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Nanoemulsions and topical creams for the safe and effective delivery of lipophilic antioxidant coenzyme Q10

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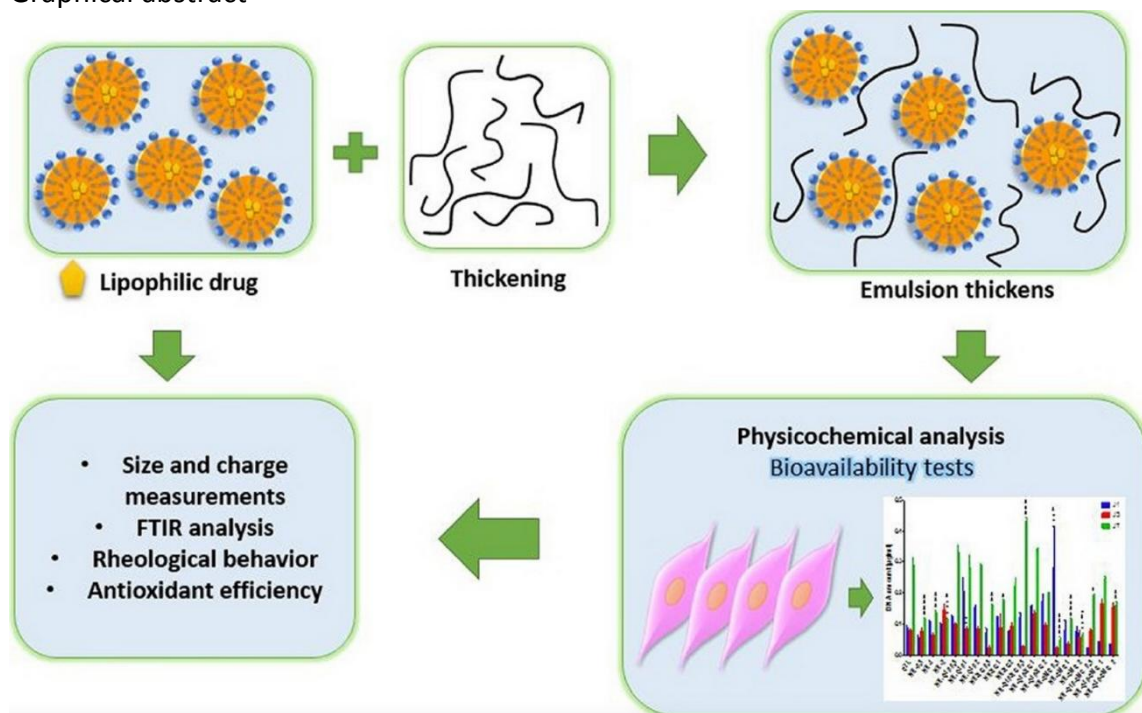
Highlights

- Natural nanoemulsion with double functionality with pure phospholipids lecithin.
- Vectorization of hydrophobic antioxidant (CoQ10) with natural nanovectors.
- Elaboration of cream with nanoemulsion and xanthan gum or carboxymethylcellulose.
- Interactions between antioxidant encapsulated and nanovectors or thickening agent.
- Nanovectors antioxidant efficiency and bioavailability on fibroblast cell.

Keywords

Nanoemulsions; Delivery systems; Lipophilic antioxidants PAOT; Coenzyme Q10; Thickener; Human skin fibroblasts

Graphical abstract



1. Introduction

Human skin is a uniquely engineered organ that permits terrestrial life by regulating heat and water loss from the body whilst preventing the ingress of noxious chemicals or microorganisms. For thousands of years, people used different substances on the skin either for therapeutic or esthetic effects and, in the modern era, a variety of topical formulations has been developed [1]. Skin drug delivery systems have been widely used nowadays for several purposes, e.g. to provide surface effects (e.g., sunscreens), dermal effects (e.g., corticosteroids), and systemic effects (e.g., nicotine patches) as well as deeper tissues (e.g. non-steroidal anti-inflammatory drugs) [[2], [3]]. Emulsion-based delivery systems have been developed to increase the topical bioavailability of lipophilic active compounds within skin membrane. These systems typically use relatively small oil droplets to encapsulate the lipophilic components [4]. The functionality of this type of delivery system can be tailored by controlling their composition and structure. For example, they can be formulated using different types and amounts of active molecules, carrier oil, emulsifier, stabilizers, and aqueous phase co-solvents. In addition, emulsions can be prepared with different particle size distributions, with diameters varying from under 50 nm to 500 nm. This gives great scope to create delivery systems with specific functional attributes for different applications [5]. Nanoemulsions (NE), as a type of emulsions with very fine droplet diameter (20–200 nm), have recently become increasingly important as potential vehicles for the controlled delivery of cosmetics and for the optimized dispersion of active principles in particular skin layers. Due to their lipophilic interior, NEs are more suitable for the transport of lipophilic compounds such as coenzyme Q10 (CoQ10). Furthermore, NEs gain increasing interest due to their own bioactive effect which is skin moisturizing by reducing trans-epidermal water loss. Nanoemulsions are often used in cosmetics because there is no inherent creaming, sedimentation, flocculation, or coalescence. Coenzyme Q10 is an important mitochondrial redox component and endogenously produced lipid-soluble antioxidant of the human organism. It plays a crucial role in the generation of cellular energy, enhances the immune system, and acts as a free radical scavenger. Ageing, poor eating habits, stress, and infection, they affect the organism's ability to provide adequate amounts of CoQ10 [6]. Often used to treat some skin conditions (e.g., facial vitiligo) or as anti-wrinkles in cosmetics and also it found in human skin with antioxidant activity. It prevents UV-induced erythema and inflammation, and has a protective role against skin cancer [7]. However, CoQ10 application in food and cosmetic formulations is limited by its instability when exposed to oxygen, light or high temperatures, and hence oil droplets are used as CoQ10 carriers. Emulsions texture properties are known to be of great importance, more particularly in cosmetics where the consumers' preference is closely connected to the texture of the product. These texture properties are given by some polymers that have the particularity to give oil-in-water (O/W) NE specific rheological behavior to ensure a good texture quality product. In this study, we used two polymers, known for their thickening properties at low concentration (2% w/w) in cold water, xanthan gum (XG) and sodium carboxymethylcellulose (CMC). XG is a polyelectrolyte with a helical secondary structure thus inducing high stabilization ability of dispersions as a result of its high viscosity at rest [8]. CMC is the most important cellulosic derivative water soluble, it is a polyanionic polymer often used as a stabilizer in food industry and cosmetics [[9], [10]]. The aim of this work was firstly to study the systematic preparation and characterization of nanoemulsions and nanoemulsion creams from natural sources (rapeseed lecithin) from enzymatic extraction

developed in our laboratory without solvents. In addition, the effects of hydrophobic molecules encapsulation and natural thickening addition on physicochemical properties, such as droplet size and physicochemical stability, were studied. The effect of CoQ10 active molecule on human skin fibroblast behavior was also observed *in vitro*. We evaluate parameters that affect the nanoemulsions properties to find an optimal formulation, subsequently characterize the nanoemulsions for physical stability and cellular proliferation. Also the aim of this study is to use a new innovative method to determine the antioxidant activity of Coenzyme Q10 in nanoemulsions and to show the effect of thickening agent addition (xanthan gum and CMC) with lecithin on cellular contact and in epifluorescence.

