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Saponin-based natural nanoemulsions as alpha-tocopherol delivery systems for dermal applications

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ABSTRACT

Nanoemulsions can be produced using simple methods and compounds from natural sources. They can increase water dispersibility and bioavailability and optimise active ingredient dispersion in particular skin layers. Lipophilic compounds of the vitamin E family (tocopherols and tocotrienols) are well-known for their high antioxidant activity and capacity to protect the skin from oxidative stress. In this context, oil-in-water (o/w) nanoemulsions with and without α-tocopherol (Vitamin E, VE) were formulated with two emulsifier alternatives, *Quillaja* saponin (QS), and a combination of QS with *Tribulus terrestris* (QSTT) (50/50, w/w). The emulsions were evaluated concerning stability, microstructure, droplet size, colour attributes, encapsulation efficiency, UV photostability, antioxidant activity, and *in vitro* permeation studies to assess the delivery potential. Results showed highly stable systems, with round-shape droplets of 80–121 nm size. QS and QSTT samples' colours were close to white and light brownish, respectively. The topical nano cream had the capacity to entrap VE, producing a protective effect from UV degradation, and very significant antioxidant activity, with IC50 values around 0.01 %wt. The skin permeation profiles showed the efficiency of the formulations in the delivery of VE, with permeabilities between 64 and 74 μg/cm^2, while the control sample showed no VE permeation.

1. Introduction

Nanoemulsions are colloidal systems with tiny droplets (20–200 nm) that have brought attention due to their enhanced functional properties over conventional emulsions, particularly their higher physical stability [1,2]. Emulsifiers play a crucial role in these systems reducing the interfacial tension between the oil and water phases and stabilising them through repulsive electrostatic interactions and/or steric hindrance [3]. In addition, the increasing interest in sustainable and environmentally friendly formulations is progressively leading to the replacement of synthetic emulsifiers with natural options, and the substitution of animal-based ingredients with plant-based counterparts, challenging researchers and industry to discover novel compounds with biocompatibility, biodegradability, low toxicity, but comparable performance [4].

In this context, saponins can be highlighted as natural emulsifiers holding a surface-active structure composed of glycosides with one, two, or three sugar chains attached to the aglycone via glycoside bonds. The sugar chains represent the molecule's hydrophilic part, and the aglycones (or sapogenins) are the hydrophobic parts, which may include steroidal or triterpene backbones [5]. *Quillaja saponaria* (QS) tree is considered the most common saponin source used as an emulsifier [6],

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implying overexploitation risks [7]. So, there is a current need to identify and apply alternative saponin sources [8], especially viable commercial samples, as is the case of the saponin-rich extract *Tribulus terrestris* (TT) from puncture vine studied in previous works of the group [9].

Nanoemulsion systems can provide a wide range of possibilities for innovative applications in the cosmetic and dermatological fields [10]. The cosmetic industry started using nanotechnology in the 1960s with liposome moisturising creams, and ever since, many advances have been observed, being one of the most competitive technologies of the 21st century [11,12]. The consumer demand for more technological and innovative products has encouraged industries to invest in the research and development of sophisticated and effective cosmetic products, such as nanoemulsions [13]. These systems have high stability and small droplet size, providing a large surface area, facilitating the uniform distribution on the skin. Thus, easy absorption, increased water dispersibility of functionalities and bioavailability, optimised dispersion in specific skin layers, better occlusiveness, film formation, and pleasant skin feel, are among the advantages [10,13–15]. The versatility of this type of delivery system is also advantageous once it can be tailored by controlling composition and production process, and can comprise all natural-based ingredients [12,16]. Therefore, the main application of nanoemulsions is for delivery systems, being oil-in-water (O/W) considered potential vehicles for the encapsulation, protection, and delivery of lipophilic bioactive compounds [17–20]. For example, vitamin E (VE) can be highlighted as a powerful antioxidant [21–23] whose poor water solubility and miscibility limit its chemical stability to light, oxygen, heat, and bioavailability. The encapsulation of this vitamin through saponin-based nanoemulsions can be an attractive strategy to promote its protection and dispersibility in aqueous-based systems [21].

The term "vitamin E" corresponds to a group of lipid-soluble compounds, including the tocopherols and tocotrienols (α-, β-, γ-, and δ-), all with a common structural feature: a chromanol ring and a phytol side chain [24]. Moreover, all these tocopherols and tocotrienols were assessed by the Cosmetic Ingredient Review (CIR) Expert Panel, concluding their safety use in cosmetics and their function as antioxidant and skin conditioning agents [25]. In these pharmaceutical-cosmetic applications, VE can also protect the skin from UV light damage, and reduce the appearance of wrinkles and fine facial lines, helping delay the progression of skin ageing [26,27]. To protect the skin from oxidative stresses, the VE should be supplemented topically, aiming to replenish the antioxidant in the upper skin layers [28]. Also, VE exhibits anticarcinogenic activity [29] and potential disease-preventive effects [30], making it widely used in supplements, pharmaceuticals, food, and cosmetic preparations. So far, some authors have successfully reported the incorporation of VE in nanoemulsions for cosmetic purposes [31–36]. For example, Abla and Banga effectively incorporated alpha-tocopherol in nanoemulsions stabilised by Tween 80, resulting in an aqueous, non-irritant, stable, and cosmetically appealing product, but not all-natural based [35]. Tween 80 is, in fact, frequently used to stabilise nanoemulsions to deliver VE. In this context, Teo et al. [34] and Chong et al. [36], developed effective cosmetic formulations with small-size droplets (80–200 nm), resulting in highly stable products. Moreover, Harun et al. [31] produced and tested VE-loaded nanoemulsions preceded by skin microwave treatment, enhancing penetration and improving therapeutic responses for dermatitis-like inflammation symptoms. In a different approach, core–shell nanoparticles based on sodium oleate (NaOl) and rebaudioside A (RebA) were tested to deliver vitamin E, resulting in systems with significantly enhanced antioxidant activity, corroborating its potential to supplement VE to both food and cosmetic products [32]. This approach was reported to be GRAS (generally recognised as safe). The approach of reaching all-natural-based nanoemulsions and cosmetics is still scarce in the literature. This includes the example of self-emulsifying drug delivery systems based on surfactin from Bacillus subtilis, which was successfully applied in nanoemulsions produced from sunflower and loaded with alpha-tocopherol, resulting in reduced discolouration and depth of wrinkles on skin tests [37].

