Journal of Molecular Liquids 306 (2020) 112908

Contents lists available at ScienceDirect

Journal of Molecular Liquids

journal homepage: www.elsevier.com/locate/molliq

Structure, activity and dynamics of extra virgin olive oil-in-water nanoemulsions loaded with vitamin D3 and calcium citrate

S. Demisli [a,b], I. Theochari [a,b], P. Christodoulou [a], M. Zervou [a], A. Xenakis [a], V. Papadimitriou [a,*]

[a] Institute of Chemical Biology, National Hellenic Research Foundation, 11635 Athens, Greece
[b] Department of Biochemistry & Biotechnology, University of Thessaly, 41500 Larissa, Greece

ARTICLE INFO

Article history:
Received 7 January 2020
Received in revised form 11 March 2020
Accepted 13 March 2020
Available online 14 March 2020

Keywords:
O/W nanoemulsions
Vitamin D3
DLS
EPR
DOSY NMR
Radical scavenging activity

ABSTRACT

Extra virgin olive oil-in-water (O/W) nanoemulsions loaded with vitamin D3 and calcium citrate were developed and studied in terms of structure, activity and dynamics. The nanoemulsions were formulated with a two-step emulsification procedure using water as the continuous phase, extra virgin olive oil (EVOO) as the dispersed phase and mixtures of food grade surfactants as stabilizers. The nanoemulsions were investigated for their particle size, polydispersity and stability over time by Dynamic light scattering (DLS) analysis. Among the different nanoemulsions studied, those based on polysorbate 20 in combination with lecithin produced systems owing EVOO droplets of 285 ± 5 nm diameter and 0.202 ± 0.01 PdI that were stable for 37 days. These stable nanoemulsions were loaded with calcium citrate and vitamin D3 to result formulations containing both water and oil soluble micronutrients. The presence of calcium ions in the aqueous phase strongly affected the stability of the nanoemulsions whereas vitamin D3 addition in the dispersed oil phase affected the size of the oil cores by several nanometers. Interfacial properties were investigated using Electron Paramagnetic Resonance (EPR) spectroscopy employing an amphiphilic spin probe. The nature of the surfactant and the presence of vitamin D3 and calcium citrate affected the properties of the surfactants' layer in terms of rigidity, local viscosity and polarity. More specifically, upon encapsulation vitamin D3 resulted in more ordered and viscous interfaces. Antioxidant potential of the proposed nanocarriers was investigated with an EPR procedure based on the scavenging the 4-hydroxy-TEMPOL. EPR signal inhibition was observed due to the scavenging activity of hydrogen donating moieties present both in empty and loaded nanoemulsions. Diffusion ordered NMR spectroscopy (DOSY NMR) successfully revealed the solubilization of the lipophilic vitamin D3 in the dispersed oil phase of the nanoemulsion formulation.

1. Introduction

In recent years, there has been considerable interest in developing fluid delivery vehicles at the nanoscale for the encapsulation and protection of lipophilic bioactive substances for food applications. Several micronutrients, phytochemicals and other biologically active compounds can be loaded into biocompatible and food grade systems to improve stability, aqueous solubility and bioavailability [1–6]. As encapsulation provides a physical barrier between the internal phase of the nanocarrier and the environment, reactive substances are protected from unfavorable environmental conditions such as oxidation and hydrolysis. Furthermore, reaction of the active ingredients with other components in the food system is avoided, thus reducing food deterioration and spoilage. As a consequence, the nutritional content of the enriched foods is improved [4,6]. Moreover, many bioactive compounds are highly hydrophobic and poorly soluble in aqueous systems thus showing low bioavailability during gastrointestinal passage [7].

The most studied liquid nanostructured systems for the encapsulation of bioactive compounds include liposomes, nanoemulsions, microemulsions, solid lipid nanoparticles and biopolymeric nanoparticles [1,8]. In general, nanocarriers intended for use in dietary supplements and functional foods must be cost effective, easy to prepare and based on FDA and EFSA permitted ingredients.

In this study, nanoemulsions were selected among other fluid vehicles as promising delivery systems for food applications, due to their unique physicochemical properties. Nanoemulsions are kinetically stable dispersions of liquid droplets (oil or water) in another nonmiscible continuous liquid phase (water or oil). The diameter of the emulsion droplets reaches approximately 50–200 nm. In general, the applicability of nanoemulsions for industrial purposes strongly depends on their particle size, size distribution and stability. Typically, very small droplets with narrow size distribution are required to ensure stability and increased bioavailability upon oral intake and digestion.

* Corresponding author at: Institute of Chemical Biology, National Hellenic Research Foundation, 48, Vassileos Constantinou Ave., 11635 Athens, Greece.
E-mail address: vpapa@eie.gr (V. Papadimitriou).

https://doi.org/10.1016/j.molliq.2020.112908
0167-7322/

Nanoemulsions for food applications consist of oils and surfactants approved for human consumption. They can be transparent, translucent or milky depending on the size of the dispersed droplets. Nanoemulsions are prepared either through high-pressure homogenization, microfluidization, ultrasonication or phase inversion. Depending on the composition, two types of nanoemulsions are formed: oil-in-water (O/W) type and water-in-oil (W/O) type [9,10].

In nanoemulsions, the coexistence of hydrophobic, hydrophilic and amphiphilic domains, enables the solubilization of a wide range of substances. Therefore, nanoemulsions could contain bioactive molecules of different hydrophilicities in the same formulation. Apparently, O/W nanoemulsions wherein oil droplets are dispersed in the continuous aqueous phase, could offer a good potential for the encapsulation of lipophilic or amphiphilic compounds in various applications [11–17].

In spite of the promising potential of nanoemulsions related to the effective delivery and protection of bioactive compounds, structural characterization of these formulations in terms of size, shape, size distribution, stability, interfacial tension and dynamics remains a fundamental challenge for many research groups.

The objective of the present work was to develop and structurally characterize oil-in-water (O/W) nanoemulsions for the encapsulation of vitamin D (D3: cholecalciferol) in the dispersed oil phase. Cholecalciferol is the most biologically active and more stable to oxidation form of vitamin D, necessary for the proper functioning of the organism [18]. Furthermore, vitamin D3 is involved in calcium homeostasis affecting active intestinal calcium absorption [19]. In this sense, calcium citrate, a source of elemental calcium, could be solubilized in the continuous aqueous phase of the nanoemulsions to result vitamin and mineral enriched formulations for food supplementation. In addition, citrates present in the aqueous phase may prevent lipid oxidation, an interfacial phenomenon mainly taking place at the interface where different non-polar and polar compounds can interact [20].

To this end, a series of nanoemulsions was developed using a two-step emulsification procedure [15,16]. The nanoemulsions were based on extra virgin olive oil (EVOO) as the dispersed phase, water or calcium citrate solution as the continuous phase and mixtures of surfactants as stabilizers. Extra virgin olive oil is one of the oldest known vegetable oils containing a variety of minor components such as phospholipids, polyphenols and partial glycerides. It is an unrefined and inexpensive natural product that can be used as a constituent for the construction of various micro- and nanoemulsions [21,22]. All systems were stabilized with soybean lecithin, a natural emulsifier widely used in food applications and other more hydrophilic food-grade nonionic surfactants. The combination of surfactants with high and low hydrophilic–lipophilic balance (HLB) values has been reported to be more effective for emulsion stabilization as compared to single surfactants [23].

The extra virgin olive oil-in-water nanoemulsions were studied for their potential to provide stable dispersions at the nanoscale using Dynamic Light Scattering (DLS). Interfacial properties of both empty and loaded nanoemulsions were investigated using Electron Paramagnetic Resonance (EPR) spectroscopy employing an amphiphilic spin probe. This technique can provide valuable information about the microenvironment of the probe in terms of viscosity, rigidity and polarity. As a consequence, the degree of vitamin's embedment in the surfactants' monolayer can be evaluated through the detection of structural modifications [16,24]. Then, diffusion-ordered NMR spectroscopy (DOSY-NMR) was applied to evaluate the encapsulation of vitamin D3 in the nanoemulsion environment [25]. Finally, the modification of an EPR technique previously developed by our group was applied to evaluate the antioxidant potential of the loaded nanocarriers. This technique is based on the scavenging reaction of antioxidant compounds present in the dispersed oil phase of the nanoemulsions against a stable nitroxide free radical [26,27].

2. Materials and methods

2.1. Materials

Organic extra virgin olive oil (EVOO) from the Amfissa Variety, Pelion, Magnesia, Greece, (acidity < 0.4%, oleocanthal 124 mg/kg, oleacein 72 mg/kg, total hydroxytyrosol derivatives 140 mg/kg, total phenols analyzed 315 mg/kg) was generously donated by "Myrolion" startup company. Cholecalciferol (Vitamin D3) ≥ 98% was supplied from Sigma-Aldrich, Germany. Soybean lecithin (L-alpha phosphatidylocholine), 90% was from Alpha Aesar, Germany. Polyethoxylene 20 (Tween 20) was purchased from Merck Schuchardt, Germany. *Quillaja* saponin (Q-Naturale®200V) was a generous gift of Ingredion, Germany GmbH. Labrasol® ALF (Caprylocaproyl Polyoxyl-8 glycerides), oral grade, was kindly donated by Gattefossé, France. Calcium citrate tetrahydrate, 99% was from Aldrich. 5-Doxyl stearic acid [5-(1-oxyl-2,2-dimethyl-oxazolidin) stearic acid] free radical was from Sigma-Aldrich, Germany. 4-hydroxy-TEMPOL was purchased from Alpha Aesar, Germany. Highly purified water was obtained from a Millipore Milli Q Plus device.

