Formulation and characterization of W/O nano-dispersions for bioactive delivery applications

To the guardian angels of my life:
My parents

Örebro Studies in Chemistry 16

MARIA D. CHATZIDAKI

Formulation and characterization of W/O nano-dispersions for bioactive delivery applications

ΕΘΝΙΚΟ ΙΔΡΥΜΑ ΕΡΕΥΝΩΝ
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Abstract

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The main objective of this study was the formulation of food-grade water-in-oil (W/O) nano-dispersions based mainly on medium or long-chain triglycerides. Two types of dispersions were formulated and structurally compared, namely emulsions and microemulsions. The systems were used as matrices for encapsulating targeted bioactive molecules with specific characteristics such as antioxidants or peptides.

The structural characterization of the formulated systems was investigated using techniques such as Electron Paramagnetic Resonance (EPR) spectroscopy, Dynamic Light Scattering (DLS), Cryogenic Transmission Electron Microscopy (Cryo-TEM) and Small Angle X-ray Scattering (SAXS). The existence of swollen inverse micelles was revealed for the case of microemulsions whereas larger droplets still at the nano-scale were observed for the case of emulsions. Structural differences in the presence of the bioactive molecules or induced by the alteration of components were also observed.

In order to study the efficacy of the formulations, the proposed loaded systems were assessed either using EPR spectroscopy or Well Diffusion Assay (WDA) depending on the bioactive molecule. It was found that the encapsulated molecules retained their claimed characteristics when encapsulated to the proposed matrices.

Finally, some of the formulated dispersions were investigated for their behavior under gastrointestinal (GI) conditions. A two-step digestion model using recombinant Dog Gastric Lipase (rDGL) and Porcine Pancreatic Lipase (PPL) was proposed to simulate lipid hydrolysis in humans. The studies revealed significant decrease of the rDGL specific activity in the presence of the microemulsion while in the presence of lower percent of surfactants (case of emulsion) no alterations were observed.

Keywords: nano-dispersions; encapsulation; food; DLS; EPR; Cryo-TEM; SAXS; pH-stat; digestion; antioxidants; gastric lipase, pancreatic lipase.

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List of papers

This thesis is based on the following papers, which hereafter will be refered to by their arithmetical numbers.

Paper 1 Amadei D., Chatzidaki M. D., Devienne J., Monteil J., Cansell M., Xenakis A., Leal-Calderon F. 2014. "*Low shear-rate process to obtain transparent W/O fine emulsions as functional foods*". Food Res. Int. 62, 533-540.

Paper 2 Chatzidaki M. D., Mitsou E., Yaghmur A., Xenakis A., Papadimitriou V. 2015. "*Formulation and characterization of food grade microemulsions as carriers of natural phenolic antioxidants*" Colloids Surf. A. 483, 130-136.

Paper 3 Chatzidaki M. D., Arik N., Monteil J., Papadimitriou V., Leal-Calderon F., Xenakis A. 2016 "*Microemulsion versus emulsion as effective carrier of hydroxytyrosol*" Colloids and Surfaces B: Biointerfaces. 137, 146-151.

Paper 4 Chatzidaki M. D., Papadimitriou K., Alexandraki V., Tsirvouli E., Chakim Z., Ghazal A., Mortensen K., Yaghmur A., Salentinig S., Papadimitriou V., Tsakalidou E., Xenakis A. "*Microemulsions as potential carriers of nisin: effect of composition on the structure and efficacy*" (Submitted)

Paper 5 Chatzidaki M. D., Mateos E., Leal-Calderon F., Xenakis A., Carrière F. "*Water-in-oil microemulsions versus emulsions as carriers of hydroxytyrosol: An in vitro gastrointestinal lipolysis study using the pHstat technique*" (Submitted)

Patent 1 Papadimitriou K., Chatzidaki M. D., Alexandraki S., Papadimitriou V., Tsakalidou E., Xenakis A. 2015. "*Water-in-oil (W/O) microemulsions as carriers of bacteriocins for the antimicrobial protection of foods*". (Submitted to the Hellenic Industrial Property Organization, OBI – 2015100227/20-5-2015)

Patent 2 Chatzidaki M.D., Mitsou E., Theohari I., Papadimitriou V., Xenakis A. 2015. "*Edible microemulsions with encapsulated plant extracts as dressing type products*". (Submitted to the Hellenic Industrial Property Organization, OBI – 2015100228/20-5-2015)

Abbreviations and nomenclature

ABTS	2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)
AOT	bis-2-ethylhexylsulfphosu
Cryo-TEM	Cryogenic Transmission Electron Microscopy
CPP	Critical packing parameter
DGL	Dog gastric lipase
DLS	Dynamic Light Scattering
DMPO	5,5-dimethyl-pyrroline N-oxide
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
EPR	Electron Paramagnetic Resonance spectroscopy
FFA	free fatty acids
FuFoSE	Functional Food Science in Europe
GRAS	Generally recognised as safe
HGL	human gastric lipase
HLB	Hydrophilic-lipophilic balance
HPH	High pressure homogenisation
HPL	human pancreatic lipase
HT	Hydroxytyrosol
ILSI	International Life Science Institute
IOM/FNB	Institute of Medicine's Food and Nutrition Board
PGPR	Polyglycerol of polyricinoleic
PIC	Phase inversion composition
PIT	Phase inversion temperature
rDGL	recombinant dog gastric lipase
ROS	reactive oxygen species
OR	Ostwald ripening
O/W	Oil in water
SAXS	Small Angle X-ray Scattering
SGF	simulated gastric fluid
SIF	simulated intestinal fluids
TEAC	Trolox equivalents antioxidant capacity
USFDA	United States Food and Drug Administration
WDA	Well Diffusion Assay
W/O	Water in oil

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1 Introduction

During the last years, there has been an increasing interest for the formulation of biocompatible nano-dispersions capable of encapsulating bioactive molecules. Due to their unique functional and physicochemical properties, these colloidal systems could be applied in the food sector and others such as pharmaceutics.

The main objective of this study was the formulation food-grade water-in-oil (W/O) nano-dispersions based mainly on medium or long-chain triglycerides. The emulsifiers used for the development of these systems were inexpensive ingredients currently utilized in the food industry such as lecithin (E322), distilled monoglycerides (DMG) (E471) and polyglycerol polyricinoleate (PGPR) (E476).

Two types of dispersions are introduced and structurally compared as part of this study, namely emulsions and microemulsions. For the case of emulsions, a novel low-emulsification method is introduced for the formation of fine emulsions with droplet size at the nano-scale. On the other hand, for the case of microemulsion development, the phase behavior of the system has been captured with the aid of the construction of psedo-ternary phase diagrams. These diagrams provide information about the monophasic region in which the investigated L_2 phases (inverted type microemulsions) exist. Following, the formulated systems were used as matrices in order to encapsulate targeted bioactive molecules with specific characteristics. Within the frame of this work, hydrophilic substances of natural origin were used. Antioxidants of plant origin were successfully introduced in the lipid-based vehicles as well as a hydrophilic bacteriocin, nisin (E234) currently used as bio-preservative in food applications.

Following, structural characterization of the systems under investigation in the presence and absence of the hydrophilic bioactive molecules was applied. In this respect, various techniques such as Dynamic Light Scattering (DLS), Electron Paramagnetic Resonance (EPR) spectroscopy, Cryogenic Transmission Electron Microscopy (Cryo-TEM) and Small Angle X-ray Scattering (SAXS) were carried out to investigate the structural characteristics of the formulated nano-dispersions.

In order to study the efficacy of the formulations, the proposed loaded systems were assessed for the claimed properties of therein encapsulated molecules. More specifically, the systems containing antioxidants were assessed and compared for the ability of therein molecules to effectively scavenge a lipophilic radical of known concentration. This was accomplished by the measurement of the integrated intensity of the radical spectra using EPR spectroscopy. For the case of nisin, a Well Diffusion Assay (WDA) was used to investigate the

claimed antimicrobial effect of the encapsulated molecule on *Lactococcus lactis*. It should be noted that some of the formulations have been submitted for two patents, with application in the food industry as salad dressing products. The proposed products are claimed either for enhanced antimicrobial or antioxidant properties.

Finally, some of the formulated systems in the presence and absence of the bioactive molecule were investigated for their behavior under gastrointestinal (GI) conditions. In the first step, the lipolytic activities of recombinant Dog Gastric Lipase (rDGL) and Porcine Pancreatic Lipase (PPL) on these colloidal systems were examined at both fasting and fed conditions and compared with the oil alone. Then, a static two-step digestion model using rDGL for the gastric digestion and PPL for the duodenal digestion was tested using the pH-stat device.

2 Nano-dispersions

In this section we focus on colloidal dispersions that can be fabricated from water, oil and surfactants and have been proposed as delivery systems in a range of industrial applications. The most common colloidal dispersions with the above characteristics are microemulsions and emulsions.

Generally, water and oil are two immiscible fluids with a high surface tension of 30-60 mN/m eventually leading to phase separation. If these fluids are subjected to mechanical stirring an emulsion will be formed, that will be separated into two discrete phases post stirring.

An emulsion could be stabilized by the addition of surface active agents (surfactants). Eventually the formed emulsion will collapse due to thermodynamic parameters that will be analyzed below (see section 2.2).

Surfactants are amphiphilic molecules composed of a hydrophobic polar "head" with high affinity to water and a hydrophobic "tail" consisting of one or more alkyl chains (Myers, 2005). These molecules are oriented with the hydrophilic head towards water and hydrophobic tails towards oil.

2.1 Microemulsions

Microemulsions are defined as colloidal systems thermodynamically stable in the sense that the formation of micelles occurs spontaneously due to the fact that the formation is the thermodynamically favorable state (Prince, 2012). The first originally reported term for these systems was "oleopathic hydro-micelles" (Hoar and Schulman, 1943). The term "microemulsions" was introduced much later, in 1959, to describe a transparent solution consisting of water, oil, surfac-

tant and alcohol (Schulman et al., 1959). Today, the most widely accepted definition for microemulsions is that proposed by Danielson and Lindman. According to that definition, "a microemulsion is a system of water, oil and an amphiphile, which is a single optically isotropic and thermodynamically stable liquid solution" (Danielsson and Lindman, 1981).

Microemulsions are isotropic and macroscopically transparent liquids due to the fact that the droplets size is smaller than the wavelength of light. In molecular scale though, heterogeneities are found depending on their composition. Hence, there are three categories of microemulsions, namely oil-in-water (O/W), water-in-oil (W/O) and bicontinuous.

In the first case, oil droplets are dispersed in the water phase while surfactants are oriented with the non-polar tails facing the oil and polar heads facing the water, forming assemblies called "swollen micelles". In the second case, droplets of water are formed surrounded by a surfactants' monolayer and are dispersed in the non-polar continuous phase. These droplets are often called "reversed micelles". The last case of microemulsions consists of similar amounts of water and oil and a bicontinuous phase is present (Figure 1).

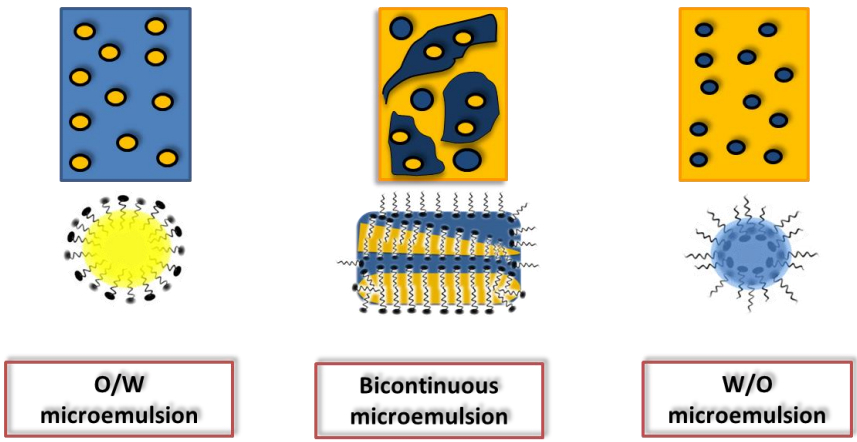

Figure 1: Different types of microemulsions

2.1.1 Surfactants

As mentioned above, the surfactants, or emulsifiers are amphiphiles that play a key role to the microemulsion formation by reducing the interfacial tension between oil and water. The presence of surfactants could cause a decrease of that interfacial tension to very small values at the range of 10^{-2} to 10^{-4} mN/m.

Sometimes, such a decrease of the interfacial tension is not feasible without the addition of small molecules such as alcohols or short chain polyols, called "co-surfactants".

On dispersal, surfactants generally self-associate into a variety of equilibrium states. When incorporated into immiscible mixtures, a variety of structures is possible namely, micellar, hexagonal, lamellar, reverse micellar and others (Lawrence and Rees, 2012).

Surfactants are classified depending on their nature to (i) non-ionic, (ii) zwitterionic and (iii) ionic. Some of the studied non-ionic surfactants are sucrose esters (Glatter et al., 2001) as well as monoglycerides of fatty acids (DMG) (Gulik-Krzywicki and Larsson, 1984). For the latter case, much attention has been paid to the self-assembled structures of pure molecules, namely monoolein (Qiu and Caffrey, 2000) or monolinolein (De Campo et al., 2004) in water. Phospholipids are the most widely used zwitterionic surfactants of soybean or egg origin, with diacylphosphatidylcholine being the most abundant constituent (Aboofazeli et al., 1994). One of the most widely investigated anionic surfactants is sodium bis-2-ethylhexylsulfphosuccinate (AOT), known to form spherical reversed micellar structures when dispersed in an oil (Bergenholtz et al., 1995). Generally for applications concerning drug or bioactive molecule delivery the selection of surfactants is very limited. For example, the utilization of ionic surfactants for such applications is limited because they have been reported to cause irritations in high concentrations (Sole et al., 2006). To this respect, Klein et al. proposed new surfactants with high water solubility based on choline, a substance of biological origin (Klein et al., 2008). The proposed amphiphiles display low toxicity levels to human cell lines. Also, good decomposition rate is reported, both inside the human body and in the environment, making it also a good candidate for environmental friendly applications (Klein et al., 2013).

