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Process optimization of nanoemulsion containing olive oil: high-pressure homogenization and D-phase emulsification

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Processes optimization of nanoemulsion preparation containing olive oil: high-pressure homogenization and D-phase emulsification

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Dedication

I would like to dedicate this work to my family, my friends and especially to my husband, who had been my joy and my support during this journey.

II

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I would like to express my sincere gratitude and appreciation to my enthusiastic supervisor, Dr. Nádia Araci Bou Chacra, for her huge support and guidance. This work would not be successful without lots of hard work, which we have experienced and overcome together. Each step of work was an apprenticeship for me. Therefore, I thank her for the patience and belief on me.

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RESUMO

YUKUYAMA, M.N. Otimização de processos de obtenção de nanoemulsões contendo óleo de oliva: homogeneização a alta pressão e emulsificação empregando fase D. 2017. 215p. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo.

O óleo de oliva apresenta elevado potencial de aplicação no desenvolvimento de produtos farmacêuticos e cosméticos. No campo farmacêutico, esse óleo tem sido utilizado para a preparação de nanossistemas de liberação de fármacos que apresentam baixa solubilidade em água. Tais nanossistemas, incluindo as nanoemulsões, podem ser obtidos empregando processos de alta (mecânico) e baixa energia (físico-químico). A proposta do presente estudo foi adquirir maior entendimento dos processos de preparação e da aplicação de nanoemulsão por homogeneização a alta pressão (HAP) e emulsificação empregando fase D (EFD), respectivamente métodos de alta e baixa energia. O conhecimento adquirido pela sistematização da pesquisa e das atividades executadas resultou em dois artigos de revisão e dois artigos de pesquisa. O primeiro artigo de revisão permitiu identificar tendo em vista o sucesso do desenvolvimento de nanoemulsão, a necessidade de conhecimento da interação entre o fármaco e os componentes da nanoemulsão; do impacto do processo de preparação nos componentes e na estabilidade da nanoemulsão; e da influência da formulação na liberação e na absorção do fármaco por diferentes rotas de administração. Além disso, apresentamos as diferentes nanoemulsões, considerando o tipo de tensoativo utilizado para sua preparação e seu respectivo uso. O segundo artigo de revisão evidenciou a adequada seleção de processo como o fator essencial para assegurar a obtenção de nanoemulsão com propriedades pré-determinadas, oferecendo aos pesquisadores alternativa realista para sua produção em escala industrial. Com referência aos artigos de pesquisa, o primeiro comprovou a possibilidade de obtenção de nanoemulsão contendo elevado teor de óleo vegetal (40% m/m) utilizando único tensoativo em

concentração de 2% m/m. Esse desempenho considerado como desafio nos processos convencionais de baixa energia, foi realizado com sucesso utilizando o processo EFD. O poliol foi confirmado como variável estatisticamente significativa para a redução do diâmetro hidrodinâmico médio (DHM) da nanoemulsão, influenciando de maneira sinérgica o comportamento do tensoativo durante a fase de transição nesse método. O estudo referente ao segundo artigo permitiu a identificação e o entendimento da relação entre as variáveis de composição e de processo e o DHM, no desenvolvimento de nanoemulsão contendo óleo de oliva, empregando os processos HAP e EFD. A abordagem estatística revelou os intervalos ótimos das variáveis de composição e de processo para a obtenção do DHM desejado, o atributo crítico de qualidade. Assim, foi adquirido maior entendimento dos respectivos processos. Adicionalmente, o conceito do espaço de design e a otimização por meio da função desejo permitu a obtenção de nanoemulsão de 275 nm com sucesso, empregando a mesma composição para ambos os processos: HAP e EFD. Os conhecimentos adquiridos nesses estudos poderão direcionar o desenvolvimento de nanoemulsão inovadora, com êxito.

Palavras-chave: nanoemulsão, óleo vegetal, homogeneização a alta pressão, emulsificação empregando fase D, processo de alta energia, processo de baixa energia.

ABSTRACT

YUKUYAMA, M.N. Processes optimization of nanoemulsion preparation containing olive oil: high pressure homogenization and D-phase emulsification. 2017. 215p. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo.

Olive oil has an extensive applicability in the development of pharmaceuticals and cosmetics. In the pharmaceutical field, this oil has been used for the preparation of poorly water-soluble drug delivery nanosystems. Such nanosystems, including nanoemulsions, can be obtained by employing high (mechanical) and low energy (physicochemical) processes. The aim of the present study was to acquire the deep understanding of the processes of preparation and applicability of nanoemulsion by high-pressure homogenization (HPH) and D-phase emulsification (DPE), respectively high- and low-energy methods. The knowledge acquired through the careful systematization of the research and the activities performed resulted in two review articles and two research articles. The first review article allowed to identify, targeting development of a high efficacy nanoemulsion, the need to understand the interaction between the drug and nanoemulsion components; the impact of the preparation process on the components and the nanoemulsion stability; and the influence of the formulation on the release and uptake of the drug by different administration routes. In addition, we described the development of different nanoemulsions, according to the selected surfactant for their preparations, and their respective application. The second review article evidenced the appropriate process selection as the key factor to ensure obtaining nanoemulsions with desired properties, offering to researchers, a realistic industrial scale alternatives for the development of nanoemulsions. Regarding the research articles, the first one confirmed the possibility of obtaining nanoemulsions containing high vegetable oil content (40% w/w) using a single surfactant at a concentration of

2% w/w. This challenge, considered as a challenge in conventional low energy processes, was successfully accomplished using the DPE process. The polyol was confirmed to be a statistical significate variable for the reduction of the nanoemulsion mean particle size (MPD), by synergistically influencing the behavior of the surfactant during the transition phase in this method. The second research article allowed identifying and understanding the relationship between composition and process variables and MPS in the development of olive oil nanoemulsion, using HPH and DPE processes. The statistical approach allowed the identification of optimal ranges of composition and process variables for obtaining the desired MPS, which is the critical quality attribute. Therefore, a better understanding of the respective processes was acquired. In addition, the design space concept and the optimization by means of the desire function allowed obtaining a 275 nm nanoemulsion with success, using the same compositions for both HPH and the DPE processes. The knowledge acquired in these studies will successfully allow directions for the future development of innovative nanoemulsion.

Key words: nanoemulsion, vegetable oil, High Pressure Homogenization, D-Phase Emulsification, high-energy process, low-energy process.

Preface

This thesis is an original work by Megumi Nishitani Yukuyama completed under the supervision of Prof. Dr. Nádia Araci Bou-Chacra at the University of São Paulo. Most of this work was performed in the Dr. Bou-Chacra lab facilities. Some experiments were also carried out at different lab facilities at the University of São Paulo, as Dr. Kato lab at Department of Pharmacy and Dr. Oliveira and Dr. Fantini of Institute of Physics, both at University of São Paulo.

Chapter 1 of this thesis is the starting chapter focused on the current review of different nanoemulsion types and their applications, emphasizing their challenges and prospects. The drug interaction with the components of the formulation, as well as the drug mechanistic interaction with the biological environment of different routes of administration was also highlighted. It was published as Megumi Nishitani Yukuyama, Edna Tomiko Myiake Kato, Raimar Lobenberg and Nádia Araci Bou-Chacra, with the title of Challenges and Future Prospects of Nanoemulsion as a Drug Delivery System in **Current Pharmaceutical Design**, 2017, 23, 495-508.

Chapter 2 of this thesis highlighted the main high- and low-energy methods applicable in cosmetics and dermatological nanoemulsion development. It also mentioned their specificities, recent research of these methods in the cosmetics, and the consideration for the process selection optimization. It was published as Megumi Nishitani Yukuyama, Daniela Dal Molim Ghisleni, Terezinha de Jesus Andreoli Pinto and Nádia Araci Bou-Chacra, with the title Nanoemulsion: process selection and application in cosmetics – a review in **International Journal of Cosmetic Science**, 2016, 38, 13–24.

Chapter 3 of this thesis aimed attention at the D-phase emulsification (DPE) process, a less explored among the low energy methods, which can overcome the drawbacks of conventional phase inversion temperature and phase inversion composition methods for obtaining a nanoscaled emulsion. This process has an advantage by not requiring strict adjustment of hydrophilic-lipophilic balance for the preparation of fine particle emulsions, nor the presence

of initial water-in-oil (W/O) phase as in the conventional phase inversion methods. The behavior of the DPE process intermediate transition phase was investigated in this study, and a nanoemulsion with mean particle size of 20 – 30 nm was successfully obtained applying the DPE process and Box-Behnken design. This study will be published as Megumi Nishitani Yukuyama, Pedro Leonidas Oseliero Filho, Gabriel Lima Barros de Araujo, Edna Tomiko Myiake Kato, Raimar Lobenberg, Cristiano Luis Pinto de Oliveira, and Nádia Araci Bou-Chacra, with the title D-phase emulsification as a unique low energy process: high internal vegetable oil nanoemulsion. It was submitted to the International Journal of Pharmaceutics.

Chapter 4 of this thesis highlighted the concept of Design Space and Quality by Design, using statistical approaches in obtaining nanoemulsions of olive oil, by high- and low-energy processes. In the high-energy process, also known as mechanical, high-pressure homogenization (HPH) was used. In the process of low-energy or physicochemical, the DPE process was employed. A deep understanding of input factors which influence the associated output responses of both methods was achieved. The optimization using the desired function provided a unique range of critical process parameters within the design space, where nanoemulsions with similar mean particle sizes of 275 nm could be achieved with equal composition, by both HPH and DPE processes. This work is under submission process for publication.

Appendix was a collaboration work for a book chapter. It summarized the principles of the formulation to develop nanoemulsions of an esthetic level and relates some specific applications, targeting cosmetic field. The chapter begins with a section on methods of obtaining nanoemulsion via high- and low-energy, pointing out their most relevant advantages, disadvantages and fundamental parameters. It also includes formulation parameters for controlling the stability and offering highly desirable sensory characteristics with functional performance. The end of the chapter provides a summary of the context and needs of the skin, hair care and scalp care, as well as the recent nanoemulsion researches on these fields. This work is under publication process.

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JUSTIFICATION

Nanoemulsion is an oil-in-water or water-in-oil nanoscaled emulsion with the advantage over other nanostructured systems by allowing a high concentration of liquid lipid phase in its core, increasing the solubility of poorly water-soluble drugs. Virgin olive oil shows extensive applicability in several fields, providing anti-oxidant properties and acting as a drug carrier in the nanoemulsion system. One of the most used high-energy processes for obtaining nanoemulsion is the High-Pressure-Homogenization (HPH), and the Phase Inversion Temperature (PIT) in the low-energy process. Nevertheless, the conversion of vegetable oil into a nanoscaled emulsion by a conventional low-energy process, as PIT, is considered a challenge, due to the complex structure of vegetable oils compared to the most used hydrocarbon oils. Therefore, the D-Phase Emulsification method (DPE) has been explored as an alternative process to overcome this difficulty. Although the first studies of this process had been presented decades ago (most in the Japanese language), the advances in understanding mechanisms of DPE were modest. Therefore, a deeper study of this process is required to elucidate this unique low-energy process. Additionally, the design space, within the quality by design environment, targets the better understanding of the product and process aiming to improve the quality and safety of the final product. Thus, the appropriate statistical approach, as the experimental design applied in this study, rationally conducted the study of process and composition variables for HPH and DPE process, as well as their preparation optimization. Therefore, the present study offers significant benefits to the formulator and to the consumer. The acquired knowledge will allow the development of innovative products, expanding the perspectives of the application of nanoemulsions in the pharmaceutical and cosmetic fields, thus justifying the present work.

1. CHAPTER 1: Challenge and Future Prospects of Nanoemulsion as a Drug Delivery System

This study was published as Megumi Nishitani Yukuyama, Edna Tomiko Myiake Kato, Raimar Löbenberg and Nádia Araci Bou-Chacra, with the title of Challenges and Future Prospects of Nanoemulsion as a Drug Delivery System in ***Current Pharmaceutical Design****, 2017, 23, 495-508.*

ABSTRACT

Nanoemulsion has the potential to overcome several disadvantages in drug formulation. Loading poor water-soluble drugs in the appropriate nanoemulsions enhances their wettability and/or solubility. Consequently, this improves their pharmacokinetics and pharmacodynamics by different routes of administration. Associated with the optimum nanodroplets size or even combined with key components, the droplets act as a reservoir of drugs, enabling nanoemulsion to be multifunctional platform to treat diverse diseases. A number of important advantages, which comprise nanoemulsion attributes, such as efficient drug release with appropriate rate, prolonged efficacy, drug uptake control, low side effects and drug protection properties from enzymatic or oxidative processes, have been reported in last decade. The high flexibility of nanoemulsion includes also a variety of manufacturing process options and a combination of widely assorted components such as surfactants, liquid lipids or even drug-conjugates. These features provide alternatives for designing innovative nanoemulsions aiming at high-value applications. This review presents the challenges and prospects of different nanoemulsion types and its application. The drug interaction with the components of the formulation, as well as the drug mechanistic interaction with the biological environment of different routes of administration are also presented.

Keywords: Nanoemulsion, drug delivery system, poor water-soluble drug, drug solubility, review.

1.1 Introduction

Nano-sized carriers are recognized as efficient drug delivery systems for poorly water-soluble drugs, which represent about 40% of newly discovered drug substances. Among the novel approaches, nanoemulsions have emerged as potential alternative drug carriers [1]. This type of surfactant-lipid-based formulation is capable of interacting with the body's natural barriers enabling drug absorption due to its composition and functionality.

Thus, oil-in-water nanoemulsions may overcome the drawback of low solubility of those drugs by improving the bioavailability, increased drug stability, and lower side effects, providing a wide range of applications [2].

In addition to the solubility enhancement, nanoemulsions are also a promising active drug targeting carrier for tumor cells [3], macrophages [4], and to overcome the blood-brain barrier [5, 6]. The flexible attribute of nanoemulsion extends through its manufacturing process options, which comprise both high and low energy processes, enabling narrow-sized droplet formation by mechanical and spontaneous physicochemical mechanisms, respectively [7].

Nanoemulsions are a resourceful platform, which can be formulated with a wide range of surfactants and liquid lipids. This enables alternatives to provide development of innovative drug delivery system with high level of applicability.

Several nanoemulsion delivery systems have been marketed since last decade including Restasis®, a non-ionic ophthalmic nanoemulsion of cyclosporine A and CationormR, a cationic ophthalmic nanoemulsion, both preservative-free and recommended for treating dry eye syndrome [8], Neoral®, non-ionic nanoemulsion of cyclosporine as oral immunosuppressive agent and Diprivan®, a propofol nanoemulsion used as an intravenous anesthetic agent [1, 9]. Despite those advantages, nanoemulsions pose several challenges to the formulators. These include identification and understanding of the sources of variability in the nanoemulsion development, which highly affects the safety and the therapeutic efficacy of the drug product and its stability. The aim of this review is to present the challenges and prospects of different nanoemulsion types and their application.

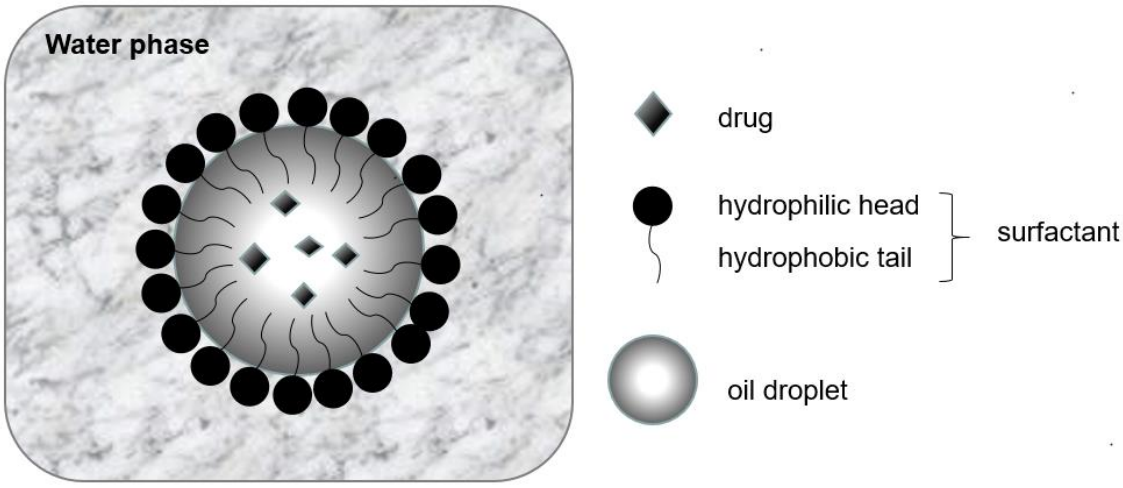
1.2 Nanotechnology and Lipid Nanoparticle Types

Nanotechnology includes engineered products or materials at the nano-scale, ranging from 1 nm to 100 nm, or even reaching a micrometer (1,000 nm) scale, exhibiting physical or chemical properties or biological effects attributable to its dimension(s), where unique phenomena enable novel applications [10]. Nano sized particles offer better stability, high efficacy and less toxicity when administered, compared to their macroscale drugs in the pharmaceutical field. Among them, lipid nanoparticles gained special attention due to their higher degree of biocompatibility, biodegradability and versatility [11]. They are classified into liposomes, solid lipid nanoparticles, nanostructured lipid carriers and nanoemulsions, according to the specific composition and physicochemical characteristics.

Nanoemulsions are nanoscale dispersions of two immiscible phases, consisting of oil-in-water (O/W) or water-in-oil (W/O) phases according to the surfactant type. For poorly water-soluble drugs, they can also be expressed as spherical nanodroplets with hydrophobic liquid cores, stabilized with surfactant shells [1] (Fig. 1-1). Differing from microemulsion which enables its spontaneous formation, nanoemulsion cannot be spontaneously formed and an introduction of some energy (mechanical or chemical) is required. Therefore, nanoemulsions are kinetically stable but thermodynamically unstable, with Ostwald ripening being the main factor of their instability [7, 12, 13]. This phenomenon of oil droplet growth over time occurs owing to the diffusion of small internal oil drops, from smaller to larger ones, in an aqueous medium [7, 14, 15]. Nanoemulsions can be classified in different types according to the surfactant selection (non-ionic, cationic or anionic). They can also be classified as, polymer associated nanoemulsions, and drug conjugated as functionalized nanoemulsions. For the non-ionic nanoemulsions, the ethoxylated non-ionic surfactants enable the curvature change of the lipid-water interface during the phase inversion process, yielding narrow droplet sizes by low energy process. The polymeric non-ionic surfactants (*e.g*. poloxamers) provide steric stabilization, also called polymeric stabilization. Cationic and anionic nanoemulsions are composed, respectively, of cationic and anionic surfactants as the main emulsifier; nevertheless, both

present higher toxicity than the non-ionic surfactants. The stabilization of cationic and anionic nanoemulsions is provided by electrostatic repulsion of the droplets due to the charged surfactant adsorbed on the lipid-water interface. Electrostatic stabilization combined with steric stabilization can be achieved using polymeric non-ionic surfactant associated with ionic surfactants [16]. For polymer associated nanoemulsion, a range of polymers can be used providing steric and/or electrostatic stabilization depending on its characteristics and method preparation. A polymer or monomer can be added into the oily or aqueous phase [14, 16, 17] or, the surface of pre-formed nanoemulsion can be coated with this component [16]. Additionally, nanoemulsion can be formulated by inclusion of conjugated drug. Usually a drug covalently coupled to other molecules such as proteins is added to the composition. The conjugated drug offers characteristics different from its original form, yielding a functionalized nanoemulsion to enable specific targeted drug delivery to the cells, overcoming the problems associated with the free drugs [18]. The advantages and disadvantages of different types of nanoemulsion, their fabrication processes and applications are described in the following sections.

Figura 1-1 - Schematic illustration of oil-in-water nanoemulsion delivery system



Source: author's own production.

1.3 Nanoemulsion Attributes in a Drug Delivery System

Nanoemulsions are versatile drug delivery systems for poorly water-soluble drugs, which exhibit low bioavailability in their original form. Several attributes of nanoemulsions to overcome those drawbacks are listed below.

1.3.1 Drug Solubilization

Under correct selection of liquid lipid and surfactants, nanoemulsions have the ability to solubilize large amounts of hydrophobic drugs, providing high drug loading in the oil core of the nanosystem, acting as a drug reservoir [1, 19, 20].

1.3.2 Bioavailability

The physicochemical properties of nanoemulsions can be easily customized by different factors such as process selection, oil/surfactant composition [8], as well as the surface modification for specific delivering to cells or organs, through active and passive targeting mechanisms [1, 21]. Nanoemulsions provide a waterbased formulation to poorly water-soluble drugs [20], and also owing to their small size, they have a large surface area that easily interacts with the body [22, 23]. This large surface area benefits the breaking rates of the formulation under oral administration, providing improved bioactive agent release, rapid and wider distribution of the drug [23], and also a prolonged efficacy by parenteral administration [21].

1.3.3 Formulation Stability

Depending on composition and manufacturing process selection, nanoemulsions may be an excellent system to overcome instability of poorly water-soluble drugs. Their nano-scaled particle size protects the formulation against sedimentation and creaming. Instability problems due to Ostwald ripening phenomenon can be

overcome by using highly water-insoluble oils, or by using steric or electrostatic repulsion elements on the droplet surface [24].

1.3.4 Drug Protection

The incorporation of a chemically labile drug into the oil core may protect it against oxidation, enzymatic degradation or hydrolysis, making nanoemulsions to an ideal platform as a drug delivery system [1, 19, 20, 23].

1.3.5 Safety

A prolonged efficacy, dose reduction due to the use of nanoemulsions can yield reduction of common side effects [21]. Owing to non-ionic surfactants that are widely employed in the nanoemulsion compositions in lower concentration compared to *e.g*. a microemulsion system, it may reduce toxicity. A study of the influence of nanoemulsions on intracellular reactive oxygen species (ROS), which normally cause deleterious oxidation of biomolecules by other nano sized formulation, showed no induced oxidative stress in human BJ5ta cells [25].

1.3.6 Sterilization

The selection of a suitable sterilization method is crucial to guarantee safe application of the nanoemulsion for ophthalmic or parenteral administration [1], since the use of filtration by a 0.22-µm-size pore membrane or moist heat sterilization are limited due to possible filter pore blockage and nanoemulsion instability, respectively [8]. Moist heat sterilization was applied to a lipid nanoparticle formulation composed of tripalmitin, lecithin, tween 20 and sodium deoxycholate, indicating the impact of the emulsifier and the liquid lipid type for good nanoemulsion stability under this sterilization process [26]. Successful sterilization of nanoemulsion containing Capmul MCM, didodecyldimethylammonium bromide, Poloxamer 188 and phospholipid was

shown by moist heat sterilization [27], and an ion-sensitive *in situ* ocular nanoemulsion gel of terbinafine by gamma radiation sterilization [28].

1.3.7 Wide Applicability

The high versatility of the nanoemulsions offers extensive potential applications which include oral delivery, parenteral, transdermal, ophthalmic and cancer targeted drug delivery. Their use as imaging components for cancer therapies have also been reported in recent studies [1]. Further data are presented in subsequent pages in this article.

One of the main disadvantages of O/W nanoemulsions is their instability mainly due to the Ostwald ripening as mentioned before. One study reported a new strategy for improving stability against Ostwald ripening by adding non-ionic amphiphilic polysaccharide (derived from dextran), without the need of ultrahydrophobic components [29]. Another recent study introduced a new biocompatible polymeric emulsifier, polyglycerol-block-poly(ε-caprolactone), which was highly effective in stabilizing O/W nanoemulsions through the formation of a semi-solid interphase. O/W nanoemulsions formed by different oils in this system presented excellent stability against mechanical stresses generated during repeated freeze/thaw cycles [30].

Another limitation of O/W nanoemulsions, i.e. compared to a highly concentrated nano sized formulations such as a nanocrystal (which is constituted primarily of the drug component itself), is the lower drug-loading capacity. Therefore, an effort to boost drug loading of nanoemulsions requires extensive understanding of the selected manufacturing process, the interactions between the nanoemulsion components, as well as the behaviour of the dissolved drug in the oil core.

1.4 Nanoemulsion Manufacturing Processes

In our previous work, we presented the different manufacturing processes and a detailed mechanism governed by high and low energy production of nanoemulsions [7]. In a general way, nanoemulsion productions are divided into two main processes: high and low energy processes. High-energy processes, (those requiring mechanical energy input), include high shear stirring, ultrasounds, high-pressure homogenisation, membrane emulsification and microfluidization. High shear stirring, used generally in combination with other processes, consists of breaking down larger droplets into the smaller droplets by mechanical force. Ultrasound is based on the implosion of the droplets by a series of mechanical depressions and compression, resulting in cavitational forces. In high-pressure homogenisation, nanodrops are produced by passage through a narrow slot of a homogenizer under high-pressure, which involves shearing, collision and cavitation force. The membrane emulsification and microfluidization involve the passage of two immiscible fluids in channels by a high-pressure pump, in which droplet sizes are controlled by the size of pores or channels, enabling formation of uniform and controlled internal phase nanodroplets [14, 16, 17].

The low energy process where physicochemical energy is required involves processes such as phase inversion temperature (PIT), phase inversion composition (PIC) and spontaneous emulsification (or Ouzo effect). Nanoemulsions formed by phase inversion temperature and phase inversion composition methods are based on the spontaneous curvature change of surfactants, by temperature or composition transition during manufacturing, respectively. The spontaneous emulsification is based on the specific and rapid diffusion of an organic solvent from the oily phase to the aqueous phase, by a dispersion and condensation phenomenon [14,16]. The D-Phase emulsification method, compared to the PIT and PIC, was presented as a recent and an alternative means for overcoming the need of strict hydrophilic-lipophilic balance (HLB) adjustment dictated in the PIT and PIC methods. D-Phase emulsification method requires the polyol as the fourth component for the creation of a low

interfacial tension phase (isotropic phase), enabling the final narrow droplet formation [31, 32].

The advantages of high-energy processes include flexibility in the process adjustment and broad formula composition choice, whereas the disadvantages include the higher cost investment on equipment. The advantages of a low energy process is the low equipment cost compared to the high energy processes, although the disadvantages consists mainly of the need for strict adjustment of the phase composition in order to lower the interfacial surface tension of the phases [14, 16, 17].

Several process application studies were also presented in our previous work, and some recent studies sparked consideration. A high-speed stirring study in an ART MICCRA DZ7 rotor-stator system was used for nanoemulsion production as an alternative for high-pressure homogenisation. Nanoemulsions with 135 nm and narrow size distribution were obtained by this system, showing to be a fast, cost-effective and suitable process for large-scale production [33].

The stability of nanoemulsions was analysed by several methods, based on drop size distribution information. The experiments were conducted using a set of alkanes with different chain lengths and physical properties. Nanoemulsions were formed by ultrasound, phase inversion composition and Ouzo methods. The authors showed that despite that, the Ostwald ripening is a dominant phenomenon for nanoemulsion at higher surfactant concentrations (i.e. 8.0 g/L), at low surfactant concentrations (i.e. $\ll$ 4.0 g/L) coalescence was identified as the dominant growth mechanism of droplet sizes. Although still vulnerable to Ostwald ripening and flocculation in the long-term shelf life, the phase inversion composition method was found to be the most stable method in this study [34].

A different method based on stirred media mills was evaluated resulting in 25-nm-sized nanoemulsions obtained in a hexane - Tween 85 - water system. The lowest oil-to-emulsifier ratio, as well as the processing temperature, below the lipid solidification temperature, was considered to be the most advantageous conditions for the small droplet sizes in this system [35].

As a complementary reflection, taking into consideration that there is no high temperature required for lipid melting nor high pressure input during

nanoemulsion production compared to solid state nano formulations, one may consider that nanoemulsions are more suitable to most manufacturing processes. This may be reflected in the optimization of the processing time, moreover the feasibility to the labile drugs, and higher reproducibility for largescale production.

1.5 Nanoemulsion - Drug Interaction with Oil Components

O/W nanoemulsion delivery systems are mainly composed of drugs solubilized in the oil phase, which is dispersed in the water phase. Different oil types can influence the drug solubilization as consequence of difference in density, viscosity, and polarity [36]. Therefore, apart from process conditions, the drug behavior in different oil components is also crucial during design of an effective delivery system. Thus, nanoemulsions possess in their core the oil phase as the main component, which may highly affect the bioavailability of the poor water-soluble drug, by increasing the drug absorption from the gastrointestinal (GI) tract and drug transportation via systemic circulation [2, 19, 22]. Several studies have been performed to evaluate the performance of drug-oil interaction in the nanoemulsion delivery systems as shown in the following sentences.

Nanoemulsion of pterostilbene, an antioxidant component of blueberries was developed for nutraceutical purpose. Two different carrier oils, flaxseed and olive oil, was investigated aiming to determine the influence of the different oil types on the metabolism and bioavailability of this natural compound pterostilbene. A Caco 2 cell permeability model was employed to evaluate the absorption

of pterostilbene from the resulting micelle phases. It has an enhanced solubility in both carrier oils, however, differences in the metabolism patterns and a higher trans-enterocyte transport were observed for this polyphenol in olive oil based nanoemulsions [23].

Aiming to design stable resveratrol nanoemulsion system against UV-light exposure, grape skin extract (GSE), rich in resveratrol, was incorporated in a mixture of grape seed oil (digestible) and orange oil (indigestible), by spontaneous emulsification process. The ratio of orange oil-to-grape seed oil of 5:5 gave the optimum arrangement between emulsion size (driven by *e.g*.

viscosity, interfacial tension and interfacial dynamics of the oils) and the stability (driven by the polarity of the oil component). The 220 nm nanomulsion, with a droplet size closest to the UV-light wavelength, showed higher resveratrol protection compared to others [37].

Ostwald ripening in O/W nanoemulsion was evaluated by the influence of lipophilicity values of different oils. The mean drop size was characterized over time (0 to 180 min), and as a general rule, a higher growth rate was shown with a short hydrocarbon chains. Nanoemulsion with C16 was stable for around 8 months [33].

In order to maintain the poor soluble drug ezetimibe (a selective cholesterol-absorption inhibitor) in the solubilised form, a screening test of combinations of six different oils and eight surfactants/cosurfactants was performed to design a suitable nanoemulsion. Oils from different categories such as long-, medium-chain triglycerides and synthetic monoglyceride oils were evaluated. Capryol 90 (proprylene glycol monocaprylate) was selected since it exhibited the highest drug solubility in the nanoemulsion system [38].

Potent anti-cancer bioactive components polymethoxyflavones (PMFs) extracted from citrus peels are highly hydrophobic molecules with poor solubility in both water and oil at room temperature. Aiming to improve their bioavailability, PMF-loaded nanoemulsions were prepared evaluating the influence of different carrier oils (corn oil, medium chain triglycerides, orange oil), emulsifiers (b-lactoglobulin and lysolecithin as highly anionic emulsifier, Tween 20 and 85 as non-ionic emulsifiers, and DTAB as cationic emulsifier), and cosolvents (glycerol and ethanol). Nanoemulsions less than 100 nm could be formed using high-pressure homogenization employing all emulsifiers, except for DTAB (dodecyl trimethyl ammonium bromide). PMF crystallization and sedimentation were observed in all prepared nanoemulsions, although the crystal morphology, size and sedimentation speed altered according to the different oil types, emulsifiers and preparation methods. These results may provide important directions for the future development of poorly water and oil soluble bioactive components in a nanoemulsion delivery system [22].

The use of the bioactive component carotenoids in nutraceulticals is currently limited due to their poor water solubility, low bioavailability and chemical instability. The permeability of carotenoids (0.5 wt% in lipid phase) in O/W nanoemulsions using different types of carrier oils was evaluated by an *in vitro* GI model that simulates the mouth, stomach and small intestine. Nanoemulsions composed of Tween 20 as non-ionic surfactant, and three different carrier oils, such as corn oil that contains long-chain triglycerides (LCT), medium-chain triglycerides (MCT) or orange oil, generated particles lower than 200 nm. Calculation from the alkali titration of the released free fatty acids (FFA) showed that the rate and extent of free acid production in the intestine was in order of LCT ~ MCT ≫ orange oil. The *in vitro* s-carotene permeability was in the order of LCT ≫ MCT > orange oil. The higher permeability of the LCT is explained by the fact that it is able to form mixed micelles larger enough to accommodate highly lipophilic molecule such as s-carotene, than MCT. Concurrently, no mixed micelles were formed to solubilise s-carotene in orange oil nanoemulsions [39].

Another recent study reported the impact of different carrier oils on the digestion of curcumin (0.15 wt% in lipid phase) O/W nanoemulsion stabilized by protein (b-lactoglobulin). An *in vitro* GI model was used to simulate the intestinal passage. Results showed the faster rate and extent of FFA release for MCT than the LCT containing nanoemulsions. This may due to the consequence of the resulting digestion products from MCT and LCT, which facilitate or hinder lipase activity, respectively. The discrepancy of these results from the precedent study may involve the difference of surfactant type (non-ionic and protein), the *in vitro* digestion conditions (fat content), and the number of stages of the *in vitro* GI model [40]. This demonstrates the importance of the right selection of oil / surfactant composition and *in vitro* digestion model for optimal nanoemulsion formulation development.

Silymarin, a hepatoprotective bioactive compound with poor aqueous solubility and low bioavailability, was incorporated into the O/W nanoemulsion to increase its oral bioavailability. Considering that higher oil solubility of this component will favour an overall stability of formulation with effective dose optimization, propylene glycol caprylate (Sefsol 218R) was selected as carrier oil, due to its high solubilization capacity of silymarin. *In vitro* dissolution studies showed higher

drug release from nanoemulsions than bulk drug suspension, and after oral administration, both maximum plasma concentration (Cmax) and area under the curve (AUC) were 4- to 6-fold higher than those of silymarin in suspension form. Pharmacokinetic studies showed better results (a 2-fold and 2.6-fold increase in the AUC and C_{max}, respectively) in nanoemulsion than the marketed formulation [2].

An interesting study as an indicative of the effectiveness of liquid core in the nanosized formulation (as the nanoemulsion) was performed comparing the behaviour of two lipid nanoparticle (LN) systems, using trilaurin and tripalmitin. Differential scanning calorimetry studies on formed LNs demonstrated that tripalmitin presented a “solid-like” state, whereas trilaurin formed a “fluid-like” core. After addition of the hydrophobic drug testosterone (TP) in the preformed LNs, from 0.2 to 1.4 wt %, it resulted in a large increase in the apparent hydrodynamic size of the tripalmitin LN. However, no change was observed in trilaurin LN even after 1- week stability, compared to their TP-free LNs. The size change in the TP-contained tripalmitin LN may be a consequence of the rearrangement in packing of the molecules comprising the LN, caused by adding the drug. The higher level of TP solubilization in the trilaurin LN compared to the solid-state LN is explained by the presence of the liquefied-lipid core. [41]. This result may illustrate the nanoemulsion as one of most promising delivey system for poorly soluble drugs.

1.6 Routes of Administration and Applications of Nanoemulsions

Aiming to accomplish the main strategy for improving the solubility of a poor water-soluble drug, by increasing its efficacy and bioavailability, a drug substance need to overcome several natural barriers in the body. Different routes of administration and applications of various nanoemulsion types are presented in the following sections. An overview of those applications is shown in the Table 1-1.

Table 1-1 - Nanoemulsion drug delivery system application (to be continued)

Route of administration	Nanoemulsion (NE) type	NE composition	Manufacturing process	Drug / active principle	Therapeutic use	Result / prospect	Reference
Oral	Non-ionic NE	1% of WBO and 7.3% of a surfactant mixture of Span 80 (37.4%) and Tween 80 (62.6%).	High-speed blender (pre-emulsification) and ultrasonication	Wheat bran oil (WBO) rich in phenolic compounds	Nutraceutical	Good antioxidant and tyrosinase inhibitory activity	[36]
Oral	Non-ionic NE	Triacetin and Capryol 90 (1:1), Tween 80 and Transcutol P	Aqueous phase titration on the pre-mixed oil / surfactant phases	Cilastazol	Antithrombotic activity	3.29-fold higher bioavailability	[51]
Oral	Non-ionic NE	Lauroglycol, Transcutol, Cremophor EL, deionized water, and λ-carrageenan	Ultrasound treatment	Aspirin	Anti-inflammatory and analgesic activities	Superior protection and less injury to the gastric mucosa, wide distribution of the drug throughout the intestinal tract, and increased drug-retention time in the desired region	[52]
Oral	Non-ionic NE	Lauroglycol™90, Transcutol HP®), Cremophore EL	Ultrasound treatment	Aspirin	Anti-inflammatory and analgesic activities	1.4- to 2.2-fold higher anti-inflammatory and analgesic effect	[53]
Oral	Non-ionic NE	Miglylol 812, egg lecithin, soyabean lecithin liquid, Poloxamer 188, glycerol, sorbitol / Capryol 90, Solutol HS 15 and Gelucire 44/14	Ultra-Turrax T 25 stirring and high pressure homogenisation / Self-emulsification	Primaquine/ clotrimazole	Malaria therapy	Efficacy at lower oral doses; increased oral bioavailability of the drugs loaded nanoemulsions	[54, 55]
Oral	Non-ionic NE	Capryol 90 (10%), Tween 20 (10%), and PEG 400 (16.67%)	Aqueous phase titration on the pre-mixed oil / surfactant phases	Ezetimibe	Cholesterol and triglyceride lowering compounds	3.23-fold increase in bioavailability compared to drug suspension and a 4.77-fold increase in bioavailability compared to the conventional tablet	[38]

Table 1-1 - Nanoemulsion drug delivery system application (continuation)

Oral	Non-ionic NE	Capryol 90 (10%, v/v), Cremophor EL (11.25%, v/v) and Transcutol P (33.75%, v/v)	Aqueous phase titration on the pre-mixed oil / surfactant phases	Ezetimibe	cholesterol and triglyceride lowering compounds	Higher release and bioavailability of ezetimibe compared to the formulation of reference [38]	[56]
Oral	Polymer Associated NE	Medium chain triglyceride, (OSA)-modified starch, curcumin, chitosan and CMC (188:57:1.2:1.5:2)	High-intensity ultrasonic homogeniser / pre-formed curcumin nanoemulsion template	Curcumin	Antitumour	Stability and shelf improvement, minimum aggregation	[57]
Oral	Polymer Associated NE	Whey protein isolate, soybean protein isolate, beta-lactoglobulin, Labrafil M 1944CS, CaCl2	Mechanical mixing and high-pressure homogenisation	Fenofibrate	Cholesterol and triglyceride lowering compounds	Excellent stability and drug-loading capacity, which can be easily freeze-dried	[58, 59]
Parenteral	Non-ionic NE	Squalane, Span-80, Tween-80, PBS, pH 7.4	High-pressure homogenisation	CHrPfs25	Malaria transmission blocking vaccine antigen	Highest antibody response; potential candidate as a Pfs25 vaccine adjuvant, as an alternative to existing aluminum salts	[64]
Parenteral	Non-ionic NE	Tween 20, phosphate buffer of pH 7.2	Ultrasound treatment	Neem oil; human serum albumin (HSA); bovine serum albumin (BSA)	Interaction of essential oil based NE with biological proteins	More stable complexes between BSA – neem oil NE compared to HSA; improving the efficacy of drug delivery, bio sensing and other clinical applications	[65]
Parenteral	Non-ionic NE	Curcuminoid extract, Tween 80, and water	Sonication	Curcuma longa Linnaeus	Inhibition mechanism of lung cancer cells (A549 and H460)	high stable NE; similar results of both NE and curcuminoid extract in the expression of proteins correlated with the cell cycle and apoptosis in these two cells.	[66]

Table 1-1 - Nanoemulsion drug delivery system application (continuation)

Parenteral	Non-ionic NE	Miglyol® 812N/Labrasol ®/Tween® 80/Lipoid E80®/water	Sonication	Fisetin	Antitumour	24-fold increase in fisetin relative bioavailability; antitumour activity at lower dose.	[68]
Parenteral	Non-ionic NE	sodium oleate, Polysorbate 80	Hot homogenisation	Risperidone	Antipsychotic	1.4–7.4-fold higher risperidone brain availability.	[69]
Parenteral	Polymer Associated NE	PEG-PDLA, perfluorocarbon	Sonication	Paclitaxel	Pancreatic anti-tumor	Higher therapeutic efficacy, lower drug resistance in tumors, lower systemic toxicity	[70]
Parenteral	Drug-conjugated NE	Vegetable oil, BSA–FA conjugate solution, PEGylated surfactant	High-pressure homogenisation	FA-functionalized PEGylated BSA + CORM-2	Antiproliferative effect on human cancer cells	FA-tagged protein NEs were preferentially internalized in the B-cell lymphoma cell line (A20 cell line)	[72]
Parenteral	Drug-conjugated NE	PEGylated surfactant, PBS, vegetable oil	High-pressure homogenisation	BSA-drug conjugates + methotrexate / BSA-drug conjugates + vancomycin	Potent anticancer agent / potent antibiotic	Effectiveness with improved half-life in systemic circulation	[18]
Parenteral	Drug-conjugated NE	PBS, vegetable oil, Poloxamer 407	High-pressure homogenisation	BSA NE + Poloxamer 407	Active targeting of folate receptor positive cells	5-fold higher internalization of tagged-nanoemulsions by cells	[25]
Topical	Non-ionic NE	Tween 20, oleic acid, propylene glycol	Aqueous phase addition on the pre-mixed oil / surfactant phases, sonication	Fennel essential oil	Prolonged antidiabetic activity	Superior permeation profiles for 24 h, high potential of reducing plasma glucose levels	[75]
Topical	Non-ionic NE	Capryol™ 90, oleic acid, Tween 20	Aqueous phase addition on the pre-mixed oil / surfactant phases, sonication	Cumin essential oil	Systemic antioxidant and hepatoprotective activities	Best in-vitro and in-vivo antioxidant efficiency, high hepatoprotective potential	[76]

Table 1-1 - Nanoemulsion drug delivery system application (continuation)

Topical	Non-ionic NE	Caprylic acid, propylene glycol, Tween 80, PEG 400, triethanolamine, Carbopol 940, Trypsin	Magnetic stirring	Meloxicam	Non-steroidal anti-inflammatory drug	Non-irritant, biocompatible, maximum inhibition of paw edema over 24 h	[77]
Intranasal	Non-ionic NE	Capmul MCM, Tween 80, PEG 400	Spontaneous emulsification process	Saquinavir mesylate	Anti-HIV	Higher permeation rate, no significant adverse effect, higher drug concentration in brain, larger extent transport of drug in the CNS	[6]
Intranasal	Polymer Associated NE	Capmul MCM, Tween 80, propylene glycol, Transcutol	Aqueous phase addition on the pre-mixed oil / surfactant phases, and subsequent addition of chitosan	Risperidone	Antipsychotic drug	Superior efficacy on brain/blood uptake ratio of risperidone	[82, 83]
Intranasal	Polymer Associated NE	Capmul MCM, Tween 80, ethanol and polyethylene glycol	Water titration method	Olanzapine	Antipsychotic agent	Highest drug targeting efficiency (DTE%) and direct nose-to-brain transport (DTP%), 2-fold higher DTP%	[5]
Ocular treatment	Cationic NE	Eutanol G, Lipoid S 100, cetylpyridinium chloride, glycerol	High-pressure homogenisation	Dexamethasone acetate and polymyxin B sulfate	Ophthalmic infection treatment	Amphiphilic cationic concept" offers highly ocular bioavailable solution for treating ophthalmic infections	[80]
Mucosal vaccine adjuvants	---	---	---	Local delivery, systemic delivery, mucosal vaccination	Mucosal and systemic immunization	Promising candidate as mucosal vaccine adjuvants: long-term release properties for antigens, non-invasive immunity and stability of antigens for mucosal and systemic immunization	[79, 84, 85]

Table 1-1 - Nanoemulsion drug delivery system application (conclusion)

Imaging-guided therapy	Non-ionic NE	Iron oxide nanocrystals, fluorescent dye Cy7	Spontaneous emulsification and sonication	Hydrophobic glucocorticoid prednisolone acetate valerate	"Theranostic" platform for image-guided therapy of cancer	Significant drug substance accumulation in tumors cells, potent inhibitory effect on the tumor growth profiles	[88]
Imaging-guided therapy	Non-ionic NE	Miglyol 810 N, DiD dye, PFPE, Pluronic® P105, Cremophor EL®	Microfluidizer	Celecoxib	Anti-inflammatory drug to target macrophages	Simultaneously delivery the drug to macrophages and monitor macrophage migration patterns by optical imaging	[4]

1.6.1 Nanoemulsions for Oral Route of Administration

1.6.1.1 Lipid Delivery System Pathway and Bioavailability

With the oral route being the most popular rout of administration, water solubility of poorly water-soluble drugs becomes a key parameter in drug formulation since it can lead to limited absorption in the gastrointestinal tract and limited bioavailability [42, 43]. Hence, understanding the complex sequences of physiological and physicochemical phenomena, which the drug faces after being administered is the first step for developing an efficient formulation. In short, these emulsions under oral administration experience structural modification, resulting on flocculation and coalescence of droplets during the gastrointestinal passage [44].

