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Master Thesis in Bioengineering

Design of an oleogel-based system for the co-delivery of hydrophilic and lipophilic bioactives in foods

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‘Structure is the ultimate expression of the complexity of nature.’

Alejandro G Marangoni

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Abstract

Oleogels have been gaining recognition in the food science field due to their easy processing, affordability, and the ability to be tailored to match specific properties. Additionally, structured emulsions using vegetable oils rich in unsaturated fatty acids may come up as an alternative for unhealthy food products that constitute today's dietary habits. The present work aims at developing a novel oleogel-based water-in-oil emulsion for the co-loading of hydrophilic and lipophilic bioactive compounds, using a γ-oryzanol-phytosterol mixture as structurant. The characterization of the emulsions demonstrated that the presence of water severely affects the gelator network, with single β-sitosterol crystals being the main contributors to the self-standing ability of the emulsions. These crystalline structures were observed in the polarized microscopy results, being later confirmed through small-angle X-ray scattering and X-ray diffraction. These analyses have shown sharp crystallographic reflections for anhydrous and hemihydrated β-sitosterol crystals, independently of the gelator concentrations and oil-to-water ratios. The samples with higher water content have shown additional reflections that could be traced back to monohydrated β-sitosterol crystals, but these were not present after 7 days. Although the X-ray analyses did not vary much according to the formulation, the rheological analysis has revealed the impact of gelator concentration and oil-to-water ratio in the emulsions' strength. The emulsion prepared with 20 % gelator and 90:10 oil-to-water ratio was the strongest, featuring a storage modulus value of 59317.50 Pa within the linear viscoelastic region. This was also the formulation that endured higher stress amplitudes, with the yield stress reaching 28.40 Pa. Although this was the strongest of the prepared emulsions, the results still contrast with the previously reported data for the oleogels, being around 30× lower than the values for oleogels. Despite the differences regarding the typical multi-component oleogels produced by oryzanol-phytosterol mixtures, the emulsions exhibited interesting textural characteristics, making them suitable for possible incorporation in food products. The addition of β-carotene and epigallocatechin gallate to the emulsions has confirmed the viability of the formulation towards incorporating bioactive compounds in their free-form, constituting a proof-of-principle for upcoming developments in the fortification of lipid-rich foods with water-soluble compounds.

Keywords: oleogel; gelator; emulsion; bioactive compounds; co-delivery.

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Resumo

Os oleogéis têm vindo a ganhar relevância na área da tecnologia alimentar devido à sua versatilidade, baixo custo e simplicidade de preparação. Para além disso, as emulsões estruturadas preparadas a partir de óleos vegetais ricos em ácidos gordos insaturados podem surgir como alternativa aos produtos menos saudáveis que integram os hábitos alimentares atuais. O presente trabalho tem como objetivo desenvolver uma nova emulsão estruturada água-em-óleo, utilizando uma mistura de γ-orizanol e fitosteróis como gelificante da fase oleosa, para incorporação de compostos bioativos hidrofílicos e lipofílicos. A caracterização das emulsões demonstrou que a presença de água influencia bastante a formação da rede tridimensional de gelificante, sendo os cristais de β-sitosterol os principais contribuintes para a sua rigidez. Estes cristais foram observados nas imagens de microscopia polarizada, tendo esta observação sido posteriormente confirmada nas análises de dispersão de raios-X em pequeno ângulo e difração de raios-X. Nesta análise, foram registados picos nítidos para cristais de β-sitosterol anidros e hemi-hidratados em todas as amostras, independentemente da concentração de gelificante e da razão óleo-água. As amostras com maior teor de água exibiram picos adicionais, que podem ser atribuídos a cristais de β-sitosterol monohidratados; no entanto, estes não estavam presentes na análise feita após 7 dias. Embora as análises de raios-X não variem muito de acordo com a formulação, a análise de reologia revelou o impacto da concentração de gelificante e da razão óleo-água na força das emulsões. A emulsão preparada com 20 % de gelificante e razão óleo-água de 90:10 foi a mais forte, apresentando um valor de módulo de armazenamento de 59317.50 Pa na região linear viscoelástica. Esta formulação foi também a que suportou tensões de corte com maior amplitude, sendo a tensão de escoamento estimada a 28.40 Pa. Embora esta tenha sido a mais forte das emulsões preparadas, os resultados são cerca de 30× menores do que os registados previamente para oleogéis preparados com misturas semelhantes de γ-orizanol e fitosteróis. Apesar das marcadas diferenças das emulsões em relação aos oleogéis, estas apresentam características texturais interessantes, que as tornam boas candidatas para uma possível incorporação em produtos alimentares. A incorporação de β-caroteno e epigalocatequina galato nas emulsões confirmou a viabilidade da formulação para a incorporação de compostos bioativos na sua forma livre, constituindo um ponto de partida para a fortificação de alimentos ricos em lípidos com compostos hidrofílicos.

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Contents

List of Figures

List of Tables

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List of Acronyms

BC	β-carotene
CMC	Carboxymethylcellulose
CSD	Colloidal Silicon Dioxide
EC	Ethylcellulose
EFSA	European Food Safety Authority
EGCG	Epigallocatechin gallate
FA	Ferulic Acid
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
HDL	High-Density Lipoprotein
HPMC	Hydroxypropyl-methylcellulose
LDL	Low-Density Lipoprotein
LMOGs	Low Molecular-mass Organic Gelators
LVR	Linear Viscoelastic Region
MAGs	Monoacylglycerols
MCT	Medium Chain Triglycerides
O:W	Oil-water [ratio]
O/W	Oil-in-water [emulsion]
O/W/O	Oil-in-water-in-oil [emulsion]
PGPR	Polyglycerol polyricinoleate
PUFA	Polyunsaturated Fatty Acids
RBX	Rice Bran Wax
RC	Regenerated cellulose
SAFiNs	Self-Assembled Fibrillar Networks
WHO	World Health Organization
W/O	Water-in-oil [emulsion]
W/O/W	Water-in-oil-in-water [emulsion]

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List of Symbols

(w/w)	Weight by weight
d	d-spacing or space between planes
G'	Storage Modulus
G''	Loss Modulus
q	Scattering wave vector
tan δ	Loss Tangent
θ	Half of the diffraction Bragg angle
λ	Diffracted wavelength
σ*	Yield Stress

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Declaration

I declare, under honour, that this work is original and that all non-original contributions were duly referenced with identification of the source.

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1 Introduction

1.1 Background and motivation

In recent decades, food systems have undergone significant changes due to advances in food processing, and the current fast-paced way of living has increased the demand for more available and affordable food products. Traditional diets, featuring whole or minimally processed foods, were gradually replaced in modern society by heavily industrialized and pre-prepared food products (1). The intake of essential fatty acids and antioxidants has given place to the overconsumption of unhealthy fats, present in many processed food products, that are deeply related to health disorders, such as coronary heart disease, inflammation, oxidative stress, and metabolic syndrome (2). The addition of fats to processed food products allows for the exhibit of interesting organoleptic properties, such as texture, mouth-feel, and flavour. This creates a barrier to its substitution in foods since it can significantly hurdle the mentioned characteristics and, consequently, hinder the overall eating pleasure. Currently, food trends have shifted towards healthy eating and plant-based diets. Consumers are increasingly aware of the negative environmental impact caused by animal food production, and such recognition regarding the dangers that are associated with the consumption of overly processed animal products is generating new challenges in the food sector, in order to meet consumers' demands (3).

The pursuit for healthy substitutes for fats is not recent; the hydrogenation process has been developed as early as the beginning of the 20th century, as a strategy for reducing the consumption of saturated fats and cholesterol intake. This process was applied to vegetable and marine oils, rich in healthy polyunsaturated fatty acids, to change the degree of saturation and confer to these oils the firmness and plasticity desired by food manufacturers and consumers (4). During the second part of the century, it took over the everyday diet in the United States and several other western countries. At first, this seemed to be a positive alternative to saturated fats, thus being promoted by health advocates. However, a side effect of the incomplete hydrogenation of fats is the isomerization of the remaining double bonds, converting them to the trans- configuration (5). In the 1990s, the first studies on the dietary impact of trans fats have appeared, associating them with unfavourable effects on the serum lipoprotein profile. Trans fats were proven to not only raise LDL cholesterol levels but also to lower HDL cholesterol levels (5). Since then, the World Health Organization (WHO) has thrived for the elimination of artificial trans fats from food supply chains, with some countries being pioneers in entirely eradicating the manufacture of food products using trans fats (6). In the United States,

trans fats are no longer considered GRAS, and the FDA has established the deadline of 1 January 2021 for products manufactured with trans fats to work their way through distribution (7).

The liquid nature of vegetable oils limits their utility in some applications since they do not share the same properties as solid fats (8). Considering this, and within the governments' frameworks for removing unhealthy fats from the market, new developments in oil structuring systems have appeared in the food industry, with oleogels being at the forefront of the scientific quest. Oil structuring is based on the formation of a gelator network, that allows the formation of a self-standing thermo-reversible viscoelastic structure, without affecting the chemical structure of the oil. Unlike the hydrogenation process, oleogels are a viable way of structuring oils that are rich in PUFA without undermining their health potential (2). The acceptability of oleogels by the consumers depends on their capability of mimicking the characteristics of solid fats. Likewise, oleogels can be tailored to match a specific purpose, with structural and textural characteristics being an essential factor. Other than the replacement of non-healthy fats by healthy oils, oleogels can also have added nutritional value through the addition of bioactive compounds to the formulation. This functionality can be an advantage not only in nutritional terms but also in making the product more attractive in terms of stability and shelf-life.

The main hindrance to the application of oleogels in food matrices is its low compatibility with water-based food products. As a way of circumventing this problem, oleogels can be transformed into emulsions by applying conventional emulsification techniques, such as high-shear homogenization (9). The interplay between oleogelation and emulsification expands the possible applications of oleogels in the food industry, making them a suitable tool for many new products. These biphasic systems are capable of constituting a structured system with the benefit of decreasing the fat content of a product and might be more suitable for some applications than pure oleogels (10). Furthermore, this approach may broaden its potential to the introduction of hydrophilic bioactive compounds, rather than lipophilic compounds, or even allowing for the co-encapsulation of bioactive compounds in both phases of an oleogel-based emulsified system.

1.2 Objectives

As a consequence of the COVID-19 pandemic, the original work plan was changed, as the start of the experimental work was inevitably postponed to early June. From this date on, the experiments had to be carefully planned out considering schedule constraints, because of the implementation of rotating shifts in INL premises. This followed an internal protocol that was established according to the safety recommendations issued by the Directorate-General of Health.

The first objective for this thesis was the development of water-in-oil emulsions with a structured oil phase, envisioning an oleogel-like system capable of incorporating a high water fraction. This included the full characterization of the emulsions based on visual appearance, microscopy, rheology, X-ray diffraction, and Small Angle X-ray Scattering.

The second objective was to repeat the developed formulation with the addition of a lipophilic compound to the oil phase and of a hydrophilic compound to the water phase, both in their free form. The characterization of these emulsions was performed only by their visual appearance and microscopy.

1.3 Research Methodology

The objectives of this thesis were accomplished through the performance of several steps, which can be divided as follows:

- A careful literature review, focusing in detail on the state of the art of oil structuring methodologies, emulsified systems with a structured oil phase, and its application as bioactive delivery systems;
- Experimental optimization of the formulation of structured emulsions and its subsequent characterization, comparing their properties to oleogels produced with similar ingredients;
- Evaluation of the designed formulation for the purpose of co-loading of hydrophilic and lipophilic compounds (namely, epigallocatechin gallate and β-carotene).

1.4 Dissertation Outline

This thesis is divided into six main parts. This introductory chapter contains the background and motivation of this work and a brief description of the problem.

Chapter 2 comprehends a brief introduction about the predominant classes of gels and where oleogels fit within this classification. This serves as a basis for the discussion of the currently known food-grade oleogelators and the state-of-the-art methodologies for the preparation of oleogels. Since the main purpose of this work is to develop an emulsified system using a structured oil phase, a literature review on these formulations is presented, as well as a brief overview of the two bioactive compounds used in this project. Lastly, it is presented a complete review of the reported approaches regarding oleogel-based systems loaded with bioactive compounds.

Chapter 3 clarifies the materials and experimental procedures used throughout this work. This encompasses the steps of development of the formulation and its characterization procedures.

Chapter 4 presents the obtained results and its critical discussion, featuring a comparison between the non-loaded emulsions and the loaded emulsions.

Chapter 5 presents the main conclusions about the topics of research and suggestions for further work on these topics.

2 Literature review

2.1 Hydrogels, oleogels, bigels and emulgels

Gels represent a class of colloids that consist of a solid-like three-dimensional network, in which a liquid phase is entrapped. A gel can be defined as a coherent system of at least two components, which exhibits mechanical properties of a solid, where both the dispersed component and the dispersion medium extend themselves continuously throughout the whole system (11). Hermans proposed this definition, as the author of the first recorded effort of connecting the macroscopic and microscopic properties of a gel, which helped to define its hybrid characteristics between liquid and solid materials (11). Gel formulations can be divided into two major classes, according to the solvent used for their production; hydrogels, which refer to the case where the liquid phase is water, and organogels, when the dispersed liquid is an organic solvent and is structured by an organogelator (12).

Hydrogels consist mainly of a hydrophilic polymeric network, which can absorb high quantities of liquid. The capability of hydrogels of absorbing fluids arises from the hydrophilic functional groups on the backbone of the crosslinked polymer chains, therefore facilitating the diffusion of liquid and important solute molecules (13). Hydrogels have certain characteristics, i.e. its hydrophilicity, flexibility, elasticity, softness, and high swelling capability, which allow them to be applied in a plethora of different applications (14). Usually, hydrogels are commonly associated with pharmaceutical applications since their features make them highly efficient for transdermal drug delivery (12). As such, many novel hydrogel-based delivery matrices have been designed for pharmaceutical and medical fields, playing a vital role in diagnosis and treatment (15–17). In recent years, hydrogels have been explored in other areas, such as tissue engineering, cosmetics, and food technology, with an increasing number of publications on the subject (18–23).

Organogels are semi-rigid formulations considered bicontinuous systems, comprising two phases: the gelator and the organic solvent. The gelator, when used in the formulation of organogels in concentrations of < 15 %, may experience physical and chemical transformations that create self-assembled structures; these structures entangle with each other, forming a three-dimensional network. The organic solvent is retained and immobilized within the spaces of the gelator network. If the used solvent is a liquid oil, then the term oleogel is also appropriate for these formulations. Considering this, oleogels allow the exploration of properties that hydrogels are not compatible with, such as hydrophobicity and antibacterial characteristics (12). One of the main advantages of oleogels is the possibility of carrying lipophilic bioactive compounds, which is of great utility in both

pharmaceutical and food applications (10). The combined action between structure and health benefits supports the important role that oleogels can have in novel food products, as they can be tailored to meet the ideal properties for a food product, acting as a healthy substitute for solid fats (24,25). Great attention from the scientific and industrial communities towards oleogels has risen since they were first suggested as a possible substitute for fats.

Certain types of gels are developed in a way that combines certain characteristics of both hydrogels and oleogels. These hybrid gels, or bigels, are systems that, in general, contain two immiscible liquid phases, that are independently stabilized by independent gelators (12). Bigels display merits of both the aqueous and the oil phase, including the ability to deliver both hydrophilic and lipophilic active agents, unique thermodynamic behaviour, and improved viscoelasticity (25). Bigels have been mainly applied in cosmetic/pharmaceutical formulations since its hybrid characteristics are proven to maximize moisturizing benefits for the skin and simplify the penetration of active agents to deeper layers of the skin tissue (26–29). These benefits may also be very valuable for food applications in the delivery of bioactive compounds (30). Despite their recognized potential, bigels are still underexplored in terms of microstructure, which has been scrutinized only in recent years (31). On the other hand, emulgels may be considered a type of hybrid between emulsions and gels. Emulgels result from an initial emulsification process followed by a gelation process, through crosslinking of the compounds that are present in the mixture. The amphiphilic behaviour of the emulgels, which is potentiated by the hydrophilic and lipophilic affinities of its constituents, makes them a good option for the delivery of active agents, in a similar way to bigels (25,32–34). In this way, emulgels have been at the forefront of the topical and transdermal delivery of drugs, due to characteristics such as its easy removability, emollient action, ease of extrusion, and spreadability. However, these are the same characteristics that hinder emulgels from being as appealing for food applications, in addition to its stickiness and its proneness to phase separation (12,13).