There have been proposed in the literature some fairly empirical approached that affect the way the surfactant molecules aggregate. Critical packing parameter (CPP) is an approach relating the geometry of the surfactant molecule to favorable self-assembled structure via the equation:

$$CPP = \frac{v}{a*l} \qquad (2.1)$$

where, v is the hydrophobic molar volume of the surfactant, α the head group area and l the length of hydrophobic tail (Israelachvili, 1994). Hydrophilic-lipophilic balance (HLB) is another way to predict surfactants' aggregation proposed by Griffin in 1949 (Griffin, 1949). HLB value is a representation of

the relationship between the hydrophilic and hydrophobic part of the molecule. Hence, molecules with low HLB values tend to form W/O microemulsions while those with high HLB values tend to form O/W microemulsions. Figure 2 represents these two different approaches in respect to the predicted surfactants' self-assemblies. In some cases, surfactants are not able to reduce the interfacial tension in such extend to enable microemulsion formation as mentioned above. Alcohols or polyols are commonly used to further reduce the interfacial tension, whilst increasing the system's entropy by increasing the fluidity of the interface (Tenjarla, 1999). Alcohols have also been reported to destabilize lamellar liquid crystalline phases resulting in an increase of the microemulsion region (Yaghmur et al., 2002).

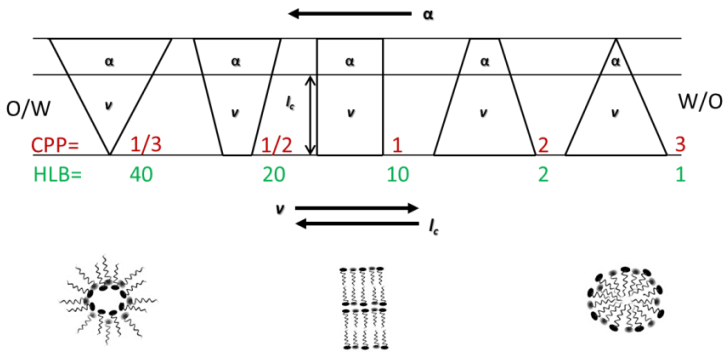

Figure 2: Parameters influencing surfactants' structure. Critical packing parameter (CPP) and Hydrophilic-lipophilic balance (HLB)

2.2 Emulsions and nanoemulsions

Emulsions are thermodynamically metastable colloids consisting also of water, oil and surfactants. This material has a relatively high interfacial tension between the two immiscible fluids, thus requiring external energy for droplets to be formed (Leal-Calderon et al., 2007). Typically a conventional emulsion has droplets ranging from a few hundreds of nm to 100 μm. Conventional emulsions are optically opaque because the formed droplets have similar dimensions with the wavelength of light.

In the recent years, much attention has been paid to the formation of nanoemulsions, a type of colloidal dispersions that, like conventional emulsions, are only kinetically stable (McClements and Rao, 2011, McClements, 2012). There has been much of a debate concerning the upper particle size limit of nanoemulsions from 500 nm (Anton et al., 2008), 200 nm (Huang et al., 2010) to 100 nm (Rao and McClements, 2011). Nevertheless, for the most cases, the particle size of nanoemulsions is much smaller than the wavelength of light

meaning that these systems are macroscopically slightly turbid. One of the main benefits of nanoemulsions over conventional emulsions is the fact that due to the smaller droplet size, they have much better stability to gravitational separation (McClements and Rao, 2011).

Like microemulsions, a system consisting of oil droplets dispersed in an aqueous medium is called O/W nanoemulsion whereas for aqueous droplets dispersed in oil phase is referred to as W/O nanoemulsion. The droplets in O/W nanoemulsions, could be considered to have a core of hydrophobic material surrounded by a shell made of surfactants (McClements and Rao, 2011).

As for the case of microemulsions, the selection of the appropriate emulsifier is also crucial for the stability of these colloidal dispersions. To this respect, especially within the food sector the most commonly used emulsifiers proposed for nanoemulsions include small surfactants, proteins, phospholipids and polysaccharides (McClements and Rao, 2011). Ionic surfactants, such as citrem, an anionic citric acid ester of monoglycerides, can be easily dispersed in the water phase without applying high energy input (Solè et al., 2006). Other emulsifiers commonly used are non-ionic surfactants due to their low toxicity levels and ease of preparation for both emulsification methods such as polyglycerol polyricinoleate (PGPR) and DMG (Benichou et al., 2001). Zwitterionic surfactants such as lecithin are also commonly proposed due to their GRAS status. These molecules have been proposed to enhance emulsification when acting in combination with co-surfactants (Hoeller et al., 2009).

2.2.1 Emulsification methods

In order for these kind of colloidal dispersions to be formed, external energy needs to be applied to the systems. There are two proposed techniques of nanoemulsions preparation involving either low or high energy input for emulsification (Acosta, 2009). High energy techniques apply high shear stresses for fine droplets to be formed. The most common techniques at this field include high pressure homogenization (HPH), microfluidization or sonication. For these kind of homogenization processes, the emulsifier has to facilitate droplet disruption under homogenization by lowering the interfacial tension (McClements and Rao, 2011).

More specifically, HPH are probably the most commonly used methods for producing conventional emulsions or nanoemulsions. Usually, a pre-mixed coarse emulsion is subjected to HPH effectively reducing its droplet size. Microfluidizers also involve the use of high pressures in order to decrease the droplet size. However, with this technique the coarse emulsion is divided in two fine streams and then directed to the same interaction chamber. The two-fast moving

streams are impinged upon each other leading to an effective droplet disruption. Another commonly used high energy emulsification technique is ultrasonic homogenization. This type of homogenizer generates intense ultrasonic waves which disrupt large droplets to smaller ones. Experiments have shown that for the latter case, protein denaturation or lipid oxidation phenomena may occur due to high local intensities produced (McClements, 2015). Generally, high-energy methods (Figure 3) could be unfavorable due to over-processing of the sample, high frequency of droplet collision and others parameters (Jafari et al., 2008).

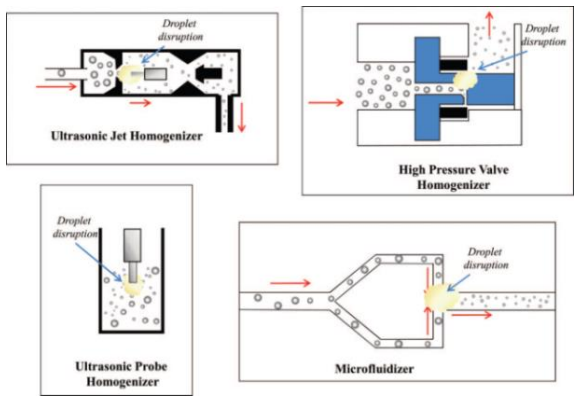

Figure 3: High energy emulsification methods (Jafari et al., 2008)

On the other hand, low energy methods are based on the spontaneous formation of droplets due to the decrease of the interfacial tension (McClements and Rao, 2011). For low energy input approaches, the emulsifier facilitates the formation of small droplets by decreasing the interfacial tension under certain conditions. Spontaneous emulsification could be applied in numerous different ways, in which the parameters such as the composition of the system, the parameters and/or the mixing conditions are varied. When two phases are brought into contact, the containing components will diffuse from one phase to the other. This will lead to interfacial turbulence and eventual formation of droplets. Another very important approach includes phase inversion methods. In that case, changing of the surfactants' molecular geometry with changing temperature (Phase inversion temperature –PIT) or composition (Phase inversion composition –PIC) leads from a W/O to an O/W (or vice versa) phase inversion.

Currently, for the formation of conventional emulsions, high energy emulsification methods are mostly employed in industrial scale, due to the easy handling towards the formation of smaller droplets.

2.2.2 Destabilization phenomena

Due to the thermodynamic instability of conventional or nano-emulsions several destabilization phenomena may occur (Figure 4). Reversible instabilities include droplets aggregation such as creaming or sedimentation. These instabilities are caused due to the association of particles via flocculation while keeping their individual integrities (McClements, 2015).

Irreversible instabilities such as Ostwald ripening (OR) and coalescence lead to droplet evolution and eventual collapse of the system (Leal-Calderon et al., 2007). More specifically, OR is caused due to the difference in the Laplace pressure between the droplets. Because of this difference, there is a diffuse transfer gradient from smaller to larger droplets leading to droplets' evolution and collapse (Petsev, 2004). This effect could be compensated by adding a solute to the dispersed phase as first proposed by Higuchi and Mistra (Higuchi and Misra, 1962). Due to osmotic pressure mismatch between the droplets, the smaller droplets tend to become enriched again with the dispersed phase thus slowing or inhibiting the OR effect.

Coalescence is caused due to random collision of droplets leading to a broadening of the size distribution (McClements and Rao, 2011). For coalescence to occur, an energy barrier has to be overcome depending on interfacial parameters or spontaneous curvature. When coalescence is the main destabilization mechanism, the size evolution of droplets is self- accelerated leading to the total phase separation of the two immiscible fluids (Leal-Calderon and Cansell, 2012).

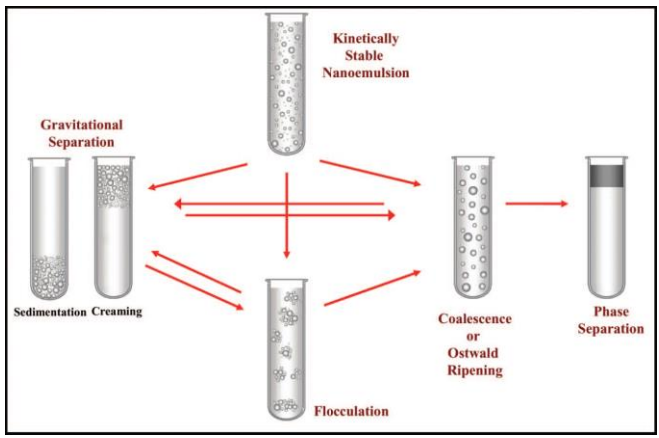

Figure 4: Destabilization phenomena occurring in emulsions. (McClements and Rao, 2011)

2.3 Microemulsion versus (nano) emulsions

The most fundamental way to distinguish between microemulsions and emulsions is their thermodynamic stability. As discussed above, microemulsions are thermodynamically stable whereas emulsions or nanoemulsions are thermodynamically unstable. As shown in figure 5, for a nanoemulsion, the free energy required for the system to be formed is higher than that of the separated phases. A nanoemulsion could be kinetically stable, meaning that it could be in a metastable situation where the energy barrier between surfactants aggregation and phase separation states, is high enough.

On the other hand, as shown in Figure 5, for the case of microemulsion, the free energy of droplet formation is higher than that of phase separation. That means that microemulsion is the favorable state, thus providing thermodynamic stability (McClements, 2012). Nevertheless, an energy barrier should be exceeded for the activation of the emulsification process.

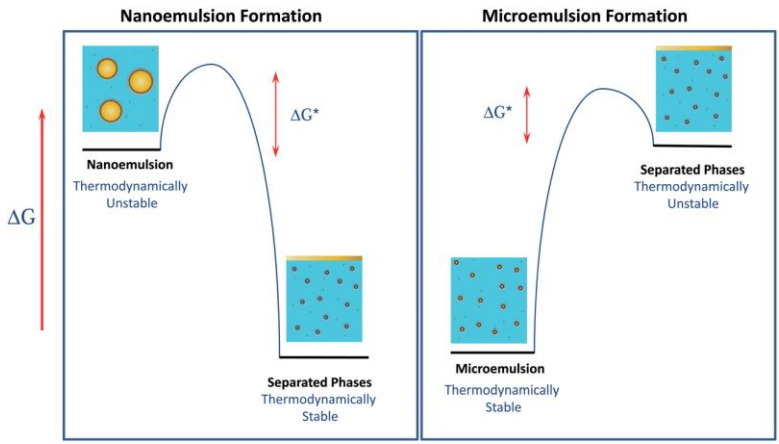

Figure 5: Schematic representation of the free energy of microemulsions and nano-emulsions when compared to the phase separation states. (McClements, 2012)

Even though microemulsions and nanoemulsions are colloidal dispersions with small droplet size there are some important aspects that clearly differentiate these two systems.

Firstly, as mentioned above, microemulsions are thermodynamically stable systems whereas nanoemulsions are only kinetically stable. Consequently, under prolonged storage conditions the structural characteristics of the microemulsion will remain unaltered while those of the nanoemulsion will change. Secondly, the type of preparation of the two systems is different in the sense that micro-

emulsions are spontaneously formed while nanoemulsions require a sufficient amount of external energy input. Another important difference between the two systems is the type of emulsifiers used. Typically, only smaller molecules are used for the formation of micelles or reversed micelles due to the ultralow interfacial tension needed for such a process. On the other hand, molecules of higher molecular weight such as proteins or polysaccharides could be used for the formation of nanoemulsions' droplets. These droplets tend to be spherical due to the relatively large Laplace pressure favoring the formation of spherical aggregates. On the contrary, micelles could have different shapes depending on the surfactant's geometry thus affecting its packing parameters (McClements, 2012).

3 Structural characterization

One of the main challenges in the formulation of colloidal nano-dispersions as effective bioactive carriers is their structural characterization. These vehicles, due to their complex composition, have to be characterized in terms of size, shape and behavior under specific conditions (Garti and McClements, 2012).

In this section, the basic principles of some of the most commonly used techniques in terms of structural characterization of colloidal nano-dispersions are discussed.

3.1 Phase behavior

The phase diagram approach is a technique used to visually assess the phase behavior between different mixtures of the system's components (water, oil and surfactant) at constant temperature and pressure (Lawrence and Rees, 2012). Within the frame of this work, phase diagrams at ambient pressure will be further analyzed.

A typical ternary phase diagram is represented in the equilateral triangle of figure 6. Every corner represents the 100 % pure component whereas the axes represent binary mixtures of the relative components. Inside the triangular diagram, every point represents mixtures of the three components (water, oil and surfactant) and thus different phases can be visually assessed. Furthermore, the phase transition from one to multi-phase region is shown. Constructing the phase diagram could be time-consuming as the equilibration time increases with approaching the phase boundary (M. Jayne Lawrence, 2000). The typical approach for phase diagram construction is the formation of binary mixtures at different compositions and titrate with the third component at constant temperature (Papadimitriou et al., 2008, Kalaitzaki et al., 2015).