2. Materials and methods

2.1. Materials

We used rapeseed oil (vegetable oil, Lesieur, Asnières-sur-Seine, France), rapeseed lecithin (Lecresoyaf F60 IP, France) and deionized water to prepare nanoemulsions. Ethanol (EtOH), tetrahydrofuran (THF), acetonitrile, and iron (III) chloride (FeCl₃) (HPLC purity grade) used for high-performance liquid chromatography (HPLC). Bioactive compound coenzyme Q10 (CoQ10) and the hydrophilic carbohydrate polymers used as thickeners, namely xanthan gum (XG) from *Xanthomonas campestris* was purchased from (Sigma-Aldrich, Munich, Germany) and carboxymethylcellulose sodium salt (CMC) was purchased from Alfa Aesar (Karlsruhe, Germany).

2.2. Nanoemulsion preparation and CoQ10 encapsulation

Two types of nanoemulsions were prepared: nanoemulsion containing CoQ10 and nanoemulsion without CoQ10. Nanoemulsion formulation was 90% w/w deionized water, 10% w/w oil phase (76.6% w/w rapeseed oil and 23.4% w/w rapeseed lecithin) and 1.64% w/w CoQ10 [11]. Initially, rapeseed lecithin and CoQ10 were solubilized in rapeseed oil at 50 °C using water bath under stirring. The aqueous phase was poured in oil phase and the mixture was homogenized with vortex. Then, the raw emulsion was more finely dispersed using Vibra Cell 75115 sonicator (20 KHz, 500 W, 13 mm diameter cylindrical titanium probe, Bioblock Scientific, Illkirch, France) at 40% full power during 6 min (1 s on and 1 s off).

2.3. Cream formulation

Two polysaccharide polymers XG and CMC were selected for their frequent use in cosmetic fields. Each polymer was incorporated directly in nanoemulsions with 2% (w/w) concentration and homogenized at 11000 “rotation per minute” during 1 min using a T25 digital ultra-turrax IKA® equipped with the dispersing element S25N-25F (rotor-stator turbine).

2.4. Particle size

Z-average hydrodynamic diameter of oil droplets was determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instrument Ltd., UK). Before measurements, samples were diluted with ratio of 1:400 (v/v) in deionized water and placed in vertical

cylindrical cuvettes (10 mm diameter). Measurements were done at a 173° angle using photodiode detector set at 25 °C. Intensity autocorrelation functions were analyzed by CONTIN algorithm. The refractive index (RI) and absorbance were respectively 1.471 and 0.01. All measurements were repeated 3 times [12].

2.5. ζ potentials and electrophoretic mobility

ζ potentials and mobility of samples were measured using a Malvern Zetasizer Nano ZS (Malvern Instrument Ltd., UK). Measurements were carried out into folded capillary electrophoresis cell equipped with copper electrodes at 25 °C (equilibration time: 45 s, number of run: 30 runs). Quoted result is the average of 6 different measurements. Measurements were performed directly in diluted samples and measurements were made in triplicate [13].

2.6. Stability

Turbidity of samples were observed using a TurbiScan Lab Expert (Formulaction Co., France) by static multiple light scattering (MLS), which operates by sending a light beam through sample cells. Samples were placed without dilution in the test cells for transmitted and backscattered lights monitoring during 30 days. The apparatus reading head consists in a pulsed light source at near infrared (880 nm) and two synchronous detectors. The transmission detectors receive the light flux transmitted through the sample. The backscattering detector measures the light backscattered by the sample. The reading head saves transmission and backscattering data either at a chosen position on the sample cell, or every 40 μm while moving along the 55 mm cell height. Data were collected automatically every 5 min.

2.7. Emulsion stability

Three creams were manufactured for each polymer and stored at different temperatures (25 °C, 37 °C and 50 °C) for simulate the storage phenomena under different conditions. Their physico-chemical stability was evaluated at 0, 15, and 30 days of storage.

2.8. Rheological properties

Rheological tests were performed using a rotational rheometer Kinexus (Malvern Instruments, MA, USA) equipped with cone-plate geometry (CP2/50, 2° cone angle, 50 mm diameter, 0.07 mm gap). The applied shear ran from 0.001 to 1000 s^{-1} on 30 points to assess flow properties of samples at 25, 37 and 50 °C. Integration and acquisition time was 10 s with automatic resolution on each measurement cycle performed in triplicate.

2.9. ATR-FTIR analysis

Fourier transform infrared (FTIR) spectroscopy equipped with diamond attenuated total reflectance (ATR) sampler (Bruker-Tensor27®, Billerica, MA, United-States of America) was used to assess chemical stability of samples. Obtained spectra were compared to non-sonicated oil and lecithin mixture. FTIR spectra of oil samples were collected in 4000–

650 cm⁻¹ frequency region by co-adding 64 scans and at 4 cm⁻¹ resolution. All spectra were rationed against a background of air spectrum. After every scan, a new reference air background spectrum was taken. These spectra were recorded as absorbance values at each data point in triplicate. Analyses were done at 0, 5 and 30 storage days, and compared with fresh emulsions, fresh creams and non-sonicated oil phase mixture.

2.10. Coenzyme Q10 encapsulation (EE)

UV-1800 Shimadzu spectrophotometer (Kyoto, Japan) was used to evaluate encapsulation efficiency (E.E.) (CoQ10 retention, %) of CoQ10 at 281 nm absorbance peak. CoQ10 was dissolved at various known concentrations in hexane and a standard curve was generated. The E.E. was determined by ultrafiltration method using centrifugal filter tubes (Amicon ultra®, Millipore, USA) the molecular weight cutoff was 30 kDa, then 1 ml of NE-CoQ10 was put into the filter tube, and centrifuged at 3000g during 30 min. Measurements were made to detect CoQ10 in the filtrate. The E.E. was calculated following the below formula:

$$E. E. = \frac{\text{Total amount of CoQ10} - \text{Free amount of CoQ10}}{\text{Total amount of CoQ10}} \times 100 \quad (1)$$

2.11. Human skin cell behavior

2.11.1. Cell culture

In vitro cell behavior was carried out on human skin fibroblasts (ATCC CRL 2522) after NE stimulation. Cells were grown in a humidified atmosphere with 5% CO₂ at 37 °C with Dulbecco's modified eagle medium (DMEM) (Gibco, France) supplemented with 10% fetal bovin serum (FBS), 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 2.5 µg ml⁻¹ Fungizone® and 2 mM glutamine. The cells used for the experiments were seeded in 12-well and 96-well plates.

2.11.2. Cell membrane integrity

Cell membrane integrity was evaluated in 96-well plates at 24 h after stimulation by evaluation of lactate dehydrogenase (LDH) leakage according to manufacturer's instruction (LDH dehydrogenase activity assay kit, Sigma Aldrich, Munich, Germany). Briefly, cell supernatants were harvested after 10 min centrifuge at 300 g. and LDH reagent was added. The mixture was kept agitating for 30 min and then measurements were performed by spectrophotometer at 540 nm (Varioskan flash, Thermo Fisher Scientific Inc., Essone, France).