2.2 Oleogel preparation for food applications

Semi-solid fat products, such as ice creams, chocolates, butter, margarine, or other spreads, owe many of its structural properties to saturated and *trans* fatty acids. These fatty acids, present in the form of triacylglycerols, form a three-dimensional colloidal fat crystal network. Upon crystallization, the triacylglycerols aggregate to form fat crystals, and these crystals aggregate into flocs. This resulting fat crystal network entrains the liquid components of the fat, preventing its exudation (35). However, the growing proof that the intake of saturated and *trans* unsaturated fats

is associated with cardiovascular disease, type II diabetes, high cholesterol, and ischemic stroke risk has led to legislative reformulation regarding these types of fats, compelling food producers to come up with alternative ingredients (36,37). The issue with replacing them is that it is very difficult to do so without compromising the overall properties of the above-mentioned products.

Hence, the process of oleogelation aims at conferring to a liquid oil the distinctive features of solid fat, without needing a large amount of saturated and trans- fat to achieve it. The goal of oleogelation is, therefore, to create an 'alternative network', envisioning an edible oil structure that mimics the fat crystal network. Several gelation methodologies have been studied, generating oleogels with very interesting properties (38).

Substances that gel edible oils can be roughly divided into two categories, based on their molecular weight: low molecular-mass organic gelators (LMOGs) and polymeric gelators (35). Most of the early work on oleogels has focused on LMOGs, such as waxes, sterol-based gelators, fatty acid derivatives, and monoacylglycerols (39). The polymeric gelation approach is still underexplored, mainly because most of the food-grade polymers at our disposal are hydrophilic, and very few can be used to produce oleogels (38). The types of structurants that are being used have different gelation mechanisms, which result in certain structural properties and macroscopic features. The used oil phase also plays a relevant role since the fatty acid profile can influence the oleogels' final rheological, textural, and visual properties (2). Based on this, the selection of components and the gelation strategy for the production of the oleogel is done envisioning the properties desired for the said product.

2.2.1 Direct Dispersion

Direct dispersion methodologies consist of a direct dispersion of the oleogelator into the liquid oil at temperatures above the melting point. This is followed by a cooling period, during which the gelator network is formed, entrapping the oil in a solid structure, thus forming the oleogel. Using a direct approach, the gelation mechanism itself can origin two different types of networks, depending on the type of structurant used: crystallite conformations or self-assembled networks (2).

Crystallite conformations

Different types of structurants can originate crystallite conformations. Lipid-based gelators, *e.g.*, waxes, monoacylglycerols (MAGs), fatty acids, and fatty alcohols are commonly used via a direct dispersion strategy and form a crystalline network. This is a very commonly used process for oleogel synthesis since it is very similar to the traditional process of oil structuring using solid fats. Steps of

nucleation, crystal growth, aggregation, and network formation are involved in both processes (10). However, conventional lipid structuring is based on creating a triacylglycerol hardstock that, in order to achieve efficient oil structuring, is required to be added in a fraction of about 20 % of the oleogel (40). The use of structurants such as waxes, wax esters, and MAGs is a very interesting and cost-effective alternative, due to the lower critical concentration needed to induce oleogelation. Typically, these compounds only need a minor purification or concentration step to convert them into functional lipid structurants (40). Moreover, the physical properties that characterize waxes range from low- to high- melting temperatures, making them a viable option for many oleogels with very different thermal and rheological properties. The conformational arrangement and crystals type also depend on the chemical composition of the gelator; for that fact, waxes, MAGs, and other fatty acid derivatives can form oleogels with different crystal morphology, even though the preparation is similar (41,42).

Self-assembled networks

Self-assembled networks can be originated by essentially four kinds of gelators: LMOGs, polymers, colloidal silicon dioxide particles, and lecithin (10,43). Regarding LMOGs, oryzanol/phytosterol-based oleogels are among the most interesting and widely studied ones, not only due to its capability of producing highly stable oleogels but also due to its health-related benefits. The combination of these two results in a tubular-shaped arrangement that can form oleogels with enhanced mechanical properties (44). These are coined self-assembled fibrillar networks (SAFiNs), as the gelation mechanism consists of the formation of a set of fibrils that present unidirectional growth and entangle with each other, forming a fibrillar network. The final length of the fibres is highly dependent on the environmental conditions, such as cooling rate and storage temperature so that these parameters could be optimized depending on the intended characteristics of the oleogel (24). Because these systems form thin fibrils, they become translucent even with high concentrations of gelator, which is an appealing characteristic for certain applications (45). Not only are these compounds approved for use in food applications, but they have been proven to actively have an impact in lowering blood LDL-cholesterol and reducing the risk of coronary heart disease, according to the EFSA (46). Other LMOGs such as 12-hydroxystearic acid and ricinoleic acid are examples of oleogelators that can be incorporated either as the only gelator or in mixed systems (43,47,48).

As of now, the only known polymer that can act as an oleogelator through the direct method is ethylcellulose (EC) (24). After complete solubilization of the EC in the liquid oil at an above glass

transition temperature (~140 ºC), the polymer softens, and there is partial solubilization in the oil. Follows the cooling period, during which the polymer returns to its rigid form, inducing the formation of hydrogen bonds that result in a three-dimensional polymer network. This network retains the liquid oil and, depending on the viscosity grade, confer higher or lower viscoelastic properties to the oleogel. If these requirements are not met, the risk of incomplete gelation is high (38).

Colloidal silicon dioxide (CSD) particles are among the inorganic particles that have relevant applications in food, cosmetic and pharmaceutical fields (49,50). They are approved for use as a food additive and were reported to function as a structurant for vegetable oils in oleogel and bigel systems. When added to the liquid oil in at least 10 %, the CSD particles result in fractal aggregates that form a network stabilized by hydrogen bonds and electrostatic interactions (51).

Another possibility for the development of self-assembled networks is the use of lecithin. This amphiphilic molecule, when surrounded by a non-polar organic liquid, like an oil, forms reverse spherical micelles. Lecithin-based oleogels require the addition of a polar solvent (like water, for example; this promotes the uniaxial growth of the micelles and turns them into elongated tubular structures that subsequentially entangle to form a three-dimensional network. These cross-linked tubules entrap the oil phase and produce a gel, very similar to the polymer oleogels (52). Moreover, lecithin has been proven to interact with other gelators, such as waxes, phytosterols and ethylcellulose, as co-gelator in direct dispersion methods. In some situations, the synergistic relationships between the gelators in these multi-component oleogels exhibited improved rheological properties when compared to the single-component oleogels (53–55).

2.2.2 Indirect Dispersion

Indirect approaches to oil structuring can be interesting and are getting recognition in recent years. The great advantage of indirect methods is the fact that it widens considerably the types of oleogelators that can be used, with recent publications introducing proteins, polysaccharides, and polymers other than EC as oil structurants. The indirect methods include biphasic emulsion-based strategies and solvent exchange methodologies (56).

Emulsion-template methodologies

The emulsion-template approach is a promising method for using hydrophilic gelators that cannot be directly dispersed in oil to achieve the network structure necessary for oleogels. This methodology involves the preparation of an emulsion stabilized by a hydrophilic gelator, followed by the removal of the hydrophilic solvent. The result is a dried structure, where the hydrophilic gelator

network acts as a building block for the oil fraction, forming an oleogel (57). This methodology broadens the range of food-grade polymers that can be used for oleogelation since most of them are hydrophilic. However, some of them exhibit amphiphilic properties (*e.g.,* proteins) that can be useful in the interaction with the oil phase.

For example, chitin is one of the polymers most present in nature and is highly biocompatible and biodegradable; however, it is reported to be inefficient as a sole gelator component, and its direct dispersion results in poorly stable gels. Adversely, it has been reported that, when used in combination with a surfactant, a stable oleogel can be produced and can acquire very interesting properties. Furthermore, the type of surfactant used heavily influences the final characteristics of the oleogel; as such, chitin is highly versatile in terms of desired properties and applications (58).

Cellulose derivatives, besides EC, can also be used in oil structuring applications by the emulsion-based approach. Well-documented, food-grade options include hydroxypropyl-methylcellulose (HPMC) and carboxymethylcellulose (CMC). HPMC is usually used as a foaming agent that, once dried, has very interesting oil sorption characteristics and consequently forms solid-like oleogels once sheared (59). CMC, in combination with regenerated cellulose (RC), was used as a stabilizer for oil-in-water emulsions that, after water removal through freeze-drying, created a structured oleogel system (60).

Another widely explored option is the use of proteins as oleogelators. Proteins are more recognized due to their potential as structurants in hydrogels rather than in oleogels, due to their predominantly hydrophilic characteristics. One of the possibilities for the synthesis of a protein oleogel is starting by preparing an emulsion using proteins as the emulsifying agent and removing the water phase. The properties of the interface can be strengthened with the addition of a polysaccharide, with gelatin and xanthan gum being highly popular. This is usually a required step, to prevent the coalescence of the oil droplets and form a stable oleogel (61).

Solvent exchange methodologies

The solvent exchange method can also be used to create protein and polysaccharide building blocks for oleogelation. This procedure is based on the formation of a protein network within an aqueous medium (hydrogel). The polarity of the solvent is decreased by the introduction of an intermediate organic solvent (i.e., acetone or tetrahydrofuran). Finally, the organic solvent is substituted by an oil, through a sequence of dipping or immersion steps (38,61). Similarly, the solvent exchange method was applied to polysaccharide-based hydrogels using κ-carrageenan hydrogels. This procedure featured an intermediate step of immersion in alcohol solutions, followed by

supercritical CO_2 drying to avoid polymeric collapse and obtain stable aerogels. These aerogels were later dipped in sunflower oil to induce the uptake of oil (62).

2.3 Oleogel-based emulsion systems using food-grade components

During the last 10 years, food-grade oleogels have been the subject of high research interest worldwide, and the variety of structuring agents that will harbour different gel-formation mechanisms has led to numerous publications (2,35,63). As an effort to expand the scope of oleogel-based food products, the development of oleogel-based systems that comprise a water fraction has started to gain attention. Moreover, the development of hybrid structures can be of great help in modulating the extent of lipolysis and increasing the lipid digestibility, while maintaining the solid-like structure that distinguishes the oleogels. Understanding the interplay between emulsification and oleogelation is fundamental (9). In this section, the focus will be laid on oleogel-derived emulsified systems, where the oil phase is structured, whereas the aqueous phase is not.

2.3.1 Single emulsions

Emulsions are colloidal dispersions comprising two immiscible liquids, in which one of them is dispersed in a continuous liquid phase of different composition. The continuous phase is referred to as the 'external phase', while the dispersed phase can be called the 'internal phase'. Considering that one of the liquids is aqueous (polar), and the other is nonpolar, typically being an oil, two types of emulsions can be distinguished:

- oil-in-water (O/W) - oil droplets are dispersed in the continuous water phase.
- water-in-oil (W/O) - water droplets are dispersed in the continuous oil phase.

Essentially, most emulsions comprise the two liquid phases and an emulsifying agent, which stabilizes the emulsion, and may form a protective film on the interface between the two liquids, keeping the droplets from coalescing and preventing the emulsion from breaking. Typically, the emulsifying agents are surfactants, which are a class of compounds that act as surface-active agents. Its amphiphilic behaviour provides them with the capability of associating in micelles (64). When added to an emulsion, the emulsifier molecules surround the dispersed droplets. In the case of an O/W emulsion, the emulsifier molecules arrange themselves with the nonpolar tails extending into the oil and the polar heads facing the water, forming micelles; in the case of a W/O emulsion, the

emulsifier's orientation is reversed, forming reverse micelles (65). These two situations are illustrated in Figure 1.

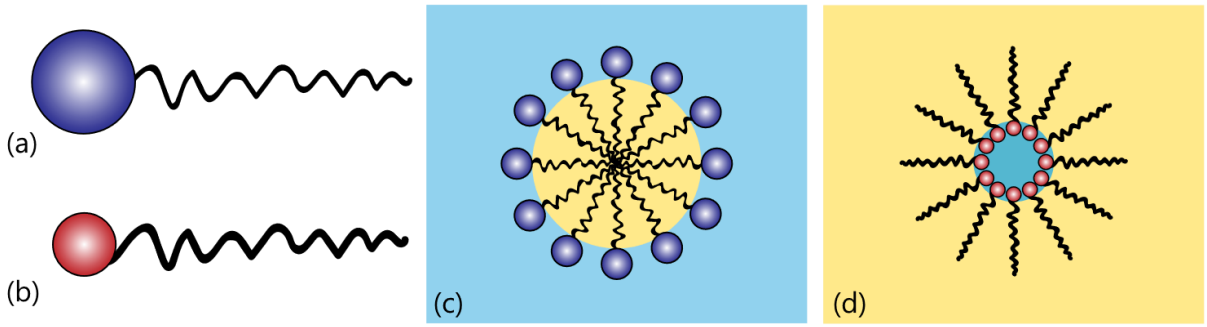


Figure 1 - Illustration of the surfactant molecules' arrangement in single emulsions.
(a) Hydrophilic emulsifier molecule (high HLB); (b) Lipophilic emulsifier molecule (low HLB);
(c) Micelle in an O/W emulsion; (d) Reverse micelle in a W/O emulsion.

There are several empirical approaches to predict the surfactant positioning at the interface, with the hydrophilic-lipophilic balance (HLB) being the most widely used parameter. The HLB refers to the hydrophilic/lipophilic ratio of a surfactant molecule and is given by a dimensionless scale, where W/O emulsifiers exhibit an HLB value below 9, being predominantly lipophilic, and O/W emulsifiers exhibit an HLB value above 11, being predominantly hydrophilic (65). It should be pointed out that not only surfactants can be used as emulsion stabilizers. Some emulsions are stabilized through adsorption of fine solid particles at the oil-water interface, called Pickering emulsions (66). Because the particles are closely packed, and due to the particle-particle interactions that occur, the stabilizing film between droplets can be quite rigid when compared to the stabilization using a surfactant. Therefore, this type of stabilization can provide enhancement of the mechanical properties of the droplets, providing a strong barrier to aggregation and coalescence. The most stable emulsions are formed when the contact angle is close to 90º so that the particles will collect at the interface (67).

Oleogel-in-water emulsions

In recent years, lipid digestion has persisted as a forefront research focus, underlining the changes that the lipid's physical state and interfacial structure induce on the digestion outcome. Regarding food applications, the suitability of oleogels to become a versatile tool depends not only on the gelation mechanism itself but also on the interaction with the surrounding matrix, that in most food products may be constituted by a water/aqueous phase.

Guo *et al.* (68) have focused on the variations that the addition of rice bran wax (RBX) to an O/W emulsion matrix incite on both the emulsion stability and the *in vitro* digestion. With increasing concentration of RBX, the melting point of the emulsions increased, and overall oil rigidity improved. The RBX crystals were formed within the oil droplets, which suggests that the length of the crystals was restricted by the interfacial film of the oil droplets. The presence of the crystals, however, impacted emulsion stability, where higher concentrations of gelator led to partial coalescence of oil droplets. As for the digestion, the presence of RBX effectively retarded lipolysis, although there was not a linear correlation with the gelator concentration. For high concentrations of RBX, the crystals pierced through the interface, exposing the oil, and becoming ineffective in the retardation of lipid digestion. In short, the addition of an oleogelator to an O/W emulsion must be accomplished in an optimum concentration, in a way that the delayed lipolysis benefits are balanced with the stability needs (68).

Comparably, Munk *et al.* (69) targeted the behaviour of EC in oil while being in contact with an aqueous phase. While approaching this situation, the authors followed a cold-temperature methodology and a hot-temperature methodology (above the melting point of EC). In both cases, the aqueous and oil phases were prepared independently, with the oil phase being prepared above the melting point in both cases. For the cold-temperature methodology, the oil phase was cooled down to room temperature and mixed with the aqueous phase, while for the alternate methodology, the mixing was performed above the melting point of EC. The hot-temperature methodology did not result in oleogel droplets, but rather in liquid oil droplets: an accumulation of EC in the surface of the oleogel was observed, forming a shell, while the interior of the shell was constituted by liquid oil. Adversely, the low-temperature methodology prompted the EC to act as an efficient oleogelator, opening up its potential use as a structurant in many O/W emulsion-based food products.

Water-in-oleogel emulsions

The current studies on the structuring of the oil droplets in an O/W emulsion foresee a variation of the product's characteristics, mainly at a microscopical level. In contrast, the structuring of the bulk oil phase in W/O emulsions, apart from microscopical changes, results in structural differences that are more evident to the consumer. This may be the reason why it is a significantly more scrutinized field rather than oleogel-in-water emulsions.