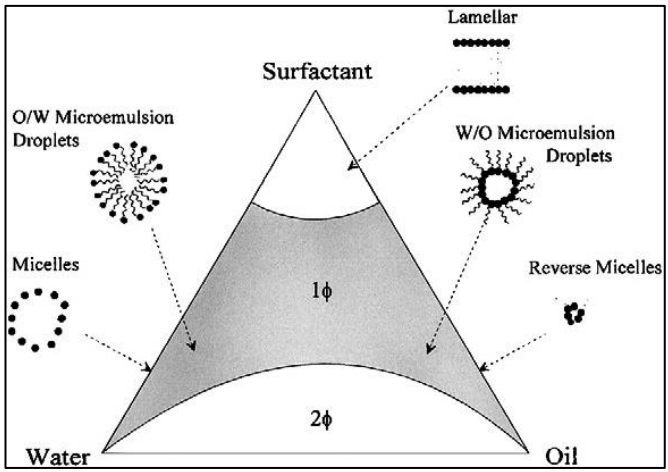

Figure 6: Schematic representation of a typical phase diagram of water-surfactant-oil system (Lawrence and Rees, 2012)

In the case where four or more components are under investigation, pseudo-ternary phase diagrams are being used. In that case, the corners of the triangular diagram represent mixtures of components such as surfactant/co-surfactant or solvent/co-solvent at given ratios. As mentioned above, (pseudo) ternary phase diagrams illustrate different phase regions. Microemulsions are placed in the one-phase region and depending on the excess of either water or oil are distinguished in water rich (oil-in-water, O/W) or oil rich (water-in-oil, W/O) microemulsions. As shown from figure 6, at the axes surfactant/water or surfactant/oil micelles or reverse micelles are formed. For compositions close to oil/water axis, the insufficient amount of surfactant induces a phase separation.

According to Garti and his team, the addition of alcohols or short chain polyols increases the monophasic region due to the destabilization of liquid crystals, sometimes leading to U-type microemulsions (Yaghmur et al., 2002). Figure 7 illustrates the increase of the monophasic region (L_2) and eventually the creation of U-type systems (panel d) with the addition of ethanol and propylene glycol (Garti et al., 2001).

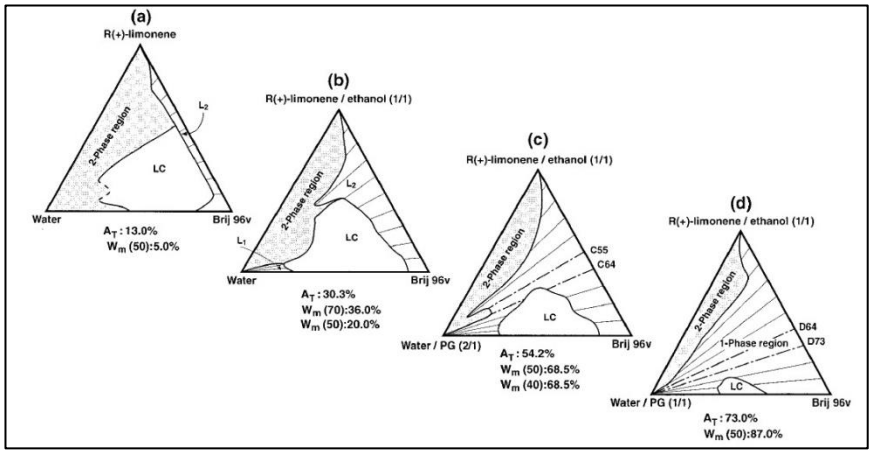

Figure 7: Pseudo-ternary phase diagrams. Formulation of U-type microemulsions with the addition of ethanol and propylene glycol (Garti et al., 2001)

To summarize, this technique is being extensively used as a phase behavior tool in many studies concerning agrochemical (Kalaitzaki et al., 2015), pharmaceutical (Constantinides, 1995), food (Garti, 2003) and other applications (Santanna et al., 2009) where microemulsions are used as the systems under investigation.

3.2 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a technique commonly used for the determination of the droplet size and polydispersity of colloids. This technique measures particles smaller that the wavelength of light taking into consideration that they are subjected to Brownian motion. When a monochromatic beam is impinged on a sample, each particle acts as a secondary source because of the scattering of radiation, with larger particles scattering more than smaller ones. Due to the relative position changes of the particles, random intensity fluctuations in time are recorded by the detector. In DLS, the time of fluctuations in the scattered intensity depends on the diffusion coefficient of the particles in the sense that larger particles diffuse more slowly than smaller ones. Autocorrelation is a signal processing technique providing quantitative information of these fluctuations in time (Figure 8).

For the assumption that the sample contains spherical particles, the hydrodynamic radius R_h, is calculated using the Stokes-Einstein relationship:

$$D = kT/(6\pi\eta R_h) \qquad (3.1)$$

where, D is the diffusion coefficient, k the Boltzmann's constant, T the absolute temperature and η the solvent viscosity. For the case that the particles are not spherical, R_h is considered as the apparent hydrodynamic radius or equivalent sphere radius (Hassan et al., 2014).

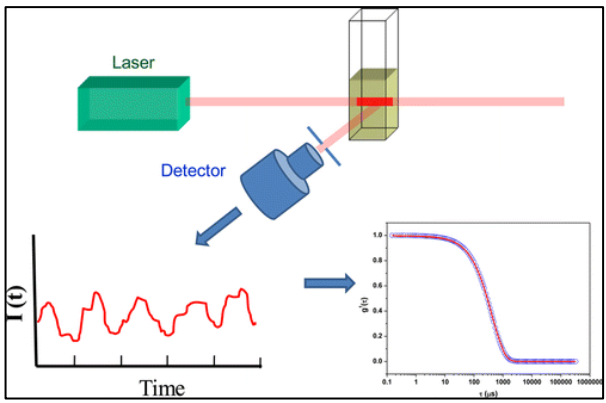

Figure 8: Schematic representation of dynamic light scattering process (Hassan et al., 2014)

Generally, DLS is a fast and easy technique to obtain information about the size of the particles or self-assembled structures in a relatively simple system under investigation. Nevertheless, it is commonly used with other complementary techniques when more complex systems are examined (Fanun, 2009, Kalaitzaki et al., 2014).

3.3 Electron Paramagnetic Resonance (EPR) spectroscopy

Electron paramagnetic resonance (EPR) is a spectroscopic method where microwave radiation is absorbed from molecules with unpaired electron spins. In other words, with this technique it is feasible to measure the energy splitting of unpaired electrons when subjected to magnetic field. This phenomenon is also referred to as electron spin resonance (ESR) and has been proposed for the study of the structure, molecular mobility and micro-polarity of physicochemical systems (Di Meglio et al., 1985, Skoutas et al., 2001, Avramiotis et al., 2007) as well as biological systems (Belle et al., 2008, Panneerselvam et al., 2013). It has also been proposed for the study of enzymes encapsulated in reverse micellar

structures (Xenakis and Cazianis, 1988, Avramiotis et al., 1999, Sereti et al., 2014).

Generally, in the absence of the magnetic field, an electron has spin of $m_s = \pm 1/2$. In the presence of magnetic field this energetic state is analyzed in a lower energy state where the magnetic moment is oriented parallel to the field and corresponds to $m_s = -½$ and a higher energy state where the magnetic moment is oriented antiparallel with $m_s = ½$.

In EPR spectroscopy the transition between two different energy states is occurring with simultaneous radiation absorbance in the microwave range.

$$\Delta E = h\nu = g\beta H_0 \qquad (3.2)$$

where, ΔE is the energy difference between the two states, h the Plank's constant, v the radiation frequency, β= Bohr magneton, H_0 the intensity of the magnetic field and g is a dimensionless magnetic moment. For free electron g= 2.0023 whereas for metal ions the so-called g-factor could be different (Carrington and McLachlan, 1967).

In principle, there are two ways in acquiring EPR spectra. Either a constant magnetic field is applied scanning all the frequencies of the microwave electromagnetic radiation or the opposite. Nevertheless, a radiation source for radar waves produces only a very limited spectral region. In EPR such a source is called "klystron". An X-band klystron has a spectral band width of about 8.8-9.6 GHz. This makes it impossible to continuously vary the wavelength similarly to optical spectroscopy. Therefore, in practice, a variation of the magnetic field occurs, until the quantum of the radar waves fits between the field-induced energy levels. A peak of the absorption will occur as soon as the difference of the two energy states (ΔE) match the radiation energy. The frequencies of the electron spins on an EPR device are in the microwave range v≈9.5GHz (X band) and the magnetic resonance field is approximately 340 mT.

Figure 9 shows an EPR spectrometer having a magnet and a "microwave bridge" (electromagnetic radiation source and detector). The sample is put in a metal microwave cavity which enhances the size of the microwave field. The detector recognizes the signal coming back from the cavity due to spectroscopic transitions. Following, the microwave power is converted to an electrical current producing the characteristic spectrum (Feher, 1957). A computer is also connected to the device in order to analyze data on the relevant acquisition program.

Figure 9: Bruker EMX EPR spectrometer (X band). National Hellenic Research Foundation, Greece

In the present work, EPR has been a valuable technique to determine the structural characteristics in terms of membrane dynamics of the proposed nano-dispersions. To this respect, the amphiphilic nitroxide 5- doxyl stearic acid (5-DSA) was used as a spin probe. 5-DSA is a radical with an unpaired electron (Figure 10). EPR spectrum of the molecule is expressed as shown in figure 11. This spectrum provides indirect information on the membrane dynamics. The mobility of the spin probe is reflected by the rotational correlation time τ_R (Kommareddi et al., 1994), while the micro-polarity of its' environment is reflected by the isotropic hyperfine splitting constant A_0. The rigidity of the membrane is expressed with the order parameter (S), with S=0 the completely random and S=1 the complete ordered state (Griffith and Jost, 1976).

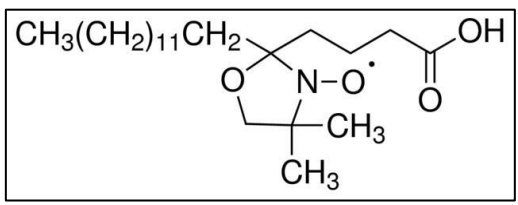

Figure 10: Chemical structure of 5 doxyl stearic acid

The rotational correlation time τ_R of the spin probe is calculated from the EPR spectra using the following relationship:

$$\tau_R = 6 \times 10^{-10} \left[\left(\frac{h_0}{h_{+1}} \right)^{1/2} + \left(\frac{h_0}{h_{-1}} \right)^{1/2} - 2 \right] \Delta H_0, (s) \qquad (3.3)$$

where, ΔH_0 is the width of the central field and $h_{+1,}\, h_0,\, h_{-1}$ are the intensities of the low, center and high field peaks of the spectrum respectively (Figure 11).

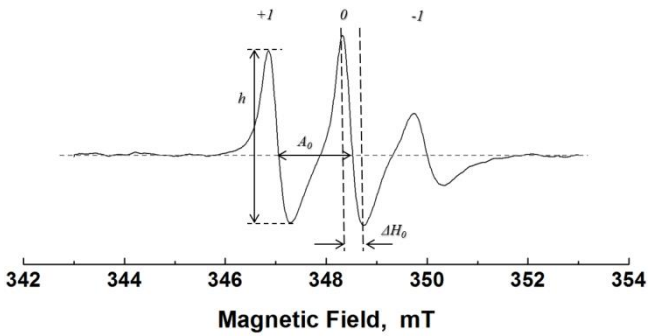

Figure 11: 5 doxyl stearic acid EPR spectrum in a microemulsion containing 49 % mixture of medium chain triglycerides and isopropyl palmitate (1:1), 49 % mixture of lecithin, ethanol and glycerol (2:1.7:3.3) and 2 % water (weight ratio). The overall concentration of 5-DSA was 10^{-4} M.

The equation 3.3 is applicable in the fast motion region, i.e. for correlation times in the range of $10^{-11} < \tau_R < 3 \times 10^{-9} s$ (Kommareddi et al., 1994).

The order parameter S is calculated from the EPR spectra using the equation:

$$S = \left(A_{\parallel} - A_{\perp}\right) / [A_{ZZ} - 1/2(A_{XX} + A_{YY})]k \qquad (3.4)$$

where, A_{XX}= 6.3*10^{-4}, A_{YY}= 5.8*10^{-4} and A_{ZZ}= 33.6*10^{-4} T the single crystal values. $A_{\parallel}$ and $A_{\perp}$the hyperfine splitting constants. $A_{\parallel}$ is the half distance of the outermost EPR lines and $A_{\perp}$ is the half distance of the inner EPR lines.

The ratio $k = A_0 / A'_0$ represents the polarity correction factor, where, $A'_0 = 1/3(A_{XX} + A_{YY} + A_{ZZ})$ is the hyperfine splitting constant for the nitroxide in the crystal state and $A_0 = 1/3(A_{\parallel} + 2A_{\perp})$ the isotropic hyperfine splitting constant for 5-DSA in the membrane. A_0 values depend on the polarity of the spin probe's environment and increase with increasing polarity.

Different methods of simulation of EPR spectra have been proposed in the literature in order to refine hypothetical parameters to optimal values and com-

pare with experimental data (Freed, 1972, Stoll and Schweiger, 2006). To this respect, in this work simulations of experimental EPR spectra were conducted using the simulation program NIHS/ NIH WinSIM versions: 0.96 and 0.98 Public EPR Software Tools (P. E. S. T.) (Duling, 1994) which allowed the calculation of the hyperfine splitting constant theoretical values.

3.4 Cryogenic Transmission Electron Micros-copy (Cryo-TEM)

Cryogenic transmission electron microscopy (Cryo-TEM) is a technique commonly used for the morphological visualization of soft nanostructured materials at near native state. This method is able to capture phase transitions and dynamic phenomena via ultra-fast cooling of a liquid sample to a vitrified specimen (Danino and Talmon, 2000). High cooling rates in the range of hundreds degrees in milliseconds are used to assure the preservation of the original nanoformulations and prevent redistribution of nanoparticles or solvent crystallization. Most commonly, liquid ethane cooled to its freezing point by liquid nitrogen is used as cryogen. Samples are prepared in closed vitrification chambers at well controlled temperatures and saturation conditions of the volatile components of the sample under study (Danino, 2012).

Blotting procedure is probably the most important step of sample preparation, determining the quality and thickness of the film. Blotting with filter paper may cause high shear stress in the liquid leading to a re-distribution of the dispersed objects or a morphological re-arrangement of the soft self-assembled nanostructures (Friedrich et al., 2010).