To validate a cosmetic product, *in vitro* release/permeation studies should be performed to understand the formulation's performance when applied to the skin, which is strongly recommended by regulatory agencies [38,39]. In this context, Franz-type diffusion cells are the most used technique to evaluate *in vitro* permeation, enabling to assess the skin permeability, and also disclosing synergetic effects between the skin, active ingredients, and formulation [40]. This skin permeation analysis is critical to establish bioavailability and, in this manner, quantify the formulation's effectiveness [41]. The Franz cell consists of two compartments (donor and receptor) separated by a membrane, where, due to structural and biochemical similarities, the pig ear skin is usually chosen to mimic the human skin [42]. This technique presents several advantages such as the needed low amount of sample, as well as tissue handling. Moreover, it presents a simple design, and its use is inexpensive [41].

This study addresses the development and testing of an all-natural topical nano cream, stabilised with saponins for delivering a lipophilic vitamin (VE). Based on previous results of the group, two saponin-based emulsions have been studied (QS and QSTT (50/50) mixture). The prepared emulsions were characterised concerning their technological (colour, morphology, stability, and VE load) and functional properties (photostability, antioxidant stability and skin permeability).

2. Materials and methods

2.1. Materials

The saponin-rich extract TT was acquired from Essência d'um Segredo, a Portuguese company specialised in the commercialisation of natural and natural-derived products. TT extract, purchased in dry form, is a hydroethanolic extract with a saponin content of 93.05 %. This extract is in good agreement with the levels of residual solvents, heavy metals, aflatoxins, benzopyrenes, and Polycyclic Aromatic Hydrocarbons (PAHs) in accordance with pharmacological standards. The microbiological parameters comply with the United States Pharmacopeia (USP) 36–61 standard. The pure QS (99.9 % wt.) was purchased from Panreac. Both saponin sources are registered as cosmetic ingredients (CosIng database) at the European Commission [43,44], and the Quillaja extract was also approved as a cosmetic ingredient on the regulation 2006/257/EC [45]. These ingredients were used as received without any further purification.

The emulsions were prepared using sweet almond oil (SAO) (LabChem), with a density of 0.916 g/cm^3 and a saponification value of 196.6 mg KOH/g. It presents palmitic acid (5.2 %), palmitoleic acid (0.8 %), stearic acid (1.9 %), oleic acid (66.4 %), linoleic acid (24.8 %), and linolenic acid (0.4 %) in its composition. The chosen VE corresponds to DL-α-tocopherol (purity > 97.0 % by GC), and was purchased from Alfa Aesar. Deionized water (resistivity of 18.2 MΩ·cm, particles with size < 0.22 μm, and total organic carbon < 5 ppb) was used. All the compounds were used as received.

2.2. Nanoemulsions preparation

The emulsifiers and formulations were selected from previous optimisation studies in the group, which indicated them as promising base

products in terms of stability [46,47]. Nanoemulsions were prepared using an O/W ratio of 10/90 and a total mass of 50 mL (oil plus water). Briefly, the mixture of SAO (5 g), the emulsifier(s) (4 % wt. of a 50/50 QS/TT mixture or 1.5 % wt. of QS; total-basis) and VE (1 % wt., total-basis) were added to the oil phase (SAO) and homogenised with a vortex for 1 min. After, the previous homogenised oil phase was pre-emulsified in water (45 g) using an Ultraturrax for 5 min at 11000 rpm stirring rate. To reduce the droplet size, the resultant coarse emulsion was subsequently subjected to high-pressure homogenisation (HPH) (Avestin Emulsiflex C3) for six cycles at a homogenisation pressure of 100 MPa. The HPH is equipped with a heat exchanger to avoid temperature increase. Four samples were prepared, the samples 'QS' and 'QS_VE', using QS as the emulsifier, without and with VE, respectively; and samples 'QSTT' and 'QSTT_VE' using the 50/50 QS/TT mixture as an emulsifier, without and with VE, respectively.

2.3. Nanoemulsions characterisation

2.3.1. Colour

Since colour is an important sensorial attribute for cosmetics, this parameter was evaluated visually and with a colourimeter (model CR-400, Konika Minolta Sensing Inc.). The results were expressed by the Commission Internationale de L'Eclairage (CIELAB) using "L*" (lightness and darkness), "a*" (redness and greenness), and "b*" (yellowness and blueness). The sensor was calibrated against a white and a black tile. The measurements were carried out in triplicate.

2.3.2. Nanoemulsions stability

Stability during storage was assessed by checking the visual aspect of the emulsions right after production and after 30 days of storage at 20 °C. These observations were complemented by optical microscopy analysis to check morphology changes and droplet size evolution.

Sample morphology was accessed at room temperature using an optical microscope (Nikon Eclipse 50i) attached to a camera (Nikon Digital Sight). The Software NIS-Elements BR was applied for the image analysis.

Droplet size distributions of the prepared samples were determined by the laser diffraction technique (Mastersizer 2000, Malvern Instruments). The refractive indexes used for the continuous (water) and dispersed (oil) phases were 1.33 and 1.47, respectively. Five consecutive measurements were carried out for each sample at room temperature. Results were expressed as median droplet size in volume (Dv50), mean droplet diameter (D[3,2]), and Span (equation (1).

$$Span = \frac{Dv90 - Dv10}{Dv50} \quad (1)$$

Where D10, D50, and D90 percentiles correspond to the size below which 10 %, 50 %, or 90 % of all particles are found.

2.3.3. VE encapsulation in the oil phase

VE encapsulation in the oil phase (VEE%) was determined as described by Sharkawy et al. [48], which was conducted by accelerated phase separation. To determine the total amount of VE (TVE), 1 mL of the nanoemulsion was diluted in 10 mL of methanol and de-emulsified by placing the sample in a sonication bath for 10 min. The sample was then filtered with a nylon syringe filter of 0.45 μm. To determine the free amount of VE (FVE), i.e., the VE remaining in the aqueous external phase, another 1 mL of the nanoemulsion was subjected to centrifugation at 12 000 rpm for 4 h, and the liquid remaining at the bottom (aqueous phase of the emulsion) collected and also filtered with a nylon syringe filter of 0.45 μm.

VE was quantified by high-performance liquid chromatography (HPLC) (Shimadzu, model LC2060 C) using a reversed-phase column C18 Nucleosil 100–5. The mobile phase consisted of a mixture of acetonitrile/methanol (95:5, v/v) following a previously reported method for VE determination [49]. The analysis was carried out at room temperature, at a flow rate of 0.8 mL/min, and UV detection at 297 nm. The calibration curve was based on eight standard solutions at a concentration range of 0.0006 to 0.04 % wt. in acetonitrile/methanol (95:5, v/v). VEE% was calculated according to equation (2).

$$VEE\% = \frac{Total\ amount\ of\ VE - Free\ amount\ of\ VE}{Total\ amount\ of\ VE}.100 \quad (2)$$

2.3.4. Emulsion photostability

The photostability was determined following well-established protocols [35,48]. For that, the VE-loaded nanoemulsions, and a VE control sample prepared in methanol, were exposed to the UV light (365 nm) using a long-wave UV lamp. Methanol was chosen due to the high solubility of VE in this solvent. The samples (15 mL) were placed in Petri dishes of 6 cm diameter and left inside an analysis cabinet (Spectroline, model CM-10). At 0.5, 1, 2, 3, and 4 h, samples were collected, extracted with methanol, and analysed by HPLC for VE quantification (see section 6.2.3.5). The experiments were carried out in triplicate.