2.2. Methods

2.2.1. Preparation of O/W nanoemulsions

Nanoemulsions were prepared in a two-step process. Initially coarse emulsions were obtained by mixing the oil phase with the aqueous phase at ambient temperature with mechanical stirring. Lecithin was solubilized in EVOO to formulate the oil phase. The aqueous phase was prepared by adding the more hydrophilic surfactants (Tween 20, Q-Naturale, Labrasol ALF) in water. Nanoemulsions were then formulated by passing the coarse emulsions through a Panda PLUS1000 (GEA, Niro Soavi) high-pressure homogenizer at 660–700 bar applying up to 12 recirculation passages. Nanoemulsions from the exit of the homogenizer were immediately cooled using an ice bath. Compositions of three different nanoemulsions are given in Table 1.

2.2.2. Addition of calcium citrate

Nanoemulsions were prepared as mentioned above by mixing the desired ingredients. Calcium citrate was added in water to result a solution of 0.15 μM. This solution was used as the continuous aqueous phase of the O/W nanoemulsions. After mechanical stirring, high-pressure homogenization was applied to obtain stable emulsions. Overall calcium concentration in the final nanoemulsions was 8 μg/g.

2.2.3. Encapsulation of vitamin D3

Vitamin D3 was encapsulated in the dispersed oil phase of the nanoemulsions following two different procedures: a) Vitamin D3 was added in an appropriate amount of EVOO and gently stirred to give a solution of 0.01% w/w (0.1 mg/g). Then, the concentrated vitamin solution was mixed with the other components to result O/W nanoemulsions as mentioned in Section 2.2.1. The nanoemulsion used for the encapsulation of vitamin D3 was System 1 (Table 1). Cholecalciferol concentration in the nanoemulsions was 2 μg/g.

b) Vitamin D3 was added in absolute ethanol to give a solution of 0.13% w/w. Then 0.5 mL of the solution was transferred to a DURAN® glass bottle and let evaporate the solvent. Following this, 250 g of nanoemulsions, prepared as mentioned in Section 2.2.1 were added in the bottle. Cholecalciferol concentration in the nanoemulsions was 2 μg/g.

2.2.4. Viscosity measurements

Viscosity measurements of the O/W nanoemulsions were performed at a shear rate of 7.5 s^{-1}, using a DV-I Prime Digital Viscometer (Brookfield Engineering Laboratories, USA), equipped with cone spindle (CPA-40Z). The temperature was kept constant at 25 °C using a water bath.

Table 1
Nanoemulsions (S1, S2, S3): composition and properties. Mean droplet diameter, polydispersity index (PdI) and viscosity were calculated immediately after preparation. All experiments were performed in triplicate and results are presented as average ± S.D.

System	Composition (w/w)	Homogenization circles	Size (nm)	PdI	Stability (days)	Viscosity (cP)
S1	EVOO 3.6% Lecithin 0.4% Tween20 4% Water 92%	12	285 ± 5	0.202 ± 0.010	37	1.3 ± 0.0
S2	EVOO 3.6% Lecithin 0.4% Q-Naturale 4%, Water 92%	12	210 ± 4	0.210 ± 0.006	7	1.0 ± 0.0
S3	EVOO 3.6% Lecithin 0.4% Labrasol 4% Water 92%	12	242 ± 2	0.102 ± 0.010	7	1.1 ± 0.0

Experiments were performed in triplicate for each sample, and results were presented as average ± S.D.

2.2.5. Dynamic light scattering measurements

Dynamic light scattering (DLS) is a technique for the characterization of different types of particles dispersed in a liquid medium. The Brownian motion of particles or molecules in suspension causes laser light to be scattered at different intensities. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein equation: $R_H = k_B T/6\pi\eta D$, where R_H is the hydrodynamic radius of the nanoparticle, k_B is the Boltzmann constant, T is the temperature in Kelvin, η is the viscosity of the sample and D is the diffusion coefficient.

Particle size and particle size distribution of the O/W nanoemulsions were measured using a Zetasizer Nano ZS (ZEN3600) from Malvern Instruments (UK) equipped with a He—Ne laser (632.8 nm) using a non-invasive back scatter (NIBS) technology. Measurements were carried out at the scattering angle of 173° and processed using the Malvern Zetasizer Nano software which fits a spherical model of diffusing particles with low polydispersity.

2.2.6. NMR spectroscopy

The nanoemulsion samples studied by NMR spectroscopy contained 3.6% EVOO, 0.4% lecithin, 4% Tween 20, 92% water and 0.17% vitamin D3. A quantity of 550 μL of the sample was further dissolved in 150 μL of D_2O for lock purposes. For the assignment of the vitamin resonances, a quantity of approx. 5 mg of the vitamin was dissolved in CD_3OD.

NMR spectra were recorded on a Varian 600 MHz spectrometer equipped with a $^1H\{^{13}C/^{15}N\}$ 5 mm PFG Automatable Triple Resonance probe using 5 mm tube. The homonuclear 2D ^1H—^1H gCOSY (gradient enabled correlation spectroscopy) and the heteronuclear 2D ^1H—^{13}C HSQC (heteronuclear single quantum coherence) experiments were run in order to facilitate the assignment of the peaks resonances.

Experimental data were processed using MestReNova 14.1.0 (Mestrelab Research — Chemistry Software Solutions).

^1H detected 2D DOSY experiments were recorded at 25 °C, using the bipolar pulse pair stimulated echo (Dbppste) pulse sequence of Varian library with diffusion delay 200 ms, gradient duration 2 ms and 25 values of gradient strength varying between 1300 and 32,500 G cm^{-1}. T1 measurements defined the relaxation delay to 8 s. Solvent presaturation has deemed necessary in order to overcome the dynamic range problem and allow for the observation of the weak vitamin signals. ^1H spectra were collected with 256 transients. Spectra were processed using the MestReNova Bayesian approximation.

2.2.7. EPR spectroscopy measurements

2.2.7.1. Membrane dynamics. Interfacial properties of O/W nanoemulsions were studied by Electron Paramagnetic Resonance (EPR) spectroscopy using the spin-probing technique. EPR measurements were performed at room temperature with a Bruker EMX EPR spectrometer operating at the X-band (9.8 GHz). Samples were contained in quartz EPR tubes Wilmad (Buena, NJ). EPR spectra were recorded with a center field of 0.349 T, scan range 0.01 T, gain of 5.64×10^3, time constant of 5.12 ms, conversion time of 5 ms, modulation amplitude of 0.4 mT. Data collection and analysis were performed using the Bruker WinEPR acquisition and processing program. All spectral simulations were performed with the EasySpin toolbox for EPR spectroscopy based on the MATLAB® platform (The MathWorks, Natick, USA) [28].

Sample preparation for EPR measurements was as follows: Initially, a stock solution of the spin probe (5-DSA) was prepared in absolute ethanol at a concentration of 7.8 mM. Then, 15 μL of the stock solution were added to Eppendorf tubes. After ethanol was evaporated, 1 mL of the nanoemulsions was added to each Eppendorf and kept for 1 day at ambient temperature to allow probe solubilization at the surfactants' layer. 5-DSA concentration in the nanoemulsions was 0.12 mM.

2.2.7.2. Free radical scavenging activity. Antioxidant properties of empty and vitamin loaded O/W nanoemulsions, were investigated by EPR spectroscopy using the stable free radical 4-hydroxy-TEMPOL.

Initially, an aqueous solution of 4-hydroxy-TEMPOL (1 mM) was prepared. Then, 0.9 mL of the free radical solution were mixed with 0.1 mL of the nanoemulsions and transferred directly to the EPR tube for analysis. In the present study, we used water (pH 5.8) as reaction medium since water is the main constituent of the O/W nanoemulsions.

EPR spectra were recorded at room temperature for 30 min with 5 min time intervals. To obtain the desired spectra, the instrument parameters were set to the following values: center field 0.349 T, receiver gain 4.48×10^3, time constant 1.28 ms, conversion time 5.00 ms modulation amplitude 0.4 mT, frequency 9.78 GHz.

EPR spectrum of 4-hydroxy-TEMPOL consists of three peaks. Upon addition of vitamin containing nanoemulsion, EPR signal intensity was decreased. The % inhibition of the EPR spectrum was calculated from the following equation:

$$\%\text{Inhibition} = (A_{\text{Control}} - A_{\text{Sample}}/A_{\text{Control}}) \times 100$$

where A_{Control} is the integral intensity of the EPR spectrum of the free radical and A_{sample} is the integral intensity of the EPR spectrum of the radical in the nanoemulsions (either loaded or empty).

2.3. Statistical analysis

All nanoemulsions were prepared in triplicate. DLS, viscosity and EPR experiments were performed in triplicate. Mean values and standard deviations are given.