The digestion process of fats, administered orally, typically begins in the stomach. This process consists of the dispersion of lipids into finely fragmented emulsion particles, by action of surface-active materials (i.e. gastric lipases) at the lipid-water interface [44-46]. At this stage, it is expected that a drug is dissolved in the lipid, preventing undesirable drug precipitation [47]. Next the gastric content is emptied into the small intestine, where in the presence of bile salts, colipase binds to the surface of fat droplets providing an attachment site for lipases. Therefore,

this process produces the final mixture for fat digestion, fatty acids and 2-monoglyceride [39, 48, 49]. During this process in the gastrointestinal tract, structural changes in the emulsion occurs, generating bile salt micelles and lamellar vesicles composed of phospholipids in the aqueous phase [45, 47, 48], and finally absorption by the enterocytes occurs [44, 46]. Therefore, those hydrophobic components of the nanoemulsion, which constitute the carrier of poor water-soluble drugs, when they are incorporated and solubilized into the mixed micelles, function like a drug reservoir to be passively transported to epithelium cells [39].

The bioavailability of nanoemulsion is expected to be higher than conventional emulsion taking into account the higher surface area-to-volume ratio of the nanoemulsion. Since lipid digestion consists of an interfacial phenomenon comprised of lipase adsorption on the lipid droplet surface, the droplet size reduction favours an increase of interfacial area, and consequent increase of lipid digestibility and release [44, 46, 50]. Other than the particle size, the nature of the interface of lipid droplet seems also to interfere with the lipid hydrolysis during gastrointestinal process. The displacement of the small surfactant components by bile salts and phopholipids allows the efficient attachment of lipase on the oil droplet surface. Therefore, the characteristics of interfacial layer components such as Tween 20, protein and phospholipids may alter those attachment phenomena, thus the lipid digestion efficiency.

However, more research on *in vitro* digestion models still needs to have better conclusions [39, 44, 49, 50]. The physical state of the fat constituting the lipid delivery system is also important regarding the rate and extent of lipid digestion. Studies with solid-state emulsions and liquid-state emulsions using an *in vitro* digestion model showed a higher rate and extent of lipid digestion in liquid-state emulsion compared to the solid-state emulsion [44, 49].

1.6.1.2 Nanoemulsion Applications in Oral Administration

Non-Ionic Nanoemulsions

For nutraceutical purposes, a formulation of O/W nanoemulsion containing wheat bran oil (WBO) rich in phenolic compounds was developed by using the response surface methodology. The emulsification method, the oil and surfactant concentration, as well as surfactant type were investigated on the droplet size and stability of the nanoemulsion. A combination of high-speed mixer (preemulsification) and ultrasonication resulted in the optimal condition reached by 1% of WBO and 7.3% of a surfactant mixture of Span 80 (37.4%) and Tween 80 (62.6%). This optimized nanoemulsion showed good stability over time, as well as good antioxidant and tyrosinase inhibitory activity [36].

A poor oral bioavailability therapeutic agent, cilastazol (CLZ), which is well known for its antithrombotic activity, was studied. Amongst various surfactants, co-surfactants and oils, the combination of triacetin and Capryol 90 (1:1), Tween 80 and Transcutol P were selected to obtain O/W nanoemulsion with droplet size of 93.72 nm and polydispersity index (PDI) of 0.278. The optimized nanoemulsion showed a 3.29-fold higher bioavailability in rats compared to CLZ suspension [51].

The effect of aspirin nanoemulsion (NE) in gastric tissue was studied compared with conventional aspirin. Conventional aspirin formulation induces pronounced oxidative damage and triggers the release of reactive oxygen species harmful to the stomach. A total of 24 male rats were used in the study. The effects of the aspirin were determined by the measuring the TNFα, iNOS, prostaglandin E2, and malondialdehyde levels, and also the glutathione, glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase. The 30-mg/kg aspirin NE showed superior protection and less injury to the gastric mucosa, which may be caused by rapid emptying of the fine oil droplets from the stomach, wide distribution of the drug throughout the intestinal tract, and increased drug retention time in the desired region [52].

Another study was using O/W nanoemulsion and water-in-oil-in-water (W/O/W) nano multiple emulsion formulations containing aspirin (60 mg/kg). Both formulations were generated by an ultrasound process, and the anti-inflammatory and analgesic activities were investigated. Compared to the reference suspension, the nanoemulsion showed a 1.4- to 2.2-fold higher anti-inflammatory and analgesic effects, while the nano multiple emulsion resulted in mild inhibitory effects in the different experimental animal model tests. These results suggest the use of nanoemulsion and nano multiple emulsion as dosage forms for treating various diseases associated with inflammation and pain [53].

An interesting research of malaria therapy, based on O/W nanoemulsion for primaquine and clotrimazole oral administration, reported considerably reduced drawbacks compared to the existing therapies. These therapies show severe side effects and emergence of resistance to anti-parasitic drugs, as a consequence of complex and prolonged drug administration regimens. The oral nanoemulsions of both drug substances showed increased efficacy at lower oral doses when compared to their suspension forms. The efficacy was attributed to an increased oral bioavailability of the nanoemulsions [54, 55].

As mentioned before in section 5 an optimized and stable O/W nanoemulsion (NE) of ezetimibe was achieve. The drug absorption of ezetimibe NE in Albino Wistar rats resulted in a 3.23-fold increase in bioavailability compared to drug suspension and a 4.77- fold increase in bioavailability compared to the conventional tablet [38].

The same authors studied in the subsequent year, a new ezetimibe formulation containing O/W nanoemulsion formulated with Capryol 90 (10%, v/v), Cremophor EL (11.25%, v/v) and Transcutol P (33.75%, v/v), as oil and surfactant phases, respectively. This new optimized version presented higher release and bioavailability of ezetimibe compared to the previous formulation. According to the authors, it may be due to the presence of Cremophor EL known to be a potent inhibitor of P-gp over Tween 80, and Transcutol P as a permeability enhancer [56]. This enhanced version of ezetimibe nanoemulsion demonstrates the versatility of the nanoemulsion as a carrier system to be easily reformulated, according to the trends or needs.

Polymer Associated Nanoemulsions

Curcumin nanoemulsions, stabilized with octenyl-succinicanhydride (OSA)-modified starch, were used as templates (core materials) and coated with an ultrathin polymeric film using a partially deacetylated chitosan (degree of deacetylation: 93.4%, with average molecular weight of 100 kDa), and Na-carboxymethyl cellulose (CMC), as cationic and anionic polyelectrolytes, respectively. A high-intensity ultrasonic homogeniser was applied to distribute these polymeric multilayer shells around the pre-formed curcumin nanoemulsion template. The aim of this invention was to overcome the drawbacks associated with the conventional nanoemulsions, such as stability and shelf life, by the creation of a barrier between the oily core and external environment. At appropriate sonication conditions, the final polymeric multilayer nanoemulsion was measured with a mass ratio of the medium chain triglyceride (density: 0.940 g/mL), (OSA)-modified starch, curcumin, chitosan and CMC of 188:57:1.2:1.5:2. The increase in mean diameter, polidispersivity index and zeta potential of this final multilayer nanoemulsion were 159.85 • } 0.92 nm, 0.140 • } 0.01 and -17.2 mV, respectively. Additionally, minimum aggregation was observed at 4 °C for 4 weeks [57].

Biocompatible biopolymers composed of whey protein isolate, soybean protein isolate or beta-lactoglobulin were employed to develop stable nanoemulsion templates [58]. Shell crosslinking was carried out by incorporation of the inorganic crosslinker (Ca2+), which binds to the adsorbed proteins at those o/w interfaces in a one-step process. Fenofibrate, a highly lipophilic drug that is clinically used to lower lipid levels, was incorporeted into this novel shell-crosslinked nanocapsule system based on nanoemulsion templates with excellent stability and drug-loading capacity. The Powder X-Ray Diffraction (PXRD) and Differential Scanning Calorimetry (DSC) test of the freeze-dried nanocapsule indicated the solubilized state of the drug in the lipid core. Upon contact with water, it was easily dispersed and re-established into the original form [59].

1.6.2 Nanoemulsions for Parenteral Route of Administration

1.6.2.1 Drug Uptake Pathway

Among the parenteral route of administration, the intravenous one directly delivers drugs into blood stream [57]. Hence, once the drug is introduced into the bloodstream, it is distributed systemically via the vascular and lymphatic systems, followed by distribution into tissues depending on the blood flow and, i.e., particle size [60].

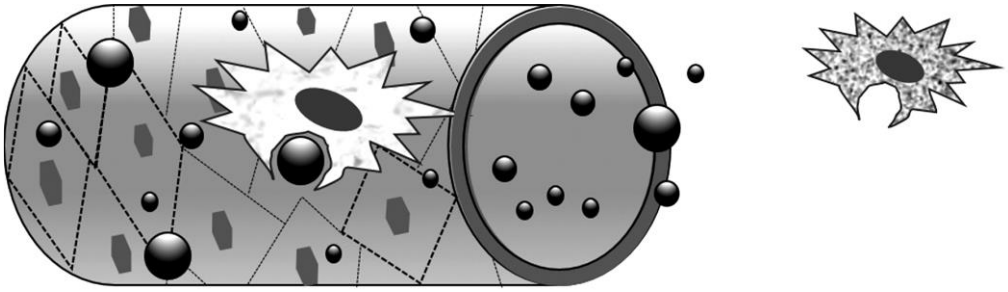
Nevertheless, upon introducing into the circulatory system, the nanoemulsion containing the drug may interact with erythrocytes, plasma proteins (opsonins), immune cells (monocytes, platelets, leukocytes, and dendritic cells), and tissue resident phagocytic cells like Kupffer cells in liver, dendritic cells in the lymph nodes, macrophages, and B cells in the spleen [1]. The opsonins (*e.g.*, immunoglobulin γ, complement factors and fibrinogen) primarily binds to the surface of foreign particles or surfaces, attracting immune cells and macrophages. The uptaked drug by *e.g*. macrophages will be forwarded to endogenous clearance mechanism, affecting its circulation time and efficacy [1, 20, 60, 61]. Opsonization can be evaded by a number of strategies within the drug formulation development, such as by modifying size, charge, and hydrophilicity [20]. Thus, to achieve the desirable extravasation of the drug into distant cells or organs, it is crucial to consider the size, the charge of the particle, surface properties (hydrophilicity), as well as the diameter of the “window” to enter in the extracellular space of the desired cells [20, 62].

Size

Smaller particles are known to escape phagocytosis in the reticuloendothelial system [1], predominantly in the liver and spleen. Concurrently the systemic circulation drug should be large enough to prevent their rapid leakage into blood capillaries [25]. Particle sizes on the order of 1 - 20 nm show improvement in circulation half-time [60, 61], and the particles about 30 - 100 nm administered by local injection avoid leakage into capillaries. [60]. Particles larger than 100 nm

are quickly captured by the cells of the mononuclear phagocyte system (MPS) [1] (Fig. 1-2).

Figura 1-2 - Nanoemulsion sizes and clearance process



Source: Faraji and Wipf, 2009.

Charge

The surface charge also influences the behavior of the drug substance, considering controlling clearance response. Positive charged particles show a greater degree of phagocytosis, followed by negative charged particles, and the lowest degree for the noncharged particles [1, 20].

Hydrophilicity

Owing to the hydrophobicity of the particle surface, the blood serum proteins easily adhere to that surface. A number of studies regarding the use of the hydrophilic PEG chain showed a substantial reduction in the rapid clearance of the particles into the MPS. PEGylation generated a steric repulsion effect, creating a neutral charged particle shield, reducing adsorption of opsonins and other serum proteins on the particle surface [1, 25, 60, 61].

Shape

Although their findings have been limited, some studies have reported on the influence of particle shape on drug delivery. Rod like structures and filamentous

micelles showed longer circulation time and higher cellular uptake efficiency compared to the other particle shapes [63].

After passing through the blood circulation barriers, nanoemulsions need to overcome multiple membranes to reach intracellular structures and to be delivered at the desired intra cellular site [1, 60]. The nanoemulsion are up taken via phagocytosis, macropinocytosis, or receptor-mediated endocytosis. Phagocytosis takes in larger particle sizes up to 10 μm, pinocytosis involves ingestion of sub-micron particles and substances in solution. Receptor-mediated endocytosis is constituted by high number of receptors generating higher selectivity for specific uptake by cells, which transduce a signal to the intracellular space, or can also lead to internalizing the ligand and its attached nanoemulsion vesicle by endocytosis [60].

Interesting phenomena occur in tumor tissues to which a drug substance can be transported both by a passive or active targeting mechanism. The tumor tissues due to the physiological mechanisms such as rapid angiogenesis generates a specific microenvironment such as more acidic pH, abnormal basement membranes, ‘leaky’ endothelial cells, enhanced vascular permeability and poor lymphatic drainage. This enhanced permeability and retention (EPR) effect of the tumor environment provides favorable distribution and extravasation of the particular drug delivery systems into the tumor tissues [1, 60]. Besides this passive mechanism, it is possible to develop active targeting strategies using specific biological markers such as Arg-Gly-Asp (RGD) peptide, an antibody, or a nanoparticle-sized bioconjugates for cancer targeting [60].

1.6.2.2 Nanoemulsion Applications in Parenteral Administration

Non-Ionic Nanoemulsions

Aiming to develop a safe and immunologically more potent novel adjuvants and vaccine delivery systems, squalane-containing nanoemulsions (NE) and poly(D,L-lactide-co-glycolide) nanoparticles (PLGA-NP) using CHrPfs25 (a malaria transmission blocking vaccine antigen) were formulated. These preparations were evaluated via intramuscular (IM) route in mice, and the

transmission blocking efficacy of antibodies was analyzed by standard mosquito membrane feeding assay using purified IgG from immune sera. Among different concentrations of NE and PLGA-NP, results showed the highest antibody response from CHrPfs25 formulated in 4% NE, compared to 8% NE and PLGA-NP. No further increases were observed by combining NE with monophosphoryl lipid-A (MPL-A) and chitosan. Further pre-clinical and clinical tests need to be performed; CHrPfs25 nanoemulsion may be a potential candidate as a Pfs25 vaccine adjuvant, as an alternative to existing aluminum salts [64].

Several spectroscopic studies were done aiming to better understand the protein-nanoemulsion interaction through their binding conformational alterations. Human serum albumin (HSA), which is considered to be the major soluble protein constituent of the circulatory system, delivering several nutrients in blood plasma, has excellent acceptor capacity with a wide range of molecules. Bovine serum albumin (BSA) has 76% sequence identity shared with HSA. Hence, the effect of neem oil nanoemulsion (NE) of different concentrations with both proteins was investigated using UV and fluorescence analysis. It was concluded that the binding mechanism of neem oil NE and serum albumins were governed by nonfluorescent ground state complex formation between them. FT-IR spectroscopy and circular dichroism spectral change studies suggest possible conformational changes in the alpha-helical and aromatic amino acid residues of the biomolecules. Also, this leads to the formation of more stable complexes between BSA - neem oil NE compared to HSA. This study provides information to develop protein-loaded nanoemulsions for improving the efficacy of drug delivery, bio-sensing and other clinical applications [65].

The inhibition mechanism of lung cancer cells (A549 and H460) by curcuminoid extracts and nanoemulsions prepared from *Curcuma longa* Linnaeus were evaluated. A high stable nanoemulsion composed of curcuminoid extract, Tween 80, and water, with 12.6 nm mean particle size was developed by sonication process. Both, curcuminoid extract and nanoemulsion treatments, demonstrated similar results in the expression of proteins correlated with the cell cycle and apoptosis in these two cells lines. However, the H460 cells were more susceptible to apoptosis than A549 cells for both treatments, and A549 cells showed a dose-dependent increase in cyclin B expression for both treatments while a reversed

trend was found for H460 cells. In this study, the two cell apoptosis pathways, mitochondria pathway (intrinsic pathway) and death receptor pathway (extrinsic pathway), seems to be responsible for apoptosis of both A549 and H460 cells [66]. Azarmi *et al* showed that A549 cells are resistant to doxorubicin while H460 cell are not. However, drug loaded nanoparticles could overcome drug resistance. A similar mechanism might play a role in the curcurmin study [67].

The natural flavonoid fisetin (3,3´,4´,7-tetrahydroxyflavone) was incorporated into a nanoemulsion to improve its pharmacokinetics and antitumour therapeutic efficacy. A nanoemulsion with 153 ± 2 nm oil droplet diameter, composed of Miglyol® 812N/Labrasol®/TweenR 80/Lipoid E80®/water, was stable at 4 ◦C for 30 days. Although no difference compared to free fisetin was observed by pharmacokinetic studies in mice, when injected intravenously, the nanoemulsion showed a 24-fold increase in fisetin bioavailability than free fisetin when administered intraperitoneally. In addition, the antitumour activity of the fisetin nanoemulsion in Lewis lung carcinoma bearing mice occurred at lower doses (36.6 mg/kg) compared to free fisetin (223 mg/kg) [68].

The design of parenteral lecithin-based nanoemulsions intended for brain delivery of risperidone, an antipsychotic drug, was performed applying a general factorial experimental design. Risperidone-loaded nanoemulsions (mean size about 160 nm, size distribution <0.15, zeta potential around -50 mV), containing sodium oleate in the aqueous phase and Polysorbate 80, Poloxamer 188 or Solutol1 HS15 as co-emulsifier, were produced by hot homogenisation. Their ability to improve risperidone delivery to the brain was assessed in rats. A promising nanocarrier for brain selective delivery purpose was shown to be the Polysorbate 80-costabilized nanoemulsion with increased risperidone brain availability (1.4-7.4-fold higher) compared to other nanoemulsions and drug suspension. These differences in pharmacokinetic results, when administrated intraperitoneally, are probably due to their different droplet surface properties (different composition of the stabilizing layer), which determined the blood-brain barrier passage of risperidone [69].

Polymer Associated Nanoemulsions

An interesting study compared the therapeutic properties of polymeric micelles and nanoemulsions generated from micelles in pancreatic tumor bearing mice. The mice were treated with paclitaxel (PTX) loaded polymeric micelles, or corresponding perfluorocarbon, a halogen-substituted carbon nonpolar oil, nanoemulsions. Two structures of the polymeric block were compared: poly(ethylene oxide)-co-poly(D,L-lactide) (PEG-PDLA) and poly(ethylene oxide)-co-poly(L-lactide) (PEG-PLLA), on which the first generated micelles with elastic amorphous cores, while micelles with solid crystalline cores were formed in the second one. Micelles and nanoemulsions stabilized with PEG-PDLA copolymer demonstrated higher therapeutic efficacy than PEG-PLLA copolymer derivative micelle or nanoemulsion. This is probably due to the elastic physical state of the micelle cores (or droplet shells), allowing drug release via diffusion and/or copolymer biodegradation. PEG-PDLA stabilized formulations showed lower drug resistance in tumors than PEG-PLLA stabilized formulations, maybe due to the presence and preventive effect of copolymer unimers that were in equilibrium with PEG-PDLA micelles. Additionally, PEG-PDLA stabilized nanoemulsions showed lower systemic toxicity than corresponding micelles, suggesting higher drug retention in circulation [70].

A similar study was conducted comparing PTX loaded PEGPDLA micelle with PEG-PDLA perfluorocarbon nanoemulsions. Polymeric micelle resulted in faster extravasation and tumor cell internalization than nanoemulsion, although the authors emphasized the need to optimize both drug retention and carrier diffusion parameters for a development of an ideal drug carrier [71].

6.2.2.3. Drug-Conjugated Nanoemulsions

Novel FA-functionalized PEGylated BSA nanoemulsions, loaded with CORM-2 (Carbon monoxide releasing molecule-2), were tested both, *in vitro* and *in vivo*. FA (folic acid) and FA conjugates are known to bind with high affinity to folate receptor (FR)- alpha and -beta and enter FR-expressing tumor cells by receptor mediated endocytosis. For the nanoemulsion preparation, firstly CORM-2, which induces an antiproliferative effect on human cancer cells, was incorporated in the

oil phase. This oil phase (vegetable oil) was emulsified with the aqueous phase containing BSA-FA (Folic Acid) conjugate solution and a PEGylated surfactant, by high-pressure homogenisation. The obtained small and stable (FA)-tagged protein nanoemulsions were then evaluated in terms of specific uptake using a lymphoma cell line (A20 cell line). Results showed that the folic Acid (FA)-tagged protein nanoemulsions were preferentially internalized in the B-cell lymphoma cell line (A20 cell line), promoting them as promising nanocarriers for the selective delivery of drugs to a target cell population that express FR. Therefore, these functionalized nanocarriers constitute attractive alternatives to ameliorate the side effects and low efficacy of conventional cancer treatments [72].

A functionalized bovine serum albumin (BSA) nanoemulsion was produced with BSA-drug conjugates, either methotrexate (MTX), a potent anticancer agent, or vancomycin (VCM), a potent antibiotic, as a drug. BSA-folic acid (FA) conjugates were also produced resulting in effective FA-tagged nanoemulsion for specific FR-mediated targeting in a KB cancer cell line. BSA-drug conjugated nanoemulsions showed by *in vitro* analysis, effectiveness with improved half-life in systemic circulation, offering a good and flexible template for a wide range of medical applications [18].

Highly stable BSA nanoemulsions were produced by high pressure homogenisation using a tri-block copolymer (Poloxamer 407). This copolymer presents a central hydrophobic chain of polyoxypropylene (PPO) and two identical lateral hydrophilic chains of polyethylene glycol (PEG). A linear correlation between this surfactant concentration and the resulting nanoemulsion’s size was observed by TEM imaging. Further, the neutral and hydrophilic surface of the generated PEGylated nanoemulsion provides stealth particles that are less phagocytized, with a longer half-life in systemic circulation. The incorporation of BSA-FA (folic acid) conjugate solution in this system generated FA-PEGylated nanoemulsions which favors specific cell uptake mediated by folate, indicating 5-fold higher internalization of these tagged-nanoemulsions by cells than non-targeted PEGylated nanoemulsions. The absence of cytotoxicity associated with these attributes provides ideal characteristics to recommend this new functionalized Folic Acid (FA)-tagged

protein nanoemulsion as a promising vehicle for targeted drug delivery into diseased tissues [25].

1.6.3 Nanoemulsions for Topical Route of Administration

1.6.3.1 Drug Uptake Pathway for Topical Administration

Drug penetration through the skin involves several challenges in this natural barrier that extends from the external to internal layers. Skin is composed of stratum corneum as the most external layer, followed by the epidermis, dermis, and subcutaneous tissue. There are three penetration pathways by topical applications, which consist of intercellular, hair follicle and transcellular pathways. The cement-like structure among the keratinized cells represents an effective natural barrier against external substances. The small particle size as well as the lipid nature of nanoemulsions favors efficient penetration of active compounds via topical application [73, 74].

1.6.3.2 Nanoemulsion Applications in Topical Administration

Non-Ionic Nanoemulsions

Fennel essential oil nanoemulsion (FEO NE) was employed as a transdermal drug delivery system aiming to achieve effective prolonged antidiabetic activity. HPLC analysis showed 64% loading efficiency for *trans*-anethole, with promising results in thermodynamic stability, conductivity, pH, particle size and zeta potential of the obtained nanoemulsion. FEO NE showed superior permeation profiles for 24 h, and also a high potential of reducing plasma glucose levels in rats which continued for 7-days after a single topical application of a dose of 120 mg/kg of FEO [75].

Cumin essential oil was loaded in transdermal nanoemulsion to acquire efficient and prolonged systemic antioxidant and hepatoprotective activities. Among the formulations that revealed good thermodynamic stability and physicochemical

properties, the most promising one resulted in the best *in vitro* and *in vivo* antioxidant efficiency, provided high hepatoprotective potential and reserved rats' body weight loss after a period of seven days of a single transdermal application [76].

A nanoemulsion (NE) gel of meloxicam (MLX), a non-steroidal anti-inflammatory drug, was developed as transdermal delivery system. Percutaneous absorption studies on rat skin demonstrated a higher permeation of meloxicam from NE gel than the drug solution. MLX-NE gel showed to be non-irritant, biocompatible, and also provided the maximum inhibition of paw edema in rats over 24 h compared to MLX solution [77].

Polymer Associated Nanoemulsions

Eucalyptus oil nanoemulsion was impregnated into chitosan todevelop a biopolymer film for wound management studies. The film with and without nanoemulsion was evaluated against *Staphylococcus aureus*, and higher antibacterial activity was obtained from the nanoemulsion-impregnated chitosan film [78].

1.6.4 Nanoemulsions for Mucosal Route of Administration/ Mucoadhesive Nanoemulsions

1.6.4.1 Drug Uptake Pathway

Composed of an epithelial layer, which varies by types, mucosal tissues yield a barrier for natural body cavities from external environment. Mucins, high molecular weight glycoproteins, heavily glycosylated (50 to 80%), which are responsible for adhesion phenomena, are the major component of mucus, usually secreted by goblet cells [43]. Intranasal and ocular routes, gained considerable attention in recent years due to its direct, efficient and non-invasive delivery system [79].

Intranasal administration pathway transports drugs by a well vasculated cavity covered by thin nasal mucosa. The drug reaches systemic circulation without

undergoing intestinal and hepatic metabolism, by the epithelial cell layer through transcellular, paracellular, carrier-mediated or transcytosis route. Targeting the bloodbrain barrier (BBB) by directly transferring the drug from the nose to the central nervous system (CNS) is one of the particular benefits of this route, aside from local and systemic drug delivery [79].

The property of mucin, a component of the tear film, can improve the retention time of ophthalmic preparations. Thus, enhancing the bioavailability of the drug [43, 80]. Nevertheless, the natural barriers and the defence mechanisms of the eye are responsible for the reduced ocular residence time and the low bioavailability of the conventional products. The cornea and lacrimal film provide an efficient barrier against ophthalmic treatment [81].

Although several limitations, such as safety approval of components and application dosages for mucosal administration still remain, progress in nanoemulsion research offers interesting uses as mucosal drug delivery system, due to their small particle size, lipophilic-hydrophilic properties and composition flexibilities.

1.6.4.2 Nanoemulsion Applications

Non-Ionic Nanoemulsions for Intranasal Route

Saquinavir mesylate (SQVM) nanoemulsion (NE) was administrated by intranasal route to enhance central nervous system (CNS) targeting of this anti-HIV drug. NE composed of Capmul MCM, Tween 80, PEG 400 and SQVM was prepared by spontaneous emulsification process. SQVM-NE showed a higher permeation rate compared to a suspension administered to sheeps nasal mucosa. The nasal route showed no significant adverse effects, a higher drug concentration in brain was also observed compared to an intravenous injection of a suspension. Gamma scintigraphy imaging showed a larger drug transport into the CNS, when this SQVM-NE was administrated by intranasal route to rats [6].

Polymer Associated Nanoemulsions for Intranasal Route

Intranasal administration of nanoemulsions was highlighted as an innovative drug delivery system, to overcome some of the known drawbacks of the oral route of administration. The presence of a direct nose-to-brain transport pathway that bypasses the normal BBB pathway via the systemic circulations and the BCSFB (bloodcerebrospinal fluid barrier) has been reported, and an interesting intranasal drug delivery system was described in a review [43]: a mucoadhesive nanoemulsion containing risperidone, an approved antipsychotic drug, was prepared by the spontaneous emulsification method and subsequent addition of chitosan. Superior efficacy on brain/blood uptake ratio of risperidone was observed when a mucoadhesive nanoemulsion was used and compared to a nonmucoadhesive ones or a drug solution [82, 83].

Olanzapine, a novel antipsychotic agent, was loaded in nanoemulsions containing Capmul MCM, Tween 80, ethanol and polyethylene glycol. This olanzapine nanoemulsion (ONE) was coated with chitosan to prepare a mucoadhesive nanoemulsion (OMNE). The OMNE showed the highest drug targeting efficiency (DTE%) and direct nose-to-brain transport (DTP%) in rats among the tested formulations, followed by ONE and thirdly by olanzapine suspension (OS). OMNE showed nearly 2-fold higher DTP% than OS. These results demonstrated the benefit of mucoadhesive nanoemulsion formulation as effective brain targeting of olanzapine [5].

Cationic Nanoemulsions for Ocular Treatment

A positive charged, cationic nanoemulsion was formulated to deliver dexamethasone acetate (DEX) and polymyxin B sulfate (polymyxin B) for treating ophthalmic infection. Narrow droplet size-distribution and average droplet size below 200 nm were obtained by high-pressure homogenisation. The *in vitro* test demonstrated the mucoadhesion efficacy by electrostatic interaction between this cationic nanoemulsion and the negatively charged mucin, which coats the corneal surface. This innovative “amphiphilic cationic concept” offers highly ocular bioavailable solution for treating ophthalmic infections and an array of ophthalmic products [80].

6.4.2.4. Nanoemulsion as Intranasal Vaccine Adjuvants

Recent research highlights nanoemulsion as a promising candidate as mucosal vaccine adjuvant, although further studies are needed to better understand its safety and the mechanisms of mucosal immune response. The mucosal membrane is a large surface area for pathogens to enter, which also makes it a promising pathway for immunization. Nanoemulsion may provide long-term release properties for antigens, non-invasive immunity and stability of antigens for mucosal and systemic immunization [79, 84, 85]. A series of nanoemulsions composed of combination of cationic and nonionic surfactants, co-solvents and soybean oil was developed as mucosal vaccine adjuvants. The physicochemical properties of formulations containing cationic surfactants demonstrated to be a key factor to modulate nanoemulsion adjuvant activities. Thus, this may support the development of customized adjuvants for specific needs to trigger appropriate immune responses [85].

1.6.5 Nanoemulsion Applications for Imaging-Guided Therapy

The application of nanoemulsion composed of, *e.g*., perfluorocarbon (PFC) in Magnetic Resonance Imaging (MRI) is an emerging concept as a non-invasive imaging analysis system. These nanoemulsions migrate to injured tissues by natural defense mechanisms such as phagocytosis, and acting then as marker agents [86, 87]. The association of targeting, therapeutic and diagnostic functions provides the so-called “theranostic” nanomedicine. These multifunctional nanoemulsions may act as a promising imaging therapy for tumor detection and treatments [88].

Non-ionic Nanoemulsions

The application of nanoemulsion in image-guided therapy showed enormous promise in cancer medicine in the past decade. A “theranostic” platform based on oil-in-water nanoemulsions, loaded with hydrophobic glucocorticoid

prednisolone acetate valerate (PAV), iron oxide nanocrystals for MRI, and fluorescent dye Cy7 for near-infrared fluorescence imaging (NIRF), was developed and evaluated in a colon cancer mouse model. All of the PAV nanoemulsion-treated animals showed a significant drug substance accumulation in tumors cells by MRI and NIRF; in addition, a potent inhibitory effect was observed on the tumor growth profiles compared to the control nanoemulsion-treated animals, representing a flexible and unique theranostic platform for image-guided therapy of cancer [88].

A stable, non-toxic, theranostic nanoemulsion of celecoxib, an anti-inflammatory drug, was developed to target macrophages. The mouse inflammation model induced with complete Freund's adjuvant (CFA) showed greater accumulation of celecoxib nanoemulsion in the inflamed vs. control paw. This innovative system is able to simultaneously delivery the drug to macrophages and monitor macrophage migration patterns by optical imaging [4].

1.7 Perspectives

Rationally designed nanoemulsion formulations with benefit cost ratio and low side effects are emerging to address the low bioavailability of poorly water-soluble drugs, allowing optimized and effective drug delivery systems. The challenges for future developments comprise the further understanding of the mechanisms that make nanoemulsion more efficient than conventional drug formulations. These challenges refer to the elucidation of the interactions between the drug and nanoemulsion’s components; the investigation of the impact of the manufacturing process on the formulation composition and drug stability. Also, a better understanding of the influence of nanoemulsion formulation on drug release and drug uptake by different routes of administration is needed. Exploring these mechanistic insights, opens opportunities to enlighten future and rising wave of advanced nanoemulsion developments. These attributes confirmed nanoemulsion as a prospective drug carrier with extensive application to a broad array of opportunities.

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2. CHAPTER 2: Nanoemulsion – process selection and application in cosmetics – a Review

This study was published as Megumi Nishitani Yukuyama, Daniela Dal Molim Ghisleni, Terezinha de Jesus Andreoli Pinto and Nádia Araci Bou-Chacra, with the title Nanoemulsion: process selection and application in cosmetics – a review in ***International Journal of Cosmetic Science****, 2016, 38, 13–24.*

ABSTRACT

In recent decades, considerable and continuous growth in consumer demand in the cosmetics field has spurred the development of sophisticated formulations, aiming at high performance, attractive appearance, sensorial benefit and safety. Yet despite increasing demand from consumers, the formulator faces certain restrictions regarding the optimum equilibrium between the active compound concentration and the formulation base taking into account the nature of the skin structure, mainly concerning to the ideal penetration of the active compound, due to the natural skin barrier. Emulsion is a mixture of two immiscible phases, and the interest in nanoscale emulsion has been growing considerably in recent decades due to its specific attributes such as high stability, attractive appearance and drug delivery properties; therefore, performance is expected to improve using a lipid-based nanocarrier. Nanoemulsions are generated by different approaches: the so-called high-energy and low-energy methods. The global overview of these mechanisms and different alternatives for each method are presented in this paper, along with their benefits and drawbacks. As a cosmetics formulation is reflected in product delivery to consumers, nanoemulsion development with prospects for large-scale production is one of the key attributes in the method selection process. Thus, the aim of this review was to highlight the main high- and low-energy methods applicable in cosmetics and dermatological product development, their specificities, recent research on these methods in the cosmetics and consideration for the process selection optimization. The specific process with regard to inorganic nanoparticles, polymer nanoparticles and nanocapsule formulation is not considered in this paper.

Keywords: emulsions, formulation, nanoemulsion, process, skin barrier, stability

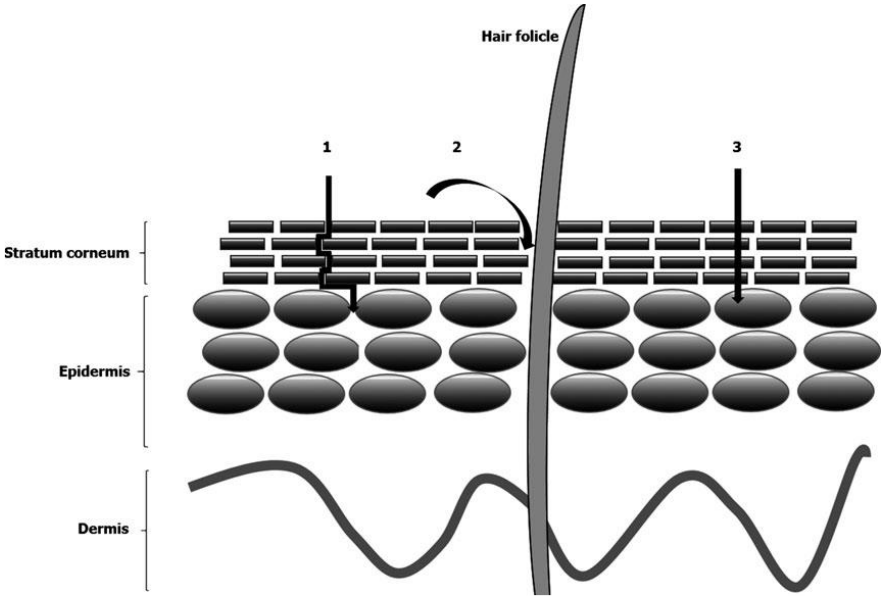
2.1 Introduction

Skin is composed of a natural barrier protecting the body from external harm such as chemical and micro-organism intrusion, UV exposure and dryness and also from mechanical damage. The skin has a multilayered structure: extending from the external layer into the internal layers, including the stratum corneum (SC) composed of dead keratinized cells, below the epidermis and dermis, and subcutaneous tissue. Among the keratinized cells, there is a lipid matrix composed of ceramides, fatty acids, cholesterol and cholesterol esters, which has a cement-like function to provide excellent barrier properties to the skin [1–3]. When a drug or active ingredient is topically applied on to the skin surface, there are, in theory, three penetration pathways though the skin barrier: (1) the intercellular pathway, (2) the hair follicles and (3) the transcellular pathway. In the first, which is the most well-known pathway, the substance diffuses through the stratum corneum via the lipid layers surrounding the corneocytes. In the second, the hair follicles serve as a relevant pathway because a dense network of blood capillaries supporting efficient penetration surrounds them, and they also act as a ‘reservoir’ of the active compound topically applied on the skin. And third, the less understood pathway is transcellular penetration by the direct transportation of drugs through the lipid layers and corneocytes to the living cells (Fig. 2-1) [1, 3, 4].