2.3.5. Antioxidant activity

The assessment of the antioxidant activity was carried out using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which is one of the most popular colorimetric assays to estimate the radical scavenging capacity. For that, the emulsions were diluted (50-, 100-, 500-, and 2000-times) using a 80 % (v/v) methanol aqueous solution. VE was used as control under the same experimental conditions and at the same concentrations. Then, 30 μL of the diluted samples were transferred to a 96-well microplate and mixed with 270 μL of DPPH methanol solution (6.10–5 mol/L). The mixture was incubated at 37 °C for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm using a microplate reader (BioTek, Winooski). The antioxidant activity was monitored over time for 30 days. The radical scavenging activity (RSA) was obtained as a percentage of DPPH discolouration using the equation (3):

$$\%RSA = \left[\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right].100 \quad (3)$$

where $Abs_{control}$ and Abs_{sample} correspond to the absorbances for the control and sample, respectively.

The results of the antioxidant activity were also expressed by half-maximal inhibitory concentration (IC50) defined as the sample concentration that led to give 50 % reduction of the initial DPPH concentration.

2.4. Skin permeation studies

VE skin absorption and permeation were evaluated by *ex vivo* tests in a multi-Franz diffusion cell apparatus (Franz cell stirrer, model V3A, PermeGear® Inc) using porcine ear skin as the membrane. The jacketed Franz diffusion cells (11.28 mm) from PermeGear® Inc had an effective diffusion area of 1.00 cm^2, a receptor chamber of 8 mL, and a jacket diameter of 30 mm. For pig skin preparation, a previously optimised procedure was adopted [50]. Briefly, the porcine ear was cleaned with ultrapure water, and the outer side of the pig auricle was carefully separated from the cartilage. For the recovered healthy skin parts, a treatment with Trypsin-EDTA solution (Sigma-Aldrich) at 4 °C for 4 h

was applied. Then, the cleaned skin membrane underwent a second treatment with Trypsin-EDTA solution overnight using the same temperature conditions. The treated skin membranes were washed with ultra-pure water and cut into round specimens of 20 mm diameter. The membranes were stored in aluminium foil at −20 °C, according to the guidelines of the "Guidance document for the conduct of skin absorption studies OECD series on testing and assessment; number 28" (n. 41, page 17) [38]. The receptor compartment was filled with ultra-pure water mixed with ethanol (50 %, v/v) and kept under magnetic stirring at 600 rpm at 32 ± 1 °C. Ethanol was added to improve the VE solubility in the aqueous medium since the solubility is lower than 0.1 mg/ml [23].

For the assays, 300 μL of nanoemulsion, or control sample (i.e., VE in sweet almond oil at the same concentration), was added to the donor chamber. The donor chambers were covered with Parafilm® to avoid water evaporation (occlusive conditions). At 0, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h, aliquots of 600 μL were withdrawn from the receptor compartment with a syringe to quantify the permeated VE. The receptor compartment was refilled with an equivalent volume of solvent to keep the same conditions. The withdrawn samples were stored at 4 °C and protected from light before HPLC analysis. The HPLC analysis was performed in duplicate and carried out according to the protocol described in section 6.2.3.5. All the samples were filtered with a nylon syringe filter of 0.45 μm before analysis.

To validate the assay, a VE mass balance was performed by summing the VE present in the donor and receptor compartments, and skin [38,51]. For that, the Franz diffusion cells were disassembled at the end of each assay. The residual sample in the donor compartment was diluted in 10 mL of methanol. The skin membrane was cut into small pieces, added to 10 mL of methanol and sonicated using an ultrasonic water bath (Sonorex, model RK52) for 30 min to extract the VE retained in the skin. According to the cosmetic-specific guidance document from the Scientific Committee on Consumer Safety (SCCS) [52], the mass balance should have an overall recovery between 85 and 115 %.

3. Results and discussion

3.1. Nanoemulsions colour

By visual inspection, the prepared formulations, both with and without VE, resulted in a milky appearance when QS was used, acquiring a slight yellowish colour when the 50/50 QS/TT mixture was used as emulsifier (Fig. 1). Table 1 shows the obtained colour parameters including "L*", which indicates the lightness and darkness (from 100 to 0), a*, the redness and greenness, and b*, the yellowness and blueness (both from positive to negative values). Regarding L*, the addition of the TT resulted in "darker" samples, reducing this parameter from 78.8 and 80.6 for QS and QS_VE, respectively, to 71.1 and 70.8 for QSTT and QSTT_VE, respectively. An analogous change was also observed in b* values, varying from 1.5 to 2.4 when QS was used, to 11.6–12.6 when the 50/50 QS/TT mixture was used. The samples, with and without the VE, presented slight variations, being the effect more pronounced in the b* parameter due to the yellow–brown colour of alpha-tocopherol. In the QS-based series, the presence of the VE raised the b* by 58.4 % compared to the base nanoemulsion (without VE), while in the QSTT-based series, only an increase of 8.5 % was observed.

Table 1
Colour parameters (L*, a* and b*) according to the Commission Internationale de L'Eclairage system (CIELAB) of the produced nanoemulsions.

Sample	L*	a*	b*
QS	78.78 ± 0.09	−1.00 ± 0.11	1.48 ± 0.09
QS_VE	80.58 ± 0.13	−1.77 ± 0.05	2.35 ± 0.04
QSTT	71.12 ± 0.15	−0.98 ± 0.19	11.58 ± 0.23
QSTT_VE	70.78 ± 0.11	−1.3 ± 0.03	12.56 ± 0.15

Table 2
Droplet size expressed as Dv50, D[3,2], and Span (nm) for the studied four nanoemulsions.

Sample	Dv50 (nm)		D[3,2] (nm)		Span	
	t0	t30	t0	t30	t0	t30
QS	153	151	86	86	2.72	2.69
QS_VE	225	212	121	116	2.53	2.52
QSTT	139	145	80	83	2.77	2.76
QSTT_VE	161	156	91	90	2.67	2.76

*The standard deviation of the measured data ranged between 10^{-4} and 10^{-5} nm.