3. Results and discussion

3.1. EVOO-in-water (O/W) nanoemulsions: formation, physical stability, viscosity and membrane dynamics

One of the main objectives of this study was to formulate physically stable O/W nanoemulsions that could be used as delivery systems of vitamin D3. We therefore prepared three different O/W nanoemulsions containing EVOO as the dispersed oil phase and different combinations of food-grade surfactants using a two -step homogenization procedure.

Initially, O/W nanoemulsions were developed using water as the continuous phase, EVOO as the dispersed phase and a mixture of lecithin with Tween 20 as surfactants (S1). The ratio of the oil phase was kept constant (3.6% w/w) while the total surfactant concentration was 4.4% w/w. Tween 20 to lecithin weight ratio was 10:1. The hydrophilic–lipophilic balance (HLB) of the surfactants' mixture was 15.5, a lower value as compared to pure polysorbate (16.7). Tween 20 is an ethoxylated sorbitan ester based on lauric acid, a natural fatty acid, highly effective at forming oil in water emulsions and nanoemulsions [16,29,30]. Soybean lecithin on the other hand is a partly water-soluble natural mixture of lipids consisted mainly of phospholipids, glycolipids, triglycerides, and sterols. As phospholipids are components of cell membranes, lecithin is considered as biocompatible and biodegradable emulsifier with good safety profile. Over the past few decades, apart from the emulsification properties, lecithin has become also very important as a nutraceutical and food supplement ingredient [31].

The nanoemulsions were investigated for their particle size and particle size distribution by DLS analysis. The day of their preparation oil droplets of 285 ± 5 nm diameter and 0.202 ± 0.010 PdI were measured after 12 homogenization circles (Table 1 and Fig. 1). The stability of the nanoemulsions was assessed by DLS analysis of droplet size over time at constant temperature (Fig. 2). S1 systems were studied for 37 days and remained stable throughout this time. In parallel, a visual control was regularly performed to detect any signs of instability.

Then, O/W nanoemulsions based on EVOO (3.6% w/w), water (92% w/w) and a mixture of lecithin with Q-Naturale (4.4% w/w) were developed (S2). For comparison reasons, the weight ratio of Q-Naturale to lecithin was kept 10:1. Q-Naturale is a similar to polysorbate 80 but natural extract containing saponin as major emulsifier. Saponins comprise a large family of structurally related compounds containing a steroid or triterpenoid aglycone (oil soluble) linked to one or more glycoside moieties (water-soluble). The HLB value of the mixture of surfactants was calculated by adding values of the individual surfactants and was found 16.7. After 12 homogenization cycles, stable nanoemulsions containing oil droplets of 210 ± 4 nm and 0.210 ± 0.006 PdI were measured (Fig. 1). Nevertheless, these systems were destabilized rapidly and eventually separated within 7 days (Fig. 2).

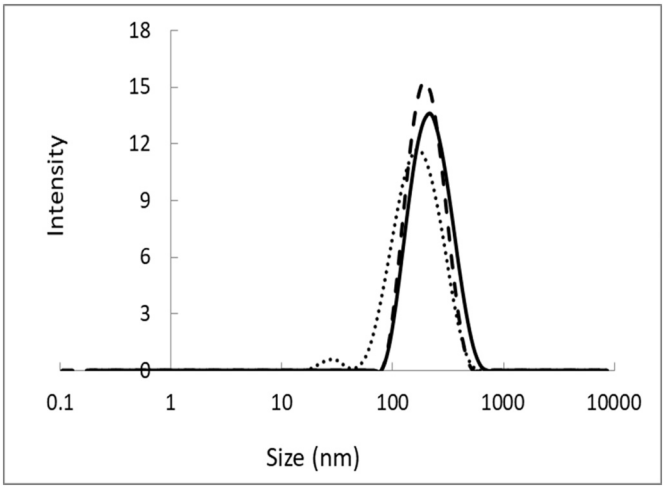


Fig. 1. Dynamic Light Scattering results of O/W nanoemulsions consisting of 92% w/w aqueous phase, 3.6% w/w oil phase and 4.4% w/w surfactants. (—): (S1) Water/EVOO/Tween 20/Lecithin; (…): (S2) Water/EVOO/Q-Naturale/Lecithin; (- - -): (S3) Water/EVOO/Labrasol/Lecithin.

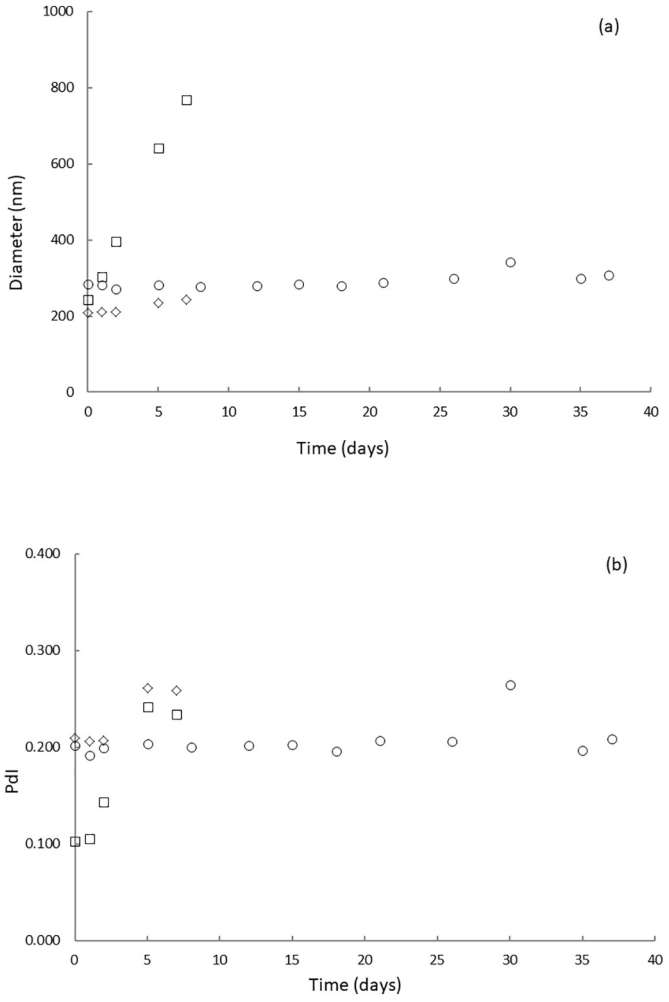


Fig. 2. Stability study of O/W nanoemulsions consisting of 92% w/w aqueous phase, 3.6% w/w oil phase and 4.4% w/w surfactants: a) Droplet size and b) polydispersity as a function of time. (○): S1, (◊): S2, (□): S3.

Finally, O/W nanoemulsions containing EVOO (3.6% w/w), water (92% w/w) and a 10:1 mixture of Labrasol ALF with lecithin were formulated (S3). Total surfactant's concentration was 4.4% w/w. Labrasol ALF (Caprylocaproyl Polyoxyl-8 glycerides, HLB 12) is a well-defined mixture of polyethylene glycol (PEG) esters, a small glyceride fraction and free PEG. The HLB of the surfactants' mixture was 11.2. Freshly prepared samples were characterized for the size and size distribution using dynamic light scattering. Oil droplets of 242 ± 2 nm and 0.102 ± 0.010 PdI were measured immediately after 12 homogenization cycles and cooling of the samples (Fig. 1). These nanoemulsions were also unstable and separated 7 days after their preparation (Fig. 2).

To summarize, formation and stability of olive oil-in-water (O/W) nanoemulsions prepared by high-pressure homogenization at constant ionic strength, surfactant-to-oil ratio and storage temperature was critically affected by the nature of the surfactant. As well established in the literature, the nanoemulsions characteristics, such as the droplet size distribution, optical and rheological stability, are governed by emulsification technologies and emulsifier types [32].

In the present study, DLS results show that the size and size distribution of the dispersed oil droplets was critically affected by the nature of the surfactants used to stabilize the emulsions. Nanoemulsions

stabilized with Tween20/Lecithin mixtures resulted in larger oil nanodroplets as compared to Labrasol/Lecithin and Q-Naturale/Lecithin mixtures. Nevertheless, all systems had droplet sizes between 200 and 300 nm and PdI values lower than 0.25. The observed close size distribution of the droplets could provide good stability to the systems as low polydispersity represent a smaller difference in chemical potential between droplets and reduce the Ostwald ripening rate [10,33]. Interestingly, nanoemulsions obtained using lecithin mixtures with Q-Naturale (S2) and Labrasol (S3) destabilized within 1 week despite the fact that they were initially smaller in size and relatively monodispersed. In S3 system, droplet size increased rapidly to result oil droplets of about 800 nm within 7 days (Fig. 2a). At the same time, the polydispersity index increased up to 0.234 (Fig. 2b) and the nanoemulsions' phases eventually separated. When S2 system was concerned, the two phases were separated within 1 week although neither the size nor the polydispersity of the oil droplets were rabidly increased (Fig. 2a and b). In a study of Yang et al. [32], the effectiveness of Q-Naturale for forming and stabilizing emulsions was compared with a synthetic surfactant that is widely used in the food industry, namely Tween 80. It was shown that Q-Naturale formed relatively small droplets ($d < 200$ nm) at surfactant-to-oil ratios <0.1 using high-pressure homogenization but the oil droplets were not as small as those produced using Tween 80 under similar conditions ($d < 150$ nm). In addition, O/W nanoemulsions had good long-term stability (one month) when stored at various holding temperatures (5, 37, and 55 °C). In a previous study of our group, O/W nanoemulsions formulated with 3:1 mixtures of Tween 20 and soybean lecithin as emulsifiers and soybean oil as oil phase had mean diameters of about 150 nm and narrow particle size distributions (PdI < 0.3). These nanoemulsions were stable for at least 30 days [16].

