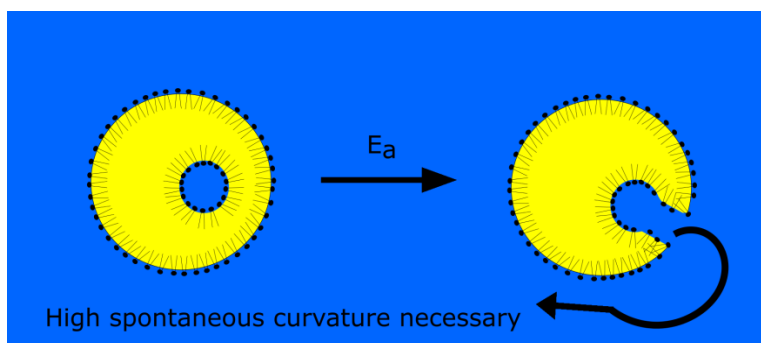


Figure 8.10: Schematic illustration showing the high spontaneous curvature needed in the monolayer when external coalescence takes place.

Secondly, the fact that the stability of the w/o/w emulsions decreased in the order PC-depleted lecithin > soybean lecithin > sunflower lecithin can be linked to their phospholipid composition, with the PE/PC ratio decreasing in the same order. Therefore, the increase in stability can again be linked to a decrease of the average spontaneous curvature of the phospholipid monolayer.

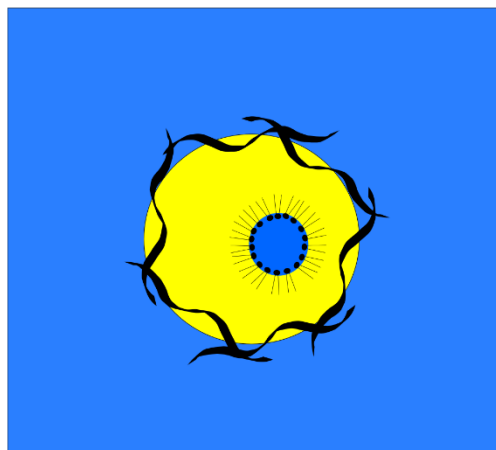
Finally, because the used oil phase and lecithin composition was the same, Table 8.2 cannot be used to explain the observed differences when only the high-HLB emulsifier was varied. Based on our results, it can be assumed that Na-caseinate was capable of (at least partly) replacing lecithin at the o/w interface. Fang and Dalgleish (1993) already showed that the surface coverage of casein on an o/w droplet was more or less independent of the used lecithin concentration. Akhtar and Dickinson (2001), however, produced w/o/w emulsions with an acceptable enclosed water volume using PC-depleted lecithin and Na-caseinate. This might seem contradictory. However, these researchers used a very low concentration of Na-caseinate (0.25 wt% in the external water phase). Considering this concentration, the ratio of multiple emulsion droplets to external water of 20/80 (assuming 100% retention of enclosed water) and the measured D_{32} of about 1.5 μm , the maximum surface coverage of Na-caseinate which could be obtained was equal to 2.5 mg/m^2 , which is still below the maximum surface coverage of 3 mg/m^2 for Na-caseinate (Fang and Dalgleish, 1993). It can thus be assumed that lecithin at the o/w interface was not fully replaced by caseinate molecules in the research of Akhtar and

Dickinson (2001) due to the low concentration. However, the concentration of Na-caseinate that we used to produce the emulsions was a lot higher (2 wt%). Na-caseinate can change the average spontaneous curvature of the o/w emulsifier layer which might enable an easier formation of holes, leading to external coalescence (Pays et al., 2001). Thus, replacement of lecithin by caseinate at the o/w interface led to the formation of an o/w rather than a w/o/w emulsion. As already mentioned in the previous chapter, another and complementary explanation of the observed adverse effect of Na-caseinate might be the adsorption of a less dense emulsifier layer (considering the sum of adsorbed protein and phospholipids) in case Na-caseinate is used. The net charge of phospholipids and Na-caseinate is negative at neutral pH. They will therefore repel each other when they are adsorbed at the interface. The difference with WPI could be explained by the globular nature of this protein as well as its lower surface activity.

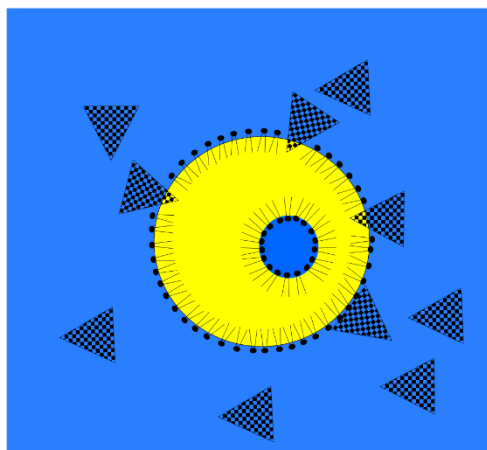
Knoth et al. (2005b) described that combining lecithin with either WPI or Na-caseinate led to a decrease in interfacial tension, whereas Na-caseinate lowered interfacial tension faster and more effectively than WPI. It might therefore be assumed that WPI is mostly replaced by phospholipids during storage. Moreover, the slightly lower rate at which enclosed water was lost when WPI was used as compared to when powdered soybean lecithin was used, could be linked to interactions between β -lactoglobulin and the phospholipid monolayer (Knoth et al., 2005b and references therein). Schuch et al. (2015) already described the higher stability of w/o/w emulsions in which both the w/o and the o/w interface are stabilized by the same emulsifier. Hereby, it can be mentioned that the frustration energy of emulsifiers with a negative curvature which are nonetheless stabilizing the o/w interface is limited. Indeed, the o/w interface of the w/o/w emulsions is less curved as compared to the w/o interface. The lower the spontaneous curvature of the o/w interface is, the less external coalescence of enclosed water droplets will take place (Kabalnov and Wennerstrom, 1996; Pays et al., 2001).

Finally, it is remarkable that using CMC, very stable w/o/w emulsions were produced. This seems to indicate that CMC is interacting with lecithin at the o/w interface. CMC can interact through hydrogen bonds as well as electrostatic interactions with the phospholipid monolayer. Hereby CMC seems to prevent the formation of holes in the w/o/w film. Schuch et al. (2015) also found CMC as the best stabilizer in combination with PGPR as the low-HLB emulsifier. They mentioned the importance of the presence of the same emulsifier (in their case PGPR) at the w/o and o/w interface. Our results confirm this statement. However, CMC also seems to be interacting with the emulsifier found at the o/w interface leading to an even higher stability. The statements above are summarized in Figure 8.11.