Fig. 1. Photo record of the produced nanoemulsions. The samples 'QS' and 'QS_VE are the emulsions prepared using QS as the emulsifier, without and with VE, respectively. The samples 'QSTT' and 'QSTT_VE' are the emulsions prepared with the 50/50 QS/TT mixture as the emulsifier, without and with VE, respectively.

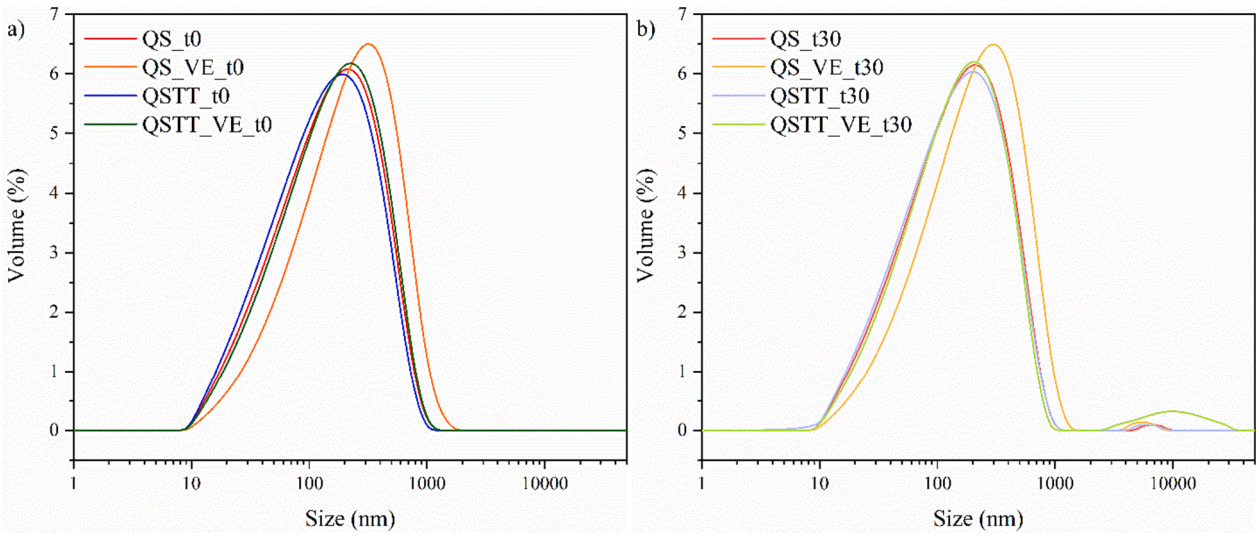

Fig. 2. Droplet size distribution of nanoemulsions for a) 0 and b) 30 days.

3.2. Nanoemulsions stability

The stability analysis of nanoemulsions was monitored for 30 days, at a storage temperature of 20 °C. By visual inspection, no visible separation was observed pointing out for a stable emulsion within the studied period. This visual evidence was corroborated by the droplet size (Table 2, which includes Dv50, D[3,2], and Span values for t0 and t30) and microscopic (Fig. 3, which includes micrographs for t0 and t30) analysis.

The produced emulsions gave rise to unimodal distributions (Fig. 2). Moreover, the distributions are similar, except for the QS_VE, whose profile slightly shifted to higher droplet size values. The peak shape was maintained after 30 days, and a small peak started to be observed in the higher droplet size region, particularly for the sample QSTT_VE. This observation suggests some degree of droplet coalescence. However, the extension of this phenomenon was not enough to give rise to substantial differences in the calculated size parameters (Dv50, D[3,2], and Span), as well in the appearance of macroscopic instability.

From the median droplet size (Dv50), it can be concluded that 50 % of the sample volume corresponds to droplets with a size lower than 139 nm for the QSTT sample. A slightly larger size (153 nm) was observed for the QS sample, probably due to the emulsifier content, which was 1.5 % wt. for QS and 4 % wt. for QSTT. These droplet size values agree with previous works reporting saponins as emulsifiers in nanoemulsions [13]. In addition, these results can also be compared with other studies dealing with nanoemulsions containing VE, but using a synthetic emulsifier (Polysorbate 80) [53]. Their droplet size varied from 136 to 148 nm. The addition of VE strongly impacted the sample stabilised by QS (QS_VE), showing an increase of ~47 %, against their analogues stabilised by the 50/50 QS/TT mixture, for which an increase of ~16 % was observed.

The D[3,2] parameter, the Sauter Mean Diameter, showed smaller values, being the samples without VE very similar (80 and 86 nm, for QS and QSTT, respectively). It was also observed that the addition of VE increased the droplet size, with a higher impact for the QS-based series.

Regarding the span analysis, reflecting the size uniformity of the droplets among samples was confirmed by the Span values, ranging from 2.5 to 2.8. This parameter is representative of the dispersion of the droplet size distribution, e.g., higher Span values reflect broader distributions. At this point, note that the droplet size substantially influences the emulsions' stability, but it should also be considered narrow distributions to prevent Ostwald Ripening destabilisation effects.

The optical micrographs of the analysed samples taken at 0 and 30 days (Fig. 3) show samples with low droplet size and a round shape as expected [9,54,55], and signs of coalescence were not evident. This indicates that the use of VE and different emulsifier systems have no impact on morphology or size. The homogeneity of the samples' size distributions was also corroborated in these analyses.

3.3. VE encapsulation in the oil phase

Concerning the VE present in the formulation, the encapsulation in the oil phase (%VEE) was 89.43 ± 0.24 wt%, and 90.04 ± 0.45 wt% for QS_VE and QSTT_VE formulations, respectively. These results agree with the published literature, where EE values around or higher than 90 wt% are usually reported for lipophilic compounds encapsulated in oil phases [31,56]. Both series presented similar %VEE, suggesting that the different emulsifiers did not directly impact this parameter. Based on these findings, it can be concluded that the emulsifier content applied in each formulation is adequate to form stable water–oil interfaces and to the VE entrapment in the oil phase.

3.4. Emulsion photostability

One of the main causes of shelf-life reduction in cosmetic products is oxidation, which can cause discolouration, unpleasant odour, degradation of the active ingredients, and even physical instability [57]. Photostability is an important requirement to ensure that an active ingredient remains chemically stable in the formulation after light exposure, which can also be impacted by the used packaging materials. In this sense, some guidelines on cosmetic stability testing indicate the importance of the product's photostability, for which different strategies might be applied aiming at achieving protection [58]. Antioxidants can help to keep the systems stable due to their ability to neutralise free radicals, thus avoiding or reducing oxidative damage. VE is a potent antioxidant with the ability to neutralise reactive oxygen species, thus preventing or slowing skin damage and ageing [59,60]. However, VE is highly sensitive to UV radiation, meaning that it is of utmost interest to protect it from UV light [35].