Due to a continuous increase in consumer demand for better product efficacy, the boundary between the cosmetics field and topical pharmaceuticals is becoming harder to distinguish. To obtain a cosmetic effect, certain penetration of the active compound into the skin is required, although the active compound should not be systemically absorbed after its topical application [5]. The penetration of the active compound through the skin is influenced by several factors, such as the molecular size, the degree of ionization, lipophilicity [3], the synergy between the base component of the formula and the active compound and the synergy between the formulation and the skin [6]. Considering the composition of the skin structure and its barrier property, it seems that a lipid-based formulation will be one of the most appropriate ones for topical application of active compounds [7]. Hence, the emulsion system specifically in the

nanoscale range and its process approach in regard to these selection variables will be discussed in detail in this review.

Figure 2-1 - Schematic illustration of the skin surface with the three possible penetration pathways for topically applied substances (1 = intercellular, 2 = follicular and 3 = intracellular).



Source: author's own production.

2.2 Nanoemulsion definition

Emulsion is a system containing two immiscible phases and composed of at least three components: water phase, oil phase and surfactant phase. The nature of the surfactant determines the continuous phase (external phase) of the emulsion. When an oil soluble surfactant is used, the continuous phase is oil, and when a water-soluble surfactant is used, the continuous phase is water. Therefore, it is called an O/W emulsion when the oil phase is dispersed into the water continuous phase, and a W/O when the water phase is dispersed into the oil continuous phase. Attention has been focused on emulsion with nanometric droplet size since 1980, and it has several potential applications in cosmetics and other topical formulations [8]. Nanoemulsions are O/W or W/O emulsions, non-equilibrium systems, with mean droplet diameters ranging from 50 to 1000 nm.

The size range varies depending on the authors, with some considering 500 nm the upper limit. Therefore, as there is not a drastic change in the physicochemical properties when emulsion droplet size reaches a nanometer range, the size limit may not be considered a key issue [9–11].

The FDA's approach to regulating nanotechnology products embraces a product-focused, science-based regulatory policy considering a material or end product which was engineered having at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm); and a material or end product engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects that are attributable to its dimension(s), even if these dimensions fall up to one micrometer (1,000 nm) outside the nanoscale range [12]. Nanoemulsions are stable against sedimentation or creaming due to their small droplet size, and the ionic and non-ionic ethoxylated surfactants are often used in the O/W nanoemulsions to stabilize against flocculation, due to electrostatic and steric stabilization [13]. The Ostwald ripening seems to be the main mechanism of stability breakdown of O/W-type nanoemulsions. Due to differences in Laplace pressure, a diffusion of molecules of the dispersed phase from small to large droplets occurs through the continuous phase. Thus, the small droplets dissolve, whereas large droplets grow, affecting the long-term stability of the system [10, 14].

The term nanoemulsion is widely used nowadays, but in some articles it is also referred to as mini-emulsion, ultrafine emulsions or submicron emulsions [15, 16]. Depending on the droplet size, a nanoemulsion can be divided into two groups: the transparent or translucent (50–200 nm) and milky (up to 500 nm) [17]. The superior property of a nanoemulsion compared to a macroemulsion is explained by the following characteristics: small droplet size for uniform distribution on the skin, large surface area, modified release and drug carrier properties, better occlusiveness, film formation on the skin, high stability, pleasant aesthetic character and skin feel [11, 17]. Moreover, some studies also report the advantage of the nanoemulsion compared to a liposome delivery system. Nanoemulsions are more stable than the liposomes, enabling even the formation of a lamellar liquid-crystalline phase around the droplets in some cases [11]. Differing from so-called microemulsion, nanoemulsion is a kinetically stable

emulsion. Being a thermodynamically unstable system, it cannot be formed alone; therefore, some energy (mechanical or chemical) input is necessary for its formation. Then, the nanoemulsion, differing from microemulsion, is highly dependent on the process for the nanoscale droplet particle formation [15].

In a preparation of emulsion, the following subjects need to be taken into account: the emulsification process, the condition, the type of components (surfactant, oil and water phases) and the amount of these in the system. For nanoemulsion preparation, there are two methods: the high-energy method in which a mechanical device is used and the low-energy method in which the chemical potential of the component is used. The high-energy method involves high-shear stirring using a rotor/stator system, with ultrasonication, a high-pressure homogenizer and, in particular, microfluidization and membrane emulsification. The low-energy method includes phase inversion temperature (PIT), phase inversion composition (PIC) and solvent diffusion (or self-emulsification or even spontaneous emulsification in non-equilibrium) methods [15]. In the cosmetics field, the O/W nanoemulsion type has been studied more than the W/O, and the high-energy method was the most reported recently in the literature, although interest in the low-energy method has grown considerably in recent studies due to its energy-saving advantages and being a less damaging process for labile bioactive molecules [16]. The differences in high-energy and the low-energy formulation methods, the advantage and the disadvantage of each process, as well as findings of studies in the cosmetics field using each process will be discussed in this paper. Nevertheless, it is also shown that similar droplet size can be achieved by both types of methods (high- and low-energy methods), depending on the system and composition variables [18].

2.3 Nanoemulsification process: high-energy mechanism

In an emulsification, the required mechanical energy exceeds the interfacial energy by several orders of magnitude. Therefore, it requires high-energy application for submicron droplet formation [19]. Thus, the high-energy process uses intense mechanical force, resulting in the development of huge interfacial areas for nanoscale emulsion formation [13]. As the applied fluid stresses

overcome interfacial tension between the two immiscible liquids, larger droplets are ruptured into smaller droplets, and as a consequence, a high total droplet surface area per volume is created [20]. In general, the high-energy process is followed by two steps: first, the deformation and disruption of macrometric droplets into the smaller droplets; second, the surfactant adsorption at their interface (to ensure the steric stabilization) [21]. High-energy methods can be categorized into four groups: (i) high-shear stirring using a rotor/ stator system, (ii) ultrasonication, (iii) high-pressure homogenization and, in particular, (iv) microfluidization and membrane emulsification [15].

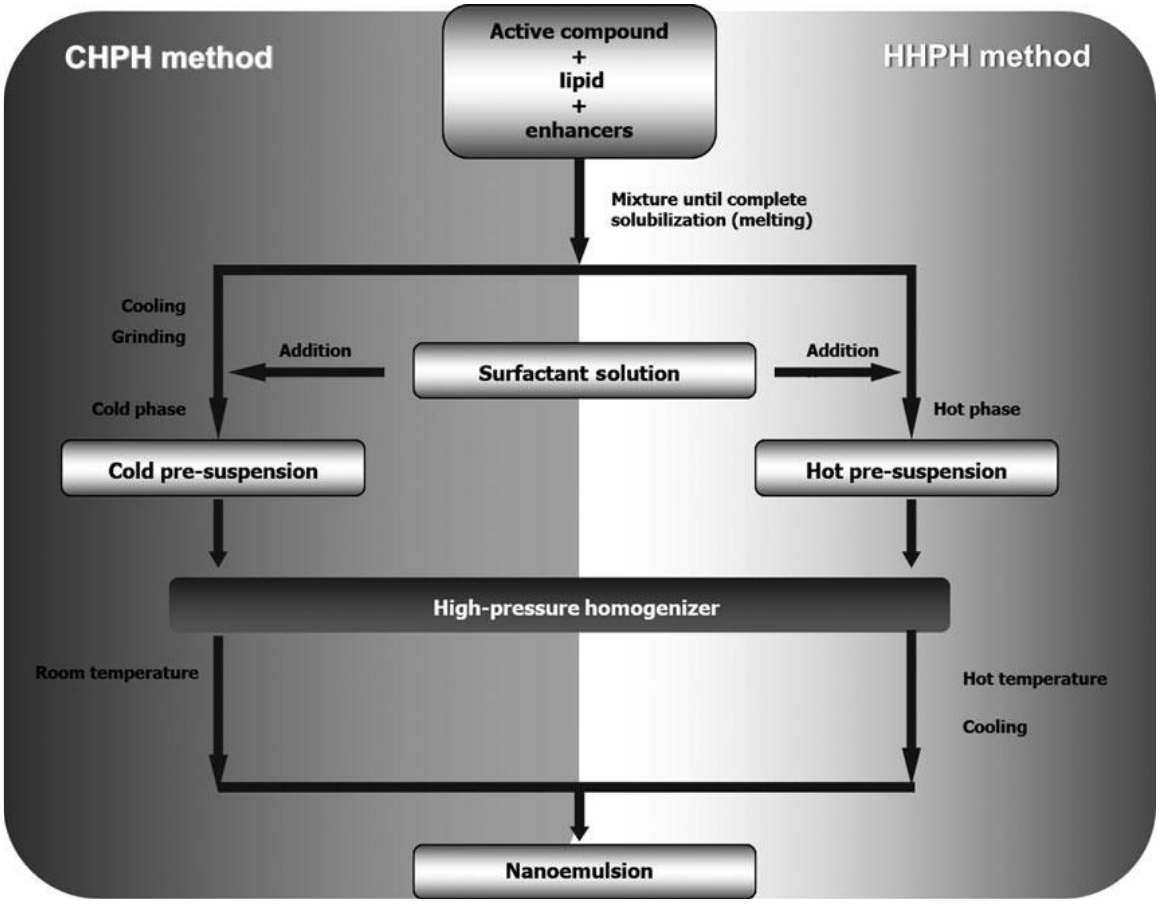
In the high-shear stirring process, the rotor/stator-type apparatuses such as Omni-mixerpsy® or Ultraturraxpsy® are usually used to breakdown the larger droplets into the smaller droplets. However, the average droplet size on a nanoscale is difficult to obtain by this process. To overcome this drawback, a multipass regime must be adopted as the maximum degree of dispersion of the system is not reached by the single-pass regime and the efficacy decreases when high viscosity systems are used [13, 16]. These apparatuses can be used in the combined methods to obtain the macroemulsion containing the active compounds as a pre-emulsion, and subsequent application of other methods as high-pressure homogenizer or self-diffusion method to form nanoscale droplets [16].

The ultrasound or sonication method is based on the cavitation mechanism. The succession of mechanical depressions and compression of the system results in an implosion. This cavitation bubble collapse results in sufficient energy to increase the interfacial area of the droplets. Some studies show that the droplet-size decreases are not proportional to the sonication power increase nor proportional time, once the optimum limit is achieved. The small droplet formation is strongly correlated with the surfactant and/or monomers used in the formulation. Although it is one of the most popular devices used in nanoemulsion research, it is most appropriate for small batches [13, 16, 21]. An industrial scale seems not to be practical as the effective emulsification only occurs in the immediate vicinity of the waveguide radiator, which impacts on the final distribution of the droplet sizes. Therefore, the additional mechanical stirring of mixture is required in large volumes [13, 16, 21].

For the high-pressure homogenization process, under high-pressure homogenization conditions ranging from 10 to 350 MPa, materials are passed through the narrow slot of a homogenizer which are affected by shearing, collision and cavitation force. Nanoemulsion is created in a continuous flow with high velocity, which can reach speeds of hundreds of metres per second [16, 21]. This process is applied to medium- to low-viscosity materials, and the final droplet size is increased when the viscosity and/or the internal phase of the system (dispersed phase) increase. To avoid the coalescence of the newborn droplets or to slow it down, it is important to use surfactants with a high adsorption rate and to increase the number of the cycles. Temperature and pressure also affect the droplet size in this process, by decreasing the droplet size once the temperature or pressure is increased [16, 21]. Hence, the droplet size depends on the emulsion composition, physicochemical condition of the emulsion and also the condition of the process (such as temperature, pressure and number of cycle) [21].

The high-pressure homogenization (HPH) method can be divided into two approaches: hot HPH technique (HHPH) or cold HPH technique (CHPH). The cold HPH technique is used for extremely temperature-sensitive compounds [22]. The active compound is solubilized, dissolved or dispersed in the melted lipid phase in both techniques. In the hot HPH technique, this mixture is dispersed into a hot surfactant solution above the melting point by highspeed stirring to obtain the so-called hot pre-emulsion. In the cold HPH technique, the mixture of active compound and lipid phase is cooled down, ground and then dispersed into a cold surfactant solution to obtain a cold pre-suspension of micronized phase. Then for both methods, the pre-emulsion or pre-suspension passes through a high-pressure homogenizer at high temperatures or room temperature, respectively, to obtain the nanoscale emulsion (Fig. 2-2) [5]. To obtain the reduced polydispersity of nanoemulsion, an additional parameter can be taken into consideration. Increasing the number of cycles of the emulsion in a homogenizer ensures that all droplets experience the peak shear rate generated by a flow-producing device during emulsification. This is a complementary condition to obtain low polydispersity nanoemulsion to a certain point [16, 20].

Figure 2-2 - Nanoemulsion production process using cold (left) and hot (right) high-pressure homogenization technique



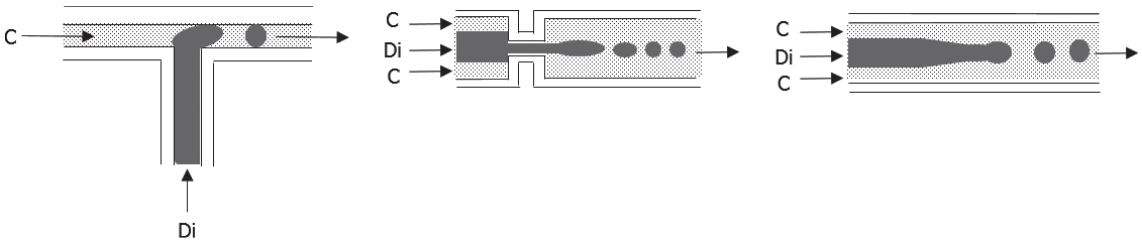
Source: Pardeike, J., Hommoss, A.; Muller, R.H (2009).

In a microfluidizer, a high-pressure pump is used to form nanoemulsion. Two immiscible fluids (oil and water phases) flow

through the microchannels under high pressure up to 2000 psi, combined together and processed in an inline homogenizer to create a coarse emulsion [16, 21]. It is also known as the ‘direct’ emulsification technique because the dispersed phase is injected into the continuous phase through microchannels without a preemulsification step, which is an advantage over the HPH method [23]. As this high performance is controlled by the size of the pores or channels, it is possible to produce emulsions with uniform and controlled droplet size of the internal phase. The stability of the emulsification regime depends on wetting the channel walls by the emulsion components. Usually hydrophobic and hydrophilic surface devices are used, for W/O and O/W emulsion preparation, respectively [16, 23]. This process can also be used for the multiple emulsion preparation as well. There are mainly three types of microfluidizers: T-junction, flow-focusing

geometries and co-flowing, as shown in Fig. 2-3 [16, 23, 24]. (1) T-Junctions: it was used in the first microfluidizers, and it is the simplest structure for droplet production. The continuous phase and the dispersed phase flow through the perpendicular channel. At the junction, the continuous phase generates a thin film between the dispersed phase and the channel of the device. The shear stresses generated by the continuous phase combined with the increasing pressure cause a squeezing of the dispersed phase to generate a droplet [16, 23, 24]. (2) Flow-Focusing Geometries: in this method, the combined dispersed and continuous phase flow is often forced through a small orifice. At this point, the pressure and shear stress from the continuous phase is generated on the dispersed phase, which enables break-up of droplets with narrow size distribution [16, 23, 24]. (3) Co-Flowing: in a co-flow microfluidic device with coaxial arrangement, the dispersed phase is injected from an inner capillary into a tube, in which there is a parallel flowing stream of the continuous phase. When both fluids flow at low rates, single almost monodispersed droplets are formed. This process is defined as dripping. When the flow rate of either fluid is increased, a wider size distribution droplet is generated, due to the jet formation [16, 23, 24].

Figure 2-3 - Different microfluidic geometries for nanoscale emulsion production: (a) T-junction, (b) flow-focusing geometries and (c) co-flowing, with dispersed phase (Di) and continuous phase (C)



Source: Choi, C.H., et al (2014).

Although microfluidizers allow narrower droplet distribution in nanoemulsion than of other emulsifying devices, they have some disadvantages such as high manufacturing costs, channels clogged by solid particles and long emulsification time, which leads to recoalescence of emulsion droplets resulting an increase in the droplet sizes [16, 21].

2.3.1 High-energy method applications in cosmetics

An ultrasound method was applied to obtain nanoemulsions composed of avocado oil, non-ionic surfactant and octyl methoxycinnamate. The sun protection factor of O/W nanoemulsion used as sunscreen, containing 5% avocado oil, 1% octyl methoxycinnamate and 0.25% titanium dioxide, was around SPF 3, and the size distribution of the system ranged from 6 to 10 nm. It showed a slow and sustained release of octyl methoxycinnamate for a period of 4 h [25].

A stable colloidal crystal structure consisting of nanodroplets with Rh_17 nm was obtained by high-pressure homogenization. This nanoemulsion does not flow and has a yield stress. The self-standing nature of nanoemulsion is due to its crystal-like ordered structure with strong electrostatic repulsion. It has a particular behaviour as it is stable against gravity, although a transition from crystal to fluid structure is observed by dilution. Due to its advantage in the percutaneous absorption, moisture retention and so forth, this colloid crystal nanoemulsion is more favourable than solid-state dispersoids for pharmaceutical and/or cosmetics applications [26].

Stable nanoemulsions in a cosmetics matrix enriched with omega-3 were obtained by a high-pressure homogenizer. The nanoemulsion was composed of a combination of oil, soy lecithin and polyoxyethylene sorbitan monooleate as the surfactant phase, glycerol and the aqueous phase. Different physicochemical properties of those nanoemulsions were measured. The mixture design approach was applied to investigate suitable cosmetics matrix systems consisting of multiple ingredients. This approach demonstrates that the nanodroplet size was dependent not only upon the physical parameters of the equipment, but also on the properties of the surfactant and the oil mixture composition. Both surfactants influenced the formulation stabilization, and the following optimal oil combination was identified: 56.5% rapeseed oil, 35.5% miglyol and 8% salmon oil (as source of polyunsaturated fatty acids). The average droplet size was 143 nm and the polydispersity index was 0.16. The formulation showed high stability, by the electrophoretic mobility measurement with readings of around -3 and -4 μmcm/Vs [27].

2.4 Nanoemulsification process: low-energy mechanism

In a low-energy method, a low quantity of energy or just a gentle mixing is applied to generate nanoemulsions. This process depends on the intrinsic physicochemical properties of the surfactants, cosurfactants and excipients composing the formulation [13].

In the early stage of studies on nanoemulsion, the high-energy methods was widely used, especially the ultrasonic emulsification and high-energy stirring to generate nanoparticles. After the diffusion of the other apparatuses such as high-pressure homogenizers, studies aiming at large-scale production were made possible. The first studies on the phase inversion temperature method were reported by Shinoda and Saito. Recently, the interest in the low-energy methods for nanoemulsion generation has grown considerably being a mild process for the sensitive molecules and energy-saving process for large-scale production [16].

The low-energy method includes the spontaneous and phase inversion methods. The phase inversion method consists of phase inversion composition (PIC) where the nanoemulsion generation is dependent upon the water or oil phase dilution process, and the phase inversion temperature (PIT) where the nanoemulsion generation is dependent upon the changing temperature [1, 16].

Going back to the basic principles of the micelle behaviour, an illustration of a sheet-like microstructure (Fig. 2-4) can describe the surfactant phase covering the water or oil phase. This sheet-like microstructure or interfacial films have an important property, which is a curvature, making it into a sphere so that the hydrophobic side (or hydrophilic side) is on the inside of the sphere and the hydrophilic side (or hydrophobic side) is on the outside (Fig. 2-4). The mean (Equation 1) and Gaussian (Equation 2) curvatures show that every point on a surface possesses two principal radii of curvature [28].

Mean Curvature

$$H = \frac{\mathbf{1}}{\mathbf{2}}\left(\frac{1}{r_1} + \frac{1}{r_2}\right) \qquad \text{Equation 1}$$

Gaussian Curvature

$k = (\frac{1}{r_1} + \frac{1}{r_2})$ Equation 2

Helfrich's identification of the membrane bending energy made an important contribution to the thermodynamic properties of sheet-like microstructures. The preference of the curvature of the interfacial film, whether to curves onto the waterside or to the oil side, or even to be flat (H0 = 0), is determined by the spontaneous mean curvature H. The bending moduli k and k measure the added energy required to deform the interfacial film from the preferred mean curvature Ho, and k and k control the response of the interfacial film to thermal fluctuations (Equation 3) [28]. Helfrich membrane bending energy for surfactant sheet-like microstructures

$E = k(H - H_0)^2 + \bar{k}K$ Equation 3

k = Bending Modulus

H = Mean Curvature

Ho = Spontaneous Mean Curvature

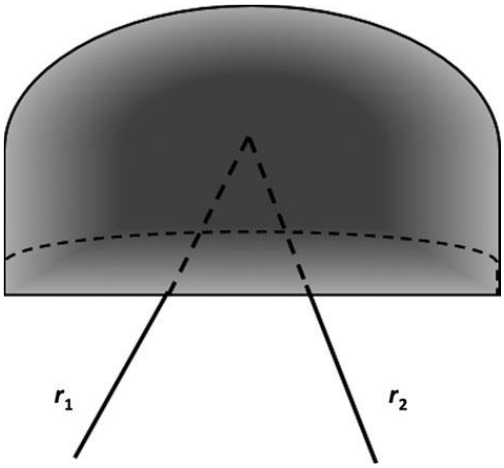
$\bar{k}$ = Gaussian Bending Modulus

K = Gaussian Curvature

As there is a strong correlation between the surfactant type and surfactant–oil–water phase behaviour, understanding the nature of the surfactant microstructure and phase behaviour is relevant to the problem of a surfactant selection for a desired emulsion.

The packing parameter of the surfactant (Ns) determines the surface curvature of the micelle, where Vc is the volume of the hydrophobic chain, a is the cross-sectional area of the hydrophobic core of the aggregate expressed per molecule in the aggregate, and Lc is the length of the hydrophobic chain. (Equation 4) [29].

Figure 2-4 - Schematic Illustration of principal radii of curvature: r1 and r2

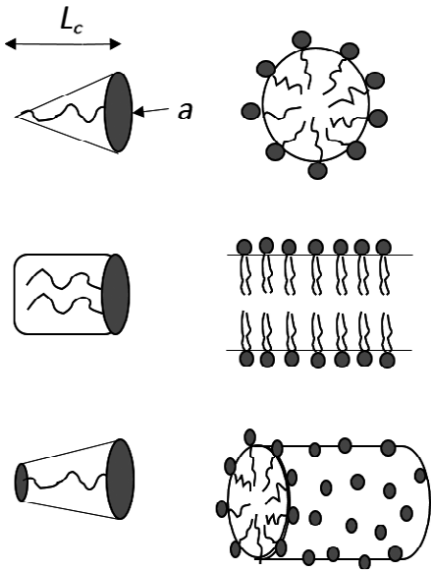


Source: Davis, H.T (1994).

$$N_s = \frac{V_c}{a * L_c} \qquad \text{Equation 4}$$

As shown in Fig. 2-5, in an aqueous environment, spherical micelles are formed by cone-like shape surfactants when Ns ≤ 1/3 (top in figure), a bilayer is formed by cylindrical shape surfactants when Ns > 1/2 (middle in figure), and cylindrical micelles are formed by wedge-like shape surfactants when 1/3 ≤ Ns ≤ 1/2 (bottom in figure). It is important to note that the packing parameter of a specific surfactant is not a constant; thus, it depends on both the composition conditions and other variables, such as temperature and process [29, 30].

Figure 2-5 - Packing parameter and micelle shape

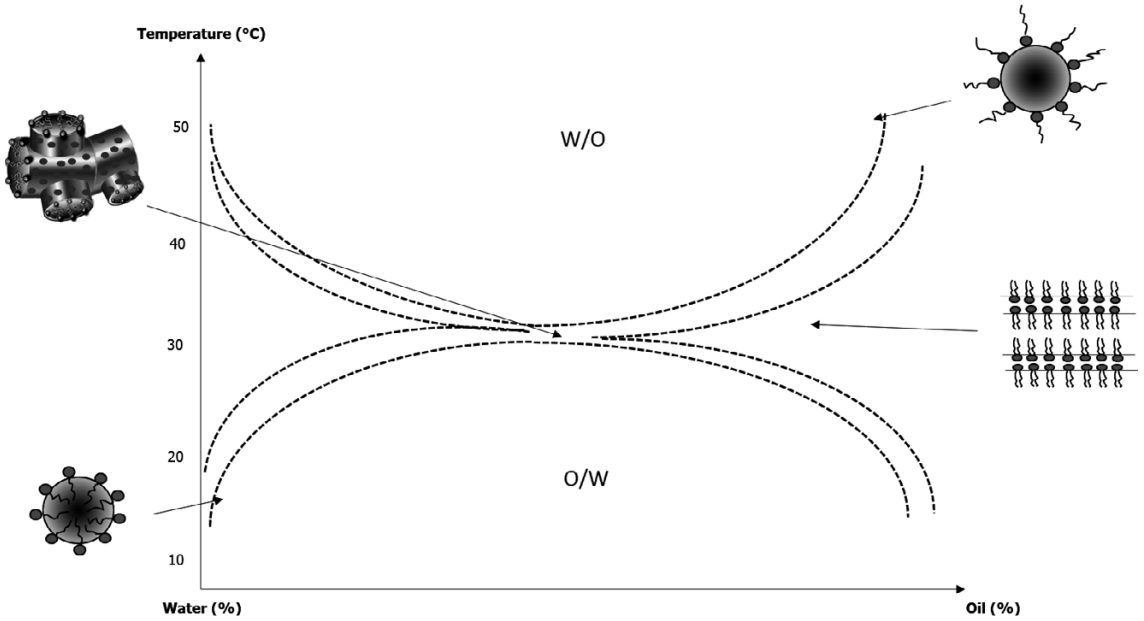


Source: Israelachvili, J. (1994); Goyal, P.S.; Aswal, V.K. (2001).

The influence of temperature as well as the surfactant/oil/water composition on the micelle structure organization is shown in the phase diagram in Figure 2-6.

Figure 2-6 shows the presence of O/W or W/O micelles below or above 30°C, respectively. At the water-rich phase in the left, the presence of a slightly oil-swollen spherical surfactant micelles is shown below 30°C, and water-rich phase containing little surfactant or oil is present above 30°C. At the oil-rich phase in the right, the presence of the oil-rich phase containing little surfactant or water is shown below 30°C, and slightly water-swollen inverse spherical surfactant micelles are present above 30°C. Near 30°C, there is the presence of lamellar liquid crystals or mid-range microemulsions, which the transitional microstructures with small mean curvature enable equal amounts of oil and water to coexist across the interfacial films formed by the surfactant [28].

Figure 2-6 - Schematic illustration of phase diagram of water–oil–non-ionic surfactant system



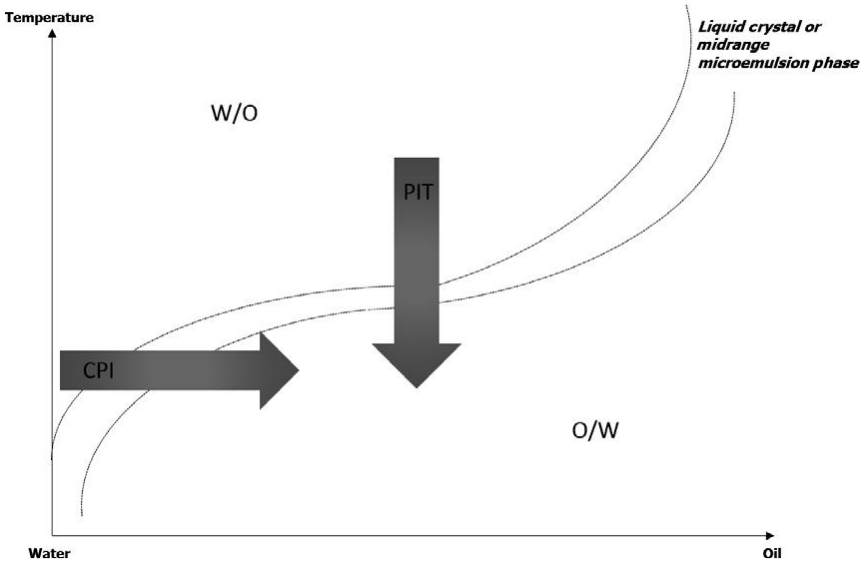
Source: Davis, H.T (1994).

The influence of the micelle or the transitional microstructures on the nanoemulsion generation is detailed in the following phase inversion methods.

The phase inversion method is based on the spontaneous change in the curvature radius of the surfactant interfacial layer, due to temperature change or composition change in the system. At a certain temperature or composition rate, the surfactant adsorbed layer reaches the state characterized by zero curvature of the surfactant monolayer, and low-interfacial tension. At this condition, with a gentle mixing, spontaneous formation of fine emulsions is obtained by the change in the interfacial properties. This average zero curvature of the surfactant film is structured as, for example, bicontinuous microemulsions or lamellar liquid-crystalline phases. The transition of all solubilized oil from this structure immediately before reaching the final two-phase region is the condition to obtain nanoemulsions with minimum droplet size and low polydispersity in an aqueous continuous phase. When the fine droplet formation is based on the composition change by increasing the volume fraction of the disperse phase, it is called a phase inversion composition or a catastrophic phase inversion method. When the

fine droplet formation is based on the temperature change, it is called a phase inversion temperature method, as shown in Fig. 2-7 [9, 10, 20, 21].

Figure 2-7 - Schematic representation of phase inversion temperature (PIT) and catastrophic phase inversion (CPI) methods in nanoemulsion process



Source: Koroleva, M.Y.; Yurtov, E.V. (2012).

The phase inversion composition (PIC) method consists of the preparation of the nanoemulsion based on the phase inversion phenomena by the progressive dilution of the oil phase with the water phase, or vice versa. It involves the changing of the hydrophilic/ lipophilic balance of the system at a constant temperature, whereby the hydration degree of the surfactant increases or decreases according to the dilute phase. The affinity of the surfactant towards the water or oil phase increases until reaching the minimum mean curvature, where thermodynamically stable microemulsion or a liquid-crystalline lamellar phase is formed. When the transition composition is exceeded, through a slight change in the water of oil

proportion, the microemulsion becomes unstable and breaks up to form an unstable but kinetically stable nanoemulsion. From this point, increasing the continuous dilution phase does not change the droplet size [10, 13, 16, 21]. The nanoemulsion formation from phase inversion composition method can be expressed by the ternary phase diagram represented in Fig. 2-8, where (A) the

oil phase was progressively added into the water phase under stirring, and (B) the water phase was progressively added into the oil phase under stirring for the O/W emulsion formation. The final droplet sizes were significantly lower in the method (B), which explains the influence of the process in obtaining the nanoscale emulsion. Only in the (B) process, the phase inversion occurred by the presence of the liquid lamellar structure formation [31].

The phase inversion temperature (PIT) method is based on the use of a temperature-sensitive non-ionic surfactant, specifically polyethoxylated surfactants. At a fixed and well-balanced HLB composition, the temperature of the system is changed, as those ethoxylated surfactants have an ability to change the affinity for water and oil as a function of the temperature. At a low temperature, due to the surface area occupied by the hydrated polar groups being larger than that occupied by hydrophobic hydrocarbon chains, the spontaneous curvature of the surfactant monolayer becomes hydrophilic and the O/W emulsion is formed. With the temperature increase, the oxyethylene groups of the surfactant are dehydrated, and the surface area occupied by the hydrocarbon chains becomes larger curvature of the surfactant monolayer becomes lipophilic and the formation of W/O emulsions becomes more favourable. At an intermediate temperature (or HLB temperature), the surfactant exhibits a similar affinity for the water and oil phases; thus, a microemulsion or lamellar liquid-crystalline phase appears as the interfacial tension and curvature of the surfactant monolayer become close to zero. In this condition, the formation of the small droplets is favoured, implying that the PIT and PIC methods are governed by the same mechanisms. However, as the coalescence rate of the droplets is enhanced under these conditions, the emulsions become very unstable and a rapid move away from this HLB temperature is necessary. By a rapid cooling or heating (to obtain O/W or W/O, respectively), a kinetically stable nanoemulsion with low polydispersity index is obtained [10, 13, 15, 16, 19, 21]. In recent studies, the sub-PIT methods were also reported for nanoemulsion generation in which the nanoscale emulsion is obtained by the phase inversion a few degrees below the PIT temperature [32,33]. The solubilization of all the dispersed phases into the microemulsion phase (at the PIT tempera ture) is linked to the optimum conditions for nanoemulsion generation [34].

In the spontaneous emulsification mechanism, spontaneous phenomenon occurs upon pouring, into water, a water-miscible solvent containing a small concentration of oil phase without the presence of surfactant. In this spontaneous emulsification method, the Ouzo effect (or solvent displacement method) is emphasized in the literature, which consists of nanoemulsion formulation due to the specific and very rapid diffusion of an organic solvent (e.g. acetone, ethanol) from the oily phase to the aqueous one [16, 21, 35, 36]. This is based on two mechanisms named dispersion and condensation. In the first mechanism, when two liquid phases that are not in equilibrium with each other are combined, the interphase instability is induced by the surface tension gradient upon diffusion of substances through the interface. The drops are created because of a quick decrease in the interfacial tension almost to zero, followed by spontaneous increase in the surface area of interface. In the second (and complementary) mechanism, which is induced by the diffusion, emulsification occurs when a new phase condenses in the local supersaturation areas. This is the result of the nucleation and growth of drops due to the spontaneous interfacial expansion [13, 16]. However, the newly formed droplets are unstable and highly subject to destabilization; therefore, the newly formed interfaces have to be stabilized by surfactant adsorption [13]. As that spontaneous emulsification occurs only under specific conditions, the use of the phase diagram makes sense. Many parameters such as the oil viscosity, the surfactant structure and the water solubility of the organic solvent are important in determining the quality of the nanoemulsions obtained by this method [13, 16, 20]. Some studies showed the influence of a high viscosity oil phase on the droplet detachment and the diffusion rate of surfactant molecules towards and through the interfacial boundary, making this detachment and diffusion more difficult [37]. The emulsification occurs spontaneously in the total volume of the system; therefore, it is quite simple to scale up. The disadvantage of this method is the limitation of the oil concentration in the dispersed phase, and the necessity of removal of the solvent by evaporation [16].

2.4.1 Low-energy method applications in the cosmetics field

Kinetically stable W/O nanoemulsions of the water/mixed Cremophor EL:Cremophor WO7 surfactant/isopropyl myristate systems have been obtained by the PIC method. Phase behaviour studies showed that nanoemulsions form in regions with a lamellar liquidcrystalline phase [38].

The phase inversion composition (PIC) method was applied for the formation of O/W nanoemulsion in the cationic system, composed of W/oleylammonium chloride–oleylamine–C12E10/hexadecane. The experimental designs were carried out, and the results were compared to an anionic system composed of W/potassium oleate–oleic acid–C12E10/hexadecane [39]. Both systems showed that the nearer the presence of cubic phase from the final nanoemulsion composition, the smaller droplet-sized emulsions were obtained. This implies that the dilution process influences the nanoemulsion size, differing from the non-ionic systems [40].

It is reported that using a PIC method, an innovative association of polysorbate 80 and palmitic ester of L-ascorbic acid with an average micellar diameter-size ranging from 100 to 300 nm was obtained [41].

The effect of vessel geometry and scale-up in the properties of nanoemulsions prepared by the PIC method was reported. A design of experiment (DoE) approach was applied in this study, and results indicated that the mixing level reached during the emulsification process seems to be the key in order to obtain small droplet-sized nanoemulsions [42].

A unique process using the PIC method at elevated temperatures was applied to obtain paraffin O/W nanoemulsions. A stable and small droplet size nanoemulsion was formed due to the enhanced surfactant adsorption at the O/W interface and reduction in the viscous resistance of the oil phase, when the preparation temperature was increased and kept during the water phase addition process [43].

Bluish and transparent W/O nanoemulsions were obtained with the PIT method, using various compositions of isohexadecane, surfactants (C12E2, C12E4) and

water. Mean droplet sizes of 21 nm were obtained with high stability and without phase separation after 200 days of storage [44].

Vitamin E-enriched nanoemulsions were obtained using spontaneous emulsification. Their variables were examined, and on the size of the droplets, the oil composition had a major impact. Other variables such as the surfactant type, its concentration, the mixing temperature and the stirring speed when the organic phase was added to the aqueous phase also had an impact on the mean particle diameter of the droplets [45].

2.5 High-energy and low-energy methods comparison: differences and similarities

The high-energy and the low-energy processes have different variables affecting the nanoemulsion formation, which were clearly described in this paper and summarized in Table 2-1. The high-energy method is governed by directly controllable formulation parameters such as the quantities of applied energy, amount of surfactant and the nature of the components. The low-energy method requires in-depth understanding of the intrinsic physicochemical properties and behaviour of the systems [13, 16].

Table 2-1 - Nanoemulsion process mechanism, advantages and disadvantages

Nanoemulsion process		Operation principles	Advantages	Disadvantages	References
High energy	Ultrasound generators	Cavitation mechanism	Less expensive than other high-energy equipment; more flexible on surfactant and internal structure selection than low-energy process	Limited to small batches	[13, 16, 21]
	High-pressure homogenization	Shear, collision and cavitation mechanism	More flexible on surfactant and internal structure selection than low-energy process; low process time	High cost; not recommended for thermo- or shear-sensitive compounds	[16, 21, 42]
	Microfluidization	High-pressure injection pump	Controlled size droplets; allows multiple emulsion preparation	High cost; not recommended for large-scale production	[16, 21]
Low energy	Phase inversion composition	Changing of the interfacial film curvature by progressive dilution of the dispersed phase	Low cost; easy to scale-up; no need to heat-up	Requires gradual addition of one phase into another; requires the presence of liquid crystal (LC) or mid-range microemulsion (ME) phases	[10, 13, 16, 21]
	Phase inversion temperature	Changing of the interfacial film curvature by temperature variation	Low cost; easy to scale-up	Limited to the non-ionic surfactants; requires the presence of LC or ME phases; heat energy is required	[13, 16, 21]
	Spontaneous emulsification	Dispersion and condensation mechanism	Low cost; easy to scale-up	Limited amount of oil phase; presence of solvent	[13, 16, 21]

The diversity of available apparatus and the methodologies and the recent advances in technology contribute to the progress of the nanoemulsion research. Due to the growing interest in nanotechnology applications in the cosmetics field in recent years, which target the final consumers, it is crucial to take into account the link and impact between the laboratory-scale and the large-scale production in a plant, such as the feasibility and cost issue.

For both laboratory and industrial scale, a high-pressure homogenizer and microfluidization can be applied. The ultrasonication method is primarily used at a laboratory scale [21].

When a high-energy process is employed, in which high energy is required to rupture droplets for the nanoscale emulsion production, the energy consumption and time of the process depend on the amount of nanoemulsion produced. When a low-energy process is used, the emulsification occurs in the whole volume of the mixture and almost simultaneously, then scaling-up with ease [13]. Thus, the low-energy process is considered an energy-saving process except for the PIT method, which is characterized for some energy consumption depending on the volume of the mixture being dispersed, as heating is required to reach a specified temperature [16].

The PIT method, being a temperature dependent process, has the flexibility of being repeated several times by increasing and decreasing the temperature to guarantee the final nanodroplet quality in the production. Thus from an industrial production point of view, it provides a remarkable advantage compared to the PIC method. However, from the stability point of view, it is key to store this final nanoemulsion in a temperature far from the PIT temperature to avoid a coalescence phenomenon [21], in contrast to the PIC method, in which the droplet formation is performed only once. For the high-energy methods, a considerable investment (i.e. equipment and high-energy consumption) is necessary for industrial-scale production [46] compared to the low-energy method (where the concept is based on the chemical energy stored in the system). However, the high-energy method has an advantage as the process time can be considered lower than the low-energy method in a large-scale production [42].

In addition to the scaling-up, it is also important to take in consideration the composition flexibility among the proposed methods for the nanoemulsion generation. The high-energy methods are effective for nanoemulsion formulation, and these methods are more flexible to introduce various compounds into droplets of the internal phase. In this case, there is no dependency of the temperature changing or adjustment of the interfacial curvature between the aqueous and oily phases [16]. The limitation of these methods is in labile drugs and macromolecules, such as proteins and nucleic acids, which can cause protein denaturation or destruction by intensive shear forces or high temperature [21]. The high-pressure homogenizer, the microfluidizer and phase inversion methods have advantages over spontaneous emulsification and some sonication methods. These methods do not require organic solvents. Some solvents are generally not recommended in most cases in the cosmetics fields. Thus in the first three methods, there is no need for their removal or use [15, 21]. It is also relevant to consider that for industrial production, the use of components with low flash points and high flammability as solvents have limitations from a safety point of view.

