Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles as green topical delivery vehicles for cannabidiol (CBD)

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HIGHLIGHTS

• Chitosan/collagen peptides Pickering emulsions were used as green vehicles for CBD.
• The produced Pickering emulsions demonstrated high CBD encapsulation efficiency.
• The stability of the developed emulsions is affected by the oil type and amount.
• The produced Pickering emulsions led to high CBD deposition in the stratum corneum.
• Higher oil volume fractions enhanced the amount of CBD delivered to the skin.

GRAPHICAL ABSTRACT

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ABSTRACT

Pickering emulsions (PEs) are attracting increasing attention in the areas of food, cosmetic and pharmaceutical applications owing to their surfactant-free and eco-friendly nature. Herein, PEs stabilized by chitosan/collagen peptides (CH/CP) nanoparticles were assessed as green surfactant-free vehicles for the topical delivery of cannabidiol (CBD), a highly lipophilic unstable drug that is finding an increasing appeal in the cosmetic market. The influence of the oil phase volume fraction (φ) and the oil type on the emulsion properties, stability, rheological properties, as well as on the ex-vivo skin absorption of CBD was evaluated. The PE prepared with olive oil (φ = 0.6) exhibited elastic gel-like properties and demonstrated long-term stability after 5 months of storage, with a CBD content of 99.45% of the initially added amount. The skin absorption studies showed that CBD was retained in high amounts in the stratum corneum, while the CBD skin permeation was extremely low, indicating that the produced formulations are suitable as topical delivery vehicles. ATR-FTIR examination of the treated skin samples confirmed that the produced PEs were able to overcome the stratum corneum barrier. These findings suggest that the PEs stabilized with CH/CP nanoparticles provide an effective surfactant-free alternative for the topical delivery of CBD.

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1. Introduction

Cannabidiol (CBD) is a non-psychoactive phytocannabinoid naturally found in Cannabis sativa L. During the last decade, CBD has attracted a growing interest in medicine due to its various therapeutic activities [1–4]. Recently, cannabis extracts, and CBD in particular, have also gained tremendous attention in the cosmetic industry sector owing to their moisturizing, anti-aging, anti-inflammatory, antioxidant and cytoprotective properties [5,6]. Moreover, recent studies have shown that cannabis extracts do not lead to cytotoxic effects on keratinocytes and skin fibroblasts, proving the safety and non-irritation of cannabis active ingredients [7,8]. CBD is a highly lipophilic drug (log P = 6.3) and, therefore, it is frequently incorporated in alcohol-based formulations or oils [9]. Additionally, CBD has been reported to be readily degraded by light, temperature and oxidation [9,10]. Hence, the development of novel and safe formulations is important for enhancing the stability, solubility and bioavailability of CBD.

Emulsions stabilized by solid particles, also known as Pickering emulsions, provide an eco-friendly alternative to the conventional emulsions stabilized by classical molecular emulsifiers [11,12]. The particles used as Pickering emulsions stabilizers possess intermediate wettability, which allows them to be irreversibly anchored at the oil-water interface providing steric hindrance and preventing coalescence, and subsequently resulting in high emulsion stability [13]. Inorganic particles have been frequently used in Pickering emulsion stabilization [13,14]. However, due to the consumers’ demands and industrial preference for natural and label-friendly ingredients, recent research has been directed towards developing particles of natural origin as Pickering stabilizers [15–19].

The use of Pickering emulsions in dermal applications is finding increasing interest due to the consumers’ demands and industry regulations to switch to green and safe formulations and to overcome the skin sensitization and irritation effects provoked by surfactants [14,20–22]. Pickering emulsions stabilized with particles of biological origin have not been frequently reported for cosmetic applications. However, this area is expected to be addressed more in future research owing to the large scale of the cosmetic market and to fulfill the biocompatibility and safety issues [23].

Among the various biopolymers utilized in the development of particles employed as Pickering stabilizers, chitosan has emerged as a promising candidate in various applications due to its biocompatibility, biodegradability and non-toxic properties [16,24,25]. Chitosan has been widely used in topical and transdermal formulations as it has been reported to have many skin benefits, such as moisturizing, antiaging, antioxidant, antimicrobial and wound healing properties [26,27]. It also enhances the skin delivery of active agents due to its cationic nature that allows its adhesion to the negatively charged skin surface [28]. Nevertheless, to date, very few studies investigated the use of chitosan-based Pickering emulsions in dermal and cosmetic applications despite the advancements these formulations proved to achieve [29]. For example, Pickering emulsions stabilized with chitosan nanoparticles have exhibited a sustained release of rutin and demonstrated an enhanced in vivo wound healing effect [30]. In another recent study, chitosan-based Pickering emulsions were reported to enhance the dermal bioavailability of resveratrol and improve its photostability upon exposure to ultraviolet radiation [31].

It is noteworthy that chitosan-based Pickering emulsions are mainly prepared by particles solely made of chitosan through self-aggregation, particles formed via the hydrophobic modification of chitosan, or by particles that are formed through the complexation of chitosan with other polymers [29]. In general, it has been reported that the latter methodologies demonstrated improved wettability of the nanoparticles, and hence enhanced Pickering emulsion stability [16,32]. Additionally, the complexation of chitosan with other polymers enhances the functionality of the produced chitosan-based Pickering emulsions when the other polymer contributes with an added value to the resulting formulation, such as adding skin benefits by complexing with collagen peptides [33] or providing nutritional advantages as in the case of chitosan/caseinophosphopeptide nanocomplexes [34].

In a recent study, we developed Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles that demonstrated high stability and enhanced ability to penetrate the stratum corneum [33]. Following these achievements, the main objective of the present work is to explore the potential of these biodegradable Pickering emulsions as green non-toxic cosmetic vehicles of CBD. The work also aims to investigate the physicochemical properties and encapsulation efficiencies of the developed CBD-loaded Pickering emulsions, and the effect of using different oils and different oil volume fractions on these properties, as well as on the skin penetration deposition of the encapsulated active agent. To the best of our knowledge, this is the first study that reports the encapsulation of CBD in Pickering emulsions and the utilization of Pickering emulsions as potential cosmetic vehicles for CBD.