Electron beam specimen interactions are required for imaging, but they cause radiolysis, i.e. beam destruction. Scattering electrons break the chemical bonds leading to the production of radical chain reactions and subsequent sample damage with organic solvents being the most sensitive (Talmon, 1987). To avoid sample damage, optimal imaging conditions are set considering that resolution increases with accelerating voltage while contrast improves at lower radiation. Images are recorded using charge-coupled device (CCD) cameras with sensitive detectors having good signal-to-noise ratio. Cryo-TEM experiments in the frame of this work were conducted in collaboration with the core facility for integrated microscopy at the University of Copenhagen, Denmark using a FEI Tecnai G2 transmission electron microscope (Figure 12).

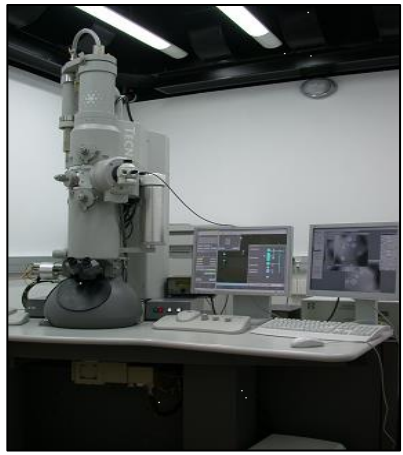

Figure 12: FEI Tecnai G2 Cryo-TEM device.
Core Facility for Integrated Microscopy,
Faculty of Health and Medical Sciences,
University of Copenhagen, Denmark

Generally, due to the direct imaging of soft matter as well as the improvement of the technique the last decades, Cryo-TEM is widely used to characterize nanostructured materials (Helvig et al., 2015). Much attention has been paid in morphological characterization of vesicles used for drug delivery applications to map "structure-activity" parameters (Klang et al., 2013). Furthermore, the food sector is also facing challenges when the addition of bioactive ingredients in liquid dispersions is suggested for improved nutritional benefits. To this respect, cryo-TEM provides a useful tool to directly visualize the morphology of the carrier as well as the molecule-matrix interactions (Semo et al., 2007, Sagalowicz and Leser, 2010).

3.5 Small Angle X-ray Scattering (SAXS)

Small angle X-ray Scattering (SAXS) is a scattering, non-invasive technique used for samples with abnormalities in the nm range. X-rays radiation (λ=0.01 - 0.2 nm) passing through the sample and the elastic radiation is recorded at very small angles (typically 0.1-10^{o}). SAXS experiments investigate the different electron densities providing information about the size and shape of the dispersed phase (Guinier and Fournet, 1955). Structural characterization of numerous materials including biological macromolecules (Putnam et al., 2007), colloids (Lutz et al., 2007) and nanocomposites (Causin et al., 2005) are continuously being reported using this technique. X-ray scattering detects particles

inside a dispersant due to the difference in electron densities (contrast) between particles and the environment. Generally liquid samples are measured inside a thin-walled capillary of around 1-2 mm thick.

SAXS experiments are performed either using synchrotrons or smaller laboratory devices. In the present study, SAXS was performed using the GANESHA-SAXS/WAXS apparatus (SAXSLAB, Denmark) (Figure 13). Measurements were performed using Cu-K_α X-ray radiation in a q range of around 0.05-0.8$Å^{-1}$, q being the magnitude of the scattering vector:

$$q = \frac{4\pi}{\lambda} sin(\theta)\,, (Å^{-1}) \qquad (3.5)$$

where, λ=1.54 Å is the X-ray wavelength and θ is half of the scattering angle.

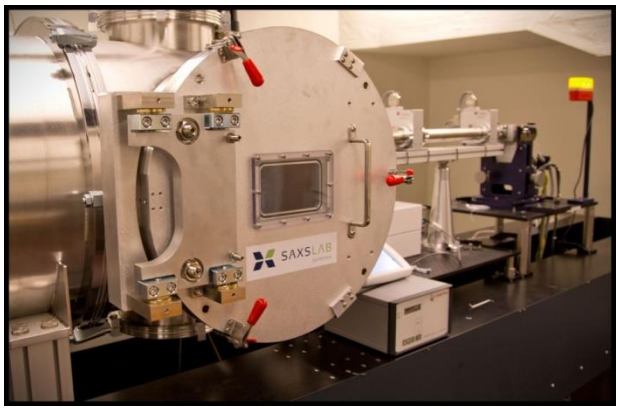

Figure 13: GANESHA-SAXS/WAXS apparatus. X-Ray and Neutron Science Section, Niels Bohr Institute, (SAXLAB JJ- X-ray, Denmark)

It would be important to note that the SAXS scattering profiles of inverted type microemulsions, like those investigated in this work, are represented by a single broad peak. Generally, when the particles align together into a highly ordered arrangement, their representation is a pronounced peak (Figure 14). The so-called "characteristic distance" (d spacing) is calculated from each curve using Bragg's law:

$$d = \frac{2\pi}{q*}\,, (Å) \qquad (3.6)$$

where, q* the scattering broad peak obtained from SAXS spectra.

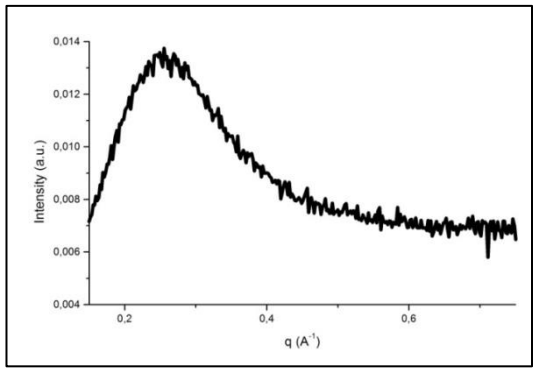

Figure 14: SAXS scattering curve of a microemulsion containing 38 % distilled monoglycerides, 58 % mixture of refined olive oil and ethanol and 4 % wt. water.

The model typically used for inverse micellar solutions is a core-shell type structure of a hydrophilic core and a hydrophobic shell (Yaghmur et al., 2004, Salentinig et al., 2014). In case of monodisperse, homogeneous and spherical particles, the SAXS scattering intensity can be expressed as following:

$$I(q) = NS(q)P(q) \qquad (3.7)$$

where, N is the number of particles, S(q) is the structure factor describing inter-particle interactions, and P(q) is the form factor describing intra-particle interactions (Guinier and Fournet, 1955). In order to determine structural characteristics of the colloidal dispersion under investigation, a mathematic model needs to be applied. For the purposes of this study, investigating the structure of swollen reverse micelles in surfactant-rich microemulsions, the form factor of spherical core-shell micelles P(q) was calculated using the Percus-Yevick approximation (Percus and Yevick, 1958). This model has been evaluated to give accurate predictions up to volume fractions of approximately 0.45 for monodispersed hard spheres and also provide good predictions for polydispersed hard spheres (Frenkel et al., 1986). This approximation could be also applied for the analysis of other small angle techniques such as SANS (Zackrisson et al., 2005).

3.6 Other techniques

Since colloidal nano-dispersions and especially microemulsions are complicated systems with unpredictable phase behavior, other techniques also have been proposed. Small angle neutron scattering (SANS) and nuclear magnetic resonance (NMR) are the most common among them.

SANS is a technique very similar to SAXS only in this case elastic neutron radiation is used for the characterization of structures or particles in the nanoscale. Neutron scattering has the advantage of a higher signal-to-noise ratio due to the technique of contrast variations (Kahlweit et al., 1987). SANS is commonly used as a complementary method to SAXS and other methods for the studying of microemulsions or other systems (Regev et al., 1996, Zackrisson et al., 2005).

NMR is an important tool used not only for the characterization of nano-dispersions but also oil- or water- surfactant solutions. Size, shape as well as the number of surfactant aggregates, critical micellar concentration (CMC) and other information could be obtained by this technique (Wennerström and Lindman, 1979, Lindman and Olsson, 1996).

Conductivity is a means of investigating whether a system is water or oil continuous. It is also a useful tool for phase transition investigations (Yu and Neuman, 1995). Kalaitzaki et al. detected the phase transition of U-type microemulsions from W/O to O/W upon dilution while remaining optically transparent (Kalaitzaki et al., 2015). Another technique for extracting structural information of the micro-domains within a sample is fluorescence quenching technique. Using a hydrophilic or hydrophobic quencher, information about the nature and dynamics of the entrapped phase could be effectively extracted (Avramiotis et al., 2007)

At the macroscopic level, viscosity measurements could also provide a helping tool providing information about the rheology of the system under investigation. Viscosity could be tailored by structural conformations or the addition of specific agents for targeted applications (Kantaria et al., 1999).

4 Nano-dispersions and bioactive molecules

Microemulsions and emulsions, as discussed above, are ideal vehicles for solubilization and transport of components due to their unique physicochemical properties. The understanding of oil recovery using surfactants and microemulsions has been well investigated over the last 30 years (Shah, 1981, Shah, 1998).

W/O or O/W microemulsions have been proposed for detergency applications being advantageous over conventional organic agents for their ability to solubilize polar, non-polar and amphiphilic components (Kumar and Mittal, 1999). Many enzymatic and bio-catalytic applications have been proposed using W/O microemulsions, due to their versatile property to encapsulate the enzyme inside the hydrophilic core while releasing products of different polarities at the continuous phase (Stamatis et al., 1999, Zoumpanioti et al., 2006). Microemulsions are currently widely used in cosmetics for personal care products with low viscosity while new are continuously being reported (Protopapa et al., 2001, Boonme, 2007).

On the other hand, nano-emulsions have been proposed for applications in many industrial fields such as agrochemicals, pharmaceutics, cosmetics and food as described from Gutiérrez and his team (Gutiérrez et al., 2008). Conventional emulsions are currently widely used in cosmetics and food in the form of viscous, fluid products.

Numerous studies have used microemulsions and emulsions as vehicles for bio-nutrients or drug delivery applications (Constantinides, 1995, Huang et al., 2010). Both colloidal dispersions have the unique properties to solubilize bioactive molecules of different polarities such as poorly water soluble functional agents and enhance their bioavailability due to the high surface-to-volume ratio (McClements, 2012). Also, they protect the encapsulated bioactive components against environmental stresses (pH alterations, oxidative damage, and protein degradation) (Flanagan and Singh, 2006, Kogan and Garti, 2006, McClements and Rao, 2011, Leal-Calderon and Cansell, 2012).

4.1 Pharmaceutical sector

Concerning pharmaceutical applications, the most widely utilized drug administration is by oral consumption. Even though recent trends in science using biotechnology or computational tools have introduced many new chemical compounds with therapeutic potentials, their poor water-solubility results to very low bioavailability at the gastrointestinal tract. Transdermal drug delivery is an alternative route for drug administration which has received considerable attention over the years. This method suggests ease of administration, the ability to avoid hepatic first pass metabolism and the possibility to remove treatment if required (Prausnitz and Langer, 2008). Nevertheless, drug absorption via transdermal routes face many challenges, due to the limited delivery rates through the skin barrier. The design of carriers that could enhance the drug absorption while decreasing possible skin irritation is thus, crucial for the pharmaceutical research. To this concern, many research groups have proposed colloidal disper-

sions for transdermal delivery applications in order to overcome such challenges (Garti et al., 2006, Kogan and Garti, 2006, Kalaitzaki et al., 2014, Fanun et al., 2011).

4.2 Food sector

In the field of food applications, research of certain bioactive compounds, mainly from natural origin, indicate their health benefits beyond nutritional value, classifying them as "nutraceuticals". Garti and McClements state that "nutraceuticals and bioactive ingredients include vitamins, minerals, phytochemicals, amino-acids and peptides, pre- and pro-biotics, healthy oils, spices and herbs" (Garti and McClements, 2012). These molecules once isolated from their natural sources face limitations mainly due to instability, thus need protection from possible environmental stresses that may occur.

In 1994, the Institute of Medicine's Food and Nutrition Board (IOM/FNB, 1994) defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains." On the other hand, the European Commission's Concerned Action on Functional Food Science in Europe (FuFoSE) in collaboration with the International Life Science Institute (ILSI) Europe stated: "a food product can be considered functional if together with the basic nutritional impact it has beneficial effect on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of evolution of diseases". Functional foods are consumed in the frame of a normal diet, similarly to conventional foods (Siro et al., 2008). The increasing market share of functional products in European and other countries indicates the importance of these products (Menrad, 2003).

A number of bioactive molecules would benefit from being encapsulated in appropriate delivery systems due to their low bioavailability, low water solubility, oxidative damage or poor chemical stability (McClements et al., 2009). Omega-3 (ω-3) fatty acids are unsaturated fatty acids present in fish oils that have been claimed for their beneficial role in cardiovascular, immune response or mental disorder diseases (Kris-Etherton et al., 2002). Food encapsulation methods have been proposed for the oxidative protection of these lipids as well as the increase of their effective consumption in order to meet the recommended health benefits (Garg et al., 2006).

4.2.1 Antioxidants

Oxidation is possible to occur during metabolism or other processes in a physiological or pathological condition of a living organism. Lipids are important

components of the biological systems as well as of the dairy nutrition, and are susceptible to oxidation due to exogenous or endogenous parameters. Auto-oxidation of lipids is a process of unsaturated and polyunsaturated fatty acids including a radical chain reaction most commonly initiated by lipid exposure to light or other form of irradiation, metal ions or enzymes. The process involves initiation (production of lipid radicals), propagation and termination (production of non-radical products) (Kohen and Nyska, 2002). Lipid oxidation is a major concern to food scientists and consumers as it causes food deterioration, thus decreasing food nutritional value.

According to the US Food and Drug Administration (USFDA) Code of Federal Regulations "antioxidants are substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation" (21CFR170.3).

Antioxidants behave as radical terminators either by directly reacting with the radicals, like phenolic antioxidants (AH) (Shahidi et al., 1992) or using other ways such as oxygen scavenging. The efficacy of antioxidants depend on many parameters including structural characteristics of the molecule, concentration, type of oxidation substrate and others (Yanishlieva-Maslarova et al., 2001).

In 1993, Porter and his team reported a paradoxical behavior of antioxidants, in the sense that polar antioxidants are more effective in less polar media, such as oils, while non- polar ones are more effective in polar media such as liposomes or O/W emulsions (Porter, 1993). Many research groups have been extensively discussed the so-called "polar paradox" in order to describe the importance of the physical location of a radical scavenger in respect to its activity (Schwarz et al., 2000, Chaiyasit et al., 2007). Early studies stated that the alterations in antioxidant activity in media of different polarity, could be related to affinities towards oil-air interfaces in bulk oils versus oil-water interfaces in emulsions (Frankel et al., 1994). However, due to the fact that air is less polar than oil, there would not be a high driving force for hydrophilic antioxidants to migrate at the air-oil interface (Chaiyasit et al., 2007).