The low-energy method has an advantage considering that optimum establishment of the phase diagram provides the generation of the minimum size nanoemulsion and ease of scale-up. But if the low-interfacial zone of the phase diagram is not large enough or flexible enough to enable additional components, to some extent, it implies restrictions of ease of handling, modifying and adapting the nanoparticle formulation to the given needs [13, 16]. The main requirement in the low-energy method for the formation of nanoemulsion with minimum droplet size and low polydispersity index is to ensure a complete solubilization of the dispersed phase in a bicontinuous microemulsion [15].

Comparing the PIC method and PIT method, the PIC method has advantages over the PIT method for the following reasons: it can use a wider range of surfactants whose hydrophilic/lipophilic balance is less dependent on temperature, and it is more suitable for thermo-sensitive active compounds as the use of temperature gradients is not necessary [15]. Nevertheless, special attention needs to be given in the PIC method such as a gradual addition of one phase into another (i.e. it can highly affect the complete solubilization of the

dispersed phase in the bicontinuous phase) [10]. Spontaneous emulsification has an advantage as an alternative to sonication and high-shear techniques in its ease to scale-up and lower process time/cost. However, it has restrictions such as the oil content limitation, the selection of the solvent soluble in water in all proportions and its removal [21].

It is also relevant to point out that in high-energy method, the influence of the preparation variables will be determinant of the nanoemulsion generation, and in the low-energy method, the composition variables will be determinant [9].

Evidently, the minimum size droplets and polydispersity are influenced by the method chosen for this preparation. However, the Ostwald ripening phenomenon, which is the main destability process that affects the final nanoemulsion, is the same, whether the method of preparation uses high-energy or low-energy method [9]. The following methods are used to decrease or slow down the Ostwald ripening mechanism: (i) the addition into the dispersion phase of, even in a small amount, a substantively lower solubility oil in the bulk phase than the main component of the droplet; and (ii) the creation of a thick steric barrier at the droplet interface by the use of surfactants, polymeric emulsifiers or stabilizers [47–49]. Therefore, the preparation of the mixture making use of these alternatives regardless of the method selected has a significant influence on the final quality of the nanoemulsion. Thus, the compatibility of the additional ingredient into the system when the apparatus or inversion is used needs to be well evaluated.

2.6 Comparative or combination study of low-/high energy methods in applications

A comparative study of high-energy method (microfluidization) and low-energy method (spontaneous emulsification) was performed for fabrication of ultrafine edible emulsions: the microfluidization method required high-energy inputs and dedicated equipment, but a lower surfactant-to-oil ratio was needed. On the other hand, the spontaneous emulsification method only required simple mixing, although it needed much higher surfactant-to-oil ratios to produce nanoscale emulsions [18]. Another comparative study was conducted simultaneously

between spontaneous emulsification (SE) and the PIC method to establish the factors that impacted droplet formation and stability. The similarities and differences in these two approaches were highlighted. Nanoemulsions with ultrafine droplets could be produced only from systems in which the surfactant phase and oil phase were mixed together prior to interaction with the aqueous phase, and in which the surfactant and oil were miscible [50].

Mini-emulsions were prepared from a combination of catastrophic phase inversion (PIC) and high-shear technique by a rotor/ stator mixer. This combination method proves to be almost four times more energy efficient than possible with a direct high-shear technique [46].

2.7 Conclusion

Nanoemulsion is a new class of dispersions that has a remarkably wide range of possibilities for innovative applications in the cosmetic and dermatological fields. This review provides the formulator a comprehensive summary of nanoemulsion from a processing techniques perspective. The high- and low-energy methods were extensively discussed, along with a brief overview of their application. It also focuses on the current trends in high productivity and robustness of the nanoemulsion manufacturing, easy scale-up, low polydispersity and size of the nanodroplets, towards higher efficacy and safety of the promising final product. This review clearly evidenced that the key factor for nanoemulsion preparation is the selection of the most suitable process, which ensure the desired properties of the final obtained nanodroplets.

This overview offers to the formulator a realistic commercial scale alternative for this emerging field of nanotechnology in the cosmetic and dermatological market. Further studies need to carry out aiming in-depth understanding in how the oil–surfactant–water phases interact during the processing time and storage, according to the composition and the process selected. This knowledge provides the formulators directions that might elucidate the performance of a particular formulation. Involving the significant challenges and multiple benefits, nanoemulsion has the potential to shape the future of topical products.

2.8 References

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3. CHAPTER 3: D-phase emulsification as a unique energy process - high internal vegetable oil nanoemulsion

This study will be published as as Megumi Nishitani Yukuyama, Pedro Leonidas Oseliero Filho, Gabriel Lima Barros de Araujo, Edna Tomiko Myiake Kato, Raimar Lobenberg, Cristiano Luis Pinto de Oliveira, and Nádia Araci Bou-Chacra, with the title D-phase emulsification as a unique low energy process: high internal vegetable oil nanoemulsion. It was submitted to the International Journal of Pharmaceutics.

ABSTRACT

Vegetable oil nanoemulsion has played a key role as an advanced delivery system for drug, bioactive and nutrients. Nevertheless, the development of nanoscale emulsions containing high vegetable oil and low surfactant concentration is considered a challenge in the conventional low-energy process such as Phase Inversion Temperature method. For the first time, a 20-30 nm nanoemulsion, containing 40.0% (w/w) of olive oil and 2.0% (w/w) of single hydrophilic surfactant, was successfully obtained applying the D-Phase Emulsification (DPE) process in association with the Box-Behnken statistical design for this proposal. DPE process doesn't require strict adjustment of hydrophilic-lipophilic balance, nor the presence of initial water-in-oil phase as in the conventional low-energy methods. The behavior of the DPE process intermediate transition phase was investigated in this study. As a variable that influences the mean particle size of the nanoemulsion, polyol was confirmed to be crucial as the fourth component in this specific system. An isotropic phase was revealed in place of liquid crystalline structure, enabling easy dispersion of high oil content into the oil-in-surfactant intermediate phase, during this process. The DPE process presented unique features as alternative low energy method for obtaining nanoemulsions with a wide range of oils, with multiple applications.

Keywords: D-Phase Emulsification, nanoemulsion, vegetable oil, low-energy, process.

3.1 Introduction

Nanoemulsions, also known as mini-emulsion, ultrafine emulsion or submicron emulsion, has a wide range of applicability for pharmaceutical, food and cosmetic products, as a potential alternative delivery system. Nanoemulsions are colloidal systems, which possesses unique properties when compared to macroscale emulsions: they can provide the bioavailability increase for poorly water-soluble drugs, lower the side effects [1], and be a promising active drug targeting carrier [2]. They are a kinetically stable but thermodynamically unstable system, diverging from thermodynamically stable microemulsion. Nanoemulsions are versatile system enabling diverse types according to their composition such as non-ionic, cationic, anionic or even polymer associated and drug conjugated nanoemulsions [3,4].

Two different mechanisms are known for nanoemulsion formation: the low energy or physico-chemical process, and the high energy or mechanical process. Although the last one, by using high-pressure homogenization, microfluidization, sonication, high-amplitude ultrasonic method, are the most explored processes in the last decades. Interest in low energy processes is emerging due to some advantages. They are considered as energy-saving processes in large-scale production at mild temperatures and suitable for shear sensitive compounds. Low energy processes include a spontaneous emulsification process, phase inversion temperature method (PIT) [5], phase inversion composition method (PIC) [6], and the less known D-phase emulsification (DPE) process.

DPE, also known as surfactant (D) phase emulsification method, presents several advantages if compared to the conventional phase inversion methods. These advantages are mentioned as: it enables the formation of fine emulsion of several oils including vegetable oil, with low surfactant concentration; there is no need of strict adjustment of hydrophilic-lipophilic balance (HLB) of the system, when compared to PIC and PIT; the use of well-balanced mixture of surfactants is not required; and an emulsion with excellent stability at high temperatures can be achieved [7]. In addition, when compared to spontaneous emulsification methods, the DPE process does not require the presence of a solvent. Employing this process, Endoo and Sagitani [8] successfully converted a 20% vegetable oil

phase into oil-in-water (O/W) nanoemulsion with 2% surfactant, using a single hydrophilic surfactant, with no need of specialized processes such as high-pressure or high-shear forces.

The basic components of an emulsion are surfactant, oil, and water, however, in DPE process, an alkyl polyol is used as a fourth component to form fine O/W emulsion [9]. Although the first studies of this process had been presented in 1983 by Sagitani et al. [10], only a limited number of studies investigating the mechanism of DPE process have been reported in the past decades. In the present study, we revisit the DPE through the systematic development of a highly concentrated olive oil nanoemulsion and explore the potentialities and advantages over conventional low energy methods.

3.2 Materials

The material consisted of polyethylene glycol monooleyl ether (Oleth-20, Croda Inc., New York, NY) as a surfactant, olive oil (Sigma Aldrich), glycerin (Sigma Aldrich) and ultrapure water.

3.3 Methods

3.3.1 Nanoemulsion development using DPE process

3.3.1.1 Nanoemulsion preparation:

The nanoemulsion was prepared based on Endoo and Sagitani [8] study with the following modifications: temperature was set to 50 °C for both aqueous and oil phases; initial water and surfactant concentration were fixed, respectively at 2.5 % (w/w) and at 2.0 % (w/w). The remaining amount of water required to obtain the nanoemulsion was calculated depending on the oil concentration (20-40%w/w). The aqueous phase consisted of initial water, oleth-20 and glycerin was heated and homogenized under stirring. The pre-heated olive oil was added

dropwise into this aqueous phase with controlled speed using magnetic stirrer (250-450 rpm). After complete addition of oil, the system was kept under stirring at 50 °C, for 20 minutes. The process was followed by dropwise addition of pre-heated remaining water. After this last step, the obtained O/W nanoemulsion was cooled down to 25 °C.

3.3.1.2 Box–Behnken experimental design:

Table 3-1 - Box-Behnken experiment for DPE nanoemulsion preparations

Formula	Olive oil (%w/w)	Glycerin (%w/w)	Speed (rpm)
1	30.0	2.0	350
2	30.0	3.0	250
3	20.0	1.0	350
4	20.0	2.0	250
5	40.0	2.0	450
6	30.0	3.0	450
7	20.0	2.0	450
8	40.0	3.0	350
9	20.0	3.0	350
10	30.0	1.0	450
11	30.0	2.0	350
12	40.0	2.0	250
13	30.0	1.0	250
14	40.0	1.0	350
15	30.0	2.0	350

The influence of nanoemulsion components and process parameter on the mean particle size was carried out using Box-Behnken experiment (three factors at three levels: +1; 0; -1). A total of 15 formulations including 3 central points

(formulas 1, 11 and 15) was prepared as shown in Table 3-1. The independent variables were olive oil (20.0; 30.0; 40.0% w/w), glycerin (1.0; 2.0; 3.0% w/w) and the stirring speed (250; 350; 450 rpm). The dependent variable or response was the mean particle size (MPS) of the nanoemulsion. The matrix was generated by Minitab 17 statistical software (State College PA, USA).

3.3.2 Optimization procedure

Minitab 17 statistical software (State College PA, USA) was used to optimize the response by composite desirability function method. The aim was to identify the highest concentration of olive oil and the lowest stirring speed, which provides nanoemulsion MPS lower than 500 nm. This was the nanoemulsion MPS value obtained by Endoo and Sagitani [8]. Composite desirability (d) ranges from zero to one. One represents the lowest MPS and zero, the highest one.

3.3.3 Model validation

Based on the result of the optimization procedure, an experiment was conducted with a new set of parameters. The MPS of new proposed formula 16 was measured and compared with the predicted value.

3.3.4 Mean particle size (MPS) and polydispersity index (PdI) analysis

The measurement of MPS and polydispersity index (PdI) were determined by photon correlation spectroscopy using Malvern Zetasizer Nano ZS90 (Malvern Instruments, UK). It is based on the principle of dynamic light scattering, and all the samples were diluted with purified water before measurement at 25 °C.

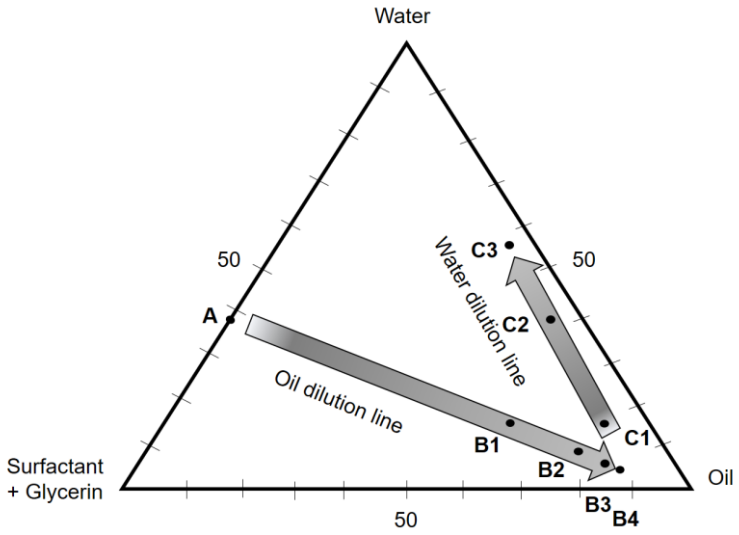
3.3.5 Stability test

The stability of the nanoemulsion was carried out by determining the MPS and by the visual inspection of the formulations during storage at the temperature of 25 °C and set time (three months). The formulations were kept in closed borosilicate glass vessels.

3.3.6 Investigation of phase transition behavior in the DPE process

Aiming to increase our understanding of the system structure behavior during DPE process, we explored some regions of pseudo-ternary phase diagram presented in Figure 3-1. Those regions were split into two dilution lines. For the first line, the samples were taken along oil dilution line where the weight ratio of surfactant/ glycerin/ initial water was held constant, and the oil content reached the maximum weight. After reaching this maximum oil content, the remaining water was added as shown in the second line, a water dilution line. In this case, the weight ratio of surfactant/glycerin/oil was held constant until the water content reached the maximum weight (Figure 3-1 and Table 3-2). This procedure allows understanding how changes in the relative amounts of oil and water influence the structure of the system during DPE process nanoemulsion preparation (e.g. water or oil continuous phase, liquid crystalline presence).

Figure 3-1 – Pseudo ternary phase diagram of the DPE process.



A to B4 is the oil dilution line where the weight ratios of surfactant/glycerin/water equal to 3/3.2/3.8, and C1 to C3 is the water dilution line where the weight ratios of surfactant/glycerin/oil equal to 0.45/0.48/9.07

Source: author's own production.

Table 3-2 - Components composition of pseudo ternary phase diagram of the DPE process

	OIL DILUTION LINE					WATER DILUTION LINE		
	A	B1	B2	B3	B4	C1	C2	C3
S (% w/w)	30.30	12.05	7.52	5.46	4.29	3.85	2.73	2.00
G (% w/w)	31.82	12.65	7.89	5.74	4.51	4.05	2.86	2.10
W (% w/w)	37.88	15.06	9.40	6.83	5.36	15.03	39.84	55.90
O (% w/w)	0	60.24	75.19	81.97	85.84	77.07	54.57	40.00
Total (% w/w)	100	100	100	100	100	100	100	100

W = water, S = surfactant, G = glycerin, O = oil

Conductivity analysis

The conductivity measurement was carried out by the equipment Conductivity-meter CG2000 (Gehaka) at 50 °C. The equipment was calibrated with Gehaka standard buffer of 1,413.0 µS/cm previously to the measurement. The samples of the pseudo ternary phase diagram of DPE process, Figure 3-1 and Table 3-2, were taken to conduct this conductivity analysis [11; 12]. The samples were kept at 50 °C.

Microscopy analysis

The presence or absence of liquid crystalline phases in the preparation was analyzed by a polarized light microscopy (Nikon Eclipse E200, Japan). The samples of the pseudo ternary phase diagram of DPE process were taken to conduct this microscopic analysis.

Small-angle x-ray scattering measurement (SAXS)

Data collection was performed in the laboratory based SAXS equipment NANOSTARTM from Bruker, which was optimized by a microfocus X-ray source, focusing mirror and collimation system provided by Xenocs. The machine is placed at the Institute of Physics, University of São Paulo. The CuKα radiation beam is generated by a GENIX3DTM microfocus source coupled to a FOX3DTM focusing optics, generating a parallel beam. The beam size is defined by using two sets of scatterless slits (Xenocs 2.0), providing a 1x1mm^2 beam size at sample position. The 2D scattering data is collected on a VANTEC2000TM detector. The samples were placed in quartz capillaries with the mean diameter of 1.5 mm. The sample to detector distance was 0.68 m, giving an accessible q range of 0.0.1 < q < 0.4 Å-1, where q is the reciprocal space momentum transfer modulus, defined as $q = (4\pi \sin\theta) / \lambda$, where 2θ is the scattering angle and λ is the radiation wavelength (λ = 1.54 Å in our experiments). For the integrated 1D data, error estimations and data treatment were performed by the program SUPERSAXS (Oliveira and Pedersen, unpublished). Then, the final SAXS data corresponds to a graph of scattered intensity $I(q)$ versus q.

3.4 Results and Discussion

3.4.1 Nanoemulsion development using DPE process

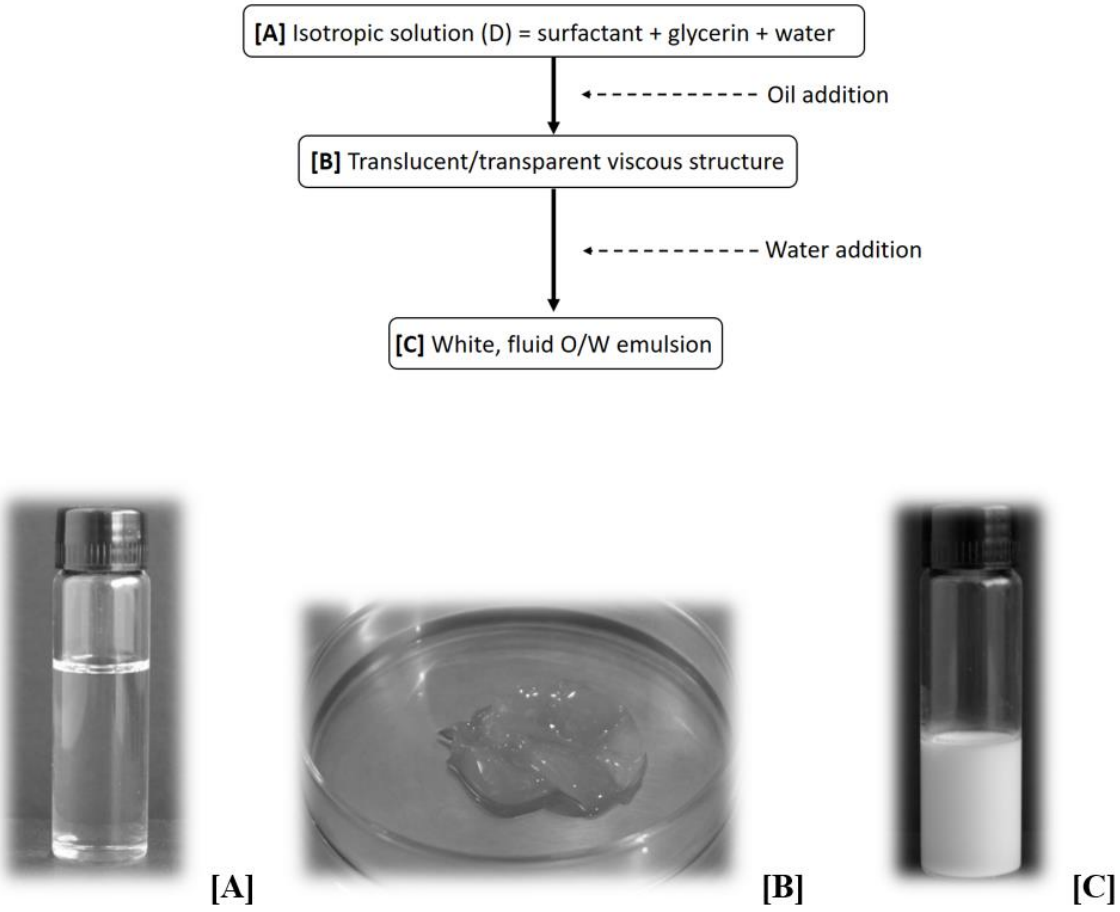
3.4.1.1 Obtaining nanoemulsion by DPE process:

The isotropic solution composed of surfactant, glycerin and water phase (structure A) showed, under stirring, a quick and easy dispersion of the added oil phase. The initial transparent isotropic solution became gradually hazy as the oil concentration was increased, followed by increasing viscosity. Progressively, the system became translucent and a high viscosity structure was formed (structure B). Even under high concentration of the oil phase, the oil was easily incorporated into this structure without phase separation. Subsequently, under continuous addition of the remaining water, the obtained fine internal oil droplets of B phase

were dispersed in the water continuous phase, to form the final O/W nanoemulsion (structure C) (Figure 3-2).

It is reported that the nanoemulsion with low MPS (under 50 nm) appears transparent/translucent [13]. In our present study, the white aspect showed in all obtained nanoemulsions may be due to the high content of the olive oil (20 to 40% w/w).

Figure 3-2 - Structures and visual aspects of DPE process at 50 °C temperature



Source: authors's own production.

Endoo and Sagitani [8] obtained an olive oil nanoemulsion with 500 nm by DPE process. They used a system composed of 20.0% olive oil, 2.0% oleth-20, 76.6 - 77.2% water and 0.8 - 1.4% glycerin (all w/w), at 70°C emulsification temperature. In our study, a 2-fold maximum oil concentration was proposed achieving a MPS approximately 20-fold lower comparing to the original study, in 1991. The Box-Behnken statistical approach allowed optimizing the formula with lower MPS,

higher olive oil content, at lower temperature preparation, compared to the original experiment.

The easy incorporation of oil content in the intermediate structure of DPE process might be a key factor for low MPS nanoemulsion achievement.

This prompt dispersion of oil phase into the isotropic solution is explained by the low interfacial tension between the added oil phase and the isotropic solution. The observed phenomena indicated the presence of oil-in-surfactant (O/D) phase, is in agreement to the procedure described by Endoo and Sagitani [8]. The transition to a translucent and viscous structure with no phase separation, when maximum oil content was added in this system, also confirmed the presence of high internal oil content O/D phase, rather than a conventional liquid crystalline (LC) phase. The LC structure is too rigid, which usually causes a phase separation under addition of high oil concentration [14]. The addition of water dispersed quickly into the intermediate O/D phase forming a white emulsion. This observation indicated that the continuous phase was not an oil phase (consequently, not a water-in-oil emulsion nor surfactant-in-oil emulsion). In brief, under continuous addition of the remaining water, the fine oil droplets previously formed at O/D phase were dispersed into the water phase, resulting in the final oil-in-water (O/W) nanoemulsion. This is also in agreement with Sagitani's report [7].

3.4.1.2 Statistical analysis and the model fitting:

Table 3-3 - Mean particle sizes and particle distributions of the formulas by DPE process (To be continued)

Formula	Mean Particle Size (MPS)	Polydisperity Index (PdI)
1	34.17 ± 0.04	0.30 ± 0.02
2	78.17 ± 1.20	0.26 ± 0.01
3	320.00 ± 11.29	0.23 ± 0.02
4	70.53 ± 1.10	0.24 ± 0.05
5	23.80 ± 0.38	0.24 ± 0.01

Table 3-3 – conclusion

6	35.88 ± 0.12	0.25 ± 0.01
7	44.39 ± 0.55	0.33 ± 0.05
8	36.87 ± 0.05	0.25 ± 0.01
9	84.71 ± 0.48	0.28 ± 0.01
10	348.90 ± 3.71	0.28 ± 0.04
11	26.97 ± 0.25	0.25 ± 0.03
12	28.17 ± 0.03	0.24 ± 0.01
13	402.60 ± 6.18	0.29 ± 0.05
14	322.70 ± 1.65	0.24 ± 0.01
15	38.39 ± 0.18	0.28 ± 0.01

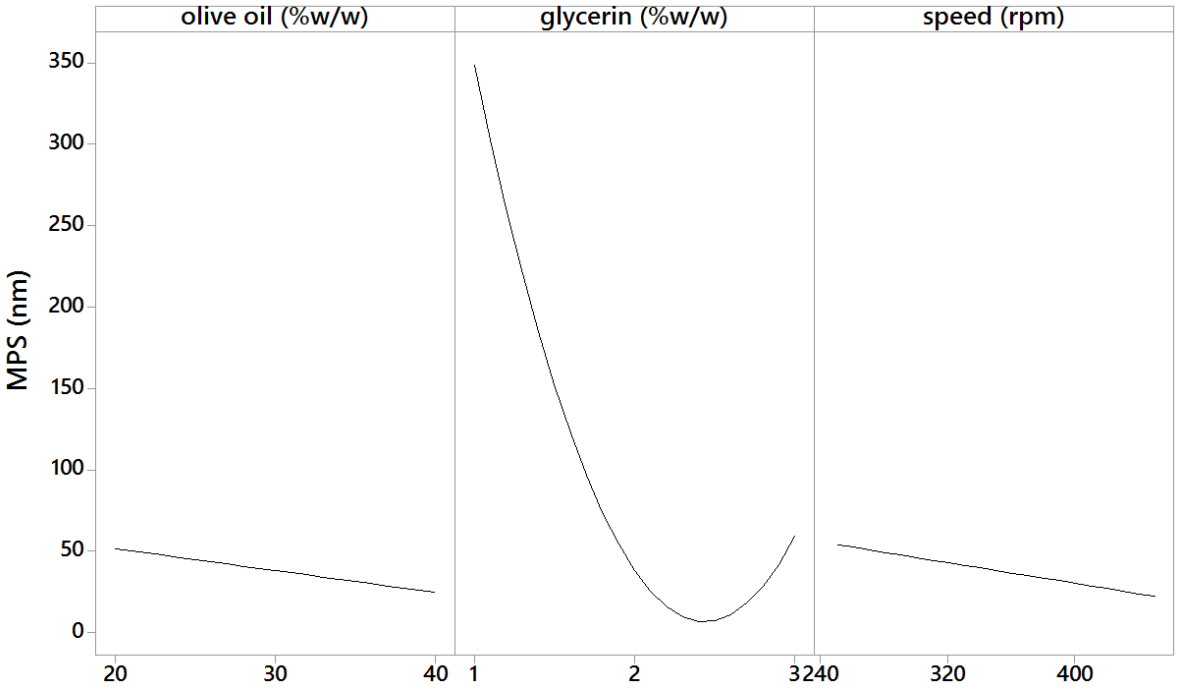
Table 3-4 - Analysis of variance for the different models fitted-response and quadratic regression model for mean particle size of nanoemulsion

Source	Degree of freedom	F-value	P-value
Regression	4	150.96	0.001
Linear	3	125.89	0.001
Oil (%w/w)	1	3.22	0.103
Glycerin (%w/w)	1	370.01	0.001
Speed (rpm)	1	4.43	0.062
Quadratic	1	226.16	0.001
Glycerin (%w/w)* Glycerin (%w/w)	1	226.16	0.001
Lack of fit	8	16.74	0.058

S = 21.29	R^2 = 98.37%	R^2 adj = 97.72%	R^2 pred =95.86%

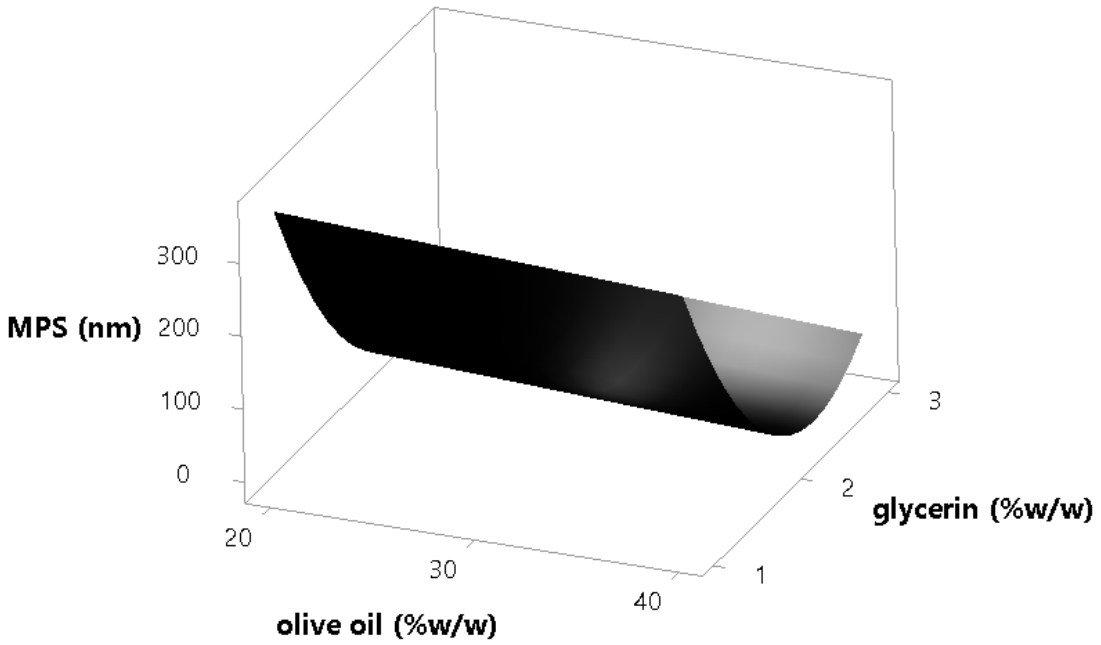
R^2 adj = adjusted coefficient of determination; R^2pred = predicted coefficient of determination

Figure 3-3 - Graph of main effects of means particle size as a function of components and preparation variables



Source: Minitab 1.7.

Figure 3-4 - Response surface of means particle size as a function of components variables



Source: Minitab 1.7.

Response surface methodology (RSM) was applied to evaluate the influence of the independent variables: olive oil concentration, glycerin concentration and stirring speed, on the MPS. Among the RSMs, Box-Behnken design provides some advantage in requiring a fewer number of runs. A total of 15 experiments was performed for optimizing the three factors. The results are shown in Table 3-3 ranging from 23.80 to 402.60 nm (PdI values between 0.23 and 0.30), showing a narrow and monomodal distribution.

The analysis of variance (Table 3-4) revealed that the only main factor, which presented statistical significance, was glycerin concentration (p-value<0.05; α=0.05). The quadratic effect of glycerin concentration was also significant for nanoemulsion MPS (p-value<0.05; α=0.05). This quadratic effect on the MPS, as shown in Figure 3-3, indicated a narrow range of the glycerin concentration (between 2 and 3% w/w), which provides the lowest MPS. The other parameters such as olive oil concentration and stirring speed demonstrated no influence on the MPS of the given nanoemulsion system (p-value>0.05; α=0.05). The effect of glycerin on the MPS is also illustrated by the response surface graph (Figure 2-4). The lack-of-fit was non-significant (p-value equal to 0.058; α=0.05) indicating the well fitness of the proposed quadratic polynomial model.

The quadratic regression model demonstrated the coefficient of determination (R^2) for the MPS of 98.37%, indicating that this response value could be attributed to the identified independent variables. The R^2adj, which reflects the correlation between the experimental and predicted values, was 97.72%. This is a closer value to R^2, indicating a good statistical model. The predicted coefficient of determination (R^2pred) was 95.86% indicating how well the model predicts responses for new observations. The regression equations for the variables in terms of uncoded factors are presented as follows:

MPS(nm) = 1086.4 – 1.351 Oil (%w/w) – 807.6 Glycerin (%w/w) – 0.1585 Speed (rpm) + 165.7 Glycerin (%w/w)* Glycerin (%w/w)

3.4.2 Optimization procedure and model validity

Table 3-5 - Theoretical and experimental value of MPS of optimized formula by Surface Response

	glycerin (w/w %)	olive oil (w/w %)	speed	Theoretical MPS	Experimental MPS
Formula 16	2.10	40.00	250	27.19	27.74 ± 0.66

The optimization condition of the nanoemulsion resulted in the highest concentration of olive oil and the lowest stirring speed, with the MPS lower than that obtained by Sagitani et al (500 nm at maximum 20% w/w of olive oil content) [8]. The optimal conditions within a desirability of 0.9910 were found combining glycerin concentration of 2.1% w/w, olive oil concentration of 40.0% w/w and stirring speed at 250 rpm yielding a theoretical MPS of 27.19 nm. The experimental value resulted from these conditions was MPS of 27.74 ± 0.66. The similar predicted and observed MPS obtained from formula 16, as shown in Table 3-5, validated the proposed model.

3.4.3 Stability of optimized nanoemulsion

The 3-month stability test at 25°C for the optimized formulation showed no change in MPS. The initial mean particle size was 27.74 ± 0.07 nm, with PDI of 0.216 ± 0.018. After 3 months at 25 °C, the MPS and PDI were 26.14 ± 0.09 nm and 0.241 ± 0.015, respectively. No phase separation was observed during this time interval, by visual aspect evaluation.

The stability test was also performed in all formulations. The nanoemulsions with the MPS larger than 60 nm separated within 2 months, and the MPS larger than 200 nm, separated within a 1 day.

As an additional outcome, the conventional low energy processes (phase inversion temperature, PIT and phase inversion composition, PIC) do not allow

obtaining a nanoemulsion with 20 – 30 nm MPS, at 40% w/w olive oil content using a single hydrophilic surfactant at 2% w/w. This is explained by the strict dependency of these conventional low-energy methods on hydrophile-lipophile balance (HLB), briefly introduced below:

The HLB concept was described by Griffin in 1949 [15], and soon the adjustment of HLB in nonionic surfactant systems became crucial for the formation of fine particle size emulsions [7]. Since 1967, the correlation of HLB with the PIT method had been extensively studied by Shinoda [16], providing insights for obtaining nanoscaled emulsions. The PIT method is based on the spontaneous transition of surfactant interfacial layer curvature, from O/W to W/O type by temperature increase, or vice-versa. At the phase inversion temperature zone, the liquid crystal or microemulsion phase is formed. At this moment, the interfacial tension between oil and water phase becomes the lowest. Hence, when emulsification is carried out at around this temperature and followed by cooling, these phases become unstable and break up to form O/W emulsion with very fine emulsified particles, under gentle mixing. When it is heated, it can be reversed to a W/O emulsion [9; 16; 17]. The phase inversion composition (PIC) method is based on the same principle of the spontaneous curvature change of surfactant interfacial layer, although affected by the progressive dilution of one phase to another, in a constant temperature [18].

Therefore, the strict adjustment of HLB is indispensable for obtaining fine emulsions by these both methods [9]. In addition, it is reported that the use of a well-balanced combination of surfactants is recommended rather than a single surfactant system [19]. One inconvenience, in particular to PIT method, is the fluctuation of the storage temperature of obtained nanoemulsion, which should be well controlled to avoid re-coalescence with the temperature increase. In other words, the phase inversion temperature of this system must be higher than the storage temperature, to guarantee the stability of obtained nanoemulsion [16]. This inconvenience can be avoided using the DPE process, since there are no temperature inversion phenomena in this process. It was also reported the difficulty to emulsify vegetable oil into nanoscale emulsion using the conventional phase inversion emulsification methods. Vegetable oils require a large amount of hydrophilic surfactant to enable proper phase inversion emulsification when

compared to hydrocarbons and monoester oils [8]. The use of a high amounts of surfactant raises safety and cost issues. Therefore, the use of DPE method with single hydrophilic surfactant at low concentration is very appropriate for development of high vegetable oil content nanoemulsions.

3.4.4 Investigation of phase transition behavior during DPE process

3.4.4.1 Conductivity of the phase transition system

The conductivity measurements were performed in order to investigate the behavior of the system surfactant/glycerin/water/oil as a function of oil volume fraction along the water/oil dilution lines (Figure 3-1); the results are summarized in Table 3-6. This dilution approach allows to study the behavior of the continuous phase of the system during the DPE process. The mixing ratio of surfactant/glycerin/initial water in A to B4 line were kept constant. After this last step, the C1 to C3 dilution line was used to investigate variations in the system as a function of water volume fraction, where the mixing ratio of surfactant/glycerin/oil were kept constant. It is noteworthy that the samples A, B1 and B2 (Table 3-2) were transparent with increasing viscosity as the oil was added. Samples B3 and B4 appeared hazy to translucent, and from C1 to C3 the samples became white with decreasing viscosity as the water was added.

High values of conductivity are characteristic of aqueous external /continuous phase (O/W or O/D phase), while values lower than 10 µS cm^{-1} or close to the zero indicate the presence of an oil as continuous phase (W/O or surfactant-in-oil, D/O phases) [12]. It was also been reported that a sharp increase in conductivity occurs when phase inversion from W/O to O/W happens during the emulsification process [20]. The conductivity of the 8 selected points indicated the absence of a phase inversion transition from W/O or D/O phase, confirming the absence of oil as a continuous phase.

As complementary information for those results, the samples were further subjected to a drop dilution test, using water to confirm the absence of oil as a continuous phase. This test was conducted by the addition of 1g sample in 200

mL distilled water, at 50°C. All the samples were easily dispersed in the water medium, with no floating or water-repellent effect, indicating that there is no oil at the external phase in those samples. The drop dilution test confirmed that the system's external phase, which was miscible with the water, is composed of water and/or the hydrophilic surfactant phase. From this study, we confirmed that the continuous phase of the oil dilution line (B1 to B4) was surfactant (D) phase during the entire process, before the addition of remaining water to form the final O/W system. The external surfactant phase allows the easy incorporation of oil in the intermediate phase of DPE process, even at high oil content.

Table 3-6 - Conductivity of intermediate phases in pseudo-phase diagram of DPE system at 50°C

Sample	A	B1	B2	B3	B4	C1	C2	C3
μS^{-1}	114.70	25.00	15.08	11.10	17.50	43.70	95.40	94.60

3.4.4.2 Microscopic observation of the phase transition system

Following the same condition as mentioned in item 3.4.4.1, the polarized light microscopic analysis was carried out to investigate the behavior of the system surfactant/glycerin/water/oil as a function of oil volume fraction and water volume fraction along the respective dilution lines.

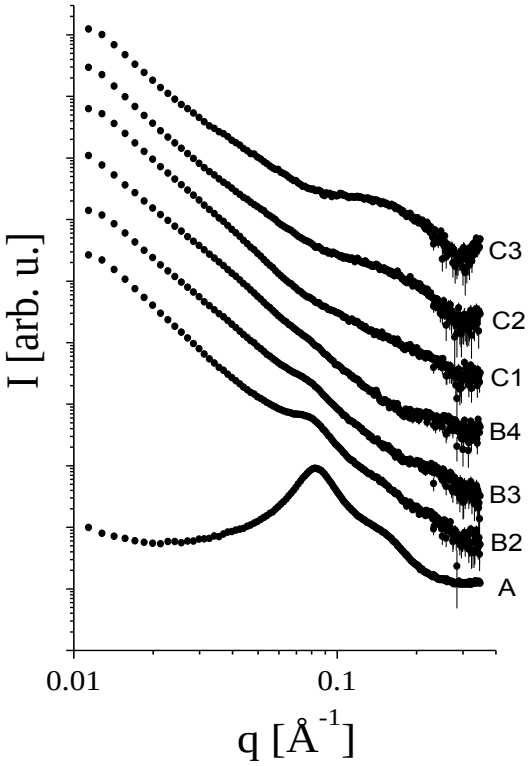
There was no observation of liquid crystalline phases in the systems. Nevertheless, this microscopic observation did not allow us to determine the presence of lyotropic cubic liquid crystalline phase or isotropic phase, which may not be observed by this method. Therefore, SAXS analysis was conducted for further investigations.

3.4.4.3 Small angle x-ray scattering measurement (SAXS)

Following the same condition as mentioned in item 3.4.4.1, the SAXS measurements were performed to investigate the behavior of the system

surfactant/glycerin/water/oil as a function of oil volume fraction and water volume fraction along the respective dilution lines. The data is presented in Figure 3-5. SAXS curves are related to the structural features of the studied system. If two SAXS curves demonstrate some differences, then it is possible to state that the observed structures are different (nevertheless, they can have some similarities). Also, when SAXS curves have sharp peaks (so-called Bragg peaks) this indicate the presence of ordered liquid crystalline structures (lamellar, hexagonal, cubic, etc).