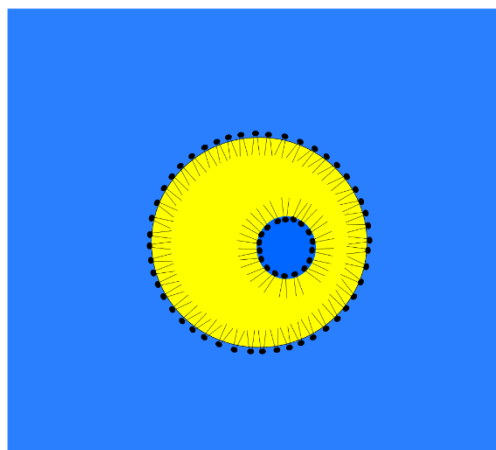
w/o/w emulsion with Na-Caseinate



w/o/w emulsion with WPI



w/o/w emulsion with soybean lecithin



w/o/w emulsion with CMC

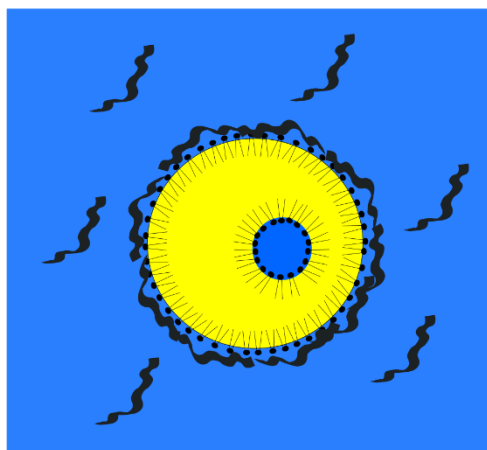


Figure 8.11: Hypothesized schematic illustration of the interfacial coverage in the w/o/w emulsions.

It is interesting to note that adding WPI and/or xanthan to the water phase of a w/o emulsion which is stabilized using lecithin has a beneficial effect while adding unfolded proteins such as gelatin and Na-caseinate leads to instability of w/o emulsions prepared using lecithin (Knoth et al., 2005b). Therefore, an interesting subject for future research would be the addition of these compounds to the enclosed water phase of a w/o/w emulsion. Moreover, adding CMC to the water phase of a lecithin-stabilized w/o emulsion could lead to even better results as compared to adding WPI and/or xanthan due to interaction between CMC and the phospholipid monolayer as well as the increase of the viscosity ratio between dispersed and continuous phase (which becomes closer to unity).

8.5 Conclusions

Lecithin samples from different sources, and thus with different phospholipid compositions, were used in the production of w/o emulsions. As described by Kabalnov et al. (1996), both the phospholipid-composition of the used lecithin as well as the used oil phase had an influence on the stability of the w/o emulsions due to their influence on the average spontaneous curvature of the phospholipid monolayer. With each of the tested lecithin samples, a stable w/o emulsion was produced when the oil phase was an MCT-oil whereas only with PC-depleted lecithin, it was possible to produce a stable w/o emulsion with Hosu (LCT-oil).

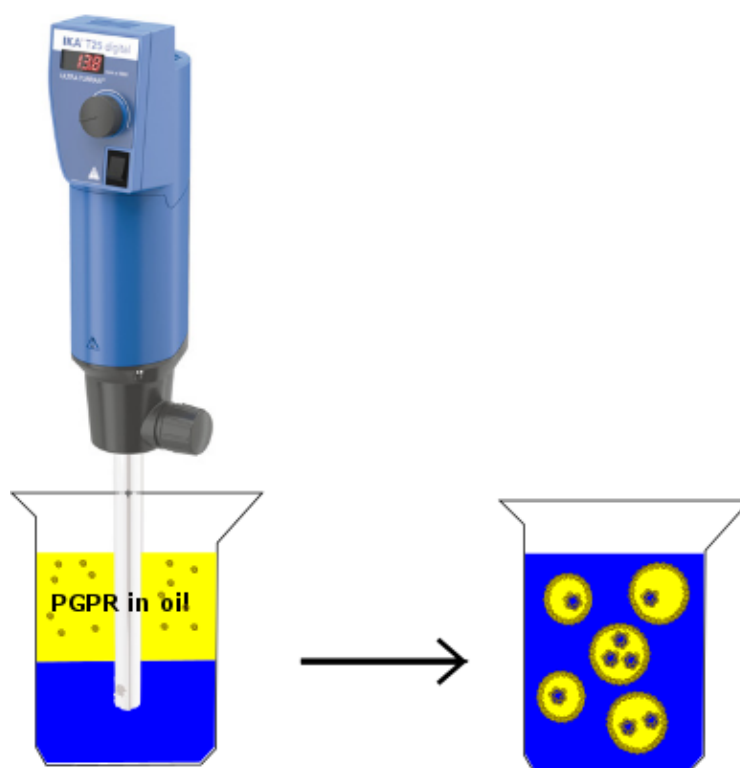
The used oil phase and lecithin composition also had an influence on the storage stability of the enclosed water volume fraction of the produced w/o/w emulsions. The highest stability was obtained using PC-depleted lecithin and the oil phase with the shortest acyl chains (MCT-oil).

W/o/w emulsions with Hosu as the oil phase, PC-depleted lecithin as low-HLB emulsifier, and varying high-HLB emulsifiers were prepared and compared. Remarkably, the results showed that using Na-caseinate as the high-HLB emulsifier in combination with lecithin as the low-HLB emulsifier, great care should be taken because this emulsifier can cause external coalescence of enclosed water. Another interesting observation was that by adding CMC to the external water phase, w/o/w emulsions were produced which had a remarkably high storage stability. Using WPI or additional lecithin in the external water phase led to intermediate results, whereby the stability was slightly higher with WPI.

Finally, the stability of a PC-depleted lecithin and WPI-stabilized w/o/w emulsion in varying aqueous dilution liquids was followed by adding a fluorescent marker to the enclosed water phase and following its concentration in the external water phase. Dilution of the w/o/w emulsion in hyper- or isotonic solution did not lead to external coalescence of the enclosed droplets. However, dilution of the w/o/w emulsion in isotonic solution to which 2 wt% Na-caseinate was added or in distilled water (hypotonic) led to a quick release of the enclosed component. This release can be linked to external coalescence due to replacement of lecithin by Na-caseinate and swelling breakdown of the double emulsion droplets, respectively.

From our experiments it follows that it was possible to produce double w/o/w type emulsions using solely clean-label ingredients.

Chapter 9: One-step production of w/o/w emulsions



- Double emulsions were produced in one step using PGPR as the only emulsifier
- In the presence of glucose, stable emulsions were formed whereas in the presence of a salt (TMACI) the o/w interfaces quickly coalesced

Abstract

During a short experiment, aimed at investigating the o/w interface stabilizing properties of PGPR, it was discovered that stable double emulsions could be produced using only PGPR as emulsifier. By placing the impeller of a rotor-stator homogenizer in the water phase, double w/o/w type emulsions were prepared in one step. Hereby, between about 9 and 13% of the volume of the produced oil droplets consisted of enclosed water droplets.

9.1 Introduction

In this chapter, it was examined whether PGPR, which is generally used in the production of w/o emulsions, might be capable of (at least partly) stabilizing an o/w interface. Therefore, it was tried to prepare an o/w emulsion using PGPR. During this experiment, it was observed that w/o/w emulsions were produced instead of simple o/w emulsions.