The photodegradation profiles of the VE present in the samples QS_VE and QSTT_VE are shown in Fig. 4, comparatively to a VE control sample (VE dissolved in methanol). As expected, a strong VE degradation was observed for the control sample. According to Sabliov et al. [61], different mechanisms may cause this phenomenon, namely the absorption of UV light by the methanol, generating methoxy radicals leading to the formation of tocopherol oxy radicals, or the break of its labile ether bonds by UV light. As a result, only 16.9 % of VE remained viable after 4 h of UV irradiation. On the other hand, the nanoemulsions

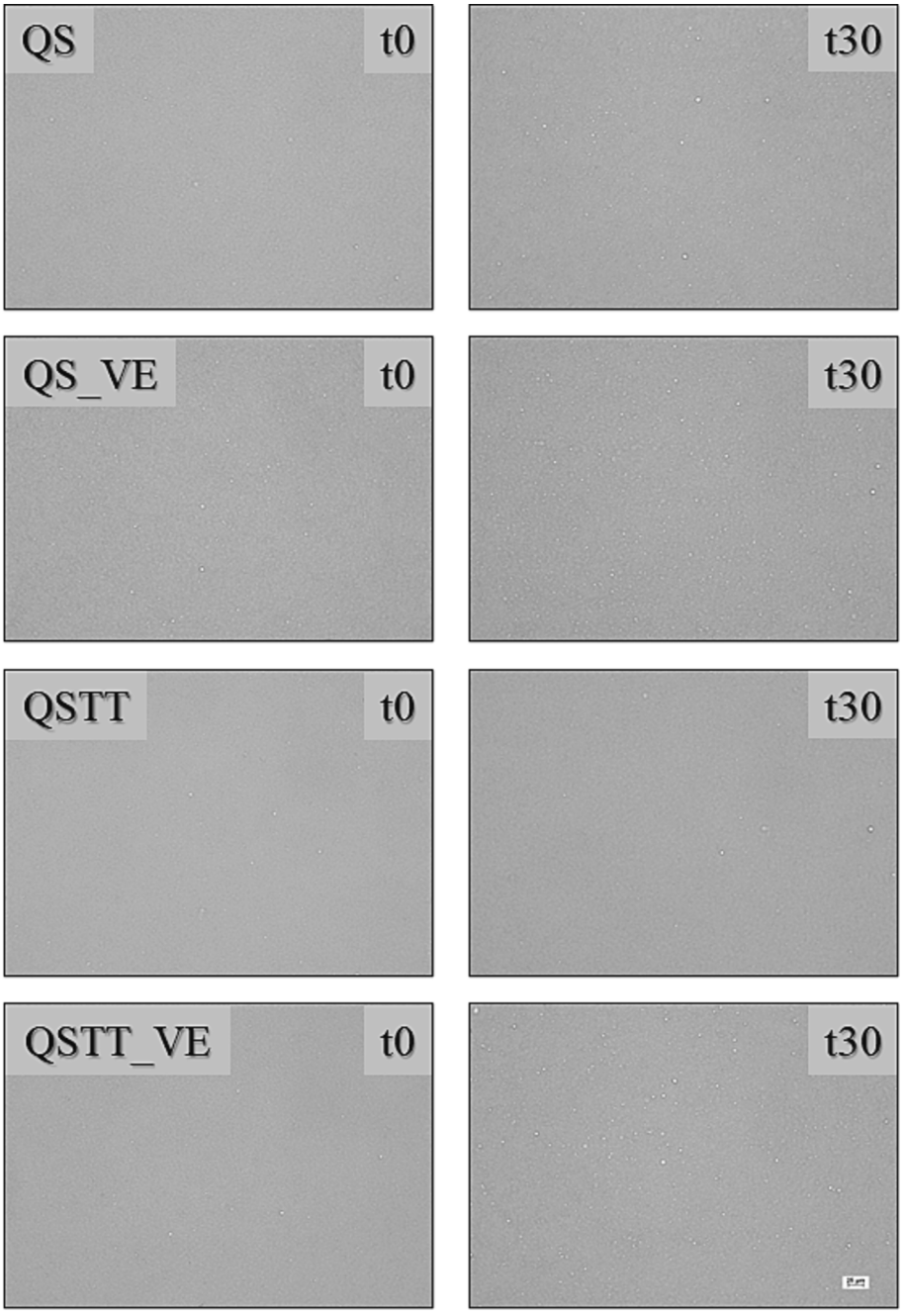

Fig. 3. Microscopic analysis (400x) of nanoemulsions with and without VE right after production (t0) and after 30 days (t30) under storage at 20 °C.

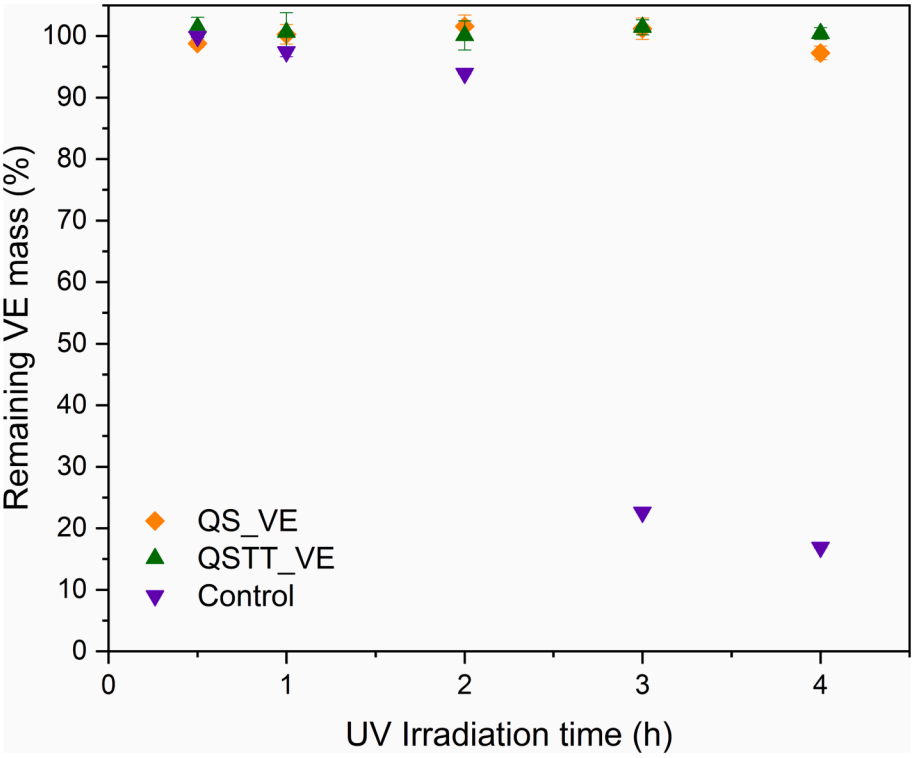

Fig. 4. Photodegradation profiles of the VE present in the samples QS_VE and QSTT_VE against VE in methanol medium (control).

could efficiently protect VE, maintaining its stability throughout the complete UV exposure time. This protection is related to the nanoemulsion properties and entrapment ability that prevents the degradation of active compounds, such as quercetin [62]. However, the composition of the emulsion formulation (emulsifier type, oil phase) and homogenisation conditions can affect the ability of nanoemulsions to control undesirable degradation [63]. In this case, both saponin-based systems had a positive effect on maintaining the VE viability, with a shield effect against UV radiation.