2. Materials and Methods

2.1. Materials

Chitosan with a molecular weight of 100–200 kDa, and a degree of deacetylation of 96% was a kind gift from Primex ehf, Iceland. Collagen peptides (the hydrolyzed form of collagen type I), with a molecular weight of 5000 Da were supplied by HiMedia Laboratories, India. Cannabidiol (CBD) was purchased from THC Pharm (Frankfurt, Germany). Olive oil (highly refined, low acidity), Nile Red, Nile Blue and sodium tripolyphosphate were obtained from Sigma Aldrich. Acetic acid (0.1 N standardized solution) was purchased from Alfa Aesar. Liquid paraffin (with the European pharmacopeia specifications) was purchased from LabChem, Portugal. Acetonitrile and methanol (HPLC grade) were supplied by Carlo Erba Reagents. HaCaT cells were obtained from American Type Culture Collection (ATCC, USA). Dulbecco’s Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Hank’s Balanced Salt Solution (HBSS), non-essential amino acids, penicillin, streptomycin and trypsin–EDTA were obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). DMSO and Triton X-100 were purchased from Sigma-Aldrich (Steinheim, Germany), respectively. The dorsal porcine skin samples were received as a gift from Grupo Primor, Portugal.

2.2. Preparation of chitosan/collagen peptides nanoparticles and Pickering emulsions

The chitosan/collagen peptides (CH/CP) nanoparticles were prepared following a method reported by Anandhakumar and co-workers [35], with some modifications described previously by our research group [33]. In brief, CH/CP nanoparticles dispersion with a polymers’ concentration of 2% w/v was prepared by dissolving 1 g of chitosan in 50 ml of 0.1 N acetic acid with continuous overnight magnetic stirring at 800 rpm. A solution of collagen peptides was prepared by dissolving 1 g in 15 ml of deionized water and was then added dropwise to the chitosan solution under magnetic stirring at 600 rpm. Thereafter, 0.018 g of sodium tripolyphosphate was dissolved in 35 ml of deionized water and added in a dropwise way to the chitosan/collagen peptides mixture over 30 min and constant magnetic stirring at 600 rpm to obtain an opalescent dispersion of CH/CP nanoparticles.

The cannabidiol-loaded Pickering emulsions were prepared by dissolving 0.6 g of cannabidiol (CBD) in the oil. Then, the oil phase with the dissolved CBD was added slowly and portion-wise to the CH/CP nanoparticle dispersion at 13500 rpm for 6 min using a high-speed homogenizer (Ultra-Turrax Digital T25, IKA, Germany). Two different oils were used separately in the formulations; olive oil and liquid paraffin. Each oil was incorporated at an oil volume fraction (φ) of 0.4 and 0.6. The final volume of the emulsion was always equal to 100 ml.
Table 1 summarises the composition and the volume fraction of the oil phase and aqueous phase in the prepared formulations. The pH of the Pickering emulsion formulations prepared with olive oil was 5.03 ± 0.01 and 5.11 ± 0.01 for oil volume fractions of 0.6 and 0.4, respectively, whereas it was 5.15 ± 0.00 and 5.16 ± 0.00 for their counterparts prepared with liquid paraffin. These values are suitable for skin applications as they are compatible with the natural pH of the skin. The pH of healthy skin has been reported to be in the range of 4.1 – 5.8 [36], which varies with age, gender and body site [36,37], and has an average value of 4.7 as reported by Lambers et al. (2006) [38]. The concentration of the CBD in the final produced emulsions was 6 mg/ml in all the formulations, which was selected based on the concentration of CBD in topical formulations present in the market.

2.3. Characterization of the chitosan/collagen peptides (CH/CP) nanoparticles and the CBD-loaded Pickering emulsions

The dynamic interfacial tension between the CH/CP nanoparticles dispersion and liquid paraffin, as well as between the CH/CP nanoparticles dispersion and olive oil was measured by the pendant drop method over 2400 s, using a Dataphysics OCA15 Plus device (Dataphysics, Germany). In brief, a drop of the CH/CP nanoparticles dispersion was formed inside a cuvette filled with liquid paraffin (or olive oil) with a submerged syringe tip. The interfacial tension was calculated by the drop shape analysis with the Young-Laplace equation.

The microstructure of the produced Pickering emulsions was assessed by optical microscopy using a Leica DM 2000 optical microscope equipped with a Leica Application Suite Interactive Measurement imaging software.

The interfacial microstructure of the produced Pickering emulsions was investigated by confocal laser scanning microscopy (CLSM). The analysis was performed using a Leica TCS SP5 CLSM (Leica Microsystems Inc., Germany). Briefly, a fluorescent dye mixture was prepared by mixing Nile Red and Nile Blue solutions in isopropyl alcohol (0.1% w/v each). The freshly prepared Pickering emulsion samples were stained by mixing 0.5 ml of the fluorescent dye mixture with 18 ml of the emulsion. The excitation wavelengths were 488 nm and 633 nm for Nile Red and Nile Blue, respectively.

The mean droplet size and droplet size distributions of the Pickering formulations were measured by laser diffraction using a Beckman Coulter LS 230 particle size analyzer. The refractive index was set as 1.33 for the aqueous phase, and 1.46 and 1.48 for olive oil and liquid paraffin, respectively.

The creaming index (CI%) was used to express the emulsion stability and was calculated according to the following equation [39]:

$$CI\% = \frac{H_s}{H_t} \times 100$$

where $H_s$ is the height of the serum layer that could appear at the bottom of the emulsion during storage, and $H_t$ is the height of the total volume of the emulsion. Lower CI% values indicate higher stability.