2.11.3. Cell metabolic activity

When cells are active the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is reduced to insoluble formazan by mitochondrial reductase. Cell metabolic activity was assessed in 96-well plates at 24, 48 and 72 h after NE stimulation using MTT. Cells were incubated with 1 mg ml⁻¹ MTT solution for 4 h at 37 °C. The blue formazan crystals formed were extracted from the cells by incubation with dimethylsulfoxide during 5 min at 37 °C. The formazan concentration was then measured by optical density determining (OD) at

570 nm by spectrophotometer (Varioskan flash, Thermofisher Scientific Inc., Essone, France) after subtracting the blank OD from the raw data.

2.11.4. Cell proliferation

Cell proliferation was evaluated by DNA concentration measurement. At day 1, 3 and 7, cells from 12-well plates were washed 3 times with phosphate buffered saline (PBS) then trypsinized. Cells were handled as previously described [14]. Briefly, cells were centrifuged during 10 min at 300g. and the pellets were suspended in Hoechst buffer. After cell lysis by thermic choc, samples were completed with 900 µl of Hoechst 33258 solution (0,1 µg ml⁻¹) (Invitrogen, molecular probes, France) then fluorescence (emission 460 nm/excitation 360 nm) was measured by spectrophotometer (Varioskan flash, Thermofisher Scientific Inc., Essone, France). To evaluate DNA concentration of samples, a standard curve was made using calf thymus DNA.

2.12. Antioxidant activity

PAOT Liquid Technology[®] (Total antioxidants power) was used for determination of total antioxidant and oxidant power in analyzed matrix such as raw materials and processed food products, cosmetic and medicinal preparations, biological fluids or extracts. The measurement is carried out in liquid medium by sample solubilization in reaction medium and measure the antioxidant activity directly in solution. The reaction medium of PAOT Technology[®] is mainly composed of physiological solutions with 6.7–7.2 of pH (to simulate the biological conditions), where 40 µl of sample are mixed with 2.5 ml of reaction medium [15]. Specific electrodes are immersed in the solution for a few minutes at temperature 25 ± 2 °C. The principle is based on variation of oxidized and reduced forms ratio of medium components. This modification results from the variation of the oxidized/reduced forms concentrations during the reaction (1) for the antioxidants and of the reaction (2) for the oxidants:

Reaction medium + AO (Antioxidant) → Reaction medium + AOOx (1)
(Antioxidant oxidation result)

Reaction medium + OA (Oxidant) → Reaction medium + OA Red (2)
(Result of oxidant reduction)

The results can be expressed with PAOT Score[®] (Total antioxidants power) or POT Score[®] units (Total Oxidative Power) per liter or per gram of analyzed sample (PAOT Score[®] or POT Score[®]/l or g of product) and can also be expressed with standard molecules (antioxidant and oxidant).

Calibration curves can be considered with pure molecules (for references), for antioxidants (trolox, vitamin C, vitamin E, polyphenols...) and for oxidizing molecules (chemical radicals: Acide 2,2'-azino-bis(3-éthylbenzothiazoline-6-sulphonique) "ABTS", Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium "DPPH", H₂O₂...).

2.13. Cell morphology analysis

Epifluorescence microscopy allowed the observation of nucleus and actin cytoskeleton of adherent cells respectively by staining with 4',6'-diamidino-2-phenylindole (DAPI) molecule and Alexa Fluor™ 488 phalloidin (Thermo Fisher Scientific Inc., Essone, France).

Cells were fixed with paraformaldehyde solution (4%) for 15 min and then rinsed during 5 min with PBS to remove non-adherent cells, and incubated for 15 min in a PBS solution containing 0.5% Triton™ X-100 to permeabilize them. This reaction is stopped by two successive washes with PBS.

The marking was carried out by diluting 50 µl of phalloidin, 50 µl of Triton™ X-100 and 0.025 g of bovine serum albumin (BSA) in 5 ml of PBS, this mixture is added to cells and is left to act 30 min. After incubation, three successive washes with PBS were performed.

For nuclei marking, DAPI solution was used by diluting of 5 µl of Triton™ X-100 and 5 µl of DAPI in 5 ml of PBS. Contact time was 20 s followed by 2 PBS rinses. Cells were covered with 100 µl of PBS before visualization by epifluorescence microscopy (Leica, Germany). The 12-well plates were protected from light throughout the preparation.

2.14. Hydration capacity measurement

The hydration capacity of cosmetic formulations was evaluated on hydrogel used as skin model. The chitosan-gelatin hydrogel was prepared by dissolving of 2 g of chitosan (Medium molecular weight, Sigma Aldrich, Munich, Germany) in 100 ml of distilled water containing 100 µl of acetic acid. Gelatin (gelatin from porcine skin, Sigma Aldrich, Munich, Germany) was dissolved in 100 ml of distilled water at 50 °C. After complete dissolution of chitosan and gelatin, the two solutions were mixed, and 2% of carbodiimide (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) was added. The mixture is poured onto petri dishes and dried at room temperature during 48 h.

For hydration tests, 1 g of each preparation (NE, NE-XG, NE-CMC) was spread and two hydration measurements were performed after 1 and 3 h using a CORNEOMETRE® CM825 (Monaderma, Monaco, France). The measurement principle is based on the dielectric capacitance. The value of water dielectric (81) is very different from many other substances contained in the skin (<7) that allows a highly selective measurement.

2.15. Statistical analysis

ANOVAs were performed using Minitab software. All tests were executed at 95% significance level to determine whether the different methods of emulsification and formulation influenced the stability and quality of the emulsions. All data are presented as the mean ± standard deviation (SD). Statistical significance was determined by one-way ANOVA with p-value < 0.05.

3. Results and discussion

3.1. Encapsulation efficiency

Several studies shown that CoQ10 encapsulation in oil/water emulsions increased its solubility and bioavailability [[16], [17], [18]]. NE allowed a good encapsulation efficiency with a high stability during storage [[19], [20], [21]]. Encapsulation ratio was 93.4%. This result was in accordance with Liu et al. results, who obtained a good CoQ10 encapsulation and high stability of nanostructured lipid carriers based on lecithin [22]. The high solubility of CoQ10 in oily phase improved encapsulation and vectorization. This solubility depended on fatty acids composition and temperature [[23], [24]]. NE used for vectorization allowed good yield due to small size distribution induced high droplets number with small volume and high stability [[5], [19], [25]].

3.2. Droplet size and electrophoretic mobility measurements

The different following formulations: NE (control), NE-XG and NE-CMC were stored during 30 days at three different temperatures: 25, 37 and 50 °C. Physical stability was evaluated by size droplets and electrophoretic mobility measurement (Table 1).

Size measurements shown that average droplets size was lower than 170 nm. In literature, nanoemulsions as a type of emulsions with very fine droplet diameter (20–200 nm), have recently become increasingly important as potential vehicles for cosmetics-controlled delivery and for optimized dispersion of active molecules in particular skin layers. So, studied emulsion were nanoemulsions.