Figure 3-5 - SAXS data of some selected points at 50 ºC. The curves were vertically and logarithmically shifted for better visualization



All curves showed in Figure 3-5 are in some aspect different depending on the water and oil content in the sample. However, there are interesting similarities among the curves. As stated before, sample preparation starts with sample A, which shows a broad peak at the position q ~ 0.08 Å-1 (corresponding to a correlation distances inside the system of ~ 40 Å) followed by an oscillation that can be considered as another weaker broad peak. The absence of sharp peaks

in the SAXS curve indicates that the formed structure is not a well-defined, ordered liquid crystalline phase. The same conclusion is applied to the other curves. Adding oil to the sample A, the broad peak is still present, as one can see from the SAXS curves for samples B2, B3 and B4; this indicates that the overall structure formed in sample A is partially preserved. However, this broad peak almost vanishes when the oil content in the sample is increased up to sample B4. Adding water, this feature was no more observed (samples C1, C2 and C3), but it was detected the formation of a bump at position q ~ 0.16 Å-1 (corresponding to a correlation distance of ~ 20 Å). In conclusion, SAXS curves showed a structural transition along the dilution lines, with absence of a well-defined ordered liquid crystalline structure in this system. In order to obtain further structural information for the system in each dilution line, a systematic SAXS investigation, data analysis and modeling is being performed.

3.4.5 Unfolding the DPE process: a critical analysis and comparison

Compared to Phase Inversion Temperature (PIT) and Phase Inversion Composition (PIC), DPE process was not given due consideration during the last three decades. Aiming to unveil the phase transition mechanism of DPE process, the following subjects will be extensively discussed in this section: (a) the relevance of polyol in this DPE system; (b) the comparison between conventional liquid crystalline phase and O/D phase in nanoemulsion preparation; (c) and the comparison between conventional microemulsion and O/D phase in nanoemulsion preparation.

- *Effect of polyol in DPE system:*

The DPE method is described by Sagitani [9] as a system composed of oil, water, polyoxyethylene-type surfactant, and the polyol as a fourth element. The author described that O/D viscous structure with high internal oil phase is formed under the presence of high oil concentration during the process. At this first study, the presence of a polyol was considered important to prevent D phase forming liquid crystals. Nevertheless, the precise structure of this viscous system is yet

unknown. Furthermore, the need of polyol for this structure construction have been questioned: Kunieda et al [21] reported that polyol is not crucial for the formation of highly concentrated phase in this method. Following these authors, the polyol is relevant only to affect the refractive index of translucent to transparent obtained high-viscous structure, and to facilitate oil introduction into the previously formed high-viscous structure.

Despite this statement, it is known that polyol as 1, 3- Butanediol, 1, 4-butanediol, and 1, 2-propanediol have properties to increase the cloud point of nonionic surfactants. Conversely, polyethylene glycol (PEG) 400, PEG 600, glycerin, diglycerol, polyglycerol 500, and D-sorbitol decrease the cloud point. The first ones render the nonionic surfactants more hydrophilic, and the second, more lipophilic [22].

The possible effect of polyols on the cloud point of polyoxyethylene type surfactant was described as following by some authors: (a) the direct mechanism, whereby dissolution of polyol occurs in the polyoxyethylene chain moiety of surfactant, replacing some of the water molecules around the micelles; or (b) indirect mechanism, whereby the polyol modifies the aqueous medium property, causing water content decreases around the polyoxyethylene chains [22; 23].

Murakami and Fukuda [24] considered that the latter possibility is the most appropriate phenomenon in a nonionic system: a decrease of hydration of surfactant head-group, generated by polyol addition in the medium.

Following this concept, it is also reported the salting-out effect of glycerin in the nonionic system, caused by competition between glycerin and surfactant for the water molecules, resulting in hydrophilicity decrease of the surfactant [23].

The cloud point is related to the HLB of the system [24]. Therefore, in a system where a highly hydrophilic surfactant is required, as in the case of vegetable oils, the glycerin introduction shifts the apparent HLB of hydrophilic surfactant toward more lipophilic. Thus, this reduction of hydrophilic surfactant affinity to the aqueous phase, by the presence of glycerin, is useful to balance the apparent HLB of the system and enables a formation of fine particle emulsion under DPE process [8].

Our study showed that glycerin significantly influenced the MPS in DPE process (Table 3-4). This result corroborated the above-mentioned impact of the glycerin on the interactions among water-oil-surfactant in the system. In addition, the experimental data conducted during the screening evaluation (data not shown) indicated that the glycerin concentration of 1.0 % or below in this system did not yield a stable emulsion. Thus, the data reinforced the effectiveness of polyol as the fourth element in DPE method for obtaining a nanoscaled emulsion, confirming its uniqueness over conventional low energy methods.

The impact of glycerin on O/D translucent viscous structure formation is also discussed in the following sections.

- *Comparison between conventional liquid crystalline phase and O/D phase for nanoemulsion preparation*

As stated previously, vegetable oils require a large amount of hydrophilic surfactants to achieve the desired results by phase inversion emulsification methods. Concurrently, as the solubility of oil in the surfactant phase further decreases with the presence of the hydrophilic surfactant, a higher amount of surfactant is required to form a nanoscale particle size emulsion [7].

However, hydrophilic surfactants tend to form a hexagonal liquid crystal in the presence of water. The structure of a hexagonal phase is too rigid and not suitable for dispersing and holding a large amount of oil [14].

According to Endoo and Sagitani (8), in DPE process, the hexagonal structure was converted to an isotropic surfactant (D) phase due to the presence of the glycerin, which reduced the hydrophilicity of the surfactant. Under addition of oil, O/D phase was formed rendering a viscous translucent to transparent structure at high oil concentration. It is reported that this structure is composed of oil-dispersed phase surrounded by isotropic surfactant continuous phase, with a very low interfacial tension [14]. Therefore, by following addition of water, this O/D emulsion is easily converted to the final O/W emulsion with fine particle sizes.

In the conventional inversion phase methods as PIT and PIC, the W/O phase formed in the initial state should pass through low interfacial tension intermediate

phase to obtain the final O/W nanoemulsion [24] (Figure 3-6). At this intermediate phase during the process, all the oil needs to be solubilized into surfactant continuous phase to enable the effective fine particle generation [25]. As vegetable oils have lower solubility in surfactant phase, a high amount of surfactant is required to form the surfactant one phase. This is the necessary condition for an effective phase inversion emulsification. This solubilization efficacy largely varies depending on HLB, the reason why the strict adjustment of HLB is required in the conventional inversion phase methods [8].

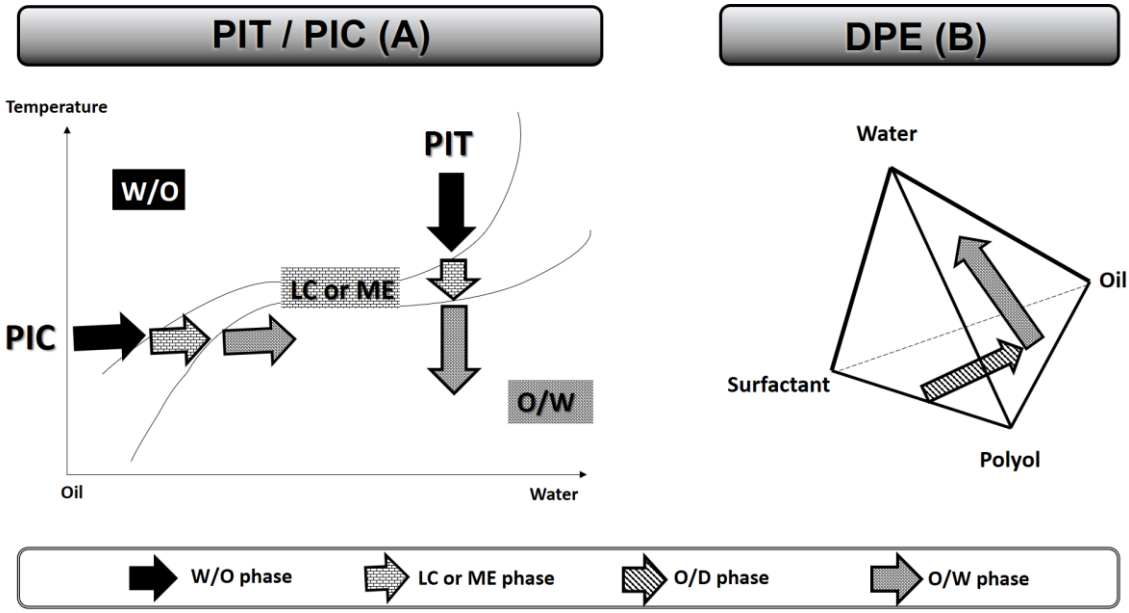
In DPE method, since the oil phase just need to be dispersed (and not solubilized) in the surfactant phase to form stable O/D phase, the strict adjustment of HLB and high amount of surfactant are not so important [7; 24]. In addition, the flexible structure of this isotropic surfactant phase easily disperses the oil phase in the inner part of O/D phase comparing to the hard structure of liquid crystals formed in the conventional phase inversion methods. In our study, microscopy and SAXS experiments, didn't identify the presence of ordered liquid crystalline structure during DPE process in the system.

The conductivity test didn't detect W/O or D/O phase during the entire process. The following addition of remained water after complete addition of oil, showed the quick conversion of the translucent/transparent structure to the white emulsion, indicating the direct transition from the O/D to O/W phase (Figure 3-6).

Nevertheless, an interesting study conducted by Kunieda et al [21] indicated that in fact, this translucent to transparent viscous structure might correspond to a lyotropic cubic liquid crystalline phase. The authors claimed that the highly concentrated cubic phase is viscous and optically isotropic. In addition, the transparency of viscous structure might be due to the addition of polyol which impacts on the refractive index of cubic phase and the internal oil phase.

Interestingly, the SAXS analysis performed in our study revealed the absence of cubic liquid crystalline structure in the system.

Figure 3-6 - (A) Phase Inversion Temperature (PIT) and Phase Inversion Composition (PIC) methods and the emulsion behavior in the colloidal system; (B) Ternary pseudo-phase diagram of DPE process



W/O = water-in-oil; LC = liquid crystal; ME = microemulsion; O/D = oil-in-surfactant; O/W = oil-in-water.

Source: author's own production.

- *Comparison between conventional microemulsion and O/D phase for nanoemulsion preparation*

The microemulsion is an optically isotropic and thermodynamically stable solution. Indeed, the microemulsion is an inappropriate term for a system that is not an emulsion, but a solubilized micellar solution that exhibits maximal solubilization capacity for oil and water phases [26; 27]. At approximately equal volumes of these two phases, the microemulsion exhibits the ultra low interfacial tension between them presenting a three-phase bicontinuous form. It can also be presented as O/W swollen micellar (or water continuous) solution, or W/O swollen micellar (or oil continuous) solution [26; 28]. Once achieved the lowest interfacial tension of the components, the spontaneously formed microemulsion enables to obtain narrow droplets, which is one of the mechanisms of conventional phase inversion methods. Likewise liquid crystalline phase, the strict adjustment and well-balanced HLB of the system are required for complete solubilization of oil phase into intermediate phase to guarantee the fine droplets formation [9].

Depending on the combination of the components, there is no microemulsion formation, which makes it in some cases impossible to achieve a nanoemulsion. Therefore, it makes the HLB adjustment a very time-consuming process in these conventional phase inversion processes.

Another well-known process for nanoemulsion preparation is the self-microemulsifying drug delivery systems - S(M)EDD. Nevertheless, the drawback of this process is the need of high surfactants concentration, usually from 20 to 80% of surfactant/co-surfactant mixture. Another drawback is, in some cases, the addition of organic solvents as ethanol in these systems [27; 29], which may not be appropriate for pharmaceutical, food or cosmetic applications.

In our study, only 2.0% of a single hydrophilic surfactant allowed the preparation of a 20 - 30 nm nanoemulsion containing 40% (w/w) olive oil. Since the added oil phase is just dispersed in the surfactant (D) phase, differing from conventional microemulsion, a wide range of oil types can be incorporated into this system, without the need of a high surfactant content.

A further study aiming to deepen the comprehension of the glycerin and isotropic D phase in the phase transition states of DPE process is still ongoing, opening possibilities to better understand the phenomenon that occurs in this system.

3.5 Summary and Conclusions

For the first time, a stable 40.0% w/w olive oil nanoemulsion with 20 - 30 nm mean particle size (MPS) was obtained using 2.0% w/w of single hydrophilic surfactant, applying DPE process with statistical optimization. It corresponds to 1:20 surfactant-to-oil ratio for obtaining nanoemulsion using a low energy process. The polyol was confirmed to be the statistically significant variable for MPS reduction of the nanoemulsion, influencing the behavior of phase transition structure in this method. The synergistic interaction between the polyol and the hydrophilic surfactant, which modified its apparent HLB, provides in this low energy process a unique phenomenon.

Differing from conventional phase inversion methods, there was no need of strict adjustment of system HLB, neither transition from W/O to O/W phase during the process. The O/D phase was able to hold a high oil content, followed by quick conversion into O/W nanoemulsion after water addition. There was no presence of liquid crystalline structures during the DPE process in this system, allowing the easy dispersion of the oil phase into the surfactant (D) continuous phase, with no separation, even at oil concentration of 40% w/w. A follow-up study of this intermediate transition phase is still ongoing.

DPE process has a myriad of potential application in pharmaceutical, cosmetic and food industry. This low energy method allows the development of nanoemulsion system with low surfactant content and no use of organic solvents. Additionally, as the conventional low energy methods, DPE process enables industrial large-scale production without specific mechanical devices. The current and ongoing future findings of mechanistic phase transition behaviors in our studies may provide nanoemulsion developments for multiple applications, with a wide range of oil types.

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4. CHAPTER 4: Olive oil nanoemulsion preparation using high pressure homogenization and D-phase emulsification – a design space approach

This study will be published as Megumi Nishitani Yukuyama and other contributors. The article is under submission process

ABSTRACT

Virgin olive oil shows extensive applicability in pharmaceutical and cosmetic industries due to its antioxidant properties. These are attributed to the monounsaturated fatty acids as oleic acid and other minor components as phenolics and triterpenic acids. Additionally, olive oil nanoemulsion may enhance the solubility of poorly water-soluble drugs, which comprises about 40% of the top 200 oral drugs marketed in the United States. However, the development of vegetable oil nanoemulsions is a challenge due to their complex composition. In this study, nanoemulsions were prepared using high-pressure homogenization (HPH) and D-phase emulsification (DPE), as high- and low-energy processes, respectively. DPE has the potential to overcome the drawbacks of the conventional Phase Inversion Methods. Aiming to achieve a deeper knowledge of HPH and DPE processes, a design of experiment approach was successfully applied. This approach allowed identifying and understanding the relationship between input factors and their associated output response, in the development stage of the nanoemulsion. Moreover, by a specific range of critical process parameters and compositions, within the design space, nanoemulsions with similar mean particle sizes of 275 nm were achieved with equal composition, regardless of using the HPH or DPE process.

Keywords: nanoemulsion, vegetable oil, low energy process, high energy process, high pressure homogenization, D-Phase emulsification, design space, quality by design.

4.1 Introduction

Virgin olive oil shows extensive applicability in pharmaceutic, food and cosmetic industries due to its wide antioxidant properties. These properties are attributed to the monounsaturated fatty acids as oleic acid, but other minor components as phenolics (oleuropein, ligstroside and oleocanthal) and triterpenic acids, present in this oil. Several studies concerning reactive oxygen species (ROS), reactive nitrogen species (RNS) scavenging, and chelating capacity of flavonoids and phenolic acids are reported [1,2]. Polyphenols gained considerable attention in the last decades as a result of their free radical and ROS scavenging properties, metal ions chelation capacity, and potential to reduce or prevent damages induced by ROS [1,3].

Additionally, olive oil is applied to reducing the risk of chronic disease development such as diabetes, atherosclerosis, cancer, cardiovascular diseases. Oleuropein and ligstroside present in olive oil are hydrolyzed in the gastrointestinal tract generating hydroxytyrosol (HT) and tyrosol, respectively, which present high antioxidant activities. Recent studies indicate the HT and other minor components of olive oil as potential therapeutically candidate to prevent neurodegenerative disorders such as Alzheimer's Disease [4].

Furthermore, olive oil has been used as a carrier of drugs in nanoemulsions - the bioavailability of active component pterostilbene, a natural component found predominantly in blueberry and several grape types, has increased significantly due to the increment in the transport of trans-enterocytes, using olive oil as a carrier when compared to flaxseed oil [5].

The oil phase of nanoemulsion is composed of liquid lipids, allowing in their core high concentrations of vegetable oils. Nanoemulsions have several benefits, including solubilization of highly lipophilic drugs and active compounds, increasing their bioavailability, drug carrier property and increased stability. Moreover, the physicochemical properties of nanoemulsions can be conveniently tailored by several processes and component selections, as well as the surface modification for specific targeting organs. Therefore, the right combination of process and composition selection is the key to the successful development of nanoemulsions with multiple purposes [6].

Two processes are used for nanoemulsion preparation: the high and low energy processes. The first one, which is attributed to the mechanical method, including high-pressure homogenization (HPH), ultrasonication and microfluidization, generate ultrafine droplets by mechanically breaking-up of oil phase by intensive disruptive forces as collision, compression, and cavitation [7]. The second one, also known as the physical-chemical method, includes phase inversion temperature (PIT), phase inversion composition (PIC), spontaneous emulsification, and the less known D-phase emulsification (DPE) methods. The low energy processes produce nanoemulsions by a spontaneous shift of interfacial curvature of oil and water phase, under specific conditions [8]. The advantage of the high-energy process is the non-dependency of hydrophilic-lipophilic balance (HLB) of the components for nanoscaled emulsion formation, although the high cost of equipment can be considered a disadvantage. The advantage and disadvantage of low-energy over high-energy process is usually the low cost in equipment and the strict adjustment of HLB, respectively [9]. Nevertheless, the DPE process has unique properties, enabling to prepare nanoemulsions without strict adjustment of HLB, and the incorporation of a high content of vegetable oils, which were considered limitations in the conventional phase inversion methods of PIT and PIC. The presence of the isotropic D phase enables the easy dispersion of the oil phase to provide the final nanoemulsion [10].

There are few publications correlating the efficacy of high- and low-energy processes in the same composition system for nanoemulsion preparation. Yang et al (2012) compared the microfluidization and spontaneous emulsification methods in the system composed of food-grade oils and surfactants (Tweens). Kotta et al [11] used Capryol 90 (Propylene glycol monocaprylate) and Transcutol HP (Diethylene glycol monoethyl ether) as oil phase and Tween 20 as the surfactant to compare HPH and PIC methods.

To the best of our knowledge, no study has been reported presenting an optimized process for the preparation of a vegetable oil and highly hydrophilic surfactant system by HPH and DPE processes using a design space (DS) approach. DS is defined as the multidimensional combination and interaction of input variables (e.g., material and process parameters) that have been

demonstrated to provide assurance of quality and process understanding. ‘Changing parameters within the design space is therefore not considered as a change and does not require any regulatory approval. The Design-of-Experiment (DoE) methods such as response surface methodology (RSM) can be used to establish the design space, which is one of the key element of quality by design (QbD)’ [12], and it is based on sound science and quality risk management.

Đorđevića et al [13] determined the design space for the nanoemulsion loaded with risperidone (RSP), a poorly water-soluble psychopharmacological drug. A general factorial experimental design was applied to evaluate the interactions of nanoemulsion formulation and process parameters on its critical quality attributes (CQA). A nanoemulsion with mean particle size of 160 nm and zeta potential around -50mV were prepared by high-pressure homogenization. By *in vivo* test in the rat, it showed 1.4 - 7.4 - fold higher risperidone brain availability compared to other nanoemulsions and the drug solution (all 1 mg/mL RSP). Using similar approach, Amasya *et al* prepared 5-Fluorouracil loaded lipid nanoparticle for non-melanoma skin cancer treatment. An artificial neural network software allowed establishing the design space and formula optimization [14]. Furthermore, a design space was successfully established in the study for the optimization of preservatives and EDTA concentration in an emulsion cosmetic product. It was possible to reveal the synergistic and the antagonistic preservative combinations as well as to determine the most effective preservative system for the microorganisms, simultaneously [15].

The aim of this study was to develop an olive oil nanoemulsion prepared by HPH and DPE processes, containing the same composition, using a design space approach to determine the critical process parameters (CPP), targeting an optimal region which offers a similar mean particle size, regardless of using the HPH or DPE process.

4.2 Materials

The material comprises oleth-20 (purchased from Croda) as surfactant, olive oil and glycerin purchased from Sigma Aldrich, carbomer 940 (Mapric, São Paulo, Brazil), and ultrapure water.

4.3 Methods

4.3.1 Nanoemulsion development using Box-Behnken statistical design

4.3.1.1 HPH process

The nanoemulsions were obtained initially using the Ultra-Turrax (IKA T25) apparatus during 5 minutes for preparation of coarse emulsion at 50 °C. This coarse emulsion was subsequently subjected to the piston-orifice type homogenizer (Nano DeBEE, BEE International, Inc. USA) to obtain the final nanoemulsion.

Table 4-1 – Input factors and levels selected for Box-Behnken design in HPH process

Input factors	Symbol	Coded levels		
		-1	0	+1
Pressure (bar)	X_1	250	350	450
Number of cycle	X_2	1	2	3
Surfactant (% w/w)	X_3	1.0	2.5	4.0
Olive oil (% w/w)	X_4	5.0	7.5	10.0
Glycerin (% w/w)	X_5	0.0	1.0	2.0

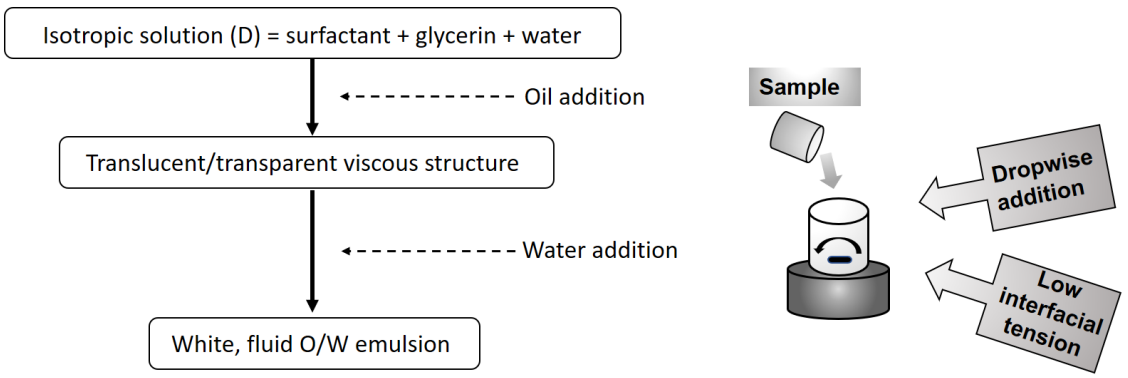
The CPP, which influence the mean particle size (MPS) and polydispersity index (PdI) were determined by a design space approach. The process parameters and the materials were the pressure and number of cycle and the concentrations of olive oil, glycerin and surfactant (input factors), respectively. The independent variables or input factors are shown in Table 4-1. Total of 46 formulas with 5 central points was prepared in randomized order using Minitab 17 statistical software (State College PA, USA).

4.3.1.2 DPE process

The preparation by DPE process was based on Endoo and Sagitani [16] study, with some modifications. Initial water, oleth-20 and glycerol were previously dissolved, at a specific temperature (Table 4-2) under stirring. The pre-heated olive oil at same temperature was added dropwise into this surfactant solution, under magnetic stirring, at 250 rpm. After complete addition of the oil, the system was kept under mixing at 250 rpm stirring speed for 20 minutes, at a constant temperature. The process was followed by dropwise addition of pre-heated remaining water, to obtain the final O/W emulsion. After complete addition of the remaining water, the emulsion was cooled down to 25 °C (Figure 4-1).

Nanoemulsion preparation by DPE process

Figure 4-1- Nanoemulsion preparation by DPE process



Source: author's own production

The process parameter and materials were the temperature and the concentrations of olive oil, glycerin, surfactant and the initial water (input factors), respectively. The independent variables or input factors are shown in Table 2. Total of 46 formulas with 5 central points was prepared in randomized order using Minitab 17 statistical software (State College PA, USA).

Table 4-2 – Input factors and levels selected for Box-Behnken design in DPE process

Input factors	Symbol	Coded levels		
		-1	0	1
Surfactant (% w/w)	Z_1	1.0	2.5	4.0
Olive oil (% w/w)	Z_2	5.0	7.5	10.0
Glycerin (% w/w)	Z_3	1.0	2.0	3.0
Initial water (% w/w)	Z_4	1.0	2.0	3.0
Temperature	Z_5	50	60	70

4.3.2 Optimization procedure

The Minitab 17 statistical software (State College PA, USA) response optimizer tool was used to identify the process parameters and the materials that provide nanoemulsion containing the same composition and presenting similar MPS, regardless of using the HPH or DPE process. Composite desirability ranges from zero to one. One represents the target MPS; zero indicates that one or more responses are outside their acceptable limits.

4.3.3 Model validation

Based on the optimization procedure, a new preparation, one for each process (HPH and DPE), was carried out. The observed and predicted values of MPS for

the obtained nanoemulsions were compared to evaluate the adequacy of the final models.

4.3.4 Mean particle size (MPS) and polydispersity index (PdI) analysis

The measurement of MPS and PdI were carried out using Malvern Zetasizer Nano ZS90 (Malvern Instruments, UK), by photon correlation spectroscopy. The samples were diluted in purified water prior to analysis to avoid multiple scattering effects. This measurement is based on the principle of dynamic light scattering.

4.3.5 Preparation of nanoemulsion gel

Nanoemulsion gels were prepared using the optimized formulations to achieve a final carbopol concentration of 0.2% (w/w) with pH adjusted to 5.0 - 5.5. These nanoemulsion gel preparations were kept in closed borosilicate glass vessels and stability test was carried out for three months at 4ºC and 25°C, by determining the MPS and by the visual inspection of the formulations.

4.4 Results and Discussion

4.4.1 Box-Behnken statistical design for HPH process

The design of experiment (DoE) allows evaluating the multiple interactions between independent variables in experiments, differing from the conventional one-factor-at-a-time method [13]. By analyzing these interactions between the input factors (material and process parameters) and associated output response (CQA) [17], it enables to enlarge product and process understanding, once integrated with mechanistic-based studies [18]. This DS approach gained attention in the last few years, considered as a powerful tool for QbD implementation [19]. In this study, a Box-Behnken design revealed the main effects and the interactions of the evaluated factors, with the advantage of requiring a fewer number of runs compared to other Response Surface Methods (RSM) (e.g. Central Composite Method). The results are shown in Table 4-3, with the MPS (output factor) ranging from 202.2 to 454.6 nm (PdI from 0.16 to 0.45).

Table 4-3 - Box-Behnken experiment for nanoemulsion preparations by HPH process (to be continued)

Order	Pressure (bar)	Cycle	Surfactant (% w/w)	Oil (% w/w)	Glycerin (% w/w)	MPS	PdI
1	450	5	2.5	7.5	2.0	287.0 ± 3.4	0.29 ± 0.07
2	600	4	2.5	10.0	1.0	265.1 ± 6.1	0.25 ± 0.08
3	300	4	2.5	5.0	1.0	236.5 ± 3.3	0.27 ± 0.06
4	450	4	1.0	7.5	0.0	386.1 ± 7.7	0.33 ± 0.12
5	600	3	2.5	7.5	1.0	239.8 ± 3.5	0.25 ± 0.03
6	450	4	4.0	7.5	2.0	283.00 ± 5.7	0.40 ± 0.02
7	450	3	2.5	5.0	1.0	238.1 ± 5.7	0.26 ± 0.02
8	450	4	4.0	5.0	1.0	202.2 ± 3.4	0.30 ± 0.04
9	450	4	4.0	7.5	0.0	248.5 ± 0.8	0.31 ± 0.07
10	600	4	2.5	7.5	2.0	241.6 ± 4.9	0.24 ± 0.04
11	450	4	1.0	7.5	2.0	376.8 ± 21.0	0.16 ± 0.10
12 (CP)	**450**	**4**	**2.5**	**7.5**	**1.0**	**297.4 ± 0.4**	**0.34 ± 0.03**
13	300	4	4.0	7.5	1.0	262.7 ± 7.5	0.36 ± 0.01
14	450	4	1.0	5.0	1.0	340.8 ± 16.7	0.22 ± 0.06
15	450	4	2.5	10.0	0.0	310.7 ± 18.7	0.23 ± 0.08
16	450	5	4.0	7.5	1.0	249.4 ± 2.5	0.30 ± 0.05
17 (CP)	**450**	**4**	**2.5**	**7.5**	**1.0**	**270.0 ± 2.5**	**0.40 ± 0.01**
18	450	3	2.5	7.5	0.0	300.3 ± 2.2	0.40 ± 0.03
19	450	4	1.0	10.0	1.0	367.7 ± 138.0	0.40 ± 0.04
20	600	4	1.0	7.5	1.0	374.4 ± 11.1	0.35 ± 0.10
21	300	5	2.5	7.5	1.0	324.0 ± 4.2	0.45 ± 0.01
22	450	5	2.5	5.0	1.0	216.9 ± 4.0	0.27 ± 0.02
23	600	4	4.0	7.5	1.0	200.9 ± 1.7	0.24 ± 0.01
24 (CP)	**450**	**4**	**2.5**	**7.5**	**1.0**	**282.3 ± 2.4**	**0.40 ± 0.01**
25	450	5	2.5	10.0	1.0	297.0 ± 4.3	0.25 ± 0.03
26	450	4	2.5	7.5	1.0	289.2 ± 8.5	0.36 ± 0.05

Table 4-3 - Box-Behnken experiment for nanoemulsion preparations by HPH process (conclusion)

27	300	4	2.5	7.5	2.0	314.5 ± 3.6	0.34 ± 0.0
28 (CP)	**450**	**4**	**2.5**	**7.5**	**1.0**	**287.1 ± 8.6**	**0.20 ± 0.09**
29	300	4	2.5	7.5	0.0	315.9 ± 4.3	0.33 ± 0.11
30	300	3	2.5	7.5	1.0	321.1 ± 8.6	0.36 ± 0.07
31	450	5	2.5	7.5	0.0	278.6 ± 5.7	0.24 ± 0.11
32	450	4	4.0	10.0	1.0	300.3 ± 11.67	0.20 ± 0.19
33	600	5	2.5	7.5	1.0	235.6 ± 2.5	0.26 ± 0.08
34	300	4	1.0	7.5	1.0	454.6 ± 12.9	0.45 ± 0.02
35	450	3	2.5	7.5	2.0	299.3 ± 0.8	0.34 ± 0.06
36 (CP)	**450**	**4**	**2.5**	**7.5**	**1.0**	**295.8 ± 7.3**	**0.29 ± 0.08**
37	450	3	4.0	7.5	1.0	241.5 ± 2.3	0.31 ± 0.04
38	450	3	2.5	10.0	1.0	308.4 ± 15.7	0.16 ± 0.12
39	600	4	2.5	5.0	1.0	204.2 ± 0.3	0.25 ± 0.02
40	300	4	2.5	10.0	1.0	313.8 ± 17.8	0.16 ± 0.10
41	450	3	1.0	7.5	1.0	400.8 ± 18.4	0.28 ± 0.17
42	450	4	2.5	5.0	0.0	236.1 ± 1.8	0.27 ± 0.01
43	450	4	2.5	5.0	2.0	227.8 ± 0.9	0.25 ± 0.02
44	450	4	2.5	10	2.0	298.4 ± 15.1	0.20 ± 0.11
45	450	5	1.0	7.5	1.0	376.6 ± 24.8	0.18 ± 0.08
46	600	4	2.5	7.5	0.0	239.7 ± 1.2	0.22 ± 0.04

CP = central point

The analysis of variance (ANOVA) of the resultant quadratic polynomial models for the MPS of olive oil nanoemulsion is shown in Table 4-4. This study was carried out to identify the significant terms (input factors) and conduct a statistical analysis of the regression model [17]. The effects corresponding to the investigated input factors (pressure [X_1], number of cycles of homogenization [X_2], concentrations of surfactant [X_3], olive oil [X_4], and glycerin ([X_5]) for the MPS was evaluated. The p-value represents the significance of the regression

coefficients for a polynomial equation, where the p-value lower than 0.05 (α=0.05) indicates that the corresponding coefficient was significant [11,13,20]. The significant input factors were the pressure (p-value equal to 0.001, α=0.05), the surfactant and olive oil concentration (p-value equal to 0.001, α=0.05) and the interaction between the surfactant and olive oil (p-value equal to 0.012, α=0.05). As observed in the main effect graph, due to the quadratic effect of surfactant, the lowest MPS was achieved in the vertex region of the parabola, corresponding to its highest concentration (3.5 to 4.0 % w/w). Differently, the influence of olive oil concentration in MPS achieved the lowest value in its lowest concentration, at 5.0 % (w/w) (Figure 4-2). The lack-of-fit was non-significant (p-value equal to 0.241, higher than 0.05), indicating minimum pure errors (e.g. experimental errors) [11,20]. This fact indicates the well fitness of the proposed quadratic polynomial model.

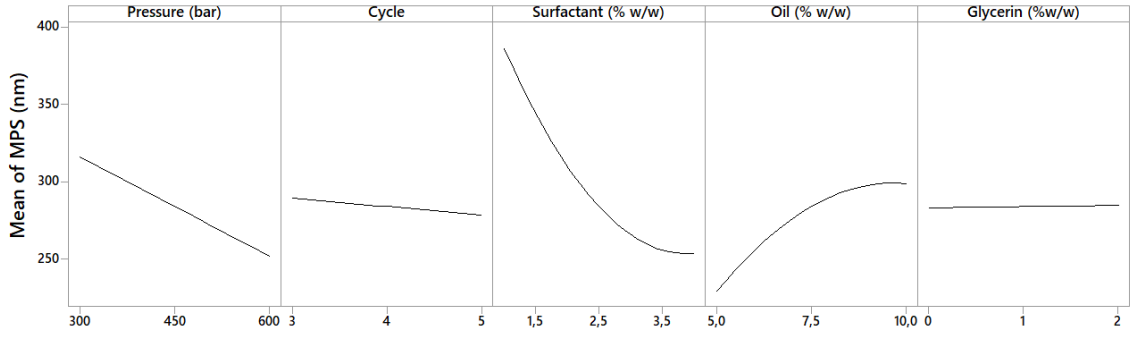
Table 4-4 - Analysis of variance for the different models fitted-response for mean particle size of nanoemulsion by HPH process

Source	DF	SS	MS	F-Value	P-Value
Model	8	127493	15936.7	88.30	0.001
Linear	5	106307	21261.4	117.80	0.001
Pressure (bar)	**1**	**16318**	**16318.1**	**90.41**	**0.001**
Number of cycle	1	440	440.0	2.4	0.127
Surfactant (% w/w)	**1**	**70022**	**70022.4**	**387.98**	**0.001**
Oil (% w/w)	**1**	**19516**	**19516.1**	**108.13**	**0.001**
Glycerin (%w/w)	1	10	10.2	0.06	0.813
Square	2	19919	9959.5	55.18	0.001
Surfactant (% w/w)* Surfactant (% w/w)	**1**	**13196**	**13196.3**	**73.12**	**0.001**
Oil (% w/w)* Oil (% w/w)	**1**	**4075**	**4074.7**	**22.58**	**0.001**
Interaction	1	1267	1267.4	7.02	0.012
Surfactant (% w/w)* Oil (% w/w)	**1**	**1267**	**1267.4**	**7.02**	**0.012**
Error	37	6678	180.5		
Lack of fit	**32**	**6176**	**193.0**	**1.92**	**0.241**
Pure error	5	502	100.3	*	*
Total	45	134171			

S = 13.43 R^2 = 95.02% R^2 adj = 93.95% R^2 pred = 90.93%

The quadratic regression model demonstrated the coefficient of determination (R^2) for the MPS of 95.02%, indicating that this response value could be attributed to the identified input factors. The R^2adj, which reflects the correlation between the experimental and predicted values, was 93.95%. This is a closer value to R^2, indicating a good statistical model. The predicted coefficient of determination (R^2pred) was 90.93% indicating how well the model predicts responses for new observations (Table 4-4).

Figure 4-2 - Main effects plots for means particle size as a function of components and preparation variables by HPH process



Source: Minitab

The number of cycles and the glycerin concentration in the evaluated range showed no significate influence on the output response. Hence, both input factors were not critical to the MPS. Thus, these factors were excluded (except for those required to support hierarchy) [11,13] and the final reduced quadratic model for MPS of olive oil nanoemulsion by HPH process was generated, as demonstrated in the following regression equation, in terms of the uncoded factor.

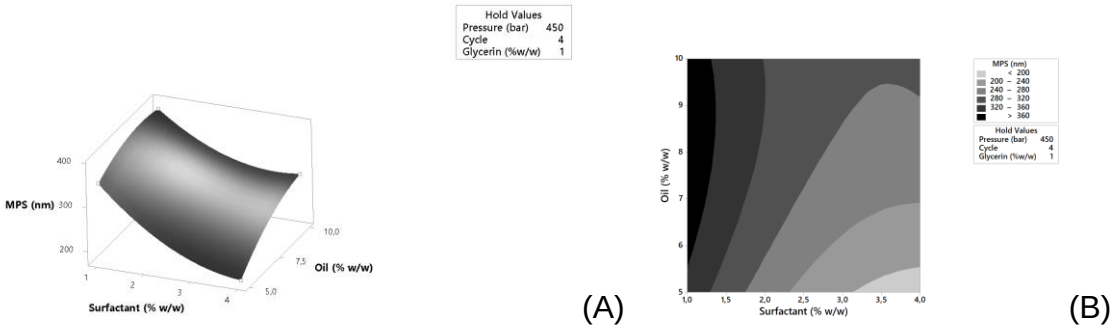
$$MPS(nm) = 414.2 - 0.2129\,X_1 - 5.24\,X_2 - 159.6\,X_3 + 50.1\,X_4 + 0.80\,X_5 + 15.99\,X_3^2 - 3.198\,X_4^2 + 4.75\,X_3X_4$$

The effect of input factor that synergistically influences the reduction of MPS is demonstrated by a negative value in the regression equation, and the inverse effect of the input factor that influences the increase of MPS is demonstrated by a positive value [11].

The interaction between input factors and output response were demonstrated under construction of three-dimensional (3D) surface response and contour plots.

The effects of the surfactant and olive oil concentration on the MPS, by HPH process, are clearly demonstrated in Figure 4-3.

Figure 4-3 - Response surface (A) and contour plot (B) of means particle size as a function of components variables by HPH process



Source: Minitab

In brief, this approach allowed identifying the pressure as a critical process parameter for MPS, a critical quality attribute of the nanoemulsion. The DS also revealed that lowest MPS was achieved using the highest surfactant and the lowest olive oil concentrations. Thus, it was possible to identify the input factors and their ranges within which consistent quality can be achieved.

4.4.2 Box-Behnken statistical design for DPE process

The Box-Behnken design used in HPH process was also applied to evaluate the influence of the input factors in DPE process. These input factors, the components and their concentration, were similar to the components used in the HPH process (Table 4-2). The results of MPS and PdI for the 46 formulas are shown in Table 4-5. However, it resulted in a wide range of outside specification nanoemulsions (separation in one day), which not allowed the subsequent data statistical analysis. Nevertheless, these results enlightened decisions for the further improvements in the process. Furthermore, it allowed us to identify and solve the unpredictability of specific process and material parameters in this exploration step of DS, in DPE process. This DS step identified the optimized ranges of the input parameter and improved the process understanding [19], as described below, bringing the output response into the specification range (CQA).

The concentration of glycerin at 1.0% (w/w) demonstrated that 7 preparations out of 8 presented separation in one day, and at 3.0% (w/w) of glycerin, 5 preparations out of 8 showed the same behavior. At 2.0% (w/w), 15 nanoemulsions out of 26 were obtained successfully. The central points, containing 2.0% (w/w) of glycerin, resulted in nanoemulsion with MPS around 300 nm. This result is in accordance with the previous study [Yukuyama *et al*, chap. 3], confirming that the glycerin concentration impacts strongly the MPS of nanoemulsion in the DPE process.

For the temperature, at 50°C and 60°C showed 6 out of 7 and 12 out of 26 of succeeded nanoemulsions, respectively. Although at 50°C the ratio was higher, we decided to use the temperature at 60°C since it generated an intermediate phase with appropriate viscosity, which allowed easier manipulate on than at 50°C.