The rheological properties of the produced Pickering emulsions were investigated using a Kinexus Pro Rheometer (Malvern, UK). The apparent viscosity ($\eta$) was measured versus the shear rate ranging from 0.1 to 100 s$^{-1}$, using a cone plate with a diameter of 40 mm, an angle of 4°, and a fixed gap of 0.15 mm. The frequency sweep measurements were conducted within the linear viscoelastic region at a constant strain amplitude of 1%, using a parallel plate of a diameter of 20 mm with a fixed gap of 1 mm. The storage modulus ($G'$) and loss modulus ($G''$) of the produced Pickering emulsions were measured versus a frequency range of 0.01–10 Hz.

2.4. Determination of the encapsulation efficiency of cannabidiol (CBD)

The encapsulation efficiency was measured to determine the amount of CBD that was encapsulated in the emulsion droplets and to investigate its distribution between the oil and water phases of the produced Pickering emulsions following previously reported methods [31,40]. Briefly, 1 ml of the emulsion was centrifuged at 10000 rpm for 15 min. The liquid layer at the bottom was then collected with a syringe and filtered (representing the aqueous phase of the emulsion). The filtrate was analyzed by HPLC (as will be described in Section 2.6) to quantify the non-encapsulated amount of CBD which will be denoted as the “Free amount of CBD”. Another 1 ml of the Pickering emulsion was diluted with 10 ml methanol and put in a sonication bath for 10 min to extract the total CBD amount (encapsulated and non-encapsulated). This procedure was done to guarantee that all the added CBD remained in the formulation. The encapsulation efficiency (EE %) was calculated based on the non-encapsulated CBD through the following equation:

$$EE\% = \frac{\text{Total amount of CBD} - \text{Free amount of CBD}}{\text{Total amount of CBD}} \times 100$$

2.5. Ex vivo skin application studies

2.5.1. Skin preparation

The preparation of the skin samples was performed in accordance with the OECD guidelines outlined for the in-vitro skin absorption studies [41]. Porcine skin samples were used in this study as a practical alternative to human skin due to the limited supply of the latter [42], and owing to the similarities in the permeability and morphological properties between both skin types [41,42]. Briefly, the dorsal porcine skin samples, obtained from pigs slaughtered at a local slaughterhouse, were shaved by an electric shaver. The subcutaneous fat was removed by a scalpel to obtain an appropriate thickness [43,44]; since excessive thickness (i.e. thickness > 1 mm) should be avoided [41]. The skin samples were washed thoroughly with water, wrapped in paraffin and aluminum foil, and stored at –20 °C until used. It is noteworthy to mention that the process of storing and freezing the skin samples at this temperature (–20 °C) does not cause alterations in the permeability characteristics of the skin after thawing [45], and is permitted as per the OECD skin preparation protocol [41]. The skin samples were taken out of the freezer and allowed to thaw for one hour before being used in the following experiments.

2.5.2. Evaluation of the skin retention and permeation of CBD

The skin retention and permeation of CBD from Pickering emulsion formulations stabilized with CH/CP nanoparticles prepared with olive oil or liquid paraffin at different oil volume fractions ($\phi$) were studied and compared. The experiments were performed according to the OECD guidelines for the conduct of skin absorption studies [41], and following methodologies described previously in the literature for the determination of the skin absorption of active agents from Pickering emulsions [43,46]. In brief, the formulations were applied to the skin samples mounted in Franz diffusion cells (PermeGear, USA), with a diffusion area of 0.64 cm² and receptor compartment volume of 5 ml. The skin samples were placed with the stratum corneum (uppermost layer of the skin) facing the donor compartment. An amount of 300 µl of each Pickering emulsion formulation was placed in the donor compartment.
and spread on the skin using a small plastic spatula. The receptor fluid consisted of a mixture of ethanol and ultrapure water (50:50 v/v) [47], and was kept under constant magnetic stirring at 600 rpm. The temperature of the Franz cells was controlled by a thermostatic water bath and kept at 32 ± 1 °C according to the OECD guidelines [41]. The experiment was conducted under occlusive conditions. A sample of 400 µl was collected from the receptor fluid after 0, 2, 4, 8, and 24 h. The sample vials were wrapped in aluminum foil to protect the CBD against potential degradation by light. After each sample withdrawal, the collected volume was immediately replaced with an equal fresh volume of the receptor medium to keep sink conditions.

The total mass recovery of CBD was determined by investigating the mass balance of CBD in the skin, as well as in the donor and the receptor compartments according to previously reported methods on the mass recovery of active agents from topically applied Pickering emulsions [43,46]. At the end of the experiment (after 24 h), the residual Pickering emulsion formulation present in the donor compartment was collected and diluted with 25 ml of methanol. Additionally, the skin surface was washed with 25 ml of methanol and the resultant washing solution was added to the previously collected solution (to ensure that all the residual Pickering formulation present on the skin surface is collected). The final solution was filtered and analyzed for CBD quantification using HPLC, representing the “residual sample” remaining on the skin surface. The skin surface was then dried with a wipe. Thereafter, the stripping of the stratum corneum was carried out using Scotch® adhesive tape (3 M, USA). Twenty tape-stripping were applied. The tapes were then cut into small pieces, added to 60 ml of methanol, and placed in an ultrasonic water bath for 30 min to extract the CBD from the stripped stratum corneum. The solution was then filtered and analyzed by HPLC to determine the amount of CBD absorbed by the horny layer of the skin (stratum corneum). The skin membrane remaining after the stripping of the stratum corneum (representing the viable epidermis and dermis) was cut into small pieces, added to 50 ml of methanol, and placed in an ultrasonic water bath for 2 h to extract the CBD present in the viable epidermis and dermis of the skin. Afterward, the solution was filtered and analyzed by HPLC. Each test was conducted four times (n = 4).