Lupi *et al.* (70) have prepared structured W/O emulsions by blending olive oil, Myverol (mainly composed of MAGs), and cocoa butter. By adjusting the oil/cocoa butter ratio, different rheological properties were obtained, without changing the oil phase/aqueous phase ratio or the total emulsifier

concentration. The structured oil phase entrapped the water droplets, similarly to the 'pure' oleogels, yielding a stable water-in-oil-emulsion. Moreover, from a macroscopic point of view, the emulsion behaved like a solid under small deformations. The obtained product was compared to commercial margarine, and its potential as a solid fat substitute was proven (70). Although this is a valid approach to successfully reduce the quantity of saturated fat in solid-like products through PUFA substitution, it is not ideal since it still features a saturated fat crystalline network. Natural waxes have a lower index of saturated fat, which is why they can present a better alternative for the production of margarine-like spreads. Toro-Vazquez *et al.* (71) developed a water-in-oleogel emulsion based on candelilla wax and MAGs, with the premise in mind that, more than just an oleogelator, the candelilla wax may help to stabilize the water droplets. In fact, it was confirmed that it also behaved as an emulsifier, adsorbing at the oil-water interface and being beneficial to the stabilization of the water droplets.

Öɨütcü *et al.* (72) have suggested a mechanism of structuring olive oil W/O emulsions using beeswax, concerning previous work using waxes in 'pure' oleogels. Despite the water droplets' size having increased after 90 days, the produced emulsions were stable, with the beeswax acting as a stabilizer along with the emulsifier Tween 80 as a droplet stabilizer. The overall conclusions of the study qualify them for an eligible alternative to margarine and spread-like products. RBX is also a natural wax, which is a value-added by-product of the rice bran oil refining process; Pandolsook *et al.* (73) hypothesized that it could be used as an efficient oleogelator for food applications, specifically as a margarine substitute in cookies. Both oleogels and W/O emulsions were prepared from RBX oleogels, and the cookies were produced by successfully substituting the margarine for the oleogelled emulsions at a rate of 50 % and 100 % of margarine content.

Fundamentally, for the oleogelation of a water-in-oil emulsion, the gelator must be combined with a suitable emulsifier, depending on the intended properties of the formulation. The water content of the formulation also influences the overall properties of the gelled emulsion, which is why Wijarnprecha *et al.* have tackled the topic of dispersed aqueous droplets acting as active or inactive fillers (74,75). These two situations were compared by using the same formulations of canola oil and RBX, altering only the type of emulsifier in each formulation. Another variable that was studied was the water content, to comprehend the effects on the water percentage in the formulations. When using monostearin, which is a MAG, the droplets behaved as active fillers, offering sites for interfacial nucleation and growth of RBX crystals, resulting in attractive interactions between the crystal network and water droplets. This induced an overall increase of the emulsions' firmness, with water droplets playing an important role; water contents of as low as 50 % were produced and remained stable for

at least 2 months. At high water percentages, however, the shear-sensibility and crystal structure recovery were hampered (74). Adversely, the use of PGPR as an emulsifier prompted the water droplets to behave as inactive fillers, where water droplets decreased the firmness of the emulsions. There was no evidence of interaction between the PGPR and the crystal network; as such, the water did not improve the emulsion rigidity. On the contrary, the increasing water volume fraction has reflected on a decrease in emulsion rigidity, which is an indication that the wax crystal network became weaker. However, even with droplets acting as inactive fillers, kinetically stable oleogelled W/O emulsions can be generated, which is a leap forward in the feasibility of swapping oil/fat with a certain amount of water, while reaching similar rheological properties.

2.3.2 Double emulsions

A double emulsion is an emulsion of a single emulsion. Double emulsions are a very suitable tool for the delivery of bioactive compounds, being actively used in the pharmaceutical industry as drug delivery vehicles. This type of emulsions allows for a slow release of the drug, and the time of release can be optimized according to the intended aim, by modulating the method of production (65). These properties are not always attained by single emulsions, since these can be very susceptible to chemical degradation, which is where the multiple emulsification method can offer superior characteristics. There are two categories of double emulsions:

- oil-in-water-in-oil (O/W/O) - a continuous oil phase contains water droplets with smaller oil droplets dispersed inside them.
- water-in-oil-in-water (W/O/W) - a continuous water phase contains oil droplets with smaller water droplets dispersed inside them.

Currently, the gelation of oil in double emulsion systems focuses on W/O/W emulsions rather than O/W/O, which is why these are the only double emulsions discussed hereafter.

Water-in-oleogel-in-water emulsions

This type of emulsions has great potential in food-related areas because its properties in drug delivery can be employed in the delivery of probiotics or protection of aromas in foods (76,77) and also because the inclusion of aqueous droplets in the oil globules of fat products can be used to reduce the fat content in these products. The gelation of the oil phase in W/O/W emulsions can provide structure to the oil phase and help to prevent destabilization phenomena, such as the coalescence of water droplets within the oil phase. Additionally, double emulsions are very prone to molecular transport of water or encapsulated compounds from one phase to another, which is very

noticeable in low-viscosity oil phases (78). Nelis *et al.* (78) monitored the water transport and permeation of manganese ions throughout the double emulsion, having observed that the presence of fat crystals in the oil phase effectively prevents the leakage of the internal water phase, therefore controlling and preventing the release of compounds from the internal water phase. This may be explained by the tortuosity of the path for molecular transport originated by the gel network, which reduces the permeability of the oil phase. The solid network also provided mechanical strength to the gel, opposing the osmotic forces.

One of the main constraints for the application of double emulsions for food purposes is the lack of food-grade emulsifiers, which are capable of stabilizing double emulsions. As such, Goibier *et al.* (79) proposed a mechanism that allows for double emulsion stabilization without using any surfactant. This feat is accomplished by the gelation of the oil phase through the addition of fat crystals, during the preparation of a single W/O emulsion. After crystallization of the bulk fat through a temperature decrease, the single emulsion was added to an external water phase. The solid fat ingredient, constituted mainly by triglycerides, stabilized the aqueous droplets and was able to impede coalescence. The process was rather versatile, and a plethora of different solid fats ingredients was applied, such as milk, cocoa, palm, and coconut.

The stabilization of double emulsions through fat crystallization has also been reported by Liu *et al.* (80). They focused on the ability of the fat crystals to provide resistance to the osmotic stress and suggested its potential utility in a temperature-triggered release of active compounds from the internal phases. Essentially, it comprises a double emulsion prepared using hydrogenated soybean oil as the oil phase, which is a semi-solid oil. Above the melting point of the fat crystals, the system was prone to osmotic pressure effects, such as an increase of the internal water droplet size. As such, the creation of an emulsion that responds to a temperature change is feasible, and the integration of active compounds on the aqueous phase can be performed for a purposeful release, responding to a temperature stimulus.

These oleogel-based emulsion systems feature very singular properties, and it is their versatility what makes them good candidates for very different applications. One system can be ideal for one application, whereas another formulation might be required for a different application, depending on the intended objective. Figure 2 displays a schematic representation of the structural organization of these types of formulation.

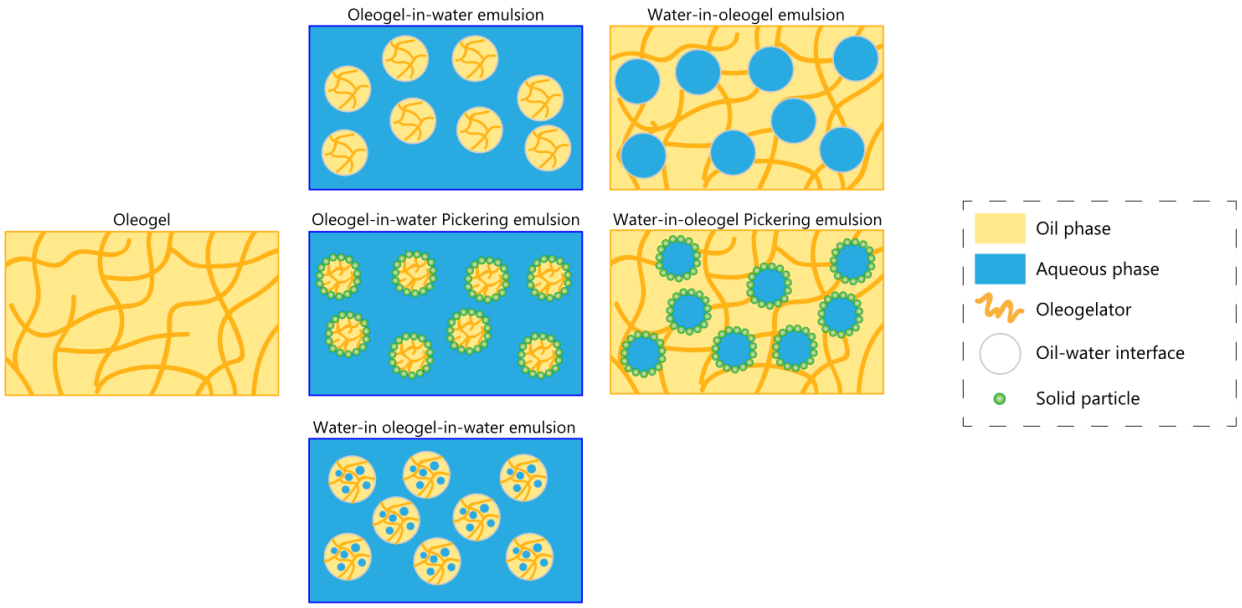
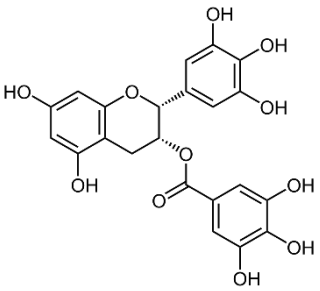


Figure 2 - Schematic representation of different types of oleogel-based emulsion systems.

2.4 Bioactive compounds

It is well conceded that a healthy diet plays a crucial role in the prevention and treatment of several diseases. According to the American Heart Association, fats can be divided into three groups: to love (*e.g.,* vegetable oils, nuts, and fish), to limit (*e.g.,* butter, cheese, and lard), and to lose (*e.g.,* artificial cakes and hydrogenated or tropical oils) (81). The first group refers to products that are rich in unsaturated fatty acids, both monounsaturated and polyunsaturated. These are present on the list of permitted health claims for food products by the European Commission, as active agents in maintaining blood cholesterol levels (82). Authors have coined the term Nutraceutics for the discipline that encompasses the fields of nutrition and pharmaceutics, which focuses on the beneficial effects of a balanced diet on human health (83). The fortification of food products with added-value bioactive compounds has numerous benefits for the consumers, and the field of Nutraceutics has been gaining relevance in modern society (83,84). Functional compounds can be hydrophilic or lipophilic; therefore, the strategies for their incorporation in food products may be different. Emulsion-based systems are increasingly being considered a good mechanism for controlling lipid uptake, with the possibility of being tailored for an envisioned specific digestion behaviour. This makes them a very good fit for the incorporation of nutraceuticals: the positioning of the compounds in micelles may result in higher bioaccessibility, which alongside the aforesaid digestibility kinetics, may lead to an improved release of the entrapped compounds (85).

Two major examples of nutraceuticals are epigallocatechin gallate (EGCG), one of the most abundant green tea polyphenols and β-carotene (BC), an organic pigment which is present in many fruits and vegetables. The chemical structure of both compounds is represented in Figure 3.

(a) 

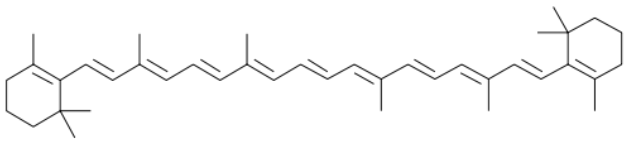
(b) 

Figure 3 - Chemical structure of (a) epigallocatechin gallate and (b) β-carotene.

Green tea is widely known by its antioxidant properties due to the presence of polyphenolic compounds, mainly catechins. EGCG is, so far, the best-studied catechin derivative and is present in high quantities in green tea (86). Other than being a strong antioxidant, EGCG is known as a strong anticancer, antidiabetic, antihyperglycemic, and anti-inflammatory agent, playing an effective role in the prevention of several chronic diseases (87–90). EGCG has also proven to be effective in modulating immune cell functions, improving the condition of many autoimmune diseases, and offering promising therapeutic potential in many applications. However, there are some hurdles to its extensive use as a nutraceutical agent. EGCG is very unstable in a neutral and alkaline environment, having low permeability under the conditions of the gastrointestinal tract. Therefore, despite its high solubility in water, the delivery of free EGCG is inefficient, and its oral bioavailability is very low. As such, efforts have been made towards the microencapsulation of EGCG for food applications, with proven evidence of increased stability and slow-release (91,92).

Carotenoids are a class of organic pigments that can be found in many fruits and vegetables, but also fungi, algae, and photosynthetic bacteria. Overall, carotenoids exhibit antioxidant properties, but individual carotenoids can display other characteristics. BC is responsible for conferring a strong red-orange colouration to plant tissues and is also a precursor of Vitamin A. Deficiency of this is a major health problem, especially in developing countries, and supplementation with Vitamin A is not an easy strategy to implement. As such, fortification of food with BC could be a viable alternative. Moreover, the consumption of a moderate dose of BC appears to have effects on eye health and cognitive performance, which may be associated with its antioxidant properties. Other than the dietary impact of BC itself, its ability to be converted to Vitamin A expands the ground of added health benefits, such as the improvement of immune function (93).

2.5 Oleogel-based systems as a vehicle for bioactive compounds

As aforementioned, the structuring of liquid oils rich in PUFA can bring significant benefits for human health since they act as fat substitutes and have a much richer constitution than typical solid fats. Furthermore, the structure of oleogels constitutes a good matrix for the delivery of bioactivity, with it being both a way of protecting the integrity of bioactive compounds against oxidation or loss of functionality and a way of controlling its release. However, this area is still scarcely explored, and most of its studies rely on the integration of liposoluble compounds in the oleogel structure, rather than hydrosoluble compounds. This seems to be the most straightforward approach due to the naturally lipophilic nature of oleogels (56). Oleogels also often serve as a starting point for the development of more complex structures, such as oleogel-based emulsions or oleogel-based Pickering emulsions, either to obtain additional benefits or to increase the formulation's versatility. Table 1 features an extensive literature review of the currently documented food-grade oleogel systems with proven effectiveness in the delivery of bioactive compounds.

Curcuminoids are a class of compounds that have been successfully integrated into oleogel structures in recent years. Its water insolubility and rapid metabolism greatly affect its bioaccessibility and bioavailability, which hinders the reaping of its health-promoting benefits. Despite previous efforts to encapsulate curcuminoids, such as regular O/W emulsions, microemulsions, and solid lipid particles, the problem of its bioavailability was not explored thoroughly. The first oleogel system established for the delivery of curcuminoids dates from 2012 (94). The gelation process was proven not to affect the bioaccessibility, and oleogels were formed with a successful loading of 2.6 % of curcuminoids with a bioaccessibility of 80 % in a fasted state. These oleogels were used for the fabrication of rapid-digestion emulsions, further proving that the delivery of poorly water-soluble nutraceuticals can be achieved through oleogel-based systems (95). Since then, other types of formulations have been developed for the delivery of curcuminoids, with authors making use of the diversity of edible gelators suitable for oil structuring. Li *et al.* (96) have developed a novel curcumin-loaded oleogel formulation, making use of the capability of β-sitosterol and lecithin to form self-assembled fibres and studied its oxidative stability and release behaviour. The structure constructed by the gelator fibres protected the curcumin from being oxidized; on the other hand, a reciprocal effect was observed, where curcumin-loaded oleogels featured higher shelf-life stability when compared to pure β-sitosterol + lecithin oleogels (96).

Table 1 - Literature review on oleogel-based systems loaded with bioactive compounds for food applications.