Table 4-5 - Box-Behnken experiment for nanoemulsion preparations by DPE process (to be continued)

Order	Surfactant (% w/w)	Oil (% w/w)	Glycerin (% w/w)	Initial Water (% w/w)	Temperature (°C)	MPS	PdI
1 (CP)	**2.5**	**7.5**	**2.0**	**2.0**	**60**	**295.8 ± 8.5**	**0.22 ± 0.03**
2	2.5	10.0	3.0	2.0	60	S	---
3	2.5	5.0	2.0	1.0	60	S	---
4	2.5	7.5	1.0	3.0	60	S	---
5	2.5	7.5	1.0	2.0	70	S	---
6	1.0	10.0	2.0	2.0	60	323.3 ± 21.5	0.23 ± 0.19
7 (CP)	**2.5**	**7.5**	**2.0**	**2.0**	**60**	**279.5 ± 8.3**	**0.17 ± 0.04**
8	2.5	7.5	2.0	3.0	70	274.7 ± 2.9	0.19 ± 0.04
9	1.0	7.5	2.0	2.0	70	S	---
10	1.0	7.5	2.0	1.0	60	S	---
11 (CP)	**2.5**	**7.5**	**2.0**	**2.0**	**60**	**334.2 ± 14.2**	**0.11 ± 0.08**

Table 4-5 - Box-Behnken experiment for nanoemulsion preparations by DPE process (continuation)

12	1.0	7.5	2.0	2.0	50	337.1 ± 13.5	0.11 ± 0.08
13	2.5	5.0	3.0	2.0	60	S	---
14	2.5	10.0	2.0	2.0	70	S	---
15	2.5	5.0	2.0	2.0	50	298.0 ± 5.2	0.16 ± 0.07
16	4.0	10.0	2.0	2.0	60	276.8 ± 23,6	0.18 ± 0.09
17	2.5	7.5	3.0	2.0	70	S	---
18	2.5	7.5	2.0.	1.0	50	S	---
19	2.5	7.5	1.0	1.0	60	S	---
20	2.5	10.0	2.0	2.0	50	371.1 ± 9.1	0.25 ± 0.08
21	4.0	5.0	2.0	2.0	60	307.2 ± 5.9	0.11 ± 0.08
22	2.5	7.5	2.0	3.0	50	327.5 ± 18.2	0.18 ± 0.12
23	1.0	7.5	2.0	3.0	60	1064.0 ± 104.0	1.00
24 (CP)	**2.5**	**7.5**	**2.0**	**2.0**	**60**	**338.8 ± 7.2**	**0.25 ± 0.04**
25	4.0	7.5	2.0	2.0	50	214.5 ± 1.4	0.17 ± 0.02
26	4.0	7.5	2.0	3.0	60	324.0 ± 10.4	0.15 ± 0.08
27 (CP)	**2.5**	**7.5**	**2.0**	**2.0**	**60**	**358.9 ± 20.6**	**0.17 ± 0.07**
28	2.5	7.5	3.0	3.0	60	356.5 ± 12.0	0.14 ± 0.10
29	2.5	7.5	2.0	2.0	60	297.8 ± 4.7	0.12 ± 0.11
30	2.5	10.0	1.0	2.0	60	S	---
31	2.5	5.0	2.0	3.0	60	242.7 ± 3.6	0.10 ± 0.04
32	1.0	7.5	1.0	2.0	60	S	---
33	2.5	7.5	2.0	1.0	70	S	---
34	1.0	7.5	3.0	2.0	60	606.0 ± 35.8	0.68 ± 0.44
35	4.0	7.5	2.0	2.0	70	S	---
36	2.5	10.0	2.0	1.0	60	S	---
37	4.0	7.5	2.0	1.0	60	S	---
38	4.0	7.5	1.0	2.0	60	S	---
39	4.0	7.5	3.0	2.0	60	S	---
40	2.5	7.5	3.0	1.0	60	S	---

Table 4-5 - Box-Behnken experiment for nanoemulsion preparations by DPE process (conclusion)

41	2.5	7.5	1.0	2.0	50	S	-----
42	2.5	7.5	3.0	2.0	50	263.1 ± 2.5	0.15 ± 0.01
43	2.5	5.0	1.0	2.0	60	237.4 ± 5.0	0.16 ± 0.03
44	2.5	10.0	2.0	3.0	60	283.1 ± 11.7	0.17 ± 0.13
45	2.5	5.0	2.0	2.0	70	S	---
46	1.0	5.0	2.0	2.0	60	751.3 ± 61.7	0.55 ± 0.43

S = Separation in one day, CP = central point

Thus, using the fixed amount of glycerin (2.0 % w/w) and set the temperature at 60 °C, tightening the surfactant concentration (2.50; 3.25; 4.00 %) and initial water (2.0; 2.5; 3.0 %) (all w/w), the subsequent and more accurate study was performed for DS building and optimization. The input factor, olive oil, was kept at the previous concentration (5.0; 7.5; 10.0 %). The output responses, MPS and PdI, for designed preparations (15 formulations with 3 central points), are shown in Table 4-6. The results showed the MPS ranging from 288.6 nm to 1399.0 nm (PdI from 0.11 to 0.87).

Table 4-6 - second phase of Box-Behnken experiment for nanoemulsion preparations by DPE process (to be continued)

Order	Surfactant (% w/w)	Oil (% w/w)	Initial water (% w/w)	MPS	PdI
1 (CP)	**3.25**	**7.5**	**2.5**	**295.0 ± 2.6**	**0.29 ± 0.01**
2	2.50	7.5	3.0	726.3 ± 4.8	0.31 ± 0.01
3	3.25	10.0	2.0	669.5 ± 5.6	0.29 ± 0.01
4	2.50	7.5	2.0	625.2 ± 4.4	0.25 ± 0.01
5	4.00	10.0	2.5	477.3 ± 9.1	0.29 ± 0.07
6	3.25	5.0	2.0	790.3 ± 20.6	0.36 ± 0.20
7	4.00	7.5	2.0	1399.0 ± 48.2	0.87 ± 0.22

Table 4-6 - second phase of Box-Behnken experiment for nanoemulsion preparations by DPE process (conclusion)

8	4.00	5.0	2.5	648.8 ± 17.7	0.14 ± 0.12
9	2.50	5.0	2.5	485.0 ± 4.9	0.21 ± 0.01
10	4.00	7.5	3.0	289.0 ± 16.6	0.20 ± 0.06
11	3.25	10.0	3.0	288.6 ± 18.6	0.17 ± 0.07
12	3.25	5.0	3.0	384.7 ± 15.0	0.11 ± 0.03
13 (CP)	**3.25**	**7.5**	**2.5**	**371.5 ± 11.3**	**0.21 ± 0.01**
14	2.50	10.0	2.5	401.4 ± 25.8	0.35 ± 0.29
15 (CP)	**3.25**	**7.5**	**2.5**	**320.0 ± 21.02**	**0.19 0.11**

CP = central point

The analysis of variance (ANOVA) of the resultant quadratic polynomial models for the MPS of olive oil nanoemulsion is shown in Table 4-7. The effects corresponding to the investigated input factors (surfactant [Z_1], olive oil [Z_2], glycerin [Z_3], initial water [Z_4], and temperature [Z_5] for the MPS was evaluated. The significant input factors were the surfactant, olive oil and initial water concentrations (p-value equal to 0.005, 0.002 and 0.001, respectively, α=0.05) and the interaction between the surfactant and initial water (p-value equal to 0.001, α=0.05). In the main effect graph, we observed that the quadratic effect of surfactant concentration on MPS provided the lowest value around 3.2% (w/w). For the initial water concentration, the quadratic effect revealed the highest MPS value around 2.8 % (w/w) (Figure 4-4). The lack-of-fit was non-significant (p-value equal to 0.489, higher than 0.05), indicating the well fitness of the proposed quadratic polynomial model (Table 4-7). Due to the quadratic effect of the surfactant, the lowest MPS were achieved in the narrow vertex region of the parabola. A similar result was obtained for the initial water concentration (Figure 4-4). Thus, the DPE process showed the higher influence of the compositions, as input factor, on the MPS (output response), compared to the HPH.

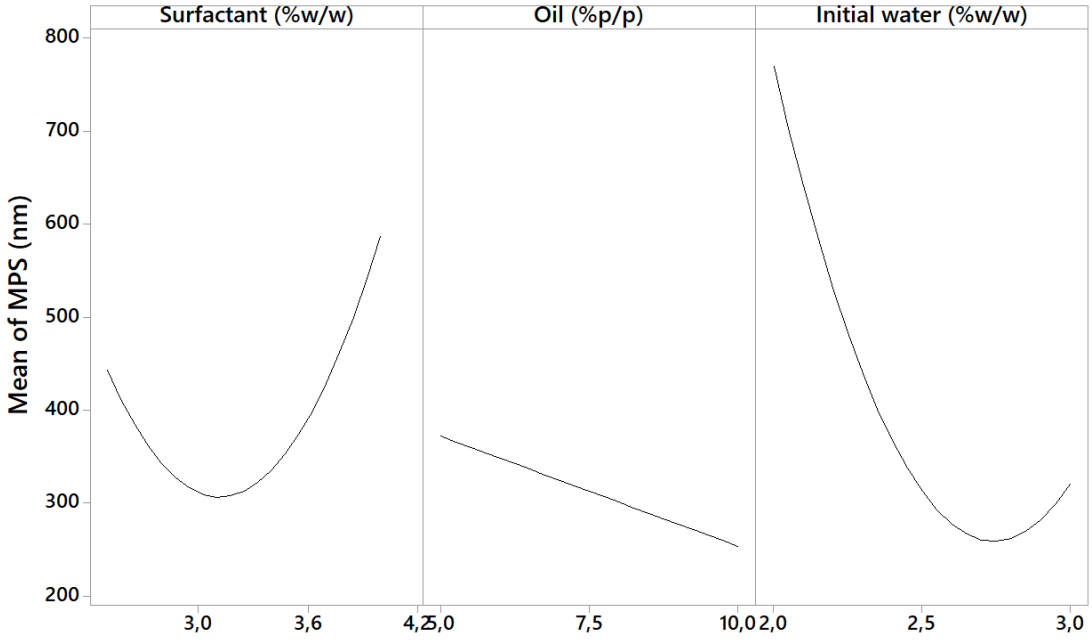
The quadratic regression model demonstrated the coefficient of determination (R^2) for the MPS of 98.72%, indicating that this response value could be attributed to the identified input factors. The R^2adj, which reflects the correlation between the experimental and predicted values, was 97.76%. This is a closer value to R^2, indicating a good statistical model. The predicted coefficient of determination (R^2pred) was 93.72% indicating how well the model predicts responses for new observations (Table 4-7).

Table 4-7 - Analysis of variance for the different models fitted-response for mean particle size of nanoemulsion by DPE process

Source	DF	SS	MS	F-value	P-value
Model	6	1168879	194813	102.60	0.001
Linear	3	472281	157427	82.91	0.001
Surfactant (%w/w)	**1**	**41501**	**41501**	**21.86**	**0.002**
Oil (%w/w)	**1**	**27848**	**27848**	**14.67**	**0.005**
Initial water (%w/w)	**1**	**402933**	**402933**	**212.22**	**0.001**
Square	2	329907	164953	86.88	0.001
Surfactant (%w/w)* Surfactant (%w/w)	**1**	**152245**	**152245**	**80.19**	**0.001**
Initial water (%w/w)* Initial water (%w/w)	**1**	**200966**	**200966**	**105.85**	**0.001**
Interaction	1	366691	366691	193.13	0.001
Surfactant (%w/w)* Initial water (%w/w)	**1**	**366691**	**366691**	**193.13**	**0.001**
Error	8	15189	1899		
Lack of fit	6	12146	2024	1.33	**0.489**
Pure error	2	3043	1522	*	*
Total	14	1184068			

S = 43.57	R^2 = 98.72%	R^2 adj = 97.76%	R^2 pred = 93.72%

Figure 4-4 - Main effects plots for means particle size as a function of components and preparation variables by DPE process



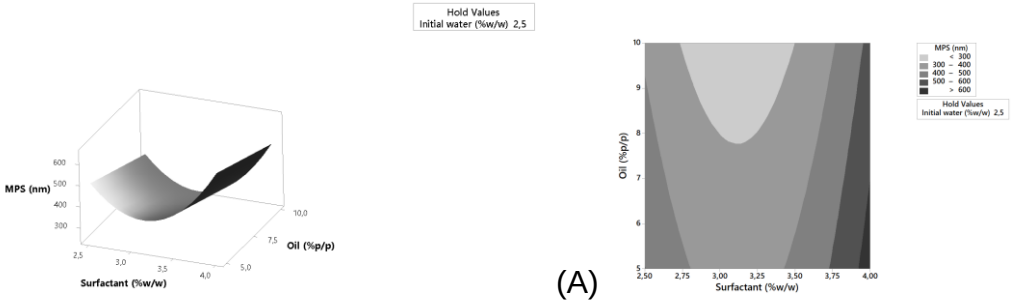
Source: Minitab

The final reduced quadratic model for MPS of olive oil nanoemulsion by DPE process was generated, as demonstrated in the following regression equation, in terms of the uncoded factor.

$$MPS\ (nm) = 4357 - 225\ Z_1 - 23.60\ Z_2 - 2477\ Z_4 + 359.9\ Z_1^2 + 930.4\ Z_4^2 - 807.4\ Z_1Z_4$$

The interaction between input factors and responses were demonstrated by the three-dimensional (3D) surface response and contour plots. The effects of the surfactant and olive oil concentration on the MPS, by DPE process, are shown in Figure 4-5. It is notable that the MPS was reduced as the concentration of olive oil was increased, in a narrow region of the surfactant concentration, which is the opposite phenomena observed in the HPH process. This behavior of olive oil in this process was also observed in the previous study carried out by Yukuyama *et al* [Yukuyama *et al*, chap. 3].

Figure 4-5 - Response surface (A) and contour plot (B) of means particle size as a function of components variables by DPE process



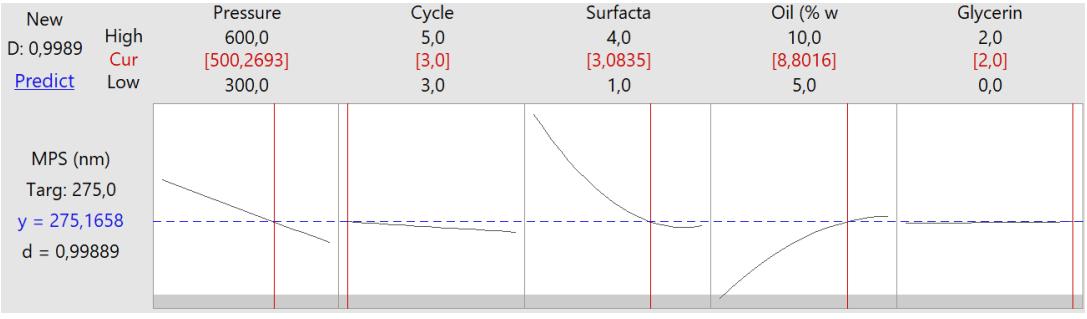
Source: Minitab

4.4.3 Optimization procedure

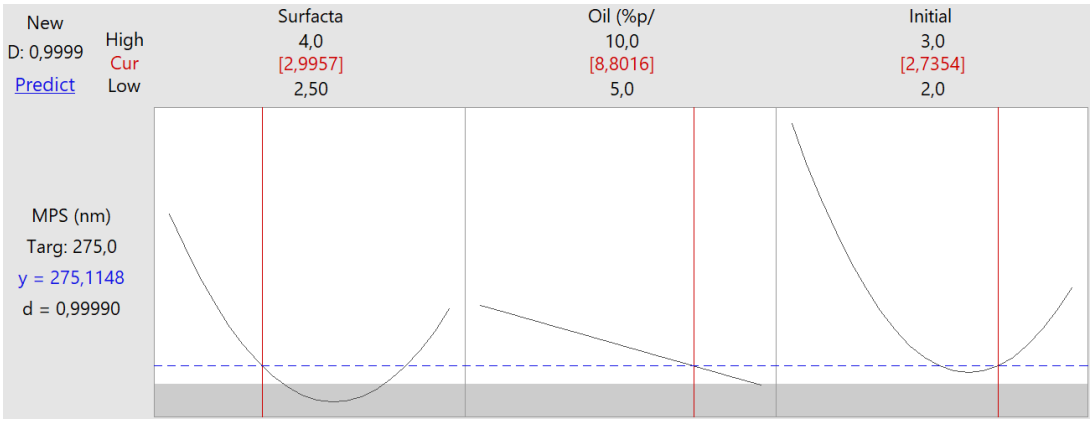
The Figures 4-1 and 4-3 revealed opposite effect of the olive oil concentration on the MPS for DPE and HPH processes. Therefore, identify optimized nanoemulsions by HPH and DPE processes with the same composition for obtaining a similar MPS, was quite a challenge. This challenge was successfully overcome using the desirability function (target 275 nm d, 0.99) which revealed a MPS of 275.1 nm for the HPH process, as well for the DPE process (Figure 4-6).

The optimization composition of the nanoemulsion for both processes was glycerin at 2.0 %, surfactant at 3.0 % and olive oil at 8.8 % (all w/w). The pressure at 500 bars and 3 cycles were fixed for HPH process. The initial water of 2.7 % w/w and temperature at 60°C were set for DPE process, as complementary preparation parameters to achieve this MPS.

Figure 4-6 - Optimization plot for HPH process (A) and DPE process (B)



(A)



(B)

Source: Minitab

4.4.4 Fitting model verification at the selected range

For both HPH and DPE processes, the similar predicted and observed MPS obtained from the optimized formulas, as shown in Table 4-8, validated the proposed models.

Table 4-8 - Theoretical and experimental value of DHM of optimized formulas by HPH and DPE processes

Formula	Theoretical DHM	Experimental DHM
HPH Opt	275.1	285.9 ± 12.8
DPE Opt	275.1	273.2 ± 10.3

4.4.5 Nanoemulsion gel stability test

The 3-month stability test for the optimized gel formulations showed no change in MPS. For nanoemulsion gel prepared by HPH process, the initial MPS was 305.9 ± 12.6 nm (PdI= 0.32 ± 0.09). After 3 months stability, the results were 284.3 ± 6.3 nm (PdI= 0.23 ± 0.07) and 294.1 ± 4.3 nm (PdI= 0.23 ± 0.11) at 25°C and 4°C, respectively. For nanoemulsion gel prepared by DPE process, the initial MPS was 328.8 ± 11.9 nm (PdI= 0.24 ± 0.05), and after 3 months stability, the results were 327.8 ± 17.7 nm (PdI= 0.10 ± 0.09) and 342.2 ± 10.4 nm (PdI= 0.18

± 0.06) at 25°C and 4°C, respectively. No phase separation was observed for both processes during this time interval, by visual evaluation.

As an outcome of this study, we observed that in the HPH process, the identification of CPP was fairly simple using one-step Box-Behnken design. For the DPE process, an additional step was necessary to identify the input factors that affect the CQA, which were represented by the narrower acceptable ranges of component and process parameters (tight concentration range of surfactant and initial water, glycerin fixed at 2% w/w and temperature at 60 °C), when compared to the HPH process. The material and process parameters of HPH method, which affect the CQA of the nanoemulsion, were pressure, surfactant and olive oil concentrations. For the DPE, these input factors were the surfactant, olive oil and initial water concentration (glycerin concentration and temperature were fixed). As a result, a deeper understanding of the HPH and DPE processes was achieved by revealing the DS for the required CQA. Additionally, a similar MPS for the same nanoemulsion composition, independently the process used, was obtained accordingly with the goal of this study. This opens a future opportunity for regulatory flexibility in the selection of these processes for the nanoemulsion olive oil manufacturing.

4.5 Conclusion

In this present study, a systematic design of experiments approach was successfully applied to identify and understand the relationship between input factors and their associated output response in the development of olive oil nanoemulsion, by HPH and DPE processes. This approach allowed identify the optimized ranges of the input parameter and improved the process understanding, bringing the output response into the specification range (CQA). Implementation applying RSM optimization provided a unique range of CPP within the design space, where nanoemulsions with similar mean particle sizes of 275 nm could be achieved with equal composition, for both HPH and DPE processes.

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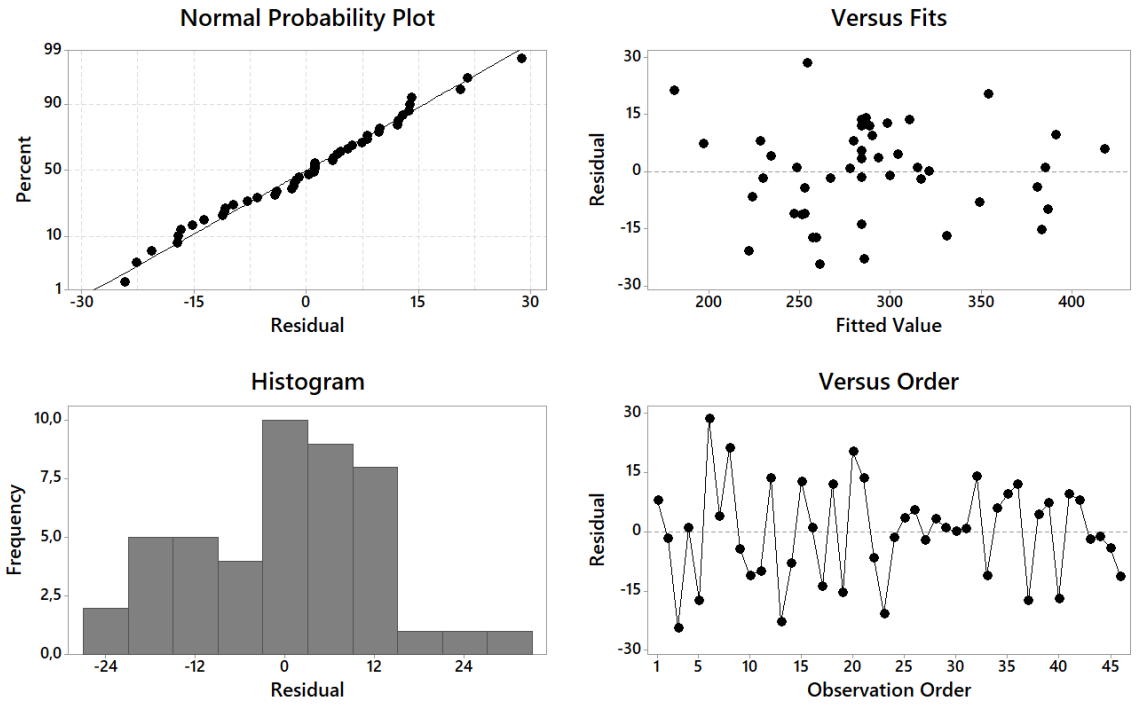
4.7 Supplementary Material

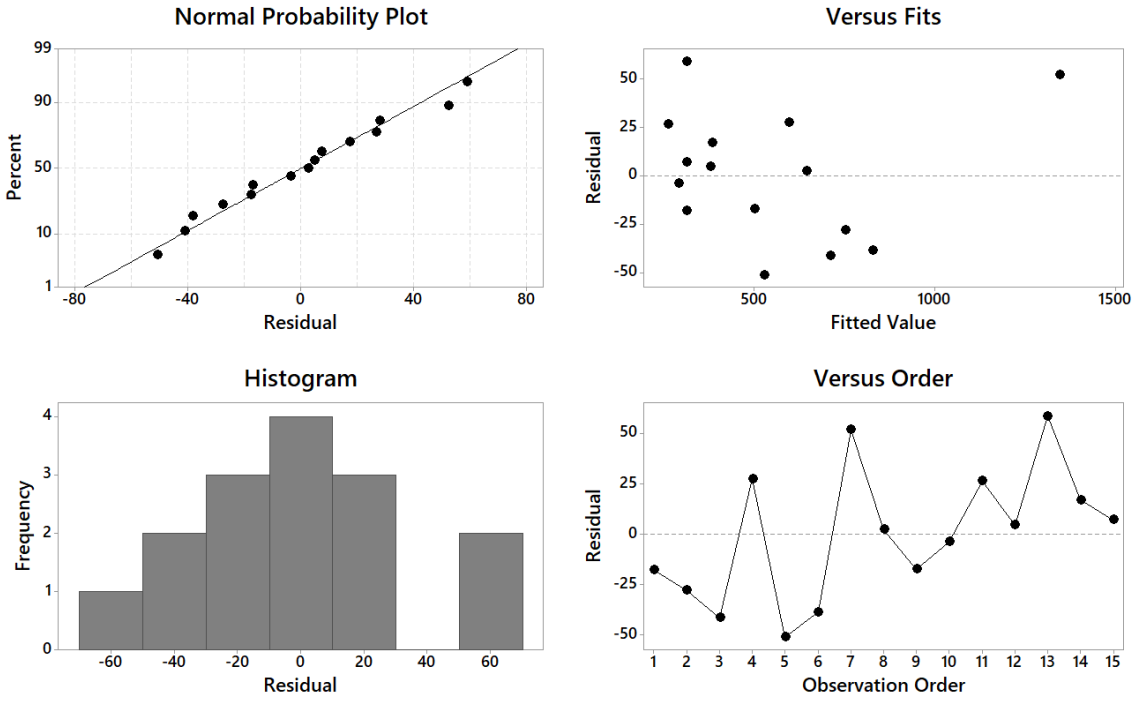
Graphs of residuals for variable of composition and preparation in HPH and DPE processes

From the residual graphs presented in Figure S1, we can observe: In the normal probability plot graph, the dot plot presents a linear behavior, and the histogram presented the normal distribution of residuals. They are in a homoscedastic linear model with normally distributed errors. The residuals versus the fitted values graph indicated the noncorrelation between the residuals model with the fitted values. The residuals versus the order of the data showed random behavior around the residual zero line, indicating that the error terms are independent, with no serial correlation.

Figure S1: Residual plots for variable of composition and preparation in (A) HPH process and (B) DPE process



(A)



(B)

Source: Minitab

5. FINAL CONCLUSION

The proposal of this present study was to achieve a deep understanding of the preparation process and the applicability of nanoemulsion by high and low energy methods. These goals were successfully accomplished through the survey and systematization of the performed activities, within the scope of new search to be undertaken in the nanostructure systems. This careful systematization of knowledge provided two review articles and two research articles, which highlight the nanoemulsion as a potential alternative drug carrier. In the first review article, we found that the challenges for the development of a high efficacy nanoemulsion refer to the elucidation of the interactions between the drug and nanoemulsion's components; the investigation of the impact of the manufacturing process on the formulation composition and drug stability. Also, a better understanding of the influence of nanoemulsion formulation on drug release and drug uptake by different routes of administration is needed. The second review article clearly evidenced that the key factor for nanoemulsion preparation is the selection of the most suitable process, which ensure the desired properties of the final obtained nanodroplets. This review offers to the formulator a realistic commercial scale alternative for this emerging field of nanotechnology. Further studies need to carry out aiming in-depth understanding in how the oil–surfactant–water phases interact during the processing time and storage, according to the composition and the process selected.

The experimental study resulted in two research articles. The first one we concluded that it is possible to obtain a nanoemulsion with high vegetable oil content with low surfactant concentration. These features emphasized the unique property of the DPE method, enabled by presence of polyol, which provided the synergistic benefit when used in combination with single surfactant. The polyol was confirmed to be the statistically significant variable for MPS reduction of the nanoemulsion, influencing the behavior of phase transition structure in this method. The synergistic interaction between the polyol and the hydrophilic surfactant modified its apparent HLB, provided in this low energy process a distinctive phenomenon. In the second research article, a systematic design of experiments approach was successfully applied to identify and understand the

relationship between input factors and their associated output response in the development of olive oil nanoemulsion, by HPH and DPE processes. This approach allowed identify the optimized ranges of the input parameter and improved the process understanding, bringing the output response into the specification range (CQA). Implementation applying RSM optimization provided a unique range of CPP within the design space, where nanoemulsions with similar mean particle sizes of 275 nm could be achieved with equal composition, for both HPH and DPE processes.

These knowledges provided directions that might elucidate the performance of a particular nanoemulsion system as drug delivery. It also indicated the significant challenges and the multiple benefits of nanoemulsion, which has the potential to shape the future of pharmaceutical and cosmetic products.

6. APPENDIX: Book chapter – Application of nanoemulsion in cosmetics, Nanoemulsions

This study will be published as Odile Sonneville Aubrun, Megumi Nishitani Yukuyama and Aldo Pizzani, with the chapter title 'Application of nanoemulsion in cosmetcis', book title 'Nanoemulsion', Elsevier. The article is in ongoing process for publication.

ABSTRACT

This chapter summarizes formulation principles to design cosmetic-grade nanoemulsions and relates some specific applications targeting cosmetic challenges. It starts with a section on high and low energy emulsification methods, pointing out the most important advantages and drawbacks of each technique, as well as key parameters to obtain nanoemulsions for cosmetic applications. It then explains rules to control nanoemulsions stability and texture through formulation parameters following cosmetic requirements, including high desirable sensory features and functional performance.

The end of the chapter provides a summary of skincare and haircare context and needs, such as active solubilization and delivery for anti-aging, whitening or moisturizing such as like plant extracts and ceramides, UV filters dispersion for suncare, as well as silicon deposition for haircare and scalp treatment.

INTRODUCTION

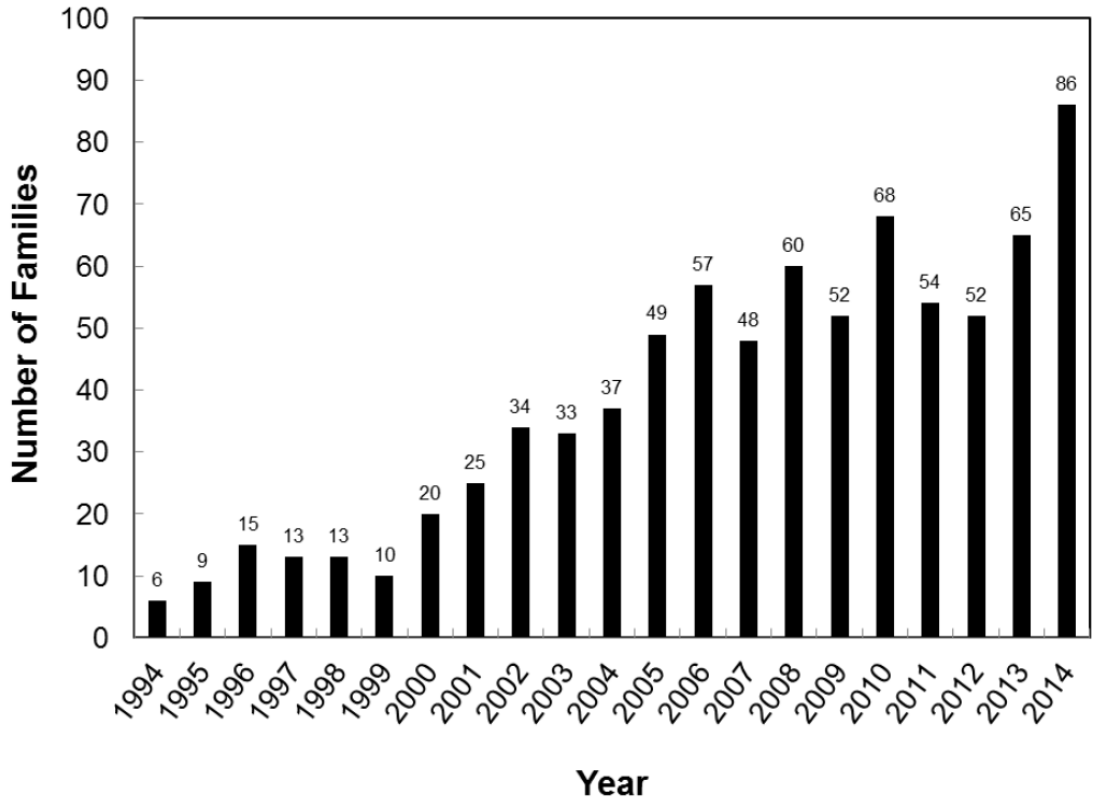
Generalities on nanoemulsions

Addressing the consumers' growing demand for high quality products, combining performance and safety, has been one of the greatest challenges for researchers of Cosmetics and Personal Care field in recent years. Nanotechnology gained a considerable attention in this field due to several advantages offered by its specific properties.

Nanoemulsions are oil-in-water (O/W) or water-in-oil (W/O) dispersions typically from a few nm to 100 nm nanoscale engineered products or materials that present novel applications. These dispersions can also be used on a micrometer (1000 nm) scale, exhibiting physical or chemical properties or biological effects attributable to their dimension(s) (US FDA, 2014). A nanoemulsion is a kinetically stable but a thermodynamically unstable system, in which Ostwald ripening is considered the main factor of its instability (Solans and Solé, 2012). Different visual appearances and textures are provided by this system, ranging from transparent to opaque, low to high viscosity as well as light to rich touch feeling when applied, enabling a wide range of applications (Ribeiro *et al*., 2015a, Kong *et al*., 2011, Sonneville-Aubrun *et al.*, 2004). 2

The growing industrial interest on nanoemulsions can also be perceived by the analysis of the industrial protection activities. From 1994, about 900 patent families (pf) can be identified in "nanoemulsions" for cosmetic applications (Figure A.1), which represents more than 1900 granted patent applications during the 20 past years. About 1/3 of these patent families are focused on hair care fields and the rest on skin, body or personal care field. L'Oréal is the most active company in the industrial protection activities on nanoemulsions, followed by Amore Pacific, Henkel, BASF and P&G companies.

Figure A.1 - Number of patent families by earliest priority years 1994 to 2014



Source: Patbase database (general research strategy based on nanoemulsions).

This section highlights the main applications of nanoemulsions in the Cosmetics and Personal Care field, as well as their prospects and challenges.

How Nanoemulsions can meet the Cosmetics needs?

Nanoemulsion is a promising system for cosmetic use due to several advantages such as easy manufacturing through high and low energy processes, controllable droplet sizes, high kinetic stability as compared to conventional emulsions and liposomes, as well as a low surfactant concentration compared to microemulsions. Its versatility in formulation allows to access to a wide range of attractive textures such as sprays, fluids, gels, creams and foams, with a variety

of visual functional and sensorial benefits (Ribeiro *et al*., 2015a, Samson *et al.,* 2016, Silva *et al.*, 2013).

The efficacy of cosmetic products is brought by their texture as well as their active and functional ingredients. Both lipophilic and hydrophilic components can be easily incorporated into the nanoemulsion owing to the efficient solubilization capacity of in the nanoemulsion. The small droplet size provides a large surface to volume ratio, enhancing the dispersed phase deposition on the hair and the active component ingredient penetration and permeability into the skin (Kong *et al.*, 2011, Mahdi *et al.*, 2011). Additionally, the topical application of nanoemulsion on the skin provides a uniform formation of lipid film, enabling higher performance and bioavailability. After several applications of cationic nanoemulsion on hair, a significant improvement was observed regarding dry hair appearance, with lingering effect (Sonneville-Aubrun *et al*., 2004). The very small size of nanoemulsions also offers the possibility to develop preservative free products, after sterilization on 0.2 µm filters.

Challenges for Cosmetics nanoemulsions

Developing safe, stable, efficient and attractive products, with optimum cost-benefit for consumers, is always a challenge for cosmetic industry. Safety and environmental aspects as well as productivity and quality have to be considered very early in the development process of new products.

Nanoemulsions offer high versatility including manufacturing process selection and compounds selection such as surfactants, lipids and active ingredients. The investigation of the impact of process or formulation component variables on final products should be performed rationally (Yukuyama *et al.*, 2016). The selection of an appropriate process for a particular system reflects on the ease of scaling-up, reproducibility and optimization of manufacturing time.

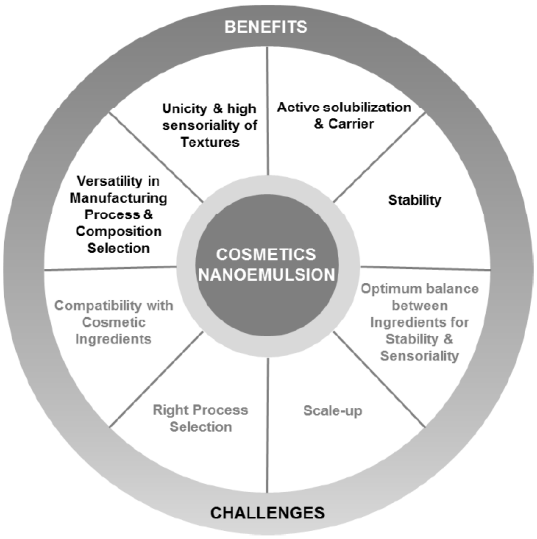
Understanding the interaction of Surfactant-Oil-Water (SOW) phases during processing time, storage and contact with skin or hair, seems also crucial. Direct nanoemulsion possesses in core the oil phase, which is the main component and may influence the solubility of lipophilic active component, and consequently its

efficacy (Yukuyama *et al.*, 2017). The role of nanoemulsion as a carrier system must also guarantee the safety of the skin, avoiding penetration of undesirable external agents (Clares *et al*., 2014).

Ensuring long term stability in standard and stressed conditions without disturbing the desirable sensory features of a product and its performance requires a good knowledge of major destabilization phenomena and triggers to control the texture (Sonneville-Aubrun *et al*., 2004). Incompatibilities with some cosmetics ingredients such as pigments, oxidants, multivalent salts, polymers…. restrict the use of nanoemulsions to specific applications and have to be mastered.

The antimicrobial activity of preservatives in the nanosystem is another concern to be considered. Parameters such as pH, water activity and ethanol are known to affect the preservation of colloidal systems. However, deeper study of other parameters such as the effect of particle size on the preservation of cosmetic nanoemulsions should be performed (Fang *et al*., 2016).

Figure A.2 - Cosmetic nanoemulsions: benefits and challenges



Source: authors' own production.

II. FORMULATION PROCESS

Nanoemulsions are thermodynamically unstable and need energy to be created. Following Laplace equation, a higher energy might be required to obtain smaller droplets. In cosmetics, nanoemulsions can be obtained either by high or low energy process (respectively external and internal energy input).

The choice of the most appropriate method mostly depends on the system composition and/or scale up requirements. The information reported in this section aim at helping the rational design of cosmetics-grade nanoemulsions using different methods. The most important advantages and drawbacks of each technique are also pointed out.

High Energy Process

In the cosmetic industry, High Pressure Homogenization (HPH) and Microfluidization are commonly used at laboratory to industrial scale to get stable sub-micron emulsions over a large range of compositions (Sharma and Sarangdevot, 2012). Droplets are forced through a very narrow channel under pressure, where they are submitted to large pressure drops, turbulent eddies and high shearing forces. The ultimate size of the emulsion is determined by the balance between two opposing processes: droplet breakup and coalescence (Wastra, 1993), promoted by the intense shear that occurs within a high shear homogenizer. The break-up of droplets occurs when the applied shear is greater than the drop Laplace pressure.

The main operating parameters that affect the final droplet size are: the pressure, the temperature, the number of passes, the valve and impingement design as well as the flow rate. Other parameters are related to the compounds selection: the surfactant concentration and their interfacial properties, the presence of co-surfactants and solvents, the viscosity ratio between the dispersed and continuous phases and the volume fraction of dispersed phase (Wastra, 1993). In terms of process and formulation selection, technical requirements for nanoemulsions are stricter compared to emulsions to be able to obtain very small droplets.

Device & process

Different devices are in use for cosmetics: Gea Niro Soavi Spa (GEA Niro Soavi Spa, Parma, Italy) by L'Oréal (Ribier *et al.,* 1988) and Boticario (Knapik et *al.*, 2010), Microfluidizer from Microfluidics Corp. (Newton, MA, USA) by Shiseido (Okamoto *et al.*, 2016). The processing temperature has to be above the melting temperature of the oil and surfactant phase.

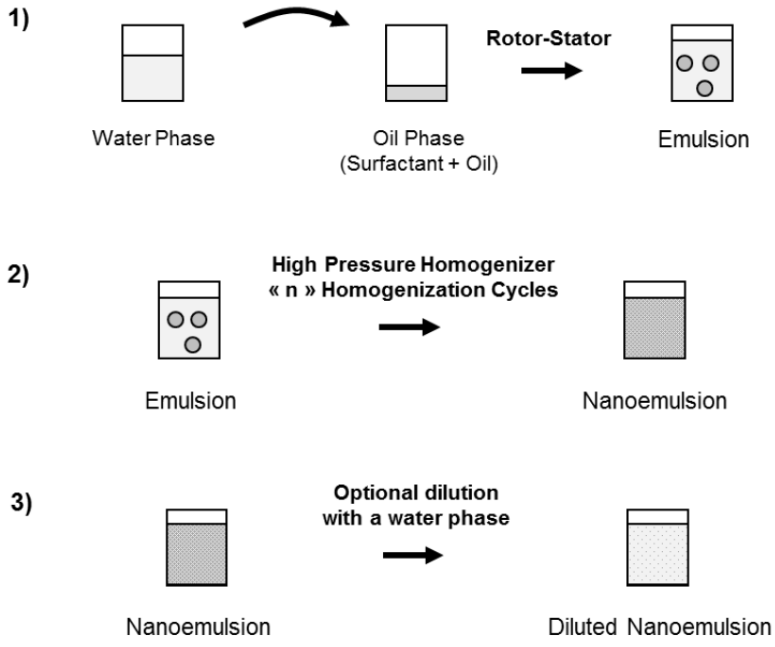
Nanoemulsions fabrication generally proceeds in two steps and sometimes in three steps, with an additional dilution step for increasing the productivity or functionalizing the product, as presented on Figure A.3.