2.6. Quantification of CBD

CBD was quantified by high performance liquid chromatography (HPLC) using a reversed phase column ACE 5 C18-pentafluorophenyl group (250 mm × 3 μm, 5 μm). The mobile phase consisted of a mixture of (7:1:2 v/v) acetonitrile/methanol/water following a previously reported method [48]. The analysis was conducted at room temperature at a flow rate of 0.8 ml/min, and UV detection at 220 nm.

2.7. ATR-FTIR skin examination

ATR-FTIR spectroscopy was used to determine the potential variations in the structure of the stratum corneum after the application of the formulations according to methods described in the literature [49,50]. Briefly, the produced Pickering emulsions were first applied to the skin samples mounted in Franz diffusion cells. The skin samples and Franz diffusion cells were prepared and arranged as per the experimental conditions previously described in Sections 2.5.1 and 2.5.2. After 24 h of skin exposure to the formulations, the skin samples were removed and rinsed with water to remove the residues of the formulation present on the surface and air-dried for 15 min. Afterward, the ATR-FTIR measurements of the surface of the stratum corneum treated with the Pickering emulsion formulations were carried out using a Perkin Elmer FTIR spectrometer equipped with a Universal ATR Sampling Accessory. The spectra were recorded at 32 scans with a resolution of 2 cm⁻¹.

![Fig. 1](image1.png)

**Fig. 1.** Dynamic interfacial tension at the oil/water interface measured over 2400 s between CH/CP nanoparticles and liquid paraffin, and CH/CP nanoparticles and olive oil.

2.8. Cell viability assessment

The 3-(4,5-dimethylthiazol-2-yl)– 2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the effect of the different Pickering emulsion formulations (with and without CBD) on HaCaT cell line (passages 89–90). Briefly, cells (25 × 103 cells per ml) were incubated for 24 h with a fresh medium in the absence or presence of the formulations (0.1, 1, 10, 100 and 1000 µg/ml) dissolved in cell culture medium. Following the formulations removal from each well, cells were washed with HBSS. The number of viable cells was determined by adding MTT reagent and incubating for 3 h at 37 °C. DMSO was used to solubilize the crystals. The positive control used was DMEM and the negative control was 1% (w/v) Triton X-100. Cells were grown according to the methodology described by Pinto et al. (2021) [51]. The absorbance was read at 590 nm with background subtraction at 630 nm. Results were expressed as percentages of cell viability.

2.9. Statistical analysis

The results were presented as the mean ± standard deviation using Microsoft Excel 365. The statistical comparisons were performed using the Student’s t-test, and one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test (with p values < 0.05 denoting significant differences). The cell viability results were analyzed using one-way ANOVA followed by Tukey’s HSD test for the post hoc comparisons of the means (with p values < 0.05 denoting significant differences) through the IBM SPSS Statistics 26.0 software (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Characterization of the chitosan/collagen peptides (CH/CP) nanoparticles

The CH/CP nanoparticles were prepared by the polyelectrolyte complexation method followed by ionic gelation, and were reported to have hydrophilic properties leading to the formation of O/W Pickering emulsions as discussed in our previous work [33]. In the present study, the CH/CP nanoparticles are used to stabilize Pickering emulsions containing olive oil or paraffin oil as oil phases. The dynamic interfacial tension was used to assess the degree of the adsorption of the CH/CP nanoparticles at the oil/water interface using the two different oils. Fig. 1 shows that the CH/CP nanoparticles are capable of decreasing the interfacial tension at the oil/water interface of both liquid paraffin and olive oil in a time-dependent way, indicating that the nanoparticles become adsorbed at the oil/water interface, and form an interfacial
particles are well-adsorbed at the oil/water interface. The images also reveal that all the formulations have spherical droplet morphology. The optical microscope images also suggest that the CH/CP nanoparticles have a better interfacial activity towards olive oil than liquid paraffin [54].

### 3.2. Characterization of the CBD-loaded Pickering emulsions

#### 3.2.1. Microstructure

The microstructure of the Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles (at a constant nanoparticles concentration of 2% w/v) was examined by optical microscopy. It was observed that the oil volume fraction influences the morphological and microstructure of the formulations. According to Fig. 2, the Pickering formulations prepared with an oil volume fraction (φ) of 0.6 had a more dense microstructure than those prepared with a φ of 0.4. The microstructure of the latter is less compact (Fig. 2C and D). The optical microscope images also reveal that all the formulations have spherical droplet morphology.

The interfacial microstructure of the produced Pickering emulsions was visualized by the CLSM. The images (Fig. 3) reveal that the nanoparticles are well-adsorbed at the oil/water interface. The images also confirm that all the formed emulsions are o/w emulsions. It can be also observed that the Pickering formulations produced with an oil volume fraction of 0.6 (Fig. 3A and B) have larger droplet sizes and a more compact microstructure than the Pickering emulsions prepared at lower oil volume fractions (Fig. 3C and D). Moreover, it can be noticed that the formulations containing a lower volume fraction have denser interfacial layers of adsorbed nanoparticles.

#### 3.2.2. Visual appearance, creaming index (CI %), and mean droplet size

Fig. 4 A shows that all the freshly prepared Pickering formulations had no phase separation (CI% = 0). However, it can be observed that the CI% of the formulations after storage for 30 days was affected by the type of oil present in the emulsion as well as by the oil volume fraction (Fig. 4B). The formulations prepared with olive oil exhibited a lower phase separation (lower CI% values) than those prepared with liquid paraffin, contributing to higher emulsion stability. Fig. 4B also shows that the formulations with higher oil volume fraction (φ = 0.6) presented a lower phase separation and higher emulsion stability than the formulations prepared with an oil volume fraction of 0.4. The formulation prepared with olive oil (φ = 0.6) possessed the highest storage stability (CI% = 0). Besides, it can be noticed that the bottom serum layer in the formulations with lower oil volume fraction (φ = 0.4) has a more opaque appearance, which is likely due to the presence of a higher amount of nanoparticles in the formulation that separated from the emulsion to the bottom layer during storage. A similar observation was reported for Pickering emulsions prepared with kafrin colloidal nanoparticles [55].