9.2 Materials and Methods

9.2.1 Materials

Polyglycerol polyricinoleate (PGPR 4175) was kindly provided by Palsgaard A/S (Juelsminde, Denmark). High oleic sunflower oil (Hoso; Iodine Value= 87; 82% C18:1) was acquired from Contined BV (Wageningen, The Netherlands). Two water phases were used which both contained 0.2M glucose (VWR Chemicals: BDH Prolabo, Leuven, Belgium) and 0.02 wt% of the anti-microbial agent NaN_3 (Sigma-Aldrich, Steinheim, Germany). To the second type of water phase, 0.2 wt% of the stabilizer carboxymethylcellulose (CMC; Degree of substitution=0.65-0.90; Viscosity 2% solution=1500-3100 mPa.s; Carl Roth, Karlsruhe, Germany) was additionally added. Another test was performed in which the water phase contained 0.1M TMACl (Sigma-Aldrich, Steinheim, Germany) as marker which could potentially be followed using high-resolution NMR.

9.2.2 Emulsion preparation

2.5 wt% PGPR 4175 was added to Hoso after which this phase was heated to 60 °C and stirred to obtain a homogeneous solution. After cooling to room temperature, this oil phase, which contains the emulsifier, was used in the production of a water-continuous 50/50 (m/m) emulsion. As a control, emulsions were prepared using each type of water phase but without emulsifier in the oil phase. To prepare the emulsions, a small Ultra-Turrax (type S25-10G, IKA®-Werke, Germany) at 24000 rpm was used.

The water phase was brought in a beaker, followed by the oil phase. The impeller of the Ultra-Turrax was placed in the water phase (thus piercing through the oil phase) after which mixing was started.

9.2.3 Determination of the w/o/w droplet size distribution

The volume weighted average diameter (D_{43}) of the double emulsion droplets was determined using a Malvern Mastersizer 3000 (Malvern Instruments) with Hydro MV accessory. Hereby, the Mie theory was used and the refractive indices were set to $1.53+0.01i$ for the dispersed phase

and 1.33 for the continuous phase. The stirring speed was set to 1500 rpm and sample was added until an obscuration between 10 and 20 % was reached. Measurements were performed in 0.2M glucose solution.

9.2.4 Light microscopy

When the emulsions were observed under the microscope after tenfold dilution in the water phase, 'half-spheres' were formed at the microscopy glass surface due to coalescence of the emulsion on the glass. This effect can probably be attributed to the fact that the emulsifier is only present in the oil phase.

Therefore, the emulsions were not diluted during the light microscopy observations. The emulsion was brought on a microscopy slide using a capillary after which a cover slip was gently applied. Image acquisition was conducted using an optical microscope (Olympus Cx40) with 40-fold objective coupled with a digital camera (AxioCam ERc 5S).

9.2.5 Analytical photocentrifugation

To determine the enclosed water volume fraction, analytical photocentrifugation was conducted at room temperature (using air cooling) with a LUMiFuge 116 instrument (LUM GmbH, Germany). Hereby, the enclosed water volume fraction was calculated as described in chapter 5.

The enclosed water volume fraction was determined in duplicate after the separation process was complete; three hours of centrifugation at the maximum speed (3000 rpm; 1142g) was sufficient.

During centrifugation, an oil layer formed on top of the cream layer. Therefore, the top of the cream layer was determined after 5 minutes of centrifugation instead of at the end of centrifugation. Also, the 20% transmission value was used to determine the position of the top and bottom of the cream layer.

9.2.6 Density measurements

Because mass ratios were used during the preparation of the (double) emulsion and analytical photocentrifugation is considering the volumes (Chapter 5), the mass ratio needed to be converted to a volume ratio. Therefore, the temperature dependency of the density of the used oil phase as well as water phase (without CMC) were determined. Using the density at the temperature which is reached during centrifugation, it was possible to calculate the volume ratios.

Using a density meter (DMA 5000M, Anton Paar, Germany) the temperature was varied between 25 and 40 °C, whereby density measurements were conducted every 3 °C. A linear relation between density and temperature was found for the oil phases ($R^2 > 0.99$) while a determination coefficient larger than 0.99 was found if a second order polynome was fitted to the density as a function of temperature for the water phase. Mostly, a temperature around 30 °C was reached at the end of centrifugation.

9.3 Results and Discussion

9.3.1 Visual observations

When no emulsifiers were added, opaque, oil continuous systems were produced which completely phase separated within 1 hour after preparation. In contrast, if the emulsions contained PGPR and the water phase contained 0.2M glucose, whether or not with CMC, stable w/o/w emulsions were prepared. Finally, if the water phase consisted of 0.1M TMACI, an unstable w/o/w emulsion was formed which quickly separated into a w/o emulsion layer floating on top of a water layer (Figure 9.1).

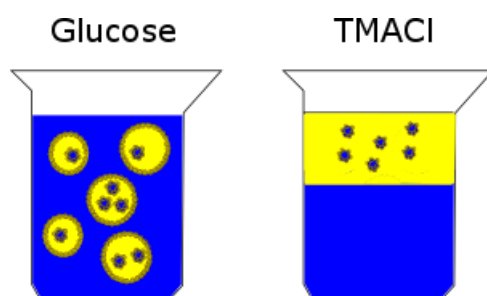


Figure 9.1: Schematic representation of the systems prepared by mixing an oil phase containing 2.5 wt% PGPR with a water phase containing 0.2M glucose or 0.1M TMACI after one hour at room temperature.

It therefore appears that the o/w emulsifying capacity of PGPR is higher if no ions are added to the water phase. A possible explanation might be the presence of charged impurities (such as fatty acids) in PGPR and in the oil phase, which aid in stabilizing the o/w interface. In that case, the addition of a salt would indeed decrease the repulsion between the (slightly) charged o/w interfaces. In order to check the validity of this hypothesis, a zeta-potential measurement should be conducted to determine the surface charge of the PGPR-stabilized o/w interface.

9.3.2 Light microscopy

As shown in Figure 9.2, light microscopy revealed that the produced emulsions were w/o/w type double emulsions. Hereby, water might have been encapsulated solely due to the shear which was used to produce the emulsion. However, Bahtz et al. (2016) also described the

spontaneous formation of water droplets in an oil phase containing PGPR. This effect might also (partly) explain the presence of water droplets inside the oil droplets. However, because of the high Laplace pressure inside water droplets, an osmotically active compound has to be present in the water droplets in order to avoid release of the water. In this context, it should be noted that the permeability of the oil phase is a lot higher for glucose than for a salt (Herzi et al., 2012). In fact, TMACl can be considered to be practically insoluble in the oil phase (Chapter 2). Due to the fact that glucose diffuses much faster through the oil phase, spontaneous formation of water droplets might be expected to contribute more to the water content of the oil phase as compared to when a salt is used. The water droplets can form around glucose molecules or the glucose molecules can reach the spontaneously forming water droplets through diffusion.