Bioactive compound	Oil	Gelator	Gelator concentration (%)	Type of structure	Main conclusions	Reference
Curcumin	MCT Oil	MAGs	20	Oleogel	Improved oral bioavailability of curcumin in both structures. The emulsions had faster lipolysis than the oleogels.	Yu *et al.* (94)
	MCT Oil	MAGs	20	Oleogel-based emulsion		Yu *et al.* (95)
	Corn Oil	β-sitosterol + lecithin	12	Oleogel	The curcumin did not interfere with the gel network assembling; its bioaccessibility at the intestinal level was enhanced in a fasted state.	Li *et al.* (96)
	Fish Oil	Fully Hydrogenated Rapeseed Oil	3 - 7	Oleogel	The gel structure and curcumin content helped to retard the oil oxidation.	Vellido-Pérez *et al.* (97)
	Sunflower Oil	Saturated MAGs, Rice bran wax, γ-oryzanol + β-sitosterol	5	Oleogel	The nature of the oleogelator affected the bioaccessibility of the curcumin during *in vitro* digestion, which was higher in the β-sitosterol + γ-oryzanol oleogel. However, the extent of lipolysis was lower on this oleogel.	Calligaris *et al.* (98)
β-carotene	Canola Oil	Ethylcellulose	10	Oleogel	Increased stability of β-carotene in the oleogel and protection against oxidation.	O'Sullivan *et al.* (99)
	Coconut Oil, Corn Oil, MCT Oil	MAGs	18.2	Oleogel, Oleogel-based emulsion	Cellular uptake and bioavailability of β-carotene were higher in the emulsion than in the control (liquid oil).	Fan *et al.* (100)
	High Oleic Sunflower Oil	Beeswax	2, 4, 6, 8	Oleogel	β-carotene improved strength and oil-binding capacity of the oleogels; higher beeswax concentration improved oxidative stability of the oleogels.	Martins *et al.* (101)
	Corn Oil	MAGs	10, 15, 20, 25	Oleogel	The oleogel structure improved the heat/light stability and solubility of β-carotene.	Cui *et al.* (102)
D-limonene	MCT Oil	Stearic Acid	5, 10, 15	Oleogel-based emulsion	Increased storage stability regarding conventional emulsions.	Zahi *et al.* (103)
Nisin + D-limonene	Peanut Oil	Stearic Acid	70	Oleogel-based emulsion	The combined use of D-limonene and nisin improved the antimicrobial properties and supported its use as a food preservative.	Bei *et al.* (104)

Volatile Aromas, Vitamins	Hazelnut Oil	Beeswax, Sunflower wax	5	Oleogel	The addition of flavourings and vitamins did not undermine the gelation process and its concentration was intact after 3 months storage.	Yilmaz *et al.* (105)
Volatile Aromas	Sunflower Oil	β-sitosterol + MAGs	10	Oleogel	The combination of 2 gelators resulted in stable oleogels, with controlled release of volatiles.	Yang *et al.* (106)
Volatile Aromas	Sunflower Oil	γ-oryzanol + β-sitosterol	10	Oleogel-based emulsion	Successful delay of volatile release by entrapment in an oleogel network.	Chen *et al.* (107)
Betulin, Curcumin, Quercetin	Canola Oil, Coconut Oil	MAGs	10	Oleogel-based emulsion	The bioaccessibility and permeability of the bioactive compound depend on the type of molecule and not only on the oleogel system.	Ojeda-Serna *et al.* (108)
Capsaicin	MCT Oil	Sucrose stearate S-370	20	Oleogel-based emulsion	Enhancement of the bioavailability of capsaicin and *in vivo* proof of the reduced irritability of the capsaicin.	Lu *et al.* (109)
Ferulic Acid	Olive Oil	Policosanol	3	Oleogel	The addition of gelator to the FA-loaded oil helped to control the release in stomach conditions.	Lupi *et al.* (110)
Hesperidin	Soybean Oil	MAGs	3	Oleogel-Pickering emulsion	Both lipolysis rate and bioaccessibility of hesperidin were improved in the Pickering emulsion regarding the oleogel.	Wei *et al.* (111)
Lutein Ester	Sunflower Oil	MAGs	4, 6, 8, 10, 12	Oleogel	The oleogel structure successfully protected lutein ester from UV radiation.	Jiang *et al.* (112)
Quercetin	Canola Oil, Corn Oil, Soybean Oil	MAGs	8	Oleogel	Oleogels prepared with canola oil featured better bioaccessibility of the loaded quercetin.	Rocha-Amador *et al.* (113)
Phytosterols, Vitamin D_3, Vitamin B_{12}	Soybean Oil	Trimyristin	15	Oleogel-based double emulsion	Higher extent of release of the bioactive compound and increased lipid digestibility, when compared to non-gelled double-emulsions.	Andrade *et al.* (114)
Tea Polyphenols	Peanut Oil	Stearic Acid	5 - 30	Oleogel	The tea polyphenols helped to extend the storage stability of the oleogel.	Shi *et al.* (115)

BC was first tested out as proof of principle for the capability of ethylcellulose oleogels to deliver bioactive compounds effectively (99). In this first approach, properties such as mechanical strength, *in-vitro* digestibility, BC accessibility, and stability in the oleogel matrix were assessed, reinforcing oleogels' value as carriers. Fan *et al.* (100) have developed both an oleogel and an oleogel-based emulsion for the delivery of BC. Although oleogel-based emulsions featured some benefits when compared to oleogels concerning the loaded amount of bioactives, bioavailability, and biological activity of said compounds, little information was established for the case of BC. This work encompassed the preparation of oleogels using different liquid oils and the assessment of the BC accessibility in these oleogels; corn oil oleogels showed the most interesting results, which can be related to its length and unsaturation degree, serving as a starting point for the oleogel-based emulsion.

One important property that should be modulated in the delivery of nutraceuticals via oral administration is the kinetics of the release, which is why oleogel structures are useful since they are capable of controlled release. Ferulic acid (FA) is known for its widespread use in anti-ageing creams, featuring strong antioxidant and anti-inflammatory properties. Its application in edible oleogels met its start in 2013 when an oleogel formulation was defined using olive oil as a vehicle. FA was submitted to a drastic acidic ambient to mimic stomach conditions, and the oleogel structure was capable of protecting the integrity of the FA to fulfil its nutraceutical function, with great rheological properties. *In vitro* release tests proved that the control samples, prepared without policosanol, were almost completely released after 2 hours in stomach conditions, while by adding as little as 1 % of policosanol, the delivery was controlled and delayed (110).

Tea polyphenols are known for their antioxidant properties and free-radical scavenging capability. However, due to its low solubility in oils, its use in food products with high lipid content is hindered. Shi *et al.* (115) came up with an approach to include tea polyphenols in emulsion-based oleogels, to benefit from its properties for preservation of the oleogels during storage. To allow the dispersibility of the tea polyphenols in the oil matrix, initially, a stearic acid-surfactant-tea polyphenol complex was prepared through emulsification and lyophilization, that was later dissolved in liquid oil in different concentrations. The tea polyphenols' antioxidant activity was comparable to chemically synthesized food additives, and this approach seemed to be effective in delaying the onset of oxidative rancidity, serving up as a good strategy of combining the potential of water-soluble ingredients with lipid-rich food products.

3 Materials and Methods

3.1 Materials

The corn oil used was from the brand Fula (Sovena, Algés, Portugal). Polyglycerol polyricinoleate (PGPR), which was kindly given by Palsgaard (Juelsminde, Denmark), was used as the emulsifier. γ-oryzanol was purchased from Oryza Co. (Ichinomiya, Japan), and the mixture of pine-tree derived phytosterols Vegapure® 867 G was purchased from BASF (Lampertheim, Germany). This phytosterol mixture has a guaranteed minimum of 99 % sterols and is composed by β-sitosterol (60 - 80 %), β-sitostanol (0 - 15 %), campesterol (0 - 15 %), campestanol (0 - 5 %), stigmasterol (0 - 5 %), brassicasterol (0 - 3 %) and other sterols/stanols (0 - 3 %). β-carotene (BC) with 95 % purity from Sigma-Aldrich (St. Louis, USA) and SunPhenon Epigallocatechin Gallate (EGCG) from Taiyo Green Power Co. (Jiangsu, China), which comprises > 94 % EGCG and < 0.1 % caffeine.

3.2 Preparation of oleogel-based water-in-oil emulsions

Oleogel-based water-in-oil emulsions were prepared following the method of Sun *et al.* (116) with some modifications. The oil phase was prepared at 85 ºC with constant stirring at 350 rpm and comprised corn oil, PGPR, and a mixture of γ-oryzanol and phytosterols as the solid gelator of the oil phase. Following previous works, a 60:40 (w/w) γ-oryzanol-phytosterol ratio was used (44). At the same time, ultrapure water was heated up to 85 ºC, forming the water phase. The aqueous phase was dispersed in the melted oil phase, under magnetic stirring at 800 rpm for 15 min at 85 ºC using a magnetic stirrer from Heidolph Instruments GmbH (Schwabach, Germany), and further processed using a high shear homogenizer T 18 digital ULTRA-TURRAX® from IKA (Staufen, Germany) at 10000 rpm for 2 min. The resulting emulsions were transferred to 10 mL centrifuge tubes and stored until characterization analyses.

These samples were, at first, prepared with a fixed concentration of 10 % γ-oryzanol-phytosterol mixture, varying the PGPR quantity from 0.5 % to 6 % (w/w), in order to assess the stability of the obtained emulsions. After this screening, the emulsions were prepared using a fixed emulsifier concentration and varying the quantity of the γ-oryzanol-phytosterol mixture from 10 % to 20 % (w/w), while using different oil-water (O:W) ratios: 60:40, 80:20, and 90:10 (w/w).

3.2.1 Preparation of bioactive-loaded oleogel-based water-in-oil emulsions

The oleogel-based water-in-oil emulsions loaded with bioactive compounds were prepared similarly to the method described above. BC in a concentration of 0.01 % (w/w) was added to the oil phase in all oleogels, before the addition of the γ-oryzanol and phytosterols; this concentration was selected to ensure total solubilization of the BC as described for LCT oil (117). EGCG was added to the aqueous phase in a concentration of 5 % (w/w), according to the information of solubility in water provided by the manufacturer. Figure 4 represents a schematic representation of the methodology.

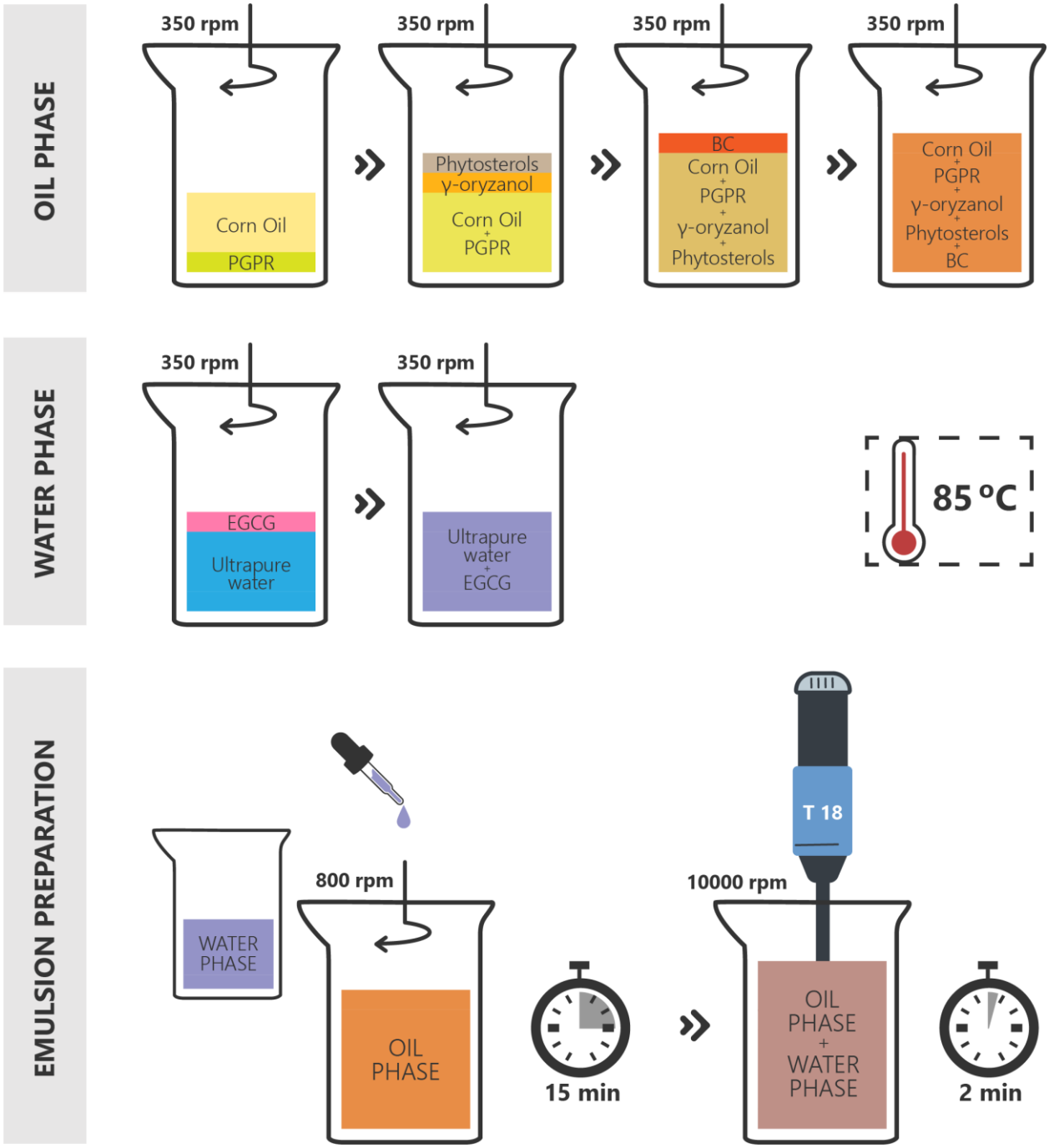


Figure 4 - Schematic representation of the methodology for the preparation of the oleogel-based emulsions.

3.3 Characterization of the emulsions

3.3.1 Visual appearance

After a minimum of 24 hours of storage, the tubes were inverted, and the self-standing ability of each sample was assessed visually. For the samples prepared with varying concentrations of PGPR, the inversion of the tubes was relevant for addressing the existence of phase separation; for this evaluation, two sets of samples were prepared, with one of the sets being stored at room temperature (20 ± 2) °C and one of the sets being stored at low temperature (4 ± 2) °C. This assay was performed after 24 hours and 48 hours of storage. For the samples prepared with varying concentrations of gelator and oil-water ratio, as well as for the bioactive-loaded samples, the storage conditions were fixed at room temperature (20 ± 2) °C. In addition to the inversion of the tubes, a portion of each sample was extracted from the tube and placed onto a Petri dish for visual assessment of the textural appearance and consistency. This was performed after 24 hours and 7 days of storage. This analysis was performed in triplicate.

3.3.2 Microscopy

A drop of each emulsion was collected immediately after the high-shear homogenization and placed on a slide, covered by a glass slip. After cooling to room temperature (20 ± 2) °C and approximately 24 hours after, the samples were analyzed with a Wide-Field Upright Fluorescence Microscope – Nikon – Ni-E equipped with a polarizer and a Nikon DS-Fi2 camera (Nikon, Japan), for bright-field imaging, and a monochrome camera for low-intensity fluorescence measurements (Orca R2, Hamamatsu, Japan). Pictures were taken with a magnification of 120× and 300×.

3.3.3 Rheological analysis

Oscillatory rheometry was performed using a Discovery HR-1 rheometer from TA Instruments (New Castle, USA), using the software TA Instruments Trios 4.1.1.33073 for equipment control and data treatment. Cone-plate geometry of 60 mm with an angle of 2.006º was used. 24 hours after the preparation of the emulsions, the samples were carefully placed onto the plate to avoid significant structural damage. The viscoelastic properties of the emulsions were studied at (22 ± 2) °C, using a stress amplitude logarithmic sweep at a fixed frequency of 1.0 Hz, five points per decade, and an oscillation strain up to 5.0 %.

Four parameters were calculated with this analysis for all the samples: the storage and loss moduli, the loss factor, and the yield stress. The storage and loss moduli were estimated in the

steady-state region of the stress amplitude logarithmic sweep graphs, and the loss factor was calculated with the two moduli values. By its definition, the yield stress is the stress value at which the sample undergoes permanent deformation, and there is a departure from the elastic response. As there is no obvious way of defining this point, there are several ways of calculating the yield stress. In this work, the calculation of the yield stress was performed by using the stress amplitude sweep data through the tangent analysis method. This method defines the yield stress as the intersection between the line with a unit slope on logarithmic coordinates that is tangent to the data at low deformations and the horizontal steady-state stress. The graphical analysis for the calculation of the yield stress is presented in Appendix A.

3.3.4 Small-angle X-ray scattering (SAXS) and X-ray diffraction (XRD)

SAXS analyses were performed at room temperature (20 ± 2 °C), using an Anton Paar SAXSess dmc2 model (Anton Paar, Graz, Austria), at 40 kV and 50 mA. The control of the device and the data acquisition were performed with SAXSquantTM software. TCS sample stages were used (kapton scattering revealed no interference). Data collected with an image plate detector (2D data acquisition) and SaxsQuand2D software was used to normalize the profile masks of samples after profile integration. The data were further analyzed using OriginPro 2018 software.