Firstly, nanoemulsions droplets sizes in emulsions containing CoQ10 were compared with free CoQ10 nanoemulsions to determine the effect of CoQ10 incorporation on droplets size and shown that no significant variation on size was observed.

The polydispersity index (PDI) of NE, NE-GX et NE-CMC was 0.2. A small PDI below 0.2 indicates a narrow droplet size distribution and thus better stability against destabilization phenomena such as Ostwald ripening [26]. Therefore, electrophoretic mobility was $-5.60 \pm 0.04 \mu\text{m cm/Vs}$ at 25 °C for CMC-NE systems, $\approx -4.9 \pm 0.1 \mu\text{m cm/Vs}$ for GX-NE and $\approx -3.0 \pm 0.03 \mu\text{m cm/Vs}$ for NE. These values were stable during storage. Anionic thickening addition influenced the electrophoretic mobility but does not affect size distribution. Average size droplets and PDI values were stable for all storage time. The addition of polyanionic thickening in NE promoted negative surface charge that increased electrostatic repulsive forces between droplets avoiding destabilization phenomena such as coalescence and Ostwald ripening [20]. In addition, rapeseed lecithin used in the formulation has hydrophilic and lipophilic properties and forms a protective shield at the oil/water interface and helps to reduce interfacial energy to prevent coalescence [16]. Rapeseed lecithin emulsifier used for emulsification has hydrophilic and lipophilic properties that induced the formation of layers at oil/water interfaces reducing interfacial energy by creating a barrier against coalescence phenomena. Nanoemulsions have the ability to greatly increase the bioavailability of highly lipophilic encapsulated substances [28] stabilization of the newly formed interface is of great interest.

3.3. Turbidity

During the process of emulsion destabilization, light transmission through emulsion increased while retrodiffusion of oil phase decreased according to Turbiscan Lab principal and allowed emulsion stability measurement [29] at different temperatures. Measurement turbidity results shown stable emulsions at different temperature until 30 days and this was in agreement with size measurements results (result not shown). No sedimentation, creaming or flocculation was registered that indicate a good stability.

3.4. Chemical stability and oxidation

FTIR spectroscopy was used for characterization of interactions between NE compounds on molecular level. This technique was usually used for detection of any interaction or variation of polymorphic form to another by intensity of absorption band or wavelength change corresponding to chemical groups or new peaks appearance [30].

3.4.1. Lipid oxidation

FTIR spectroscopy was used to observe sonication effect on lipids in oily phase and the interaction between CoQ10 and oily phase. FTIR spectroscopy shows the presence or absence of specific functional groups which variations indicated the formation of primary oxidation products [31]. The evaluation of chemical stability of samples by FTIR analysis need identification of characteristics vibration related to the chemical functions of lipids sensitive to oxidation. Fig. 1A shows the FTIR Spectra of oily phase before and after sonication (3500–500 cm^{-1}) and different characteristic bands were identified using previous studies [[32], [33], [34], [35]]. The presented profiles indicated that sonication process had no alteration effect on lipids of oily phase. Peaks at 722 and 1461 cm^{-1} corresponding to cis double layer and aliphatic chains of fatty acids respectively did not change [[33], [36]]. According to Rohman and Che Man (2013), at 1744 cm^{-1} , peak increased when carboxylic compounds were formed during thermal oxidation which is not the case here. The two peaks at 2853 and 2923 cm^{-1} were not changed. The vibration band position of cis double stretching of unsaturated fatty acids between 3009 and 3006 cm^{-1} , which characterize the non-oxidized oils, had a same intensity before and after sonication [[31], [37]].

On the other hand, results shown that CoQ10 vectorization protected lipids from alterations due to sonication process. According to Talpur et al., a peak at 3471 cm^{-1} shows the presence of hydroperoxides and free fatty acids due to lipids oxidation. This peak did not appear on tested oily phases.

Peaks characterizing oxidation phenomenon had a same profile before and after sonication process, so, no oxidation was registered. We also observed that CoQ10 incorporation had no effect on lipids composition. This result can be explained by the natural antioxidant activity of CoQ10 and lecithin that promoted resistance of lipids against oxidation [38].

3.4.2. Chemical stability

FTIR spectroscopy was used for evaluation of chemical interactions could be produced between matrix compounds. FTIR spectra of studied emulsions shown similar profiles that indicated the chemical stability. In Fig. 1B, common bands between xanthan gum and carboxymethylcellulose was presented at 990–1100 cm^{-1} and 1530–1650 cm^{-1}

corresponding to C single bond H stretching vibration of alkanes groups (CH₂) and stretching vibration of C double bond O double bonds of β -dicetones, respectively [39]. C single bond H and C double bond O groups correspond to osidic residues that make up the thickening agents used in the formulation of cosmetic creams. These results shown the absence of denaturant interactions between Thickening and creams compounds.

3.5. Rheological characterization

3.5.1. Flow measurement preparations

Emulsion flow allows to predict their behavior under shear stress processes, packaging, and at using. Fig. 2 shows apparent viscosity and shear stress depending on shear rate for cream with xanthan gum and carboxymethylcellulose.

The tested emulsions had shear-thinning properties. For a given temperature and concentration (2%), XG cream shown viscosity at rest of 0.001 s⁻¹ and shear stress 10 times higher than CMC cream. Curves had the same appearance with a small decrease of viscosity when temperature increased. The estimation of viscosity at function of temperature and shear rate was made after testing different models: Herschel–Bulkley, Casson, Power law and Moore. Coefficient of correlation obtained is shown in Table 1 (supplementary data). Herschel–Bulkley model could not be applied to any system. Carboxyl groups provided to CMC an anionic charge in aqueous solution, furthermore, the nature of solubilization medium influenced CMC behavior due to important interchain association. CMC had a high solubility in water due to intermolecular interactions reduction that provided rheological properties of CMC solution (viscosity, thixotropy, pseudoplasticity). Results shown that CMC had pseudo-gel behavior in water and pseudo-plastic behavior in nanoemulsion.

3.5.2. Thixotropy cream analysis

Evaluation of emulsions thixotropic nature allowed evaluation of rheological properties depending on time and stress. Fluid is thixotropic when the stress at a constant shear rate decreases with time. Fig. 3 shows hysteresis loops obtained for each polymer. Xanthan gum and CMC solutions (2% w/w in distilled water) were used as controls to see templating effect acquired in O/W emulsion and in water. The CMC had a similar profile in both tested temperatures. CMC solubilization in an O/W emulsion did not allow restructuration after shearing compared to the CMC solution.

3.5.3. Viscoelastic properties determination

This measure allowed prediction of emulsions behavior under hard stress such as spreading on the skin or output from cream tube.

Viscous modulus G' and elastic modulus G'' did not changed by initial stress increasing. Structure disruption of sample under stress gradient could be determined when G' decreased and G'' increased above the certain stress value. During scanning distortion, G' modulus was constant until certain deformation value. Outside this range, G' became depending of deformation and decreased. This phenomenon could be interpreted as

material first breaking consequence. Linearity output can be determined when G' loses 10% of its set value [40]. Elastic and viscous moduli values of deformation and stress (G' , G'') are shown in Table 2.