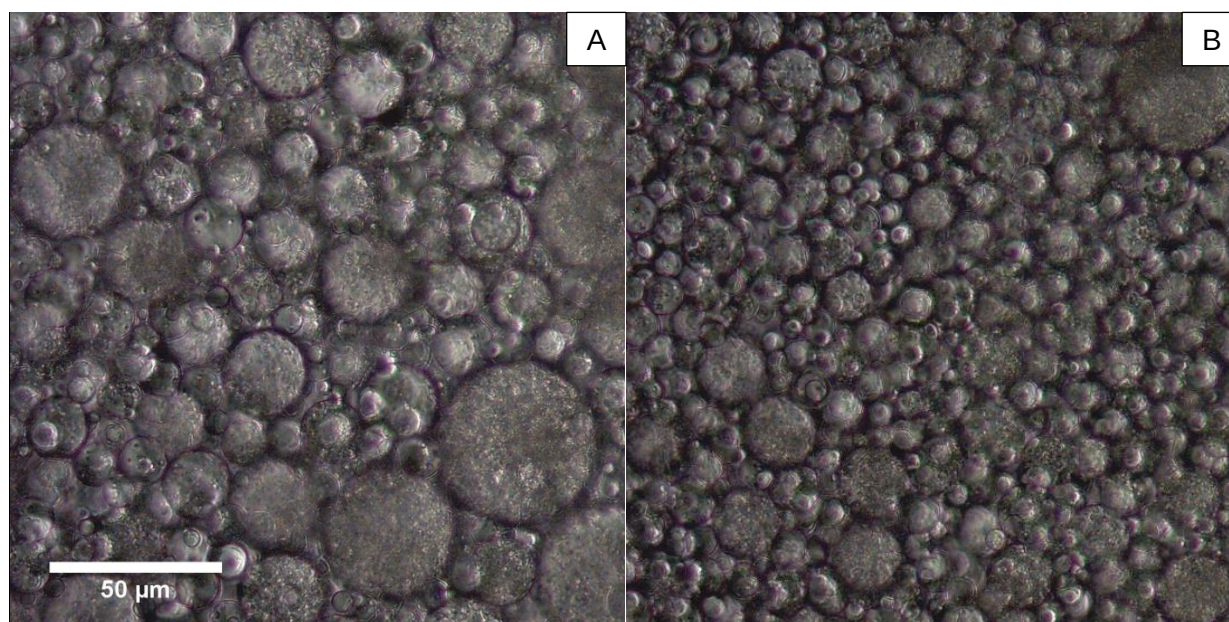


Figure 9.2: Pictures of the undiluted emulsions containing 2.5 wt% PGPR in the oil phase and 0.2M glucose in the aqueous phase in the absence (A) and presence (B) of 0.2 wt% CMC in the aqueous phase obtained from light microscopy using a 40x objective.

9.3.3 Enclosed water volume fraction

The enclosed water volume fraction was determined using analytical photocentrifugation. One day after preparation, the enclosed water volume fraction was equal to $5.3 \pm 0.6\%$ and $7.5 \pm 0.6\%$ for the emulsions with and without CMC, respectively. This means that, considering the fact that the emulsion contains about 52.5 volume% of oil, about 9 to 12.5 % of the volume of the droplets consisted of water (Figure 9.3).

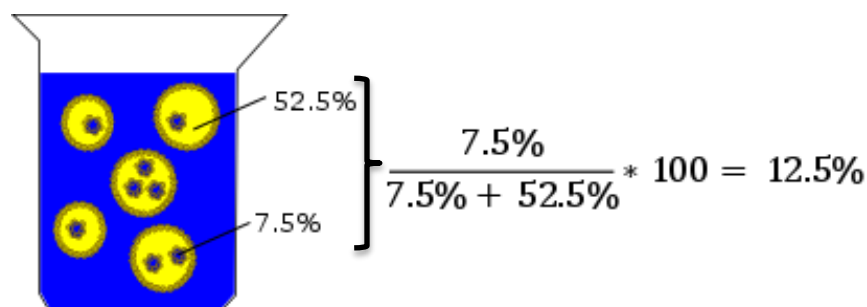


Figure 9.3: Calculation of the volume fraction of water in the double emulsion droplets in case a water phase with 0.2M glucose without CMC was used.

As already mentioned, an oil layer formed on top of the emulsions during centrifugation which shows that the emulsions, specifically the o/w interfaces, were not stable towards coalescence under the pressure which was caused by the centrifugal field. In fact, centrifugal compression has already been exploited as analytical technique to estimate the coalescence stability of o/w emulsions (Cheetangdee et al., 2011; Tcholakova et al., 2002).

9.3.4 Double emulsion droplet size

The volume weighted average droplet size (D_{43}) of the emulsions with and without CMC was equal to 14.7 ± 0.2 and 26.4 ± 0.4 μm , respectively. The addition of CMC to the water phase causes an increase of the viscosity of the water phase, due to which the viscosity ratio between the dispersed and continuous phase is decreased. It is well known that a decrease in the ratio of the dispersed (η_D) and continuous phase viscosity (η_C) to a value between 0.05 and 5 favors the formation of smaller droplets (McClements and Rao, 2011). According to the Taylor curve, a minimum is reached at a η_D/η_C of 1 to 3 depending on the shearing mode (Iancu et al., 2009). It should also be noted that, even though a moderate production process was used, relatively small double emulsion droplets were obtained. Also, more surprisingly, PGPR, which is categorized as typical w/o emulsifier, could stabilize the o/w interface of these small oil droplets.

9.4 Conclusions

Using a water phase containing 0.1M of the salt TMACI and an oil phase containing PGPR, an unstable w/o/w emulsion was produced which separated into a w/o emulsion layer floating on top of an aqueous serum layer within minutes.

However, when the water phase contained 0.2M glucose, a stable w/o/w emulsion was produced in one step. It thus appeared that PGPR was a better o/w interface stabilizer in the presence of glucose as compared to in the presence of a salt. This effect might be due to

charged impurities (e.g. fatty acids) in the PGPR and/or in the used oil phase which might act as emulsifiers at the o/w interface.

The produced water-continuous emulsions were no conventional o/w emulsions but double w/o/w type emulsions. Interestingly, the results shown in this chapter indicated that it was possible to produce double emulsions with a relatively small droplet size which contain a considerable amount of enclosed water in one step. It should be emphasized that this chapter presents preliminary observations and further research is needed to determine the storage stability of these double emulsions as well as the parameters which determine the enclosed water volume fraction. Moreover, the influence of the PGPR-concentration should be examined.

Chapter 10: Conclusions and future perspectives

The research and industrial interest towards w/o/w type double emulsions is high and increasing. Indeed, there are a lot of possible applications for this type of double emulsions. Due to the replacement of part of the oil phase by water, they can be used to prepare o/w type food products with a lower fat content. Moreover, they can be used to encapsulate hydrophilic as well as lipophilic ingredients in order to protect them from the environment or to mask their unpleasant taste. Finally, w/o/w emulsions can also be used to separate two water-soluble but incompatible substances in both water phases.

Our research work contributed to the characterization and production of both w/o and w/o/w emulsions. In this work and most of the work published by others, double emulsions are produced in a two-step process in which a w/o/w emulsion is prepared from a previously prepared liquid w/o emulsion. Therefore, also the characterization and production of w/o emulsions was of major interest.

The first part of this work concerned the characterization of w/o (Chapter 2-4) and w/o/w emulsions (Chapter 5). Chapter 2 and 3 both focused on the characterization of the water droplet size distribution of liquid w/o emulsions. These chapters showed that, depending on the water droplet size, different techniques should be used in order to determine the water droplet size distribution of w/o emulsions. Moreover, both chapters indicated that it should be kept in mind that part of the water, used to prepare the w/o emulsion, in fact dissolves into the continuous oil phase. This fraction depends on the temperature, the droplet size distribution and the properties of the continuous phase. Also, as shown using DLS in chapter 3, it should not be forgotten that the water which dissolves into the continuous oil phase, can evaporate which further stimulates water droplet dissolution.