The mean emulsion droplet size of the fresh formulations versus the stored ones are shown in Fig. 4C. It can be observed that formulations with higher oil volume fractions exhibited larger droplet sizes than the ones prepared with low oil volume fractions. This observation is attributed to the decreased amount of the nanoparticles present during emulsification due to the decreased volume of the aqueous phase, which subsequently results in decreasing the number of the nanoparticles that are expected to be adsorbed at the oil/water interface of the droplets leading to the formation of larger emulsion droplets [56]. Fig. 4C also shows that the oil volume fraction (φ) had a more dominant effect on the mean droplet sizes than the oil type. It can be observed that the mean droplet sizes of the formulations prepared with an oil volume fraction of 0.6 (containing either olive oil or liquid paraffin) are not significantly different. Additionally, no significant difference was observed between the mean droplet size of formulations prepared with low oil volume fraction (φ = 0.4). These results are in alignment with the optical microscopy results (Fig. 2), and the CLSM images.
images (Fig. 3) which demonstrated that the morphology of the produced formulations was influenced by the oil volume fraction more than the oil type. Although the difference in interfacial tension and viscosities between oils is expected to result in significant variations in the emulsion droplet sizes, high particle concentrations lead to insignificant differences in the droplet size among the emulsions prepared with different oils [57]. In the current study, it was observed that CH/CP nanoparticles had a higher dynamic interfacial tension with liquid paraffin than olive oil (as was shown in Fig. 1). It is also noteworthy that the viscosity of liquid paraffin equals 148 mPa.s at 20 °C (as specified by the manufacturer), while the viscosity of olive oil is 74.1 mPa.s at 22 ± 1 °C [58]. Therefore, it was expected that formulations prepared with liquid paraffin would have larger droplet sizes than their counterparts prepared with olive oil. However, in the present work, the CH/CP nanoparticles have been incorporated at a high enough concentration (2% w/v) in all the prepared formulations which allowed them to sufficiently cover the produced emulsion droplets at a specific oil volume fraction giving rise to similar droplet sizes when different oils were used [57].

Fig. 4C shows that the formulations that exhibited phase separation after storage demonstrated a reduction in the mean droplet size. The decrease in the mean droplet sizes after storage of Pickering emulsions has been reported in some studies [59-61]. It usually occurs when the original dimension distribution of the emulsion droplets is not monomodal [59,61]. Zafeiri et al. (2017) suggested that it could be linked to the potential desorption of some particles from the oil-water interface during storage for 30 days, which eventually causes a drop in the overall mean sizes of the emulsion droplets [61]. In another study, Marefati et al. (2020), stated that the decrease in the mean droplet sizes could be due to the possible dissociation of aggregated emulsion droplets after storage for 2–8 years [59].

3.3. Rheological properties of the produced Pickering emulsions

The steady flow curves of the formulations are shown in Fig. 5A and B. It can be observed that the formulations demonstrated a non-Newtonian fluid behavior, where the viscosity decreased by the increase in the shear rate. This shear-thinning behavior was more prominent in the formulations prepared with a higher oil to nanoparticles ratio (i.e., \( \varphi = 0.6 \)). This phenomenon happens because when the oil content increases, the Pickering formulation obtains a higher ability to overcome the Brownian movement when subjected to shear force, and hence the viscosity decreases more rapidly [62]. It can be also noticed from the steady flow curves (Fig. 5A and B) that the Pickering emulsions prepared with olive oil had higher viscosities than the ones prepared with liquid paraffin and formulated with the same oil volume fractions. It has been reported that the apparent viscosity of the produced emulsion depends on the hydrodynamic inter-particle interactions between the oil droplets and the solid particles, and not just on the viscosity of the oil.
Therefore, it is suggested that the higher viscosity of the emulsions prepared with olive oil than those prepared with liquid paraffin (at the same oil volume fraction) is related to the greater interaction existing between the CH/CP nanoparticles and olive oil, than that present between the nanoparticles and liquid paraffin due to the structural differences of the two oils. Olive oil is mainly composed of triacylglycerols and free fatty acids (such as oleic, linoleic and linolenic acids), which has been reported to be able to interact with...
chitosan directly through the N-acetyl-D-glucosamine units, or indirectly by the aid of the free fatty acids through electrostatic binding to the amino groups of the D-glucosamine units of chitosan [64]. In contrast, liquid paraffin is a mixture of high molecular weight alkane derivatives, which makes it incapable of any electrostatic binding with the amino groups of chitosan or collagen peptides in the CH/CP nanoparticles. Therefore, the interaction of the CH/CP nanoparticles and liquid paraffin was relatively low which resulted in lower apparent viscosities.

The oscillatory frequency sweep curves of the Pickering formulations prepared with olive oil (Fig. 5 C) show that the emulsion containing a higher oil fraction had higher storage modulus (G’) values than the loss modulus (G’’), indicating the elastic gel-like characteristics of the formulation with \( \phi = 0.6 \), while the one produced with \( \phi = 0.4 \) showed a liquid behavior as G’’ was always greater than G’ over the tested range of frequency (0.01–10 Hz). This observation points to the presence of the emulsion droplets in a tightly packed way and close proximity to one another in the formulation with \( \phi = 0.6 \), suggesting the formulation does not possess the elastic gel properties as its counterpart formulated with olive oil. However, it is still not purely liquid-like compared to the formulation prepared with liquid paraffin but with lower oil content. It has been reported that the formation of an elastic gel network structure in Pickering emulsions (which is indicated by higher values of G’) is mainly related to the interaction of the particles on the oil droplets [66], droplet-droplet interaction, droplet-bridging and partial aggregation [67]. Therefore, a higher interaction leads to the formation of a more compact three-dimensional gel-like structure that restricts (or lowers) the droplet movement and migration rate, preventing creaming (i.e., reducing CI%) and coalescence, and thus results in higher emulsion stability [66,67]. In the present work, it is suggested that the structural differences between olive oil and liquid paraffin (that were discussed in the previous paragraph) resulted in variations of such interactions (at the same oil volume fraction), which resulted in a lower CI% and thus enhanced emulsion stability in the case of the Pickering emulsions prepared with olive oil (as was shown in Fig. 4 B).