3.5. Antioxidant activity

The antioxidant capacity of all nanoemulsions was evaluated based on the absorbance change produced by the reduction of the DPPH radicals in the samples. The obtained results, shown in Fig. 5, were expressed as the percentage of radical scavenging activity (%RSA). Fig. 5a shows the results for all dilutions (50, 100, 500, and 2000×) and Fig. 5b evidence the results for 50x dilution.

The base nanoemulsions (without VE) did not show significant antioxidant activity, being the formulation QSTT the one presenting a higher value when compared to QS. The samples containing VE (QS_VE and QSTT_VE) showed higher values, which evidences their potential for cosmetic application, namely stable formulations against oxidative stress [57]. Comparing both formulations with VE, the antioxidant activity is very similar, which can be attributed to the performance of VE independently of the presence of the other ingredients. After 30 days, the activity increased for QS_VE and QSTT_VE (50× dilution) and decreased for samples without VE. This behaviour can be related to the controlled release capacity of nanoemulsions and the effective entrapment of VE in the oil phase. Nevertheless, this behaviour is not observed for higher dilutions, with values remaining close or even decreasing, which can be attributed to the reduction in the concentration of the systems that might impact their main characteristics.

Table 3 shows the results of the half-maximal inhibitory concentration (IC_{50}). Based on the results, the emulsifier system had no negative effect on the antioxidant activity, since similar values were obtained for the samples QS_VE and QSTT_VE. Also, after 30 days, just a slight increase in the concentration was observed, corroborating the stability of VE in the systems.

3.6. Skin permeation studies

Ex vivo skin permeation studies were performed using Franz cells to assess the VE-loaded nanoemulsions behaviour. Fig. 6 shows the treated ear pig skin membranes and the Franz cells apparatus to carry out the assays in occlusive conditions.

As previously mentioned, the VE was quantified in the *stratum corneum*, the receptor fluid (corresponding to the dose crossing the skin), and in the residual sample remaining in the donor compartment. The mass balance results obtained were found to be 87 and 108 % (Table 4), it means, in agreement with the OECD guidelines (85–115 %).

The *stratum corneum* is the outermost layer of the skin, identified as the main barrier restricting the permeation of active ingredients through the skin, thus limiting their function [48]. In this work, the VE retained in the *stratum corneum* was higher for the sample QS_VE, around 21 μg/cm^2, while QSTT_VE gave rise to a retention of just 11 μg/cm^2. One reason for this difference might be the presence of the extract, which increases the complexity of the formulation. Also, the variety of compounds present in the extract can modify the interactions of VE with the barrier of the *stratum corneum*. Compared with similar results in the literature, vitamin E acetate (a derivative of VE with higher aqueous solubility) loaded in an O/W base cream gave rise to retentions in the *stratum corneum* of 12 μg/cm^2, even though a higher load of the active agent was used (2 %) [64]. Considering the VE concentration in the developed formulations (1 %), the evidenced stronger capacity to diffuse into the *stratum corneum* is an advantage to potentiate the VE effects in the skin. In fact, it is known that topically applied antioxidants constitute important pharmacological active agents to prevent and reduce UV-induced skin damage and ageing [65].

The control sample (VE dissolved in sweet almond oil), was not identified in the receptor fluid, indicating that it was not able to permeate through the skin. By contrast, values around 74 and 64 μg/cm^2