In chapter 2, it was shown that PFG-NMR water diffusometry was not capable of measuring the water droplet size from a certain (sub)micron size downwards. Due to the increasing solubility of water droplets with decreasing droplet size (Kelvin effect), the contribution of diffusion between droplets (through the oil phase) to the total diffusion signal of the water molecules, increased with decreasing droplet size. Therefore, due to the measurement of free diffusion of water molecules between water droplets instead of hindered diffusion within droplets, PFG-NMR water diffusometry could not be used to determine the droplet size distribution from a certain size downwards. In practice, when an average droplet size of a few micrometer (in our case about 3 μm) is measured using PFG-NMR water diffusometry, it should be realized that the lower boundary of this technique is reached and the droplet size has to be determined using an alternative technique. Chapter 2 also showed that T_2 -relaxometry, high-resolution PFG-NMR

diffusometry and DLS are alternative techniques which can be used to determine the droplet size of these (sub)micron sized w/o emulsions.

If qualitative results are sufficient, T_2 -relaxometry can readily be used: this technique, which can be performed on a low-resolution NMR-device, can detect a droplet size difference (in fact a difference in specific surface area) between differently sized (sub)micron w/o emulsions.

If qualitative results do not suffice, high-resolution PFG-NMR diffusometry and DLS can be used to determine the droplet size distribution of (sub)micron sized w/o emulsions. Using high-resolution PFG-NMR diffusometry, it is possible to follow the diffusion behavior of an oil-insoluble marker. However, the minimum measureable droplet size is limited by the maximum magnetic gradient strength of the device, which mostly limits this technique to droplets with a size of about 1 μm . Moreover, a marker has to be added to the water phase prior to production of the w/o emulsion. Therefore, this technique cannot be used to determine the droplet size distribution of an existing w/o emulsion.

If the droplet size of an existing (sub)micron sized w/o emulsion has to be determined, DLS can be used. As shown in chapter 3, the measurement parameters have to be carefully selected as the obtained results are highly dependent on the used degree of dilution of the water droplets and on the used dilution liquid. It was shown that dilution of the w/o emulsions in 1-bromododecane led to robust results as the water solubility in this phase appeared to be much lower than in sunflower oil. Because interaction effects between the water droplets lead to the measurement of a higher apparent diameter, the used concentration should be as low as possible. Typically a concentration of about 0.01% water in the continuous phase gave results which were no longer strongly influenced by interactions between the droplets. If a more exact value for the droplet size is required, measurement of this parameter at different degrees of dilution followed by extrapolation to infinite dilution is a possibility. No lower boundary for the measurable droplet size was found. However, it should be noted that the droplets have to be small enough in order to avoid too long measurement times.

Finally, it can be noted that it should also be possible to obtain a water droplet size distribution using T_2 -relaxometry. However, the surface relaxivity parameter, which links the droplet size to the relaxation time distribution, is low for PGPR-stabilized w/o emulsions. Future work should optimize this NMR-based technique in order to characterize (sub)micron sized edible w/o emulsions. Optimizing the measurement parameters, finding a paramagnetic compound which increases the surface relaxivity or combining T_1 - with T_2 -relaxometry might aid in using

relaxometry in order to characterize the droplet size distribution. For samples containing water droplets which can be characterized using both low-resolution NMR-techniques (relaxometry and PFG-NMR water diffusometry), it should be possible to measure the droplet size without prior knowledge of the surface relaxivity parameter and without assumptions on the droplet size distribution.

Chapter 4 describes methods which enable the estimation of the residual PGPR-concentration in the oil phase of w/o emulsions. If the equilibrium PGPR-concentration is known, the processing procedure and/or used PGPR-concentration can be optimized. Indeed, in order to use w/o/w emulsions in food products, PGPR should be avoided or at least limited. To be able to select the minimal PGPR-concentration, the residual concentration of PGPR in the oil phase of the w/o emulsion should be known. It was shown that it is possible to estimate the residual PGPR-concentration in the oil phase using either ^1H -NMR or viscometry after separation of the w/o emulsions using ultracentrifugation. Both techniques hereby led to similar results. (Sub)micron sized w/o emulsions, similar to the ones prepared in chapter 2 and 3, were used in these experiments and a decreasing residual PGPR-concentration in the oil phase, with increasing shear intensity during production, was measured. This observation therefore again indicated a droplet size distribution difference between these samples. Indeed, increasing the specific surface area (linked to the D_{32}), increases the amount of PGPR which adsorbs at the surface and therefore decreases the residual PGPR-concentration in the oil phase. The advantage of ^1H -NMR is the fact that only a very limited amount of oil phase is required. However, viscometry requires a less expensive and more simple device. Both aforementioned techniques relied on separation of the aqueous and the oil phase. It should be kept in mind that the separation process should not influence the residual PGPR-concentration. For example, coalescence of water droplets due to centrifugation could lead to erroneous results.

In chapter 5, a simple and straightforward method was developed in order to measure the enclosed water volume fraction of w/o/w emulsions. Hereby, two simple principles were taken into consideration: the used oil volume is known and the double emulsion droplets have a lower density than the external water phase. Therefore, the double emulsion droplets can be separated from the external water phase by centrifugation. The cream layer hereby mainly consists of the double emulsion droplets which themselves consist of oil, of which the volume is known, and enclosed water, from which the volume fraction can therefore be calculated. It was shown that measuring the cream volume during rather than after centrifugation led to more accurate results as the amount of interstitial water is lower when the measurement is conducted

during centrifugation. This newly developed method was used in a lot of the subsequent chapters concerning the formulation of w/o/w emulsions (Chapter 7-9).

The second part of this manuscript was about the optimization of the composition and production process of w/o and w/o/w emulsions (Chapter 6-9). First, it was tried whether gelation of the water droplets in the w/o emulsion could make them more resistant towards the second emulsification step of the w/o/w emulsion production process (Chapter 6). Hereby, T_2 -relaxometry was used to prove the fact that the encapsulated droplets were indeed gelled. It was also shown that in case the T_1 - and T_2 -relaxation time of the enclosed and external water phase is different, care should be taken when using PFG-NMR water diffusometry to determine the enclosed water volume fraction. Indeed, a factor which corrects for the decrease of the signal due to relaxation has to be taken into account. This observation again shows that the use of analytical photocentrifugation in order to determine the enclosed water volume fraction is an interesting alternative for PFG-NMR water diffusometry.

Afterwards, it was shown that gelation indeed led to the retention of a higher enclosed water volume fraction when double emulsions were prepared under the same conditions. Therefore, it was shown that the resistance towards shear (which frequently occurs during industrial production processes) was increased when the enclosed water phase of the double emulsion was gelled. However, the sensitivity towards hypotonic dilution was also increased: droplet-globule (external) coalescence occurred at a lower maximal degree of swelling in case the enclosed water droplets were gelled.