Instead, nowadays, it is well known that natural oils contain minor components such as mono and di-glycerides, fatty acids, antioxidants, phospholipids, sterols, aldehydes, ketones and others produced during lipid oxidation processes (Xenakis et al., 2010). Traces of water due to moisture are also present in vegetable oils and since water is practically immiscible with oil, moisture is probably entrapped in colloidal associations formed from endogenous amphiphilic molecules (Figure 15). This was confirmed by the structural characterization of olive oil samples in a recent study (Papadimitriou et al., 2013). Thus, there is a high probability that radical polar scavengers accumulate to the hydrophilic core of

association colloids (reverse micelles or lamellar structures) (Chaiyasit et al., 2007).

Generally, it has been widely accepted that the antioxidant activity in bulk oils as well as colloidal structures such as liposomes, emulsions or microemulsions depend on many parameters such as scavenger concentration, polarity, oxidation substrate, structure, molecular weight and others. This suggests that the polar paradox may be a case of a much wider global rule (Shahidi and Zhong, 2011), not fully understood so far.

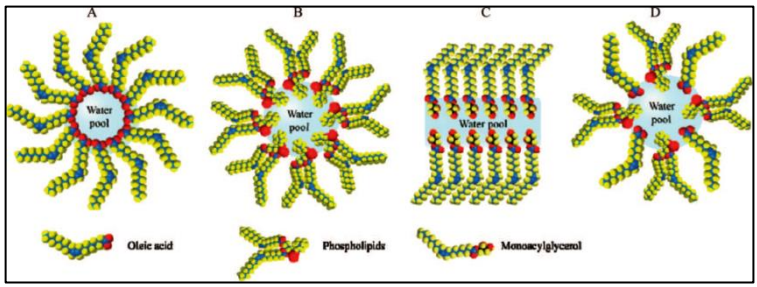

Figure 15: Different associations formed in a vegetable oil from endogenous amphiphiles (Chaiyasit et al., 2007)

In the present study, phenolic compounds of plant origin were encapsulated to the polar cores of the nano-dispesions and were examined for their scavenging activity against known radicals as will be further analyzed below (see section 5.1). More specifically, several epidemiological studies suggest that the Mediterranean diet and especially the consumption of olive oil decrease the incident of degenerative diseases including cancer and coronary heart diseases (Trichopoulou et al., 1995, Psaltopoulou et al., 2004, Covas et al., 2006).

Hydroxytyrosol (HT) and tyrosol are found in the leaves of the olive tree (Olea europaea), in extra virgin olive oil and are also abundant in olive oil millwastewaters. HT and tyrosol are metabolites of oleuropein, the major bioactive compound of Olea europaea causing the characteristic bitter taste of immature olives and also fresh extra virgin olive oil (EVOO) (Boskou, 1996). HT is not only claimed for protecting against oxidative stress but also for other properties such as anti-inflammatory (Haloui et al., 2011) and anti-cancer (Fabiani et al., 2002). In 2011, the European Food Safety Authority (EFSA) approved the claim that LDL particles are protected from oxidative damage by consuming hydroxytyrosol and its derivatives. Furthermore, it established a daily intake of 5 mg of these antioxidants (oleuropein complex and tyrosol) in

order to effectively obtain the claimed characteristics (EFSA, 2011). Gallic acid is another antioxidant widely studied for many beneficial properties found in extra virgin olive oil (Boskou, 1996) and green tea (Wang et al., 2000) extracts. Generally, EVOO is a natural source rich in various phenolic compounds such as vanilic, syringic, protocatechuic and p-hydrobenzoic acids as well as many others (Boskou, 1996). Mustard and rapeseeds have also been reported as an antioxidant food source containing ferulic, caffeic, p-hydrobenzoic and protocatechuic acids (Shahidi et al., 1992). In general, there is a variety of natural sources rich in phenolic compounds that could have a beneficial effect via scavenging radicals (Shahidi et al., 1992).

4.2.2 Peptides

Proteins are natural polymers made from amino acids linked together by peptide bonds. As food ingredients, apart from energy providing molecules some proteins or peptides have been also claimed for their antimicrobial, antioxidant or immune regulatory properties (Playne et al., 2003). Proteins could be part of complex foods such as milk or egg, or may be found in isolated forms such as whey protein or gelatin. The structural characteristics of a protein or a peptide (molecular mass, type and number of amino acid sequence), determine its molecular characteristics and thus its functional abilities (McClements et al., 2009). Some type of functional proteins, peptides or amino acids may lose their bioactivity during production, storage, transport and utilization of food (Chatterton et al., 2006).

Some peptides are incorporated in foods not for direct health benefits but for serving against food deterioration. An example of this category is bacteriocins, antimicrobial substances from bacterial origin produced by lactic acid bacteria (LAB). Tagg et al. have defined bacteriocins as "proteinaceous compounds which kill closely related bacteria" (Tagg et al., 1976). Nisin is an approved by EFSA biopreservative (E234) (EEC, 1983) of Class I bacteriocins of low molecular weight (<5 kDa) (Figure 16) (Sobrino-López and Martín-Belloso, 2008). It is also approved by the FDA (1988) as GRAS for its' antimicrobial activity against some gram positive and gram negative food borne pathogens. To date, it is the only approved bacteriocin by the World Health Organization (WHO) for use in dietary products and it is usually commercialized as a dried concentrated powder (Sobrino-López and Martín-Belloso, 2008). Interestingly, "free" nisin incorporation to foods face challenges such as lower activity and stability due to its sensitivity to environmental parameters (Delves-Broughton et al., 1996).

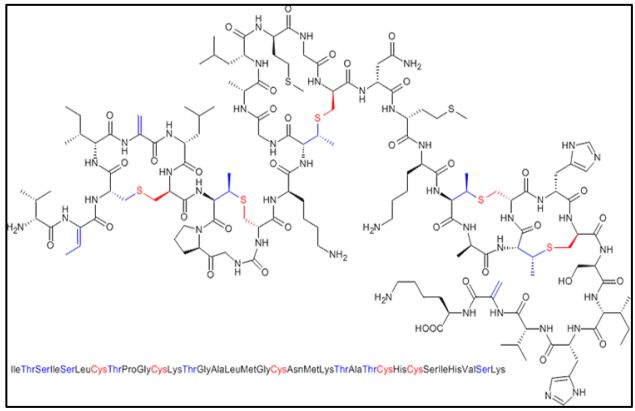

Figure 16: Chemical structure of nisin

5 Assessment

The bioactive molecules analyzed in the previous section (see section 4: Nano-dispersions and bioactive molecules) have been claimed to possess specific characteristics. Depending on the characteristic properties of these substances, targeted assessment techniques have been used namely antioxidant efficacy or antibiotic strength against bacterial strains. In this section, the different methods of assessment used in this study will be analyzed.

5.1 Antioxidant efficacy

It is widely known that oxidative rancidity is the main cause of deterioration in vegetable oils due to the oxidation of unsaturated fatty acids (FAs) leading to off-taste effects (Boskou, 1996).

As reported above (see section 3.3), EPR is one of the main techniques used to detect radicals due to its unique ability to directly measure and distinguish already present or reaction generated radicals (Diplock et al., 1991). Nevertheless, not all radical intermediates can be detected, due to the fact that some give very large, undetectable peaks, or have very small half-life.

Spin trapping EPR is usually used to detect unknown radicals in a given sample. In principle, a chemical compound, used as a “spin trap”, is covalently bound on an unstable radical forming a long-lived radical easily detected by EPR (Finkelstein et al., 1980). In most studies, nitrones such as DMPO (5,5-

dimethyl-pyrroline N-oxide) are used to detect unknown radicals in a sample (Skoutas et al., 2001).

A wide range of *in vitro* methods is being used to assess the scavenging activity of antioxidants against different reactive oxygen species (ROS) (Halliwell et al., 1995). The capacity of the antioxidants could be measured using a reference stable radical such as DPPH (1,1-Diphenyl-2-picryl-hydrazyl) (Butkovic et al., 2004) or galvinoxyl (Shi et al., 2001). They are commercially available, stable, relatively cheap and easy to handle molecules. The 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) cation ($ABTS^{\bullet+}$) is also a radical widely used for the antioxidant capacity assessment (Re et al., 1999). While DPPH and galvinoxyl are stable radicals per se, $ABTS^{\bullet+}$ is produced by the oxidation of ABTS molecule before measurement.

Many antioxidants react with DPPH or galvinoxyl either by hydrogen transfer or just electron transfer followed by proton transfer depending on the antioxidant and the reaction environment. Scavenging reactions could be measured either by the decrease of their absorption at 520 and 430 nm respectively, or by their EPR spectra. The relative reactivity can be assessed from the decrease in absorption or by the decrease in the integrated intensity in the presence of antioxidants. DPPH and galvinoxyl are lipid soluble, but hydrophilic antioxidants may be assessed in microscopically heterogeneous environments such as liposomes and micelles (Tsuchiya et al., 1985). Many studies have reported measurements of the radical scavenging activity of a mixture including extra virgin olive oils (Papadimitriou et al., 2006), tea (Polovka et al., 2003), coffee brews (Cämmerer and Kroh, 2006), fruit juices (Tzika et al., 2007) and others (Polovka, 2006). In this respect, in order to observe the scavenging efficacy of an antioxidant in time, stable radicals are accumulated to be then studied by EPR. In this study, the antiradical properties of the encapsulated antioxidants were assessed using the EPR technique and the lipophilic galvinoxyl (Figure 17) as a stable radical.

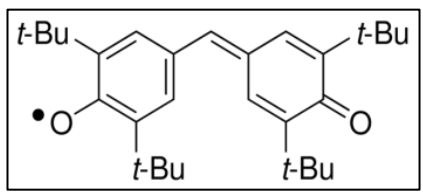

Figure 17: Chemical structure of galvinoxyl stable radical

The scavenging activity of the antioxidant was measured and expressed as % inhibition of the radical. The method was based on the ability of the scavenger to interact with the radical as shown in the following reaction and quench its EPR signal.

$$\boldsymbol{Galv - O \cdot + A - OH \leftrightarrow Galv - OH + A - O \cdot}$$

The EPR signal of the stable radical was obtained and analyzed in terms of integrated area delimited by the spectrum. In the presence of the scavenger, the intensity of galvinoxyl was increased in time as observed in Figure 18.

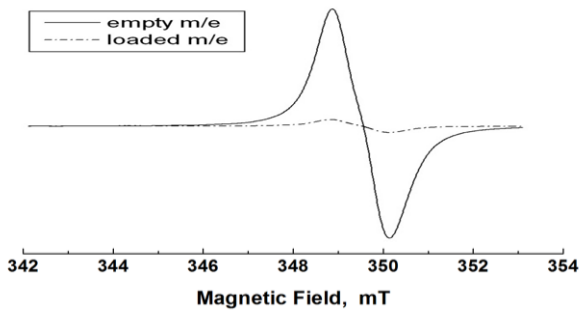

Figure 18: EPR spectra of galvinoxyl stable radical. Microemulsion containing 49 % mixture of medium chain triglycerides and isopropyl palmitate (1:1), 49 % mixture of lecithin, ethanol, glycerol (2:1.7:3.3) and 2 % water (weight ratio). Solid line: sample in the absence of antioxidant, dashed line, sample containing 0.7 mM of gallic acid at 30 minutes.

The percent of inhibition is calculated by the equation:

$$\%\, Inhibition = (A_0 - \frac{A}{A_0}) \times 100 \qquad (4.1)$$

where, A_0 is the integrated intensitiy of the control sample at a specific time point and A the integrated intensity of the sample containing the antioxidants at the same time point.

Regardless of the radical or the method used to determine the scavenging capacity of a sample, a standarized technique is applied commonly using the 6-Hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid (Trolox). Trolox is the water-soluble analogue of vitamin E and is widely taken as reference for antioxidant capacity (Tzika et al., 2007).

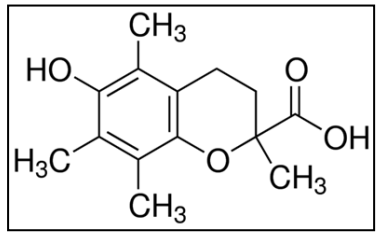

Figure 19: Chemical structure of Trolox

The TEAC (Trolox equivalents antioxidant capacity) is a unit of activity based on Trolox in order to express the scavenging efficacy of a sample. The assay was first introduced by Miller and co-workers, measuring the absorbance of $ABTS^{\bullet+}$ radical cation in the presence of antioxidants in plasma (Miller et al., 1993b). TEAC is defined as the concentration (mM) of Trolox having the equivalent antioxidant capacity to a 1.0 mmol per liter solution of the substance under study. This method measuring TEAC value strongly depends on the nature and the solubility of the substance (Miller et al., 1993a). Generally the method provides a direct comparative measure of various molecules investigated for their potential antioxidant capacity.

5.2 *In vitro* antimicrobial assessment

Accurate determination of microorganisms' susceptibility to antibiotics is essential for the discovery and design of new drugs as well as for the comparative study of different proposed antimicrobial agents. This could be accomplished by a variety of techniques such as diffusion assay, broth dilution assay and others. The diffusion technique involves the application of different antimicrobial solutions to wells into agar plates seeded with the bacterial strains under investigation (Bonev et al., 2008).

In principle, the petri dish is engrafted with the strains under investigation and left to solidify as shown in figure 20. Following, wells are manually created and different antibiotic agents are engrafted into them.