This behaviour could be possibly related to different steric interactions of the surfactants adsorbed on the oil droplets. Normally, due to the adsorbed layer of surfactant molecules on the droplet, steric interactions increase the repulsive maximum which in turn stabilize the emulsions against flocculation and coalescence. Ethoxylated esters of sorbitan and lauric acid (Tween 20) in combination with lecithin seems to produce more stable interfacial films due to stronger anchoring or formation of thicker layers as compared to Labrasol ALF and Q-Naturale, resulting in increased steric repulsion between droplets.

In another aspect, the choice of surfactant in an emulsion is made on the basis of attaining philic-phobic equilibrium, which in turn is influenced by the specific purpose of the emulsification. In general, the emulsions used to encapsulate lipophilic bioactive substances are of the O/W type and the surfactants used have a hydrophilic inclination. In the present study, the combination of lecithin with Tween 20 could have efficiently moderated the hydrophobicity of the phospholipid molecule to facilitate a greater philic-phobic homogenization [33].

To extend the DLS findings, Electron Paramagnetic Resonance (EPR) spectroscopy using the spin-probing technique was applied to study the interfacial properties of the surfactants monolayer in O/W nanoemulsions (systems S1–3). For this purpose, 5-DSA, a spin-labeled fatty acid analog consisting of stearic acid and an N–O• moiety attached to the C-5 position of the hydrocarbon chain, was used. Due to its amphiphilic nature, 5-DSA was preferably localized at the oil/water interface interacting with the surfactant molecules [16,22].

As can be seen in Fig. 3, EPR spectra of the nitroxide spin probe 5-DSA consists of a triplet which is due to the hyperfine interaction between the spin of the nitrogen nucleus ($I = 1$) and that of the unpaired electron ($S = 1/2$). EPR spectra of 5-DSA in solution are characterized by three narrow peaks of almost the same intensities [34]. However, EPR spectra of unequal heights and widths are indicative of a restrictive motion of the spin probe in the environment where it is located.

To express the mobility of the probe and the rigidity of the interface quantitatively, the rotational correlation time (τ_R) and the order parameter (S) of the spin probe were calculated by computer-aided analysis of the EPR spectra using the Easy Spin simulation models for the "chili" function [35].

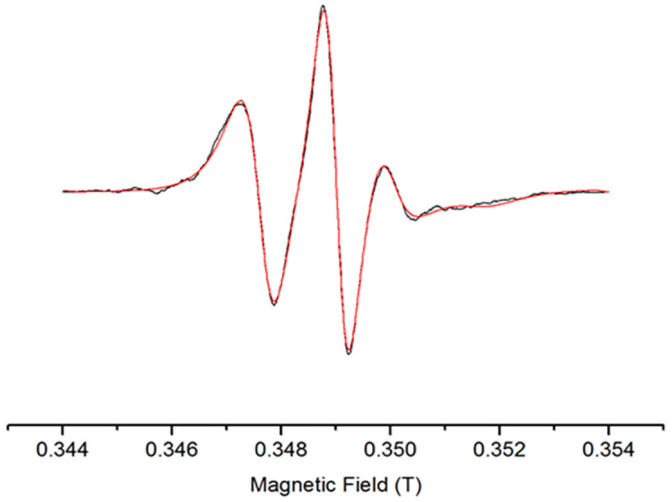


Fig. 3. Electron Paramagnetic Resonance (EPR) spectra (experimental-black line and simulated-red line) of 5-DSA in O/W nanoemulsions consisting of 92% w/w aqueous phase, 3.6% w/w oil phase and 4.4% w/w surfactants (S1).

The correlation time for the rotational motion (τ_R) is increased with the decrease in 5-DSA mobility, which reflects an increase in the microviscosity of the spin probe's environment. Increases of the order parameter reflect increases in the rigidity of the interface. The order parameter varies from 0 to 1, with $S = 1$ for the completely ordered state and $S = 0$ for the completely random state [36].

Table 2 presents the τ_R and S values of 5-DSA in systems S1–3. As can be observed, the nature of the surfactant used to disperse EVOO in water affected spin probe's mobility expressed as τ_R values. In other words, the addition of Q-Naturale together with lecithin in the O/W nanoemulsions (S2) leads to a more viscous environment near the spin probe resulting in the decrease of 5-DSA mobility. At the same time, order parameter value was considerably increased as compared to systems S1 and S3, indicating the formation of a more rigid surfactants' monolayer. System S3 that was formulated using a 10:1 mixture of Labrasol ALF with lecithin was characterized by the lowest τ_R and S values showing the formation of a rather fluid and low viscous layer of surfactants. Finally, S1 system, consisting of a 10:1 mixture of Tween 20 with lecithin as surfactants, had intermediate values of both τ_R and S indicating the formation of membranes with corresponding intermediate properties.

Polarity variations of the surfactants layer for the three different systems were assessed by the hyperfine splitting constant (α_N) which was calculated from the experimental EPR spectra as reported elsewhere [34]. As can be seen from the values of Table 2, α_N variations for systems 1 and 3 are small. However, when saponins were used as surfactants, the doxyl group of the spin probe was located in an environment with increased polarity.

To summarize, EPR spectroscopy findings indicate that O/W nanoemulsions using Tween 20, Q-Naturale and Labrasol, exhibited surfactant stabilized films of different local viscosities, polarities and

Table 2
Rotational correlation time (τ_R), order parameter (S) and hyperfine coupling constant (α_N) of 5-DSA in O/W nanoemulsions of different compositions revealed by EPR measurements.

System	τ_R (ns)	S	α_N (mT)
S1	5.28 ± 0.33	0.22 ± 0.10	13.72 ± 0.80
S2	6.62 ± 0.003	0.56 ± 0.01	17.14 ± 1.72
S3	4.40 ± 0.15	0.15 ± 0.02	13.59 ± 0.28

rigidities, depending on the nature of the surfactant. Taking into consideration that 5-DSA is localized in the area below the polar head groups and between the lipophilic tails of the surfactant molecules, we can conclude that each surfactant results in the formulation of layers with varying properties.

To conclude, O/W nanoemulsions of EVOO containing saponin-based amphiphilic molecules (S2) demonstrated the less flexible surfactants' monolayer, the lowest overall viscosity and low physical stability (Tables 1, 2 and Fig. 2). These characteristics can be probably related to the aglycone (oil soluble) part of the surfactant and also the oligosaccharide moieties extended in the aqueous phase of the emulsions and their interaction with the phosphoglycerides. On the contrary, nanoemulsions containing a mixture of polyethylene glycol esters, glycerides and free PEG (S3) showed very flexible membranes but the stability was also low as proved by DLS measurements. Finally, O/W nanoemulsions stabilized by mixtures of polysorbate 20 and phosphoglycerides (S1) were characterized by relatively fluid and moderately viscous interfaces. The viscosity of these nanoemulsions was 1.3 cP, a value which is close to that of most commercial fruit juices, energy drinks and ready-to-drink coffees and teas. What is more important, they were monodispersed (PdI = 0.2) and remained stable for 37 days having average droplet diameters of about 290 nm. From these results, it was suggested that O/W nanoemulsions based on polysorbates and lecithin were more appropriate as encapsulation vehicles of lipophilic compounds and were therefore applied for the encapsulation of vitamin D3.

3.2. Addition of health promoting compounds: vitamin D3 and calcium

Stable EVOO-in-water (O/W) nanoemulsions developed and characterized for their size, size distribution, viscosity and membrane dynamics were loaded with 8 μg/g calcium and 2 μg/g cholecalciferol (80 IU) to result a liquid, emulsions-based formulation. Water-insoluble compounds, such as vitamin D3, were incorporated into the O/W nanoemulsions either by dissolving the vitamin in the oil phase prior to emulsification or through addition to pre-prepared nanoemulsions as described above (Section 2.2.3). At the same time, water-soluble micronutrients were instantly solubilized in the continuous aqueous phase of the nanoemulsions before the homogenization step. Following this, multiple advanced instrumental techniques based on spectroscopy and scattering such as Electron Paramagnetic Resonance (EPR) spectroscopy, NMR spectroscopy, and Dynamic Light scattering (DLS) were applied to elucidate interactions between the encapsulated compounds and the constituents of the nanoemulsions.