In the two subsequent chapters (Chapter 7 and 8), it was tested if PGPR could be replaced by an alternative low-HLB emulsifier. Hereby, a low-HLB sucrose ester, ammonium phosphatide and lecithin with a varying composition, were tested. Both chapters led to the conclusion that none of the tested alternatives were as performant as PGPR. Nonetheless, it was shown that it was possible to obtain stable w/o and w/o/w emulsions using these low-HLB emulsifiers. When ammonium phosphatide or lecithin were used as low-HLB emulsifier, careful selection of the high-HLB emulsifier was required. Hereby, the use of Na-caseinate as high-HLB emulsifier led to the release of almost all of the enclosed water (thus, the formation of an o/w emulsion), whereas when WPI was used, w/o/w emulsions were prepared with a high enclosed water volume fraction. Our results also pointed towards the importance of the interaction effects between low- and high-HLB emulsifiers at the o/w interface which surely should be examined in future work. Also a combination of the principles used in chapter 6 with chapter 7 and 8 i.e. gelation of the water droplets in the w/o emulsions, stabilized using alternative low-HLB emulsifiers, might be a

promising path towards increasing the w/o and therefore w/o/w emulsion stability when PGPR is replaced. Finally, chapter 8 also showed the importance of the used oil phase on the stability of lecithin stabilized w/o and w/o/w emulsions: when a medium chain triglyceride oil was used, w/o emulsions could be prepared using either of the lecithin samples whereas when a long chain triglyceride (sunflower) oil was used, a w/o emulsion could only be obtained with PC-depleted lecithin.

In the final experimental chapter (Chapter 9), it was shown that w/o/w emulsions could be produced in one step. This might be the most cost-effective way to incorporate water into the dispersed oil phase of o/w type emulsions. However, this approach cannot be used in case sensitive ingredients are to be encapsulated: as only one water phase is used during production of the w/o/w emulsion, the sensitive ingredient will also largely be present in the external water phase.

Summary

This manuscript consists of a general introduction on the production and characterization of w/o and w/o/w type emulsions (**Chapter 1**), followed by two parts which are related. The first part was focused on the characterization of w/o and w/o/w emulsions (**Chapters 2-5**) while the second part was focused on the production process and composition of w/o and w/o/w emulsions (**Chapters 6-9**).

In **Chapter 2**, liquid w/o emulsions were produced using PGPR as the low-HLB emulsifier. PFG-NMR water diffusometry was used to follow the diffusion behavior of water molecules in the w/o emulsions. It was shown that when the droplet size of the water droplets decreased, the diffusion behavior gradually changed from restricted diffusion within towards free diffusion between the water droplets. Using high-resolution PFG-NMR diffusometry, the diffusion of the oil-insoluble marker TMACI and water could be followed separately. It was shown that the restricted diffusion character of TMACI increased with decreasing droplet size whereas this was not the case for water. This effect was ascribed to the increasing water solubility with decreasing droplet size (Kelvin effect) and it was shown that most of the free diffusion could probably be attributed to water bound to PGPR. Finally, it was shown that both dynamic light scattering and T_2 -relaxometry were capable of detecting a difference in size distribution which was not detectable using PFG-NMR water diffusometry.

Due to the fact that DLS was capable of determining a difference in droplet size distribution between the (sub)micron w/o emulsions which were produced in **Chapter 2**, a more profound examination of the used measurement parameters was conducted in **Chapter 3**. First, it was shown that care should be taken when diluting the w/o emulsions. If the continuous phase was not saturated with water prior to dilution of the sample, part of the water droplets dissolved. Also, due to interactions between the water droplets, the measured droplet size was highly dependent on the used water concentration. Finally, it was shown that care should be taken when selecting an appropriate dilution liquid, as the interaction effects varied as a function of the used dilution liquid. Moreover, the water solubility also depended on the used dilution liquid. To summarize, it was shown that dilution in 1-bromododecane led to the most robust results: the water solubility in this phase appeared to be much lower than in sunflower oil and the interaction effects between the droplets were similar. Moreover, the viscosity of 1-bromododecane is lower as compared to sunflower oil (decreasing the measurement time) and the density is similar to the density of water, which decreases the risk of sedimentation during the measurement.

In **Chapter 4**, two techniques were developed which enabled the estimation of the residual PGPR-concentration in the oil phase of a w/o emulsion. Hereby, the water and oil phase were separated using ultracentrifugation. Afterwards, the PGPR-content in the oil phase was estimated using ^1H -NMR and viscometry. By comparing the height of the NMR-peak specific for the proton next to the polymerized OH-groups of polyricinoleic acid to the height of this peak in a sample with a known concentration of PGPR, the PGPR-concentration in the oil phase could be estimated. In order to use the viscosity of the oil phase, a standard curve was constructed which linked the viscosity of the oil phase to its PGPR-content. Both aforementioned techniques led to similar results. However, it should be noted that PGPR is a mixture of compounds with a varying degree of polymerization. Both techniques therefore only give an estimation of the residual PGPR-content.

In the final chapter concerning characterization (**Chapter 5**), a simple and straightforward method to measure the enclosed water volume fraction of double emulsions was developed. Using centrifugation, the double emulsion droplets and external water phase were separated. Because analytical photocentrifugation was used, it was possible to determine the height of these phases during the centrifugation process. Considering the used volume of oil, it was therefore possible to estimate the enclosed water volume fraction: the cream-layer consists mainly of double emulsion droplets which themselves consist of oil and the enclosed water phase.

In **Chapter 6**, it was tested whether gelation of the water phase of w/o emulsions, before production of the corresponding w/o/w emulsion, led to an increase of the stability of the w/o/w emulsions towards shear stresses and osmotic pressure gradients. Hereby, it was shown that w/o/w emulsions with a gelled enclosed water phase had a higher resistance to shear stresses as compared to their non-gelled counterparts. However, the maximal increase in double emulsion diameter due to osmotic swelling was much lower in case the enclosed water droplets were gelled. This is probably due to the occurrence of external coalescence of the water droplets at a lower degree of swelling when the droplets are gelled.

Ammonium phosphatide and a low-HLB sucrose ester (O-170) were tested as alternatives for PGPR in **Chapter 7**. It was shown that it was possible to produce stable w/o emulsions using either of these emulsifiers. However, the obtained w/o emulsions were always highly flocculated. When the w/o emulsion, stabilized using ammonium phosphatide, was used to prepare the corresponding w/o/w emulsion, the used high-HLB emulsifier had a huge impact on the enclosed water volume. When Na-caseinate was used, almost all of the enclosed water volume was

released whereas the use of WPI led to w/o/w emulsions with a high enclosed water volume fraction. The double emulsion which was prepared using a low-HLB sucrose ester was less sensitive towards the used high-HLB emulsifier. Finally, it was also tested whether dilution of the double emulsions in different liquids led to release of an enclosed compound. Hypotonic dilution led to partial release of the enclosed compound, which did not occur when PGPR was used. Moreover, dilution of the ammonium phosphatide- and WPI-stabilized emulsion into an isotonic solution containing Na-caseinate also led to external coalescence of enclosed droplets with rapid release of the enclosed compound.