3.4. Encapsulation efficiency (EE %) of CBD

The EE% of CBD in the Pickering emulsions prepared with oil volume fraction (\( \phi \)) of 0.6 was found to be 99.66 ± 0.12% and 99.04 ± 0.10%, for olive oil and liquid paraffin formulations, respectively. For Pickering emulsions prepared with lower oil volume fraction (\( \phi = 0.4 \)), the EE% values were 99.40 ± 0.05% and 99.28 ± 0.13% for olive oil and liquid paraffin formulations, respectively. The high values of EE% of all formulations, which were above 99%, are due to the high hydrophobicity of CBD, as well as the formation of a strong barrier of chitosan/collagen peptides nanoparticles surrounding the oil droplets, which guarded against the leakage of CBD to the surrounding phase during the centrifugation step.
The amount of CBD is shown in emulsion stabilized with CH/CP nanoparticles is an excellent vehicle for overall stability in terms of the visual appearance and creaming index. One as could be seen in Fig. 6D. However, this change did not affect the compact microstructure after storage. Moreover, no phase separation occurred (CIP = 0) during this long storage period as shown in Fig. 6C. Nevertheless, there was a slight change in the emulsion droplet size distribution for the stored formulation compared to the freshly prepared formulation prepared with olive oil (φ = 0.6), while it was 97.26 µg/cm² and 80.16 µg/cm² for their counterparts prepared with lower oil volume fraction (φ = 0.4) as can be calculated from Table 2. The amounts reported in the current study are comparable with the amounts of CBD absorbed from topical formulations that have been recently reported in the literature. For example, in a recent study, Vanti et al. (2021) reported that the amount of topically absorbed CBD was 151.35 ± 16.99 µg/cm² after 24 h of applying novel microemulsion gels loaded with 1% (w/w) CBD to an artificial skin membrane, while the amount absorbed was low when rabbit ear skin was used [70]. In another work, Casiraghi et al. (2020) investigated, for the first time, the influence of different vehicles and formulation composition on the skin permeation and absorption of CBD [47]. In that study, the amount of CBD delivered to the skin after 24 h of topical administration of 1% (w/w) solutions of CBD in liquid paraffin and virgin olive oil was reported to be 15.15 ± 4.02 µg/cm² and 6.45 ± 3.23 µg/cm², respectively, while it increased to 37.81 ± 9.20 µg/cm² by incorporating CBD in a mixture of propylene glycol/water (80/20, v/v) [47]. The present work shows that the encapsulation of CBD in Pickering emulsions has enhanced its topical delivery.

### 3.6. Ex-vivo skin absorption and permeation of CBD

Formulations designed for the dermal (topical) delivery of cosmetic and cosmeceutical active agents involve targeting the skin sites, ensuring minimal or no systemic absorption [69]. Fig. 7 shows that CBD permeation after 24 h of exposure to the formulations is very low in comparison to the amount of CBD absorbed in the stratum corneum, and the viable epidermis and dermis. It can be also observed that the highest amount of CBD was retained in the stratum corneum rather than the viable epidermis and dermis, owing to the high lipophilicity of CBD (log P = 6.3). It has been reported that highly lipophilic agents have high affinity to the skin horny layer (stratum corneum) than the hydrophilic ones, and thus they have a lower ability to achieve high permeation to the receptor fluid [46]. Therefore, the stratum corneum is considered as a “reservoir” for highly lipophilic drugs encapsulated in Pickering emulsions, which allows their sustained release from the stratum corneum to deeper layers of the skin [43,46].

The effect of the volume fraction of the oil phase (φ) on the skin penetration of active agents from Pickering emulsions is scarcely reported in the literature. Fig. 7 shows that the amount of penetrated CBD is enhanced by the increase of oil volume fraction from 0.4 to 0.6. Moreover, it was observed that the total amount of CBD that was absorbed in the skin (in the stratum corneum, viable epidermis and dermis) were 4.42% and 4.78% of the total applied dose for Pickering emulsions with an oil volume fraction of 0.6, whereas it was found to be 3.11% and 2.58% for the ones prepared with an oil volume fraction of 0.4 (as summarized in Table 2).

The long-term stability (after 5 months of storage) was evaluated for the formulation prepared with olive oil (φ = 0.6) only as it did not exhibit any phase separation after the initial short storage period of one month. Fig. 6A and B shows that there is almost no difference in the microstructure of the freshly prepared formulation and after being stored for 5 months. The figures show that the formulation maintained its dense/compact microstructure after storage. Moreover, no phase separation occurred (CIP = 0) as can be seen in Fig. 6D. However, this change did not affect the overall stability in terms of the visual appearance and creaming index. This high stability is attributed to the high viscosity of the formulation and the remarkable gel-like structure which guarded against the movement and potential coalescence of the emulsion droplets.