1 - At first, a coarse emulsion is made by mixing the surfactant system with the oily and aqueous phase with a classical shearing tool (a rotor-stator or a mixer).

2 - Secondly, the coarse emulsion is recirculated several times in a High-Pressure Homogenizer to get a stationary size.

3 - At third, the nanoemulsion can be diluted with an additional aqueous phase or incorporated as a preparation into another composition.

Figure A.3 - Schematic representation of the Homogenization steps process



Source: authors' own production.

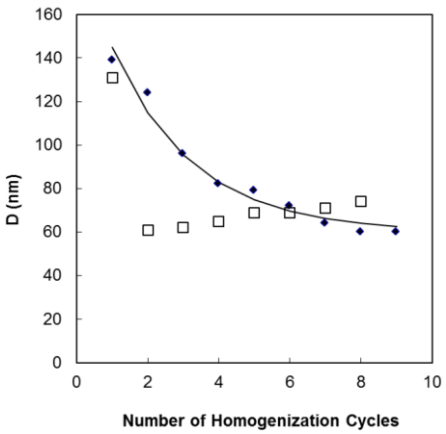
The number of recirculation cycles to obtain an optimum size depends on the homogenizer, the applied pressure and the composition. On the one hand, 3 homogenization cycles seem sufficient to yield a stationary size at 60 MPa and 120 MPa (Knapik *et al*., 2010; Lietdke *et al*., 2000). On the other hand, a nanoemulsion with a stationary size was only obtained after at least 6 homogenization cycles (Sonneville-Aubrun *et al*., unpublished; Kentish *et al*., 2008; Qian *et al*., 2011). The number of recirculation steps has to be checked experimentally and to be adapted to the composition and the homogenizations conditions.

An example is presented on Figure A.4 comparing data from Sonneville-Aubrun *et al.*, with a Niro-Soavi Panda at 120 MPa (Isostearate of PEG-8 / Stearoyl glutamate / Isopropyl myristate / Isocetyl stearate / Dipropylene glycol / Glycerin / Water), to data from Knapik *et al.* (2010) with a Niro-Soavi SpA model NS100 at 120 MPa (Steareth-2 / Steareth-20 / Stearyl Alcohol / Cetearyl Alcohol / Almond Oil / Capric-Caprylic Triglycerides / Tocopherol Acetate / Mineral Oil / Water).

In the first case, the size evolution can be fitted by an exponential function of the number of recirculation cycles as presented in Eq. A.1, where DF describes the emulsion final diameter and N the characteristic decay number, as what has been found with sonication (Delmas *et al.*, 2011).

$$D(n)=DF+A\cdot e^{-n/N} \qquad \text{Eq. A.1}$$

Figure A.4 - Nanoemulsion droplets diameter as a function of number of Homogenization cycles. Diamond symbols correspond to experimental data from Sonneville-Aubrun *et al.* and square symbols from Knapik *et al*. (2010)



Source: Sonneville-Aubrun et al. (2010); Knapik et al. (2010).

Forming nanoemulsions generally requires pressure above 60 MPa. Very thin emulsions of 170 and 190 nm median droplet diameter were obtained with an ethoxylated surfactant and an oligomeric surfactant at 60 MPa, respectively. With the same surfactants, increasing the pressure to 120 MPa led to nanoemulsions of 61 nm and 82 nm size, respectively (Knapik *et al*., 2010).

Formulation Parameters

Besides process, compositions have to be adapted to obtain nanoemulsions, especially the surfactant/oil weight ratio. Other parameters play also a key role in optimizing the droplet size and nanoemulsion transparency such as surfactant structure, oil phase viscosity as well as additives solubilized in aqueous phase, such as polyols.

- Surfactant/Oil Ratio

The droplets size of a nanoemulsion formed by a High Pressure Homogenizer results from a succession of fragmentation and coalescence events, where the surfactant play a key role. On one hand, by lowering the interfacial tension, it helps to the droplets break-up.

On the other hand, its adsorption at oil/water interface prevents the droplets coalescence (Walstra *et al*., 1993).

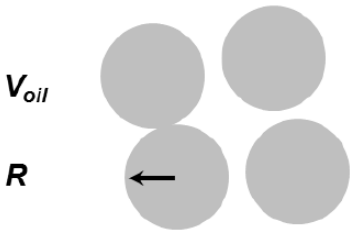
Two emulsification regimes can be found depending on the surfactant/oil ratio and energy input (Taisne *et al.*, 1996). First, in the ‘‘surfactant-poor’’ regime, the surfactant is in default and coalescence occurs till the surfactant coverage is high enough to stabilize the droplets under homogenizing conditions. In this case, the droplet size is determined by the surfactant/oil ratio. Second, in the ‘‘surfactant-rich’’ regime, the surfactant is in excess and coalescence remains rare. The droplet size is then mostly determined by the applied pressure.

Assuming that all the surfactant is located at oil/water interface, simple calculations allow estimating the minimum amount of surfactant ms needed to form a nanoemulsion of a given radius R for a given oil volume, knowing the average area per surfactant molecule at an interface and the surfactant molecular weight, as presented in Figure A.5.

Figure A.5 - Theoretical estimation of area developed by oil droplets and area developed by surfactant at interface of oil droplets

Area developped by Oil Phase

$$\begin{cases} A_E = n_D . 4\pi R^2 \\ n_D = \dfrac{V_{oil}}{4/3\,\pi R^3} \end{cases}$$



$$\boldsymbol{A_E = \frac{3V_{oil}}{R}}$$ Eq. A.2a

$\boldsymbol{n_D}$ = Number of Oil Droplets
$\boldsymbol{\rho_{oil}}$ = Oil Volumic Weight (kg/cm^3)
$\boldsymbol{V_{oil}}$ = Oil Volume (m^3)
$\boldsymbol{m_{oil}}$ = Oil Weight (kg)
$\boldsymbol{R}$ = Droplet Radius (m)

Area developped by Surfactant

$$\begin{cases} A_E = n_S . a_0 \\ n_S = \dfrac{m_S}{M_S} \mathrm{N}_A \end{cases}$$

$$\boldsymbol{A_E = \frac{m_S}{M_S} . N_A . a_0}$$ Eq. A.2b

$\boldsymbol{m_S}$ = Surfactant Weight (kg)
$\boldsymbol{M_S}$ = Surfactant Molar Mass (kg/mole)
$\boldsymbol{n_S}$ = Number of Surfactant Molecules
$\boldsymbol{a_0}$ = Area / Surfactant Molecule (m^3)
$\boldsymbol{N_A}$ = Avogadro Number

Source: authors' own production.

The equality between the two equations Equation A.2a and Equation A.2b gives a simple relation (Eq. A.3) between surfactant weight and droplets size, to estimate either the amount of surfactant needed for a given droplet size or the theoretical droplet size for a given oil/surfactant ratio.

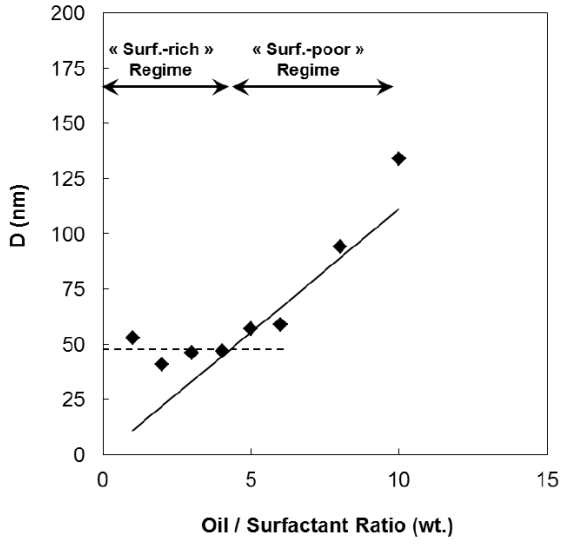
$$m_S = 3 \cdot \frac{M_S}{a_0} \cdot \frac{m_{oil}}{\rho_{oil} \cdot R} \cdot \frac{1}{N_A}$$ Eq. A.3

As an example, the surfactant needed to obtain a nanoemulsion of a 40 nm droplet diameter with 15% hydrocarbon oils, is estimated at about 4.7% for a nonionic surfactant (MTA ~ 600 g/mol, a0 ~ 60 Å²/molecule), corresponding to an oil/surfactant weight ratio of about 3.2.

To determine the transition between the two regimes, nanoemulsions were produced with 5% surfactant (PEG8-isostearate / stearoyl glutamate 9/1) and increasing weight fractions in oil (avocado oil / cyclopentasiloxane 2/1) between 5% and 50% with 15% ethanol with a Niro-Soavi OBL20 at 1200 bar (Sonneville-Aubrun, unpublished).

As presented in Figure A.6, two regimes can be distinguished on the result obtained. For low oil/surfactant weight ratio (<4) corresponding to “surfactant-rich” regime, the droplets diameter is almost constant around 45 nm. Surfactant excess in water phase is expected. For higher oil/surfactant weight ratio (4-10) corresponding to “surfactant-poor” regime, the droplets size increases linearly with the oil/surfactant weight ratio in good agreement with the estimated droplet diameter based on Equation A.2.

Figure A.6 - Evolution of the nanoemulsion droplets diameter as a function of oil/surfactant weight ratio. The straight line represents the estimated diameter (mTA ~ 600 g/mol, a0 ~ 60 Å²/molecule)and dashed line, the saturated diameter



Source: authors’ own production.

The determination of the transition between the two regimes allows to optimize the surfactant concentration for a given targeted size and oil content.

- Surfactants

In cosmetics, a wide variety of surfactants has been used to obtain nanoemulsions through homogenization (Table A.1): (i) Classical nonionic surfactants such as ethoxylated fatty alcohols or fatty acids, sucrose fatty esters, alkyl polyglucosides, polyglycerol esters…, generally forming monolayers at oil/water interface, (ii) amphiphilic oligomers and (iii) association of fatty alcohol and surfactant or phospholipids forming crystalline lamellar phase

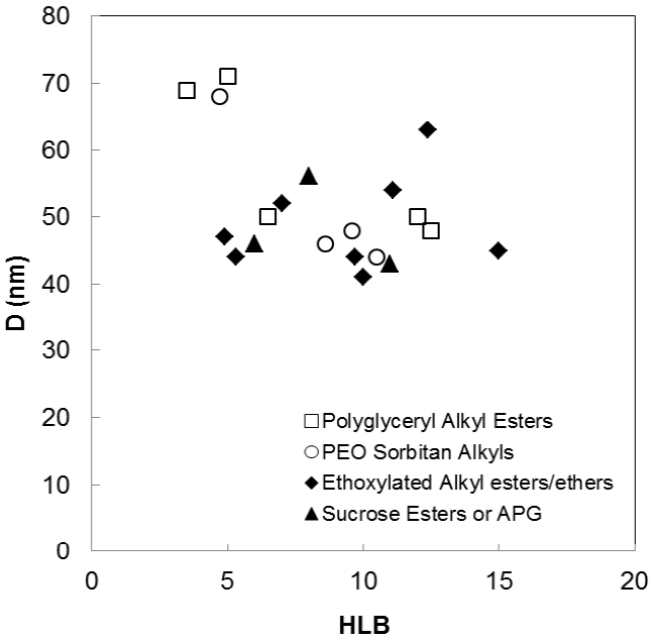
Table A.1 - Examples of surfactants used to stabilize cosmetic nanoemulsions

Surfactant type	Examples	References
PEO alkyl ether **PEO alkyl ester**	PEO-8 Isostearate PEO-20 Stearate Steareth-10	Ribier *et al.*, 1995b Simonnet *et al.*, 1998e Franco *et al.*, 1999 Quemin, 2002 Gesztesi *et al.*, 2006 Knapik *et al.*, 2010
Alkyl Polyglucoside **Sucrose alkyl ester**	Cetearyl Glucoside Sucrose Distearate	Simonnet *et al.*, 1998a
Sorbitan alkyl ester **PEO sorbitan alkyl ester**	Polysorbate 61	Simonnet *et al.*, 1998d
Polyglycerol alkyl ester	Decaglycerol Monostearate	Simonnet et al, 1998c
Phosphoric alkyl **Citric alkyl ether**	K Cetyl Phosphate Trilaureth-9 citrate	Knapik *et al.*, 2010 Simonnet *et al.*, 1998b
Lecithin **Gemini**	Soybean Lecithin Dilauramidoglutamide lysine	Zhou *et al.*, 2010 Klang *et al.*, 2011 Tian *et al.*, 2016
Alkyl Trimethyl Ammonium **Alkyl Amido Propyl Trimethyl Ammonium**	Behenyl trimethyl ammonium Chloride	Dubief *et al.*, 2009
Amphiphilic oligomer	Poloxamer 231 Inuline Lauryl Carbamate	Simonnet *et al.*, 1999a, b Knapik *et al.*, 2010
Alpha-gel with fatty alcohol and surfactant	Stearyl alcohol / Behenyl alcohol / K stearate / K behenate	Ribier *et al.*, 1995a Okamoto *et al.*, 2016

A comparison between classical surfactants has been made by keeping the other parameters constant (Figure A.7): 4.5% Surfactant / 0.5% Stearoyl Glutamate /

10% Isocetyl Stearate / 5% Isopropyl Myristate / 10% Dipropylene glycol / 5% Glycerin / Water with a Niro-Soavi OBL20 device at 120 MPa (Sonneville-Aubrun *et al.*, Unpublished). Droplet diameters between 40 and 60 nm could be obtained on a wide (5-15) HLB range.

Figure A.7 - Nanoemulsion droplets diameter as a function of Surfactant HLB



Source: authors' own production.

For surfactant with HLB under 5, slightly larger diameter were obtained with very poor emulsion stability, even in the presence of an anionic surfactant at a low concentration.

Many systems described in the literature contain both nonionic and ionic surfactant to ensure a good stability on long term. Some authors perform potential Zeta measurement to verify the droplet surface charge and to anticipate aggregation phenomena (Tian *et al.*, 2006).

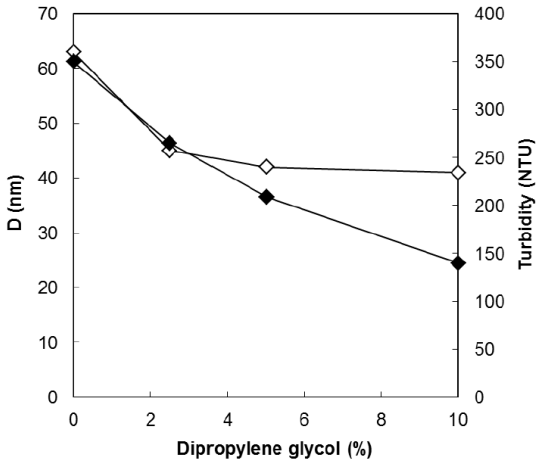
Some examples may contain specific functional amphiphilic lipids, which bring skin or hair benefits, such as phytosphingosine (Yilmaz & Borchert, 2006). These will be developed in the following sections of this chapter.

- Polyols

Polyols are classical ingredients in cosmetics, used as humectants, penetration enhancers for active ingredients, solvents…. They can also be seen as formulation aid to reduce droplet size and the formula-product transparency during the homogenization process.

An experiment has been conducted with formulae by increasing the amount of dipropylene glycol from 0 to 10% (Sonneville-Aubrun *et al.*, Unpublished). The nanoemulsions contained 5% surfactant (PEO-8 Isostearate/Stearoyl Glutamate 9/1) and 25% oil (Isocetyl Stearate / Isopropyl Myristate 2/1). The Niro-Soavi OBL20 at 120 MPa device was used to perform the experiments. As shown in the Figure A.8, the nanoemulsion droplet size and turbidity decrease by increasing the concentration of dipropylene glycol (Hach 2100).

Figure A.8 - Nanoemulsion droplets diameter (Empty Diamond Symbols) and turbidity (Full Diamond Symbols) as a function of dipropylene concentration



Source: authors' own production.

Different factors may explain the positive impact of dipropylene glycol dose effect on nanoemulsion transparency: (i) Decrease in refractive index difference between oil and water phase. (ii) Increase in aqueous phase viscosity from 1.8

cP without dipropylene glycol to 4 cP with 10% dipropylene glycol, with a theoretical reduction in droplet size by a factor of 0.8 according to power law found in literature (Walstra, *et al.* 1993), close to experimental data. (iii) Decrease in interfacial tension from 43 mN/m to 25 mN/m following addition of 10% dipropylene glycol, (for isocetyl stearate/isopropyl myristate oil phase).

Positive impact on droplet size and transparency has also been observed for lecithin nanoemulsions with a glycerin dose-effect (Zhou *et al*., 2010).

Glycols can easily be added to tune the aspect and the properties of nanoemulsions, being aware of their negative impact on sensoriality (stickiness) at higher dosage.

- Oils

A large variety of oils are used in cosmetics depending on the targeted benefits; emolliency and rich feeling, active solubilization and bioavailability, make-up remover, volatility and light feeling, shininess …. Table A.2 assesses some oils families used to prepare cosmetics nanoemulsions.

Table A.2 - Current oils used in cosmetics

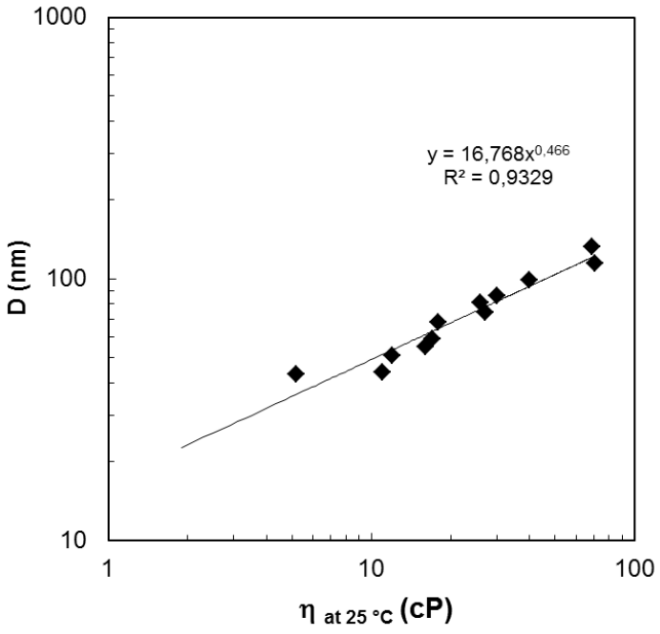
Family	Oils	Chain Lengths	Mw (g/mol)	$\eta_{at\ 25\ °C}$ (cP)
Triglyceride	Caprylic/capric triglyceride	$C_{8\text{-}10}$	542	27
	Avocado oil	$C_{16}C_{18:1}$ 20/60	930	71
	Apricot Kernel oil	$C_{18:1}C_{18:2}$ 66/28	930	69
Esters	Isopropyl myristate	$C_{17}C_{34}O_2$	270	5.7
	Isopropyl palmitate	$C_{19}C_{38}O_2$	298	12
	Isostearyl neopentanoate	$C_{23}C_{46}O_2$	354	18
	Isocetyl stearate	$C_{34}C_{68}O_2$	508	30
Alkanes	Isododecane	$C_{12}C_{26}$	170	1.9
	Isohexadecane	$C_{16}C_{34}$	226	4.5
	Vaseline	-	345	26
	Parleam	$C_{30}C_{62}$	422	40

According to Walstra *et al*. (1993), the droplet size increases with oil phase viscosity, following a power-law $D \sim \eta^{0.37}$. The experimental data presented on

Figure A.9 shows a power-law D ~ η $^{0.47}$, with a slightly higher exponent. Long chain triglycerids tend to give larger droplets compared to esters.

The viscosity of the oil phase plays a key role to reach nanoemulsions and can be optimized by mixing different oils. High pressure homogenization remains limited to low to average oil viscosity which, in fact, covers most of cosmetics applications.

Figure A.9 - Nanoemulsion droplets diameter D (full diamond symbols) as a function of oil phase viscosity η at 25 °C



Source: authors' own production.

In conclusion, high shear homogenization may yield to a wide range of compositions and offers to formulators the opportunity to create new and attractive products that respond to technical, toxicological and environmental requirements of cosmetic products. However, high shear homogenization requires costly and specific equipments. The following section will focus on an interesting alternative provided by a Low Energy Process.

Low Energy Process

Nanoemulsions can be obtained by using the internal system energy during the emulsification process (phase inversion and/or phase transition). For industrial

applications, these methods require a well understanding of the equilibrium phase behavior, the evolution of changes in the system physicochemical properties under emulsification and the key process variables. Nanoemulsions can also been obtained by spontaneous emulsification, which is mainly driven by the surfactant chemical potentials and mass transfer (Solans and Solé, 2012). These methods are known as low-energy methods and require relatively simple process devices for the scale-up.

PIT-Method

The most widely known low-energy method to obtain nanoemulsions in cosmetics is based on the Phase Inversion Temperature (PIT) introduced by Shinoda in the 60's (Shinoda and Saito 1968; Shinoda and Arai, 1967, 1964). The PIT is a transitional phase inversion (Salager *et al.*, 2004) induced by specific temperature sensitive non-ionic polyethoxylated surfactants. Even though this method is associated with the emulsion morphological changes during the phase inversion process, O/W nanoemulsions (respectively W/O) are obtained thanks to change in surfactant spontaneous curvature at the interphase and at some degrees below (respectively above) the PIT, i.e., not so far from the minimum interfacial tension locus. Salager and co-workers showed that the optimum domain to get the smallest droplets size corresponds to the best compromise between droplets break-up and coalescence in a SOW dispersion. This is favored by the extremely low interfacial tensions near and on both sides of the optimal formulation where Hydrophilic Lipophilic Deviation (HLD) is nil (Forgiarini *et al.*, 2015; Salager *et al*, 2010, 2000, 1996; Tolosa *et al.*, 2006; Shinoda and Saito, 1969). These physico-chemical phenomena are well described and explained in specific literature (Solans *et al.*, 2003).

In the 90's, Förster obtained 130 nm nanoemulsions composed by typical cosmetic ingredients, ceteareth-12 or polyoxyethylene eicosyl/docosyl ether were studied at different proportions and concentrations to ensure the nanoemulsion stability. Förster also showed the influence of the oil polarity, using decyl oleate, 2-octyl dodecanol or isopropyl myristate and the mixtures with mineral oil (Förster, 1997; Förster *et al.*, 1995, 1990). From these results, some formulation

rules can be identified to reach stable nanoemulsion at room temperature, i.e. surfactant concentration and hydrophilicity, as well as the oil nature should be adapted to fix the PIT system between 70 – 90 °C. Within this range, nanoemulsions are stable according to standardized long-time stability tests at 45 °C (Förster *et al.*, 1992).

The following equation obtained from the HLD expression shows some physicochemical variables that influence the PIT value (Salager *et al.*, 2001), notably the surfactant and oil nature and the presence of the alcohols, that can be considered as co-surfactants.

$$PIT = 25 + \frac{[EON - \alpha - b \cdot S + k \cdot EACN - a \cdot A]}{t} \qquad \text{Eq. A.4}$$

EON corresponds to the number of Ethylene Oxide group of surfactant, EACN to Equivalent Alkane Carbon Number linked to the polarity and carbon number of the oil, S to the salt concentration in water phase and A to the presence of alcohols or co-solvents; α, k and t are surfactant characteristic parameters, “a” and “b” represent respectively the specific constants of the alcohol and salt (Ontiveros et al., 2013).

During emulsification, to ensure nanoemulsion stability, the gap between PIT and storage temperature is important (Förster *et al.*, 1990; Shinoda and Saito, 1969), as well the cooling rate to ensure a narrow drop size distribution and the translucent appearance of the emulsion. Obtaining monodisperse drop size distributions also helps to prevent Oswald ripening phenomenon.

Surfactant concentration and water/oil ratio are important parameters to formulate nanoemulsions. Tadros and co-workers showed how increasing the surfactant concentration, the PIT value of industrial grade of non-ionic surfactant decreases (Tadros *et al.*, 2004). This behavior allows to ensuring that the system is provided by a sufficient amount of amphiphilic molecules to stabilize nanodroplets, and to eventually cross the Liquid Crystal (LC) zones (in the Winsor IV behavior). It has been shown that higher surfactant concentration and dynamic emulsification conditions allow the droplets life time to being increased reach translucent nanoemulsions. Furthermore, better results can be obtained if the formulation is performed near to the X point in the limit of the Winsor III (WIII) and

Winsor IV (WIV) in the gamma cut phase behavior. This point is currently associated with the surfactant efficacy. Pizzino *et al.* show the evolution of the gamma cut phase behavior for non-ionic polyethoxylated surfactants at different water/oil ratio (Pizzino *et al.*, 2009). From these results, water/oil ratio can be used to slightly increase the surfactant efficacy and to shift the X point and the WIV LC zone towards lower concentrations.

Sub-PIT process was presented by Roger *et al.* as a new process to obtain nanoemulsions (Roger *et al.*, 2009). The latter work signals the existence of a clear boundary and the advantages offered by this zone. These results correspond to the same observations pointed out by Shinoda 50 years before (Shinoda and Saito, 1969) and extensively explained during the 30 past years for many authors (Tolosa *et al.*, 2006; Solans *et al.*, 2005, 2003; Salager *et al.*, 1996). From Shinoda original work *"better, stable and fine emulsion can be obtained if a system is shaken at first close to the PIT (about 2–4 °C below the PIT is the best) to fine dispersion and then rapidly cooled down to storage temperature, at which the coalescence rate is slow. We designate this process as emulsification by the PIT-method* (Shinoda and Saito, 1969)*".* The scale up of this method however is not easy to transpose as explained latter.

Recent works in the cosmetic domain show how PIT method allows obtaining nanoemulsions devoted to skin and hair products to being obtained. Dario *et al.* present a stable quercetin-loaded cationic sub-micrometric emulsion composed mainly of 5.6% PEO-20 oleyl and 3.4% PEO-3 oleyl, 5% caprylic/capric triglyceride and 1% cetyl trimethyl ammonium chloride, a cationic surfactant (Dario *et al.*, 2016a, 2016b). In these works, authors claim that nanoemulsions were obtained by a sub-PIT process.

At an industrial scale, PIT method consists in heating the chosen surfactant/oil/water combination above the PIT temperature under stirring. The system is further cooled down by adding cold water or by circulation of ice water in the double-wall reactor. Working at a temperature higher than PIT allows to being ensured high cooling kinetics during the phase transition that favors nanoemulsions mono-dispersity and stability. However, if the cooling process is started at PIT or near PIT, the thermal inertia due to the reactor volume can be high enough to quickly lose the small droplet size and increase the polydispersity

(Tolosa *et al.*, 2006; Solans *et al.*, 2003). The same results are obtained for lower cooling rates (Ozawa *et al*., 1997; Kunieda *et al.*, 1996). Under these process conditions, opaque emulsions are formed.

On one hand, adding ice water into the reactor mass helps to homogenize the temperature of the system; however, it also promotes strong composition changes, principally by decreasing the surfactant concentration or by changing the water/oil ratio. On the other hand, cooling down the system by external ice water circulation allows the system composition to remain constant even if the heat transfer into the reactor mass is low and with the same consequences as exposed in the previous paragraph.

For cosmetic purposes, PIT method is specific to thermal sensitive surfactants such as PEO derivatives; thereby strongly restricting the choice in surfactants. To overcome this constraint, other low-energy methods can be used, as described in the following section.

PIC-Method

Another low-energy process takes advantage, at constant temperature, on the phase transition that occurs during a dilution process. This method studied by Forgiarini *et al.*, is known as Phase Inversion Composition PIC (Solans *et al.* 2003; Forgiarini *et al.,* 2001). In general, the PIC-method consists in the dilution by water (or oil) of an initial binary surfactant/oil (or surfactant/water) mixture under stirring. The dilution provokes an inversion of surfactant curvature as in a PIT-process. The way by which the emulsion is produced is still unclear as pointed out by Sonneville-Aubrun *et al.*, even if detailed SANS and NMR studies during dilution steps helps to understand the changes in evolution of the system structure, and the link with the SOW phase behavior (Solé *et al.* 2012; Sonneville-Aubrun *et al.*, 2009; Forgiarini *et al.* 2001). Obtaining nanoemulsions by the PIC-method cannot be explained solely by the system equilibrium properties. Transient mesomorphic phases, lamellar or cubic, have been observed during the inversion process (Sonneville-Aubrun *et al.*, 2009; Maestro *et al*. 2008). Low interfacial tension may also be a necessary condition to form nanodroplets, as well as inversion kinetics.

This method allows to extending the surfactant types used in the PIT methods as long as the amphiphilic/oil system has to spontaneously form lamellar or cubic phase in contact with the dilution phase, thus ionic surfactant and non-ionic surfactants other than polyethoxylated ones can also be used (Yu *et al.* 2012; Solé *et al*., 2010, 2006a, 2006b).

Formulation and composition variables are not the only factors to take into account. The vessel geometry and other process parameters such as addition rates and the adapted stirring energy are also key factors to reach translucent, fine and monodispersed nanoemulsions (Solé *et al*., 2010, 2006b; Solans *et al.* 2003).

In 2010, Solé studied the PIC method scale up from 100 mL to 600 mL vessel through experimental design. The main process factors identified for scaling-up are the vessel geometry, the dilution phase rate addition and the energy of stirring used during the emulsification. In this report, Solé *et al.* obtained translucent nanoemulsions through various systems (Solé *et al*., 2010). For industrial devices, these results are promising but should nevertheless be considered as laboratory-scale trials. More complex process parameters need to be considered when developing devices for scaling up to 1 ton. As previously mentioned a lower addition rate of the dilution phase and stirring geometries limit the possibility to obtain translucent, fine monodisperse nanoemulsions. At such a scale, lower addition rate involves specific geometric changes in conventional devices, in addition, the latter are usually provided with high shear turbines that can perturb the phase transition and the emulsification kinetics. Other main industrial factor resides in the processing time, slow addition rates, i.e. time consuming and cost estimate.

In cosmetics, this technology was applied to develop O/W nanoemulsions for hair care, and for topical application as reported by Dubief *et al.* (2009), Sonneville-Aubrun and Guiramand (2014). Other compositions were also studied and reported in the literature (Yu *et al.,* 2012; Solé *et al.*, 2010, 2006a).

Spontaneous Emulsification

Spontaneous emulsification may occur by merging two immiscible liquids with different free energy levels. This phenomenon is triggered by gradients of chemical potential between the phases, which under certain conditions lead to negative values of free energy of emulsification (Solans *et al.* 2016). This method is generally based on solvent, co-surfactant or surfactant diffusion from the initial oily (or water) phase to the water (respectively oil) dilution phase. Considerable efforts have been made to better understand the mechanisms for spontaneous emulsification, as summarized by Solans *et al.* (2016). Even though some questions still remain (Saberi *et al.*, 2015), we can assume that the phenomenon is driven by diffusion followed by nucleation, with the contribution of other factors such as interfacial turbulences (Rayleigh instabilities), local low interfacial tensions… (Solans *et al.* 2016; Forgiarini *et al.*, 2015; Anton *et al.,* 2008; Miller, 2006; Ganachaud and Katz, 2005; Lopez-Montilla *et al.* 2002). The diffusion path can be plotted on the equilibrium ternary phase diagram (Anton *et al.*, 2008), that constitutes an important tool to understand and control these systems. Without surfactants this phenomenon was also studied and is known as the Ouzo effect (Ganachaud and Katz, 2005).

Nonionic surfactants such as Span 80 and polyglycerol polyricinoleate (PGPR) were used to obtain nanoemulsions underlying the high interest of PGPR in spontaneous method (Mehrnia *et al.*, 2016). Vitamin E-enriched nanoemulsions were also obtained using this low energy method. The study shows that the oil composition has a major impact on the nanoemulsion droplet size. Other variables, such as the surfactant concentration and type, the mixing temperature and the stirring energy when the organic phase is added to the aqueous phase, also impact the mean droplet size (Yukuyama *et al*. 2016; Saberi *et al.,* 2014, 2013a, 2013b, 2013c).

In cosmetics, this method is very promising to obtain nanoemulsions by simply mixing ingredients. However, the fine control of the diffusion path may represent a challenge to obtain homogeneous and reproducible nanoemulsions. In terms of formulation parameters, spontaneous emulsification method allows low internal phase nanoemulsions to being prepared, typically below 5% after solvent evaporation (Ganachaud and Katz, 2005; Solans *et al.* 2005). Many parameters

such as the oil viscosity, the surfactant structure and the water solubility of the organic solvent are important in determining the quality of the nanoemulsions obtained by this method (Yukuyama *et al*. 2016). Use of solvents and the low concentration in oil volume fraction may represent a limit for some applications.

III. Mastering nanoemulsion stability & texture

Most legal frames, worldwide, constrain cosmetics products to remain stable for at least 3 years, irrespective of local climates, ranging from tropical to cold conditions. Accelerated stability tests are performed to guarantee a constant quality of the products to consumer, including temperature cycles and long term cold (4 °C) and hot (45-55 °C) storage. As all other formulations, nanoemulsions have complied with these stability requirements.
Furthermore, consumers do not use the same products, according to climate, skin/hair types, product function as well as cultural or fashion habits. For instance, for face products, Japanese women apply different layers of very fluid textures from cosmetic waters to milky lotions, whereas European women tend to use light to rich creams. Being able to fine-tune texture, visual appearance and sensory feel, as well as functional benefits pleas for a large deployment of nanoemulsion technology in cosmetic products.

Stability control

Emulsions are by nature metastable systems that encounter well known instability phenomena such as aggregation, sedimentation/creaming, coalescence and Ostwald ripening. However, the expression of instability phenomena strongly depends on the type of emulsion. For instance, in the case in W/O emulsions, Ostwald ripening and coalescence are generally favored by short distances between droplets and a significant water solubilization in oil phase.

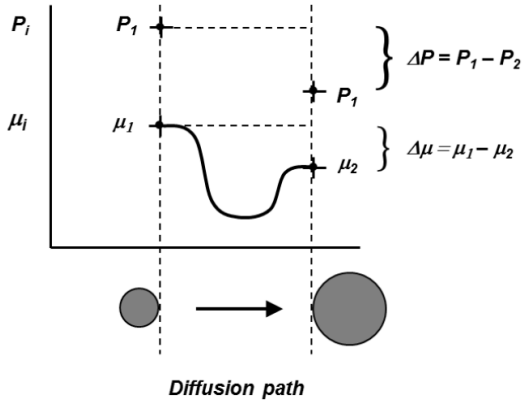
In nanoemulsions, very small droplet size and rather low volume fraction are unfavorable to coalescence and sedimentation/creaming. However, numerous

examples show the dramatic effects of Ostwald ripening and aggregation phenomena in cosmetic compositions that appear to being the dominant destabilization mechanisms (Sonneville-Aubrun *et al*., 2004; Wooster *et al*., 2008).

Ostwald Ripening

Ostwald ripening is characterized by a coarsening of emulsion droplets with time due to molecular diffusion of dispersed phase between droplets through the continuous phase from smaller droplets to larger ones. For an oil-in-water emulsion, the process is driven by the difference of the oil chemical potential between droplets of different sizes: the lower the droplet size, the higher the Laplace pressure as well as the oil chemical potential (Figure A.10).

Figure A.10 - Expression of differences in oil chemical potential according to emulsion droplet size



$$\mu_1 = \mu^\circ + \frac{2\gamma}{r_1} \cdot V^m > \mu_2 = \mu^\circ + \frac{2\gamma}{r_2} \cdot V^m$$

μ_i = Chemical potential of Oil in droplet *i*
μ° = Reference chemical potential of oil
γ = Interfacial Tension (mN/m)
r_i = Radius of droplet *i*
V^m = Molar volume of Oil drop (m^3/mol)
P = Pressure of drop *i*

Eq. A.5

Source: authors' own production.

Ostwald Ripening Rate "ω" is proportional to the oil solubility "*s*" and the oil diffusion coefficient "*Df*" in the continuous phase according to the LSW theory (Liftshitz and Slyozov, 1961; Wagner, 1961).

$$\omega = \frac{32}{27}\pi\frac{\gamma \cdot V^m \cdot D_f \cdot s}{R \cdot T}$$ Eq. A.6

Depending on oil polarity, oil molecular weight as well as aqueous phase composition, Ostwald Ripening Rate can vary by several orders of magnitude. Table A.3 exhibits calculated Ostwald ripening rate for 3 alkanes with increasing molecular weight, taking 10mN/m as interfacial tension value. Time needed to double the size of an emulsion for two initial diameters was also calculated.

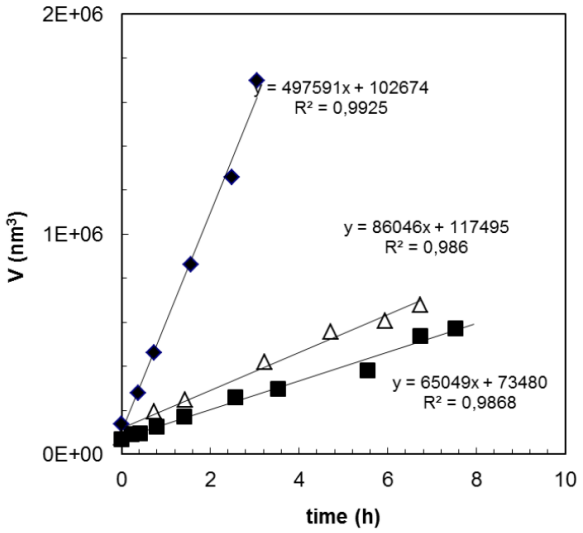
Table A.3 - Solubility data for alkanes and calculated Ostwald Ripening Rates

Oil	C_i	Mw (g/mol)	Solubility/Water (ml/ml)	Ripening rate (nm^3/h)	Time needed to double the initial Droplet size	
					$D_{init.}$= 50 nm	$D_{init.}$= 1 µm
Dodecane	12	170	3.40×10^{-9}	2.1×10^{4}	22 hours	19 years
Tetradecane	14	198	2.10×10^{-10}	1.4×10^{3}	14 days	300 years
Hexadecane	16	226	1.30×10^{-11}	9.0×10^{1}	7 months	4000 years

Dodecane nanoemulsions appear very unstable, as compared to micron size emulsions. The increase in droplet diameter is directly linked to a given transferred volume of oil, which is relatively higher for smaller droplets. A few hours or days suffice to double the initial droplet size for nanoemulsions, as compared to years for micron size emulsions

Ostwald ripening rates for cosmetic oils were obtained by measurement of droplets size upon time by Dynamic Quasi-Elastic Light Scattering (Sonneville-Aubrun *et al*., 2004). Figure A.11 presents data for 3 nanoemulsions containing 4.5% PEO-8 Isostearate / 0.5% Stearoyl Glutamate / 15% Oil / 10% Dipropylene glycol / 5% Glycerin / Water made with a Niro-Soavi OBL20 device at 120 MPa. 3 different oils have been used: Isopropyl Myristate, Isohexadecane and Isopropyl Palmitate. For Isohexadecane, measured Ostwald ripening rate was found 1000 times larger than calculated rates for hexadecane on a model emulsion. Discrepancy may come from the glycols present in aqueous phase that favor oil solubility in the continuous phase.

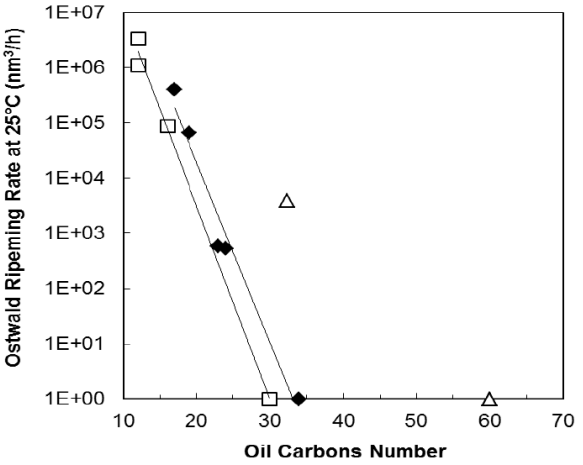
Figure A.11 - Evolution of droplets volume upon time for different cosmetic oils: Full Diamond symbols – Isopropyl Myristate, Empty Triangle symbols – Isododecane, Full Square symbols – Isopropyl Palmitate.



Source: authors' own production.