Experiments gave the viscoelastic linear region (LVR), corresponding to the region where rheological properties do not depend on frequencies and viscoelastic moduli. Table 2 shows that studied samples had predominant elastic behavior ($G' > G''$) in viscoelastic linear region.

3.6. Bioavailability evaluation

3.6.1. Membrane integrity, metabolic activity and cell proliferation

Cell viability was determined by LDH test to check cell membrane integrity indicating the absence of cell lysis. Cellular metabolic activity was performed by MTT test consisting in tetrazolium reduction measurement to estimate cell metabolic activity [41].

Cells were stimulated for 7 days with three emulsions containing coenzyme Q10 and their respective (controls without coenzyme Q10) which are: NE, NE-XG and NE-CMC. Each preparation was tested with 0.5; 1 and 2 mg ml⁻¹ concentration by adding an appropriate volume of culture medium.

The metabolic activity of fibroblast was not dose-dependent and tested samples did not reduce the viability of fibroblasts, as shown in Fig. 4B. The decrease of metabolic activity was observed at 3 days for all systems. This could be due to stimulation after-effects where the cells try to adapt to prolonged exogenous stimulations.

Hoechst test allowed measurement of cellular DNA which is used as cell proliferation parameters. The results of this analysis were linked to metabolic activity results (Fig. 4C) and confirmed that tested formulations were dose-dependent. The peak increase observed after 7 days was due to cell proliferation indicating a good metabolic activity inducing cell growth.

After 7 days of incubation, a higher increase of DNA amount was registered for CoQ10 xanthan gum nanoemulsions than for CMC nanoemulsions. The addition of xanthan gum to nanoemulsions increased DNA amounts. The same observation was registered when CoQ10 was added to CMC nanoemulsions but the value was significantly lower than control and CoQ10 xanthan gum nanoemulsions. Addition of CMC to nanoemulsion with or without CoQ10 decreased significantly ($p < 0.0001$) DNA amount compared to xanthan nanoemulsions and tested emulsions.

These observations can be attributed firstly to CoQ10 which protected the DNA from reactive oxygen species [11], and participated in maintaining cell membrane integrity [6]. On the other hand, xanthan gum improved glucose metabolism inducing a better cell growth [42], while carboxymethylcellulose – being complex polysaccharide (cellulose derivative) – was not metabolized by fibroblast cell inducing lower growth rate [43]. CoQ10 addition improved DNA amount that remain lower than control. Thus, CMC was not suitable for cosmetic formulations and developed nanoemulsions shown no cytotoxicity. The presence of active agent favored cell proliferation confirming biological activity which confirm that CoQ10 is as key compound in the respiratory chain and DNA protector against free radicals [6]. Furthermore, it has been demonstrated that the thickening agents used were not toxic

for cells [44], and that nanoemulsion components and CoQ10 had beneficial effects on cell physiology [[6], [16], [45]]. Cell proliferation results were consistent with those observed with the LDH assay and with literature.

So, NE use for vectorization allowed good encapsulation yield due to small size distribution inducing high droplets number with small volume and high stability [[5], [19], [25]].

3.7. Antioxidant activity “PAOT score”

Fig. 5(A) shows total antioxidant activity expressed with PAOT Score® for studied nanoemulsions. Results shown that CoQ10 nanoemulsions with xanthan gum had a highest antioxidant activity. CoQ10 free emulsion shown a little antioxidant activity due to small fraction of rapeseed lecithin used as emulsifier and known as an antioxidant. Xanthan gum addition did not alter the total antioxidant activity of tested emulsions. Conversely, CMC significantly reduced antioxidant activity. This can be explained by masking action of functional groups of CoQ10 by the carboxymethylcellulose gum. The same ascertainment was registered for CMC addition to CoQ10 free emulsion. CMC decreased significantly ($p < 0.05$) the antioxidant activity compared to nanoemulsions samples, while XG addition. Antioxidant activity results were in accordance with biological results. CMC addition induce the decrease of antioxidant activity giving rise to DNA amounts decrease for human fibroblast cells.

3.8. Cell morphology analysis

After 1 and 7 days of stimulation with various samples at 0.5; 1 and 2 mg ml⁻¹ concentration, morphological characteristics of typical fibroblasts were observed by epifluorescence (labeling of nuclei with DAPI and actin filaments with phalloidin).

Fibroblasts preserved their original fusiform morphology. The images shown that the presence of CoQ10 has an effect on cell proliferation. This is in agreement with the previous observations.

As shown in Table 3, the three tested ratio shown different results. Firstly, Epifluorescence image analysis shown that 1 mg mL⁻¹ was a better ratio (emulsion/culture medium) and cell proliferation was higher than 0.5 and 2 mg mL⁻¹ ratio except for NE-XG-Q10 at 7 days, when 181 ± 09 cells was registered, who is a maximum of cells number, these results are in agreement with DNA amount results, which shown that xanthan gum and CoQ10 combination give a best proliferation. These observations are attributed firstly to CoQ10 which protected the DNA, and secondly, to xanthan gum which improve glucose metabolism inducing a better cell growth. On the other hand, the increase of cell number was registered for all tested formulation especially when CoQ10 was added, who means that all emulsion are safe vectors. Correlation of Epifluorescence images with DNA quantification measurement (Fig. 4). Epifluorescence was used for show the morphological aspect of treated cells. It is not usually used as quantitative analysis and it is different from live/dead assay.

3.9. Hydration capacity measurement

Nanoemulsions and creams were applied to hydrogel to simulate the application on skin. According to Corneometre® CM825, the threshold value of the capacitance indicating that proper hydration was 30 (no units). Measurement showed that the capacitance of the single hydrogel was 120. After nanoemulsion application, hydration value was 70 and did not change after 3 h. NE-XG cream application-maintained hydration of hydrogel at 120 after 3 h, while NE-CMC cream application showed a 120 capacitance value but decreased down to 110 after 3 h. Studied emulsions contained 95% water explaining the high values of capacitance. Furthermore, the cream prepared with the xanthan gum has an improved hydration property compared to NE-CMC cream. Nanoemulsions used has a hydration reduction at the surface which can be due to water evaporation or product penetration on hydrogel.

4. Conclusion

In many cases, it is advantageous to deliver bioactive lipids in an aqueous medium because they increase their palatability, and bioactivity. Emulsions are systems widely used for vectorization especially for hydrophobic substances such as CoQ10. The active agent release is very strongly related to the systems composition used for vectorization. In this paper, stable nanoemulsions was developed with natural components by sonication process at low-frequency ultrasound. The present paper studies the effect of CMC and XG Thickening in vectorization of highly hydrophobic biomolecules (CoQ10) using nano-structures which are nanoemulsions. Physico-chemical characters was studied and compared to systems without Thickening. Antioxidant activity was evaluated and was in accordance with human cells biocompatibility. Studied systems products exhibited good physical stability on one month storage. Cosmetic matrices showed no cytotoxicity, demonstrating their safety for an in vivo use. Thus, NE use for vectorization allowed good yield due to small size distribution inducing high droplets number with small volume and high stability. Since the nanoemulsions could successfully encapsulate the hydrophobic active molecule (CoQ10), therefore, their bioavailability, safety, and effectiveness were studied using LDH, MTT and DNA quantifying assays for potential cosmetic applications. For a better evaluation of sensorial quality of cosmetic matrices, test would be necessary with consumers' panel. The results shown that CoQ10 which protected the DNA, and xanthan gum which improve glucose metabolism inducing a better cell growth, while carboxymethylcellulose – being complex polysaccharide (cellulose derivative) – was not metabolized by fibroblast cell inducing lower growth rate. CoQ10 addition improved DNA amount that nevertheless remained lower than control. Thus, CMC was not suitable for cosmetics formulations. Studied cosmetic matrices shown no cytotoxicity. The presence of active agent favored cell proliferation confirming cream biological activity. CoQ10 is as key compound in the respiratory chain and DNA protector against free radicals