Chapter 8 is an extension to **Chapter 7**, whereby three different lecithin samples (soybean, sunflower and PC-depleted lecithin) were tested as low-HLB emulsifiers. In this regard, ammonium phosphatide can be seen as a model compound for PC-depleted lecithin and the results between these compounds indeed corresponded quite well. However, it was shown that it was possible to produce stable w/o emulsions using either of the lecithin samples when the oil phase was a medium chain triglyceride oil. In contrast, in case a long chain triglyceride oil phase was used, a stable w/o emulsion could only be produced with PC-depleted lecithin. It was again shown that, as with ammonium phosphatide, careful selection of the high-HLB emulsifier was necessary. Remarkably, the best results were obtained when carboxymethylcellulose was added to the external water phase as this compound cannot be considered to be a high-HLB emulsifier. However, CMC does increase the viscosity of the external water phase. Therefore, it appears that the most stable w/o/w emulsion was obtained when only one (PC-depleted lecithin) surface active compound was added to the mixture.

Finally, **Chapter 9** showed the possibility of producing w/o/w emulsions in one step and using only one (low-HLB) emulsifier. It was also shown that this was only possible in case glucose and not a salt was added as the osmotic agent in the water phase.

Samenvatting

Dit manuscript bestaat uit een algemene inleiding rond de productie en karakterisatie van w/o en w/o/w emulsies (**Hoofdstuk 1**) gevolgd door twee aan elkaar gerelateerde delen. Het eerste deel gaat over de karakterisatie van w/o en w/o/w emulsies (**Hoofdstuk 2-5**) terwijl het tweede deel over het productieproces en de compositie van w/o en w/o/w emulsies gaat (**Hoofdstuk 6-9**).

In **Hoofdstuk 2** werden vloeibare w/o emulsies geproduceerd gebruik makende van PGPR als de lage-HLB emulgator. PFG-NMR water diffusometrie werd gebruikt om het diffusiegedrag van de watermoleculen in de w/o emulsies te volgen. Er werd aangetoond dat, wanneer de waterdruppels kleiner werden, het diffusiegedrag gradueel veranderde van gehinderde diffusie in de waterdruppels naar vrije diffusie tussen de waterdruppels. Via hoge-resolutie PFG-NMR diffusometrie, waarmee het diffusiegedrag van zowel de olie-onoplosbare merker TMACI als van water afzonderlijk gevolgd kon worden, werd aangetoond dat de diffusie van TMACI steeds meer gehinderd werd met afnemende druppelgrootte wat niet het geval was voor de watermoleculen. Dit effect werd toegeschreven aan de toenemende oplosbaarheid van water met afnemende waterdruppelgrootte (Kelvin effect). Er werd ook aangetoond dat het grootste deel van de vrije diffusie waarschijnlijk kan toegeschreven worden aan watermoleculen die gebonden zijn aan PGPR. Ten slotte werd aangetoond dat via dynamische licht verstrooiing en T_2 -relaxometrie wel een verschil in druppelgroottedistributie kon gedetecteerd worden wat via PFG-NMR water diffusometrie niet mogelijk was.

Omdat het via DLS wel mogelijk was om een verschillende druppelgroottedistributie te meten voor de (sub)micron w/o emulsies die geproduceerd werden in **Hoofdstuk 2**, werd in **Hoofdstuk 3** een meer diepgaand onderzoek van de gebruikte parameters uitgevoerd. Eerst werd aangetoond dat verdunnen van de w/o emulsies zorgvuldig dient te gebeuren. Indien de continue fase, waarin de w/o emulsie verdunt dient te worden, niet gesatureerd werd met water, dan loste een deel van de waterdruppels op in deze continue fase. Ook was het zo dat, omwille van interacties tussen de waterdruppels, de gemeten druppelgrootte afhankelijk was van de gebruikte verdunningsgraad. Ten slotte werd ook aangetoond dat de keuze van de verdunningsvloeistof zorgvuldig moest gebeuren omdat de interacties tussen druppels varieerden als functie van de gebruikte verdunningsvloeistof. Samenvattend kan gesteld worden dat verdunning in 1-bromododecaan de meest robuuste resultaten gaf. De wateroplosbaarheid in deze fase bleek geringer te zijn dan in zonnebloemolie. Bovendien waren de interactie-

effecten tussen waterdruppels in 1-bromododecaan gelijkaardig aan de effecten in zonnebloemolie. Ten slotte was de viscositeit lager dan die van zonnebloemolie (wat de meettijd verkort) en de densiteit gelijkaardig aan die van water wat het risico op sedimentatie van waterdruppels tijdens de meting reduceert.

In **Hoofdstuk 4** werden twee technieken ontwikkeld die het mogelijk maakten om de residuele PGPR-concentratie in de oliefase van een w/o emulsie te schatten. Hierbij werden de water- en de oliefase gescheiden via ultracentrifugatie waarna de PGPR-concentratie in de oliefase geschat werd via ^1H -NMR en viscometrie. Door de hoogte van de NMR-piek die specifiek is voor het proton naast de gepolymeriseerde OH-groepen van polyricinoleïnezuur te vergelijken met de hoogte van deze piek voor een staal met een gekende PGPR-concentratie, kon de PGPR-concentratie in de oliefase geschat worden. Om via de viscositeit van de oliefase de PGPR-concentratie te schatten, werd een standaardcurve opgesteld voor viscositeit van de oliefase als functie van de PGPR-concentratie. Beide technieken gaven gelijkaardige resultaten. Er dient hierbij opgemerkt te worden dat PGPR eigenlijk een mengsel is van componenten met een variërende polymerisatiegraad. Dit impliceert dat beide technieken slechts een schatting geven van de residuele PGPR-concentratie in de oliefase.

In het laatste hoofdstuk rond karakterisatie (**Hoofdstuk 5**) werd een eenvoudige en directe methode ontwikkeld om de fractie aan ingesloten water van dubbele emulsies te bepalen. Via centrifugatie werden de dubbele emulsie druppels en de externe waterfase van elkaar gescheiden. Omdat analytische fotocentrifugatie gebruikt werd, was het mogelijk om de hoogte van deze fases te volgen gedurende het centrifugatieproces. Omdat de roomlaag voornamelijk bestaat uit druppels dubbele emulsie, die zelf bestaan uit olie en ingesloten water en omdat het olievolume gekend is, was het mogelijk om de fractie aan ingesloten water te schatten.

In **Hoofdstuk 6** werd getest of gatering van de waterfase van de w/o emulsie, voor de productie van de corresponderende w/o/w emulsie, tot een toename van de stabiliteit van deze w/o/w emulsies ten opzichte van afschuifkrachten en osmotische drukgradiënten zorgde. Het werd aangetoond dat de w/o/w emulsies met een gegeleerde ingesloten waterfase minder gevoelig waren voor afschuifkrachten dan wanneer de ingesloten waterfase niet gegeleerd was. Daarentegen was de maximale toename van de diameter van de dubbele emulsiedruppels in een hypotone waterfase veel lager wanneer de ingesloten druppels gegeleerd waren. Dit is waarschijnlijk omwille van externe coalescentie van ingesloten druppels bij een lagere zwellingsgraad wanneer de ingesloten druppels gegeleerd zijn.