Figure A.12 presents the Ostwald ripening rate for different oils (Sonneville-Aubrun *et al.,* 2004). As obtained by different authors, Ostwald Ripening Rate decreases with oil carbon number or oil molecular weight, following a semi-logarithmic law (Taylor *et al*., 2003). Furthermore, for comparable molecular weight or carbon number, Ostwald Ripening Rate increases with oil polarity (V alkane < V ester < V triglyceride).

Figure A.12 - Evolution of droplets volume upon time for different cosmetic oils: Empty Square symbols – Alkanes, Full Diamond symbols – Fatty Esters, Empty Triangles Diamond symbols – Triglycerides

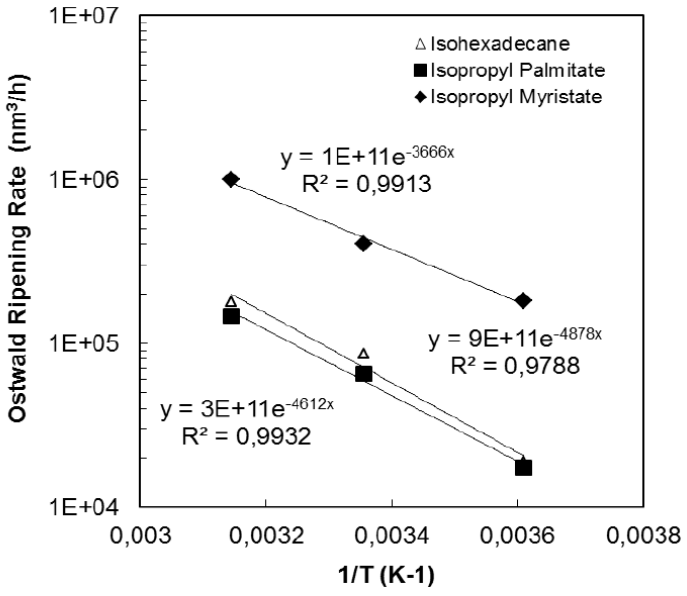


Source: authors' own production.

Cosmetic formulations must be stable at different temperatures encountered in real life. Stability tests are usually performed at 4 °C, Room Temperature (~25 °C) and 45 °C for several months.

According to previous studies, Ostwald ripening rate should follow an Arrhenius law with temperature (Delmas *et al*., 2011). Figure A.13 presents experimental data on nanoemulsions containing 4.5% PEO-8 Isostearate / 0.5% Stearoyl Glutamate / 15% Oil / 10% Dipropylene Glycol / 5% Glycerin / Water made with a Niro-Soavi OBL20 device at 120 MPa (Sonneville-Aubrun *et al.*, Unpublished). The graph shows an exponential decrease of Ostwald ripening rate with 1/T, confirming the former results. From 4 °C to 45 °C, the Ostwald ripening rate was found to increase by about one order of magnitude.

Figure A.13 - Evolution of Ostwald Ripening Rate with 1/T for 3 different oils : Full Diamond Symbols – Isopropyl Myristate, Empty Triangle Symbols – Isohexadecane, Full Square Symbols – Isopropyl Palmitate



Source: authors' own production.

Amongst each oil family, it is still possible to find oils with no Ostwald Ripening phenomenon to reach stability. However, to better optimize the sensoriality and performance of the products, it is important to find solutions that stabilize nanoemulsions with low molecular weight oils and polar oils.

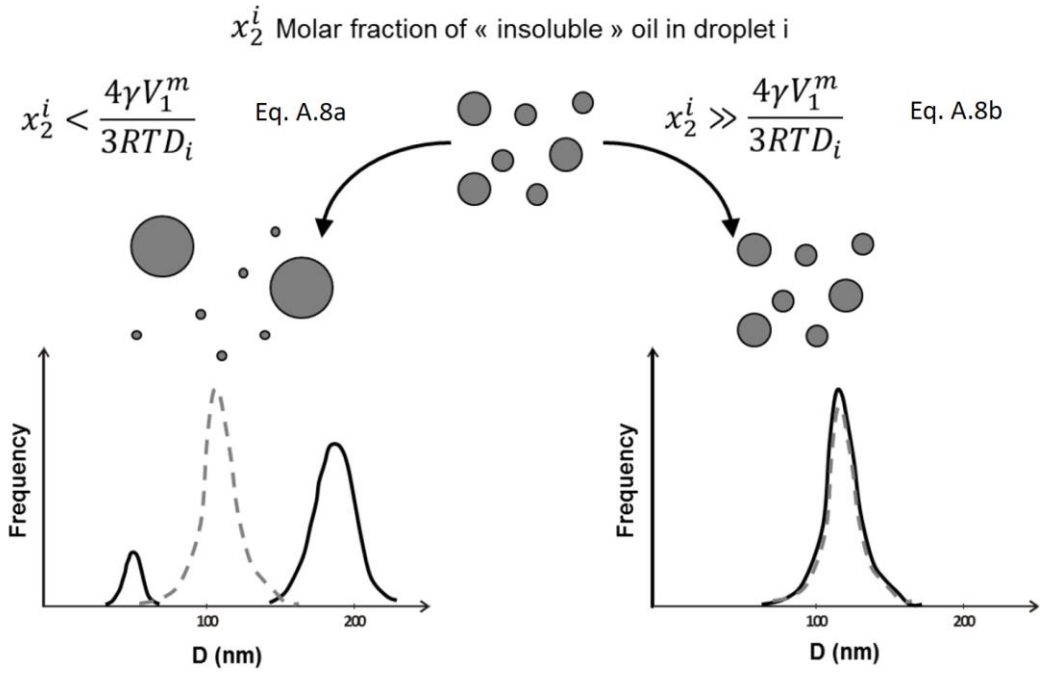
Higuchi and Misra (1962) first proposed guidelines to stabilize emulsions sensitive to Ostwald ripening by using mixtures of oils. According to theory, two scenarii may occur for an emulsion prepared with two oils of different nature, oil1 “soluble” and oil2 “insoluble”. In equation A.7, an additional term of entropy of mixing can compensate for the Laplace pressure term to obtain a stable system.

$$\mu_1 = \mu_1^{\circ} + \frac{4 \cdot \gamma \cdot V_1^m}{D} - R \cdot T \cdot x_2 \cdot \left(\frac{D^i}{D}\right)^3 \qquad \text{Eq. A.7}$$

The stability condition is linked to the molar fraction of the ”insoluble” oil in a droplet, that has to be above a critical value depending on interfacial tension, oil molar volume, droplet diameter and temperature as expressed by equations A.18a and A.18b presented on Figure A.14.

If the stability condition is not satisfied, the size distribution evolves towards a bimodal nanoemulsion with smaller droplets enriched in “insoluble” oil and larger droplets enriched in “soluble” oil. The critical molar fraction of “insoluble” oil is all the more important that the initial droplet size is small.

Figure A.14 - Evolution of droplets size according to an Ostwald Ripening mechanism for an emulsion containing two types of oils, “soluble” and “insoluble”, as a function of molar fraction (xi) in insoluble oil



Source: authors’ own production.

The critical weight fraction in “Insoluble Oil” has been determined theoretically and compared to experimental data for nanoemulsions containing mixtures of “Insoluble Oil” and Soluble Oil”. Theoretically, 10% of Isocetyl Stearate in weight relative to oil phase could stabilize a nanoemulsion with 40 nm diameter containing Isododecane, taking a value of 10 mN/m for Interfacial Tension. Experimentally, 50% Isocetyl stearate in weight relative to oil phase was necessary to stabilize a 40nm nanoemulsion containing Isododecane (Sonneville-Aubrun *et al.,* 2004).

Textures: from lotions to gels

The texture of cosmetic products is a complex and multidimensional notion that drives the consumers’ liking/acceptance by conveying an immediate hedonic perception, uniqueness, a surprise or an excitement.

Nanoemulsions cover a wide range of textures, starting from lotions to stiff gels. Three major factors triggers the product consistency: the dispersed fraction, the presence of polymeric thickener or gelling agents and the presence of crystalline lamellar phases.

Dispersed fraction

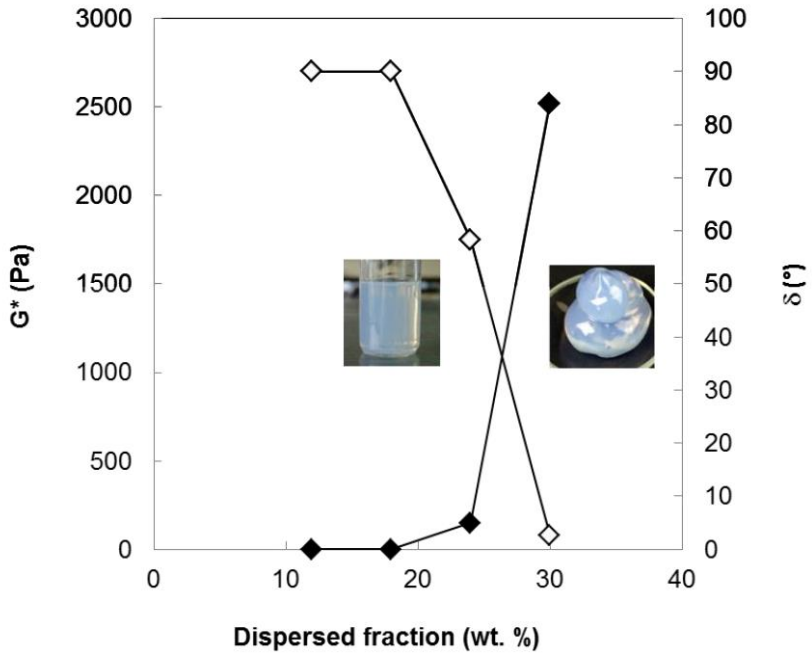
By increasing the droplet volume fraction, an emulsion can be changed from a simple viscous liquid to an elastic solid of a substantial shear modulus (Mason, 1999). For classical emulsions, the droplets become transiently caged by their neighbors by an approx. 0.58 volume fraction (hard sphere glass transition), and become compressed with deformed interfaces of above 0.64 volume fraction (random hard sphere close packing).

Nanoemulsions were made with increasing volume fraction with 5% surfactant (PEO-8 Isostearate / Stearoyl Glutamate 9/1), oil (Isocetyl Stearate / Isopopyl Myristate 2/1) from 10 to 25%, Dipropylene Glycol, Glycerol and Water, with a Niro-Soavi OBL20 at 120 MPa. Oscillatory measurements have been performed

to characterize the change in viscoelasticity with volume disperse fraction with a Haake RS150 (Stress Sweep at 1 Hz, 25 °C) (Arnaud-Roux and Sonneville-Aubrun, Unpublished). Complex Modulus and Loss Angle as a function of disperse phase fraction are presented on Figure A.15.

Comparably to classical emulsions, the consistency of nanoemulsions sharply increases with increase of the dispersed phase volume fraction. The liquid-solid transition happens at lower volume fractions for repulsive nanoemulsions. Stiff gels have been observed between 0.25 and 0.3 volume fraction in disperse phase in accordance with Kawata (2010).

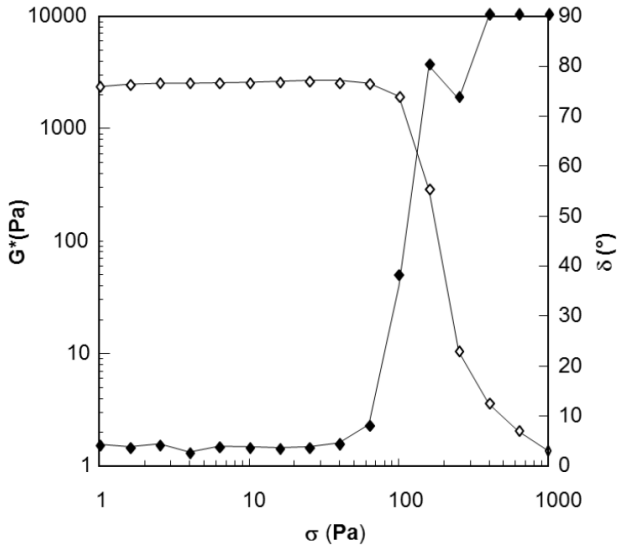
Figure A.15 - Complex modulus G* (Full Diamond Symbols) and loss angle (Empty Diamond Symbols) as a function of disperse phase weight fraction, in the linear domain



Source: authors' own production

Figure A.16 represents the complex modulus and loss angle as a function of stress for a 25% oil nanoemulsion. It shows a large linear domain with an abrupt transition around 1000 Pa in relation to the sensorial characteristics of the product: a stiff gel that melts upon shearing.

Figure A.16 - Stress sweep for a concentrated nanoemulsion of 25% oil



Source: authors' own production

This gel texture is ascribed to the crystal-like lattice structure of nanodroplets, as observed by transmission electron microscopy of a freeze-fractured surface of the specimen (Kawata, 2010). Small-angle neutron scattering (SANS) revealed the presence of an ordered structure in addition to spherical domains with a radius of 17 nm. This long-range order is, in principle, due to electrostatic repulsive interaction between charged nanodroplets.

A simple calculation of inter-droplets distances "d", assuming a Centered Cubic Lattice, shows distances smaller than 10 nm that can be reached by nanoemulsion for 25% oil in weight (table A.4). This distance shall be in the range of repulsive distance between droplets.

$$d = \left(\left(\frac{1}{2}\right)^{1/6} \cdot \left(\frac{\pi}{3 \cdot \emptyset_{oil}}\right)^{1/3} - 1 \right) \cdot D$$

Eq. A.8

Table A.4 - Droplets interdistances for different droplet sizes and oil volume fraction

Oil fraction (wt.%)	Oil + Surfactant fraction (v.%)	D = 40 nm d (nm)	D = 60 nm d (nm)	D = 300 nm d (nm)	D = 1µm d (nm)
25	34.2	8.5	14.3	84.9	290.6
23	31.9	9.6	16.1	93.8	320.3
21	29.7	11	18.1	103.6	353.2
19	27.4	12.4	20.3	114.7	390.0

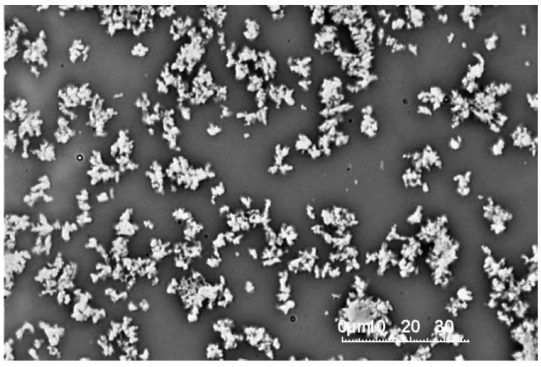
The gel textures appear extremely shear-thinning, leaving a melting feeling upon application. This rheological behavior might be an issue in picking-up the product from a jar. In that case, the texture is linked to the oil volume fraction. Obtaining gel textures with lower oil content for lighter products would be desirable. Thickeners or gelling agents can give access to other textures and sensory feelings.

Thickeners or gelling agents

Cosmetic O/W emulsions are classically thickened by polymers that offer a wide range of structures and cosmetic benefits. Usually, the addition of polymers is performed at the end of the emulsification process, to avoid disturbing droplets fragmentation and degrading the polymer. A pre-gel can be prepared and added to a concentrated nanoemulsion. In some cases, it might be favorable to add the polymer during the emulsification process (i.e. low molecular weight polymers with low sensitivity to shear).

Microgels such as carbomers are very commonly used in cosmetics and especially in skincare products for their appreciated texturing and sensorial properties. Some authors used them to thicken nanoemulsions at low oil volume fraction (Oliveira, 2011). However, they are sensitive to formulation ingredients such as ionic species (salts, actives, preservatives…), with a dramatic decrease in consistency. In case of nanoemulsions at medium to high volume fraction, as soon as they are introduced, the system turns white and micron size irregular structures are visible through microscopic observations (Figure A.17).

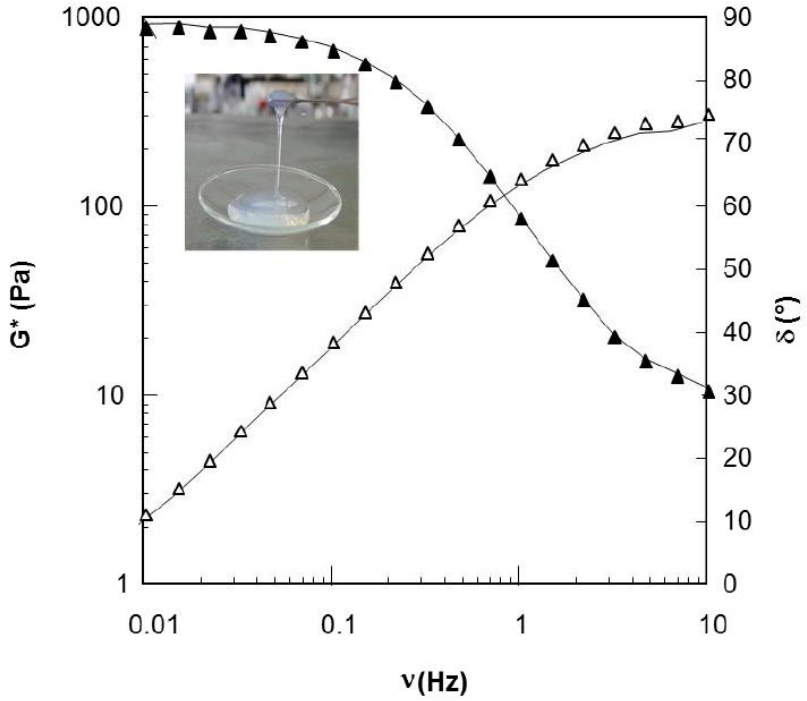
Figure A.17 - Optical Microscopy image from a thickened nanoemulsion with carbomer



Source: Sonneville-Aubrun, 2004.

Associative polymers such as Hydrophobically modified Ethoxylated Urethane polymers (HEUR) can easily thicken nanoemulsions even at low oil dispersed volume fraction. The droplets participate to the tri-dimensional network formed by the polymer in water. A thickened nanoemulsion may present a fluid character at low frequency, turning to solid at high frequency as presented on Figure A.18 for a nanoemulsion with 5% surfactant and 15% oil and 0,5% HEUR (Arnaud-Roux and Sonneville-Aubrun, Unpublished).

Figure A.18 - Complex modulus G* (Full Triangle Symbols) and loss angle (Empty Triangle Symbols) as a function of frequency



Source: authors' own production.

Linear and non-associative polymers can also be used, such as Hydroxyethyl Cellulose, Xanthane Gum (Samson, 2016; Ribeiro, 2015a), to slightly thicken the nanoemulsions. Depletion flocculation mechanisms may happen even at rather low concentration in dispersed phase and polymer, with a dramatic effect on stability and transparency of the nanoemulsion.

Crystalline Lamellar Phase

Fatty alcohol/surfactant associations have been used for a long time to texture emulsions without polymer, through the formation of a crystalline lamellar phase

in the bulk and at interface (Barry, 1968; Nakajima, 1998). These structures also play a role in nanoemulsion efficacy by creating an occlusive and protective layer onto the skin for hydration. Okamoto *et al.* (2016) have demonstrated the interest of a decreased droplet size to get new milky lotion, lighter upon application without compromising on hydration benefits. The nanoemulsion is broken during its application onto the skin, thereby releasing the fatty alcohol and surfactant molecules into the aqueous phase.

IV. EXAMPLES OF COSMETIC APPLICATIONS

Besides the noticeable interest in nanoemulsions for their unique texture, other benefits of nanoemulsions have been valued in many cosmetic applications, especially in skincare for the solubilization and delivery of active ingredients. In the haircare domain, they play an important role in the silicon deposition onto hair fiber or scalp treatment. The following lines give an overview of different cosmetic applications.

Skin care

Skin structure and penetration pathways

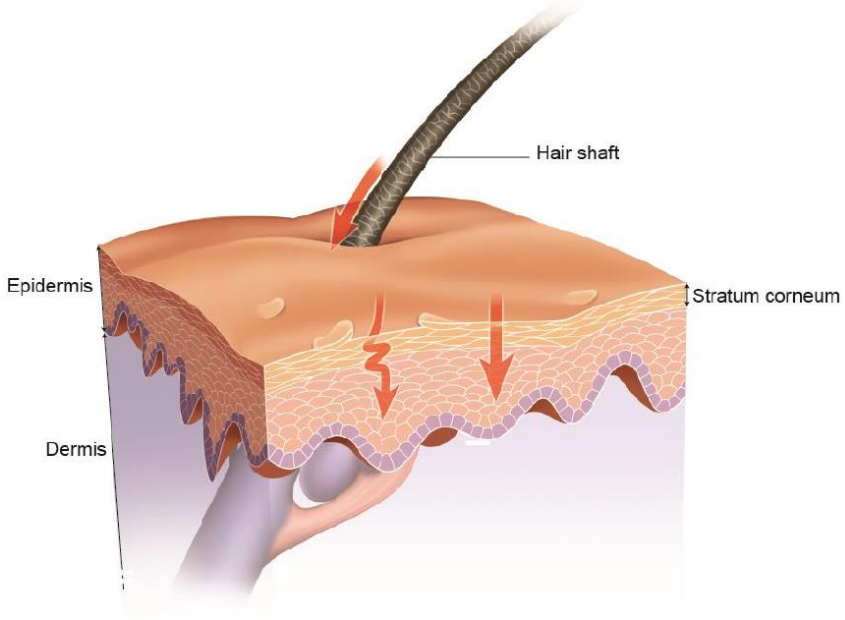
The human skin is the largest organ in the body, consisting of distinct layers, providing, as its main function, a protection to the body from environmental harms (Ribeiro *et al.*, 2015a, Wu and Guy, 2009). Starting from the outermost layer of skin, as shown in Figure A.19, the *Stratum Corneum* (SC) of epidermis is composed of dead cell, i.e. of corneocytes. Below the SC, the viable epidermis comprises keratinocytes, melanocytes and Langerhans cells. Keratinocytes cells are continuously regenerated from the basal cell layer of epidermis then differentiating upward to towards the surface. Underlying the epidermis, the dermis contains fibroblasts, collagen, elastin, hair follicles, sebaceous glands, sweat glands, nerve, blood and lymphatic vessels, and it is considered the major support and feeding component of the skin. The subcutaneous fat layer or hypodermis is mainly made of hypertrophic adipocytes and provides a thermal

barrier to the body (Wu and Guy, 2009, Ammala, 2013, Kong *et al.*, 2011, Alzorqi, 2016).

At the skin surface, the flat corneocytes are assembled in parallel and overlapped structure as “bricks”, and surrounded by a “cement-like” structure of lipid matrix composed of ceramides, fatty acids and cholesterol. Despite its permanent losses in corneocytes though the regular process of desquamation, this structure acts as an excellent barrier against penetration of external agents by topical application administration or exposure, and the components loss from internal to external part of the skin (Kong *et al.*, 2011, Wu and Guy, 2009).

There are a number of possible pathways for molecules to penetrate through the skin barrier, and three main routes are generally considered. First, the intercellular pathway that is most well-known occurs by the molecule diffusion via lipid layers among the interface of corneocytes. The second pathway is the follicular pathway with its dense network of blood capillaries, also acting as an active component ‘reservoir’. Third is the transcellular pathway where molecule transportation occurs directly through the corneocytes and lipid layers (Morrow, 2007, Mihranyan *et al.*, 2012).

Figure A.19 - Schematic illustration of skin structure and active components penetration pathways. 1 = intercellular, 2 = follicular and 3 = intracellular.



Source: authors’ own production.

Skin disorders and needs

Disturbance of the *Stratum Corneum* barrier function caused by a disorder in the corneocyte arrangement or delipidation may increase Transepidermal Water Loss (TEWL), leading to a decrease in skin hydration and a dry skin syndrome, a common complaint among the population, particularly among older subjects (Bernardi, 2011, Yilmaz and Borchert, 2006, Ribeiro *et al.*, 2015a). Another undesirable phenomenon for consumers is brought by the global skin aging process of two types: intrinsic aging and extrinsic aging. Intrinsic (chronological) aging is a natural process caused by genetic factors. It is an irreversible degeneration process into which a progressive alteration of dermal extracellular matrix network leads to the loss of skin firmness and elasticity. Extrinsic aging manifests in deep lines and wrinkles, rough and hyper-pigmented skin, as a consequence of exposure to sun/ultraviolet (UV) rays or overexposure to oxidative stresses (Samson *et al.*, 2016). Several chromophores in the skin (such as melanin, DNA, RNA, lipid and aromatic amino acids) absorb UV, leading to photochemical reactions and free radicals, otherwise known as Reactive Oxygen Species (ROS). The excess of ROS induces the oxidative stress state caused by the deterioration of the natural antioxidant defense mechanisms, leads to protein denaturation and lipid peroxidation, and finally to cell and tissue death (Alzorqi, 2016, Mahdi *et al.*, 2011). These oxidative damages contribute (Krutman *et al.*, 2017) to several skin disorders such as photoaging, inflammation and photo carcinogenesis. These incidences of skin disorders spur the development of high quality products, targeting high performance, safety and cost-benefits for consumers.

Ceramides Solubilization and Delivery

Ceramides are major *Stratum Corneum* lipids, representing 40% to 50% of its intercellular lipids. They play crucial roles in the skin barrier function, cell adhesion, epidermal differentiation and induction of tumor cell apoptosis. A ceramide comprises a fatty acid and a sphingoid base, and nine sub-classes of ceramides have been identified in the *Stratum Corneum*, according to acid chains and sphingoid base. Ceramide compositions vary according to the skin conditions such as aging and dry skin. Previous studies reported the correlations between

decrease in ceramide content and dry skin or skin with and atopic dermatitis, indicating the relevance of topical application of lipid mixtures for these conditions treatments (Choi and Maibach, 2005).

Nonetheless, ceramide application is limited due to their difficult solubilization into the water-based systems. Some researches, aiming at reducing and overcoming these limitations, are listed below. In some cases, *In vivo* benefits of ceramide nanoemulsions have been demonstrated.

Aiming at restoring these disturbed skin barriers, different nanoemulsions containing specific lipids were prepared and compared (Yilmaz and Borchert, 2006): positively charged oil/water nanoemulsion (PN), positively charged oil/water nanoemulsion containing ceramide 3B and naturally found SC lipids such as ceramide 3, cholesterol, and palmitic acid (PNSC) and a negatively charged O/W nanoemulsion containing SC lipids (NNSC). The positive charge was achieved by incorporating phytosphingosine (PS), an amphiphilic sphingoid base naturally found in the human body. Carbopol 940 was further added after the nanoemulsion formation by high-pressure homogenization at 500 bars. The droplet size was medium, around 200 nm.

In Vivo study of skin hydration, elasticity, and erythema was performed on 14 healthy female volunteers, by instrumental measurements, using Corneometer® 825 (SC water content), Cutometer® SEM 575 (skin firmness) and Mexameter® 18 (skin darkness). Results showed that all formulations increased skin hydration and elasticity. However, PNSC demonstrated significantly higher efficacy for skin hydration and elasticity, as compared to PN and NNSC. These results indicate that phytosphingosine plays an important role to deliver SC lipids to the skin. However, the impact of emulsion size on the efficacy still has to be investigated.

Plant extracts for skin protection and moisturizing

Plant extracts are widely used in cosmetic preparations due to several associated properties such as antioxidant activities and moisturizing properties (Bernardi *et al.*, 2011, Zhong *et al*., 2010). Generally speaking, the natural antioxidants comprise three important groups: phenolic acids, flavonoids and high-molecular

weight polyphenols (Svobodová, 2003). Numerous *in vitro* studies have demonstrated that antioxydant compounds can directly scavenge ROS as well as Reactive Nitrogen Species (RNS), chelate potentially pro-oxidant metal ions (Fe3+, Al3+, Cu2+) and complex with pro-oxidant proteins (Mahdi *et al.*, 2011, Munin and Edwards-Lévy, 2011, Svobodová, 2003). Other categories of natural components such as water-soluble polysaccharides are also of great interest.

One of the main constrains of the natural compounds' application, mainly the polyphenols, lies on their instabilities along exposure to external factors during processing and storage, and their limited solubility. The stability problem may result in changes in the color and odor of the final products, as well as considerable activity loss (Munin and Edwards-Lévy, 2011).

Different works describe the feasibility of nanoemulsions loaded with different polyphenols-rich plant (Mahdi *et al*., 2011, Ribeiro *et al*., 2015a) using PIC method and/or high shear device. Small sizes to 30nm could easily be obtained with classical surfactants such as Tween® 80/Span® 80 mixtures. Comparison with regular emulsions would be necessary to demonstrate the advantage of nanoemulsions either for delivery or hydration benefits.

Another study was performed with a quercetin-loaded nanoemulsion (flavonoid) prepared by the sub-PIT method with a PEO-oleyl ether mixture (Dario *et al.*, 2016). The active was found to act as a co-surfactant with a strong positive impact on droplet size and nanoemulsion stability. In nanoemulsions, the quercetin was mainly located at the oil/water interface, whereas in coarse emulsions it was mainly dispersed in the aqueous phase in a crystallized form due to its low solubility in water. In this case, Nanoemulsions appear superior to deliver poorly soluble amphiphilic antioxidant with a good photostability.

Rice bran oil nanoemulsion was developed by Emulsion Phase Inversion (EPI) method (Bernardi *et al.*, 2011) to provide a stable, low irritant and high moisturizing product. The unsaponifiable fractions of rice (*Oryza sativa*) bran oil are rich in tocopherols/tocotrienols and gamma-oryzanol, well known for their antioxidant properties. The obtained nanoemulsion composed of 10% rice bran oil, 10% surfactants sorbitan oleate/PEO-30 castor oil with a droplet size of 70 nm. I*n vivo* assessment of this nanoemulsion demonstrated good tolerance and

increased skin hydration with satisfactory results, considered as superior to commercial products according to the authors.

UV filters dispersions for Suncare

Preventing and protecting the skin from the harmful effect of UV rays require the development of efficient topical products, which that cover a wide range of UV radiation levels. UVA waves range from 320 to 400 nm, whereas UVB rays range from 290 to 320 nm. UVA's are mostly responsible for extrinsic aging process by their penetration into the dermis, whereas UVB's cause sunburns and promote skin malignant process (Pillai *et al*., 2005). Photo protective agents are usually classified into organic and inorganic/mineral absorbers. Organic UV filters act by chemical absorption and comprise para-aminobenzoic acid derivatives, salicylates, cinnamates and camphor derivatives as main UVB sunscreens, and Avobenzone, Ecamsule, drometrizoletrisiloxane… as UVA sunscreens. Inorganic absorbers prevent the radiation by physical blockage (reflection), and include Titanium dioxide as UVB absorber and zinc oxide as UVA absorber (Coutinho *et al.*, 2015, Puglia *et al*., 2014). The development of an efficient colloidal system that overcomes the limitations of current sunscreen formulations as photostability, water resistance, risk of penetration, high Sun Protection Factor is a challenge (Puglia *et al.*, 2014).

O/W nanoemulsions containing both chemical and physical UV filters (Octyl Methoxycinnamate and lipophilic nano-TiO2) were processed by ultrasound (US) with classical ethoxylated alkyl ethers (Silva et al., 2013). Without physical UV filters, very fine and stable nanoemulsions (3-10 nm) could be obtained with limited quantity in chemical UV filter providing *in vitro* SPF as low as 3. Incorporation of physical UV filters lead to a huge increase in droplet size to 400-500 nm, due to the presence of lipophilic TiO2 in the internal phase. This example illustrates the limit in using nanoemulsions for mineral UV filtration.

Other authors studied the impact of structuring oil internal phase on nanoemulsions containing chemical UV filters. Nanoemulsions and Nanostructured Lipid Carriers (NLC) were formulated with different UV filters in oil phase (Puglia *et al.*, 2014). Nanoemulsions only contained liquid oil in

dispersed phase whereas NLC contained a mixture of liquid oil and waxy material (NLC). They were stabilized by Pluronic F68 and prepared using an ultrasonication method. Droplet size between 120 nm and 320 nm were successfully obtained. No significant differences in the spectral profile were observed between nanoemulsions and NLC. *In vitro* percutaneous absorption studies showed reduces permeation ability of sun filters for NLC compared to nanoemulsions. However, the epidermal penetration values were low and below the accepted minimum safety limit accepted, for both nanoemulsions and NLC.

Skin cell Targeting with amphiphilic ligands

Direct ligand incorporation into nanoemulsions was explored to target specific skin cells such as keratinocytes and fibroblasts (Atrux-Tallau *et al.*, 2013). The authors studied two different amphiphilic ligands: a polypeptide lysine–threonine–threonine–lysine–serine (KTTKS) lipophilized with palmitic acid (palmitoyl-KTTKS) and a trisaccharide chain glucose–glucose–rhamnose, asiaticoside. The peptide KTTKS is a sub-fragment of collagen I with potent activity on fibroblasts, improving extracellular matrix synthesis and inhibiting collagenase activity. Asiaticoside may link to lecithin type receptors on skin cells. Direct emulsification of all components (the oil/wax mixture, surfactants and targeting ligand at 1% w/w of dispersed phase) by using ultrasonication process enabled formation of 50 and 120 nm nanoemulsions. *In vitro* cell absorption assay using HaCaT cell line showed a preferential binding of asiaticoside-targeted nanoemulsion droplets to the keratinocytes. When tested on fibroblasts, palmitoyl KTTKS-targeted nanoemulsions demonstrated specific interaction with fibroblasts. This study shows that different skin cell types might be targeted with selected ligands by increasing their adhesion onto cells.

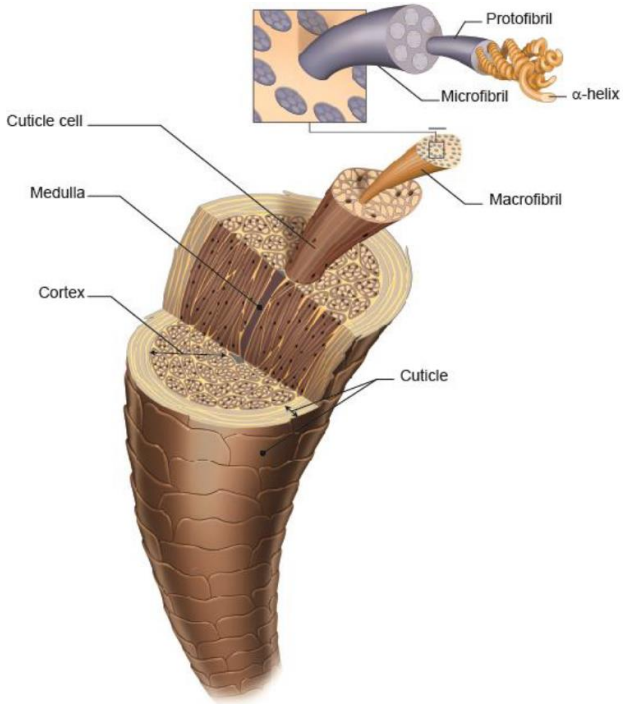
Further studies have been performed to test ligand-targeted nanoemulsions for whitening active delivery (licorice) to melanocytes (Attrux-Tallau *et al.*, 2014). Four ligand candidates were tested: a palmitoyl-peptide (palmitoyl-GQPR), a lipidized hyaluronic acid (caproyl-HA) and two amphiphilic actives (polydatin and isopilosine). The use of caproyl-HA significantly improved the ability of licorice-loaded nanoemulsion to reduce melanin concentration on melanocytes *in vitro*. *In vivo* efficacy has to be demonstrated.

Hair fiber and Scalp

Hair structure

Hair is formed by dynamic, complex processes of structural alignment and keratinization of proliferated cells originating from hair matrix at the base of hair follicle and soon entering a multistep differentiation process upwards (Franbourg and Leroy, 2005). As shown in Figure A.20, the outermost layer of the hair is composed of a cuticle, a protective layer that tightly surrounds the cortex, the major component of the hair. The continuous phase named the cell membrane complex (CMC) acts as cement between the cells. Cuticle CMC contains polysaccharides and a lipid, 18-methyleicosanoic acid (18-MEA) that confers hydrophobic properties to the hair surface. The cortex mainly consists in highly-arranged keratin filaments called microfibrils, which are packed into larger units called macrofibrils. The fiber is made of 60% keratins and 40% keratin associated proteins, composing the matrix that stabilizes the microfibrils in macrofibrils. Among the structure of cortex, melanin pigments are dispersed, providing color characteristics to hair. Finally, located in the center of the fiber, are the medulla cells.

Figure A.20 - schematic illustration of hair structure



Source: authors' own production.

Hair care and needs

The condition of hair surface is daily altered by weathering, which may encompass a number of factors, aerial pollution included. But the main damage inducer is exposure to UV rays that causes photo oxidative cleavage of cystine linkages, free radical production, generating as a result cysteic acid end groups that confer a high anionic character to the hair surface and increased porosity. Among 18 aminoacids that compose hair keratins, cystine amounts to about 16% and even 20 to 30% in the outer cuticle. Hence, the chemical behavior of the hair surface may be highly impacted.

Another highly damaging process is daily handling namely frequent or excessive brushing of hair for dry untangling, mainly in women with mid-long or long hair as seen by the condition of hair from root to distal end: cuticle scales are eroded, uplifted, scratched, torn out, the cuticle becoming increasingly thinner up to being erased in split ends. Chemical processes such as hair straightening alter cuticles in order to achieve the desired visual effect (Lohani *et al.*, 2014). These external events lead to brittleness, loss of shine, impairing smoothness, high friction level of hair.

Thus, the development of efficient hair conditioning products aiming at an optimal repair and softness of the hair surface, easy combing and brushing, improved control, gloss, manageability, body, volume, or bounce balance between rinsing / active compound deposition, penetration / safety and delivery of active compounds to the right destination without systemic circulation absorption, presents are paramount challenges and opportunities in the Cosmetics and Personal Care field.

Another common hair concern is hair loss, either caused by *Androgenetic Alopecia* or *Alopecia Areata*, where hair follicles on a small scalp region are affected by a chronic inflammatory process (Aljuffali *et al.*, 2014).

Silicon Deposition for hair conditioning

Silicon-in-Water nanoemulsion, stabilized by nonionic surfactants (Span 80/Tween 80) and a cosurfactant (n-Butanol), was developed and compared to

classical emulsions regarding silicon deposition on hair (Hu *et al.*, 2012). Nanoemulsions of mean droplet size of 300 nm were obtained by phase inversion composition method and dispersed in a shampoo. They were compared to micron size emulsions. Experimental results demonstrated a significant increase in silicon oil deposition on hair treated with a direct correlation of droplet size: the smaller the size, the greater the adsorption onto the hair. Nanoemulsion provides potential formulation with effective combination of washing and hair care properties, into a one-step process, with a higher performance compared to classical emulsions.

Scalp treatment for hair loss

Two lipid nanosystems (Squarticles) composed of squalene (sebum lipid) were developed by Aljuffali et al. (2014) to deliver active material against hair loss to the hair follicles: NLC and nanoemulsions. Diphencyprone (DPCP) is a topical drug for *Alopecia Areata* treatment that induces hair regrowth through local immune response. Minoxidil is another drug (OTC at 2% in some countries) for treating *Androgenic Alopecia* by promoting vasodilation and hair regrowth. These drugs may cause side effects such as skin irritation and risk of systemic absorption. Drugs encapsulation in the nanosystems possibly reduces these drawbacks, by drug targeting into the hair follicles, controlling drug release, and increasing drug stability. As sebum is secreted by the sebaceous duct, which connects to the hair follicles, good interaction of squarticles with the follicles is expected. Particle sizes of 177 nm for NLC and 194 nm for nanoemulsions were obtained by sonication method, with concentration of 0.15% w/v (7.3 mM) and 0.8% w/v (38.2 mM) of DPCP and Minoxidil, respectively. *In Vitro* and *In Vivo* tests demonstrated a reduced systemic absorption of drug and a higher follicular uptake for Squarticles vs control with free active.

Preservative system for cosmetic nanoemulsions

Aiming at investigating the relationship between the nanoemulsion droplet size and the anti-microbial activity, a new alternative combination of preservative

system composed of caprylyl glycol, phenethyl alcohol and glyceryl caprylate was developed (Fang *et al.*, 2016). The anti-microbial activities were analyzed through MIC/MBC/MFC methods and the results demonstrated a synergic effect of the combination of these preservative ingredients in five common microorganisms, as compared to their use alone. A broad-spectrum anti-microbial activity was observed with in this blend, meeting the PCPC and European Pharmacopoeia requirements at a concentration of 1.4%. The effect of size of emulsion droplets on preservative activity was observed, indicating the gradual enhancement of this activity with the increase of emulsion droplets in the range of 100–900 nm. According to this study, nanoemulsion droplet size is a factor that influences the preservation of cosmetic products. As a consequence, preservative dose has to be adapted to nanoemulsions to guarantee a good preservation.

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