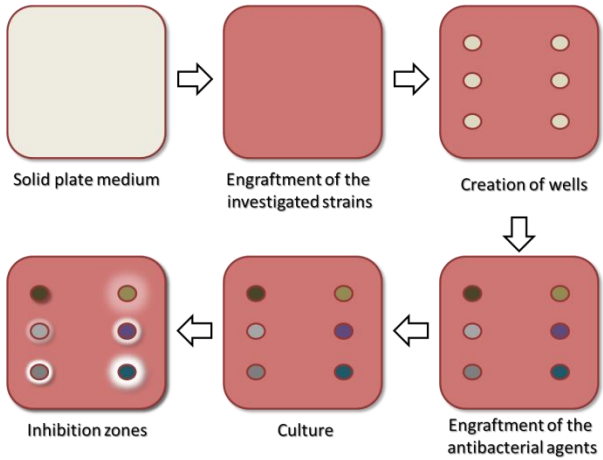

Figure 20: Schematic representation of the well diffusion assay process

Bacterial strain and antibiotic agents are incubated under specific conditions (temperature, time). The diffusion of the antibiotic agent in the aqueous medium enables the inhibition of the bacterial growth in the vicinity of the source. This finally leads to the formation of "inhibition zones" around the wells (Figure 20). Depending on the antibacterial agent, the zones could have different "clearness" and size. The diameter increases with antibiotic concentration (Bonev et al., 2008). For the quantification of the results, the inhibition zones are measured in terms of dimensions in mm and a comparative study between antimicrobial agents is then conducted. The quality of the antibiotic increases with increasing diameter of the inhibition zones.

6. Digestion

There is an increasing interest in the food and pharmaceutical sector in understanding and controlling the digestibility of different emulsified lipids and poorly water soluble substances (Porter and Charman, 2001, Porter et al., 2007, Hur et al., 2009). So far, many analytical tools have been proposed to provide information on the lipid digestion process, from *in vitro* to *in vivo* techniques and human trials as well as *in vitro- in vivo* correlations (Hur et al., 2011). Generally, different models have been proposed to study food digestion. Unfortunately, digestion times, pH and other parameter alterations as well as differences in the origin of enzymes have resulted in significant variations within the studies.

These variations are so large often impeding the comparison across research groups.

Trying to solve this problem, in 2014, an international consensus within the COST Infogest network, proposed a standardized static *in vitro* digestion model based on physiologically relevant conditions (Minekus et al., 2014). A frameset of parameters for the oral, gastric and small intestinal digestion is being proposed. Specifically for the gastric digestion, the addition of simulated gastric fluid (SGF) and porcine pepsin at pH 3 for 2h under mild agitation is suggested. Moreover, for the intestinal digestion, pH 7 for 2h and simulated intestinal fluids (SIF) along with bile salts and enzymes such as trypsin, chymotrypsin, pancreatic lipase and co-lipase are proposed.

The pH-stat technique is an *in vitro* static method proposed from many research teams for pharmaceutical and food research in lipid digestion (Fernandez et al., 2007, Fernandez et al., 2008, Li et al., 2011). This method is designed to simulate gastric, intestinal or both digestion steps and is based on the determination of the amounts of free fatty acids (FFAs) released during lipolysis. The sample under investigation is placed at a chamber usually at controlled temperature (37^{o} C) with the addition of the relevant digestive juices and lipases (Figure 21). The pH is adjusted at a specific end-point depending on the lipolysis step and remains constant due to the titration with alkali that neutralizes the FFAs produced by lipid digestion in time. The pH stat method is a simple and rapid process that enables the quantitative analysis of released lipids under the same digestion conditions.

Figure 21: pH stat device

Static models for simulation of gastrointestinal digestion represent the 95 % of the overall *in vitro* digestion models mainly due to their low cost and ease of

sample handling (Sams et al., 2016). From this 95 %, only 5 % use lipase for mimicking the intra-gastric lipolysis. Moreover, the pH for this step varies from 1.4-5.5 and the enzyme concentration from 17 μg/mL to 2,700 μg/mL depending on the lipase origin (Sams et al., 2016). As gastric lipase (GL) either human (HGL) or alternatives are not commercially available, the step of gastric lipid hydrolysis is overlooked in the Infogest and other proposed digestion models. This omission might not be full adequate for mimicking gastrointestinal conditions though, as gastric lipolysis reaches 10-30 % of the overall lipolysis in human digestion (Singh et al., 2009). It is also known that gastric lipase further triggers pancreatic lipase activity in *in vitro* experiments (Gargouri et al., 1986) thus altering the overall lipid hydrolysis of foods. Gastric lipase is characterized as an extremophilic enzyme due to its ability to be active at acidic conditions (pH as low as 2) in the presence of pepsin (Aloulou and Carrière, 2008).

It is widely known that lipases do not act on a specific free substrate but on lipid aggregates, mixed micelles or lipid monolayers interfacing with an aqueous medium (Reis et al., 2009). HGL exhibits some unique biochemical characteristics in terms of stereospecificity, optimum pH and other parameters. More specifically, although the enzyme is able to hydrolyze the three ester bonds of TAGs, it shows a preference for the hydrolysis of sn-3 position (Ransac et al., 1990, Rogalska et al., 1990). This is important for its biological function since the release in the stomach of short or medium FAs specifically found in this position could find a faster route for energy production when absorbed in the stomach (Sams et al., 2016). Whatever the substrate, the optimum activity of HGL is at the range of pH 4-6 (Carriere et al., 1991). Another specificity of HGL is the fact that it is active in the presence of physiological concentration of bile salts. Also, the critical surface pressure for HGL penetration into a model phosphatidylcholine (PC) monolayer is 23-25 mN/m whereas for HPL is 15-18 mN/m. HGL is thus as amphiphilic as the HPL –colipase complex (1:1 molar ratio), that penetrates to the monolayer of PC with a critical surface pressure of 27 mN/m (de La Fournière et al., 1994). Dog gastric lipase (DGL) is one of the most commonly used lipases to replace HGL due to its 85.7% amino acid sequence identity, sn-3 stereospecificity and resistance to pepsin (Carriere et al., 1991). What is more, DGL shows the highest hydrolytic activity on long chain triglycerides (LCTs) at low pHs with an optimum activity at pH 4 (Roussel et al., 2002, Aloulou and Carrière, 2008). Figure 22 shows the 3D structures of HGL. The structure provided in B panel of open HGL was built from the HGL and rDGL sequence alignment and the known X-ray structure of rDGL at the open-lid conformation (Sams et al., 2016).

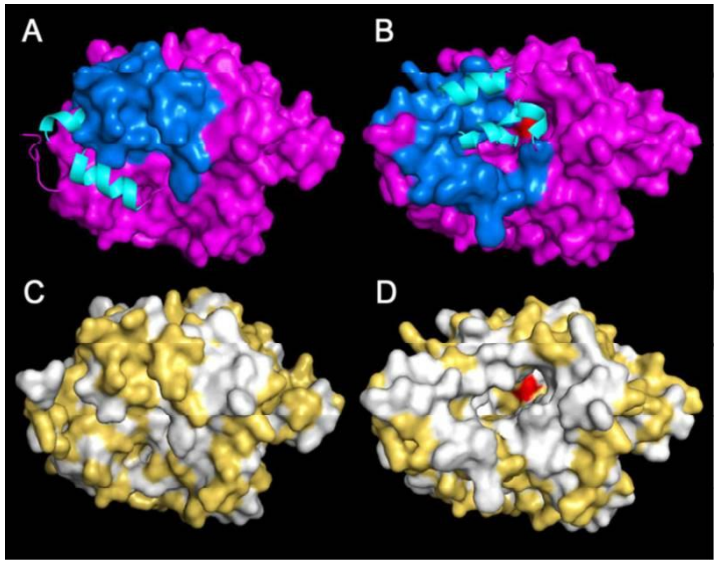

Figure 22: Human gastric lipase (A) closed lid and (B) open lid conformation. All residues are colored in purple except the lid that is colored in blue. The active site serine residue (Ser153) is shown in red. (C) and (D) same views as A and B, respectively but hydrophobic amino acids are colored in white while polar amino acids in yellow. (Sams et al., 2016)

For the duodenal digestion, HPL, co-lipase and bile salts excreted from the pancreas are mainly involved in *in vitro* digestion simulations. The first crystal structure of HPL was proposed by Winkler and his team more than 20 years ago (Winkler et al., 1990). Pancreatic lipase has an sn-1, 3 specificity on TAGs, but acts also on the sn-2 bonds at a very low rate. However, it cannot act without the aid of an 11kDa protein, the co-lipase (Lowe, 2002). As mentioned above, the surface pressure for penetrating the phospholipids monolayer is increased for the lipase- co-lipase complex increasing the amphiphilic properties of the HPL. Bile salts are surfactants that compete with the lipases for the binding at the lipid-water interface thus preventing the enzyme from reaching the substrate. To overcome this problem, HPL forms a complex with the co-lipase (van Tilbeurgh et al., 1993) as shown in figure 23. Also, co-lipase stabilizes PL to its open, active conformation (Chapus et al., 1975).

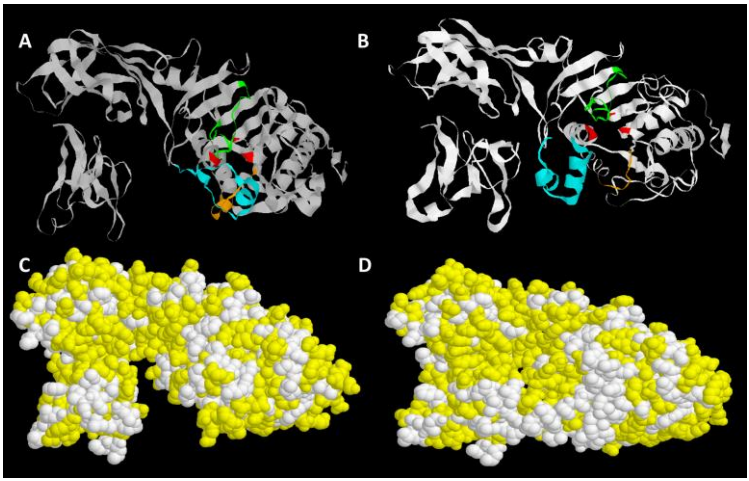

Figure 23: Human pancreatic lipase co-lipase complex in a closed (A), (C) (RCSB Protein Data Bank ID: 1N8S)(van Tilbeurgh et al., 1992) and open (B), (D) (RCSB Protein Data Bank ID: 1LPA) (van Tilbeurgh et al., 1993) conformation as produced by RasMol computer program (Sayle and Milner-White, 1995). In panels (A), (B) the lid and catalytic triad are represented in cyan and red respectively. The β5and β9 loops are represented in orange and green respectively. In panels (C) and (D) hydrophobic residues are colored in white while polar residues in yellow.

While PL and co-lipase are commercially available at low cost mainly via porcine pancreatin, which contains both lipase and co-lipase, replacing GL by other lipases may sometimes be arbitrary and thus misleading. In a recent review, Sams et al. report that lipases from different origins have been proposed to mimic the action of HGL, namely bacterial, fungal or mammalian lipases (Sams et al., 2016). Unfortunately, these replacements are not always significant, in terms of sn-3 specificity, or pepsin resistance thus indicating a low relevance for simulating the intragastric lipid hydrolysis.

GL has been proposed as a good candidate for pancreatic enzyme replacement therapy (PERT) in patients with pancreatic exocrine insufficiency (PEI) (Moreau et al., 1991, Junien, 1993). Unfortunately, the low availability of HGL from gastric juice along with ethical considerations, have limited the use of such enzymes. On the other hand, recombinant HGL from different organisms have been explored from the 1980's using expression systems with low industrial potentials (Sams et al., 2016). To overcome the cost challenge for PERT patients, the company Meristem Therapeutics SA, Clermont-Ferrand in France started producing rDGL from transgenic plants at a reasonable cost (US $ 5.90-

43/g) (Zhong et al., 2006). The production and clinical development of rDGL was however seriously damaged due to concerns about GMO and transgenic plants and the company stopped its activities in 2008. Although rDGL is not being produced today, within the frame of the present work, a batch of rDGL produced from transgenic maize from the above company was used for mimicking the gastric step.

In 1993, Carrière and his team measured the specific activities o HGL and HPL on dietary TAGs of a test meal in healthy volunteers (Carriere et al., 1993). They found that the 17.5 % of the overall lipolysis is accomplished due to the gastric lipase in humans pointing out the importance of the gastric step to the hydrolysis of lipids. Some years later, in 2000, the same team observed that at 50 % of liquid meal emptying, the pHs at gastric and duodenal phases were different than those supported from *in vitro* studies so far (Carrière et al., 2000). For the case of gastric step, the pH at 50% of a liquid meal emptying on healthy volunteers was reported to be 5.5, while for the duodenal step under same conditions the pH was 6.25. Figure 24A represents the experimental device for the *in vivo* studies of the ingestion of a solid meal (Carrière et al., 2001).

To this respect, they established an *in vitro* two-step digestion model based on the above experiments in healthy volunteers. More specifically, the samples under investigation are mixed with gastric lipase and gastric juice under constant stirring. The pH for this step is set to 5.5 and FFAs release is recorder for 30 min using the pH stat device. Following, the pH-endpoint is shifted to 6.25 while pancreatic lipase and co-lipase, bile salts and phospholipids are added to the solution. The FFAs release is being recorded for an additional 60 min period for the duodenal step. The findings are calculated and the overall lipolysis level or the μmoles of FFAs released during the 90 min assay are expressed in a graphical representation as illustrated in Figure 24B.

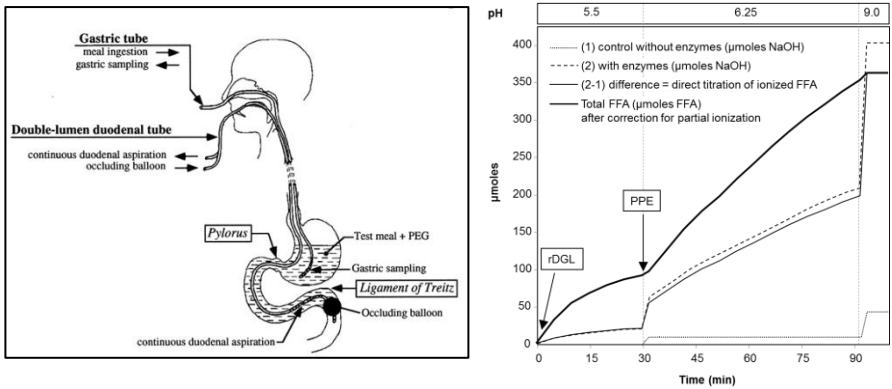

Figure 24: A. Experimental device for in vivo studies in healthy volunteers. Gastric and 2-lumen duodenal tubes were used with the solid test meal to collect gastric and duodenal samples (Carrière et al., 2001). B. Graphical representation of two-step in vitro *lipid hydrolysis expressed as µmoles of free fatty acids (FFAs) versus time.*

During a lipid digestion, lipases are exposed to a variety of emulsifiers, either occurring naturally in the gastrointestinal tract, or added with foods, or produced with lipolysis. Lipase-surfactant interactions have been extensively discussed in the literature (Reis et al., 2008, Delorme et al., 2011, Li et al., 2011). It is observed that lipase activity is proportional to the available lipid-water interface. Thus, the existence of surfactants at specific concentrations enhances lipase binding at the interface through increasing the surface-to-volume ratio. On the other hand, surfactants could compete with lipases for interfacial adsorption (Figure A) (Delorme et al., 2011). Depending on their structure or concentration, these surface active molecules may promote or decrease the activity of the lipases.