The XRD analysis was employed to understand the differences between oleogels at molecular length scales. The study of the crystalline polymorphic structure was performed by scattering methodology, using an X-Ray Diffractometer X'Pert PRO MTD system from PanAnalytical. XRD diffractograms were acquired at room temperature, using angular scans from 5º to 50º (2θ), performed with a Cu source, X-ray tube (λ = 1.54056 Å) at 45 kV and 40 mA. The fine calibration offset for 2θ = -0.0372º. PanAnalytical X'Pert HighScore Plus was the software used to gather data and analyze peak diffractions. Determination of the lattice parameter d was performed using Bragg's law, where λ is the wavelength of the used X-ray, θ is the half of the diffraction Bragg angle (2θ), and d is the space between planes.

$$n\lambda = 2d \sin\theta \quad (1)$$

4 Results and Discussion

4.1 Influence of the PGPR fraction in the emulsions' stability

As mentioned in Section 3, the formulation of the oleogel-based emulsions was optimized following an iterative approach. Preliminary testing was performed, starting with an O:W ratio range from 50:50 to 90:10 and with a fixed concentration of 10 % PGPR and 15 % of gelator mixture. These emulsions registered high variability among the same formulation between replicates. For this reason, the approach was revised, and it was decided that the first step to be performed should be the study of the necessary emulsifier concentration. This study encompasses the determination of the minimum PGPR concentration for stabilization of the emulsion, preventing the samples from suffering phase separation and ensuring their stabilization. Within the range of O:W ratios and gelator concentrations to be considered, emulsions with a 60:40 (O:W) ratio and a 10 % concentration of gelator were expected to be the most challenging samples to stabilize. Key factors would come in play such as the high water content that implies a high volume of water to be stabilized in droplets, and therefore it requires a high concentration of emulsifier; and a low gelator concentration that results in a small role of stabilization of the droplets by the gelator network. For this fact, this assessment was performed only for emulsions with a fixed O:W ratio of 60:40 and a gelator concentration of 10 %. The influence of the storage temperature was also appraised. The observations are presented in Figure 5. As can be observed, in both room temperature and cold temperature conditions, the samples with the same concentration of PGPR exhibited similar properties. The samples which were prepared with 0.5 % PGPR displayed phase separation. This phase separation was more evident under room temperature conditions than in refrigerated conditions, where a very distinct water phase is placed above the oil phase in the inverted tubes. This can also be observed for cold temperature storage conditions, even though to a lesser extent, where a smaller volume of the water phase is separated from the emulsion. The separation of the two emulsion phases separation can take place either by creaming or sedimentation, depending on the relative density of the two phases. For most food-grade emulsions, prepared with edible oils, the oil phase has a lower density than the water phase; thus, it is typical for the oil phase to move upwards. In this situation, as the oil phase is the continuous phase, the water droplets, which constitute the dispersed phase, move downwards, being a sedimentation phenomenon (118).

The presence of the emulsifier in an adequate concentration can successfully prevent sedimentation (among other destabilization phenomena) since its density is typically more than either oil or water phase. By surrounding the water droplet, the overall density of the dispersed phase

increases, and there is a reduction of the density difference between the two phases (118). Regarding the samples with 1 % PGPR and above, all the samples seemed homogeneous and analogous to the unaided eye. Additionally, these samples were very liquid-like and dropped upon inversion of the tubes; this may be related to the low concentration of gelator, that may not be enough to self-support the water droplets within its matrix against gravitational force. As can be observed, this did not happen for the samples prepared with 0.5 % PGPR; given that the two phases are separated in this sample, the gelator matrix in the oil phase is deemed to be stronger since it retains a lower water volume than the others, resembling the matrix of a pure oleogel. For this reason, it did not drop upon inversion of the tube. Therefore, a concentration of 0.5 % is not enough to prevent phase separation. This conclusion was fundamentally the same for both storage conditions, which is why it was decided that, for the following experiments, the samples were to be stored at room temperature.

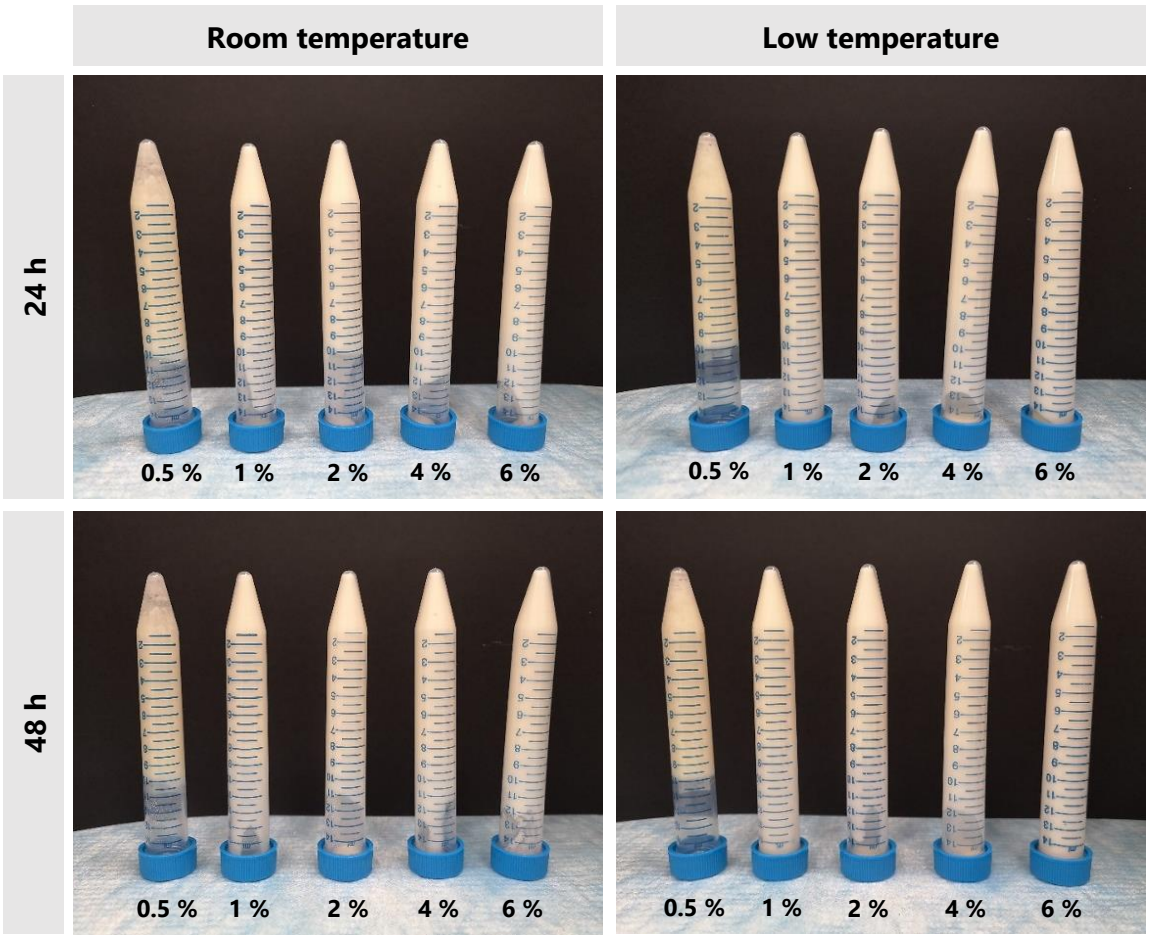


Figure 5 - Inverted tubes from the oleogel-based emulsions prepared with 10 % gelator, 60:40 (O:W) ratio, and varying concentrations of PGPR, stored at different temperatures.

Considering the visual appearance, 1 % PGPR could be enough to stabilize the emulsions. However, microscope observation of the emulsions was carried out to gauge the variation of the

emulsions' organization with the concentration of PGPR. Figure 6 and Figure 7 represent the bright-field microscopy images and the polarized microscopy images taken of the emulsions, respectively.

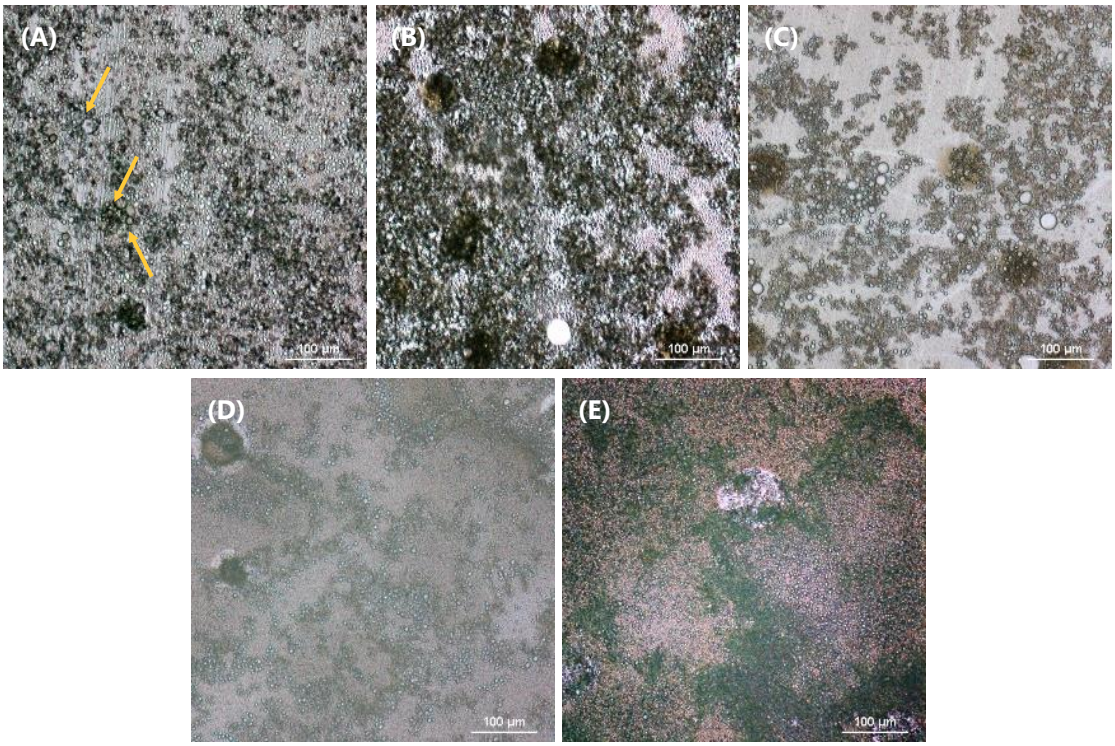


Figure 6 - Bright-field micrographs of the oleogel-based emulsions, taken with 120× magnification. A, B, C, D, and E correspond to emulsions with 0.5 %, 1 %, 2 %, 4 %, and 6 % of PGPR, respectively.

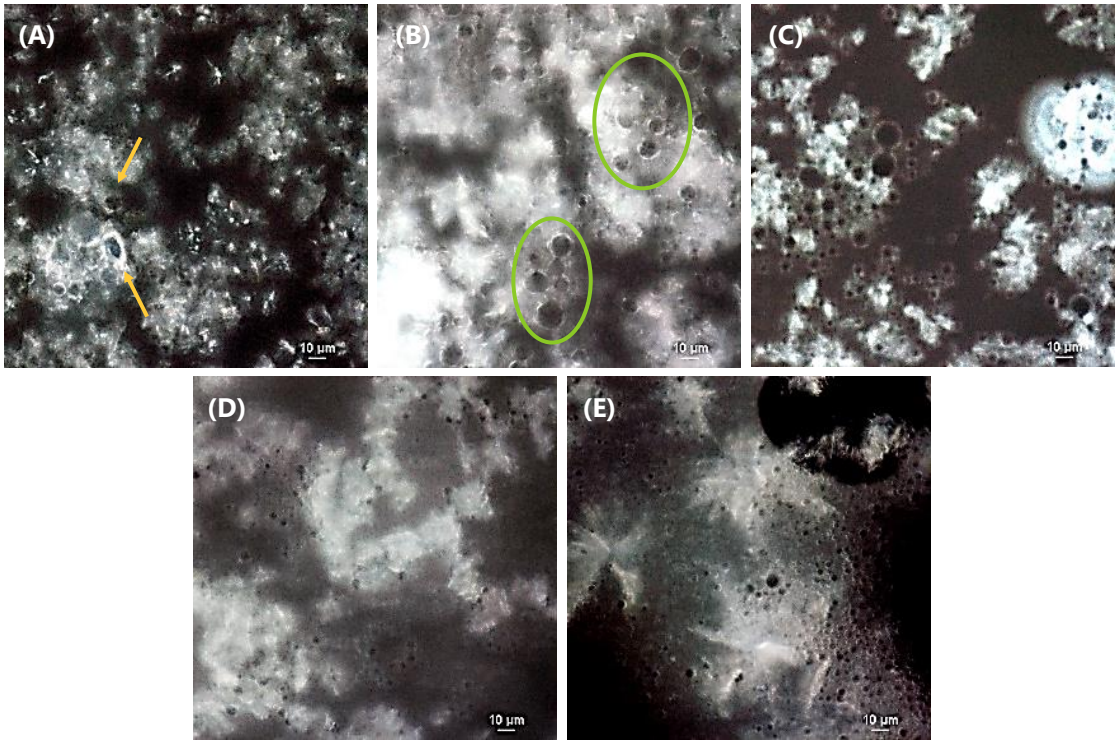


Figure 7 - Polarized micrographs of the oleogel-based emulsions, taken with 300× magnification. A, B, C, D, and E correspond to emulsions with 0.5 %, 1 %, 2 %, 4 %, and 6 % of PGPR, respectively.

The system composed of 0.5 % PGPR (images 6A and 7A) presents some non-spherical structures, which is indicative of the failure of the preparation of a stable emulsion. This corroborates the previous observations of this emulsion, where the sedimentation phenomenon is indicative of the lack of emulsifier to stabilize the droplets, resulting in the irregular structures that can be seen during microscopy (and are pointed out with yellow arrows). Considering the emulsion prepared with 1 % PGPR (images 6B and 7B), the main difference comparing to images 6A and 7A is that, overall, the shape of the water droplets is spherical and the overall droplet size seems bigger. This suggests that the interfacial film is more efficient in stabilizing the droplets than in the system with 0.5 % PGPR. However, as is circled out in green, some of the water droplets are present in agglomerates, and therefore are not evenly distributed on the oil phase. This represents another mechanism of physical instability of the emulsion, named flocculation (118). The emulsion prepared with 2 % PGPR, however, features a largely dispersed placement of the water droplets in the oil phase (images 6C and 7C). Furthermore, although there can be seen some visibly larger water droplets, its overall size seems to be homogeneous, which is an improvement in comparison to the previous observations. This tendency was maintained for emulsions with 4 % and 6 % PGPR. The bright-field images (6D and 6E) are very dark, which may suggest the presence of small, densely packed water droplets (119).

For PGPR concentrations above 2 %, the droplet size seemed to slightly decrease with the increase in PGPR, and the droplet distribution remained approximately the same. This is coherent with previous results, where the critical micellar concentration of PGPR was found to be between 0.76 % and 1.5 % (w/w) on the oil phase, as determined by the Wilhelmy plate method (120). Bahtz *et al.* (119) focused on the quantification of spontaneous W/O emulsification by PGPR and studied the characteristics of the dispersed droplets with the increasing concentration of PGPR. Although this experiment was performed with no application of external energy, such as stirring or shear, these results may be useful as a comparison. Below the concentration of 1.5 %, there was a steady increase in the droplet size; above this concentration, there was a decrease of droplet size as more PGPR was added to the formulation. These results are similar to the present observations. For this reason, 2 % was the selected concentration of PGPR to be used in every subsequent experiment. Although 4 % and 6 % PGPR resulted in emulsions with similar properties, an excess of PGPR may also result in instability problems, which should be avoided (118). Additionally, it has a low limit of acceptable daily intake, which is why its consumption must be regulated (119).

4.2 Oleogel-based emulsions' analysis and characterization

As mentioned in Section 4.1, as a consequence of the screening of the influence of PGPR concentration on the emulsions' stability, a 2 % PGPR concentration and storage conditions at room temperature were established as the most stable and used for further analysis. The gelator concentrations of 10 %, 15 %, and 20 % and O:W ratios of 60:40, 80:20, and 90:10 (w/w) in the structured emulsions' stability was evaluated.

4.2.1 Visual appearance

The visual appearance of the new emulsions is presented in Figure 8. The gelator concentration and water content showed to have an impact on both the self-sustaining ability and the textural appearance of the samples.