3.2.1. DLS measurements

Initially, DLS measurements were carried out to investigate the hydrodynamic diameter, the polydispersity and the stability of the proposed formulation. Fig. 4 shows the stability study of empty and vitamin loaded O/W nanoemulsions. As can be observed, the presence of calcium ions in the aqueous phase strongly affected the stability of the nanoemulsions. Calcium ions bind to the hydroxyl groups of sorbitan head groups and also to the phosphate groups of lecithin resulting in weaker electrostatic repulsive forces between oil nanodroplets. Droplet size remained practically stable, 231 ± 0.4 nm, for 18 days whereas PdI values slowly increased from 0.215 to 0.225 within the same time period.

In the presence of vitamin D3, droplet size increased up to 265 ± 3 nm the day of emulsion preparation and remained stable for 18 days. The polydispersity of vitamin loaded emulsions was slightly decreased (0.194) as a result of vitamin addition in the oil phase and increased slowly up to 0.204 before phase separation.

As already discussed in a previous study, vitamin D3 addition in the dispersed phase of food-grade O/W nanoemulsions containing water, vegetable oils and mixtures of food grade emulsifiers affected the size of the oil cores by several nanometers [16].

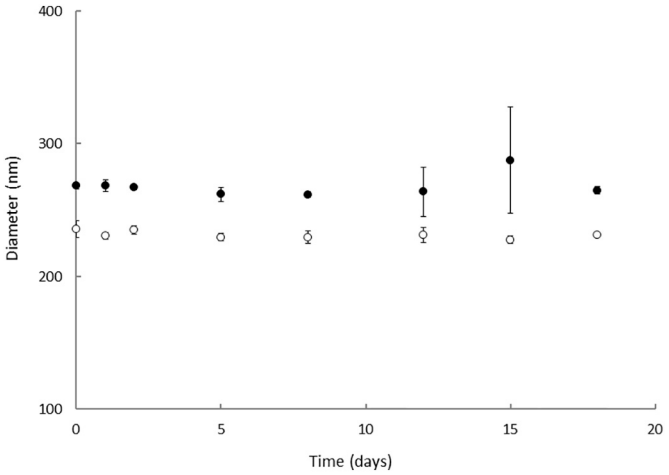


Fig. 4. Stability study of O/W nanoemulsions (S1 system) in the presence of calcium and Vitamin D3 (2 μg/g). (●): vitamin loaded, (○): empty nanoemulsions.

3.2.2. EPR measurements

In order to get deeper understandings of the structural changes occurring upon vitamin D3 addition in O/W nanoemulsions, EPR spin-probing spectroscopy was applied. For this purpose the amphiphilic spin-labeled fatty acid analog, 5-DSA, was used. Consequently, EPR spectra of the spin-labeled fatty acid in empty and loaded O/W nanoemulsions were obtained and analyzed using an anisotropic rotational model based on the slow-motion theory [35].

Fig. 5a and b shows experimental and simulated EPR spectrum of 5-DSA in empty and vitamin loaded nanoemulsions. Both spectra were characterized by the presence of three lines of unequal heights and widths. The observed EPR spectra anisotropy is indicative of spin probe's preferential rotation around the long molecular axis when located among the oriented surfactant molecules.

The mobility (τ_R) and order parameter (S) values obtained from computer simulations are reported and discussed below. In the empty system, both $\tau_R = 5.28 \pm 0.33$ ns and $S = 0.22 \pm 0.10$ values indicate a rather viscous but moderately ordered environment of the spin probe. When calcium ions were added in the continuous aqueous phase, local viscosity ($\tau_R = 5.50 \pm 0.08$ ns) and order parameter ($S = 0.27 \pm 0.05$) were increased. In the presence of cholecalciferol (vitamin D3) both values were increased up to $\tau_R = 5.61 \pm 0.23$ ns and $S = 0.32 \pm 0.05$ indicating a considerable change of both local viscosity and surfactants' layer order over vitamin's addition. Additionally, local polarity was examined by the hyperfine splitting constant (α_N) which was calculated from the experimental EPR spectra as reported elsewhere [34]. In the empty system, the polarity was $\alpha_N = 13.72 \pm 0.80$ mT a relatively low value, indicating that the nitroxide group of the spin probe is localized in the hydrophobic environment of the surfactant tails. Upon vitamin and calcium addition the polarity slightly increased up to $\alpha_N = 13.86 \pm 0.19$ mT since the presence of the oil soluble secosteroid molecules did not displace 5-DSA in an environment with different polarity.

Overall, EPR results show that in the presence of cholecalciferol, a steroid molecule with one open ring, there was no modification of local polarity although both local viscosity and order were increased at room temperature. In other words, cholecalciferol seems to be closely packed with the surfactant hydrophobic tails affecting both local viscosity and fluidity.

3.2.3. DOSY-NMR measurements

DOSY NMR has emerged as a valuable tool to describe intermolecular interactions between different molecular species and more specifically to study the encapsulation of pharmaceuticals and bioactive

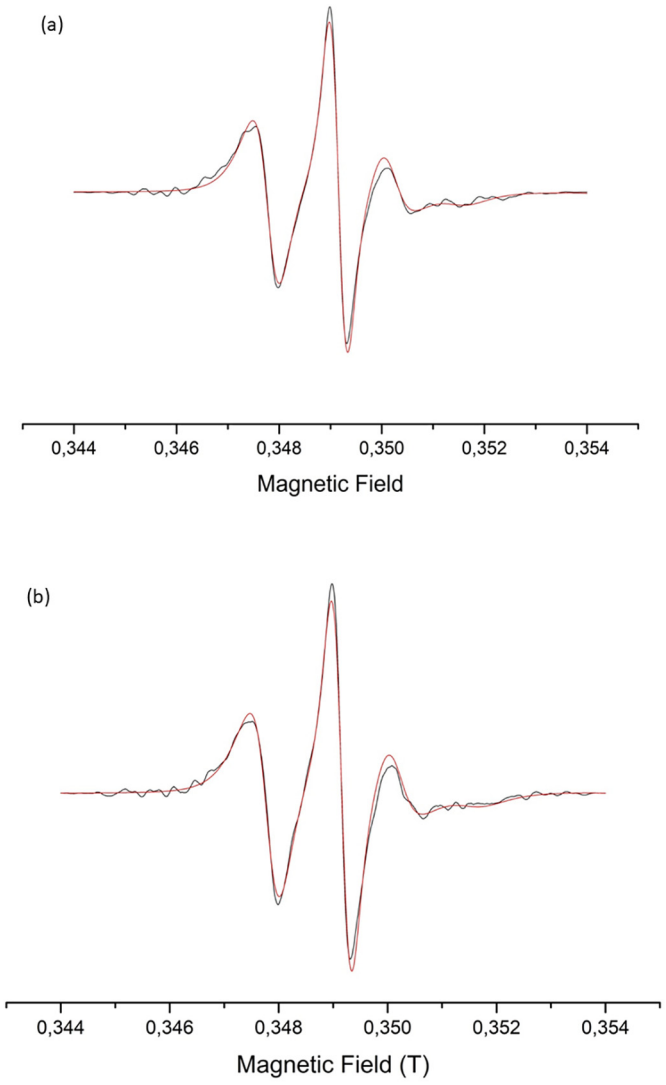


Fig. 5. Electron Paramagnetic Resonance (EPR) spectra (experimental-black line and simulated-red line) of 5-DSA in (a) empty and (b) loaded nanoemulsions. The system used for the EPR studies was the S1. D3 concentration was 2 μg/g.

natural products in molecular host-guest systems [37–39]. The role of nanoemulsions for the effective delivery of functional compounds has been recognized [40]. Yet, extensive literature search reveals that no study has up to now utilized 2D DOSY map to resolve the encapsulation of bioactives in such vehicles.

The current study has evaluated the characteristics of the O/W nanoemulsion and successfully probed the encapsulation of the lipophilic vitamin D3 in the emulsion environment.

The 2D DOSY map is displayed in Fig. 6a with the ^{1}H spectrum shown in the horizontal projection, enlarged to assist interpretation. Fig. 6b depicts the assignment of the emulsion components and Fig. 6c includes the assignment of the vitamin peaks. Resonances assignment has been assisted by literature data and the use of homonuclear and heteronuclear 2D spectroscopy.

The 2D DOSY map clearly indicates a major component associated with a diffusion coefficient of ($D_{diff} = 1.06 \cdot 10^{-7}$ cm^2 s^{-1}) including traces corresponding to the resonance peaks of EVOO, lecithin and Tween 20 and confirming the formation of the emulsion consisting of these components. Importantly, the sole vitamin resonances which are not overlapped and are attributed to the olefinic protons 6 and 7 and the methyl are clearly assembled with the nanoemulsions components indicating its encapsulation. Minor components are also observed in the map indicated by an area with faster diffusing components ($D_{diff} = 5.5–9.5 \cdot 10^{-7}$ cm^2 s^{-1}). Those are attributed to assemblies of Tween 20 which is evidenced mainly by the presence of the polyethylene oxide protons at 3.7 ppm. Of note, these minor assemblies are however unloaded.

To resume, the current DOSY NMR study confirmed the solubilization of the vitamin D3 in the nanoemulsion droplets.