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Table 1

Particles charge and average diameter during 30 days of storage at 25, 37 and 50 ° C for emulsions and creams made with Xanthan gum (XG) or carboxymethylcellulose (CMC) with or without CoQ10. Measurement was made on diluted emulsions (1:400), d: days, PDI: Polydispersity index

Storage temperature	Sample	Average diameter (nm)			PDI			Electrophoretic mobility ($\mu\text{mcm/Vs}$)		
		0d	15d	30d	0d	15d	30d	0d	15d	30d
25	NE-CoQ10	144.0 ± 0.8	144.5 ± 1.5	146.3 ± 1.3	0.18 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	-3.10 ± 0.08	-3.40 ± 0.80	-3.10 ± 0.06
	NE-XG-CoQ10	123.6 ± 0.8	135.3 ± 0.5	142.6 ± 1.3	0.20 ± 0.01	0.18 ± 0.01	0.24 ± 0.01	-4.70 ± 0.08	-4.40 ± 0.04	-4.20 ± 0.12
	NE-CMC-CoQ10	158.6 ± 1.7	147.1 ± 1.5	152.3 ± 1.6	0.22 ± 0.01	0.21 ± 0.01	0.21 ± 0.01	-5.60 ± 0.04	-5.50 ± 0.18	-5.60 ± 0.09
37	NE-CoQ10	144.0 ± 0.8	137.1 ± 0.8	144.1 ± 2.2	0.18 ± 0.01	0.15 ± 0.01	0.20 ± 0.01	-3.10 ± 0.08	-3.10 ± 0.02	-3.00 ± 0.07
	NE-XG-CoQ10	123.6 ± 0.8	136.0 ± 0.7	140.0 ± 2.1	0.20 ± 0.01	0.23 ± 0.03	0.22 ± 0.01	-4.70 ± 0.08	-4.70 ± 0.86	-4.50 ± 0.11
	NE-CMC-CoQ10	158.6 ± 1.7	160.7 ± 1.0	164.8 ± 1.2	0.22 ± 0.01	0.21 ± 0.01	0.15 ± 0.01	-5.60 ± 0.04	-6.10 ± 1.11	-6.20 ± 0.08
50	NE-CoQ10	144.0 ± 0.8	150.7 ± 1.0	152.6 ± 1.7	0.18 ± 0.01	0.22 ± 0.01	0.23 ± 0.01	-3.10 ± 0.08	-3.30 ± 0.02	-3.10 ± 0.04
	NE-XG-CoQ10	123.6 ± 0.8	142.1 ± 2.0	149.2 ± 0.7	0.20 ± 0.01	0.21 ± 0.01	0.23 ± 0.01	-4.70 ± 0.08	-4.80 ± 0.03	-4.90 ± 0.02
	NE-CMC-CoQ10	158.6 ± 1.7	161.4 ± 2.1	151.9 ± 2.5	0.22 ± 0.01	0.26 ± 0.01	0.20 ± 0.01	-5.60 ± 0.04	-5.80 ± 0.10	-6.30 ± 0.02

Table 2

Elastic and viscous modulus of with xanthan gum (XG) and carboxymethylcellulose gums (CMC) emulsions and solution (solubilized in water) at 25, 37 and 50 ° C

Samples	Temperature (°C)	Viscosity (Pa.s)	G' (Pa)	G'' (Pa)	Tangent delta
NE-XG	25	16.60 ± 0.01	102.40 ± 0.01	19.58 ± 0.01	0.19 ± 0.01
	37	20.50 ± 0.02	126.13 ± 0.02	26.15 ± 0.04	0.20 ± 0.02
	50	16.48 ± 0.04	101.71 ± 0.02	19.49 ± 0.02	0.19 ± 0.01
NE-CMC	25	0.36 ± 0.07	1.79 ± 0.21	1.39 ± 0.03	0.77 ± 0.01
	37	0.45 ± 0.10	2.59 ± 0.16	1.25 ± 0.03	0.48 ± 0.01
	50	313.36 ± 0.01	1939.27 ± 0.11	339.64 ± 0.01	1.08 ± 0.01
XG-water	25	17.75 ± 0.00	109.39 ± 0.01	21.86 ± 0.00	0.19 ± 0.04
	37	43.61 ± 0.00	268.28 ± 0.01	55.73 ± 0.00	0.20 ± 0.03
	50	435.42 ± 0.03	2712 ± 0.01	362.20 ± 0.02	0.13 ± 0.06
CMC-water	25	3.40 ± 0.01	14.23 ± 0.01	15.92 ± 0.05	1.11 ± 0.02
	37	2.64 ± 0.02	10.77 ± 0.00	12.68 ± 0.01	1.17 ± 0.01
	50	10374.16 ± 0.03	5692 ± 0.01	3174.00 ± 0.01	0.55 ± 0.07

Table 3

Estimation of Number of cell nuclei in the Epifluorescence images of cells in contact with the tested emulsions: (i) NE without CoQ10 , (ii) CoQ10 NE, (iii) NE-XG and NE-CMC with and without CoQ10 , Results are mean +/- SD (n = 3). D1: 1 day of contact, D7: 7 days of contact. NE: Nanoemulsion, CMC: Carboxymethylcellulose, XG: Xanthan gum, Q10: CoQ10.

Ratio			
Nanoemulsion	0,5 mg/ml	1 mg/ml	2 mg/ml
NE-D1	10 ± 02	94 ± 04	14 ± 03
NE-Q10-D1	10 ± 01	57 ± 04	16 ± 02
NE-Q10-D7	23 ± 03	100 ± 03	75 ± 05
NE-XG-D1	51 ± 03	56 ± 02	21 ± 02
NE-XG-D7	84 ± 05	57 ± 04	11 ± 02
NE-XG-Q10-D1	36 ± 02	12 ± 02	10 ± 01
NE-XG-Q10-D7	135 ± 06	110 ± 05	181 ± 09
NE-CMC-D1	49 ± 03	53 ± 05	25 ± 03
NE-CMC-D7	94 ± 04	74 ± 06	43 ± 04
NE-CMC-Q10-D1	64 ± 03	74 ± 04	57 ± 05
NE-CMC-Q10-D7	123 ± 06	130 ± 05	81 ± 04