The increase in gelator concentration to 15 % and 20 % has resulted in self-sustained emulsions, which was, once again, not observed for emulsions prepared with 10 % gelator, where the emulsions dropped immediately after inversion of the tubes. Previously, it has been reported that the minimal concentration of gelator for obtaining a self-sustained γ-oryzanol-phytosterol oleogel is 6 % (w/w) (44). However, this has been determined for a pure oleogel formulation, comprising only oil and the gelator mixture. Adversely, it is known that the presence of water is inherently a factor of instability for oleogels; this instability is due to the tendency for water to bind to the hydroxyl group of sitosterol, promoting the formation of hydrate crystals and hindering the hydrogen bonds from being formed between the sitosterol and oryzanol molecules (121). This is damaging to the γ-oryzanol-β-sitosterol network, and, therefore, the minimal concentration for obtaining a self-standing emulsion is higher than the reported for sterol-based oleogels. This might be the reason why at a concentration as high as 10 % gelator, the emulsions dropped upon inversion of the tubes. After 7 days, the observations remained the same as after the first 24 hours, with the emulsions prepared with 10 % gelator dropping upon inversion of the tubes. As such, the storage for 7 days did not affect the self-standing ability of the emulsions.

A portion of each tube was carefully extracted and placed in a Petri dish to better assess the textural appearance of the sample when in contact with a flat surface. Concerning the three samples prepared with 10 % gelator, all of them were liquid-like and had spread out immediately when placed in the Petri dish. This was confirmed both 24 hours after preparation and 7 days later, with the behaviour of the sample remaining the same.

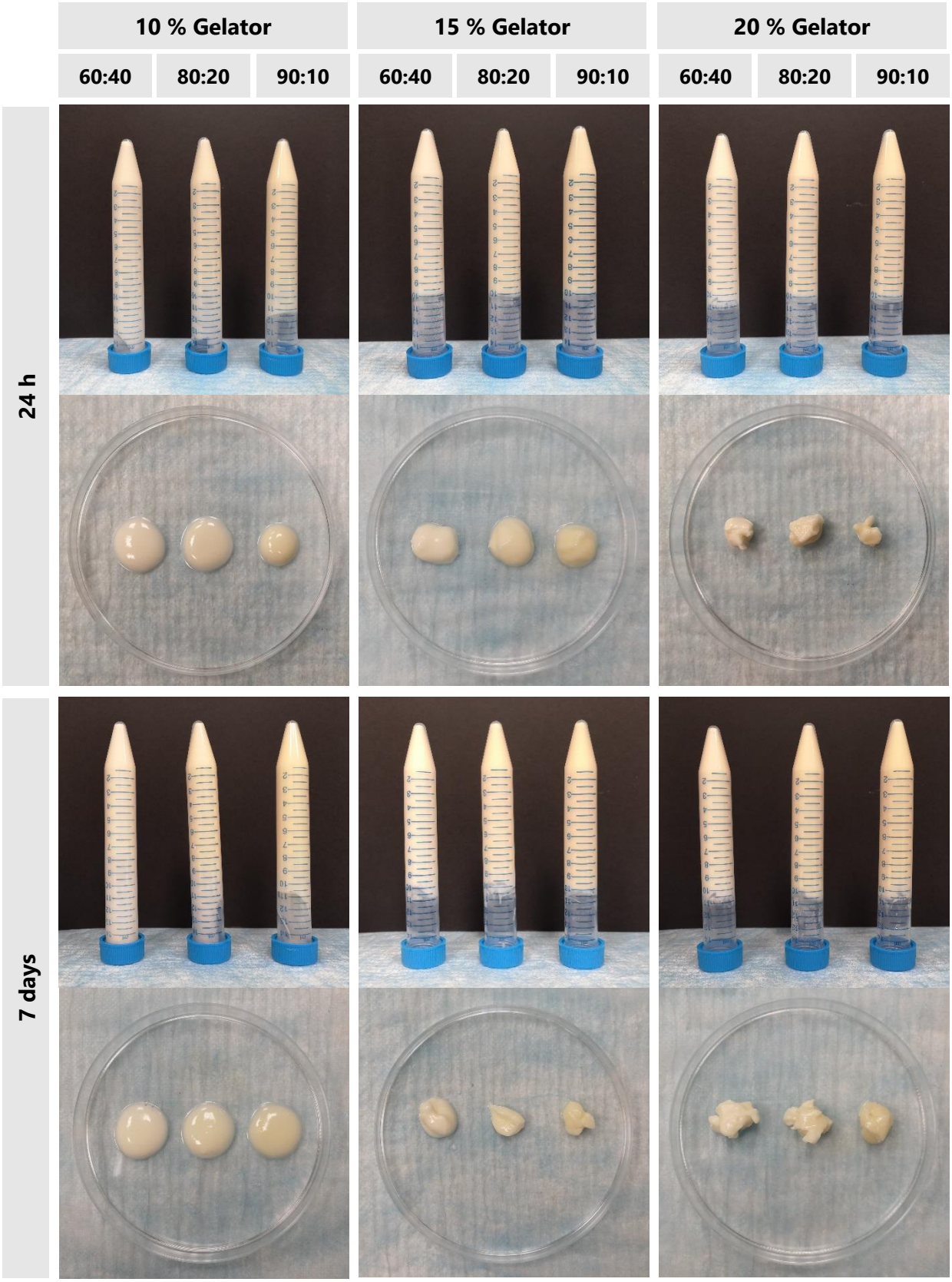


Figure 8 - Inverted tubes and extracted portions from the oleogel-based emulsions prepared with varying gelator concentrations and O:W ratio.

Concerning the samples prepared with 15 % gelator, there was an evident improvement texture-wise, since in this case, the emulsion did not spread out immediately, so it was more resemblant of cream instead of a liquid. Additionally, the influence of the O:W ratio was noticeable, with the 90:10 sample being more reminiscent of a solid gel than the 80:20 and 60:40 samples. Moreover, after 7 days, differences at the textural level were observed: the three samples seemed more solid after this storage period when compared to the observations after the first 24 hours. It has been reported for similar formulations that the ageing of the emulsions causes changes at the network level, with thin tubules giving place to bigger structures (122). These changes can be the source of these textural appearance differences. Similar results were observed for the samples prepared with 20 % gelator. These emulsions did not spread out at all when placed in the Petri dish, which is much similar to a sterol-based oleogel than all the previous samples, and, once again, the solid-like behaviour was more noticeable 7 days after preparation (44). In terms of transparency, the previously reported oleogels were shown to have a large degree of transparency with a yellowish tonality. Although the prepared emulsions had an identical tonality, which was more intense on emulsions with a higher oil-water ratio, they seemed opaque. This might be justified by the presence of the highly dispersed water phase, that affected the microstructure of the emulsion and, therefore, changes its overall appearance.

It is established that the rheology of colloidal aggregates depends not only on the solid content but also on the composition, average size, shape, and orientation of the crystals, solid-liquid surface energy, and microstructural organization (123). As above said, the ageing of the emulsions can induce changes at a microstructural level, which manifests as an increase in the emulsions' hardness. It has been reported for candelilla wax oleogels that the microplatelet units that formed the oleogel aggregated as a function of the storage time at room temperature, a process that resulted in firmer oleogels (124). Although this is a topic to be further developed, specifically in sterol-based oleogels, it might also be a suitable explanation for the textural changes over 7 days.

4.2.2 Microscopy

A typical oryzanol-sitosterol system, when prepared under certain conditions, is composed of intertwined hollow tubules formed by the co-crystallization of the two compounds (125). The formation of tubules is driven by an enthalpy change, and the physical and mechanical properties of the tubules have been associated with the γ-oryzanol:β-sitosterol ratio, with a molar ratio of 1:1 presenting the firmest gels (126,127). As aforementioned, some other phytosterols are present in the

mixture, not only β-sitosterol; however, being β-sitosterol the one which has a more widely reported and well-known interaction with γ-oryzanol for the formation of these tubular conformations. Figure 9, Figure 10, and Figure 11 show the polarized light microscopy images obtained from the emulsions prepared with 10 %, 15 %, and 20 % gelator, respectively.

Polarized light microscopy imaging allows for the identification of crystalline structures in edible oils. In Figure 9, particularly in images 9B and 9C, these structures are very well distinguished, and the branching of the crystals is evident. It has been reported that, when oryzanol and sitosterol mixtures are used for structuring water-in-oil emulsions, the outcome is very different than when structuring pure oil, resulting in crystalline fibres, rather than in hollow tubular fibrils. These are thought to be attributed to the formation of monohydrate crystals of β-sitosterol (122). As was previously discussed, the water molecules can occupy the binding spots between γ-oryzanol and β-sitosterol, weakening the tubules and possibly reducing the firmness of the emulsion (121,128). These fibres are more resemblant of those observed in emulsions with β-sitosterol, but not with γ-oryzanol, which suggests that the fibres consist mainly of β-sitosterol (129). This might be due to the high solubility of γ-oryzanol in triglyceride oil, which results in few oryzanol crystals (130).

As the gelator concentration increases, some of the crystalline structures are similar to those reported for β-sitosterol in oil. Still, the more noticeable change is the presence of plate-like crystals, which can be related to the ones previously observed for γ-oryzanol dispersed in triglyceride oil (131). These crystalline structures become denser with the increase in gelator concentration, making it more difficult to fully distinguish them. This may come about because of the relative position of the crystals that may be in different morphological planes within the structure (44). Overall, there is an increased homogeneity of the gelator distribution across the plane with the increase in concentration; these observations are comparable to the obtained results by Xiao *et al.* (132). The fact that the crystals are placed closer to each other creates a more compact network, increasing the mechanical properties and resulting in a self-standing and opaque emulsion. However, the impact of the γ-oryzanol-β-sitosterol binding capability was not detected in the microscopy observations.

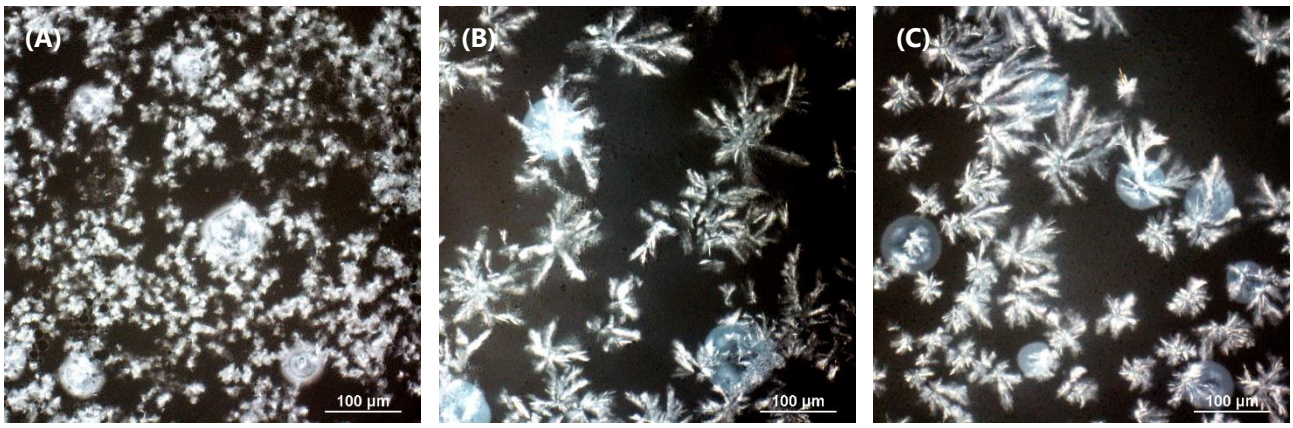


Figure 9 - Polarized micrographs (120× magnification) of the oleogel-based emulsions prepared with 10 % gelator. A, B, and C correspond to emulsions with 60:40, 80:20 and 90:10 O:W ratios, respectively.

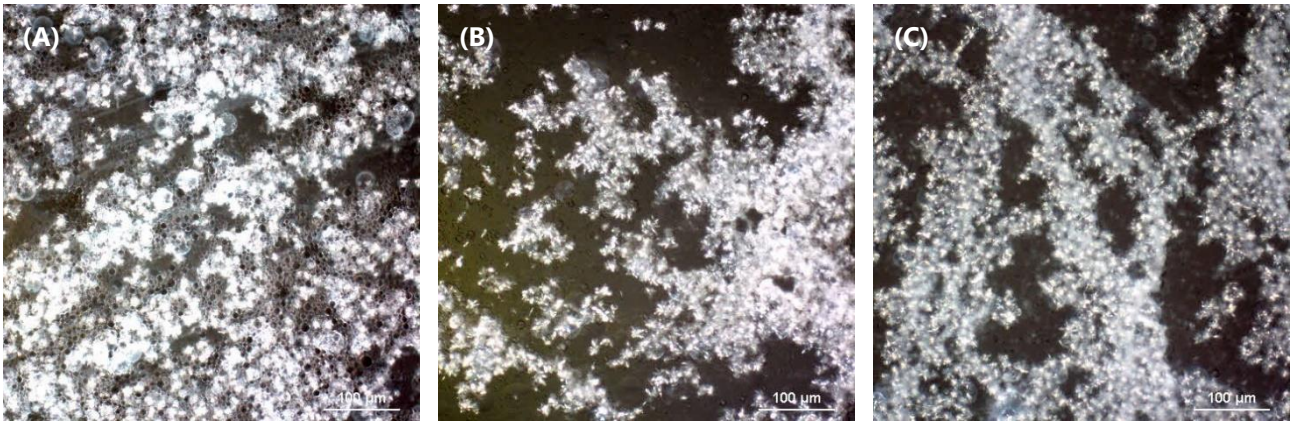


Figure 10 - Polarized micrographs (120× magnification) of the oleogel-based emulsions prepared with 15 % gelator. A, B, and C correspond to emulsions with 60:40, 80:20, and 90:10 O:W ratios, respectively.

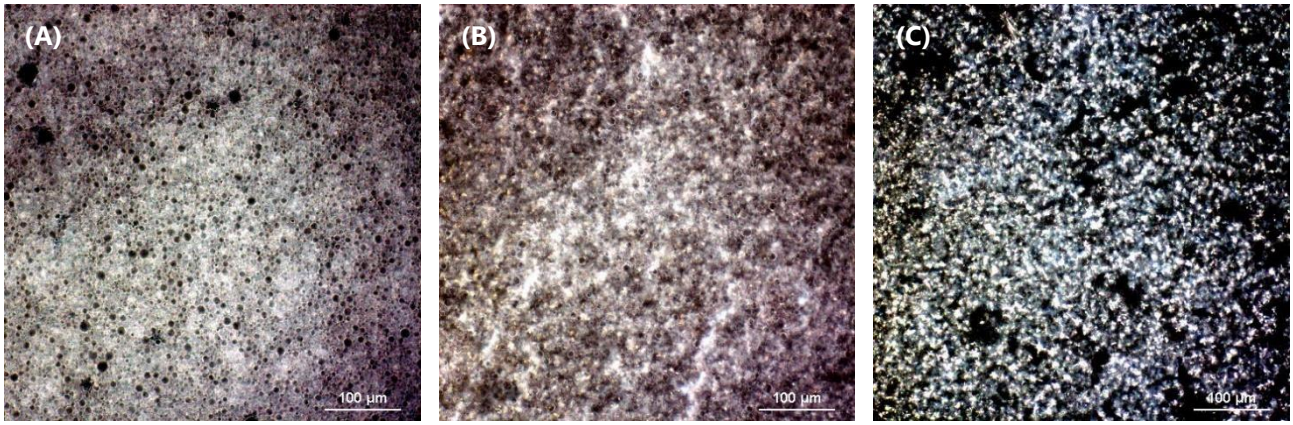


Figure 11 - Polarized micrographs (120× magnification) of the oleogel-based emulsions prepared with 20 % gelator. A, B, and C correspond to emulsions with 60:40, 80:20, and 90:10 O:W ratios, respectively.