3.3. Free radical scavenging activity

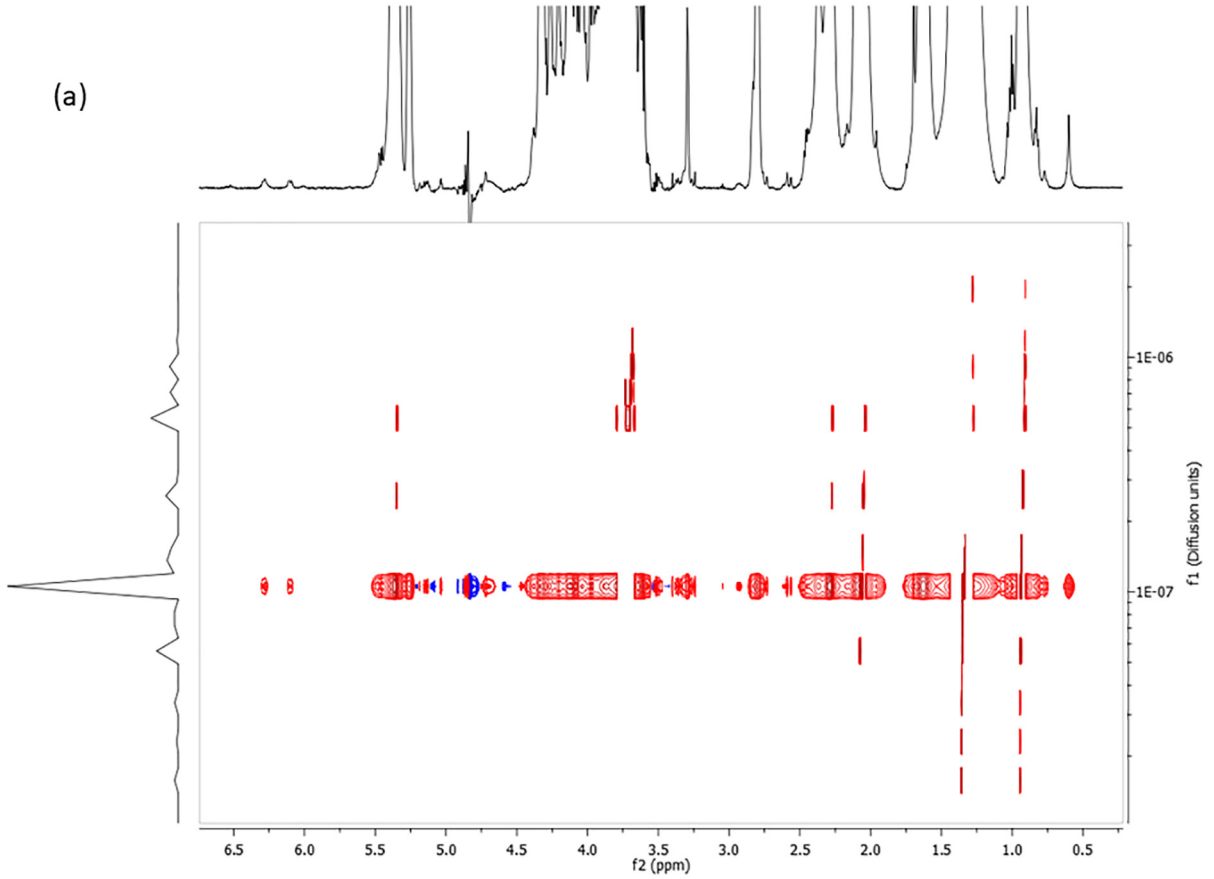
EPR spectroscopy using the stable free radical 4-hydroxy-TEMPOL was applied to study the potential antioxidant properties of the proposed nanocarriers. More specifically, vitamin loaded and vitamin free nanoemulsions (S1) were examined by an EPR procedure based on the principle of scavenging the 4-hydroxy-TEMPOL stable free radical. Previous studies have shown that EPR spectroscopy can assess the scavenging activity of both hydrophilic and hydrophobic antioxidant compounds, either free or encapsulated, against adequate free radicals [14,25,26]. In general, hydrogen-donating compounds react with 4-hydroxy-TEMPOL to generate EPR silent hydroxyl amines and unstable radicals. The molecular structure of 4-hydroxy TEMPOL and the rearrangements upon reduction to 4-hydroxy TEMPOL-H (change of angles between C—N—O, change in the value of electric dipole) have been reported. The energy released in the reduction reaction of TEMPOL (-261.1 kJ mol^{-1}) indicated a greater difficulty in reacting with antioxidant molecules as compared to other radicals [41]. On the other hand, in the presence of surfactant molecules (Tween 20) in the O/W nanoemulsions, a considerably higher H^+ release for efficient scavenging of 4-hydroxy TEMPOL electron could be observed, in accordance with proton-coupled electron transfer effect [33].

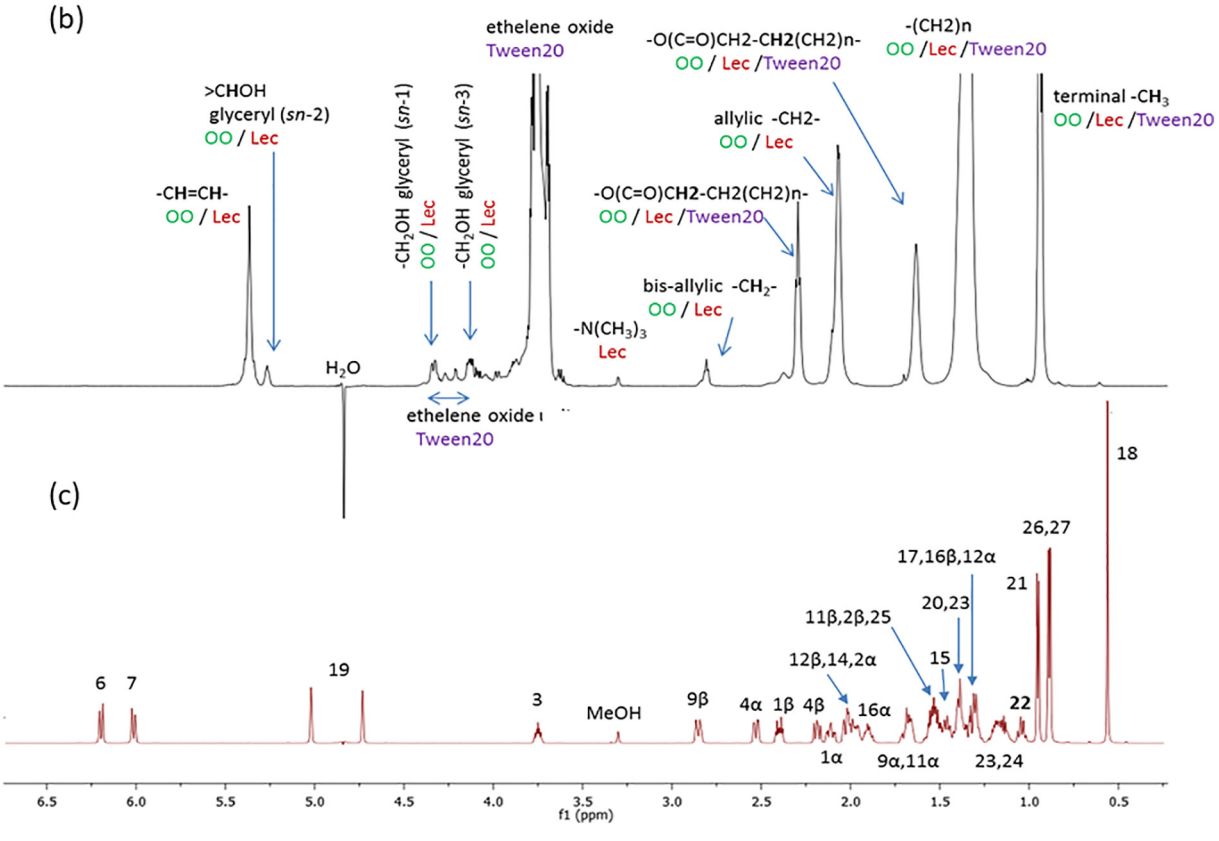
4-hydroxy-TEMPOL is a metal-independent and membrane-permeable cyclic nitroxyl radical, largely applied in studies of antioxidant activity [42]. Since 4-hydroxy-TEMPOL has a characteristic EPR spectrum whereas respective hydroxyl amine is diamagnetic and EPR-silent, its reduction by hydrogen-donating substances such as HNO has been demonstrated using EPR spectroscopy [43]. In a previous study of our group, the antioxidant activity of some commercially available fruit and vegetable juices was evaluated with regard to their radical scavenging activity against 4-hydroxy-TEMPOL monitored by EPR spectroscopy [27]. Moreover, the radical scavenging activity of carotenoids extracted from halophilic *Archaea* encapsulated in micro- and nanoemulsions was examined by EPR spectroscopy using the hydrophilic 4-hydroxy-TEMPOL. Interestingly both encapsulating systems showed similar capability to retain the carotenoids antioxidant capacity [14].

In these series of experiments, we examined the influence of incubation time on the 4-hydroxy-TEMPOL inhibition percentage. The scavenging reaction was studied for 30 min. Fig. 7a shows EPR spectra of 4-hydroxy-TEMPOL and its decrease upon addition of vitamin loaded nanoemulsions. The observed decrease of the integral intensity of the EPR spectrum of 4-hydroxy-TEMPOL indicates the ability of the nanocarriers to quench water-soluble cyclic nitroxyl radicals in the presence of water-oil interfaces.