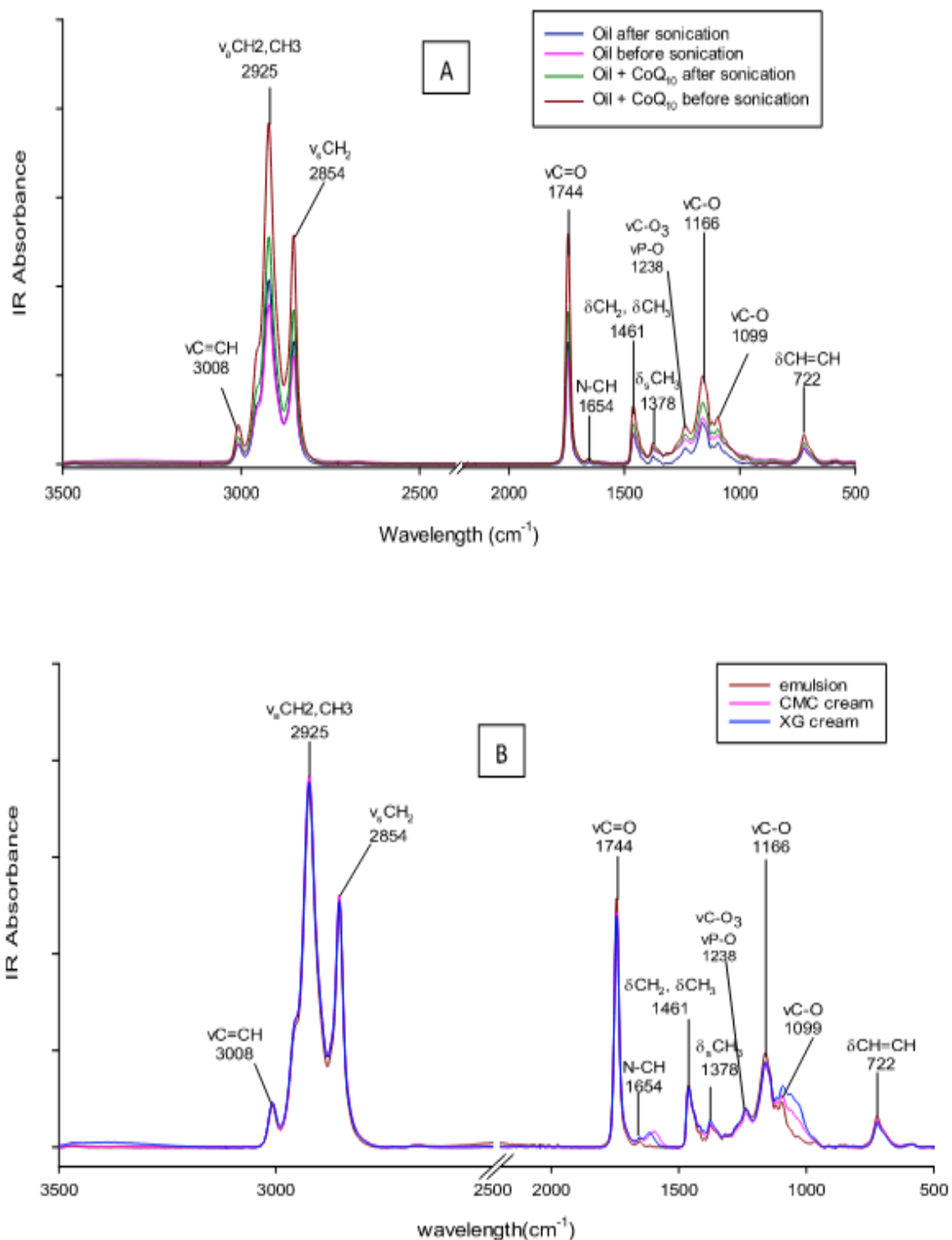


Fig. 1. (A) FTIR spectra of lipid phase before and after sonication, with and without CoQ₁₀ at 3500–500 cm⁻¹ region. V: stretching vibration,

: bending vibrations, s: symmetric, a: asymmetrical. (B) FTIR spectra of emulsion and cream with xanthan gum (XG) and Carboxymethylcellulose (CMC), and assignment of the bands associated at components

in 3500–500 cm^{-1} region. Graph legend: v: stretching vibration,

: bending vibrations, s: symmetric, a: asymmetrical.

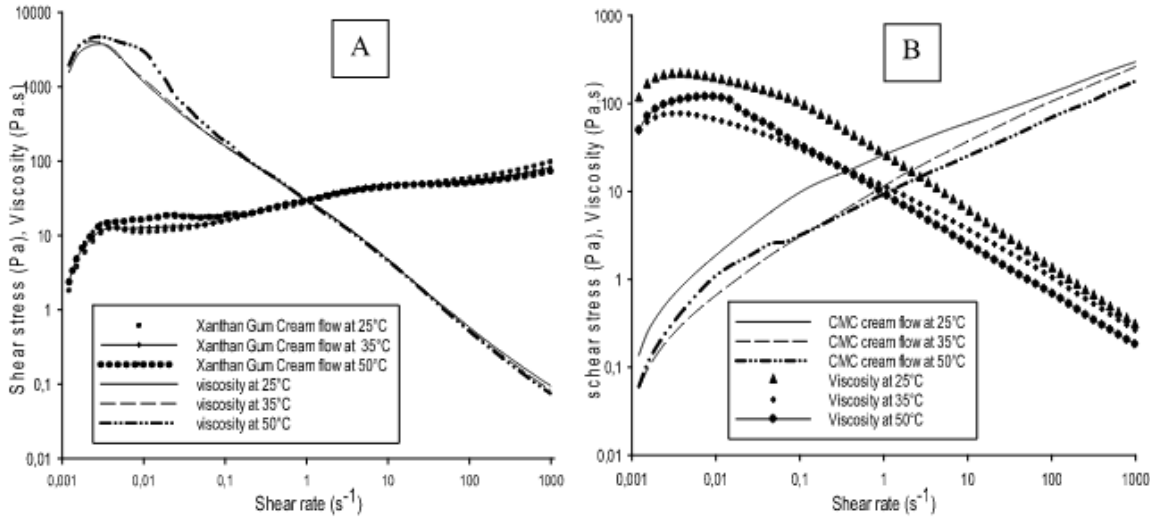


Fig. 2. Emulsion flow characterization with xanthan gum (XG) (A) and with carboxymethylcellulose (CMC) (B) at 25, 35 and 50 °C. Each curve shows the average values of three tests. The standard deviations are less than 5%, and are not shown for readability.