4.2.4 Rheological analysis

Rheology tests allow inferring on the interaction between particles and the structural strength of the selected materials. The oscillatory rheological analysis was performed to assess the effect of the gelator content and O:W ratio in the oleogel-based emulsions' stability against deformation. Usually, the rheological properties of a viscoelastic material are independent of the stress that is applied, up to a critical level. Below this critical level, the material is said to be fully elastic, and above this level, the structure of the material breaks, and it flows as a liquid. The Linear Viscoelastic Region (LVR) is designed as the range in which the sample endures the applied stress without structural breakdown (133). As such, it is essential to carry out an 'amplitude sweep', where the sample undergoes sinusoidal stress with increasing amplitude. The complex modulus, *G**, which is determined experimentally by applying sinusoidal stress, is resolved into two components: storage modulus *G'* and loss modulus *G''*. *G'* represents the elastic component of material behaviour, and it is directly proportional to the energy storage in a cycle of deformation. *G''* represents the viscous component of material behaviour, and it is directly proportional to the loss of energy as heat in a cycle of deformation (133). The variation of the Storage Modulus (*G'*) and the Loss Modulus (*G''*) is monitored during the amplitude sweep, as a way of pinpointing the limit of the LVR. The ratio that describes the two portions of the viscoelastic behaviour (*G''/G'*) is called loss factor, represented as *tan δ*. The results of the amplitude sweeps performed for all the samples are presented in Figure 12.

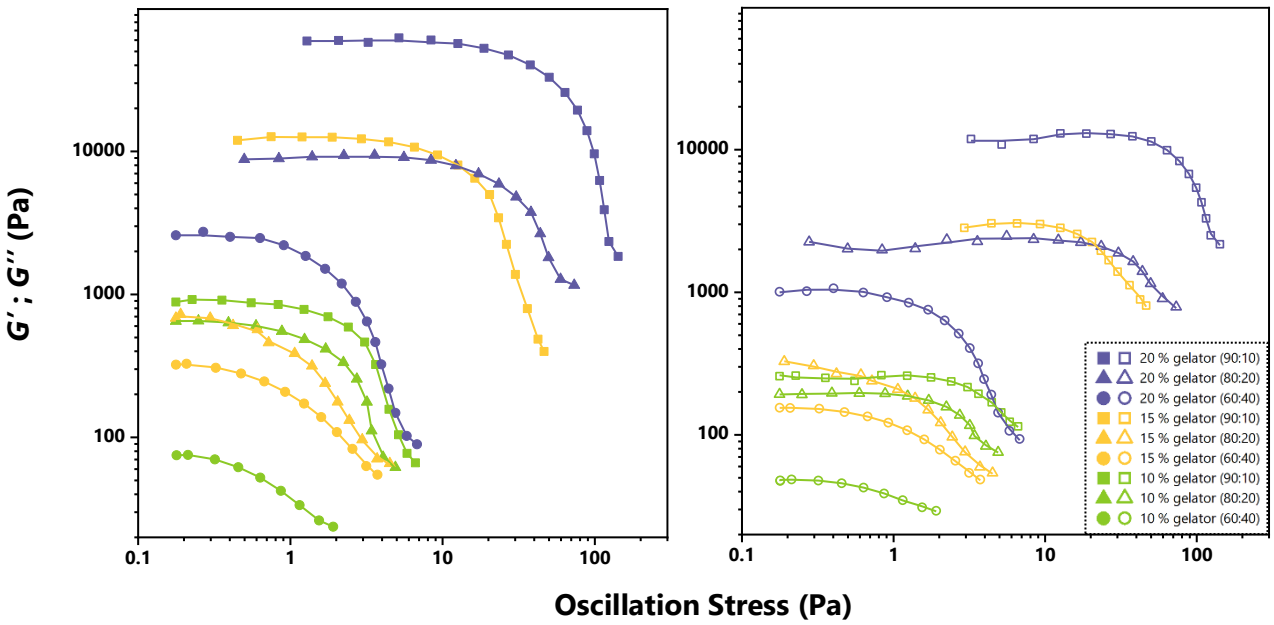


Figure 12 - Storage (*G'* - full symbols) and loss (*G''* - empty symbols) moduli as a function of oscillation stress for emulsions prepared with different gelator concentrations and different O:W ratios. The different gelator concentrations are represented in different colours (20 % - violet; 15 % - yellow; 10 % - green) and the different O:W ratios are represented in different symbols (■□ - 90:10; ▲△ - 80:20; ●○ - 60:40).

The data from the logarithmic plots above revealed that all of the samples displayed gel-like behaviour in the LVR, given that the storage modulus (*G'*) was, in all samples, superior to the loss modulus (*G''*), which can be observed by the value of *tan δ* (less than 1). This means that the elastic response is dominant, hence the gel-like behaviour. However, there was a considerable variation in both moduli values as a function of the content in gelator and the O:W ratio. As can be observed in the plots, per each fixed gelator concentration, the *G'* and *G''* demonstrated to increase with the O:W ratio. This difference is understandable since higher water content is expected to favour a liquid-like behaviour (134). The values of *G'*, *G''* and *tan δ* for the analyzed samples within the LVR are summarized in Table 2.

Table 2 - Storage (*G'*) and loss (*G'*) moduli of the samples in the LVR.

Samples	10 % Gelator			15 % Gelator			20 % Gelator		
	60:40	80:20	90:10	60:40	80:20	90:10	60:40	80:20	90:10
G' (Pa)	75.19	645.78	887.89	324.78	704.16	12283.30	2583.94	9129.99	59317.50
G'' (Pa)	47.55	193.163	254.80	316.63	151.60	2841.93	1022.02	2346.22	12630.46
tan δ	0.63	0.30	0.29	0.97	0.22	0.23	0.40	0.26	0.21

The tendency observed is of an increase in the viscoelastic properties of the emulsion with the increase in gelator concentration. Such behaviour is coherent with the previously observed for oleogels and is indicative that a desired macroscopic functionality can be achieved by manipulating the amount of gelator (127). The highest *G'* value was recorded for the emulsion produced with 20 % gelator and an O:W ratio of 90:10; thus, this can be considered the 'strongest' emulsion. Overall, the obtained values are much lower than the ones obtained for γ-oryzanol-β-sitosterol oleogels. As previously reported, oleogels produced with 10 % of a similar oryzanol-sitosterol mixture have registered a *G'* value of 1.759×10^6 Pa, which is about 30× superior to the strongest emulsion undergoing analysis (20 % gelator, 90:10 O:W) (135). The second strongest emulsion was the one prepared with 15 % gelator and an O:W ratio of 90:10, which confirms the impact of the water fraction in the rheological behaviour of the emulsions.

Within the same gelator concentration, although the *G'* values were increasingly higher with the decrease of the water fraction, *tan δ* does not exhibit a similar pattern. This indicates that, although the emulsions are stiffer when the water content is lower, the same cannot be said for their elasticity. The emulsions prepared with a 60:40 O:W ratio presented higher *tan δ* values across all gelator concentrations, confirming that the water content will cause the emulsions to pull away from exhibiting solid-like behaviour. The elastic properties of the network are dependent on the amount

and spatial distribution of mass, as well as the nature of the crystals and crystal-crystal interactions (136). The scarce dispersion of the crystals and its assembly in bigger structures may be associated with a decrease in emulsion strength (134). These observations can be related to the microscopic images, particularly for the emulsions prepared with 10 % gelator, where large crystal structures were observed. This may also explain the narrowness of the LVR in these emulsions, meaning that these emulsions are not capable of enduring higher oscillation stresses. The yield stress (σ^*) indicates the stress value where non-reversible structural changes occur to the sample, and the liquid-like behaviour overpowers the solid-like behaviour. The σ^* values determined for the emulsions are presented in Table 3.

Table 3 - Yield stress (σ^*) determined for the samples.

Samples	**10 % Gelator**			**15 % Gelator**			**20 % Gelator**		
	60:40	**80:20**	**90:10**	**60:40**	**80:20**	**90:10**	**60:40**	**80:20**	**90:10**
$\boldsymbol{\sigma^*}$ (Pa)	0.53	1.15	1.59	0.64	0.66	8.21	1.02	15.84	28.40

As expected, for the same gelator concentration, the increase in O:W ratio resulted in a progressive increase of the σ^* value for all the samples. An increase in gelator concentration results in higher σ^* values for emulsions produced with the same O:W ratio (except for the sample prepared with 15 % gelator and an 80:20 O:W ratio, which was not a part of this tendency). As such, the sample that exhibited the highest level of internal structure strength was the one prepared with 20 % gelator and O:W ratio of 90:10. For the samples prepared with 20 % gelator, the increase in the σ^* value with the O:W ratio is more noticeable than for samples prepared with 10 % gelator, where the σ^* values are very similar.

When comparing these results to γ-oryzanol-β-sitosterol oleogels produced with 10 % gelator, these exhibit a σ^* value of 1547 Pa, which is coherent due to the stronger gel network that is formed (135). Moschakis *et al.* (137) prepared oil-in-water emulsions using γ-oryzanol-phytosterols (60:40) as the oleogelator and added xanthan gum as a gelator of the aqueous phase. When compared to the results presented on Table 3, they registered σ^* values for emulsions with an O:W ratio of 50:50 in the same order of magnitude (σ^* = 0.4 Pa for emulsions with 10 % gelator; σ^* = 1.5 Pa for emulsions with 20 % gelator). On the other hand, they noticed that a higher γ-oryzanol-phytosterols ratio (70:30) leads to stronger emulsions, which implies that the equimolar ratio of 60:40 that is ideal for oleogels might not work in the same manner when structuring emulsions.

4.2.4 SAXS and XRD

Small-angle and wide-angle X-ray scattering measurements were performed to evaluate the influence of the gelator concentration and the O:W ratio in the microstructure of the emulsions. Figure 13 represents the SAXS pattern and XRD spectra of the samples retrieved 24 hours after preparation. Vertical shifts were applied to the curves to improve the observation.

Regarding the SAXS pattern, none of the analyzed samples exhibited the interference pattern that was previously associated with tubules. As was assessed by Bot *et al.* (122), the effect of water is a hindrance to the formation of hollow cylinders, and the necessary concentration of gelator to obtain an emulsion gel with these structures would have to be above 30 %. Nonetheless, two sharp crystallographic reflections can be observed across all samples, being the lattice of these crystalline arrangements at 1.75 nm^{-1} and 3.56 nm^{-1}. These correspond to Bragg spacings of 35.90 Å and 17.65 Å, respectively. As previously reported in the literature, one of the most pronounced and characteristic peaks of both anhydrous and monohydrate β-sitosterol crystals relate to a Bragg spacing of 17.6 Å, which may be associated with Peak A (138). Peak B, referring to a Bragg spacing of 35.90 Å has been previously reported in γ-oryzanol-β-sitosterol emulsions with 30 % water; however, this is thought to be associated with β-sitosterol crystals in the oil phase, and not the assembly of tubules (130).

Regarding the XRD spectra, in similarity with the SAXS, two peaks were found across all the samples, at approximate angles of 14.9º and 19.3º (Peaks C and D). These correspond to Bragg spacing values of 5.91 Å and 4.60 Å, which were listed by Christiansen *et al.* (138) among the main peaks for β-sitosterol crystals in the hemihydrated form. It should be, however, noted that the Bragg spacing of 5.91 Å might also be associated with other forms of β-sitosterol crystals.

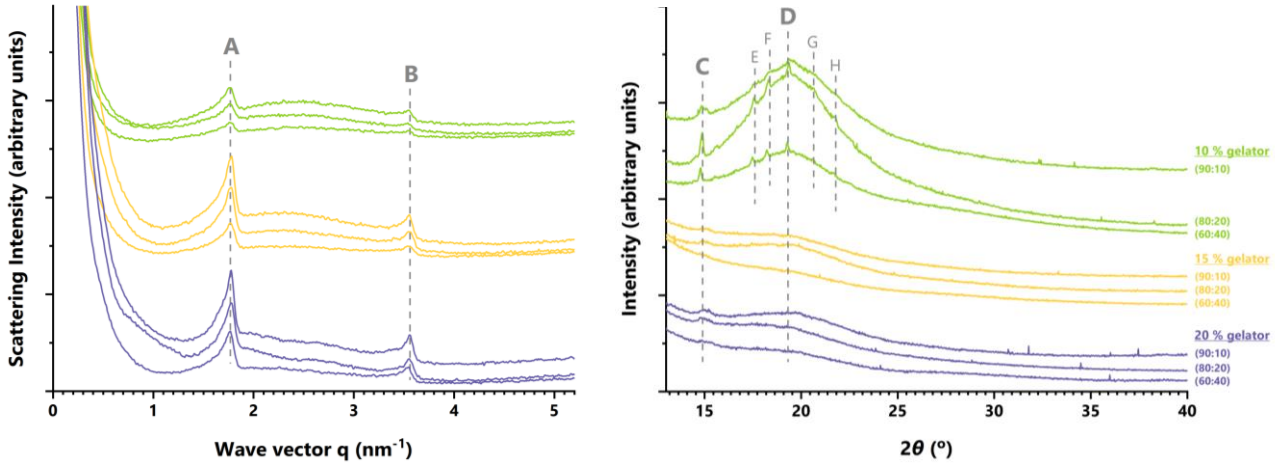


Figure 13 - SAXS pattern and XRD spectra for the emulsions after 24 hours.

Moreover, for the XRD spectra of emulsions prepared with 10 % gelator, four other peaks were identified, corresponding to Bragg spacings of 5.03 Å, 4.82 Å, 4.30 Å, and 4.07 Å (Peak E, F, G, and H, respectively). Peaks E and F are typically associated with the presence of monohydrated β-sitosterol crystals (138). Peaks G and H can be traced back to previous reports of β-sitosterol crystallites dispersed in oil and of oryzanol hydrates, even though as minor peaks (130). A similar situation was reported previously by von Bonsdorff-Nikander *et al.* (129) for β-sitosterol suspensions in oil, where a lower concentration of sterol (5 %) resulted in monohydrated crystals. In contrast, higher concentrations resulted in hemihydrated crystals.

After 7 days of storage, the same analysis was performed; the SAXS pattern and XRD spectra obtained for all the samples are represented in Figure 14. Once again, vertical shifts were applied to the curves to improve the observation. The two major peaks observed in the SAXS pattern after 24 hours are still identifiable after 1 week of storage, and the same can be said for the two peaks identified in all samples on the XRD spectra. However, the four minor peaks present in the XRD spectra for the samples prepared with 10 % (Peak E, F, G, and H) are not present after 7 days. According to these observations, the peaks that are not present are the ones associated with monohydrated crystals. This may be due to dehydration of the sample during a week; in fact, hydrated β-sitosterol crystals have been reported to undergo a process of dehydration in two phases (139). As was assessed by Christiansen *et al.* (138), the dehydration of monohydrated crystals to hemihydrated crystals of β-sitosterol is very common and happens at low temperatures. The migration of the water molecules along the tunnels may explain the ease with which these crystals dehydrate. Approximately half of the water leaves the structure, leaving a hemihydrated form of the crystal. This form is more stable, and therefore does not dehydrate so easily, but rather at higher temperatures, which is possibly why the peak corresponding to a hemihydrated crystal is still visible.

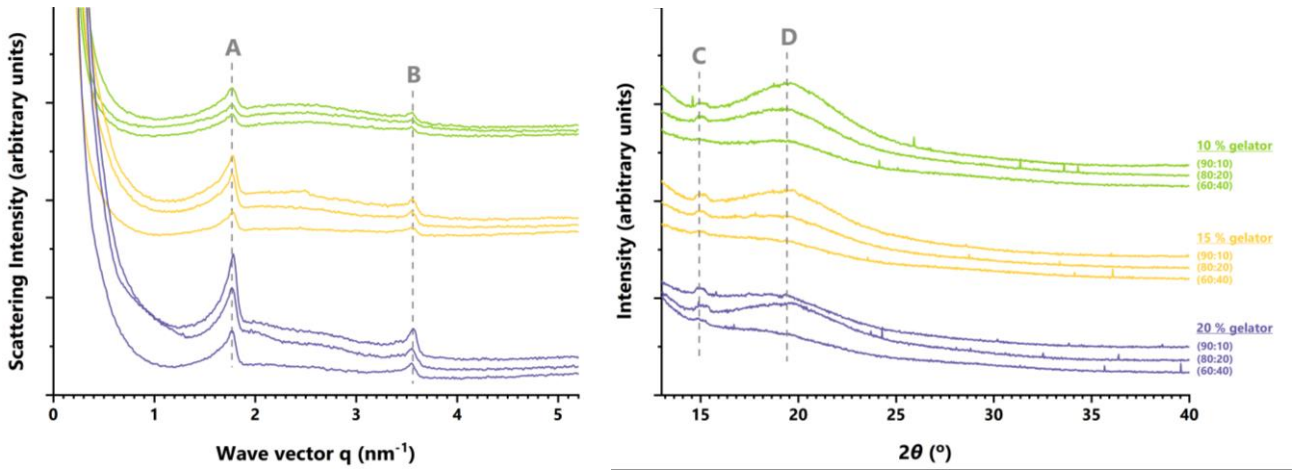


Figure 14 - SAXS pattern and XRD spectra for the emulsions after 7 days.