Interestingly, the total amount of CBD in this formulation after 5 months of storage (protected from light), was found to be 99.45% of the initial incorporated amount (i.e., 5.996 mg/ml) compared to the initial amount of 6.03 mg/ml. In a recent study [68], it has been reported that the total amount of CBD in this formulation after 5 months of storage (protected from light), was found to be 99.45% of the initial amount of 6.03 mg/ml. It can be also observed that the highest amount of CBD was retained in the stratum corneum rather than the viable epidermis and dermis, owing to the high lipophilicity of CBD (log P = 6.3). It has been reported that highly lipophilic agents have high affinity to the skin horny layer (stratum corneum) than the hydrophilic ones, and thus they have a lower ability to achieve high permeation to the receptor fluid [46]. Therefore, the stratum corneum is considered as a “reservoir” for highly lipophilic drugs encapsulated in Pickering emulsions, which allows their sustained release from the stratum corneum to deeper layers of the skin [43,46].

### 3.5. Effect of long-term storage on emulsion properties, and on the total amount of CBD

The total amount of CBD absorbed in the stratum corneum, viable epidermis, and dermis after 24 h of application was 143.98 ± 117.29 µg/cm² and 99.39 ± 123.11 µg/cm² for Pickering emulsion formulations prepared with higher oil volume fraction (φ = 0.6), while it was 97.26 µg/cm² and 80.16 µg/cm² for their counterparts prepared with lower oil volume fraction (φ = 0.4) as can be calculated from Table 2. The amounts reported in the current study are comparable with the amounts of CBD absorbed from topical formulations that have been recently reported in the literature. For example, in a recent study, Vanti et al. (2021) reported that the amount of topically absorbed CBD was 115.30 ± 16.99 µg/cm² after 24 h of applying novel microemulsion gels loaded with 1% (w/w) CBD to an artificial skin membrane, while the amount absorbed was low when rabbit ear skin was used [70]. In another work, Casiraghi et al. (2020) investigated, for the first time, the influence of different vehicles and formulation composition on the skin permeation and absorption of CBD [47]. In that study, the amount of CBD delivered to the skin after 24 h of topical administration of 1% (w/w) solutions of CBD in liquid paraffin and virgin olive oil was reported to be 15.15 ± 4.02 µg/cm² and 6.45 ± 3.23 µg/cm², respectively, while it increased to 37.81 ± 9.20 µg/cm² by incorporating CBD in a mixture of propylene glycol/water (80/20, v/v) [47]. The present work shows that the encapsulation of CBD in Pickering emulsions has enhanced its topical delivery.

### Table 2

Distribution of CBD in skin layers (stratum corneum, viable epidermis (VE) and dermis (D)), receptor fluid, and skin surface after 24 h of exposure to the formulations.

<table>
<thead>
<tr>
<th>Applied dose</th>
<th>Stratum corneum</th>
<th>VE and D</th>
<th>Receptor fluid</th>
<th>Residual sample (skin surface)</th>
<th>Mass recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/cm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation 1 (φ = 0.6, olive oil)</td>
<td>3257.30 ± 117.29</td>
<td>105.92 ± 12.95</td>
<td>38.06 ± 8.05</td>
<td>4.06 ± 1.01</td>
<td>2846.02 ± 104.41</td>
</tr>
<tr>
<td>% of total applied dose</td>
<td>3.25</td>
<td>1.17</td>
<td>0.12</td>
<td>0.41</td>
<td>87.37</td>
</tr>
<tr>
<td>Formulation 2 (φ = 0.6, liquid paraffin)</td>
<td>319.68 ± 99.39</td>
<td>123.11 ± 17.59</td>
<td>35.67 ± 7.50</td>
<td>3.78 ± 1.00</td>
<td>2783.30 ± 139.75</td>
</tr>
<tr>
<td>% of total applied dose</td>
<td>3.71</td>
<td>1.07</td>
<td>0.11</td>
<td>0.33</td>
<td>83.90</td>
</tr>
<tr>
<td>Formulation 3 (φ = 0.4, olive oil)</td>
<td>312.02 ± 20.64</td>
<td>77.47 ± 12.33</td>
<td>19.79 ± 4.67</td>
<td>0.80 ± 1.03</td>
<td>2579.72 ± 55.07</td>
</tr>
<tr>
<td>% of total applied dose</td>
<td>2.48</td>
<td>0.63</td>
<td>0.03</td>
<td>0.24</td>
<td>82.68</td>
</tr>
<tr>
<td>Formulation 4 (φ = 0.4, liquid paraffin)</td>
<td>301.37 ± 15.75</td>
<td>64.82 ± 9.77</td>
<td>15.34 ± 5.37</td>
<td>0.64 ± 0.77</td>
<td>2546.36 ± 104.93</td>
</tr>
<tr>
<td>% of total applied dose</td>
<td>2.09</td>
<td>0.49</td>
<td>0.02</td>
<td>0.21</td>
<td>82.10</td>
</tr>
</tbody>
</table>
The type of oil has been described to have a significant effect on the dermal delivery of lipophilic active agents incorporated in cyclodextrin-based Pickering emulsions [71], but it did not impact the transdermal diffusion of active agents from starch-based Pickering emulsions [72]. Furthermore, it has been reported that the solution of CBD in liquid paraffin results in higher skin permeation and higher skin retention of CBD than when it is dissolved in olive oil [47]. However, in the present work, the oil type does not seem to have a remarkable impact on the total amount of CBD delivered to the skin. The results presented in Fig. 7 show that the oil phase volume fraction has a predominant effect on the skin absorption of CBD more than the oil type. These results suggest that the chitosan/collagen peptides nanoparticles (2% w/v) present in high amounts in the formulations prepared with an oil volume fraction of 0.4 retarded the diffusion of the CBD from the oil droplets to the surroundings, and hence hampered its availability in the skin. This observation also indicates that the presence of smaller droplet mean size of the produced Pickering emulsions is not a crucial factor in enhancing the amount of CBD delivered to the skin. In the present study, the highest amount of CBD delivered to the skin was achieved by the formulations with larger mean droplet size (the formulations prepared with olive oil and liquid paraffin having \( \phi = 0.6 \)).