In **Hoofdstuk 7** werden ammonium fosfatide en een lage-HLB sucrose-ester (O-170) getest als alternatieven voor PGPR. Er werd aangetoond dat het mogelijk was om stabiele w/o emulsies te maken met elk van deze emulgatoren. De geproduceerde w/o emulsies waren echter steeds sterk geflocculeerd. Wanneer de w/o emulsie, met ammonium fosfatide als lage-HLB emulgator, gebruikt werd om de corresponderende w/o/w emulsie te produceren, had de gebruikte hoge-HLB emulgator een grote impact op de bekomen fractie aan ingesloten water. Wanneer gebruik gemaakt werd van Na-caseïnaat werd bijna alle ingesloten water vrijgegeven. Wanneer daarentegen WPI gebruikt werd, werden w/o/w emulsies bekomen met een hoge ingesloten waterfractie. De fractie van ingesloten water in de dubbele emulsie die geproduceerd werd met de lage-HLB sucrose-ester als emulgator was minder gevoelig voor de gebruikte hoge-HLB emulgator. Ten slotte werd ook getest of verdunning van de dubbele emulsies in verschillende verdunningsvloeistoffen tot de vrijgave leidde van een ingesloten component. Hypotone verdunning leidde tot gedeeltelijke vrijgave van de ingesloten component, wat niet voorkwam wanneer PGPR werd gebruikt als lage-HLB emulgator. Bovendien werd de ingesloten component snel vrijgesteld wanneer de dubbele emulsie die gestabiliseerd werd met ammonium fosfatide en WPI, verdund werd in een isotone oplossing die Na-caseïnaat bevatte.

Hoofdstuk 8 vormt een uitbreiding bij **Hoofdstuk 7** waarbij drie verschillende lecithine stalen (soja, zonnebloem en PC-verlaagde lecithine) getest werden als lage-HLB emulgatoren. Hierbij kan ammonium fosfatide gezien worden als een model voor PC-verlaagde lecithine. De resultaten van deze verbindingen kwamen inderdaad goed overeen. Wanneer een middellange keten triglyceride olie gebruikt werd, kon met elk van de lecithine stalen een stabiele w/o emulsie geproduceerd worden. Daartegenover staat dat, wanneer een langketen triglyceride olie gebruikt werd, er enkel een stabiele w/o emulsie kon geproduceerd worden met de PC-verlaagde lecithine. Ook werd opnieuw aangetoond dat, zoals met ammonium fosfatide, de hoge-HLB emulgator zorgvuldig moest worden geselecteerd. Merkwaardig genoeg werden de beste resultaten bekomen wanneer carboxymethylcellulose toegevoegd werd aan de externe waterfase. Deze verbinding kan niet als een hoge-HLB emulgator beschouwd worden maar verhoogt wel de viscositeit van de externe waterfase. Het lijkt er daarom op dat de meest stabiele w/o/w emulsie bekomen werd wanneer slechts één (PC-verlaagde lecithine) oppervlak actieve component werd toegevoegd aan de emulsie.

Ten slotte werd in **Hoofdstuk 9** aangetoond dat het mogelijk is om w/o/w emulsies te produceren in één stap en met slechts één (lage-HLB) emulgator. Er werd ook aangetoond dat

dit slechts mogelijk was wanneer glucose gebruikt werd als osmotisch agens in de waterfase in plaats van een zout.

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Curriculum Vitae

Personal information

Name: Mathieu Balcaen
Date of birth: 05-11-1989
Address: Pijkstraat 35, 8790 Waregem
Telephone: +32 472 80 99 80
E-mail: mathieu_balcaen@hotmail.com

Education

2012-2019: PhD in Applied Biological Sciences, Faculty of Bioscience Engineering, Department of Green Chemistry, Particle and Interfacial Technology Group (PalnT), Ghent University, Belgium

2010-2012: Master of Science in Bio-Engineering: Chemistry and Bioprocess Technology, Ghent University, Belgium
Graduated with greatest distinction

2007-2010: Bachelor of Science in Bio-Engineering: Chemistry and Food Technology, Ghent University, Belgium
Graduated with great distinction

International publications with peer review

Vermeir, L., **Balcaen, M.**, Sabatino, P., Dewettinck, K., Van der Meeren, P., 2014. Influence of molecular exchange on the enclosed water volume fraction of W/O/W double emulsions as determined by low-resolution NMR diffusometry and T₂-relaxometry. Colloids Surf. Physicochem. Eng. Asp. 456, 129–138. <https://doi.org/10.1016/j.colsurfa.2014.05.022>

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Papers in conference proceedings

Vermeir, L., **Balcaen, M.**, Dewettinck, K., Van der Meeren, P., 2014. Water droplet size analysis of emulsions by pfg-NMR diffusometry. *Proceedings of Particulate Systems Analysis. PSA 2014*, Manchester, UK.

Vandewiele, S., **Balcaen, M.**, Brans, T., Beunis, F., Van der Meeren, P., Neyts, K., 2015. Optical tweezers: A tool for the stability investigation of double emulsions. *Proceedings of Optics in the Life Sciences, OSA 2015*, Vancouver, Canada.

Conference abstracts

- International Workshop Dispersion Analysis & Material Testing 2014, Berlin, Germany (Oral presentation)
- European Colloid and Interface Society (ECIS) Conference 2015, Bordeaux, France (Poster presentation)
- Food Colloids Conference, 2016, Wageningen, The Netherlands (Poster presentation)
- Delivery of Functionality in complex food systems (DOF) 2017, Auckland, New-Zealand (Oral presentation)

Master thesis supervision

Yessica Reckziegel Kressin, 2014-2015: Characterization and comparison of the functionality of fractionated lecithin from different sources

Dias Mandala Nurhutama, 2015-2016: pH effects in the application of WOW-type double emulsions as liquid membranes for removal of trace organic compounds from wastewater streams

Josephine Van Den Bossche, 2015-2016: Bereiding van laag-calorische koude sauzen met behulp van W/O/W technologie

Maarten Mattheeuws, 2016-2017: Invloed van PGPR concentratie en productiecondities op de stabiliteit van W/O en W/O/W emulsies

Ricardo Uribe Alvarez, 2016-2017: Encapsulation and release of amino acids in double (W/O/W) emulsion

Bachelor thesis supervision

Andreas Simoens, Eline Raeman, Laurens Desot & Robbert Joye, 2016-2017: Polyglycerol polyricinoleaat (PGPR): Ideale emulgator voor light minarines kampt met slecht imago

Internship supervision

Cecilia Callizo, 2013-2014: Preparation and characterization of low-fat dressings based on water-in-oil-in-water emulsion technology

Paolo Demicheli, 2017-2018: Emulsifying and stabilizing properties of whey protein : pectin conjugates prepared by dry heat treatment

Teaching experience

Colloïd- en oppervlaktechemie
Practical and computer exercises

Proceskunde 2
Computer exercises and grading

Reactiekinetiek en reactoren
Computer exercises and grading

Food Colloids
Practical and computer exercises

Review activity

Manuscript review for Food Research International (Elsevier)

Manuscript review for Journal of Colloid and Interface Science (Elsevier)

Manuscript review for Food Hydrocolloids (Elsevier)

Manuscript review for Comprehensive Reviews in Food Science and Food Safety (Wiley)