Fig. 7b shows the inhibition effect of both empty and vitamin loaded olive oil-in-water nanoemulsions as a function of time. Both nanoemulsions presented relatively small EPR signal inhibition. As can be observed in Fig. 7b, the percentage inhibition of the stable free radical was linearly increased for the first 15 min before a plateau was reached.

Fig. 6. (a) 2D DOSY map of the emulsion loaded with vitamin D3. The ^{1}H spectrum shown in the horizontal projection is enlarged to enable the visualization of the small vitamin resonances (encircled at the DOSY map). (b) ^{1}H NMR spectrum of the emulsion showing the resonance peak assignment of the distinct components in a color coding format (EVOO peaks in green, lecithin peaks in red and Tween 20 in purple). (c) ^{1}H NMR spectrum of the vitamin D3 in MeOD with the assignment of the peaks. ^{1}H chemical shift scales in all three spectra are aligned to assist interpretation.


(a)
1E-06
1E-07
f1 (Diffusion units)
6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f2 (ppm)


(b)
ethelene oxide
Tween20
-O(C=O)CH2-CH2(CH2)n-
OO / Lec /Tween20
-(CH2)n
OO /Lec /Tween20
terminal -CH3
OO /Lec /Tween20
>CHOH
glyceryl (sn-2)
OO / Lec
allylic -CH2-
OO / Lec
-CH=CH-
OO / Lec
-CH2OH glyceryl (sn-1)
OO / Lec
-CH2OH glyceryl (sn-3)
OO / Lec
-O(C=O)CH2-CH2(CH2)n-
OO / Lec /Tween20
bis-allylic -CH2-
OO / Lec
-N(CH3)3
Lec
H2O
ethelene oxide
Tween20
(c)
18
26,27
17,16β,12α
21
20,23
11β,2β,25
15
12β,14,2α
6 7
19
3
MeOH
9β
4α 1β 4β
16α
22
1α
9α,11α
23,24
6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

More specifically, 38% of 4-hydroxy-TEMPOL signal was reduced within the first 15 min of the reaction before a plateau was reached at about 40% inhibition after 20 min. When vitamin loaded nanoemulsions containing 2 μg/g vitamin D3 were added in the reaction mixture, the scavenging effect was not further increased most probably due to the low vitamin concentration as compared to the phenolic compounds already present in the EVOO.

The concentration of the vitamin was kept at low levels (2 μg/g) since the proposed system was designed as an emulsion-based ready-to use food supplement (5 g) containing 0.01 mg (400 IU) of encapsulated vitamin D3. This formulation could be easily and safely consumed as a shot drink on a daily basis.

The results reported in this work are in accordance with previous studies dealing with the antioxidant activity alterations in media of different polarities, where such activation was related to affinities towards oil-air interfaces in bulk oils versus oil-water interfaces in emulsion [14,16]. Phenolic compounds present in EVOO, cholecalciferol itself and also surfactant molecules possess moieties with radical scavenging properties thus resulting in EPR signal reduction [16,27]. Since quenching agents of both lipophilic and amphiphilic nature are present in the reaction medium it can be assumed that the scavenging reaction most likely occurs in the oil-water interface.

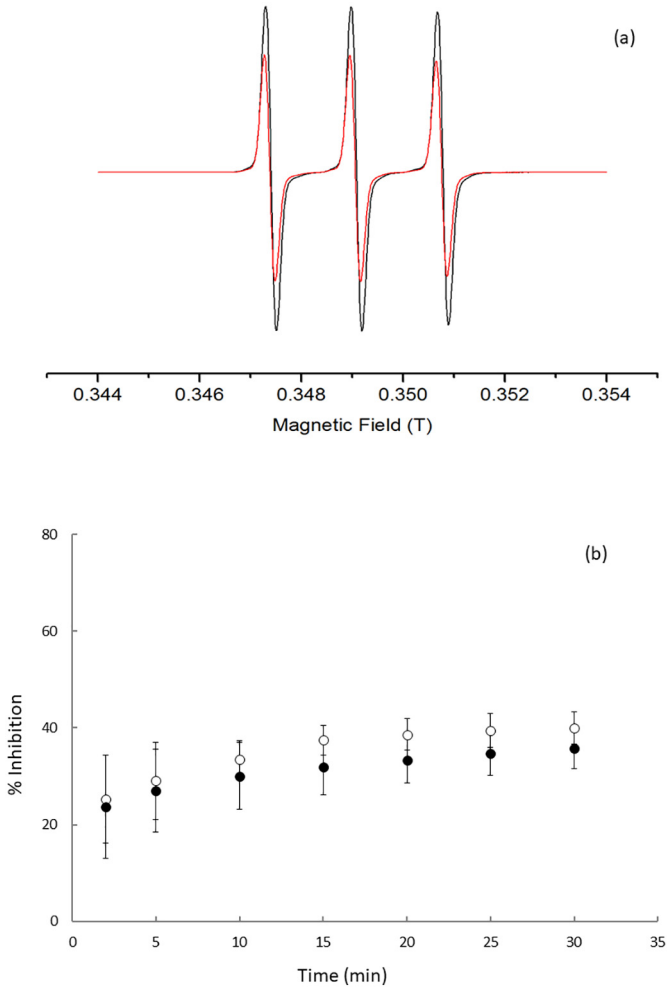


Fig. 7. a) EPR spectra of 4-hydroxy-TEMPOL (black line: EPR spectrum of the free radical, red line: EPR spectrum of the free radical in addition of full nanoemulsions after 30 min) and b) Scavenging effect of EVOO-in-water (O/W) nanoemulsions on 4-hydroxy-TEMPOL stable free radical as a function of incubation time using EPR spectroscopy. Vitamin D3 concentration was 2 μg/g. (●): vitamin loaded, (○): empty nanoemulsions.

4. Conclusions

In the present study structural changes in stable extra virgin olive oil-in-water nanoemulsions loaded with vitamin D3 and calcium citrate to be used for food supplementation were investigated by means of DLS, EPR, and DOSY-NMR.

DLS results show that the size and size distribution of the dispersed oil droplets at constant surfactant-to-oil ratio was affected by the nature of the surfactants used to stabilize the systems. When vitamin D3 was added in the dispersed oil phase of the nanoemulsions, the size of the oil cores was increased by several nanometers. On the other hand, the presence of calcium ions in the continuous phase affected the stability of the nanoemulsions, yet the formation of stable formulations was still possible.

EPR results indicate an increase of local viscosity and surfactants' layer rigidity over vitamin's addition most probably due to vitamin's participation in the interface. The addition of vitamin D did not have any significant effect on the antioxidant potential of the systems.

Finally, DOSY NMR results clearly revealed the formation of the surfactant based oil-in-water assemblies and the effective encapsulation of the lipophilic vitamin D3 in the emulsion environment, as expected.

Overall, our study has shown that the proposed ready-to use nanoemulsions may have important implication for the design and utilisation of food supplements that can be easily consumed as a shot drink on a daily basis.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

S. Demisli: Investigation, Formal analysis, Visualization, Conceptualization.**I. Theochari:**Investigation.**P. Christodoulou:**Investigation, Formal analysis.**M. Zervou:**Formal analysis, Writing - original draft.**A. Xenakis:**Conceptualization, Writing - review & editing.**V. Papadimitriou:**Conceptualization, Writing - original draft, Writing - review & editing.

Acknowledgements

This work was supported by the Interinstitutional Program of Postgraduate Studies (MSc) in Bio entrepreneurship which is co-organised by the Department of Biochemistry and Biotechnology, University of Thessaly and the Institute of Chemical Biology, National Hellenic Research Foundation.

References

[1] H. Sing, Nanotechnology applications in functional foods; opportunities and challenges, Prev. Nutr. Food Sci. 21 (2016) 1–8.
[2] L. Salvia-Trujillo, R.C. Soliva-Fortuny, M.A. Rojas-Graü, O. Martín-Belloso, D.J. McClements, Edible nanoemulsions as carriers of active ingredients: a review, Annu. Rev. Food Sci. Technol. 8 (2017) 439–466.
[3] I. Katouzian, S.M. Jafari, Nano-encapsulation as a promising approach for targeted delivery and controlled release of vitamins, Trends Food Sci. Technol. 53 (2016) 34–48.
[4] R. Tolve, F. Galgano, M. Caruso, F. Tchuenbou-Magaia, N. Condelli, F. Favati, Z. Zhang, Encapsulation of health-promoting ingredients: applications in foodstuffs, Int. J. Food Sci. Nutr. 67 (8) (2016) 888–918.
[5] M.A. Augustin, Y. Hemar, Nano- and micro-structured assemblies for encapsulation of food ingredients, Chem. Soc. Rev. 38 (2009) 902–912.
[6] N. Garti, I. Yuli-Amar, Micro- and nano-emulsions for delivery of functional food ingredients, in: N. Garti (Ed.), Delivery and Controlled Release of Bioactives in Foods and Nutraceuticals, Woodhead Publishing Limited, Cambridge 2008, pp. 149–183.
[7] Y. Li, J. Zheng, H. Xiao, D.J. McClements, Nanoemulsion-based delivery systems for poorly water-soluble bioactive compounds: influence of formulation parameters on polymethoxyflavone crystallization, Food Hydrocoll. 27 (2012) 517–528.