Generally, in the presence of surfactants, lipases undergo conformational changes, adapting to the new environment without losing their catalytic properties (Delorme et al., 2011). For example, HPL was observed to open the so-called "lid" thus giving access to the active site of the lipase in the presence of bile salts and phospholipids (van Tilbeurgh et al., 1993). Tiss and co-workers reported the increase of lipolysis and lipase adsorption in the presence of arabic gum (acacia), an amphiphilic substance (Tiss et al., 2001).

Lipase inhibitors could form aggregates or be present in lipid-water interfaces (Figure 25B). The amphiphilic lipase inhibitor Orlistat (tetrahydrolipstatin, THL) is the only globally licensed anti-obesity drug currently on the market. The drug can be found in two different products: Xenical^{TM} (Roche) which is given to patients on medical prescription and Alli^{TM} (GSK) which contains a

lower drug dosage and is available without prescription. The inhibitor covalently reacts with the serine residue of the catalytic triad thus inactivating the GI lipases (Carrière et al., 2001).

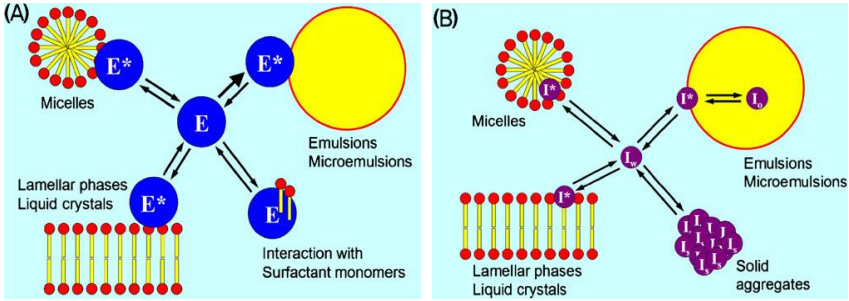

Figure 25: Possible location of (A) a lipase or (B) a lipase inhibitor in the presence of surfactants and lipids (Delorme et al., 2011)

Interestingly, it was observed that the efficacy of Orlistat strongly depends on the type of meal ingested. The TAGs of a liquid meal, pre-emulsified in the presence of phospholipids, were efficiently digested before the HPL was inhibited by the drug, resulting to a poor drug efficacy. On the other hand, when a solid-liquid meal was given to volunteers eating TAGs in a non-emulsified form, HPL was sufficiently inhibited by the drug leading to poor lipid hydrolysis (Carrière et al., 2001).

To conclude, amphiphilic molecules may strongly affect the behavior of lipases either by enhancing or decreasing and inhibiting their activity. Depending on the nature and concentration of the surfactants and other molecules, they could activate or deactivate them. This reversible effect could be beneficial for pharmaceutical applications for the treatment of type 2 diabetes (Petry et al., 2004), atherosclerosis (Jin et al., 2003) and others. Static models for simulating GI lipolysis have been found to be a relatively cheap and reproducible model for understanding the GI conditions as well as the behavior of naturally occurring molecules used as food additives under specific conditions. Also, especially for encapsulated bioactive molecules in heterogeneous matrices with applications in the pharmaceutical or food sector, the investigation of lipid hydrolysis and enzyme behavior is crucial for the design of such systems.

7 Purpose of the study

The aim of the present study was to formulate W/O nano-dispersions to be used as carriers of hydrophilic nutrients with uses mainly at the food sector. Generally, for the proposed microemulsions and emulsions, the continuous phase used was mostly based on medium- or long-chain triglycerides. The edible emulsifiers under study were mainly lecithin, DMG or their combination in accordance with low cost industrial requirements. More specifically, the present study was divided in three sub-projects. In all cases, microemulsions or emulsions were effectively formulated. Following, structural characterization of the formulated systems was investigated using various techniques as will be thoroughly analyzed below. Specific bioactive molecules were chosen to be encapsulated in the systems. The loaded systems were assessed for the efficacy of therein encapsulated molecules in order to qualitatively verify the release of these molecules under specific circumstances. Finally, some of the colloidal dispersions were investigated for their behavior under gastrointestinal conditions using a two-step digestion model. As shown in the diagram on the next page, the present study was divided in three sub-categories that will be analyzed in the next chapter.

PhD Objectives
PAPER 2
• Microemulsion Formation
• Antioxidants encapsulation and assessment
• Structural characterization (DLS, EPR, Cryo-TEM)
PAPER 1
• Emulsion formation
• Stability measurements
• PGPR fraction determination
• RI matching
PAPER 3
• Microemulsion formation
• Structural comparison with Emulsion (DLS, EPR, Cryo-TEM)
• HT encapsulation and antioxidant Assessment
PAPER 5
• HT-loaded dispersions
• Assessed under GI conditions
• 2-step digestion
• rDG and PP lipases
PAPER 4
• Microemulsion formation
• Structural characterization (SAXS, EPR)
• Nisin encapsulation and antimicrobial assessment
PATENTS 1, 2

8 Description of the experimental data - Papers

8.1 Sub-project 1 (Paper 2)

The aim of the second paper (PAPER 2) was the formulation of a microemulsion made of food grade compounds. This colloidal structure has been used as a matrix of four antioxidants found in a variety of natural sources. The antioxidant-loaded microemulsions were examined for their structural characteristics using DLS, EPR spin probing technique and Cryo-TEM. Following, a comparative study was conducted in order to assess the radical scavenging activity of the encapsulated antioxidants.

More specifically, microemulsions from water as the dispersed phase and a binary mixture of isopropyl myristate and medium chain triglycerides as the oil phase were formulated. Also, a mixture of ethanol, glycerol and lecithin was used as the emulsifier. The bioactive molecules used for this study were known antioxidants of natural origin such as gallic acid, tyrosol, p-hydrobenzoic acid and protocatechuic acid (Shahidi et al., 1992).

First, a phase diagram was conducted in order to determine the monophasic area of the inverse microemulsions (L_2 region). A specific point within this monophasic area, namely 49:49:2 (emulsifier: oil: water) was chosen for further investigation. The antioxidants under study were first solubilized in ethanol at desired concentrations. The solution was transferred in tubes and let until ethanol evaporation. Finally, 1 g of microemulsion was added to the tubes where the antioxidant had been previously deposited having an overall concentration of 0.7 mM. Solubilization was accomplished by gentle stirring.

Particle size measurements were performed for the empty and antioxidant-loaded microemulsion using DLS. It was observed that in the presence of gallic acid, tyrosol and protocatechuic acid, the inverse micellar size was slightly affected. The higher difference was observed in the presence of p-hydrobenzoic acid where the apparent hydrodynamic diameter was recorder from 5 nm (empty microemulsion) to 11 nm (p-hydrobenzoic acid encapsulation) resulting to swollen inverse micellar structures.

Interfacial properties were studied by EPR spin probing technique employing the nitroxide 5-doxyl stearic acid (5-DSA) following the procedure established elsewhere (Papadimitriou et al., 2007). The determined order parameter (S) (Griffith and Jost, 1976) showed no significant alterations in the membranes rigidity in the presence of antioxidants. The rotational correlation time (τ_R) (Kommareddi et al., 1994) was slightly decreased in the presence of antioxi-

dants indicating possible interactions of the antioxidants with the emulsifiers' polar head groups.

The microemulsion was observed using Cryo-TEM in order to determine the structure of the reversed micelles in the absence of antioxidants. The observation of the soft self-assembled structures indicated the existence of thread-like reversed micelles.

Finally, radical scavenging activity of the encapsulated natural antioxidants was estimated using EPR towards the stable radical galvinoxyl. The method was based on the ability of the entrapped bioactive molecules to react with galvinoxyl quenching its EPR signal (Niki, 2010). The scavenging reaction was followed for a 30 min period. Gallic acid showed a greater scavenging activity towards galvinoxyl compared to the other antioxidants under investigation. Following, in order to evaluate and compare the radical properties of the encapsulated bioactive molecules, their scavenging effect was expressed as Trolox equivalents. This was accomplished by encapsulated Trolox in the matrix of the microemulsions and constructing its standard curve at a specific time point. Experimental results revealed that after 10 min of incubation, gallic acid quenched galvinoxyl EPR signal at 78.8 % of its value in the absence of the antioxidant. This result could be expressed as 0.93 mM Trolox equivalents.

8.2 Sub-project 2 (PAPER 1, PAPER 3 and PAPER 5)

The first paper (PAPER 1) describes a novel method to formulate W/O emulsions with relatively small particle size which could be used in the food industry as carrier of hydrophilic compounds. The method involves the preparation of coarse emulsions which are then submitted to shear under laminar flow to reduce mean droplet size with subsequent negligible heat generation. The systems were able to successfully incorporate a range of 5-10 % aqueous phase retaining their mean droplet size for at least two months.

The basic principle concerning the formation of emulsions with small droplets is the fact that in order for the fragmentation to occur, a relatively large viscosity would lead to droplet rupture under low shear rates. In this work, a W/O emulsion was formulated starting from a coarse highly viscous emulsion with a high percent of surfactants. The aqueous solution contained sucrose or sucrose and $CaCl_2$ in order to increase the refractive index and thus, match it with that of the continuous phase (sunflower oil- SO). What is more, with this addition, the OR effect could be compensated due to an osmotic gradient tending to oppose to the OR (Kabalnov, 2001). The binary mixture of emulsifiers used and tested at different weight ratios were PGPR and DMG, two well-

known lipophilic emulsifiers extensively used in food industry (Benichou et al., 2001). Viscous emulsions were then subjected to strong shear using the Couette's cell to produce emulsions' droplets in the sub-micron size range. Finally, once the coarse emulsions were formed; they were simply diluted with SO to set the final aqueous fraction to 5 or 10%. For comparative reasons, a microfluidizer was also used to form the emulsions.

In the first set of experiments, different PGPR weight fractions at the premix were tested in order to obtain W/O emulsions with an average diameter lower than 250 nm. It was observed that the average droplet diameter was decreased with the increase of PGPR concentration which could be explained by two main reasons. Firstly, the rate of diffusion is increased with surfactant concentration leading to a decrease for coalescence phenomena to occur. Secondly, according to the Laplace pressure equation, the droplet size is inversely proportional to the viscosity of the system. Consequently, the increase of surfactant concentration increases the overall viscosity thus facilitating the rupturing of the droplets.

In the second set of experiments PGPR was partially replaced by an emulsifier with a higher daily acceptance intake for the use in food industry, namely DMG. It was found that for PGPR weight fractions lower than 55% in the PGPR/DMG mixtures, emulsions were impossible to be formed. What is more, the lowest mean droplet size was accomplished for a PGPR fraction 65-70 % for an overall 2.5 and 2 % emulsifier amount.

Following, the stability of the final emulsions was studied by measuring the average droplet evolution in time using the DLS technique. It was found that for a total 1.3 % of emulsifier content, the mean droplet size gradually evolved from 170 nm to 200 nm within the first 80 days. All the above formulated emulsions remained macroscopically turbid.

To overcome this problem, different solutes were added at the aqueous phase in order to increase the refractive index (RI) of the water to match with that of the SO (1.470). To this respect, three possible compounds were used, namely glycerol, sucrose and $CaCl_2$. Glycerol was found to reach the required RI at its 98% in water resulting to a very viscous solution that was not compatible with the desired application requirements (Mabille et al., 2000). Finally, a mixture of sucrose or $CaCl_2$ was used to obtain the required RI. It was finally found that even though the RIs of oil and aqueous phase were not fully matched, partial transparency was accomplished at 65% overall solutes concentration. Comparing Couette's cell technique with manual stirring and Ultra Turrax device, it was found that the macroscopic transparency was only obvious with emulsions with relatively low average diameters, like the ones obtained using the Couette's cell. To point out the importance of the emulsion fabrication using the Coutte's cell

technique, a comparative study using a microfluidizer was assessed. It was found that using the latter device, the emulsion droplets were initially lower (150 nm) and the system transparent. However, a fast droplet evolution, probably due to coalescence, occurred. This was probably due to a chemical degradation of PGPR under high pressure homogenization process under the given oil environment.
Finally, HT was effectively solubilized to the aqueous phase of the emulsions containing 65 % sucrose. The emulsion contained 5 % of aqueous droplets and 1.3 % of PGPR/DMG mixture in the 65/35 ratio. The HT fraction relative to the overall mass was 500 ppm.

The aim of the third paper (PAPER 3) was the formulation of two different edible W/O colloidal dispersions, an emulsion and a microemulsion and their comparative study in terms of structural characteristics and effectiveness of therein encapsulated bioactive molecule. They both contained medium chain triglycerides as the continuous phase and were used as matrices of hydroxytyrosol (HT), an antioxidant present in extra virgin olive oil (EVOO) (Boskou, 1996). Particle size distribution and membrane dynamics were examined using DLS and EPR respectively. Furthermore, the proposed systems were investigated for the scavenging activity of the encapsulated antioxidant towards galvinoxyl stable radical.

The designed emulsion (as described in PAPER 1) was formulated using of edible emulsifiers, namely DMG and PGPR. Sucrose was solubilized in water in order to potentially stabilize the emulsion against OR and to match the refractive index of the oil phase (PAPER 1). The proposed microemulsion also contained a mixture of food grade emulsifier, namely, lecithin and DMG. The polar phase in this case was a mixture of water and propylene glycol, a practice commonly followed for the enlargement of the mono-phasic region (Yaghmur et al., 2002).

For the case of microemulsion a pseudo-ternary phase diagram was constructed in order to determine the monophasic region. A specific point within this L_2 area, namely 4.9:93.1:2 (emulsifier: oil: polar phase) was chosen for further investigation. For the case of emulsion, the final composition was 1.3: 93.7: 5 (emulsifier: oil: aqueous phase). Hydroxytyrosol was solubilized at the polar phase of each system and then incorporated to the emulsifier: surfactant mixtures to finally achieve 500ppm and 600ppm for the emulsion and microemulsion respectively.