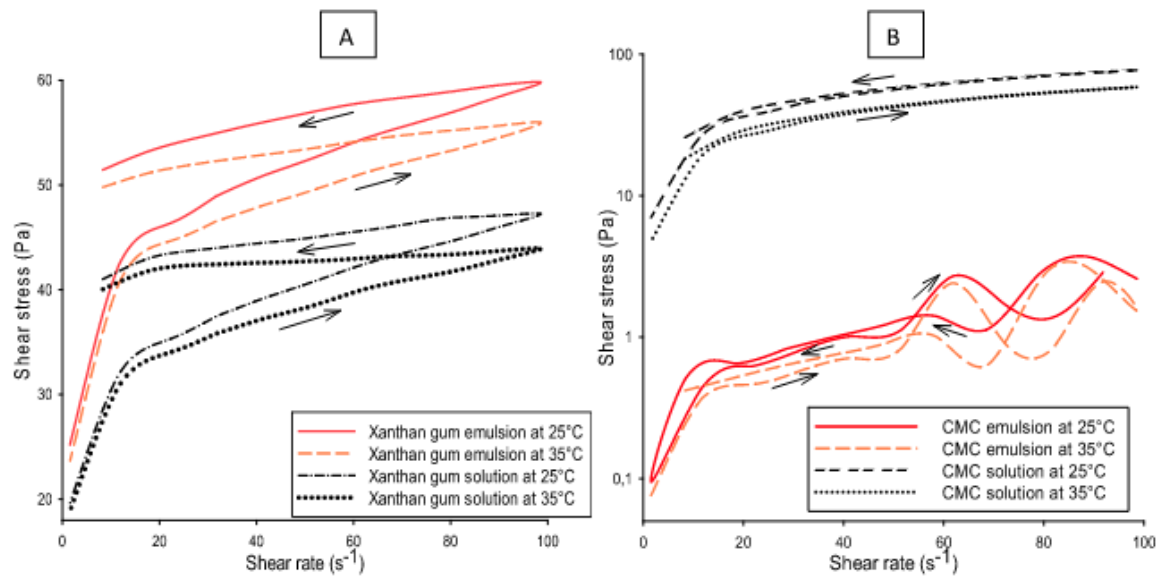


Fig. 3. Temperature effect on xanthan gum (XG) (A) and carboxymethylcellulose (CMC) (B) emulsion and solution flow at 25 ° C and 35 ° C. XG and CMC solution (solubilized in water) was used as control.

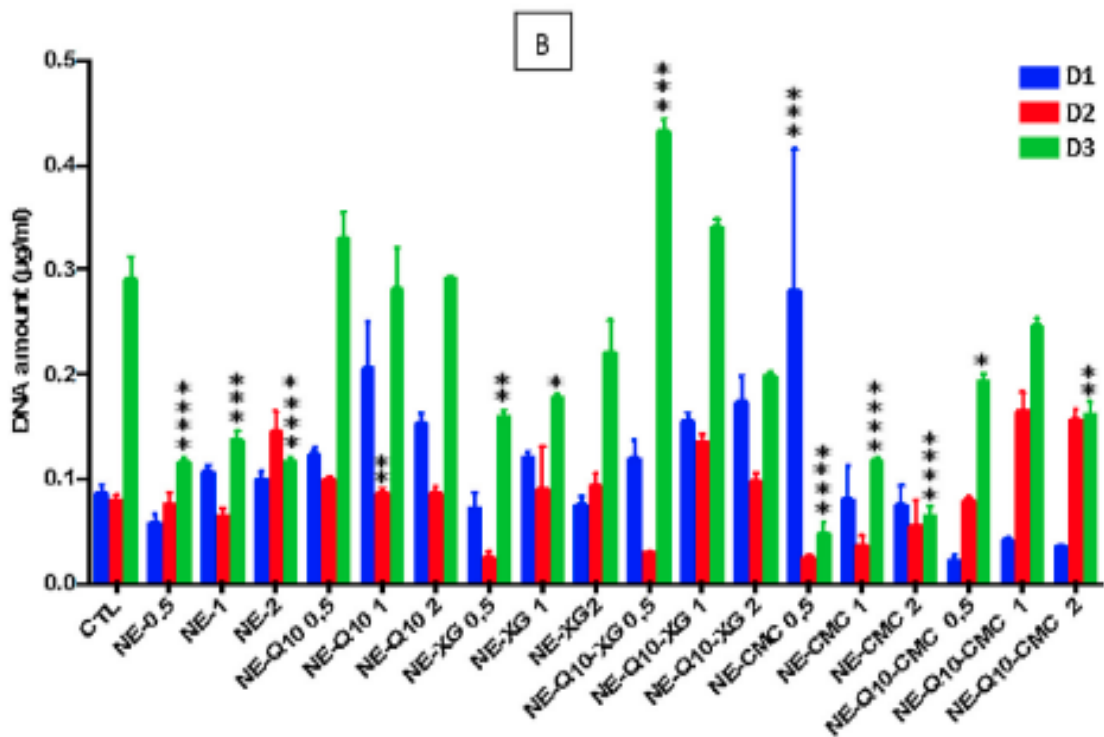
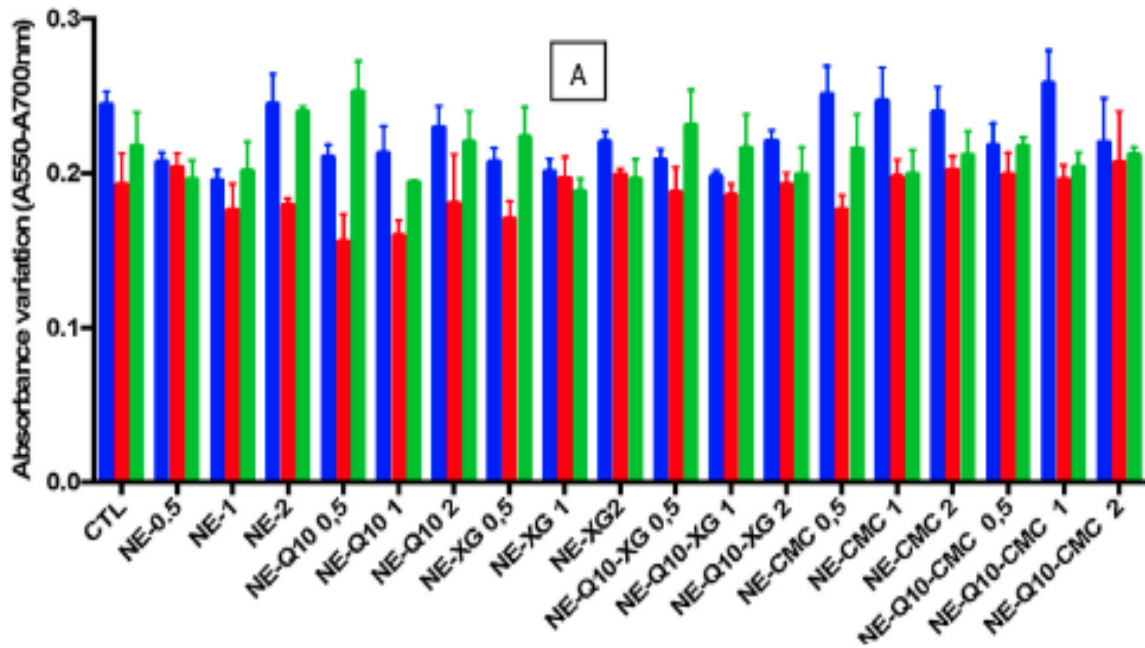


Fig. 4A shows LDH results observed after 24 h of incubation with different concentrations of preparations in cells stimulated with emulsions. Compared to the control (unstimulated cells), the different preparations shown no cytotoxic effect. For each stimulation (nanoemulsions with or without active molecule and with or without thickener), cell viability was not dose dependent. Furthermore, the best viability results were obtained by combining xanthan gum and CoQ10 (XG-CoQ10).