The overall mass recovery % of CBD ranged between 91.91% and 84.70% (as shown in Table 2) which is a satisfactory percentage for active agents that are susceptible to degradation and oxidation during handling and conducting the experiments [41,43].

### 3.7. ATR-FTIR skin analysis

ATR-FTIR spectroscopy is a non-invasive technique normally used to provide insight into the molecular structure and the barrier function of the *stratum corneum* [73,74]. The main bands associated with the alkyl chain of the *stratum corneum* lipids vibration include the asymmetric (~2900 cm\(^{-1}\)) and symmetric (2850 cm\(^{-1}\)) stretching CH\(_2\) vibrations (as shown in Fig. 8). It has been reported that even slight changes (slight shift to higher wavelength) in the asymmetric and symmetric CH\(_2\) stretching vibrations (caused by vehicles or permeation enhancers) have a profound impact on the conformation of the lipids present in the *stratum corneum* which affects the drug penetration through the skin [75]. Additionally, the amide I and amide II bands at ~1640 cm\(^{-1}\) and ~1540 cm\(^{-1}\) (denoted by numbers 3 and 4 in Fig. 8) provide information about the secondary structure of proteins present in the *stratum corneum*. For instance, wavenumbers of amide I band ranging between 1638 and 1645 cm\(^{-1}\) indicate a random coil structure of keratin in the horny layer [50]. Furthermore, the two amide bands have also been reported to be sensitive to new hydrogen bond formation [76].

Fig. 8 highlights the main structural changes in the molecular vibrations of the components of the *stratum corneum* after the application of Pickering emulsion formulations. It can be observed that there are changes in the asymmetric and symmetric vibrational wavelength values between the control skin samples and the other skin samples treated with the Pickering emulsion formulations. These shifts are indicative of changes in the lipid order which contribute to increasing the fluidity of the *stratum corneum* lipids and consequently, increasing skin penetration [73]. Nevertheless, the wavenumbers of the amide I band reveal that the keratin in the *stratum corneum* maintained its coil structure with all formulations.

The FTIR spectra also show that the changes in the stretching vibrations were greater as the oil volume fraction decreased from 0.6 to 0.4. This could be attributed to the presence of greater amounts of chitosan/collagen peptides nanoparticles in the formulations with \( \phi = 0.4 \). Chitosan and chitosan-based nanoparticles have been reported to promote the skin absorption of drugs in topical formulations owing to their positive charges which allow their adhesion to the skin surface which is naturally negatively charged [28,77,78]. Chitosan is also known to increase the water content in the *stratum corneum* enhancing the cell membrane fluidity [28]. Chitosan/collagen peptides nanoparticles have

![Fig. 8. FTIR spectra of porcine skin](image-url)
been reported to have a high positive zeta potential (59.7 mV) [33]. In the present work, it is suggested that the presence of cationic chitosan/collagen peptides particles in higher amounts (when $\phi = 0.4$) resulted in greater interactions with the lipids of the stratum corneum. Nevertheless, these interactions did not result in increased skin penetration of the CBD (compared to formulations having $\phi = 0.6$) since high amounts of nanoparticles present at the oil/water interface surrounding the emulsion droplets (as was revealed by the CLSM images in Fig. 3) retard the diffusion of the active agent from the oil phase to the surrounding medium as has been discussed in Section 3.6.

3.8. Effects of the produced Pickering emulsions towards keratinocytes

HaCaT cells are considered a good model for the evaluation of skin toxicity since they possess the functional and morphological properties of the normal epidermal keratinocytes [79]. This cell line is the most common one used to assess the cytotoxicity of topically applied Pickering emulsions. For instance, it has been used to evaluate the cytotoxicity of Pickering emulsions stabilized with Quercus suber bark (i.e., cork) particles, poly(lactide-co-glycolide)-based nanoparticles, and calcium carbonate particles [15,21,80]. In this work, the cell viability of chitosan/collagen peptides Pickering emulsions (prepared with olive oil

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**Fig. 9.** Effect of chitosan/collagen peptides Pickering formulations prepared with olive oil ($\phi = 0.6$, with and without CBD) on the viability of keratinocytes (HaCaT) cells at different concentrations, as measured by the MTT assay ($n = 3$). Different letters mean significant differences between concentrations of the same formulation ($p < 0.05$).

**Fig. 10.** Effect of chitosan/collagen peptides Pickering formulations prepared with liquid paraffin ($\phi = 0.6$, with and without CBD) on the viability of keratinocytes (HaCaT) cells at different concentrations, as measured by the MTT assay ($n = 3$). Different letters mean significant differences between concentrations of the same formulation ($p < 0.05$).
Pickering emulsions stabilized with the biocompatible and biodegradable chitosan/collagen peptides nanoparticles were efficiently assessed as green biodegradable topical vehicles of CBD. The physico-chemical properties and stability of the produced CBD-loaded Pickering emulsions were influenced by the amount of the oil and water phases, as well as the oil type. The formulation produced with olive oil ($q = 0.6$) has shown long-term stability during the testing period that lasted for 5 months. The in vitro skin absorption studies revealed high skin deposition of CBD in the stratum corneum, while very low amounts permeated to the receptor fluid. The skin deposition was enhanced by increasing the oil volume fraction in the formulations regardless of the oil type. The ATR-FTIR results have shown that the Pickering emulsion formulations caused changes in the microstructure and molecular vibrations of the stratum corneum which enabled them to overcome its barrier function. The findings obtained in this work prove the successful utilization of Pickering emulsions stabilized by biopolymeric nanoparticles, namely chitosan/collagen peptides nanoparticles, as environmentally-friendly and non-toxic topical delivery vehicles of a highly lipophilic active agent, and thus contribute to the ongoing research in the area of sustainable cosmetics. Future work will focus on comparing the skin delivery of the CBD-loaded Pickering emulsions developed in the current study with different skincare CBD-based formulations that are available in the market.

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.

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