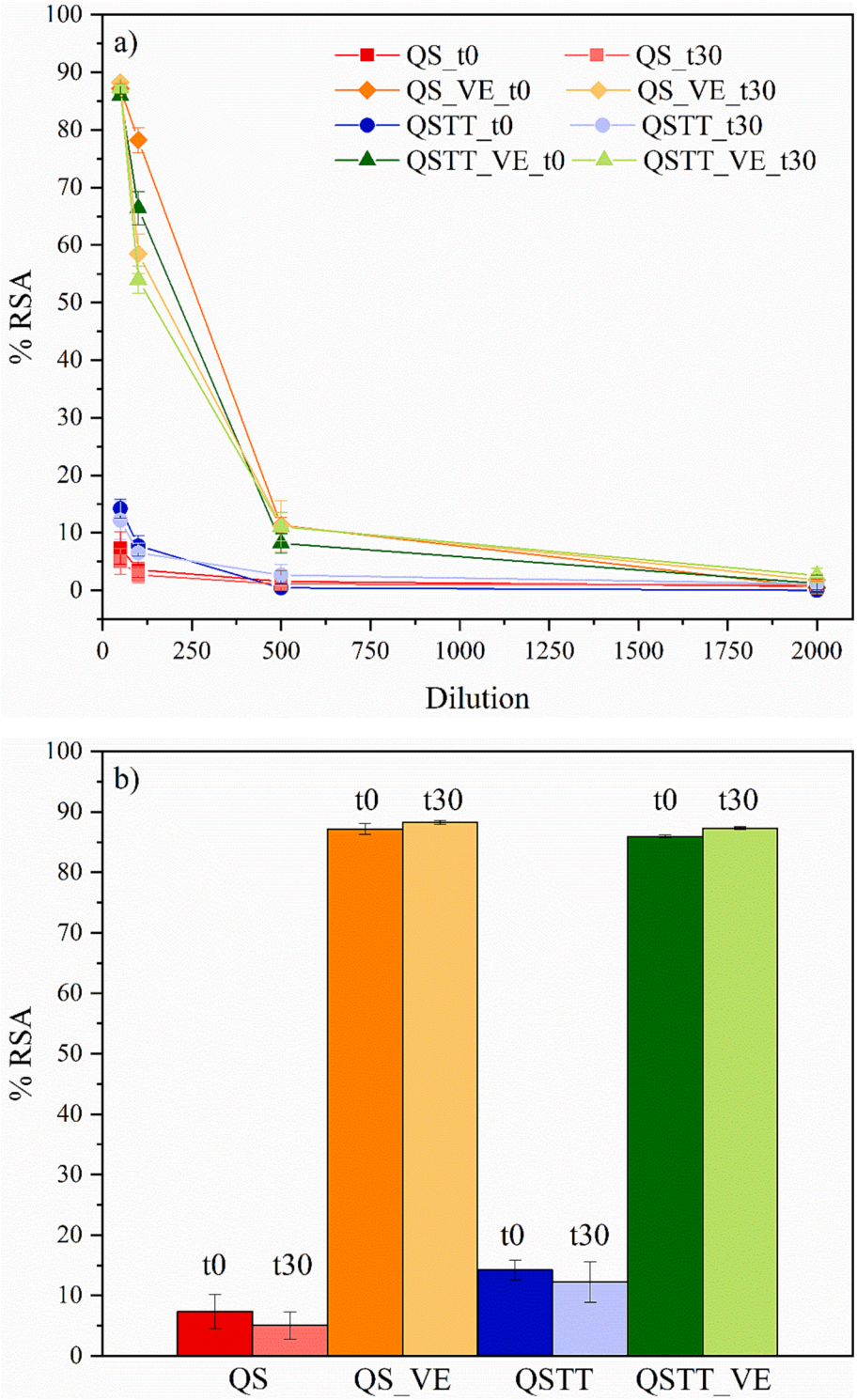

Fig. 5. Antioxidant activity expressed as the percentage of radical scavenging activity (%RSA) for the nanoemulsions produced with and without VE right after production (t0) and after 30 days (t30): a) Results for all dilutions; b) Results for the 50x dilution.

Table 3
IC_{50} values of nanoemulsions with VE at 0 and 30 days.

Sample	IC_{50} (%wt.)	
	t0	t30
QS_VE	$0.0099 \pm 2.7 \cdot 10^{-4}$	$0.0106 \pm 9.7 \cdot 10^{-5}$
QSTT_VE	$0.0105 \pm 2.0 \cdot 10^{-4}$	$0.0109 \pm 1.2 \cdot 10^{-4}$

were achieved for QS_VE and QSTT_VE, respectively, which are very significant if compared with other works that attempted the permeation of VE through the skin. For example, Gabbanini et al. [66] showed the difficulty of VE diffusion using a base O/W cosmetic cream containing 3 % VE, with values below 0.5 μg/cm^2 after 22 h. Rangarajan et al. [67] performed *in vitro* studies using microemulsions based on Tween 20 (synthetic emulsifier) and 1 % of VE, resulting in permeation values between 3 and 8 μg/cm^2. In addition, in vivo permeation tests in rats,

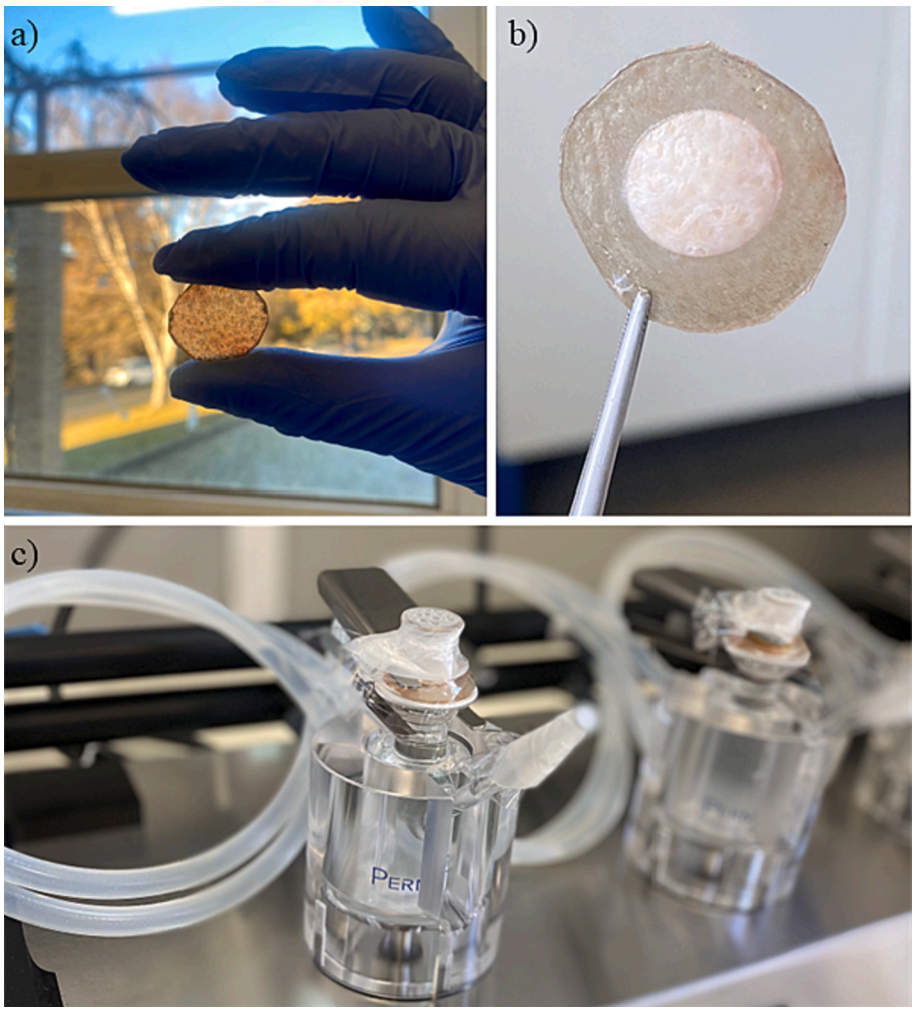

Fig. 6. Franz cell apparatus. a) Skin membrane before the experiment, b) Skin membrane after the experiment, c) Franz-cells during the experiment in occlusive conditions.

Table 4
Distribution (after 24 h) of VE within stratum corneum, receptor fluid, donor residual sample, and the calculated mass recovery.

	Applied Dose (μg/cm^2)	Stratum Corneum (μg/cm^2)	Receptor Fluid (Absorbed dose, μg/cm^2)	Residual Sample (Donor dose, μg/cm^2)	Mass Recovery (% wt.)
QS_VE	2754 ± 0	20.7 ± 0.6	74 ± 7	2315 ± 58	87 ± 2
Total %		0.86 %	3.06 %	96.08 %	
QSTT_VE	2773 ± 0	11 ± 5	64 ± 3	2388 ± 28	88.8 ± 0.7
Total %		0.45 %	2.62 %	96.94 %	
Control	3380 ± 0	15.86 ± 0.03	0 ± 0	3625 ± 69	108 ± 2
Total %		0.44 %	0 %	99.56 %	

such as reported in the study of Nada et al. [68], values of 27.7 μg using formulations containing 0.5 % VE applied to a dorsal area of 7–8 cm^2, were achieved.