[8] M. Fathi, M.R. Mozafari, M. Mohebbi, Nanoencapsulation of food ingredients using lipid based delivery systems, Trends Food Sci. Technol. 23 (2012) 13–27.
[9] T.G. Mason, J.N. Wilking, K. Meleson, C.B. Chang, S.M. Graves, Nanoemulsions: formation, structure, and physical properties, J. Phys. Condens. Matter 18 (2006) R635–R666.
[10] A. Gupta, H. Burak Eral, T.A. Hatton, P.S. Doyle, Nanoemulsions: formation, properties and applications, Soft Matter 12 (2016) 2826–2841.
[11] G. Davidov-Pardo, D.J. McClements, Nutraceutical delivery systems: resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification, Food Chem. 167 (2015) 205–212.
[12] R. Walker, E.A. Decker, D.J. McClements, Development of food-grade nanoemulsions and emulsions for delivery of omega-3 fatty acids: opportunities and obstacles in the food industry, Food Funct. 6 (2015) 42–55.
[13] D.J. McClements, Nanoemulsion-based oral delivery systems for lipophilic bioactive components: nutraceuticals and pharmaceuticals, Ther. Deliv. 4 (2013).
[14] M. Chaari, I. Theochari, V. Papadimitriou, A. Xenakis, E. Ammar, Encapsulation of carotenoids extracted from halophilic *Archaea* in oil-in-water (O/W) micro- and nanoemulsions, Colloids Surf. B: Biointerfaces 161 (2018) 219–227.
[15] B. Ozturk, S. Argin, D.J. McLements, Nanoemulsion delivery systems for oil-soluble vitamins: influence of carrier oil type on lipid digestion and vitamin D_3 bioaccessibility, Food Chem. 187 (2014) 499–506.
[16] I. Golfomitsou, E. Mitsou, A. Xenakis, V. Papadimitriou, Development of food grade O/W nanoemulsions as carriers of vitamin D for the fortification of emulsion based food matrices: a structural and activity study, J. Mol. Liq. 268 (2018) 734–742.
[17] B. Ozturk, Nanoemulsions for food fortification with lipophilic vitamins: production challenges, stability, and bioavailability, Eur. J. Lipid Sci. Technol. 119 (2017) 1–18.
[18] M.F. Holick, High prevalence of vitamin D inadequacy and implications for health, Mayo Clin. Proc. 81 (2006) 353–373.
[19] S. Christakos, P. Dhawan, A. Porta, L.J. Mady, T. Seth, Vitamin D and intestinal calcium absorption, Mol. Cell. Endocrinol. 347 (1–2) (2011) 25–29.
[20] C.C. Berton-Carabin, M.-H. Ropers, C. Genot, Lipid oxidation in oil-in-water emulsions: involvement of the interfacial layer, Compr. Rev. Food Sci. Food Saf. 13 (2014) 945–977.
[21] M. Katsouli, V. Polychniatou, C. Tzia, Optimization of water in olive oil nanoemulsions composition with bioactive compounds by response surface methodology, LWT 89 (2018) 748–749.
[22] V. Papadimitriou, T.G. Sotiroudis, A. Xenakis, Olive oil microemulsions: enzymatic activities and structural characteristics, Langmuir 23 (2007) 2071–2077.
[23] L.C. Peng, C.H. Liu, C.C. Kwan, K.F. Huang, Optimization of water-in-oil nanoemulsions by mixed surfactants, Colloids Surf. A Physicochem. Eng. Asp. 370 (2010) 136–142.
[24] A. Kalaitzaki, M. Emo, M.J. Stébé, A. Xenakis, V. Papadimitriou, Biocompatible nanodispersions as delivery systems of food additives, Food Res. Int. 54 (2013) 1448–1454.
[25] E.D. Tzika, M. Christoforou, S. Pispas, M. Zervou, V. Papadimitriou, T.G. Sotiroudis, E. Leontidis, A. Xenakis, Influence of nanoreactor environment and substrate location on the activity of horseradish peroxidase in olive oil-based w/o microemulsions, Langmuir 27 (2011) 2692–2700.
[26] V. Papadimitriou, T.G. Sotiroudis, A. Xenakis, N. Sofikiti, V. Stavyiannoudaki, N. Chaniotakis, Oxidative stability and radical scavenging activity of extra virgin olive oils: an electron paramagnetic resonance spectroscopy study, Anal. Chim. Acta 573–574 (2006) 453–458.
[27] E.D. Tzika, V. Papadimitriou, T.G. Sotiroudis, A. Xenakis, Antioxidant properties of fruits and vegetables shots and juices: an electron paramagnetic resonance study, Food Biophys. 3 (2008) 48–53.
[28] S. Stoll, A. Schweiger, EasySpin, a comprehensive software package for spectral simulation and analysis in EPR, J. Magn. Reson. 178 (1) (2006) 42–55.
[29] Y. Yuan, Y. Gao, J. Zhao, L. Mao, Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions, Food Res. Int. 41 (2008) 61–68.
[30] J.S. Komaiko, D.J. McClements, Formation of food-grade nanoemulsions using low-energy preparation methods: a review of available methods, Compr. Rev. Food Sci. Food Saf. 15 (2016) 331–352.
[31] G.R. List, Soybean lecithin: food, industrial uses, and other applications, in: M.U. Ahmad, X. Xu (Eds.), Polar Lipids, Biology, Chemistry and Technology, Academic Press and AOCS Press, Elsevier 2015, pp. 1–33.
[32] Y. Yang, M.E. Leser, A.A. Sher, D.J. McClements, Formation and stability of emulsions using a natural small molecule surfactant: *Quillaja* saponin (Q-Naturale®), Food Hydrocoll. 30 (2013) 589–596.
[33] P. Malik, G.K. Inwati, T.K. Mukherjee, S. Singh, M. Singh, Green silver nanoparticle and Tween-20 modulated pro-oxidant to antioxidant curcumin transformation in aqueous CTAB stabilized peanut oil emulsions, J. Mol. Liq. 291 (2019), 111252.
[34] S. Avramiotis, V. Papadimitriou, E. Hatzara, V. Bekiari, P. Lianos, A. Xenakis, Lecithin organogels used as bioactive compounds carriers. A microdomain properties investigation, Langmuir 23 (2007) 4438–4447.
[35] J.H. Freed, Theory of slow tumbling ESR spectra for nitroxides, in: L.J. Berliner (Ed.), Spin Labeling, Theory and Applications, Academic Press, New York 1976, pp. 53–132.
[36] O.H. Griffith, P.C. Jost, Lipid spin labels in biological membrane, in: L.J. Berliner (Ed.), Spin Labeling, Theory and Applications, Academic Press, New York 1976, pp. 454–484.
[37] G. Pagès, V. Gilard, R. Martino, M. Malet-Martino, Pulsed-field gradient nuclear magnetic resonance measurements (PFG NMR) for diffusion ordered spectroscopy (DOSY) mapping, Analyst 142 (2017) 3771, https://doi.org/10.1039/c7an01031a.
[38] M. Verbrugghe, P. Sabatino, E. Cocquyt, P. Saveyn, D. Sinnaeve, P. Van der Meeren, J.C. Martins, Solubilization of flurbiprofen with non-ionic Tween 20 surfactant micelles: a diffusion ^{1}H NMR study, Colloids Surf. A Physicochem. Eng. Asp. 372 (2010) 28–34, https://doi.org/10.1016/j.colsurfa.2010.09.020.
[39] Abhishek Dubey, Dae Won Park, Jung Eun Kwon, Yong Joon Jeong, Taegeun Kim, Inhye Kim, Se Chan Kang, Ki-Whan Chi. Int. J. Nanomedicine 2015:10 (Special Issue on diverse applications in Nano-Theranostics) 227–240 http://dx.doi.org/https://doi.org/10.2147/IJN.S88289.
[40] Jamuna Bai Aswathanarayan and Ravishankar Rai Vittal. Nanoemulsions and their potential applications in food industry. Front. Sustain. Food Syst. 3:95. doi: https://doi.org/10.3389/fsufs.2019.00095.
[41] A. B. dos Santos, D. H. S. Silva, V. da Silva Bolzani, L. Á. Santos, T. M. Schmidt, O. Baffa. Antioxidant properties of plant extracts: an EPR and DFT comparative study of the reaction with DPPH, TEMPOL and spin trap DMPO. J. Braz. Chem. Soc. 20 (2009) 1483–1492. http://dx.doi.org/https://doi.org/10.1590/S0103-50532009000800015.
[42] N.D. Yordanov, K. Ranguelova, Quantitative electron paramagnetic resonance and spectrophotometric determination of the free radical 4-hydroxy-2, 2, 6, 6-tetramethylpiperidinyloxy, Spectrochim. Acta A 56 (2000) 373–378.
[43] Y. Samuni, U. Samuni, S. Goldstein, The use of cyclic nitroxide radicals as HNO scavengers, J. Inorg. Biochem. 118 (2013) 155–161.