Particle size measurements were obtained using DLS for the empty and HT-loaded systems. In was observed that the mean reversed swollen micelles size

was 20 nm and remained unaltered in the presence of HT. For the case of emulsion, mean droplet size was measured to be at 384 nm with a slight decrease to 354 in the presence of the bioactive molecule. It should be mentioned that stability studies were conducted for the latter case and it was found that the emulsion in the presence and absence of HT was stable for 12 days.

Membranes' dynamics were studied using the amphiphilic molecule 5-DSA. It was observed that for both dispersions the order parameter S was not affected in the presence of HT indicating no significant changes at the rigidity of the membrane. On the other hand, the rotational correlation time parameter slightly decreased indicating an increase of the spin probe's mobility in the presence of the antioxidant. Also, the viscosity of the systems did not significantly changed compared to that of the MCT alone.

Finally, the radical scavenging activity of encapsulated HT was measured against galvinoxyl stable radical. HT was found to be a very strong scavenger of galvinoxyl inhibiting its EPR signal at more than 90 % of its initial value within the first 5 min for both carriers. It should be noted that the proposed method involves the dilution of the HT-loaded systems to an isooctane solution containing galvinoxyl. From DLS measurements we observed that the particle size of the microemulsion remained unaltered whereas for the case of emulsion the droplet size reduced to 193 nm.

The aim of this work (PAPER 5) was to measure the digestibility of two W/O dispersions based on MCT and containing HT, an antioxidant found in EVOO and oil leaves. As described in a previous paper (PAPER 3), a microemulsion containing 5% of emulsifiers (lecithin/DMG) and 2% of aqueous phase and an emulsion containing 1.3% emulsifiers (DMG/PGPR) and 4.9% of aqueous phase, were successfully formulated. HT was also added to the aqueous phases with an overall concentration of 600 ppm and 500 ppm for the microemulsion and emulsion respectively. The lipolytic activities of recombinant gastric lipase (rDGL) and porcine pancreatic lipase (PPL) were examined using the pHstat technique at different pHs. A two-step *in vitro* digestion model was then introduced mimicking the physiological conditions found in the human gastrointestinal tract (Carrière et al., 2000).

The preparation of W/O microemulsions and emulsions as well as the incorporation of HT has been extensively discussed previously (PAPER 3). It should be mentioned that no significant changes were observed in the presence of HT on the mean droplet size of the systems under investigation.

The lipase activity of rDGL was measured at pHs ranging from 2-8 using MCT and W/O colloidal dispersions in the presence and absence of HT as sub-

strates. rDGL was active on all colloidal dispersions examined at examined pHs 3-7. Differences of the rDGL activity were observed depending on the substrate. More specifically, while the maximum specific activity on MCT (714 ± 26 U/mg) was recorded at pH 6, rDGL activity on W/O microemulsion was 4–fold lower and optimum was shifted to pH 4 while the activity slightly decreased in the presence of HT. On the other hand, the W/O emulsion was a better substrate with the highest specific activity (726 ± 8 U/mg) recorded with the HT-loaded emulsion. Although the activity level of the latter system was identical to that measured with MCT, the optimum pH was shifted to a more acidic pH value (5.0). In the absence of HT, rDGL activity on W/O emulsions was reduced 1.6-fold and optimum pH was 6.0.

Similarly, PPL was active on all systems used at examined pHs ≥ 4. As previously observed with rDGL, differences in optimum pH and activity level were observed with W/O dispersions compared to MCT. Namely, the maximum specific activity on MCT (8,600±141 U/mg) was recorded at pH 7 while the activity on W/O microemulsion was reduced 3.4–fold in the absence of HT and 5-fold for the HT-loaded microemulsion. For both in the presence and absence of HT in the microemulsions, the maximum activity of PPL was shifted to a more acidic pH (pH=6). The W/O emulsion was a much better substrate, with the highest specific activity of PPL (6,245 ± 530 U/mg) recorded in the absence of HT. HT loading had no significant effect under these conditions and maximum activity remained at pH 7.

Furthermore, the estimation of FFAs ionization (%) at various pH values was obtained for the calculation of the ratio between back and direct titration. Similar results were obtained with all dispersions and enzymes and thus the mean apparent of MCT was estimated to be 6.48, a value in accordance with previous findings (Bakala-N'Goma et al., 2015).

In vitro two step digestion model was finally estimated for MCT and the colloidal dispersions in the presence and absence of HT. The selected pH values namely 5.5 for the gastric phase (30min) and 6.25 for the duodenal phase (additional 60min) were those observed in gastric and duodenal contents of healthy volunteers at 50% gastric emptying of a liquid test meal based on previous studies (Carriere et al., 1993). The full titration of FFAs was impaired by the partial ionization of FFAs at pH 5.5 and 6.25. A back-titration at pH 9 was performed at t=90 min to ensure the full ionization of the FFAs release during the whole incubation period to determine the correction factors for the gastric (4.0±0.7) and duodenal (1.8±0.2) phases, and finally to estimate the total amounts (μmoles) of FFAs released as a function of time.

It is worth noticing that the overall decrease in lipolysis mainly results from the lower activity of rDGL during the gastric phase. Gastric lipolysis of empty and HT-loaded W/O microemulsion only represent 17.4 and 19.6 % of controls with MCT, while duodenal lipolysis of MCT and W/O dispersions are all found in a similar range.

During the first 10 min of gastric digestion, the rate of FFA release from W/O microemulsions (0.6-0.8 μmole FFA/min) was 6 to 9-fold lower than the rates recorded with MCT (4.9 μmole FFA/min), empty (5.1 μmoles FFA/min) and HT-loaded (5.0 μmoles FFA/min) W/O emulsions. At the end of the digestion period (75 to 90 min), the rates of lipolysis of empty (4.3 μmoles FFA/min) and HT-loaded (3.9 μmoles FFA/min) W/O microemulsions slightly exceeded those of MCT (3.7 μmoles FFA/min), empty (3.5 μmole FFA/min) and HT-loaded (3.5 μmole FFA/min) W/O emulsions.

8.3 Sub-project 3 (PAPER 4 and PATENTS 1, 2)

In the present study (PAPER 4, PATENTS 1, 2) W/O microemulsions based on refined olive oil (ROO) and SO were used as carriers of nisin and the effect of composition on the structure and efficacy were investigated. More specifically, DMG was used as emulsifier, and was held constant at 40% at all compositions while vegetable oil and ethanol mixtures were tested at specific weight compositions. Different ROO/ethanol mixtures were examined with the ethanol mass fraction ($W_{ethanol}$) ranging from 0.00-0.50.

SAXS experiments revealed a shift of the scattered broad peak to higher q-values with the addition of ethanol indicating a decrease in the characteristic distance of the inverse swollen micelles. A spherical core-shell model in combination with a hard sphere structure factor model was suggested for further SAXS analysis using the Percus-Yevick approximation (Percus and Yevick, 1958). It was found that for ethanol weight fractions ranging from 0.00-0.33 the dimensions of micelle-core and shell, as well as the overall size of the micelles were almost identical. Increasing ethanol weight fractions to 0.50 resulted to a significant overall decrease of the inverse micelle radius from 24.5 to 18.5 Å leading to an increase of the number of inverse swollen micelles. Similarly, the investigated systems were examined for the effect of hydration on the structure at a constant $W_{ethanol}$= 0.50. It was found that hydration induces a shift of the position of the detected broad peak to lower q values indicating that the inverse self-assembled nanostructures become larger with increasing the amount of solubilized water (swelling of the inverse micelles). A more detailed SAXS analysis using the same model as above on the samples 0 and 6 %, indicated that the radius of the hydrophilic core increased from 4.6 to 6.1 Å while the thick-

ness of the hydrophobic shell decreased slightly from 13.1 to 12.3 Å resulting to enlarged hydrophilic domains.

According to the above measurements the effect of vegetable oil used, resulted to more ordered structures when ROO was the vegetable oil used probably due to the larger contribution of the monounsaturated fatty acids (oleic acid) compared to the polyunsaturated ones (linoleic acid).

EPR spin probing technique using the amphiphile 5-DSA was used to determine the membrane dynamics of the reverse swollen micelles. EPR experiments were in accordance with SAXS data revealing an increase at the membranes fluidity at increasing ethanol fractions. Moreover, the mobility of the spin probe expressed by the rotational correlation time (τ_R) was also increased. EPR spectra simulations (Duling, 1994) suggested a good fitting ($R^2 \geq 0.996$) when two distinct 5-DSA populations at environments with different polarities were assumed. More specifically, the isotropic hyperfine splitting values (A_0) are sensitive to spin probes micro-polarity and increase with increasing polarity. It was found that for low ethanol content systems ($W_{ethanol}$= 0.08 Å) two states of 5-DSA "facing" a more polar (A_{01}=147.5 mT) and a less polar (A_{02}=137.1 mT) environments were detected. For the microemulsions with higher ethanol content ($W_{ethanol}$=0.50) the two states seem to converge to one polar environment.

Finally, the WDA was used for the determination of nisin-loaded microemulsions activity against *L. Lactis* MG1363 culture. WDA in inverse microemulsions at increasing ethanol revealed an increasing to the inhibition zones. More specifically, for the low ethanol content ($W_{ethanol}$=0.08) the inhibition zones were calculated to be 9.5±0.0 and 9.0±0.0 mm for ROO and SO-based systems respectively. For higher ethanol content ($W_{ethanol}$=0.50) inhibition zones were calculated 12.5±0.1 and 13±0.0 mm for the ROO and SO-based systems respectively. This could be due to the higher elasticity of the membrane leading to easier nisin diffusion to the outer aqueous culture medium.

This paper resulted in two patents. For patent 1, the formulated microemulsions are being proposed as carriers for the encapsulation of bacteriocins in a proportion of 0.5-5 % wt. for the antimicrobial protection of foods. In patent 2, the proposed systems could be used to encapsulate bioactive substances that are isolated by extraction from natural products. Both inventions could result in salad dressing type products for the food industry.

9 Conclusions

In PAPER 2, natural phenolic antioxidants such as gallic acid, tyrosol, p-hydrobenzoic and protocatechuic acids were encapsulated in food grade W/O

microemulsions. Scattering and spectroscopic techniques revealed perturbations in the presence of the added compounds possibly due to interactions between the encapsulated molecules and the surfactant monolayer. Cryo-TEM images indicated the existence of entangled reverse thread-like micellar structures. Finally, the systems were assessed for the scavenging activity of therein encapsulated molecules. The antioxidant activity was expressed in terms of activity of a known antioxidant, Trolox, commonly used as standard for antioxidant capacity studies. It was found that gallic acid had the higher scavenging effect against galvinoxyl stable radical possibly due to its structure. EPR spectroscopy could be used as a simple and sensitive method to provide information about the scavenging effect of targeted molecules even when encapsulated in a microheterogeneous medium. The information generated in this study could potentially facilitate the design and development of novel systems for food or cosmetics industrial applications.

In PAPER 1 we developed a novel strategy for the preparation of edible W/O emulsions based on low shear rate processes favorable for the preservation of systems containing thermo-sensitive compounds or bioactive molecules. The minimum average droplet size was close to 175 nm while the viscosity was identical to that of the continuous phase. The emulsions could be macroscopically transparent by dissolving hydrophilic solutes which concomitantly offered stability against Ostwald ripening. Moreover, HT was effectively solubilized in the aqueous phase of the dispersion. The present approach could be applicable not only in the food sector but also in the pharmaceutical and cosmetics design.

In PAPER 3, a comparative study of two different W/O dispersions, an emulsion and a microemulsion was carried out. The dispersions contained MCT as the continuous phase and were able to effectively encapsulate HT. Generally the systems were tested for their structural characteristics using scattering and spectroscopic techniques. Even though the emulsions droplet size was approximately 18-fold higher than that of microemulsions, the information about membrane dynamics revealed an almost identical behavior. Generally, both systems showed good potential as carriers of HT as revealed from the high scavenging activity of the bioactive when encapsulated in the dispersed phase. In this paper, we were able to formulate a stable emulsion by applying manual stirring and obtaining a relatively small droplet size. Furthermore, we were able to fabricate a microemulsion with a relatively small amount of edible emulsifiers that could be an ideal candidate for long-storage food applications. It is interesting that the above dispersions could be also used for pharmaceutical or cosmetics applications for the encapsulation of hydrophilic substances.

PAPER 5, discusses the fact that the lipolysis of the triglycerides contained in W/O dispersions can be significantly impacted by the emulsifiers used to form these systems, and to a lower extent, by the bioactive molecules encapsulated in them. The lower surfactant concentration of emulsions did not seem to alter lipases' activity while microemulsions resulted in a much lower digestive process. This finding may assist the need of slowing down the digestion processes in pathological conditions such as the ileal brake and obesity. It is worth noticing that within the frame of this work, for the case of W/O microemulsions the gastric lipolysis was drastically reduced. Although the rate of duodenal lipolysis was not changed, the specific inhibition of gastric lipolysis allowed a delay in the overall lipolysis process that could be beneficial under specific pathogenic circumstances.

In PAPER 4 & PATENTS 1, 2, W/O microemulsions containing DMG of plant origin and two edible vegetable oils, ethanol and water were investigated for their structural characteristics. The structural investigation comparing the two oils revealed a relatively more ordered structure for the ROO-based systems while for the SO-based ones a relatively more flexible membrane was apparent. The effects of hydration and ethanol concentration were also investigated. Analysis of the SAXS data revealed changes in the micellar size and shape with increasing ethanol concentration. Hydration, on the other hand, induced an enlargement of the reverse swollen micelles due to water uptake in their hydrophilic cores. EPR spectra simulation revealed the existence of two distinct 5-DSA populations that seem to converge to one more polar environment upon ethanol increase. Finally, the developed microemulsions were loaded with nisin and tested for its antimicrobial activity against *L. lactis*. It was observed that the inhibition zones were increased with ethanol addition, probably due to the fact that ethanol induces a more flexible DMG monolayer thus facilitating nisin's diffusion. Overall, by studying the structural modulations of the proposed W/O microemulsions in terms of microstructure properties, one is able to tune liquid isotropic phases to design systems with desired characteristics. The patents 1, 2 are based on these systems proposing salad dressing type products with enhanced characteristics such as antioxidant or antimicrobial properties. What is more, further experimentation of these systems either by the replacement of ethanol or by introducing new components with known antimicrobial activity such as essential oils would also be interesting.

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