The scattering data obtained for the emulsions revealed reflections that can be traced back mainly to β-sitosterol crystals, and possibly to some weak γ-oryzanol crystals. This may be due to the high solubility of γ-oryzanol in the corn oil, which translates into very weak reflections, leading to a greater contribution of the β-sitosterol crystals to the diffraction pattern. The X-ray data also confirmed that tubule formation does not occur, proving the susceptibility of this multi-component system to the presence of water molecules. In Section 4.2.1, it was observed that, after the storage period of 7 days, there is a modification of the textural appearance of the emulsions, which was associated with possible partial evaporation of the water fraction. The SAXS and XRD results may confirm this assumption and explain the improved firmness of the emulsions after 7 days of ageing.

4.3 Bioactive-loaded emulsions' analysis

The intermediate gelator concentration of 15 % was chosen for the preparation of the oleogel-based emulsions with bioactive compounds, to assess the differences with the non-loaded emulsions. The visual appearance of the loaded emulsions is presented in Figure 15.

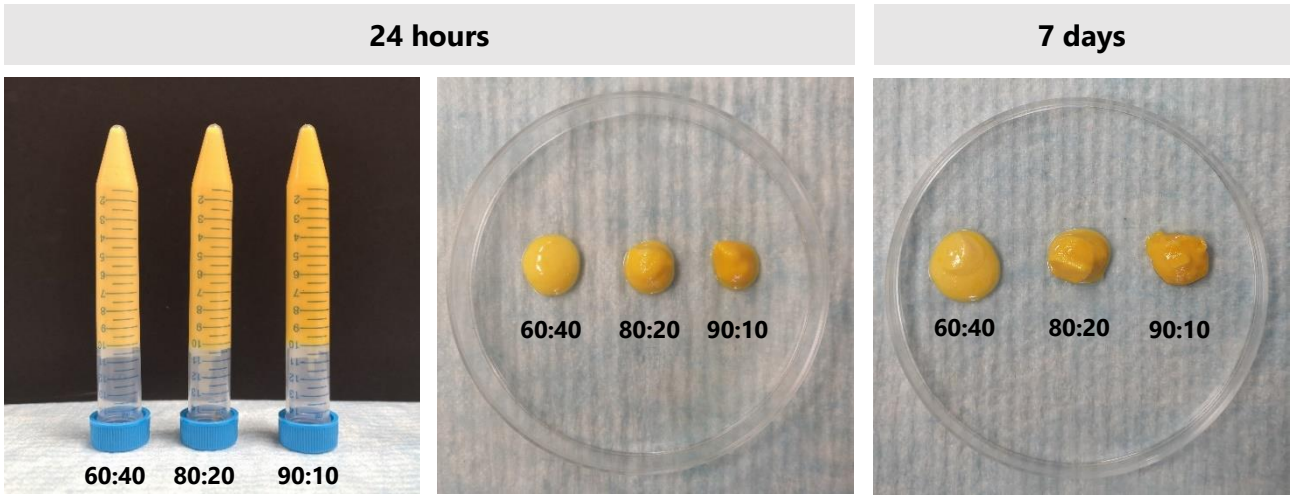


Figure 15 - Inverted tubes and extracted portions from the oleogel-based emulsions prepared with 15 % gelator and co-loaded with BC and EGCG.

The presence of the bioactive compounds did not impact the self-standing ability of these emulsions, as none of the samples dropped upon inversion of the tubes. The orange colour of the emulsions is more intense as the O:W ratio increases, which is explained by the increasing amount of BC in the samples. Although the samples did not spread out when placed in the plate, the textural appearance seems less solid-like than the non-loaded emulsions, both after 24 hours and 7 days. Other oleogel-based systems designed for nutraceutical purposes have recorded alterations in their

structural properties with the addition of a bioactive compound, such as presented by Yu *et al.* (94), that concluded that a higher concentration of MAGs was necessary to gel MCT oil when curcuminoids were added to the formulation.

Key factors such as the gelation mechanism, polarity of the oil phase, and the chemical composition of the introduced bioactive compounds may or may not influence the physicochemical properties of the emulsion (99). Microscopical analysis of the emulsions was performed to assess the microscopical characteristics and compare them to the non-loaded emulsions. The obtained fluorescence micrographs are presented in Figure 16.

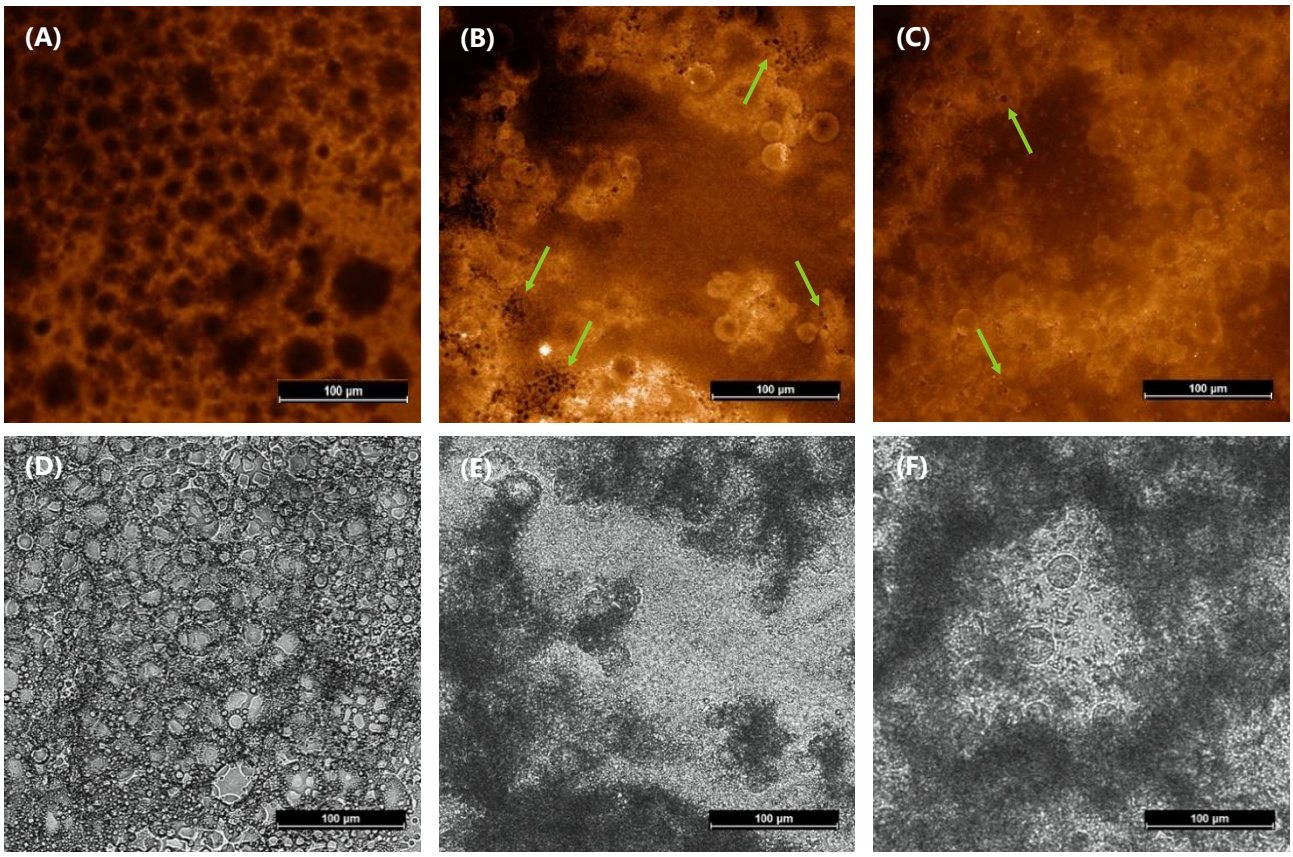


Figure 16 - Fluorescence (above) and bright-field (below) micrographs with 120× magnification of the structured emulsions prepared with 15 % gelator and co-loaded with BC and EGCG. A, B, C, and D, E, F correspond to emulsions with 60:40, 80:20, and 90:10 O:W ratios, respectively.

As can be observed in the images, the BC is present in the oil phase. Some regions feature a more intense orange colour, which may be associated with BC precipitation on the β-sitosterol crystals, coherently with the bright-field images since the darker regions are related to crystalline structures. On Figures 16B and 16C, the water droplet size (pointed out with green arrows) was approximately the same as in the non-loaded emulsions with the same gelator concentration. Figure 16A was the only that was appreciably different than the obtained for the non-loaded emulsion; the

water droplet size seems to be very irregular, as well as the shape that seems to be non-spherical. These differences might be associated with the composition of the water phase: it has been observed that the behaviour of PGPR at the interface is deeply affected by the composition of the water phase, and the presence of EGCG might have led to destabilization phenomena (140). Previously, it has been reported that the presence of green tea catechins in O/W emulsion systems leads to the coalescence of droplets, changing the droplet size distribution and, consequently, decreasing the specific surface area (141). Although it is not clear if this is the reason for the instability of the 60:40 emulsion, it constitutes a good hypothesis for further analysis.

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5 Conclusions

The main question to be assessed in this study was the practicality of oleogel components as structurants in water-rich matrices, considering the interplay between emulsification and oleogelation. The inclusion of free-form bioactive compounds served as a first experiment for checking the potential of these novel formulations as nutraceutical tools.

From these preliminary studies, it was concluded that a 2 % fraction of PGPR is needed to obtain oleogel-based emulsions, which were resistant to phase separation and were microscopically stable. The visual assessment of the emulsions has shown that a gelator concentration below 15 % does not result in self-standing emulsions; above this critical concentration, an increase in gelator concentration and O:W ratio resulted in more solid-like emulsions. These observations were confirmed by rheology data, where the samples prepared with higher gelator concentrations and O:W ratios were revealed to be the strongest emulsions. This improved strength of the emulsions is related to a more compact organization of the crystalline structures, which consist mainly of β-sitosterol crystals. The incorporation of the BC and EGCG in the novel formulations resulted in some textural and microscopical changes that may be related to the interaction of the emulsifier with the water phase.

The evaluation of the novel formulations' properties and its comparison with state-of-the-art oleogels prepared with the same gelator mixture was an important part of this study. It was anticipated that the presence of highly dispersed water might influence the microstructure of the emulsions, and this premise was confirmed from the first analyses. The yellowish and opaque tonality of the emulsions contrasted with the translucent aspect of the oleogels and the microscopy observations have confirmed the absence of tubular fibrils. Accordingly, the SAXS pattern usually connected with tubules was not observed, and the sharp reflections present in both the SAXS patterns and the XRD spectra were linked to anhydrous, hemihydrated, and monohydrated β-sitosterol crystals.

The results obtained in this work establish a proof of concept for the co-loading of hydrophilic and lipophilic compounds in oleogel-based emulsions. Although it was observed that some structural changes might be induced by the presence of these compounds, this work represents one of the first attempts of entrapping hydrophilic compounds in an oleogel matrix, which may constitute a significant step in the fortification of food products.

5.1 Future work

As was originally planned out, the scope of the project was to fully characterize the non-loaded emulsions as well as the bioactive-loaded emulsions, as a way of understanding the suitability of the formulation for nutraceutical purposes. According to the observations, there were some differences in the microstructure, which is why further research to this extent is required.

A key objective of the original work plan was to assess the bioaccessibility of the entrapped bioactive compounds through *in vitro* digestion of the emulsions. This would confirm if the novel formulation effectively protects the compounds throughout the gastrointestinal tract for controlled delivery and absorption at the intestinal level. Considering that this step could not be performed now, it is a paramount goal for further works.

Additionally, it would be interesting to modulate the water activity of the emulsions and assess its influence in the formation of tubular fibrils of γ-oryzanol and β-sitosterol. Furthermore, the substitution of the corn oil by different oils with lower capacity for solubilizing γ-oryzanol could be an interesting experiment.

The assembly of the γ-oryzanol-β-sitosterol network was heavily influenced by the presence of water, which led to the gelator structure being attributed mainly to β-sitosterol crystals. Since this was the case, a possible study would be to produce the same emulsions only with phytosterols or develop a new formulation with β-sitosterol and lecithin, benefiting from the capability of these compounds to form self-assembled fibres.

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Appendices

Appendix A: Yield Stress Calculation

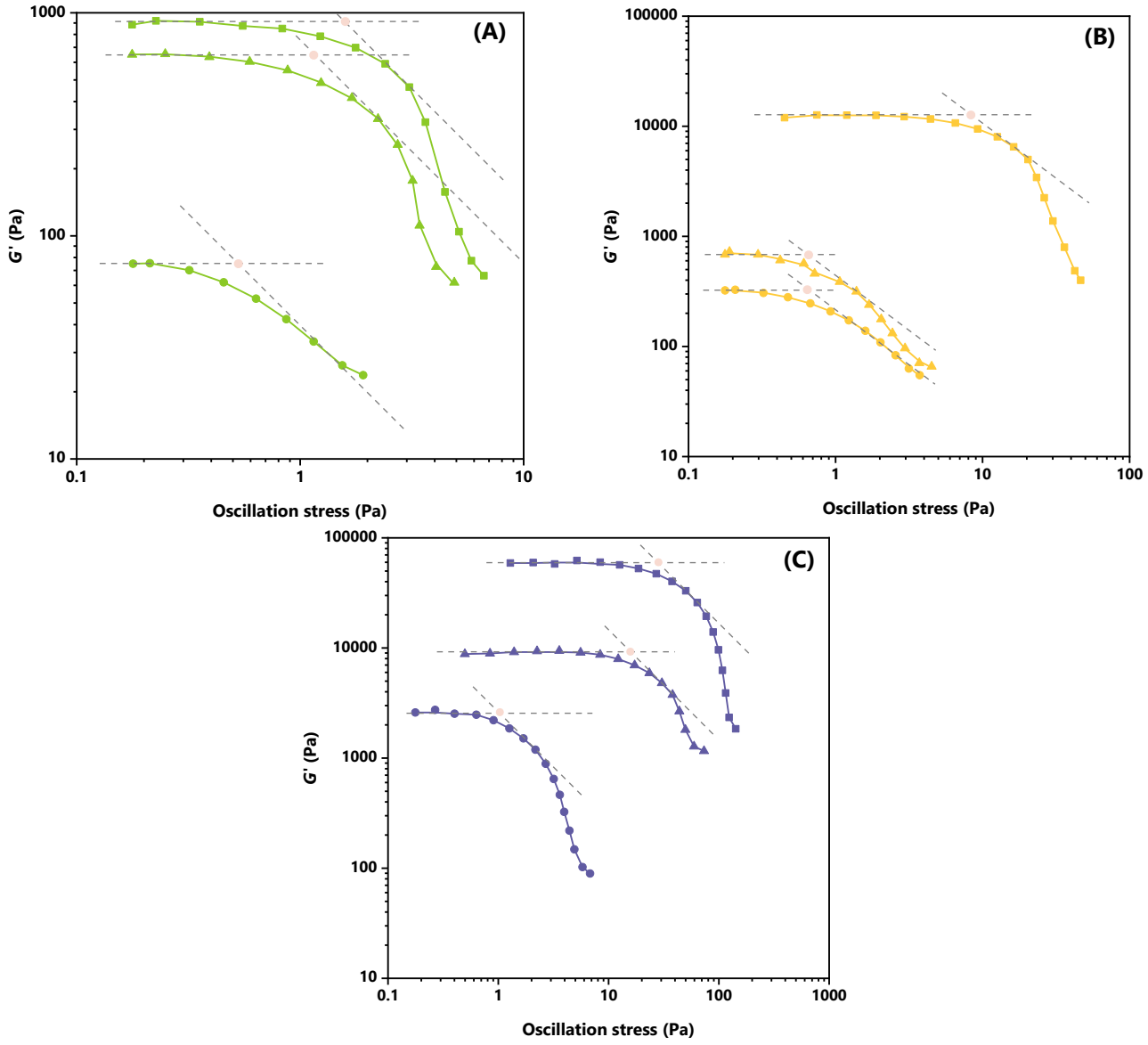

Figure I – Logarithmic plots of the storage modulus (*G'*) as a function of oscillation stress for emulsions prepared with different gelator concentrations and different O:W ratios. The different gelator concentrations are represented in different colours (20 % - violet; 15 % - yellow; 10 % - green) and the different O:W ratios are represented in different symbols (■□ - 90:10; ▲△ - 80:20; ●○ - 60:40). The yield stress (represented in salmon) is defined as the intersection between the line with a unit slope on logarithmic coordinates that is tangent to the data at low deformations and the horizontal steady-state stress (both represented in a dashed grey line).