The VE release profiles for both systems (QS_VE and QSTT_VE), i.e., the VE crossing the *stratum corneum* to the receptor fluid, are shown in Fig. 7. The release profile for the control (VE in sweet almond oil), is not presented since VE did not cross the skin membrane. QS_VE permeation started to be observed 1 h after the product application in the donor compartment, while QSTT_VE started to permeate immediately after the application. Both formulations showed similar VE release profiles until 7 h, with the QS_VE showing higher releases at 8 and 24 h (approximately 13 and 9 μg/cm^2 higher, respectively). This trend was also observed for the *stratum corneum* retention, where the lower values for the QSTT_VE were associated with the extract complex composition, favouring the barrier effect of the skin. It is important to highlight that the correlation between formulation composition and active agents release mechanism was rarely determined, possibly due to its high complexity [69].

The promising obtained results regarding the VE permeation must be emphasised, which can be associated with the nanoemulsions' intrinsic characteristics. In fact, it has been reported that the small droplet size of nanoemulsions improves the permeation of the active ingredients through the skin, as well as controlled release profiles [35,70]. Such effects can be related to increased surface area, higher solubility, and stability. Generally, two hypotheses are advanced concerning the penetration of active agents guided by nanocarriers, including nanoemulsions. The first is the appendageal route, which consists of the penetration via hair follicles, pilosebaceous, and sweat gland pores. The

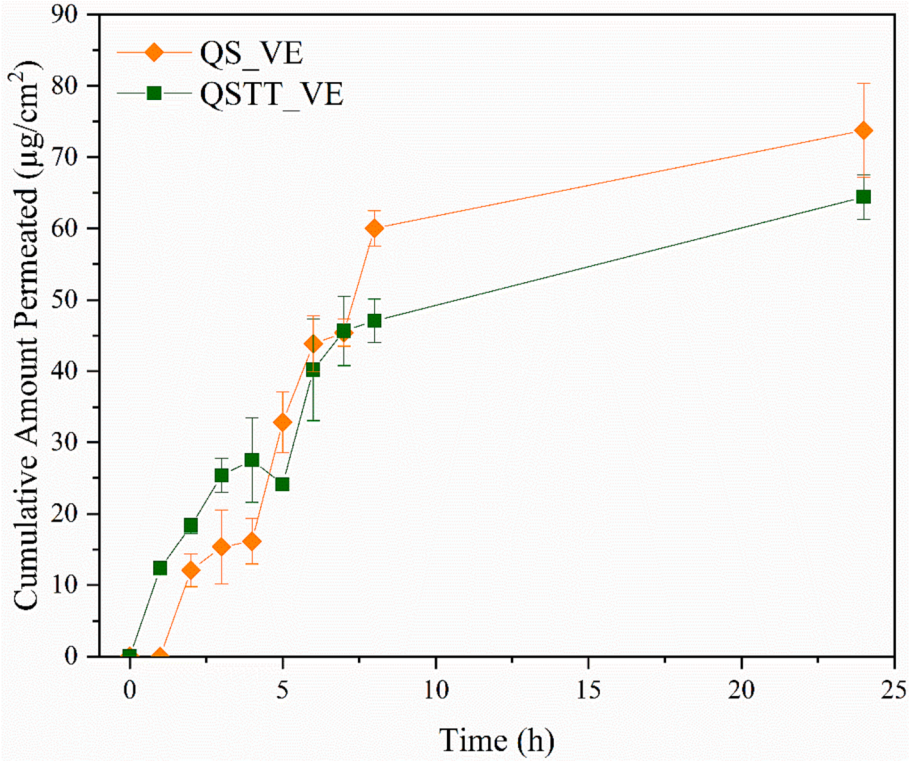

Fig. 7. Cumulative amount permeated of VE through time.

second, the intercellular route, is based on the effect caused by the nanocarriers at the *stratum corneum* level, modulating its organisation and decreasing its barrier capacity. For tocopherol, it has been previously reported that the intercellular route [35], which can probably be associated with these formulations, and responsible for producing an occlusive effect on the *stratum corneum*, thereby increasing the skin hydration, and leading to higher VE nanoemulsions permeation. This assumption is also corroborated by the differences found between samples since the emulsifier, which can act as a penetration enhancer, can have different interaction effects with the *stratum corneum* barrier. In addition, both studied samples present different concentrations of emulsifiers that can contribute to the differences found. It was previously stated that the two main factors determining the penetration capacity of lipophilic compounds by nanoemulsions are the concentration of penetration enhancers and of the active ingredient in the donor [71].

Additionally, the W/O ratio could favour the VE absorption in the nanoemulsion formulations. It was indicated that higher water contents facilitate the release and penetration of lipophilic active agents, while systems with high oil content decrease their permeation effectiveness [23,72].

4. Conclusions

The present study proposes a successful topical nanoemulsion with and without VE and natural-based emulsifiers: *Quillaja saponin* (QS) isolated and combined with *Tribulus terrestris* (TT) at 50/50 (w/w). All tested systems produced by HPH resulted in natural, stable, and cosmetically appealing aqueous formulations. The nanoemulsions showed small droplet sizes (between 80 and 121 nm) and stability over time, exhibiting monomodal size distributions. Moreover, the presence of the extracts induced colour brownish in the final formulation, to light brownish. The functional properties, particularly those showing satisfactory encapsulation efficiencies, exhibited an outstanding outcome regarding UV photostability. In terms of UV photostability, the nanoemulsions protected VE from degradation. As expected, the systems containing the active ingredient showed higher antioxidant activity. The same trend was observed in the skin permeation studies, where the QS_VE and QSTT_VE systems showed higher permeation values (~74 and 64 μg/cm^2, respectively) after 24 h of exposure. A fraction of the vitamin was retained in the *stratum corneum*, being highly advantageous considering its antioxidant capacity. Both systems appear as beneficial for topical applications:

Overall, both proposed formulations - QS_VE and QSTT_VE - are great candidates for the delivery of VE, presenting nanodroplet size, photoprotective capacity, high antioxidant activity, efficient entrapment, and good penetration and skin retention of the active, postulating them as potential green-labelled cosmetical products.

CRediT authorship contribution statement

Tatiana B. Schreiner: Conceptualization, Methodology, Investigation, Writing – original draft. **Arantzazu Santamaria-Echart:** Methodology, Investigation, Writing – original draft. **Giovana Colucci:** Investigation. **Paula Plasencia:** Investigation. **Patrícia Santos Costa:** Methodology, Writing – review & editing. **Madalena M. Dias:** Conceptualization, Supervision, Writing – review & editing. **Simão P. Pinho:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Maria Filomena Barreiro:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

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.

Data availability

No data was used for the research described in the article.

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